CENTRO INTERNACIONAL DE ESTUDOS
DE DOUTORAMENTO E AVANZADOS
DA USC (CIEDUS)

TESIS DE DOCTORADO

SEARCH FOR BIOMARKERS RELATED TO RHINITIS AND DIFFERENT ASTHMA PHENOTYPES BY SERUM PROTEOMICS AND IMMUNOASSAYS

Juan José Nieto Fontarigo

ESCUELA DE DOCTORADO INTERNACIONAL

PROGRAMA DE DOCTORADO EN MEDICINA MOLECULAR

SANTIAGO DE COMPOSTELA

2019

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

DECLARACIÓN DO AUTOR/A DA TESE

SEARCH FOR BIOMARKERS RELATED TO RHINITIS AND DIFFERENT ASTHMA PHENOTYPES BY SERUM PROTEOMICS AND IMMUNOASSAYS

D./Dna. Juan José Nieto Fontarigo

Presento a miña tese, seguindo o procedemento axeitado ao Regulamento, e declaro que:

1) A tese abarca os resultados da elaboración do meu traballo.
2) De selo caso, na tese faise referencia ás colaboracións que tivo este traballo.
3) A tese é a versión definitiva presentada para a súa defensa e coincide coa versión enviada en formato electrónico.
4) Confirmo que a tese non incorre en ningún tipo de plaxio doutros autores nin de traballos presentados por min para a obtención doutros títulos.

En Santiago de Compostela, 26 de Marzo de 2019.

Asdo. Juan José Nieto Fontarigo

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

AUTORIZACIÓN DO
DIRECTOR / TITOR DA TESE

SEARCH FOR BIOMARKERS RELATED TO RHINITIS AND DIFFERENT ASTHMA PHENOTYPES BY SERUM PROTEOMICS AND IMMUNOASSAYS

D./Dna. Francisco Javier Salgado Castro
D./Dna. Montserrat Nogueira Álvarez

INFORMA/N:

Que a presente tese, correspóndese co traballo realizado por D/Dna. ***Juan José Nieto Fontarigo****, baixo a nosa dirección, e autorizamos a súa presentación, considerando que reúne os requisitos esixidos no Regulamento de Estudos de Doutoramento da USC, e que como directores desta non incorre nas causas de abstención establecidas na Lei 40/2015.*

En Santiago de Compostela, 26 de Marzo de 2019.

Asdo.
Francisco Javier Salgado Castro

Asdo.
Montserrat Nogueira Álvarez

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CONFLICTO DE INTERÉS

SEARCH FOR BIOMARKERS RELATED TO RHINITIS AND DIFFERENT ASTHMA PHENOTYPES BY SERUM PROTEOMICS AND IMMUNOASSAYS

D. Juan José Nieto Fontarigo

Declaro no tener ningún tipo de conflicto de intereses, ni ninguna relación económica, personal, política, interés financiero ni académico que pueda influir en este trabajo.

En Santiago de Compostela, 26 de Marzo de 2019.

Fdo. Juan José Nieto Fontarigo

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Esta tesis y los trabajos derivados se han realizado en el Centro de Investigación en Biología de la Universidad de Santiago de Compostela (CIBUS) – Facultad de Biología, Campus vida, Universidade de Santiago de Compostela gracias al financiamiento de los proyectos (121/2012) por la Sociedad Española de Neumología y Cirugía Torácica, (SEPAR) y el (PI13/02046) del Instituto de Salud Carlos III, Ministerio de Economía y Competitividad (Fondo de Investigación Sanitaria, FIS; co-financiado por fondos FEDER de la Unión Europea)

El autor de esta tesis, D. Juan José Nieto Fontarigo, ha sido benficiario de la beca predoctoral de la Xunta de Galicia (DOG Núm. 122, Páx. 27453, Mércores, 29 de xuño de 2016), Modalidad B (Cofinanciadas por Fondo Social Europeo, FSE).

Todos los trabajos realizados en esta tesis cuentan con la aprobación del Comité de Ética de Investigación Clínica de Galicia (CEIC)(2011/001), y todos los participantes en los mismos han firmado un consentimiento informado.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

"No es el más fuerte de las especies el que sobrevive, tampoco es el más inteligente el que sobrevive. Es aquel que es más adaptable al cambio"

Charles Darwin

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

ACKNOWLEDGEMENTS

Esta tesis y los trabajos derivados de ella no podría ser posibles sin el financiamiento de los proyectos por la Sociedad Española de Neumología y Cirugía Torácica, (SEPAR) (121/2012) y el Instituto de Salud Carlos III, Ministerio de Economía y Competitividad (Fondo de Investigación Sanitaria, FIS; co-financiado por fondos FEDER de la Unión Europea) (PI13/02046). Tampoco sin el contrato pre-doctoral de la Xunta de Galicia que he recibido (DOG Núm. 122, Páx. 27453).

Dicho esto, en primer lugar me gustaría agradecer el apoyo de todas las personas que participaron en el desarrollo de esta tesis. Entre ellas, a **Montse y Pilar** ya que sin ellas no sería posible el desarrollo de este trabajo, tanto por su aporte tanto a nivel profesional, como por su aporte moral y los buenos momentos que pasé con ellas a lo largo de estos 4 años. También agradecer a todo el servicio del Hospital Clínico de Santiago de Compostela (CHUS) y en especial al Dr. **Francisco Javier Barcala**, sin el cual hoy no podría estar escribiendo esta tesis. Agradecer además el aporte de los demás participantes en los artículos que deriban de esta tesis. En segundo lugar quiero dar las gracias a todos mis compañeros de laboratorio durante todos estos años. **Ana, Adriana, Tamara, Jaime, Ángel, Pablo, Eva, María, Alba,** y en especial a **Luis,** por su ayuda para que esta tesis llegara a buen puerto.

A special thanks to **Lena,** who allowed me to be in her laboratory for 3 magnificent months, learning many things about research, but also meeting many people to whom I have an appreciation for the treatment received during that stay, especially to **Samuel, Sangeetha, Hamid, and Cecilia.**

En especial, agradecerle a **Fran** todo el apoyo, que ha sido mucho, a lo largo de estos 4 años; las charlas, las cañas, las risas, los

agobios, el compañerismo, la humildad, y sobre todo las ganas con las que trabaja y vive cada día. Gracias por tratarme como un igual, por tener en cuenta todas mis opiniones, por no enfadarte nunca a pesar de las veces que he metido la pata. Aparte de un director de tesis, me llevo un amigo.

Darles las gracias a mis amigos, **Andrea, Edgar, Tere, Luis,** por esas noches en el Rudi de desconexión que tanto me hacían falta. O a **Jose Ángel, Carla, Iván, Fátima**, y demás compañeros a los que también tengo un especial cariño. También a los chicos de **Biosant,** por esas charlas/reuniones tan divertidas que siempre mantenemos.

Tengo que agradecer a **mis padres, mi abuela**, y a toda mi familia por estar siempre ahí, por darme el apoyo que he necesitado sin pedir nada a cambio, por darme esta educación, criarme con humildad y con valores, por permitirme hacer esta carrera tan apasionante, por darme cariño, por quererme.

Por último, y no menos importante, **Mónica**. A ti te debo todo. Gracias por tu risa. Gracias por pasar día a día a mi lado, dándome el cariño y la fuerza que tanto necesito. Gracias por pasarte horas escuchando mis problemas. Gracias por estar ahí en los momentos buenos, pero sobre todo por evitar que me caiga. Gracias por permitirme ser yo mismo. Gracias por ser tú. Sin ti mi carrera en Biología sería un papel en blanco. Sin ti, esta tesis no existiría.

INDEX

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

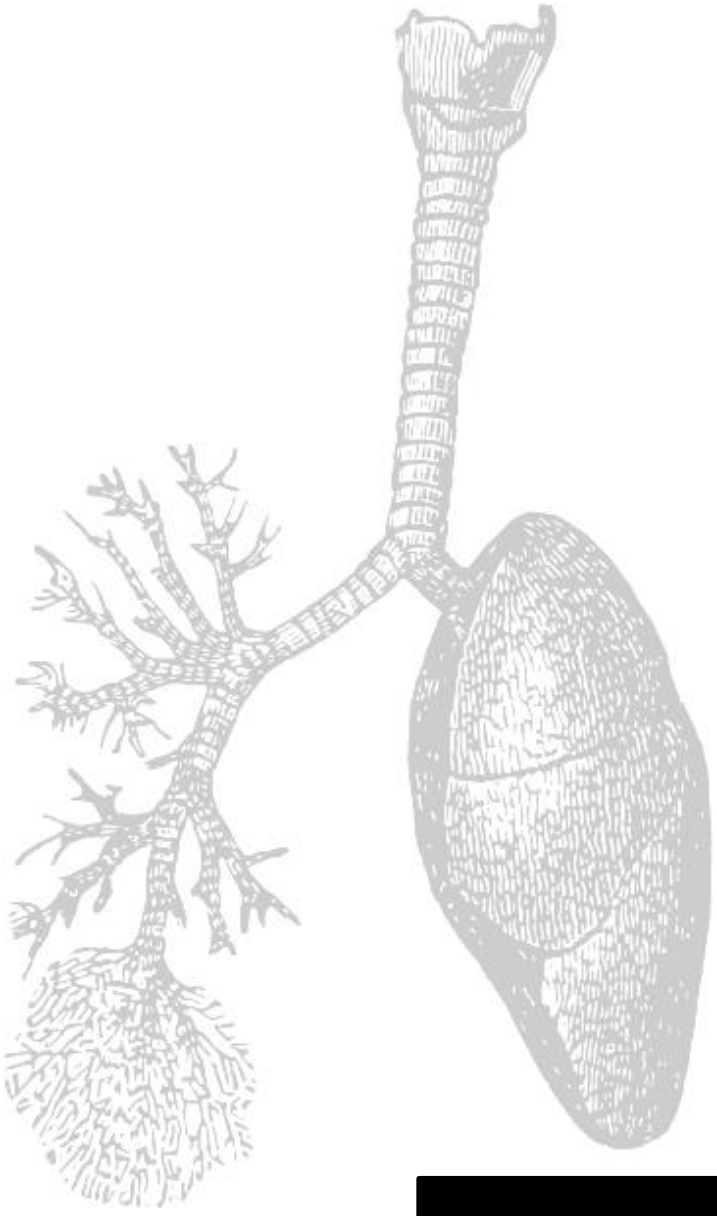
ABBREVIATIONS

2-DE:	*Two dimension electrophoresis*
2D-DIGE:	*2D Fluorescence Difference Gel Electrophoresis*
AA:	*Allergic asthma*
ADA:	*Adenosine deaminase*
AECs:	*Airway epithelial cells*
AHSG:	*α-2-HS-glycoprotein*
AHR:	*Airway hyperresponsiveness*
APCs:	*Antigen presenting cells*
BALF:	*Bronchoalveolar lavage fluid*
BB:	*Bronchial biopsies*
BCR:	*B-cell receptor*
BIM:	*Bcl-2 interacting mediator*
CFH:	*Complement factor H*
CFI:	*Complement factor I*
CPLLs:	*Combinatorial peptide ligand libraries*
CSF:	*Cerebrospinal fluid*
DAMPs:	*Damage associated molecular patterns*
DPP:	*Dipeptidyl peptidase protein*
EBC:	*Exhaled breath condensate*
ELISAs:	*Enzyme-linked immunosorbent assays*
ESI:	*Electrospray ionisation*
EU:	*European Union*
FeNO:	*Fractional exhaled nitric oxid*
GWAS:	*Genome-wide association studies*
HDM:	*House dust mite*
HSPG2:	*Heparan sulphate proteoglycan 2*
ICS:	*Inhaled costicosteroids*
IGFs:	*Insuline-like growth factors*
IGFALS:	*Insulin-like growth factor binding protein, acid labile subunit*
IGFBPs:	*Insulin-like growth factor binding proteins*

ILCs:	*Innate lymphoid cells*
iNKT:	*Invariant natural killer T cells*
iTRAQ:	*Isobaric tag for relative and absolute quantitation*
iTregs:	*Induced regulatory T cells*
LAP:	*Low abundant proteins*
LBP:	*Lipopolysaccharide binding protein*
LC:	*Liquid chromatography*
LC-MS/MS:	*Liquid chromatography coupled to mass spectrometry*
LMW:	*Low molecular weight proteins*
LPS:	*Lipopolysaccharide*
LTQ:	*Linear trap quadrupole*
MALDI-TOF:	*Matrix-Assisted Laser Desorption/Ionization-time-of-flight*
mCD14:	*Membrane CD14*
MHC:	*Major histocompatibility complex*
MS:	*Mass spectrometry*
NAA:	*Non-allergic asthma*
NB:	*Nasal brushing*
NK:	*Natural killer*
NLF:	*Nasal lavage fluid*
NSE:	*Neuron-specific enolase*
nTregs:	*Natural regulatory T cells*
ORM1/AGP:	*Orosomucoid/α-1-acid glycoprotein 1*
OVA:	*Ovalbumin*
PAMPs:	*Pathogen associated molecular patterns.*
Protein AMBP:	*α-1-microglobulin/bikunin precursor*
PRRs:	*Pathogen recognition receptors*
PCs:	*Principal components*
RP:	*Reverse phase*
sCD14:	*Soluble CD14*

sCD25:	*Soluble CD25*
sCD26:	*Soluble CD26*
SCX:	*Strong cation exchange*
SNPs:	*Single nucleotide polymorphisms*
T_{CM}:	*Central-memory T cells*
TCR:	*T-cell receptor*
T_{EM}:	*Effector-memory T cells*
T_{EMRA}:	*Terminally-differentiated effector T cells*
Teff:	*Effector T cells*
TfH:	*Follicular helper T cells*
TH:	*Helper T cells*
TLRs:	*Toll-like receptors*
T_N:	*Naïve T cells*
Treg:	*Regulatory T cells*
T_{SCM}:	*Stem cell memory T cells*
UPLC:	*Ultra performance liquid chromatography*
US:	*United States*
WT:	*Wild type*

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA



ABSTRACT

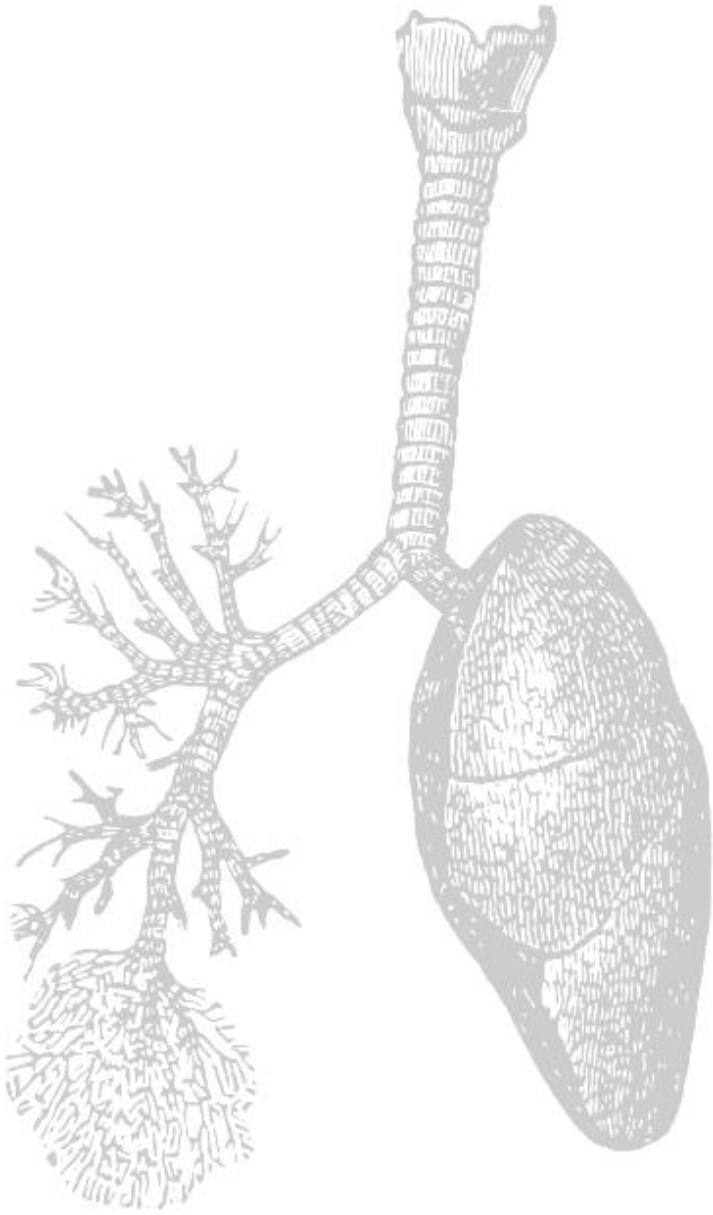
USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Asthma is a heterogeneous disease with several clinical phenotypes and molecular endotypes. However, the specific connection between asthma phenotypes and the underlying pathological features is difficult to explain. Thus, the overall aim of the present thesis was to search for biomarkers associated with rhinitis and different phenotypes (allergic and non-allergic) and severities (intermittent-mild and moderate-severe) of asthma, which could have an application in the diagnosis, prognosis or treatment of this disease. The mechanisms underlying asthma are multiple and complex, but the immune system plays a key role in the pathophysiology of this disease. Therefore, we decided to further study the role of the immune system in different phenotypes or severities of asthma through the analysis of certain biomarkers previously related to this disease: CD14 (innate immune system) and CD26/CD126 (adaptive immune system).

CD14 is a receptor mainly expressed on monocytes, which participates in the lipopolysaccharide (LPS) signalling. Our results show that both the expression of CD14 on monocytes and the normalized levels of soluble CD14 (sCD14) in serum are reduced in allergic asthma *vs.* healthy controls. This reduction can be explained by the expansion of $CD14^{low}$ cells, probably non-classical monocytes with a high capacity to differentiate into M2 macrophages. In addition, sCD14 levels are associated with the promoter SNP of *CD14* (-159 C/T). Thus, subjects with the C allele and CC genotype have lower levels of sCD14, as well as increased risk of allergic asthma. On the other hand, CD26 is a peptidase mainly expressed by helper T (TH) lymphocytes. Our results evidence a high correlation between the expression of CD26 on those cells and DPP4 activity (~ soluble CD26/sCD26) *in vitro*. Moreover, the expression of this molecule differentiates subtypes of T cells (TH17>>TH1>TH2>Treg), as well as discriminates between cells with different stages of differentiation: central-memory T cells (T_{CM}, $CD26^{high}$, $CD45RA^{-}CCR7^{+}CD28^{+}$), naïve T cells ($T_N$, $CD26^{int}$, $CD45RA^{+}CCR7^{+}CD28^{+}$), and terminally differentiated or effector-memory T cells (T_{EM} or T_{EMRA}, $CD26^{low}$, $CD45RA^{+/-}CCR7^{-}CD28^{-}$). In addition, there is an

expansion of different subpopulations with low expression of CD26 in asthma: Tlow cells ($CD25^{low}CD26^{low}CD127^{low}$) in allergic asthma and $CD26^{-}$ γδ-T cells in non-allergic asthma. This expansion could explain the decreased levels of sCD26 in serum in both allergic and non-allergic asthma. The reduction of CD26 levels in asthmatics could lead to a higher proliferation capacity of their lymphocytes, as well as to an increased migration to inflammatory sites. The expression of CD26 and CD126 in $CD4^{-}$ T lymphocytes is highly correlated. Thus, the population of T_{EM} or T_{EMRA} cells increased in non-allergic asthma is also $CD126^{-}$. CD126 is also related to asthma severity, since patients with a moderate-severe profile displayed reduced levels of this molecule on the surface of monocytes, neutrophils and lymphocytes, compared to intermittent-mild asthmatics. This could imply the role of *trans*-signalling (activation of $CD126^{-}$cells by sCD126) in the severity of asthma.

Finally, a non-target study aimed to identify new biomarkers associated with different phenotypes of asthma was also performed. Firstly, a new methodology for the analysis of the low abundance proteins based on the reduction of serum proteome complexity was created. This implies the use of combinatorial peptide ligand libraries (CPLLs) and the identification and relative quantification of serum proteins by iTRAQ and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Through this methodology, 26 proteins with differential changes between groups were described, many of them possible biomarkers of allergic asthma (IGFALS, protein AMBP, or HSPG2) or non-allergic (CFI, CFH, or MASP1) and severities (e.g., IGFALS in moderate-severe allergic asthma).



SUMMARY IN SPANISH

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

El asma es una enfermedad crónica con una alta prevalencia (>300 millones de individuos a escala mundial), especialmente en zonas urbanas, y con un impacto cada vez mayor en nuestras sociedades. Esta enfermedad afecta al 5% de la población adulta, un porcentaje que se puede incrementar hasta al 10% en el caso de la población infantil. Además, muchos pacientes asmáticos presentan una enfermedad mal controlada o son refractarios a los tratamientos convencionales, generando un gran consumo de recursos sanitarios tanto directos como indirectos.

El asma es una enfermedad respiratoria caracterizada por una obstrucción de las vías aéreas recurrente y reversible. Dicha obstrucción está asociada a hipersensibilidad y remodelado de las vías aéreas con presencia de fibrosis y edema, mayor permeabilidad vascular e infiltración de células inflamatorias. Precisamente esta última característica otorga al sistema inmunológico un papel central en esta afección. Sin embargo, el término "asma" supone una especie de paraguas bajo el cual se recogen diversas patologías, ya que esta enfermedad es muy heterogénea, resultado de la interacción entre multitud de factores genéticos y ambientales. Dicha interacción da lugar a una capa superficial observable de distintos fenotipos clínicos, por debajo de la cual subyacen diversos endotipos moleculares o causas fisiopatológicas. Sin embargo, la conexión específica entre estos fenotipos específicos y los mecanismos patológicos/moleculares latentes es difícil de explicar. Esto hace que el asma presente un tratamiento complejo y con un grado de respuesta al mismo muy variable entre los distintos pacientes. Por todo ello, es más que necesaria la búsqueda de nuevos biomarcadores que permitan un refinamiento de los fenotipos y endotipos y que tengan una aplicación en los procesos de diagnóstico, pronóstico o tratamiento de la enfermedad. En esta línea se enmarca el objetivo general de la presente tesis, que es la búsqueda de biomarcadores de diversa índole

(moleculares, celulares) asociados a rinitis y a distintos fenotipos (asma alérgica y asma no alérgica) y severidades (asma intermitente-leve y asma moderado-grave) del asma. La búsqueda de estos biomarcadores es importante para llegar a entender las características clínicas de los diversos pacientes, agruparlos, y tomar decisiones, basadas en evidencias objetivas, sobre la forma de abordar el tratamiento de una manera específica y personalizada; la llamada medicina de precisión.

Los mecanismos que subyacen al asma son múltiples y complejos, pero como se mencionó previamente el sistema inmune tiene un papel clave en la fisiopatología de la enfermedad asmática. Esta incluye tanto mecanismos inmunológicos innatos como adaptativos, empezando por el epitelio bronquial, continuamente expuesto a alérgenos, patógenos y polución. Durante un ataque de asma, los alérgenos desencadenan la producción de citoquinas como TSLP, IL-25, o IL-33, muy importantes en la patogénesis del asma, especialmente en asma alérgica. La presencia de estas citoquinas conduce a la activación de células linfoides innatas de tipo 2, que producen citoquinas de tipo 2 (IL-4, IL-5, e IL-13) y conducen al desarrollo de una inflamación dominada por linfocitos adaptativos efectores de tipo T cooperador/"helper" (TH) con fenotipo TH2.

Sin embargo, cada vez se hace más evidente que otros subtipos de linfocitos adaptativos efectores, como los TH1 o los TH17, son muy importantes en el desarrollo de diversos fenotipos asmáticos, como por ejemplo el asma neutrofílica, o en la generación de una mayor severidad. Además, aparte de los linfocitos TH, las diversas investigaciones en el campo del asma también han puesto el foco sobre otras poblaciones leucocitarias muy importantes en las etapas tempranas del asma, como los monocitos, los eosinófilos, los neutrófilos o los linfocitos T-γδ. Debido a la complejidad de la patogénesis asmática, en la presente tesis hemos decidido profundizar

en el estudio del papel del sistema inmune en los distintos fenotipos y severidades del asma, mediante el estudio de ciertos biomarcadores previamente relacionados con esta enfermedad: CD14, un receptor presente mayoritariamente en células del sistema inmune innato (los monocitos), y CD26/DPP4, una molécula preferentemente y diferencialmente expresada por células del sistema inmune adaptativo, los linfocitos TH: TH17>>TH2>TH1>Treg; TH memoria > TH naïve.

Por otro lado, los estudios proteómicos han supuesto un gran avance en el descubrimiento de nuevos biomarcadores. Estos estudios utilizan diferentes muestras biológicas, como biopsias de tejido, lavado broncoalveolar o esputo inducido, si bien la muestra más utilizada sigue siendo el suero o el plasma, dos hemoderivados obtenidos por procedimientos no invasivos y en la que se realizan numerosas determinaciones en los estudios clínicos rutinarios. Sin embargo, el análisis proteómico de este tipo de biofluidos representa un enorme reto tecnológico debido fundamentalmente a dos factores: la media-baja abundancia de las proteínas liberadas por los tejidos afectados por la enfermedad y el elevado rango dinámico de abundancias de proteínas. Por tanto, es necesario el desarrollo de nuevos métodos analíticos que nos permitan eliminar las proteínas altamente abundantes y acceder al denominado proteoma de media-baja abundancia, enriquecido en proteínas de bajo peso molecular y potenciales biomarcadores. Actualmente existen diversas metodologías desarrolladas con ese propósito, pero la mayoría de los estudios realizados en asma siguen detectando proteínas que pertenecen al proteoma de alta-media abundancia. Además, ninguno de los estudios proteómicos encaminados a entender esta enfermedad tenía como objetivo comparar simultáneamente distintos fenotipos asmáticos o severidades. Por tanto, la segunda parte de esta tesis tiene como objetivo el desarrollo de un método de análisis de proteínas de media-baja abundancia en suero aplicable a la búsqueda de

biomarcadores séricos en asma (aplicable a otras patologías también), así como la realización de un estudio prospectivo, no dirigido, para identificar nuevos marcadores biológicos asociados con distintos fenotipos y/o severidades de esta enfermedad. Para ello, tras probar diferentes metodologías, se ha desarrollado un protocolo que consiste en una eliminación de lipoproteínas del suero, el enriquecimiento en proteínas de media-baja abundancia mediante librerías de hexapéptidos combinatorios (CPLLs) (compresión del rango dinámico de abundancias), la generación de péptidos trípticos, su marcaje mediante reactivos iTRAQ, y el fraccionamiento, identificación y cuantificación relativa mediante cromatografía líquida en fase reversa acoplada a espectrometría de masas en tándem (LC-MS/MS).

Capítulo I

El estudio que se expone en el capítulo I tuvo como objetivo el estudio de la expresión de CD14 en la superficie de monocitos, así como el análisis de los niveles de CD14 soluble, su relación con el SNP del promotor de *CD14* (-159 C/T) y la susceptibilidad de sufrir asma alérgica. Todo esto en una población bien definida (adultos, caucásicos, bajos niveles de endotoxina) y con un tamaño muestral relativamente elevado (277 pacientes con asma alérgica y 277 controles sanos). Los resultados de este estudio demostraron un aumento del número de monocitos en pacientes con asma alérgica pocas veces descrito previamente. Sin embargo, la expresión de CD14 en la superficie de estas células, así como la concentración de CD14 soluble (normalizado por el número de monocitos) en suero estaban reducidos en los pacientes con asma. Dicha reducción podría deberse a la expansión de monocitos con fenotipo $CD14^-$, probablemente de tipo "no clásico" ($CD14^-CD16^+$) y con alta capacidad de diferenciarse a macrófagos tisulares M2. Pero además, a ello se podría sumar el efecto del SNP *CD14* (-159 C/T) sobre los niveles de sCD14. De

hecho, nuestros resultados apuntan a mayores niveles de sCD14 en individuos portadores del alelo T y del genotipo TT. Además, tanto el alelo T como el genotipo TT de dicho SNP están asociados a un menor riesgo de sufrir asma alérgica. O dicho de otro modo, nuestros resultados sugieren un papel para el alelo C o el genotipo CC del SNP (-159 C/T) a la hora de generar bajos niveles de CD14 soluble y aumentar el riesgo de desarrollar asma alérgica de mayor gravedad. Los menores niveles de CD14/CD14 soluble en individuos con asma alérgica tienen sentido biológico, ya que esta molécula constituye junto con "Toll-like Receptor 4" (TLR4) un complejo responsable de la señalización del lipopolisacárido bacteriano (LPS) en monocitos/macrófagos. Dicha vía desencadena la producción de citoquinas como IL-12, promoviendo la diferenciación TH1 en detrimento de la TH2.

Finalmente, debido al aumento de monocitos circulantes detectado en pacientes con asma alérgica, durante la realización de este estudio también se analizaron los niveles de la enolasa neuronal específica (NSE), una molécula que se ha visto aumentada tras activación de eosinófilos y macrófagos en algunas enfermedades. Nuestros resultados muestran una correlación positiva entra las concentraciones séricas de esta molécula e IgE total, ambas aumentadas en pacientes con asma alérgica. De hecho, la eficiencia diagnóstica evaluada a través de los valores de "area under the curve" AUC de las curvas "Receiver Operating Characteristic" (ROC) demuestran unos valores muy similares de efectividad diagnóstica para NSE e IgE, subrayando un posible uso como biomarcador de este fenotipo asmático.

Capítulos II y III

Del mismo modo que sucede con CD14 en monocitos, CD26 es una peptidasa con actividad DPP4 que muestra una especial abundancia en linfocitos T colaboradores ($TCD4^+$; sistema inmune adaptativo), que presenta una forma soluble, y que también ha sido asociada al asma por diversos autores. Los estudios llevados a cabo en los capítulos II y III tienen como objetivo profundizar en esta última asociación en relación con los distintos fenotipos asmáticos y severidades. CD26/DPP4 es una proteína "pluriempleada" ("moonlighting"), lo que hace que su papel en asma sea complejo de estudiar. Además, la práctica ausencia de estudios de revisión bibliográfica sobre las funciones de esta molécula y sus posibles consecuencias en asma impulsó el trabajo que se muestra en el anexo I de la presente tesis (Anexo I). Este proporciona una visión general y estructurada de las distintas funciones pro- y anti-inflamatorias de CD26, así como de las posibles implicaciones en la fisiopatología asmática. Estudios previos en nuestro grupo habían demostrado una mayor abundancia de CD26 en las células T efectoras que en células T reguladoras. Además, otros autores habían demostrado que los niveles de CD26 dentro de las células efectoras eran bastante variables: TH17>> TH1> TH2> Treg. Con esa idea en mente, nuestro trabajo (Capítulo II) demostró por primera vez en cultivo *in vitro* un aumento de expresión de CD26 en linfocitos TH efectores humanos tras activación, especialmente en condiciones que promovían una diferenciación TH17, pero también una correlación positiva con la actividad DPP4 soluble (una medida indirecta de CD26 soluble). Por tanto, nuestros resultados sugerían que los niveles de CD26/DPP4 soluble *in vitro* estaban influenciados por el número de linfocitos T $CD4^+$ así como por su grado de activación y fenotipo (TH1, TH2, o TH17), de modo que los niveles de CD26 soluble en suero podrían estar reflejando, como una "huella dactilar", la contracción o expansión de unas subpoblaciones TH sobre

otras; dicho de otro modo, podría haber diferencias en la expresión de CD26 en los linfocitos TH y en los niveles de su isoforma soluble dependiendo del fenotipo y severidad asmáticas. Por tanto, esta hipótesis inicial fue examinada en estudios con pacientes.

En consonancia con dicha hipótesis de partida y también con los resultados de un estudio previo de 2007 publicado por Samantha Wei-Man Lun y colaboradores, la expresión de CD26 en linfocitos TH fue mayor en pacientes con asma alérgica en comparación con los controles sanos. Sin embargo, los niveles de CD26 estaban, contrariamente, reducidos en el suero de estos mismos pacientes; lo mismo ocurría con CD25 soluble, otro marcador de activación. Dado que no se observaron cambios en los porcentajes de células T reguladoras (una población $CD26^{low}$), estos menores niveles de CD26/CD25 solubles fueron relacionados con la expansión de una subpoblación TH efectora con fenotipo $CD25^{low}/CD26^{low}/CD127^{low}$ (células "triple low" o Tlow) en asma alérgica. Además, dicha expansión fue confirmada en el Capítulo III, donde además se profundizó en la caracterización fenotípica de esta población. Así, se vio que las células Tlow eran células TH en un avanzado estado de diferenciación, con fenotipo "efector de memoria" (T_{EM}) o "terminalmente diferenciado" (T_{EMRA}), caracterizadas por la pérdida de marcadores de superficie como CD27, CD28, CCR7 o CD127. Por tanto, la expresión de CD26 en células TH y sus niveles en suero son indicativos sobre todo de las distintas etapas de diferenciación naïve-memoria: células de memoria central (T_{CM}, $CD26^{high}$), células naïve (T_N, $CD26^{intermediate}$) y células efectoras de memoria o terminalmente diferenciadas (T_{EM} o T_{EMRA}, $CD26^{-/low}$).

De igual forma que en pacientes con asma alérgica, también hemos visto un descenso de CD26 soluble en pacientes con asma no alérgica. Sin embargo, no se observó una expansión significativa en estos pacientes de poblaciones TH con fenotipo T_{EM}/T_{EMRA} (Tlow).

Por tanto, decidimos enfocar el análisis sobre los linfocitos T $CD4^-$, que incluyen células B, NK, NKT y linfocitos T-γδ. Además, se analizaron no sólo los niveles de CD26, sino también los de CD126/IL-6Rα, ya que IL-6 tiene un papel importante en la generación de linfocitos TH17, ambos con relevancia en asma no alérgica. En el conjunto de linfocitos T $CD4^-$, se observó una elevada correlación entre la expresión de CD26 y CD126. También se documentó en pacientes con asma no alérgica un aumento del porcentaje de linfocitos T $CD4^-$ en avanzado estado de diferenciación (T_{EM}/T_{EMRA}) y con fenotipo $CD26^-$ o $CD126^-$. Además, a pesar de no observar cambios en los porcentajes de los distintos tipos celulares dentro de los linfocitos T $CD4^-$, nuestros resultados sí evidenciaron un aumento de la proporción de células T-γδ con bajos niveles de CD26 en estos pacientes. Por tanto, ambos fenotipos asmáticos (asma alérgica y asma no alérgica) presentan un paralelismo en relación con la expansión de células $CD26^{-/low}$ altamente diferenciadas ($CD27^{low}CD28^{low}CCR7^{low}CD127^{low}$) pertenecientes a diferentes linajes: TH en asma alérgica y $CD4^-$ T-γδ en asma no alérgica; la reducción de los niveles de CD26 solubles en ambos fenotipos asmáticos sería una "impronta" de dicha expansión en el suero de los pacientes.

La reducción de CD26 de membrana o soluble en asma, especialmente el fenotipo no alérgico, debe tenerse en cuenta a la vista del papel modulador de CD26 sobre citoquinas (p.ej., IL-3, GM-CSF) o quimioquinas (p.ej., RANTES o eotaxina); especialmente teniendo en cuenta el papel inhibidor que dicha función tiene sobre la atracción quimiotáctica de células efectoras importantes en asma, como los linfocitos TH o T-γδ. Por ejemplo, eotaxina es una quimioquina muy potente en la atracción de eosinófilos y células TH2 a lugares de inflamación, cuya función se ve truncada por el corte proteolítico producido por CD26. Lo mismo pasa con SDF-1α/CXCL12 o las

quimioquinas inducidas por IFN-γ (CXCL9-11), que son quimioatrayentes de células TH1 a lugares de inflamación. Estas funciones inhibidoras de CD26 deben sobre todo tenerse en cuenta en pacientes con diabetes tipo-II que están recibiendo o van a recibir gliptinas (inhibidores de la actividad DPP4 de CD26) y que además presentan asma (p.ej., asma no alérgica asociada a obesidad), ya que se podría agravar el estado del paciente.

El estudio expuesto en el capítulo III también mostró una reducción de los niveles de CD126/IL-6Rα en monocitos, neutrófilos y linfocitos TH en pacientes con asma moderado-grave en comparación con los pacientes con asma intermitente-leve. Este resultado es indicativo de un papel de CD126/IL-6Rα en la severidad del asma, posiblemente a través de un mecanismo denominado *trans*-señalización en células que son $CD126^{-}gp130^{+}$. En dicho mecanismo interviene una versión soluble de CD126, que es generada por procesos de procesamiento alternativo del mRNA o mediante proteólisis de la forma anclada a membrana. Finalmente, este estudio también sugiere un defecto funcional (no numérico) en las células T reguladoras de pacientes conforme aumenta la severidad de la enfermedad. Este defecto dependería de una menor producción neta de adenosina, un nucleósido inmunosupresor, como consecuencia de la presencia de mayores niveles de CD26 y menores de CD39 en las células T reguladoras de pacientes con asma moderado-grave comparado con las correspondientes a pacientes con asma intermitente-leve.

Capítulo IV

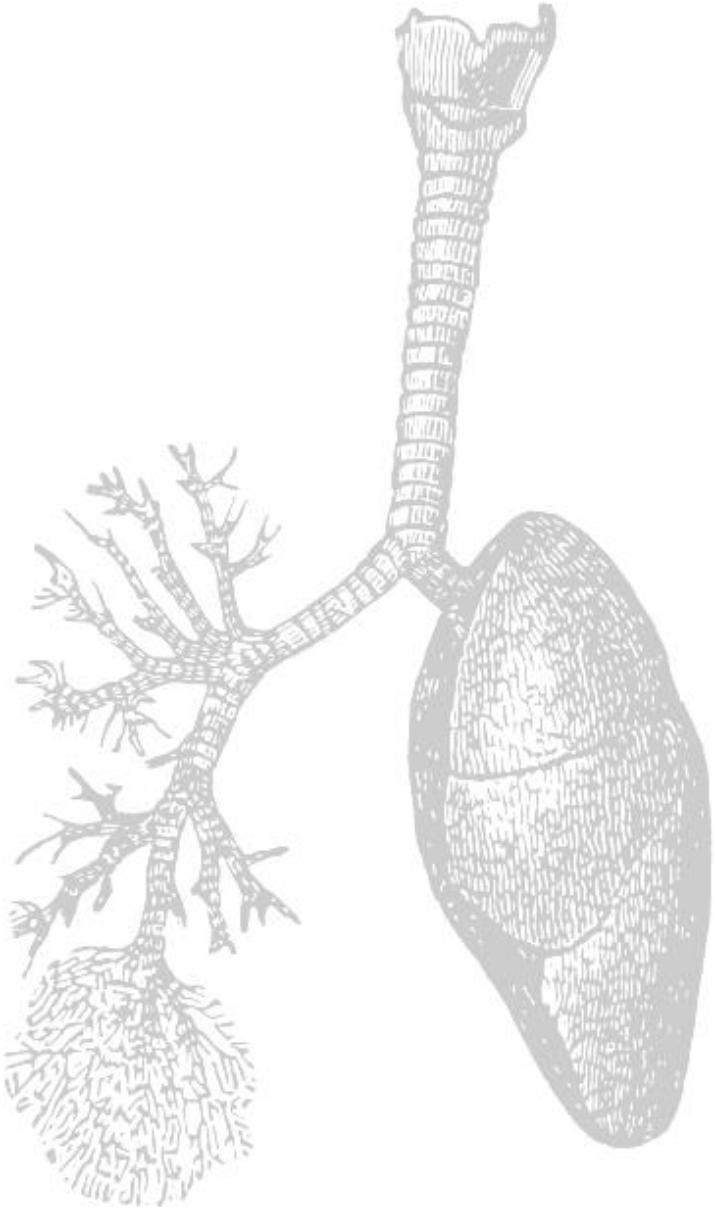
En la última parte de esta tesis se planteó como objetivo la identificación de proteínas de baja abundancia de suero que sirviesen como biomarcadores potenciales de fenotipos o severidad asmática. Para ello se desarrolló un método basado la eliminación de

lipoproteínas del suero y el enriquecimiento de proteínas de baja abundancia mediante el uso de librerías peptídicas aleatorias (CPLLs). Posteriormente, estas muestras enriquecidas en proteínas poco abundantes fueron tripsinizadas, los péptidos fueron etiquetados mediante marcajes isobáricos (iTRAQ) y las proteínas de las distintas muestras (asma alérgica moderado-grave, asma alérgica intermitente-leve, rinitis, asma no alérgica moderado-grave, asma no alérgica intermitente-leve, sanos) fueron identificadas y cuantificadas mediante LC-MS/MS (LTQ-Orbitrap). Para poder tener en cuenta la variabilidad biológica, los sueros de cada uno de los seis grupos muestrales fueron aleatoriamente separados en dos subgrupos y combinados ("pooles biológicos"). A su vez, cada uno de esos "pooles biológicos" fue analizado por triplicado mediante LC-MS/MS. También fue empleado un control interno con el objetivo de normalizar las señales.

En conjunto, el estudio proteómico cuantitativo detectó 217 proteínas séricas, dentro de las cuales 26 mostraban una abundancia diferencial entre grupos. Los estudios funcionales ("gene ontology", GO) han demostrado que estos biomarcadores potenciales desempeñan procesos clave en la patogénesis del asma, tales como activación del complemento, respuesta inmune, respuesta a estímulos bióticos, o respuesta inmune mediada por células B. Los estudios de enriquecimiento (Reactome), por su parte, muestran un enriquecimiento en la vía "Regulación del transporte de factores de crecimiento similares a insulina (IGFs) y captación por proteínas de unión a factores de crecimiento similares a insulina (IGFBPs)" en asma alérgica. A esta vía pertenece IGFALS, un posible biomarcador de asma alérgica (especialmente la moderado-grave) que hemos confirmado ya mediante ELISA. Otras proteínas, en cambio, están pendientes de validación, como por ejemplo HSPG2 o la proteína AMBP, cuya concentración sérica ha sido encontrada aumentada en

pacientes con asma alérgica. Por otro lado, también se han visto cambios en diversas proteínas relacionadas con la activación y regulación de todas las vías del complemento en asma no alérgica. Entre ellas cabe destacar los factores de complemento I (CFI) o H (CFH), así como la proteína MASP1; todas ellas están elevadas en el fenotipo no alérgico, y también pendientes de una posterior validación. Junto con lo anteriormente comentado sobre las células T-γδ, el papel de los factores del complemento habla nuevamente de las implicaciones del sistema inmune innato en el asma no alérgica. Ello asemeja a esta enfermedad a una patología autoinmune y explica, a su vez, su edad más tardía de aparición y la mayor incidencia en mujeres. Por tanto, estos resultados preliminares muestran cambios en un grupo de proteínas séricas que podrían ser útiles para diferenciar diferentes fenotipos asmáticos, servir para pronosticar la severidad de la enfermedad, o ser dianas de futuras terapias dirigidas.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA



INTRODUCTION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. ASTHMA DEFINITION

Asthma is a chronic respiratory disease characterized by reversible expiratory airflow limitation associated with mucous cell hyperplasia, bronchial/airway hyperresponsiveness (AHR), airway remodelling with fibrosis, higher vascular permeability, airway edema and inflammatory cell infiltration into the airway such as $CD4^+$ T cells (TH cells), regulatory T cells (Treg), mast cells, and eosinophils. Asthma symptoms include wheezing, coughing, shortness of breath, and chest tightness [1-4].

2. EPIDEMIOLOGY OF ASTHMA

Nowadays, there are millions of people with asthma over the world, and its prevalence is increasing. Global Burden of Disease Study Collaborators in 2015 has estimated that asthma prevalence was increased by 12.6% from 1990 to 2015, having more than 358 million patients in 2015 [5]. Moreover, ISAAC Phase Three study noticed that 11.7% of 6-7-years age children and 14.1% of 13-14-years age children had asthma [6].

Asthma is more frequent and severe in boys than girls under the age of 18 years. After this age, both prevalence and severity increase significantly in women leading to a shift in the prevalence pattern [7, 8], as studies in both European Union (EU) [9] and United States (US) [8, 10] have shown.

Data from 28 countries of EU reflect that 5 out of 78 million children (0-14 years old) and 16 out of 204 million adults (15-44 years old) had asthma in 2010. From these subjects, there were 167,000 hospital admissions per year of children and 82,000 of adults because of asthma. Furthermore, 40 children and 380 adults (<44 years old)

passed away due to asthma in the EU (Figure 1) [11]. On the other hand, the overall prevalence of this disease in the US increased from 7.3% in 2001 to 8.4% in 2010, with 25.7 million asthmatics: 18.7 million were adults, and 7 million were children [12]. The Centers for Disease Control and Prevention of US (CDC) evidenced more than 1.9 million medical visits and almost 500,000 hospitalizations due to asthma in 2009, more than 17 million work days lost per year, and extremely high economic costs; the US spend an annual amount of 56 million dollars due to asthma [13]. Overall, these data reflect the high relevance of studying asthma for Health Care Systems of different countries all over the globe.

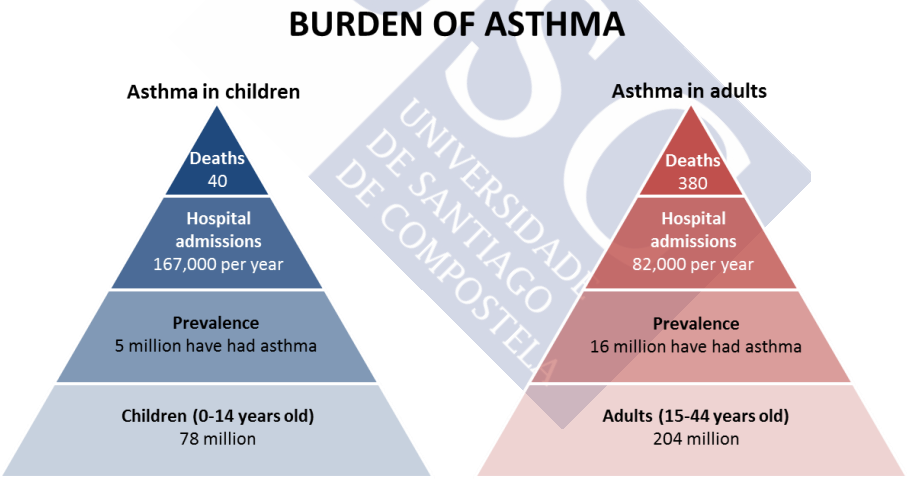


Figure 1. The burden of asthma in the European Union (EU) in 2010. Data from ERS, EUROPEAN LUNG *white book*, https://www.erswhitebook.org/ [Accessed 2019-02-15].

3. RISK FACTORS FOR ASTHMA

3.1. Genetic factors implicated in asthma

Asthma is a complex disease. As such, many genetic and environmental factors confer susceptibility and increased severity to this pathology. In the last years, a huge amount of genetic studies was developed in order to unravel the genetic basis of asthma. Most of them were genome-wide association studies (GWAS) (Figure 2), which have reported a wide variety of genes associated with asthma (e.g., *IL-6R, RAD50-IL-13, DENND1B, HLA-DQ, IL-33, TSLP, SMAD3, PDE4B, IL-1RL1, IL-18R1, IL-2RB, PDE4D, LRRC32, ORMDL3-GSDMB, PDCD4, PDE11A,* or *DPP10*) [14-17]. Many of these genes encode proteins with immunological functions (e.g., the IL-1 cytokine family and its associated receptors, IL-2RB, LRRC32/GARP, IL-6R, IL-33, TSLP, HLA-DQ, or SMAD3) [14-17].

Besides GWAS, other genes such as *ADAM33, HLA-G, PLAUR, PCDH1,* and *NPSR1* were found to be associated with asthma through positional cloning studies [18]. In addition, other single nucleotide polymorphisms (SNP) located in genes with immunological functions have been connected to asthma, with both protective and negative roles. These genes encode proteins such as IL-1β [19, 20], IL-17a [21], IL-23R [22], IL-13 [23], IL-4 [24], or CD14 [25]. Most of these genes have only weak effects on asthma, but the overall evidence available suggests a strong polygenic component for this disease.

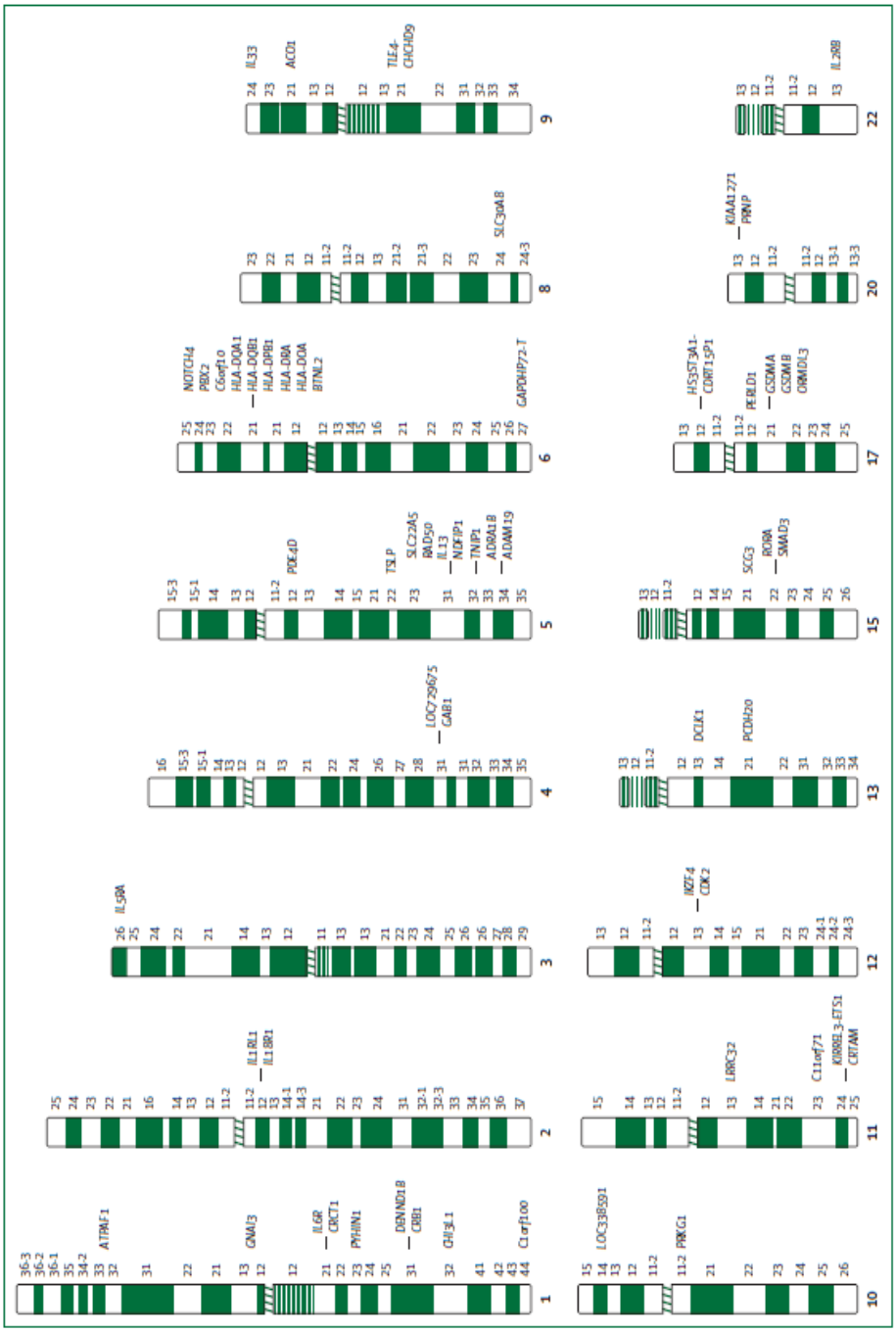


Figure 2. Genes associated to asthma by genome-wide association studies (GWAS). "Reprinted from The Lancet, Vol. 382, Martinez FD, Vercelli D, Asthma, 1360-72, Copyright (2013), with permission from Elsevier."

3.2. Environmental factors implicated in asthma

Besides genetic factors, environmental factors are also involved in the development and severity of asthma. There are a handful of environmental factors found to raise the risk of developing asthma. These factors include damp housing/mould and inhaled allergens (e.g., house dust mite/HDM, pets, pollens), smoke from different sources (e.g., tobacco, industrial combustion/incinerators, fireworks, bonfires), inhaled chemicals derived from domestic or outdoor sources (e.g., air pollution), dietary exposures (e.g, maternal and child diet, breast feeding), medications (e.g., antibiotics, paracetamol), or exposure to allergic triggers linked to vacuuming or air-modification systems (e.g., air conditioning, humidifiers).

However, one of the most important environmental factors implicated in asthma is microbial exposure during childhood (i.e., "hygiene hypothesis") [26, 27]. Some epidemiological studies show that the decreased exposure to environmental microbes in early life is associated with augmented risk to asthma and allergy later in life. Indeed, children growing up on farms with animals displayed a lower risk for asthma than those living in the countryside in the bosom of a family not doing farming [26]. The same happened in the case of babies fed with breast milk as compared to those being formula-fed, [28], or in children exposed to other children at home (large families) compared to smaller family entities [29]. There are also some studies proving the association between the presence of pets in early childhood and the decreased risk of allergen sensitization late in life [30]. Moreover, the protection from asthma development could begin even before birth, as maternal exposure to a farming environment also protects children from asthma [31, 32].

Recently, it has been shown that the bronchial tree is not sterile. Thus, more than two thousand bacterial genomes per cm^2 can

be found in samples from the left upper lobe of the lung [33]. Interestingly, asthma susceptibility has also been associated with changes in the colonization of lower airways by microbes. Indeed, some works have shown that asthmatics displayed a higher predominance of Proteobacteria (a phylum of gram-negative bacteria) than subjects without asthma, where Bacteriodetes (another phylum of gram-negative bacteria) are more frequent [1, 33, 34].

There is a disagreement about the nature of the underlying mechanism that explains the higher relative abundance of phylum Proteobacteria in asthmatics, but it is broadly accepted that the immune system is implicated. Microbes produce substances such as LPS, flagellin, or lipoteichoic acid. These exogenous and highly conserved molecules are collectively called pathogen-associated molecular patterns (PAMPs). PAMPs are recognized by pattern recognition receptors (PRRs), which are membrane-bound (e.g., Toll-like receptors/TLRs) or cytoplasmic (e.g., NOD-like receptors, RIG-I-like receptors) receptors mainly expressed by innate immune cells (e.g., monocytes, macrophages, dendritic cells, neutrophils, eosinophils, epithelial cells) [35]. PRRs also identify damage-associated molecular patterns (DAMPs) or alarmins, another subset of molecules released this time by host cells during inflammation or when they are damaged or dead. Activation of PRRs leads to innate cells to release soluble factors (e.g., cytokines, chemokines) and express co-stimulatory molecules, which in turn unchain the adaptive immune response.

CD14 is a co-receptor that allows the detection of bacterial LPS along with LPS binding protein (LBP), TLR4 and MD2. Triggering of CD14/TLR4 leads to the release of IL-12/IL-18 by monocyte/macrophages, two cytokines that favour the TH1 differentiation, in detriment of the TH2-mediated adaptive responses [36-38]. This shift in the TH1-TH2 axis was the classical explanation

of the hygiene hypothesis. However, certain helminthic infections (e.g., Schistosomiasis) trigger TH2-type immune responses and prevent asthma [39]. Moreover, TH1 cells could even increase the pathogenicity of TH2 cells, which makes it difficult to explain the hygiene effect through a simple TH1-TH2 cell counterbalance. Nowadays, it is believed that there is a loss of Treg cells in subjects living in "hygiene environments", which turns into increased TH2 cell immunity and higher susceptibility to develop asthma/allergy [39, 40]. CD14/TLR4 is at the crossroads where naïve lymphocytes have to make the decision between TH1 and TH2 effector responses so that polymorphisms in these genes could impact seriously to asthma development or severity [41].

4. INNATE IMMUNITY IN ASTHMA

The innate immunity is the first line of defence against pathogens. This is an immediate and relatively non-specific response in which different cellular populations and soluble factors are implicated. Between them, we are going to put special emphasis on the role of airway epithelial cells (AECs), innate lymphoid cells (ILCs), γδ-T lymphocytes, and monocytes in asthma.

4.1. Airway epithelial cells (AECs) and innate lymphoid cells (ILCs) in asthma.

The first cellular component implicated in the pathogenesis of asthma is the airway epithelial barrier. AECs are continuously exposed to different pollutants, particulate matter, allergens and pathogens. PAMPs from microorganisms (e.g., LPS) and enzymes derived from pathogens or allergens (e.g., dust mite allergen Der p1) interact with

AECs through PRRs (e.g., TLRs) and protease-activated receptors, respectively. This interaction unleashes the potential of epithelial cells to produce a set of cytokines and chemokines implicated in asthma pathogenesis [42, 43], whose best examples are TSLP (thymic stromal lymphopoietin), IL-25 and IL-33. PRRs activation by DAMPs or protease-activated receptors cleavage by endogenous proteinases (e.g., thrombin, plasmin, kallikreins, matrix metalloproteinases, elastases) play a complementary role in AECs activation.

As we state above, the genes encoding both IL-33 and its receptor (ST2/IL-1R1L) are asthma susceptibility genes [17, 44, 45]. IL-33 is a member of the IL-1 superfamily of cytokines, but it is also considered an alarmin or DAMP; i.e. an intracellular molecule released in response to cell injury which is discharged to extracellular space after cell damage (necrosis or trauma) [46]. IL-33 is constitutively expressed by alveolar type-II pneumocytes, and its expression is enhanced upon airway inflammation [47, 48], particularly in allergic asthma (AA) [49, 50]. Additionally, macrophages are both the source and target of this cytokine [51]. IL-33 is involved in the response to both helminths and viral infection [52, 53], through its binding to its receptor (ST2/IL-1R1L). ST2 is expressed by a myriad of immune cells, including mast cells, basophils, eosinophils, dendritic cells, and ILCs [51]. Binding of IL-33 to transmembrane ST2 triggers different signalling routes. For example, IL-33 induces the activation of the NF-κB pathway in human mast cells stimulating the release of different cytokines (e.g., GM-CSF, TNF-α, IL-1β, IL-6, IL-8, or IL-13) and chemokines (e.g., CXCL8, CCL1) [54].

Similarly, TSLP is a cytokine encoded by a gene associated with a higher risk of AA [17]. TSLP (IL-2 family of cytokines), as well as IL-25 (IL-17 family of cytokines; IL-17E), are released by AECs in response to allergens (e.g., HDM) that possess protease

activity and are capable of activating protease-activated receptors like PAR2 [55]. Both cytokines display increased levels in asthmatic patients [56, 57]. TSLP is critical for the maintenance of allergic airway inflammation through inducing TH2-type cytokines production, boosting TH9 cells differentiation, inhibiting Treg function, and activating basophils/mast cells [43, 58-60]. Moreover, rhinovirus infection boost levels of TSLP in a mouse model of AA, underlying the potential role of this cytokine in asthma exacerbation [61]. IL-25, for its part, is mainly associated with eosinophils activation. However, it is worth highlighting that the heterodimeric receptor of this cytokine (IL-17RA/IL-17RB) is expressed in many immune cell types such as antigen presenting cells (APCs), invariant natural killer T cells (iNKT), AECs, and ILC2/nuocites [43]. Altogether, IL-25, TSLP, and particularly IL-33 are responsible for the early allergic response in asthma, driving the activation of ILC2 cells [43, 52, 55].

Upon activation by IL-25, TSLP and IL-33, these ILC2 cells become one of the major producers of IL-5 and IL-13 [62], two cytokines with a pivotal role in the induction and maintenance of AA [63]. Recently, several works in both mice and the human system gave support to an important role of this subset in asthma. These studies have shown that activated ILC2 cells not only produce IL-5 and IL-13, but also IL-3, IL-4, IL-6, IL-8, or GM-CSF [reviewed in 62]. Moreover, this function of ILC2 cells is enabled by human mast cells via prostaglandin D2 [64-66]. Therefore, ILC2 lymphocytes might be a bridge between the innate and the adaptive immune system.

Apart from ILC2 cells, other innate immune cells are becoming more relevant for asthma pathogenesis, especially in the light of asthma heterogeneity and the presence of different disease phenotypes, such as non-allergic asthma (NAA). For example, alternative ILCs subsets such as natural killer (NK) cells, ILC1, or

ILC3 cells could be relevant to asthma. Both NK and ILC1 cells have been linked to the pathogenesis of this disorder [67, 68], but more interesting is the role of IL-17-producing ILC3 cells in obesity-related asthma [69]. However, more studies are necessary to ascertain the possible roles of ILC1 and ILC3 in this disease.

4.2. Monocytes in asthma pathogenesis

Monocytes represent about 10% of circulating leukocytes and their half-life is around 3 days in human blood [70]. These cells are "circulating macrophages" that can turn into tissular inflammatory cells also capable of generating IL-33. This is illustrated in mouse models, where circulating monocytes are recruited to the lung upon HDM-induction to become active producers of IL-33 [71].

Human blood monocytes have been subdivided into three discrete subpopulations based on the surface expression of CD14 and CD16: "classical" $CD14^{++}CD16^{-}$ cells, representing ~ 90% of circulating monocytes; "non-classical" $CD14^{+}CD16^{++}$ monocytes; and "intermediate" $CD14^{++}CD16^{+}$ monocytes [72-74]. However, the last subset is considered a transitional subpopulation more than a true subtype [75]. There is an increase in maturity from classical monocytes, which display an anti-apoptotic and proliferative state, to non-classical subsets, having an anti-proliferative and apoptotic state [75].

$CD14^{++}CD16^{-}$ classical monocytes respond to LPS via TLR2 and TLR4, producing reactive oxygen species and a set of cytokines and chemokines (e.g., IL-6, IL-8 or CCL-3) [76]. Mukherjee *et al.* have shown that these cells display high levels of CD36 and CD163 (scavenger receptors), which evidence the phagocytic nature of this subset [76]. Additionally, intermediate monocytes and non-classical

monocytes are characterized by a high co-stimulatory ability, as they express CD80 and CD86 [77]. Non-classical monocytes respond to nucleic acids (damaged cells) and viruses via TLR7 and TLR8, secreting TNF-α and IL-1β [76] and they are also increased during sepsis [77].

All types of monocytes are able to differentiate into monocyte-derived macrophages or dendritic cells. Indeed, non-classical monocytes display high levels of CD11a and CTSL1, which allow them to infiltrate and differentiate in M2-type macrophages [78]. Macrophage-derived monocytes have both pro-inflammatory and anti-inflammatory roles. M1 macrophages or classically-activated macrophages are mainly pro-inflammatory cells which release cytokines and chemokines such as IL-1β, IFN-γ, IL-8, IP-10, IL-23, TNF-α, or RANTES after pathogen infection. These cytokines are potent inducers of TH1 and TH17-driven inflammation [79], which is important in non-allergic lung inflammation. Moreover, higher levels of M1-type macrophages have been described in less severe AA compared to severe allergic disease, highlighting their possible anti-allergic role [80]. On the other hand, M2 macrophages consist of four major groups of cells: M2a cells, which secrete IL-4 and IL-13; M2b cells, which secrete pro-inflammatory (IL-6, IL-1β, and TNF) and anti-inflammatory (IL-10 and TGF-β) cytokines; M2c cells, mainly IL-10 producers and Treg cell-inducers; and M2d cells (Figure 3) [80]. M2a-type macrophages have been related to AA in several works. They are IL-13 producers, a cytokine implicated in mucus production, tissue remodelling, and fibrosis that drives the characteristic TH2-type inflammation in AA [81]. These cells are also IL-33 producers (see above) [82], and they express a wide variety of chemokines (CCL-17, CCL-18, CCL-22, and CCL-24), which attract eosinophils and TH2 cells to the inflamed tissue [80, 83].

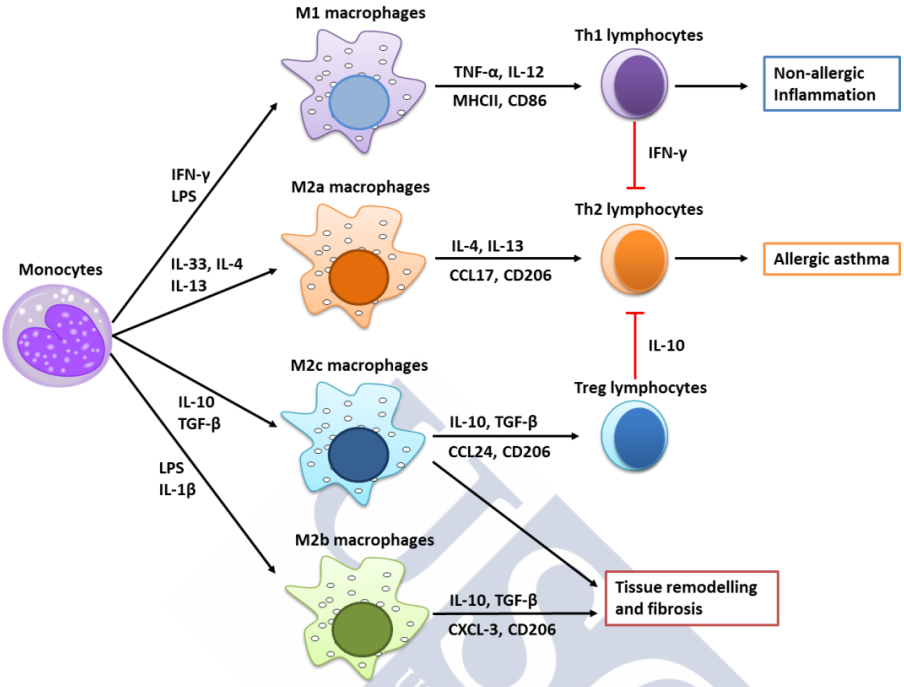


Figure 3. Schematic representation of subtypes of monocyte-derived macrophages during inflammation. In the presence of a different stimulus, monocytes can be differentiated in M2a, M2b, M2c, and M2d cells, which have either proinflammatory or anti-inflammatory roles in asthma through the secretion of different cytokines and chemokines.

4.2.1. CD14 and asthma

As commented above, some pathogens display several types of PAMPs, such as LPS in gram-negative bacteria or lipoteichoic acid in gram-positive. These molecules are recognized by PRRs, which are localized on innate immune cells such as monocytes or macrophages. [35]. There are different types of PRRs but the most studied are the TLRs. There are 10 types of TLRs (TLR1 to 10) in humans, each of them capable of recognizing different pathogen components. For

example, TLR2 recognizes microbial lipopeptides, whereas TLR3 recognizes dsRNA from the viral genome [84].

TLR4 forms part of the receptor for bacterial LPS and is mainly expressed by monocytes, macrophages and dendritic cells [84, 85]. LBP captures LPS monomers and transfers them to CD14, a co-receptor and a susceptibility gene for asthma and allergy [86, 87]. LPS is then delivered to the complex MD2/TLR4, which form a hexamer complex (two LPS/TLR4/MD2). Although there are MyD88-independent TLR-4 signalling pathways, the main route begins with the sequential recruitment of two adaptor proteins: TIRAP (toll-interleukin 1 receptor domain containing adaptor protein) and MyD88 (Myeloid differentiation primary response 88) [85, 88, 89]. Then, IRAK kinases (IRAK4 and IRAK1 or 2) are recruited and phosphorylated, allowing the binding of the ubiquitin protein ligase TRAF6 and the subsequent recruitment of TAK1 and IKK complex. Finally, both MAP kinases and early NF-κB pathways are activated (Figure 4) [90-92].

The hexamer complex $(TLR4\text{-}MD2\text{-}LPS)_2$ can be internalized via endosomes, which induces a signal transduction pathway dependent on the recruitment of TRIF/TRAM. This pathway ends up with the activation of NF-κB and IRF3 (Figure 4) [91]. Finally, all these signal transduction pathways promote the expression of genes encoding TH1 and TH17-type cytokines and chemokines, such as type-I Interferon, IL-12, IL-1, IL-6, TNF-α, IL-8, or RANTES [90-92]. This set of cytokines drives the induction of a TH1 and TH17-type inflammation, in detriment of a TH2-type polarization. Indeed, MyD88-deficient mice had a compromised activation of antigen-specific TH1 cells, but not TH2 [93].

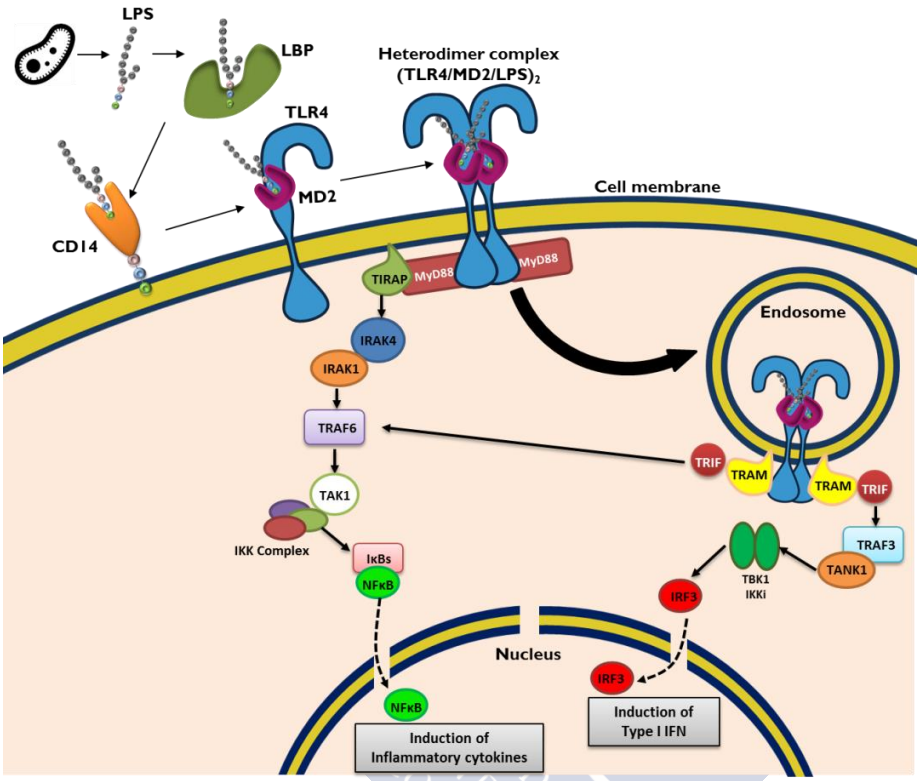


Figure 4. TLR4-CD14 signalling pathway. The formation of the heterodimer complex $(TLR4/MD2/LPS)_2$ triggers two sequential signalling pathways. The fist of them starts with the recruitment of MyD88 and TIRAP and finish with the early-phase activation of NFκB, its translocation to the nucleus and the induction of inflammatory cytokines such as IL-12 or IL18. Secondly, the TLR4/MD2/LPS-hexamer complex can be internalized into endosomes, where through the recruitment of TRAM and TRIF, leads to the activation of late-phase NFκB, and thus, the induction of type I IFN.

In addition, membrane-anchored CD14 (mCD14) facilitates the response of TLRs (TLR2, TLR3, TLR4) to other PAMPs (e.g., viral nucleic acids and components from Gram-positive and negative

bacteria) or DAMPs (e.g., endogenous molecules like ceramide, urate crystals, or amyloid peptides) [94]. Hence, changes in the abundance or defects in the function of CD14 (e.g., genetic factors like SNPs) could be relevant for the development of allergen-specific TH2 cells and AA pathogenesis.

4.2.1.1. Soluble CD14 (sCD14) and asthma

mCD14 is attached to the cell surface by means of a glycophosphatidylinositol anchorage, but this protein is also detectable as a soluble form (sCD14) in plasma. sCD14 is released by a controlled mechanism (e.g., LPS, CpG, TNF-α) involving either enzymatic cleavage (e.g., elastase, phosphatidylinositol phospholipase C) or direct secretion by exocytosis [94-96]. sCD14 can display opposite functions. On the one hand, sCD14 can mimic the function of the mCD14 by transferring LPS from LBP to TLR4; therefore sCD14 potentiates the induction of type-1 or type-17 proinflammatory cytokines by endotoxin [96]. In addition, sCD14 qualifies CD14-negative cells (e.g., endothelial or epithelial cells) to respond to LPS [97]. Reversely, sCD14 can act as a scavenger receptor transferring LPS to plasma lipoproteins and preventing the activation of TLR4-NF-κB pathway [98].

sCD14 is released from monocytes upon stimulation with TLR ligands (e.g., LPS, CpG, flagellin) or pro-inflammatory cytokines (e.g., IL-6 or IL-1β) [38]. Moreover, plasma sCD14 also behaves as a positive acute phase protein (APP+). This means that sCD14 levels increase during infection, inflammation or upon exposure of hepatocytes to IL-6 [94]. Hence, sCD14 can be considered a non-specific monocyte activation marker [38]. sCD14 features two serum isoforms, with 49 and 55 kDa, and is also cleaved in plasma by proteases like cathepsin D to yield 13 kDa N-terminal

fragments named sCD14 subtype (sCD14-ST) or presepsin, a sepsis marker like CRP or procalcitonin [99].

sCD14 has been widely studied in asthma. As expected for an acute phase protein, children with asthma exacerbations show higher sCD14 levels than in the recovery phase [100, 101]. In the same line, adult patients with asthma displayed augmented levels of sCD14 compared to healthy controls [102], and the amount of sCD14 in bronchoalveolar lavage fluid increased after allergen exposure [103]. In contrast, some works have shown unchanged sCD14 levels in asthmatic children compared to healthy subjects [95, 101]. Additionally, releasing of mCD14 is decreased by TH2 cytokines (IL-4 and IL-13) [104, 105]. This could explain the negative correlation between sCD14 levels and the production of IgE, IL-4, or asthma severity [106, 107]. Hence, these different results could rely on the presence of confounding factors such as SNPs.

4.2.1.2. CD14 (-159 C/T) SNP and asthma

SNPs in the *CD14* gene are one of the modulation factors for CD14 expression and function so that they are risk factors for asthma development or severity [108]. In 1999, Baldini *et al*. described the first SNP of the *CD14* gene: rs2569190 [106]. This SNP is localized in the 5q31.1 chromosome region (*CD14* promoter), which has been associated with asthma and atopy in GWAS [86]. Since those landmark studies, other sets of *CD14* SNPs have been described, but most of them non-related to asthma [101].

rs2569190 is described as a C-to-T transition in the position -159, upstream of the transcription start site of the *CD14* gene [106]. Several works, including the work of Baldini *et al.*, showed an increment of asthma susceptibility and lower sCD14 levels in subjects carrying the C allele and the CC genotype in the *CD14* C-159T SNP,

maybe due to a lower transcriptional activity [36, 109, 110]. Moreover, T allele seems to be protective and associated with low IgE levels and less atopy and asthma [101, 111, 112].

In contrast, other studies have shown no association between this *CD14* C-159T SNP and asthma or atopy, or even a protective role for the C allele [41, 87, 108, 113-115]. According to different authors, there are several factors that could explain these different outcomes: racial/ethnic origin (Caucasian, African or Asiatic population), age of the study population (early childhood vs. adulthood asthma), population stratification, statistical power, linkage disequilibrium/haplotypes, gene-gene interactions, or gene-environment interplay (e.g., levels of endotoxin in dust and degree of CpG island methylation) [41, 87, 101, 108, 111-118]. In addition, the majority of these studies have focused on sCD14 and almost none have measured mCD14 or both variables at the same time. Therefore, it is necessary to perform new studies that address the measurement of both mCD14 and sCD14 parameters with high sample size and a well-defined set of patients (according to age or ethnicity).

4.3. The role of γδ-T lymphocytes in asthma pathogenesis

γδ-T cells account for the 5-10% of human peripheral blood lymphocytes [119], but they are more abundant in mucosal and epithelial surfaces, within a group of cells named intraepithelial lymphocytes. These lymphocytes are at the frontier between innate and adaptive cells. Thus, γδ-T cells allow rapid responses to different challenges, but they also rearrange the T-cell receptor (TCR) genes and can develop a long-lived memory phenotype [120]. γδ-T lymphocytes are typically subdivided into two major subpopulations based on the expression of the variable TCR-δ region: $V\delta2^-$ and $V\delta2^+$.

Vδ2$^-$ cells constitute a heterogeneous subset (mainly Vδ1$^+$, and less likely Vδ3$^+$ or Vδ5$^+$) that can be found in mucosal tissues, such as gut or lung [121, 122]. These resident cells are mostly CD8$^+$ and express a γδ-TCR with broad reactivity, which is a little bit more restricted during adulthood after different microbial encounters. This TCR works as a PRR to respond to stress signals from epithelial cells (e.g., heat shock proteins, annexin A2) [123]. Besides, Vδ2$^-$ T cells have both IFN-γ-secretion and cytotoxic capacities; i.e., they show a TH1/cytotoxic T cell (TC)1-like phenotype [124].

On the other hand, up to 90% of peripheral blood γδ-T cells express Vδ2 TCR, normally in combination with the Vγ9 region to create a semi-invariant receptor (natural or innate-like cells). Vδ2$^+$ T lymphocytes respond to molecules derived from invading microbes such as prenyl pyrophosphate metabolites (e.g., (E)-4-hydroxy-3-methyl-but2-enyl pyrophosphate), which are presented by butyrophilins [125]. These unconventional T cells can be recruited from blood to inflamed tissues [125], and display phagocytic, professional antigen-presenting, and cross-presentation capacities [126, 127]. Vδ2$^+$ T cells are responsible for the response against viruses and other intracellular pathogens, although their responses are non-restricted by major histocompatibility complex (MHC) [121, 122, 128, 129]. Peripheral blood γδ-T cells could be also differentiated according to which type of cytokine they produce after antigen stimulation: i.e., IL-4, IFN-γ, or IL-17. Therefore, circulating γδ-T cells mirror the adaptive T helper subsets (TH1, TH2 and TH17) [130-132]. Indeed, asthmatic patients display decreased levels of IFN-γ-producing cells and enhanced numbers of IL-4-secreting γδ-T lymphocytes compared to healthy subjects [133]. Moreover, γδ-T lymphocytes have been described as early producers of IL-17 [134].

Similar to it happens in the adaptive immunity, these innate lymphocytes (both Vδ2$^-$ and Vδ2$^+$) can be subdivided into several

subpopulations according to their naïve-memory status: naïve T cells (T_N; $CD45RA^+CD27^+$; lymph nodes homing), central-memory T cells (T_{CM}; $CD45RA^-CD27^+$; lymph nodes homing), effector-memory T cells (T_{EM}; $CD45RA^-CD27^-$; peripheral blood) and terminally-differentiated effector T cells (T_{EMRA}; $CD45RA^+CD27^-$; sites of inflammation) [119, 135, 136]. Most of the peripheral blood (Vγ9/Vδ2) γδ-T cells are T_{CM} or T_{EM} cells. In contrast, T_{EMRA} lymphocytes ($CD27^-CD45RA^+$) are almost absent in circulation (less than 7% of peripheral blood γδ-T cells), whereas they are abundant on inflammatory sites [119, 135, 136]. This latter subset has no expression of CCR7 and CD62L, displays high levels of chemokine receptors (CCR5 and CXCR3) and cytotoxic molecules (perforin and granulysin), and is not able to proliferate [135].

γδ-T cells have been widely studied in tumours and autoimmune diseases, tuberculosis, malaria, and other viral and bacterial infections [reviewed in 137]. However, only a few studies have been performed in asthma, most of them a long time ago. From studies in mice, Tamura-Yamashita *et al.* have observed that γδ-T lymphocytes are critical for late asthmatic responses [138]. In the human system, some works have reported decreased numbers of γδ-T lymphocytes in peripheral blood from allergic asthmatics compared to healthy controls [119, 133, 139-141]. In contrast, others have found an increased proportion of γδ-T cells in bronchoalveolar lavage fluid (BALF) or induced sputum (IS) from asthmatics vs. healthy individuals [142, 143]; these expanded γδ-T cells in BALF primarily belong to the $V\delta1^+$ subset. In contrast, other works with BALF [144] or bronchial biopsies (BB) [145] found no difference in the number of γδ-T cells in allergic asthmatics. However, despite all the knowledge accumulated over the years, almost no data exist regarding the possible implications of this subset in different asthma phenotypes

(e.g., AA vs. NAA) or the abundance of different memory γδ-T subsets in this complex disease.

5. ADAPTIVE IMMUNE SYSTEM IN ASTHMA

Contrary to the innate immune system, the adaptive or acquired immunity is highly antigen-specific and adaptable. The specificity and diversity of adaptive cells come from the existence of genetic recombination (V-D-J recombination) of antigen receptor genes (TCR and B cell receptor/BCR), as well as somatic hypermutation (BCR). Moreover, this antigen specificity is inherited by all progeny of that cell by a process called clonal selection. After the first contact of antigen with one of these pre-existing antigen-specific clones, adaptive cells are activated, proliferate, and generate a huge number of cells with the same antigen specificity; this process is called clonal expansion. Furthermore, adaptive immunity is also characterized by the generation of long-lived memory cells, which are responsible for the secondary immune response against the same antigen, a faster and quantitatively more intense immune response.

Lymphocytes are in charge of adaptive immunity. These cells are subdivided into B and T cells. B lymphocytes secrete antibodies capable of recognizing and neutralizing microbes or toxins by inducing the activation of the complement system or pathogen-phagocytosis. T lymphocytes consist of different subsets that can be segregated according to their phenotype and specialized function [146]:

- **$CD4^+$ or TH cells.** Upon MHC-II-dependent antigenic peptide presentation by APCs, T_N cells initiate the activation programme, proliferate, and differentiate into different TH subsets according to the array of cytokines present in the

extracellular milieu. TH lymphocyte subsets (effector T cells/Teff or Treg cells) release cytokines that either activate or inhibit innate or adaptive leukocytes (target cells). Teff lymphocytes can be segregated in three major subpopulations according to the expression of transcription factors, surface receptors, and cytokines. These specialized Teff subsets have been named TH1, TH2, and TH17 lymphocytes. TH1 cells (T-bet+, IFN-γ^+) are highly effective in the defence against intracellular pathogens (e.g., bacteria, viruses, protozoa), and has been related to autoimmunity diseases. TH2 cells (GATA3$^+$, IL-4$^+$, IL-5$^+$, IL-9$^+$, IL-13$^+$) have been linked to allergic diseases and the protection against helminths and venoms. Finally, TH17 cells (RORγT$^+$, IL-17$^+$) have been related to autoimmunity (as TH1 cells) but also in the defence against other extracellular pathogens, such as bacteria or fungi.

- **CD8$^+$ or TC cells.** They recognize antigenic peptides presented by MHC class I molecules, which induces the differentiation of naïve CD8$^+$ T lymphocytes into effector TC cells. The main function of these TC cells is to kill infected cells by inducing cytotoxicity. However, in an analogous way to TH cells, TC effector subsets can produce cytokines and be differentiated into TC1, TC2 or TC17 cells.

All of these lymphocyte subsets have been implicated in asthma immunopathology. For example, B cells are well-known cell-mediators of AA pathogenesis by means of the production of antigen-specific IgE [147, 148]. Studies on the role of CD8$^+$ T cells in asthma have received relatively little attention and yielded contradictory results. For example, transcriptome analysis of circulating CD8$^+$ T cells in patients with severe asthma shows an activated status of these cells [149]. Moreover, it has been reported that certain CD8$^+$ T cell subpopulations (TC2) produce type 2 cytokines (IL-4 and IL-5) in the

airways [150, 151] and show a strong association with asthma disease severity [151]. Furthermore, it has also been detected in peripheral blood of asthmatics the expansion of a subset of circulating effector memory IL-6Rα/CD126^{high} CD8^{+} T cells that produce TH2 cytokines [152]. However, most of the studies in asthma have been focused on CD4^{+} T cells, especially TH2 cells and AA.

5.1. The role of TH2 cells in asthma

TH2 cells have been considered since a long time ago major players in AA. GATA3 and STAT6 are key transcriptions factors for the development of TH2 cells, and their effector type-2 cytokines (IL-4, IL-5, IL-9, IL-13, and IL-25) are important for humoral immune responses, defence against extracellular parasites (e.g., helminths), and AA pathogenesis.

IL-4 is cytokine produced, amongst others, by basophils and ILC2 cells, essential for proliferation, survival, and differentiation of T_N cells to TH2 lymphocytes [153]. This interleukin induces B-cell proliferation, B-cell class switching to IgE synthesis, and plasma cell differentiation [154]. Therefore, IL-4 plays an important role in the early allergic phase. This cytokine acts via the IL-4Rα, which can be in complex with the common cytokine receptor γ-chain (IL-2Rγc; type-I IL-4 receptor) or with the IL-13Rα1 (type-II IL-4 receptor [154, 155]. The last one is also a receptor for IL-13, which shares some functions with IL-4.

However, IL-13 binds to another receptor with higher affinity, IL-13Rα2 [155]. Compared to IL-4, IL-13 is a late-acting (effector phase) pro-inflammatory cytokine that induces mucus production, AHR, and lung fibrosis. Indeed, blockade of IL-13 prevents and reverses mucus secretion [156]. IL-13 is responsible for the transition

of Clara cells (also known as bronchiolar exocrine cells) to globet cells (metaplasia), whose main role is to secrete a group of glycoproteins called mucins capable of attracting water molecules [157, 158]. IL-13 also leads to goblet cell hyperplasia (cell proliferation) and induces the expression of several genes related to mucus hypersecretion: MUC5AC, MAPK13, GABAA-R, or TMEM16A [159-162]. When the amount and speed involved in mucus production are excessive, this generates a thick secretion in the airways that cannot be easily removed by cilia or cough, driving to an airflow obstruction (together with AHR) characteristic of asthma. Different studies in mice have reported that IL-13 could affect directly to AECs or/and smooth muscle cells to produce AHR [163, 164]. Moreover, the induction of MUC5AC or certain contractile mediators such as nitric oxide by IL-13 appears to contribute to the development of AHR in asthmatics [165, 166]. Another function of IL-13 is the stimulation of myofibroblasts proliferation and their recruitment to airway tissues, leading sub-epithelial fibrosis [167].

IL-5, for its part, promotes the maturation, differentiation, and survival of eosinophils. Moreover, this cytokine act as a chemokine for this leukocyte subset and enhances its effector function [168]. Indeed, the overexpression of IL-5 in mice induces the expansion of eosinophils both in bone marrow and peripheral blood [168, 169], whereas the absence of IL-5Rα abolishes the eosinophilia and AHR compared to wild-type (WT) mice [168, 170]. In addition, some studies have noticed reduced eosinophils influx to the lung epithelium and less AHR after the use of anti-IL-5 antibodies [168]. Recruitment of eosinophils and TH2 cells to lung epithelium drives the late-phase allergic response in asthma due to the release of effector mediators such as the major basic protein, eosinophil-derived neurotoxin or peroxidase [171, 172].

5.2. Participation of other effector TH subsets in asthma: TH1 and TH17 lymphocytes

TH2 cells have been the most studied $CD4^+$ T subset in asthma over time, particularly in the allergic phenotype. However, other TH subpopulations display important roles in the pathology of this complex disease, such as TH1 or TH17 cells. As we have stated above (see section 3.2), both TH2 and TH1 cells were believed to have opposite roles promoting or inhibiting asthma, respectively.

TH1 cells differentiate from T_N lymphocytes in response to IL-12 (an APC-derived cytokine). This subset is characterized by the expression of the transcription factors T-bet and STAT4, and the secretion of IFN-γ and other effector cytokines such as IL-2, TNF, or LT-α [146]. TH1 cells are responsible for the protection against viruses and other intracellular pathogens [146]. The first studies made on the role of TH1 lymphocytes in asthma described this subset as protective. For example, children living on farms and exposed to high endotoxin (LPS) levels were found to be less predisposed to asthma or allergies. Indeed, LPS exposure by means of CD14-TLR4 drives the differentiation towards TH1- instead of TH2-type responses, as stated above. Furthermore, IL-12 prevents the production of IL-4 and inhibits the development of TH2 cells, downmodulating AA inflammation [37]. However, a potential proinflammatory role for TH1 cells in asthma is now emerging. Indeed, some viruses like human rhinoviruses induce the secretion of IL-33 and TSLP, which also trigger TH1 responses and are responsible for acute asthma attacks [173]. Additionally, adults with treatment-refractory severe asthma have increased levels of TH1 cells in their lungs, highlighting the possible role of this subset on the severity of this disorder [174].

TH17 keep mucosal barriers fit and facilitate pathogen clearance at mucosal surfaces [175]. In the presence of IL-1β, IL-6, and IL-23, T_N cells differentiate into TH17 lymphocytes; TGF-β also appears to play a role in TH17 differentiation [146]. Through its interaction with IL-6R (gp130 + IL-6Rα/CD126), IL-6 induces the up-regulation of retinoic acid receptor-related orphan receptor gamma (ROR-γt). ROR-γt and ROR-αt are two transcription factors that drive the development of TH17 cells [176-178]. Moreover, IL-6 can also interact with soluble IL-6Rα, activating $CD126^{-}gp130^{+}$ cells [179, 180]. Indeed, this process, also known as *trans*-signalling, is important for the maintenance of several inflammatory diseases such as asthma [181-184]. Apart from ROR-γt, TH17 lymphocytes can be distinguished by the expression of another transcription factor (STAT3) and the surface receptors CCR6 and IL23R. TH17 cells carry out many of their activities by releasing several effector cytokines from the IL-17- (IL-17A, IL-17F), the IL-10- (IL-22), and the IL-12-family (IL-23) [185, 186].

Some IL-17 polymorphisms have been associated with higher asthma risk, like for instance IL-17A rs4711998(A/G), IL-17F rs1889570(C/T), and IL-17A rs3819024(A/G) [187]. IL-17 has been related to airway diseases due to it is directly connected with the production of pro-inflammatory (e.g., IL-8) and pro-fibrotic cytokines (e.g., IL-6, IL-11), as well as matrix metalloproteases from inflammatory cells (e.g., eosinophils), AECs, and fibroblasts [188-190]. These pro-inflammatory cytokines promote lung inflammation by means of the recruitment of macrophages and neutrophils [191, 192]. Additionally, Chang *et al.* described that IL17-A, IL-17F, and IL-22 can induce airway smooth muscle cells proliferation and therefore promote airway remodelling [193].

Several works have been suggested a possible role of TH17 cells in asthma pathogenesis [194]. For example, the absence of either IL-17A or IL-17F is enough to suppress AA induced by HDM in mice [195]. Both IL-17 and neutrophils levels are augmented in asthmatic subjects with higher body mass index [196], highlighting the role of IL-17 in worse control in obese adults with asthma [69]. On the other hand, Zao *et al*. reported an increase in both the percentage of TH17 cells and the production of TH17-derived cytokines (IL-17 and IL-25) in peripheral blood from patients with AA and in activated peripheral blood mononuclear cells (PBMCs) from allergic asthmatics compared to healthy controls. Moreover, they also evidenced higher levels of both IL-17 and IL-22 cytokines as disease severity increases (i.e. Controls < Mild asthma < Moderate asthma < Severe asthma), highlighting the possible implication of this subset in asthma severity [197, 198]. Furthermore, TH17 cells have also been implicated in steroid-resistant asthma [199]. Glucocorticoids have been able to induce BIM (Bcl-2 interacting mediator), a pro-apoptotic protein which antagonizes Bcl-2. The predominance of BIM over Bcl-2 induces the activation of BAX, and the subsequent activation of caspases, leading to apoptosis [200, 201]. However, contrary to TH1 lymphocytes, TH17 cells express high levels of Bcl-2, which makes this subset insensitive to glucocorticoids. Indeed, short hairpin RNA (shRNA) mediated silencing of *Bcl-2* gene in TH17 cells makes them sensitive to glucocorticoid-induced apoptosis [202]. Furthermore, TH17 cells were augmented in the lungs of a murine model of severe asthma [202].

5.3. Other TH subsets implicated in asthma pathogenesis: TH9, TH22 and TfH lymphocytes

At least 3 more TH cell subsets have implications in asthma pathogenesis: TH9, TH22 and follicular helper T (TfH) cells. TH9 lymphocytes differentiate from T_N cells when IL-4 and TGF-β are present in the media. Both STAT6 and PU.1 are transcription factors important for the development of TH9 cells; they also express GATA3, but to a lesser extent than TH2 cells. Apart from IL-10 and IL-21, TH9 cells are a major source of IL-9, a cytokine which gives them their name. IL-9 has several relevant effects in AA pathogenesis [203]. For example, this interleukin induces the production of histamine, proteases and cytokines from mast cells, promotes TH2-differentiation and eosinophils development, induces IgE class-switching in B cells, and favours bronchoconstriction, globet cell metaplasia, and mucus production [203, 204]. In addition to TH17 cells, TH9 lymphocytes are resistant to glucocorticoids and can be mediators of steroid-resistant asthma [205].

In the presence of IL-6 and TNF-α, T_N cells can differentiate into TH22, which are characterized by the secretion of IL-22 and the expression of CCR6, CCR4, and CCR10 [206]. The function of this cytokine is controversial, as it has been implicated in both anti-inflammatory and pro-inflammatory processes [207]. Indeed, some works have described higher levels of IL-22 in serum from asthmatics compared to healthy individuals [207], or in severe asthma compared to moderate disease [207-209]. In contrast, other *in vitro* or *in vivo* studies suggest that IL-22 have a protective role in the late-phase of allergic induced airway inflammation by decreasing eosinophils levels, IL-5, IL-13, and CXCL10 [207].

TfH cells are another subset of TH cells important for the maintenance of germinal centres and the generation of long-lived

plasma cells and memory B cell responses [210]. These cells display a high dependency of IL-2 for its generation and are identified by the high expression of ICOS, PD-1, and CXCR5, whose mRNA has been found augmented in asthmatics compared to healthy subjects [211]. The ligand of CXCR5, CXCL13, is produced by follicular stromal cells and helps TfH cell to find the way to germinal centres (B-cell follicles), where somatic hypermutation of BCR and T-B cells interaction leading to class switching and B-cell differentiation takes place [212]. B-cell lymphoma 6 protein (Bcl-6) is a transcription factor expressed by TfH cells that suppresses the expression of transcription factors involved in the differentiation of other TH subsets (e.g., TH1, TH2, TH17) [213]. Moreover, TfH cells release IL-21, which is also increased in asthmatic lungs [211]. Therefore, more studies are needed to clarify the function of the different TH lymphocyte subsets, their relationship and their implication in asthma pathogenesis.

5.4. The role of regulatory T cells (Treg) in asthma

The above-mentioned effector functions of human TH lymphocytes are counteracted by Treg cells [214, 215]. There are two major types of Treg cells, with different biological functions: natural Treg cells (nTregs) and induced Treg cells (iTregs). The first subtype is responsible for tolerance to self-antigens. These $FoxP3^{+}CD25^{+}CD304/Neuropilin\text{-}1^{+}$ nTreg cells are generated in the thymus, where they manage to escape the negative selection. Lineage-specific transcription factors like FoxP3, Helios, or RUNX are important regulators of the development of nTregs and their immunosuppressive functions [215, 216]. In contrast, iTregs are generated in the periphery from other TH subsets under tolerogenic conditions: i.e., strong signalling via TCR, low co-stimulation, and

high levels of TGF-β and retinoic acid [215]. iTregs appear to control immune responses to "non-self" antigens (e.g., allergens, commensal microbiota, diet), and are classically divided into three major subtypes: FoxP3^{+} iTregs, IL-10^{+} Tr1 cells, and TGF-β^{+} TH3 cells [217]. Loss of iTreg cells leads to allergic-type-2 inflammation at mucosal interfaces (for example, in the lung) [218].

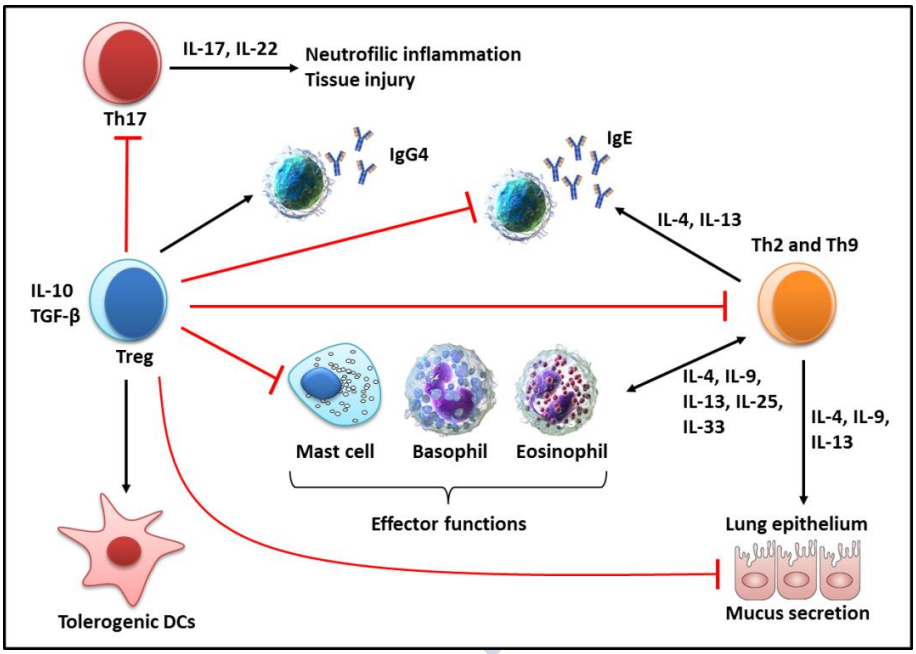


Figure 5. Schematic representation of the multitude of regulatory T cells (Treg) functions implicated in asthma pathogenesis. Treg cells control the effector functions of several innate system mediators such as mast cells, basophils, and eosinophils, as well as TH subsets from the adaptive immune system. They also control immunoglobulin (Ig) class switching through the induction of IgG4 production in detriment of IgE production. Stimulatory functions are represented by a solid arrow, whereas inhibitory signals are represented by blunt arrows.

Treg cells can be distinguished from Teff cells according to the levels of CD25 (IL-2Rα) and CD127 (IL-7Rα). Thus, contrary to Teff

cells ($CD25^{low}CD127^{high}$), most of Treg lymphocytes display high levels of CD25 and low levels of CD127 ($CD25^{high}CD127^{low}$) [219]. CD25 is considered an activation marker released by TH lymphocytes during inflammation in the form of soluble CD25 (sCD25) [220-222]. Indeed, sCD25 is augmented in BALF of asthmatics compared to healthy subjects [221, 222]. Moreover, sCD25 is increased in serum during asthma exacerbation [223] and has been correlated with AA severity [224]. On the other hand, CD127 is a subunit of both IL-7 and TSLP receptors [225]. Together with IL-15, IL-7 is cytokine important for homeostatic survival of TH cells, and therefore CD127 is also a marker for naïve and certain memory subpopulations (stem-cell memory T cells/T_{SCM} and T_{CM}). Other markers expressed on Treg cells, like CTLA-4 or GITR, are also up-regulated upon activation [215]. Therefore, this means that the Treg phenotype displays characteristics of activated and T_{EM} cells (see below).

Treg cells play an important role as negative regulators of inflammatory disorders and tissue repair [215]. This function requires signalling via TCR and IL-2R, and is exercised by means of multiple cell-contact dependent or independent mechanisms involving soluble (e.g., adenosine, granzyme, TGF-β, IL-10, and IL-35) or membrane-associated (e.g., CTLA-4, CD39, CD73, CD25, perforin) molecules [215]. Treg lymphocytes deprive the environment of IL-2, reduce the activation status of dendritic and myeloid cells, and restraint Teff functions by directly inhibit (or killing) TH1, TH2, and TH17 cells. Moreover, this Treg subset switches immunoglobulin isotypes from IgE to IgG4 in B cells and prevents the activation and migration through the airway epithelium of mast cells, eosinophils, and basophils (Figure 5) [1, 217]. Therefore, a functional defect in Treg cells or a change in the effector/regulatory equilibrium may be important in asthma pathogenesis [214]. Indeed, the percentage of IL-4 producing cells was augmented in allergic subjects, whereas IL-10-

producing Treg cells were decreased in allergic individuals (in contrast, they were the dominant allergen-specific subset in healthy subjects) [226]. In the same line, children with asthma display low levels of Treg cells in BALF compared to control subjects. Furthermore, contrary to $CD4^{+}CD25^{high}$ T cells isolated from control subjects, Treg cells from asthmatic subjects fail to suppress proliferation and TH2-cytokine production by responder T cells [227].

5.5. Naïve-memory differentiation of T cells and asthma

As stated above, the adaptive immune system is characterized by the generation of long-lived memory cells, even though it has been found recently that ILC2 cells, NK cells, or monocytes display an 'innate memory' or "trained immunity"; i.e., a long-term improvement of the function of innate immune cells after infection or vaccination due to epigenetic causes [228].

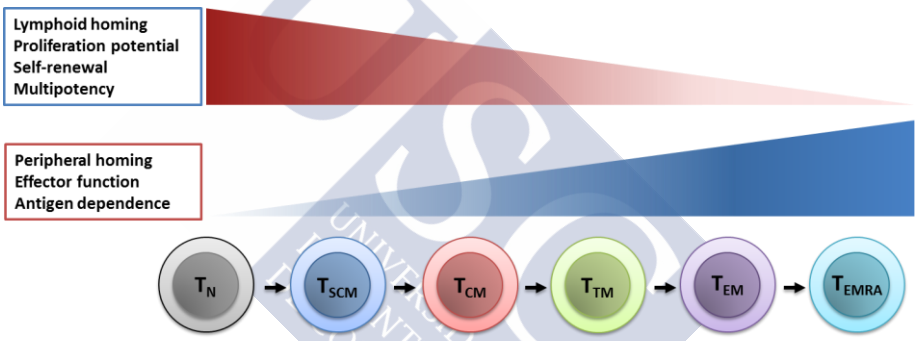
Following Ag encounter, T_N cells proliferate and differentiate into cells with different phenotypes and functions following a linear model (Figure 6). These six subsets of T cells (T_N, stem cell memory T cells/T_{SCM}, T_{CM}, transitional memory T cells, T_{EM}, and T_{EMRA}) can be characterized according to combinations of 4 markers: CD45RA or CD45RO, CD62L or CCR7, CD27 or CD28, and CD95 (Figure 6) [229]. T_N cells display the highest expression of CD45RA and no expression of CD45RO, two splicing variants of the *CD45* gene. CD45RO define memory T cells, whereas T_{EMRA} cells re-express the high molecular weight protein CD45RA. CD62L (L-selectin) and CCR7 allow T cells to migrate to secondary lymphoid tissues. Thus, these two molecules are highly expressed in T_N, T_{SCM} and T_{CM} cells. T_{SCM} cells retain stem cell properties and they share some genes with T_N cells, but they display high expression of CD95 (as the other

memory subsets). Finally, both CD27 and CD28 are co-stimulatory molecules which are lost in the last differentiation steps (T_{EM} and T_{EMRA}) (Figure 6) [229]. Both T_{EM} and T_{CM} cells can be distinguished according to the expression levels of chemokine receptors. For example, CXCR3 is mostly expressed on TH1 cells, CCR4 on TH2 cells, and CCR6 on TH17 cells [230, 231]. Most of the chemokine receptors (CCR5, CCR6, CCR10, and CRTh2) increase from less differentiated subsets (T_N) to more differentiated subpopulations (T_{EM}), which allow them to migrate into inflamed tissues such as the asthmatic lung. T_{EMRA} cells lost almost all chemokine receptors (except CCR5) as they do not need to migrate. Both T_{EM} and T_{EMRA} cells secrete different inflammatory mediators into inflamed tissues such as IL-4, IL-13 or IFN-γ ($CD4^+$ T cells) or cytotoxic molecules such as granzymes A/B or perforin ($CD8^+$ T cells) [229].

In addition to the previously mentioned naïve-memory T cells, it has been shown that in chronic airway inflammation, some memory T cells can permanently reside into airways. These cells are called tissue-resident memory T cells, they do not circulate, and they have a protective role against pathogens in mucosal tissues because of their localization and their elevated activation status (high expression of CD69 and CD25) [232].

In 2002, Mojtabavi *et al.* described the existence of long-lived TH2 memory cells in a mouse model of chronic AA (ovalbumin/OVA-induced). These mice present a quick development of IgE production, TH2-cytokines expression, eosinophilic inflammation, lymphocytic lung infiltration, mucus overproduction, and airway hyperresponsiveness (in some cases after more than 400 days from the acute disease) [233]. After these initial studies, the number of works about memory T cells in asthma has increased, especially in chronic airway inflammation [234, 235]. It has been described in both mouse and human that the presence in the lower

airway of memory T cells within $CD4^+$ lymphocytes, which are ready to respond against allergens, is necessary for the recurrence of inflammatory episodes in asthma [234-238]. However, less is known about which specific subsets of memory T cells are responsible for chronic asthma, maybe due to the different phenotypes of T cells involved in asthma pathogenesis and pathophysiology. Therefore, new studies are needed focusing on which T cell subpopulations, especially within the memory T cell subset, are associated with the different phenotypes of asthmatic patients during a chronic state of the disease.



CD45RA	+	+	-	-	-	+
CD45RO	-	-	+	+	+	-
CCR7	+	+	+	-	-	-
CD62L	+	+	+	-	-	-
CD27	+	+	+	+	-/+	-/+
CD28	+	+	+	+	-/+	-
CD95	-	+	+	+	+	+

Figure 6. Naïve-memory differentiation of T cells. T cells differentiate from naïve (T_N) to terminally-differentiated effector subsets (T_{EMRA}) following a sequential model in which T_N cells progressively lost lymph node homing capacity and multipotency properties to acquire effector functions and antigen dependence. The expression of 4 molecules (CD45RA or RO, CCR7 or CD62L, CD27 or CD28, and CD95) allows differentiating cells from different stages. T_{CM}, central-memory T cells; T_{EM}, effector-memory T cells; T_{SCM}, stem-cell memory T cells; T_{TM}, transitional-memory T cells.

5.6. S9B serine proteases subfamily and asthma: CD26

CD26/Dipeptidyl peptidase (DPP) 4 is a prolyl-oligopeptidase that belongs to the S9B subfamily of serine proteases. This subfamily consists of several peptidases (e.g., DPP4, DPP8, DPP9, and fibroblast activation protein/FAP/Seprase), some of whose encoding genes are located on chromosome 2: *FAP*, *DPP4* and *DPP10*.

DPP10 gene is considered an asthma susceptibility gene and a biomarker for aspirin-exacerbated respiratory disease [15, 239, 240]. Like DPP8 and DPP9, two intracellular active S9B proteases [241], DPP10 is mainly located on lung trachea and bronchi [242]. However, DPP10 is an extracellular protein with no serine-protease activity [243]. Therefore, the mode of action of DPP10 could be rather different from other members of the S9B subfamily and include the regulation of voltage-gated potassium channels [244].

CD26/DPP4, the major member of the S9B family, is a receptor for the Middle-East respiratory syndrome coronavirus [245]. This S9B protease is characterised by the presence of soluble (sCD26) and membrane forms, both of which have been found elevated in asthma, highlighting a possible role of CD26/DPP4 in the pathogenesis of this disease [246, 247]. CD26 is widely expressed in the whole organism [248] but is especially abundant in certain locations such as the lung parenchyma [247, 242]. Moreover, CD26 levels, as well as its DPP4 activity, are increased in rat lungs after allergen exposure [242], while the type-2 cytokine IL-13 promotes the upregulation of CD26 in AECs [249]. Several immune cells also display CD26 on their surface (e.g., B and NK cells, monocytes, granulocytes), but the highest expression is accounted in peripheral blood T cells [250, 251], particularly $CD4^+$ TH cells. Furthermore, the expression of this protein on T cells is highly up-regulated upon activation [251, 252], and fine-tuned by soluble molecules like

cytokines (e.g., IL-12, IL-4, TGF-β1) [253-256] or nucleosides (e.g., adenosine) [257], either increasing or down-modulating it. Indeed, CD26 was described by our group in 2012 as a negative selection marker for human Treg cells [258], whose abundance on Teff lymphocytes also appears to be rather variable: TH17>>TH1>TH2>Treg [259, 260].

In addition, CD26 levels are up-regulated upon acquisition of a memory phenotype by T lymphocytes [251, 252] and might define the stages of naïve-memory differentiation in $CD4^+$ and $CD8^+$ T subpopulations. Thus, T_N ($CD45RA^+$, $CD62L^+$, $CCR7^+$, $CD28^+$) are characterized by intermediate levels of CD26, memory T cells ($CD45RO^+$, $CD62L^{+/-}$, $CCR7^{+/-}$, $CD28^+$) show the highest expression of this serine protease, whereas T_{EMRA} cells ($CD45RA^{+/-}$, $CD62L^-$, $CCR7^-$, $CD28^-$) display the lowest levels of CD26 [261, 262].

CD26/DPP4 displays both co-stimulatory and inhibitory roles [243, 251], which could be relevant for asthma pathogenesis. Co-stimulation due to CD26 could be dependent on DPP4 activity [251, 252] and its association with molecules such as CD45 [263], adenosine deaminase (ADA) [264], CXCR4 [265], caveolin-1 [266], plasminogen II [267], collagen [268], fibronectin III [269], or glypican-3 [270]. For instance, *cis*-interaction of CD26 with CD45 on lipid rafts promotes the tyrosine phosphorylation of several signalling molecules such as ZAP-70, p56Lck, or TCR-ζ, boosting TCR signalling [263, 271]. As well, CD26 induces the up-regulation of B7.2/CD86 (a pro-inflammatory molecule) in APCs [266, 272], or the activation and nuclear translocation of NF-κB in T cells [273], upon its *trans*-interaction with caveolin-1 in lipid rafts [266, 272].

Without excluding enzyme-independent actions, many inhibitory functions of CD26 rely upon DPP4 activity, which clips N-terminal dipeptides with proline or alanine in the second position from

some cytokines (e.g., IL-3, G-CSF, or GM-CSF), chemokines (e.g., eotaxin/CCL11, RANTES/CCL5, SDF-1α/CXCL12, MDC/CCL22, I-TAC, IP-10,), or other soluble mediators (e.g., incretins, glucagon, peptide YY, bradykinin, vasoactive intestinal peptide, or neuropeptides like substance P) [243, 248, 251, 274].

An example to illustrate this inhibitory function of CD26/DPP4 is eotaxin. This chemokine is produced by monocytes and endothelial cells in response to TNF-α and IFN-γ, respectively. As its name suggests, eotaxin is an important chemokine in AA though its role in the attraction of $CCR3^+$ leukocytes to the lung: eosinophils, basophils, mast cells, and TH2 lymphocytes [275-278]. Truncation of eotaxin by CD26 induces CCR3-desensitization and reduces its chemotactic activity [278-280]. Indeed, the administration of DPP4 inhibitors enhances the mobilization of eosinophils *in vivo* [280]. Moreover, CD26-KO and OVA-challenged mice display higher levels of eotaxin and stronger infiltration of eosinophils into the lungs than WT mice [281].

Another soluble factor attracting monocytes, eosinophils, and T cells to inflammatory sites is RANTES. This chemokine acts through CCR1, CCR3 (TH2 cells), and preferentially CCR5 (TH1 cells) [282], but RANTES cleavage by CD26 results in the loss of the capacity to bind CCR1 and CCR3, but not CCR5 [283]. As CCR5 is mostly expressed on TH1 and T_{EM} cells [146, 284], the cleavage of RANTES favours their attraction toward inflammatory sites [283, 285]. Therefore, through the enzymatic cleavage of different chemokines, it seems that CD26 acts through a homeostatic mechanism to down-modulate airways inflammation mediated by TH cells, eosinophils and other inflammatory mediators.

A soluble version of CD26 (sCD26) can be found in many biological fluids (e.g., plasma, serum, BALF, synovial fluid) [286].

DPP4 activity in these biofluids is not only derived from sCD26 molecules, but also from alternative proteins such as attractin, DPP8, or DPP9. Indeed, some studies reported a low correlation between sCD26 and DPP4 activity [286-288]. In contrast, other authors have found a positive correlation between sCD26 and circulating DPP4 activity [289, 290]. CD26 molecules could be released from cells by classical exocytosis or by a process mediated by "secretases"/"sheddases" [291]. Indeed, sCD26 is released into the circulation by adipocytes, endothelial cells (e.g. lung), or epithelial cells from liver or kidney [292]. However, immune cells are perhaps the most likely source for sCD26, especially $CD4^+$ T lymphocytes [286, 293, 294]. As stated above, $CD4^+$ T lymphocytes are particularly enriched in CD26 molecules, and this is further enhanced in adults with AA, suggesting the activated status of these cells [246]. Thus, the concentration of sCD26 in plasma might be influenced by the number of lymphocytes [295] and/or mirror the phenotype of circulating TH lymphocytes in a pathological situation such as asthma.

CD26 has been related to asthma pathogenesis in several studies performed with experimental models of this disease and with human samples. The first studies evaluating the role of CD26 in asthma were performed in OVA-challenged F344 rats. The authors of these works reported that a loss of CD26/DPP4 resulted in a protective effect in experimental asthma, with reduced eosinophils and T cell recruitment into the BALF, and decreased airway inflammation and IgE levels. These findings were partly explained by increased activity and influx of $FoxP3^+$ Treg cells into de airways [296, 297]. However, rat Treg cells display a $CD26^+$ phenotype [298, 299], in contrast to the above commented $CD26^{-/low}$ phenotype in humans [258]. In addition, it has been shown that the outcome in experimental models of asthma in rats could be totally different according to the genetic background of the animals [300], or the dose, route, or timing of DPP4-inhibitor

administration [301]. Moreover, a model of OVA-induced experimental asthma in $CD26^{-/-}$ mice showed increased levels of IL-4, IL-5, and IL-13 in BALF, higher circulating levels of eosinophils, enhanced levels of eotaxin and RANTES, as well as augmented expression of TH2 chemokine receptors (CCR3 and CCR5), compared to WT mice, supporting that CD26/DPP4 could play a "braking" role in experimental asthma [281].

From studies in the human system, Lun *et al*. have shown higher levels of CD26 on total lymphocytes, $CD4^+$ T cells, and iNKT lymphocytes, as well as higher concentrations of sCD26 in adult allergic asthmatics [246]. Moreover, the TH2-cytokine IL-13 has been identified as an important inducer of CD26/DPP4 expression in bronchial epithelial cells [302]. However, other works have shown no changes of sCD26 in asthma [303]. These results could be explained because of many potential confounders: low sample size, male/female proportion, age, or lymphocyte count. For instance, is well-known that both CD26 expression on lymphocytes and circulating sCD26 levels are higher in men than women [303, 304]. Therefore, more studies are needed to assess the relationship between CD26 and asthma, as well as to extend this area of research to different asthma phenotypes (or endotypes) and severity degrees.

***For more information see appendix I.**

6. ASTHMA PHENOTYPES AND ENDOTYPES

Asthma is a heterogeneous disease that groups different clinical phenotypes or "*observable properties of an organism that are produced by the interaction of the genotype and the environment*",

(Merriam-Webster's Collegiate Dictionary (© 2019 Merriam-Webster, Incorporated 2019)). Moreover, below this first outer and visible layer of complexity lies a second one, named asthma endotypes, which represent the pathophysiologic and molecular mechanisms that trigger and make this disease worse [2]. However, this connection between specific asthma phenotypes and the underlying pathological features is elusive [2]. Therefore, most of the research is focused on the search for a variety of biomarkers that clearly define the different asthma phenotypes and/or endotypes. Those works are intended to develop new biological drugs, necessary for personalized/precision medicine in asthma and respiratory diseases [45, 305, 306].

The most extended classification of asthma amongst clinicians relies on the presence or absence of allergic triggers: AA and NAA (Figure 7). AA is the most common phenotype (~ 60%) [307]. It is characterized by the presence of allergic sensitization, which is defined by the presence of allergen-specific IgE molecules and a positive skin prick test against common environmental allergens. Compared to the adult onset of NAA, AA often starts in childhood. Moreover, the prevalence of AA is higher in males, whereas non-allergic asthmatics are mostly women. Additionally, AA patients frequently respond well to inhaled corticosteroid (ICS) treatment compared to NAA patients, which are usually more heterogeneous regarding the underlying inflammatory pattern (neutrophilic or paucygranulocytic inflammation) [4, 308, 309]. However, this classification of asthma according to the kind of trigger is an oversimplification, as other asthma phenotypes apart from AA and NAA have been defined by the Asthma Phenotypes Task Force: aspirin-exacerbated respiratory disease, infection-induced asthma, and exercise-induced asthma [307].

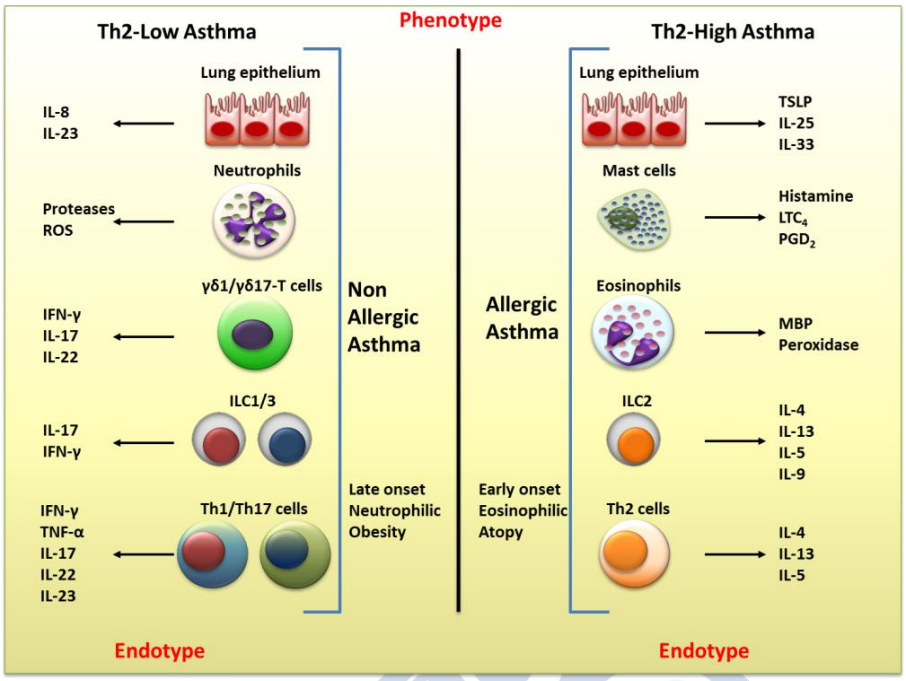


Figure 7. Cells and molecules implicated in the different asthma phenotypes and endotypes. Two major phenotypes have been described in asthma, the early onset, eosinophilic and atopic/allergic asthma, and the late onset, non-allergic asthma. Allergic asthma mainly fits into the TH2 high asthma, and it is characterized by mast cell and eosinophilic response and a TH2 driven inflammation. On the other hand, non-allergic asthma is more heterogeneous, and it could be neutrophilic or obesity-related. The non-allergic phenotype is characterized by a TH2 low endotype, where type-1 and type-17 cytokines and the cells they come from (ILC1/3, γδ-T cells, or TH1/TH17 cells), as well as neutrophils, have an important role.

Underneath the phenotypic classification of AA and NAA, the most common endotype classification is based on the predominant TH-type inflammation: i.e., TH2-high vs TH2-low asthma [310, 311]. The early-onset TH2-high asthma is the most common endotype and almost 100% of patients with this endotype fall into the AA category. The TH2-high endotype is characterized by a TH2- and mast cells-

driven inflammation, sub-epithelial basement membrane thickening, and a central role for IgE and eosinophils (Figure 7) [2, 312]. This type of asthma is also associated with atopy (i.e., a genetic predisposition to suffer allergic diseases). Indeed, diseases such as allergic rhinitis, eczema, or food and drug allergy are often comorbidities in AA patients [2, 4]. Several biomarkers of TH2-high asthma have been described: elevated sputum eosinophils levels, high fractional exhaled nitric oxide (FeNO), which is a surrogate marker of eosinophilic airway inflammation, and raised levels of periostin and total IgE in serum [311, 313, 314]. However, the main feature of this endotype is the good response to non-specific anti-inflammatory drugs (ICS), resulting in the down-modulation of TH2 cytokines (IL-4, IL-5, and IL-13) and the underlying inflammation [2].

Different biological therapies have emerged in the form of humanised antibodies that target several AA mediators such as IgE or TH2-cytokines [311]. The first humanised monoclonal antibody specifically designed to treat AA was omalizumab (Xolair®). This antibody makes a complex with free IgE and inhibits its binding to the high-affinity FcεR1 receptor on mast cells and basophils [314]. As a result of this blockade, the typical mast cell activation/degranulation event that occurs in AA is interrupted, as well as the eosinophilic inflammation [315]. Therefore, severe persistent allergic asthmatic patients treated with omalizumab undergo a reduction of asthma exacerbations [316] and an improvement in their quality of life [317]. Beyond omalizumab therapy, there are other humanised monoclonal antibodies that target several important molecules in asthma: a) TH2-cytokines such as IL-5 (mepolizumab and reslizumab) or IL-13 (lebrikizumab and tralokinumab); b) TH2-cytokine receptors such as IL-4Rα (dupilumab and AMG-317) or IL-5Rα (benralizumab); and c) epithelial cell-derived cytokines such as TSLP (AMG-157) [311, 318]. These alternative biologicals have yielded promising results in terms

of improvement of lung function and reduction of exacerbations in TH2-high AA [318].

TH2-low asthma, for its part, is more heterogeneous and poorly defined; indeed, there are no specific biomarkers for this asthma endotype. Overall, patients with TH2-low asthma are badly-responders to ICS and display a more severe disease. In addition, these type of patients are characterized by the absence of a TH2-signature, lower levels of IgE, delayed disease onset (adulthood), and non-allergic/atopic disease usually associated with neutrophilic inflammation (Figure 7) [310, 319-321]. Obesity-related asthma, which is predominant in women, is also included within TH2-low asthma [322]. TH1 and TH17 cells and their derived cytokines seem to be key players in the pathogenesis of this endotype (Figure 7). As commented above, IL-17 is the major mediator of neutrophils recruitment and activation by inducing the production of IL-8 and other chemokines (e.g., CXCL1 or CXCL5) by AECs (Figure 7). IL-17 has been associated with AHR, remodelling and asthma severity [188, 323, 324]. Although IL-17 is mainly produced by TH17 cells, certain subpopulations of innate lymphocytes also secrete this cytokine. This is the case of ILC3s, which play a central role in obesity-related asthma [69], or γδ-T lymphocytes, which are the major producers of early IL-17 (Figure 7) [134]. On the other hand, IFN-γ, the fingerprint cytokine of TH1 cells, is also implicated in the chemotaxis of neutrophils through the up-regulation of CCR1 and CCR3 on this leukocyte subset (Figure 7) [325].

Given all the above commented, and in the era of biological therapies and personalised/precision medicine, it is more necessary than ever the search for new biological markers, with application in phenotype/endotype refinement and disease diagnosis/prognosis/management. These new biomarkers will enlarge the list of currently available options, which include both cells (e.g.,

eosinophils, neutrophils) and molecules (e.g., total and specific IgE, periostin, FeNO, cytokines) in different kind of samples (e.g., peripheral blood, serum, plasma, IS) [307].

7. APPLICATION OF BIOFLUID PROTEOMICS FOR ASTHMA-BIOMARKER DISCOVERY

The emergence of Proteomics, which is the large-scale study of proteins, has meant a turning point in the world of biomarkers discovery. Proteomic studies often use samples from tissues, *in vitro* cell cultures, or biofluids such as urine, BALF, exhaled breath condensate (EBC), or cerebrospinal fluid (CSF) [326, 327]. However, the most commonly used samples in clinical studies are those derived from venous peripheral blood: i.e., serum and plasma. This easy to acquire samples are challenging in Proteomics because they are highly dynamic and complex (>10,000 proteins; http://www.plasmaproteomedatabase.org). This is because both serum and plasma samples are an open window to the proteomes of all the tissues in the body. This makes these biofluids ideal for biomarker discovery studies, especially amongst low molecular weight (LMW; < 30-40 kDa) or low abundance protein (LAP) sub-proteomes [328, 329].

Serum/plasma includes proteins that fulfil their function in the extracellular compartment, such as cytokines, hormones, or growth factors. However, they also contain "bystander" molecules such as tissue leakage products; i.e., intracellular proteins released by tissues for multiple reasons (some of them summarized in Figure 8) [328, 330, 331]. LMW cellular proteins (especially the cytosolic ones) reach easily the blood circulation during physiological cell turnover or pathological plasma membrane permeabilization due to their small

protein size [332]. Despite this ease, the serum/plasma LMW proteome is made of small proteins, protein fragments, or peptides that display a low abundance and elevated complexity. Therefore, LAP and LMW sub-proteomes from serum/plasma are difficult to investigate but represent a partly overlapping rich source of information and novel potential biomarkers related to cancer [333], cardiovascular conditions [334], infectious illnesses [335], or airway diseases [336, 337].

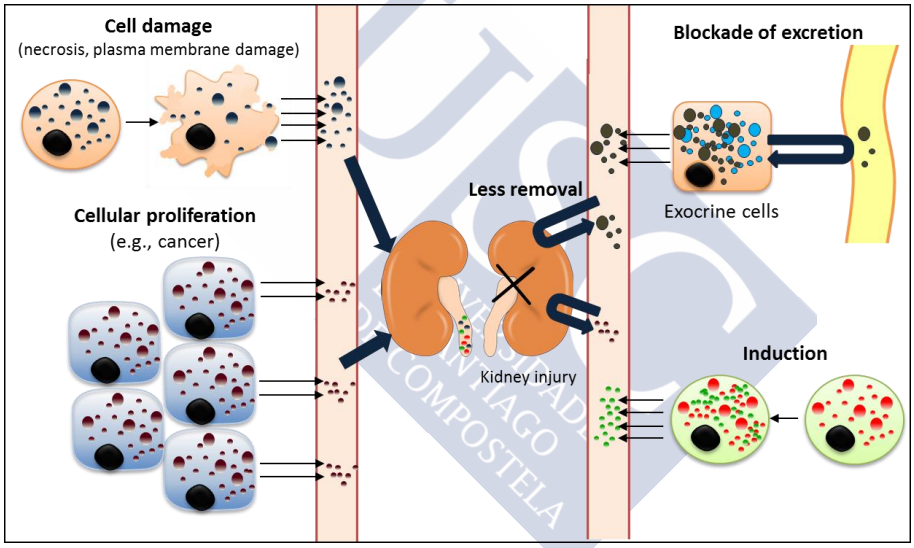


Figure 8. Cell/tissue sources and origin of low abundance serum/plasma proteins. The serum/plasma abundance of disease biomarkers is dependent on the equilibrium between secretions by cells and removal (e.g., kidney or proteolysis). Several reasons are the responsible for the increment or detriment of tissue proteins in plasma: 1) cell damage (necrosis, cell membrane permeabilization), 2) induction of protein synthesis, 3) increment of cellular proliferation/ cell replacement (e.g., cancer), 4) Reduction of protein excretion by exocrine cells (e.g., pancreatic or prostate cells) due to obstruction of the ducts, or 5) less removal of proteins from circulation due to for example renal injury.

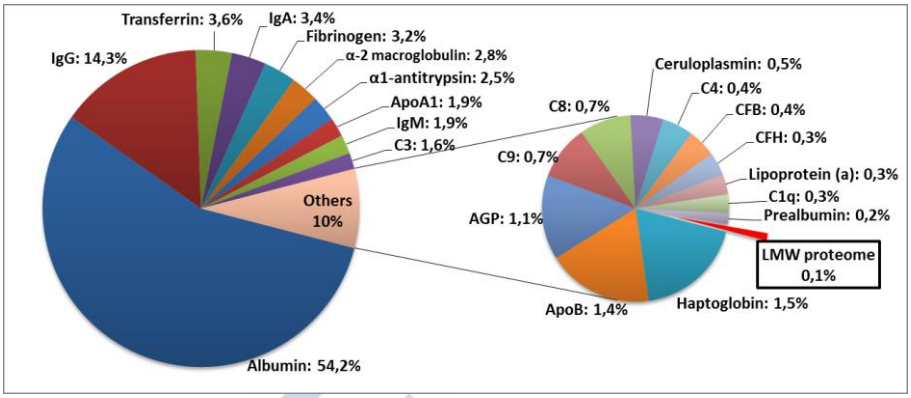


Figure 9. The dynamic range of proteins concentrations in human plasma. Human plasma proteome is composed of thousands of proteins. 22 of them represent the 99.9% and are considered high abundant proteins. Between them, only 10 represent more than 90%, and only albumin constitutes more than 50% of total protein concentration. Low abundance proteins, also called LMW proteome is compound by only the 0.1% of proteins in the human plasma.

As above commented, several challenges must be considered when analysing LAP/LMW sub-proteomes from serum/plasma samples. The most important one is the extremely high dynamic range of protein concentrations in those biofluids, of almost 10^{10} magnitude, and the fact that <0.1% of the total number of species represents almost 99% of the bulk mass of plasma/serum proteins; that is, a set of 22 protein that includes albumin, immunoglobulins, transferrin, α-2 macroglobulin or α1-antitrypsin (Figure 9). Only albumin itself constitutes ~50% of protein content in serum, with concentrations ranging between 35 and 50 mg/mL. In contrast, the low abundance proteome reaches a scarce 0.1% of the bulk mass of proteins, covers a concentration range from ng/mL to fg/mL, and is made up of thousands of LMW proteins with a specific function in plasma (e.g., cytokines, growth factors, and chemokines) or derived from tissues or

cells (Figure 9) [328, 330, 331, 338, 339]. Therefore, reduction of sample complexity is necessary to ensure access to this potential source of disease biomarkers, coined by certain researchers as the "deep-proteome" [339].

7.1. Pre-fractioning methods for plasma/serum proteins

In the last years, different experimental approaches consisting of either depletion of high-abundance proteins or enrichment of LAP/LMW species in serum/plasma samples have been developed in order to reduce the serum/plasma dynamic range of protein abundances.

Ultrafiltration is included in the first group of techniques [340]. This technique consists of centrifugal filter devices whose membranes display a specific cut-off (e.g., 40 kDa cut-off), which means that only low molecular weight proteins will be able to pass through. However, it has been shown that high molecular weight proteins like albumin still appear in the eluted fraction. Other approaches involve the depletion of high abundant protein by affinity chromatography [341]. This is the case of human serum albumin affinity columns (e.g. Cibacron Blue-based affinity chromatography) [342], immunoglobulins depletion (e.g., protein A/G) [343], Proteoprep columns (Merck Millipore) for Albumin/IgG depletion [344], the Pierce™ Top 12 Abundant Protein Depletion Spin Columns (Thermo Scientific™), or the multiple affinity removal system (MARS) (Agilent Technologies). Finally, high-abundance proteins can be depleted from serum by using organic solvent precipitation (e.g., acetonitrile precipitation) [345]. However, it has been described that some LMW proteins are found in association with carrier proteins such as albumin [346]. Therefore, depletion or precipitation of these

highly-abundant "shipper" proteins can result in the loss of interesting LMW biomarkers. More importantly, removal of the upper-layer of high-abundance proteins from serum/plasma samples only allows researchers to reach the medium abundance sub-proteome.

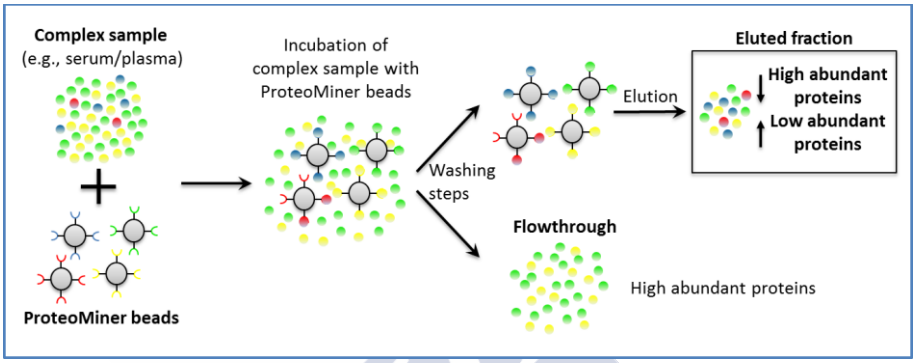


Figure 10. The use of combinatorial peptide ligand libraries (CPLL) as an enrichment method for low abundance proteins in complex samples. The ProteoMiner™ protein enrichment technology (BioRad) is based on the use of combinatorial ligand library of hexapeptides (CPLL) attached to beads. **Step 1.** The CPLL is added to a complex sample (e.g., serum or plasma) where almost all low abundant proteins bind to their specific ligand hexapeptides, but high abundant proteins saturate their ligands. **Step 2.** The washing of the sample allows the elimination of high abundant proteins, which had saturated their ligands. **Step 3.** The proteins attached to the beads are eluted. Therefore, the eluted fraction is enriched in low abundant proteins, whereas high abundant proteins are eliminated in the flowthrough fraction.

Apart from depletion methods, several approaches that enrich low-abundance/LMW proteins have been developed. This is the case of affinity capture of low-abundance proteins by means of magnetic beads [347], nanoparticles [348], antibody arrays [349], or the use of combinatorial peptide ligand libraries (CPLLs) (Figure 10) [350]. The last approach, marketed under the name of ProteoMiner™ protein

enrichment technology by BioRad, is a widely used enrichment technology. CPLLs consist of a large combinatorial ligand library of hexapeptides attached to chromatography beads. In such a way, each hexapeptide can bind to a specific serum/plasma protein by virtue of its primary sequence. However, the number of hexapeptide ligands is limited. Therefore, after the sample is added, all the low-abundance proteins in serum/plasma are retained by their respective hexapeptides, while the highly abundant proteins rapidly saturate their ligands. As a result, most of the high-abundance proteins are lost in the flow-through fraction, whereas low-abundance proteins display augmented concentrations in the resulting eluted fraction (Figure 10).

7.2. LMW proteome characterisation by mass spectrometry (MS)

Two different methods of protein identification have been developed since the term proteomics was coined in 1997: *Top-down* and *bottom-up* proteomics [351, 352]. Both strategies are differentiated based on the way of which the proteins are identified. *Top-down* proteomics is referred to as the analysis of entire proteins and is commonly used for post-translational modifications analysis, isoform analysis, and structural studies [351]. In contrast, the *bottom-up* strategy consists of the analysis by mass spectrometry of proteolysis-derived peptides (e.g., by means of trypsin). This method is useful to identify and quantify proteins, which can be procured from either simple (e.g., a two-dimension electrophoresis/2-DE spot) or complex (e.g., a crude extract; *shotgun* proteomics) starting mixtures [352, 353]. The *bottom-up* strategy is the most used for biomarker discovery approaches. However, LMW proteome characterization by *bottom-up* proteomics is challenging because these proteins generate a low number of proteolysis-derived peptides for MS analysis [354].

2-DE has been a technique of paramount importance in the development of the proteomics field [355]. This technique achieves the separation of proteins based on two independent attributes: their isoelectric point (isoelectric focusing; fist dimension) and their molecular weight (SDS-PAGE; second dimension). Then, in-gel detection of proteins by staining of gels (e.g., Coomassie brilliant blue, silver staining) is performed, followed by relative quantification through a side-by-side comparison of gels (e.g., control sample against treatment/disease sample). A variation of 2-DE named 2-D Fluorescence Difference Gel Electrophoresis (2D-DIGE) technique was created to overcome the low inter-gel analytical precision. 2D-DIGE uses up to three different charge/size-matched fluorescent dyes (Cy2, Cy3, and Cy5) to label the same number of protein samples and separate them in the same gel [356]. Afterwards, different types of imaging systems that use CCD devices or laser-based technologies can be used to scan the emitted fluorescence light at a specific excitation wavelength. After 2-DE or 2D-DIGE gels scanning, images are analysed by means of different specialised application software. Once proteins showing a differential abundance or any feature of interest are detected, proteins spots are manually or automatically excised, in-gel proteolytic digestions are performed, and the resulting peptides are analysed by either Matrix-Assisted Laser Desorption/Ionization-time-of-flight (MALDI-TOF MS) or liquid chromatography coupled to mass spectrometry (LC-MS/MS) [357, 358]. However, this "classic" gel-based strategy is very laborious for diagnostic approaches and its performance with proteins with extreme characteristics (i.e., highly hydrophobic, acid, or basic proteins) as well as low abundance/LMW species is poor. For this reason, 2-DE is being gradually replaced by *bottom-up* "gel-free" MS-based approaches [359], or a combination of gel-based technologies (1-DE or 2-DE) coupled to LC-MS/MS [360].

In the *bottom-up* strategy, proteins are digested with different proteases such as subtilisin, proteinase K, chymotrypsin, or some endopeptidases, but the most used is trypsin [353]. After protein digestion, the complex mixture of peptides derived from each sample is usually separated in several fractions by using different hyphenated chromatography methods. The most common fractionation technique stems from the sequential combination of two (2D) orthologous technologies: strong cation exchange (SCX) and reverse phase (RP) liquid chromatography (LC) [361, 362]. This strategy is called Multidimensional Protein Identification Technology (MudPIT) [362].

The first dimension, SCX, consists in the separation of peptide mixture based on the net charge of peptides, which is dependent on amino-acids such as histidine, lysine, or arginine [362]. Thus, the peptide mixture is placed in an acid buffer (pH<3) and loaded into the SCX column, resulting in the binding to the SCX column. Then, the column is washed several times and bound peptides are sequentially eluted with increasing concentrations of salts or H^+ (pH). After SCX, a set of fractions is obtained (usually 4-6), which need to be desalted before being introduced into the analytical RP column. Although other chromatography approaches can be used in MS-based analysis such as strong anion exchange (SAX), affinity, or hydrophilic interaction chromatography (HILIC) [363, 364], most 2D-LC-MS/MS studies use RPLC as a second dimension. RP-ultra performance liquid chromatography (UPLC) allows the separation of peptides based on hydrophobicity properties [365]. These RP-UPLC columns are usually coupled in tandem to nano-electrospray ionisation (nano-ESI)-mass spectrometers, with the capacity to do mass spectrometry (MS or MS^1) or tandem mass spectrometry (MS/MS or MS^2) analyses.

MS is an analytical technique based on the ionization of chemical species and the separation of these ions according to their mass-to-charge (m/z) ratio. Therefore, a prototypical mass

spectrometer consists of an ion source, a mass analyser, and a detector. There are several types of mass analysers [366], but one of the most commonly used is the linear trap quadrupole/LTQ-Orbitrap family (Thermo Scientific), which combine LTQ ion trapping for data acquisition with Orbitrap mass analyser. LTQ-Orbitrap displays a high mass accuracy (< 2 ppm), a high sensitivity (femtomole), a high resolving power (>150,000), and a high dynamic range [365, 367].

7.3. Quantitative proteomic approaches using mass spectrometry (MS)

Apart from protein identification (i.e., data mining), current proteomic biomarker discovery techniques also rely on quantification of protein abundances across different states (e.g., healthy vs. patients, treated vs. non-treated). Quantitative MS-based approaches can be divided in both absolute and relative quantification methodologies, being the last ones the most broadly used in proteomics-based biomarker discovery. Relative quantification can be carried out by either label-free or stable isotope labelling methods [368].

Label-free quantification is a simple, inexpensive, and quick technical solution for the comparison of protein levels across all the different samples of a study. This methodology uses either the peak intensity of a peptide in the MS analysis (MS^1 level) [369] or the number of MS^2 spectra for a given protein (spectral counting) [370], as an indicator of its abundance (Figure 11).

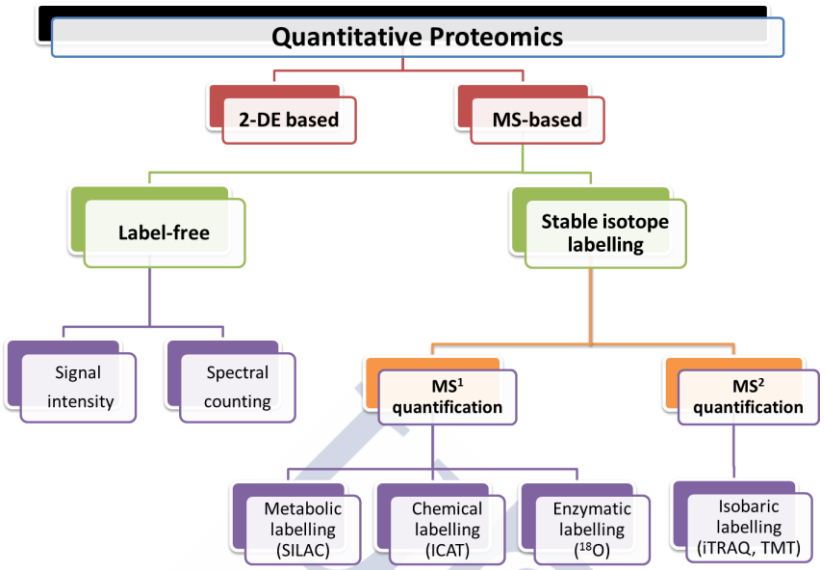


Figure 11. Schematic representation of the different methodologies used in quantitative proteomics.

Relative quantification can also be carried out by stable isotope labelling methods. The isotope-labelling quantification includes several MS-based strategies (Figure 11), which can be classified in MS^1 and MS^2 quantification techniques based on when the quantification is performed. In turn, MS^1 strategies can be classified into the following categories [353]: 1) **metabolic labelling**, including *stable isotope labelling by amino acids in cell culture* (SILAC), *culture-derived isotope tags* (CDITs), or *stable isotope labelling of mammal* (SILAM); 2) **chemical labelling**, which includes thiol groups labelling using *isotope-coded affinity tags* (ICAT), or *acid-labile isotope-coded extractants* (ALICE), and labelling of post-transcriptional modifications using *phosphoprotein isotope-coded affinity tags* (PhIAT); or 3) **enzymatic labelling using $^{18}O/^{16}O$** (Figure 11). On the other hand, MS^2 strategies include two similar

techniques based on labelling of amino groups with either *tandem mass tags* (TMT) or *isobaric tags* (iTRAQ) (Figure 11) [353, 368]. Finally, once a researcher has found several candidate biomarkers, strategies of *targeted*-proteomics with high sensitivity and selectivity such as selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) have been recently developed [371, 372].

iTRAQ protein quantification is a commonly used approach for unbiased untargeted biomarker discovery. In this method, tryptic peptides are covalently labelled with isobaric tags, which are reagents composed of three parts: 1) a reporter group, which presents variable mass (113.1-119.1, 121.1 Da), display an intense signal in MS/MS, and retains the charge; 2) a balance group (184-192 Da) to conserve a constant tag mass of 305.1; and 3) an amine-specific peptide reactive group, which binds primary amines of peptides (Figure 12). After labelling, all samples are combined and analysed by LC-MS/MS (Figure 13). As an isobaric labelling, identical peptides with different reporters (i.e., they come from different samples) are not distinguished in MS^1 step, so that tandem mass spectrometry (MS^2 or MS/MS) is necessary. After precursor peptide fragmentation by collision-induced dissociation (CID), reporter ions are released and detected by MS/MS at the low molecular mass/charge region (low m/z). Therefore, proteins can be both identified by comparing the peptide sequence obtained (peptide fragment fingerprint) with databases and relatively quantified by comparing the intensity of each reporter. Variation in the reporter intensity for each peptide is directly correlated with the change in the levels of the proteins where they come from [368].

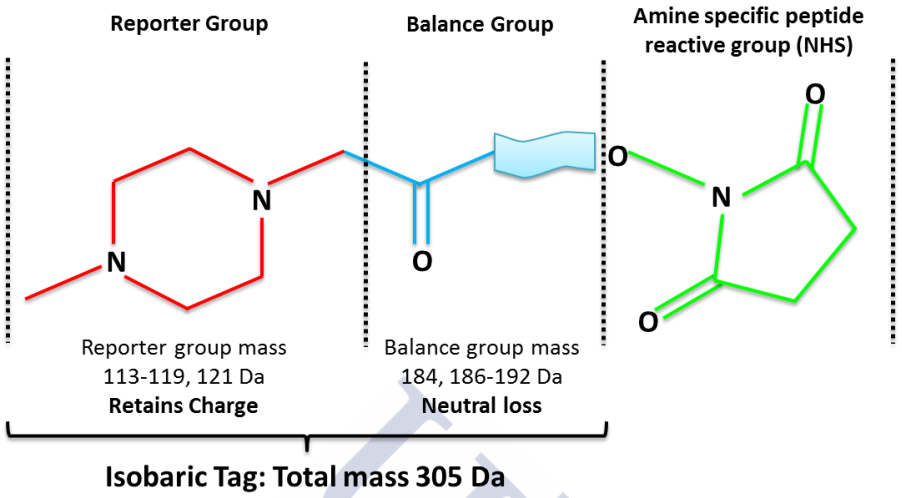


Figure 12. iTRAQ labelling. Each label of iTRAQ 8-plex is composed of 3 regions. Tryptic peptides covalently bind to the amine-specific peptide reactive group (NHS). The region next to NHS is the balance group (184-192 Da) aimed to conserve a constant tag mass of 305.1 in MS^1. Finally, the reporter group retains the charge after peptide fragmentation, presents variable mass (113.1-119.1, 121.1 Da), and display an intense signal in MS^2.

Compared to label-free strategies, which can process an unlimited number of samples in the same experiment, iTRAQ only allows us to label, analyse, and compare up to 8 samples (Figure 13). However, label-free strategies present several challenges [368, 373]: 1) all samples are prepared and analysed separately; 2) it is necessary to use internal standards in order to avoid variations in the peak intensities between different experiments; 3) quantification errors due to close mass signals can be present, so that technical replicates are mandatory (Figure 13). Therefore, label-free quantification results in a huge amount of data, which are difficult and laborious to process (Figure 13). This issue has been solved with iTRAQ technology, which is an easy-to-use method that allows the analysis and

comparison of all samples (up to 8) in the same MS/MS read out, as all labelled samples are mixed in one common sample (CS) before LC-MS/MS analysis (Figure 13).

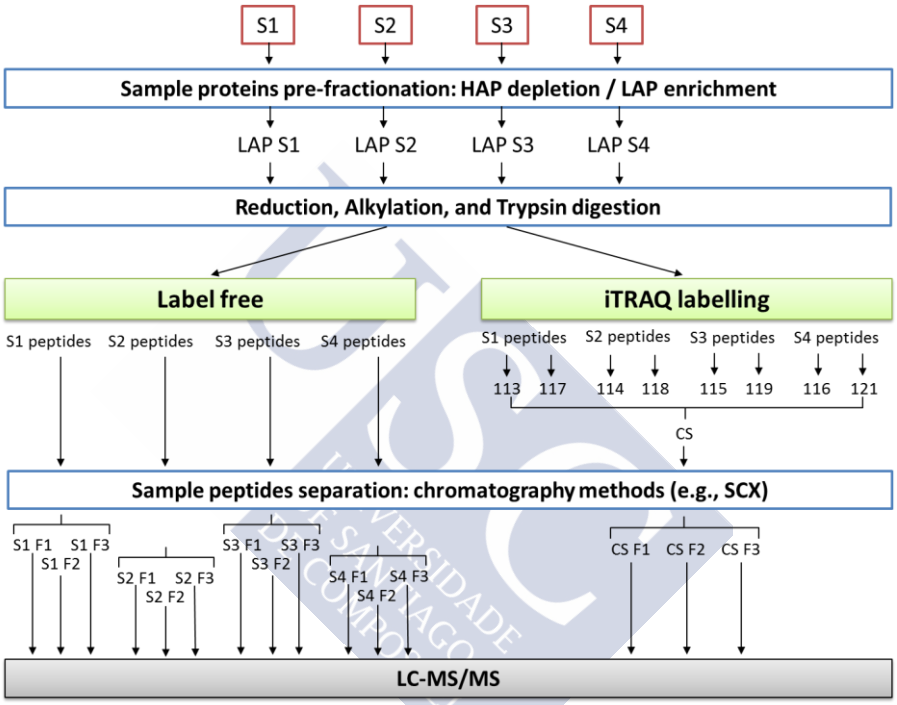


Figure 13. Comparison of label-free and iTRAQ quantification. In label-free quantification, all samples are prepared and analysed separately, which results in a huge amount of data difficult to interpret. Stable-isotope labelling by means of iTRAQ allows the comparison of proteins from each sample in the same LC-MS/MS read out. CS, common sample; HAP, high abundant protein; LAP, low abundant protein; LC-MS/MS, liquid chromatography coupled to tandem MS; S, sample; F, fraction.

7.4. Proteomic studies in asthma

As Table 1 shows, several proteomic studies have been carried out in the last years in order to search for specific biomarkers of asthma, asthma exacerbation, and treatment response. The first analysis was carried out by Lindahl *et al.* in 1999, who used 2-D electrophoresis to show the alteration of several proteins (Lipocalin-1, transthyretin, cystatin S, and IgBF) in both BALF and NLF (nasal lavage fluid) from patients with asthma compared to healthy subjects [374]. Following this initial work, a few more studies in BALF [375, 376] and NLF [377, 378] came to light. For example, Wu *et al.* in 2005 used affinity depletion of top 6 abundant proteins, 1-DE-LC-MS/MS, and a label-free approach to identify 160 BALF proteins with changes in asthmatics after allergen challenge, some of them with high importance such as IGFBPs, AMBP protein, CD5L or AGP1 [375]. Some years later, Cederfur *et al.* employed a similar MS approach to identify a set of galectin binding glycoproteins as potential asthma biomarkers (e.g., CD59, keratin 8, HP, RAIG, SBEM, attractin, CD55, AGP, or SERPINA1) [376]. In addition, FABP5 and VEGF were increased in asthmatics compared to healthy controls both in NLF and IS [378]. Apart from BALF and NLF, a few numbers of studies have been performed in BB [357, 379], nasal brushing (NB) [380], or EBC.

However, proteomics studies with IS [378, 381-386] or plasma/serum [358, 380, 386-393] samples to look for specific asthma biomarkers have been far more frequent. Moreover, most of these studies have compared asthmatics vs. healthy subjects, coming across significant changes in proteins such as S100A8/9/12, CC16, cystatin s, AAT/SERPINA1, α2M, HP, CP, Hpx, I-TAC, EGF, MMP-9, ITIH4, ORM1, or FGA/B [381, 382, 385, 390, 391]. In contrast, other proteomic studies have compared asthma with COPD [381, 383, 390] or cystic fibrosis [380], aspirin-induced asthma vs. aspirin-tolerant asthma [377, 387], the effect of exercise-induced bronchoconstriction

[382], the level of asthma control [384, 386, 394] or exacerbation [388], and the effect of different treatments [357, 358, 379]. However, none of them was aimed to compare the differences between asthma phenotypes, and only a handful reported proteomic changes in patients with different asthma severities [384, 386, 388, 394]. Moreover, most of the studies in plasma/serum were performed using 2-DE and MALDI-TOF, only one was carried out by an LC-MS/MS (label-free quantification) approach [391], and none of them used the iTRAQ technology. Therefore, further studies are needed to compare the serum/plasma proteome of different asthma phenotypes (e.g., AA vs. NAA) and severities (intermittent-mild vs. moderate-severe).

Table 1. Proteomic studies in asthma.

Authors	Sample	Methods	Results	Ref.
Lindahl M, 1999	• BALF • NLF	2-DE	• LCN1, TTR, cystatin S, and IgBF are altered in asthma.	[374]
Wu J, 2005	• BALF	AC and 1DE LC-MS/MS Label-Free	• 160 proteins were differentially expressed in asthmatic patients afte2r allergen challenge (e.g., IGFBPs, AMBP, CD5L, or AGP1)	[375]
Lee SH, 2006	• Plasma	2-DE MALDI-TOF	• C3a and C4a were higher in AIA patients than in patients with ATA.	[387]
Gray RD, 2008	• IS	CM10, Q10, or IMACNi SELDI-TOF	• 105 peaks with changes between asthmatics and controls, and 16 between asthma and COPD. • Proteins identified: S100A8, 9 and 12, CC16, lysosyme c, proline rich salivary peptide, cystatin s, and HBA1.	[381]
Nishioka T, 2008	• Plasma	2-DE (DIGE) MALDI-TOF	• 19 proteins with changes during asthma exacerbation vs. non-exacerbation: GP1BB, CLU, C7, C1s, AAT/SERPINA1, SERPINA2 and3, F2, FGB, KNG1, A1BG, AZGP1, ALB, HP, AFM, APCS, CD5L, APOA1, APOA4.	[388]

Continued on next page

Table 1 (Continued)

Rhim T, 2009	• Plasma	2-DE MALDI-TOF	• C3 increases whereas γ-fibrinogen decreases in asthma positive responders against *Dermatophagoides pteronyssinus* inhalation in comparison with negative responders.	[389]
Gomes-Alves P, 2010	• NB • Serum	CM10 pH 4 Q10 pH 10 SELDI-TOF	• 91 peaks with changes between control/asthma/cystic fibrosis • Protein identified: Hemoglobin subunit-beta	[380]
Bloemen K, 2011	• EBC	nano-HPLC MALDI-TOF/TOF	• A peak pattern of peptides was identified to discriminate between healthy controls and asthmatics. Only cytokeratin 1 was identified.	[395]
Gharib SA, 2011	• IS	LC-MS/MS Label-Free (spectral counting)	• 70 proteins with differences between asthma and HC (e.g., S100A8/9, AAT/SERPINA1, SMR3B, or SCGB1A1). • 5 differential proteins between EIB^+ and EIB^- (e.g., C3a and HPX).	[382]

Continued on next page

Table 1 (Continued)

Ko YC, 2011	• CD4+ T cells	2-DE MALDI-TOF	• 13 proteins with differential expression between controlled and uncontrolled asthmatics: HSP-70, FGB, TPM3, ATP-dependent DNA helicase II, HSP-90, ACTB, VIM, ARHGDIB, ENO1, CALR precursor, YWHAZ, PRDX2.	[394]
O'Neil SE, 2011	• BB	SCX LC-MS/MS iTRAQ	• 7 differential proteins between asthma patients and HC (ANXA5, DPT, HIST1H2AH, LMNA, PPIA, RPL7 and 8). • 7 modified proteins between budesonide pre and post-treated asthmatics (α2M, ALDOA, ATP5B, DPYSL5, RPS20, SERPINB3, and VIM)	[379]
Terracciano R, 2011	• IS	MSB MALDI TOF/TOF	• HNP1, HNP2, HNP3, and three C-terminal amidated peptides with changes between asthma, COPD, and healthy volunteers.	[383]
Verrills NM, 2011	• Plasma	2-DE MALDI-TOF	• 20 proteins with differences between asthma, COPD, and controls. 4 of them able to discriminate between the three groups: α2M, HP, CP, and Hpx.	[390]

Continued on next page

Table 1 (Continued)

Cederfur C, 2012	• BALF	LC-MS/MS Label-Free (spectral counting)	• Some galectin-binding glycoproteins were found only in asthma compared to controls: CD59, keratin 8, HP, RAIG, SBEM, attractin, CD55, AGP, and SERPINA1	[376]
Izbicka E, 2012	• Plasma	LC- MS/MS Label-Free	• I-TAC, EGF, and MMP-9 were higher in asthmatics than in controls.	[391]
Choi GS, 2013	• NLF	2-DE (DIGE) MALDI-TOF	• ApoA1, α2M, and CP were increased in aspirin-induced nasal responders compared to non-responders.	[377]
Lee TH, 2013	• IS	2-DE MALDI-TOF	• HNP-2, S100A9, β-amylase, NGAL, 4-aminobutyrate transaminase, and cystatin SA were increased whereas plunc precursor, C3, GFAP, IgM κIIIb SON, MLL-AF4 der(11) fusion protein, CK-8, and IgG4 heavy chain were decreased in severe uncontrolled asthma compared to controlled asthma.	[384]

Continued on next page

Table 1 (Continued)

Mauri P, 2014	• BB	SCX-LC-MS/MS Label-Free (Spectral count)	• 23 proteins were unregulated in omalizumab responders and 84 in omalizumab non-responders after treatment. • LGALS3, HSPG2, ELN, TGFB1, VCL, EZR, MYH9, Myl6, MYH11, actin, ACTG2, FBLN5, and FBLN2 were only identified in omalizumab responders.	[357]
Suojalehto H, 2015	• IS • NLF	2D-DIGE LC-MS/MS	• FABP5 and VEGF were increased in asthmatics compared to healthy controls	[378]
Hamsten C, 2016	• Plasma	Suspension bead array	• Children with asthma display less CL5 and HPGDS levels and high NPSR1 levels.	[392]
Jiang H, 2016	• Serum	2-DE (DIGE) MALDI-TOF	• 7 proteins were differentially expressed in SRA vs. SSA: Up (MTMR9, VDBP, HP precursor, C4a) Down (ALB precursor, MASP2, ApoA1)	[358]
Xu H, 2016	• Plasma	Antibody array	• EPO and sGP130 were found augmented in childhood asthma compared to controls.	[393]

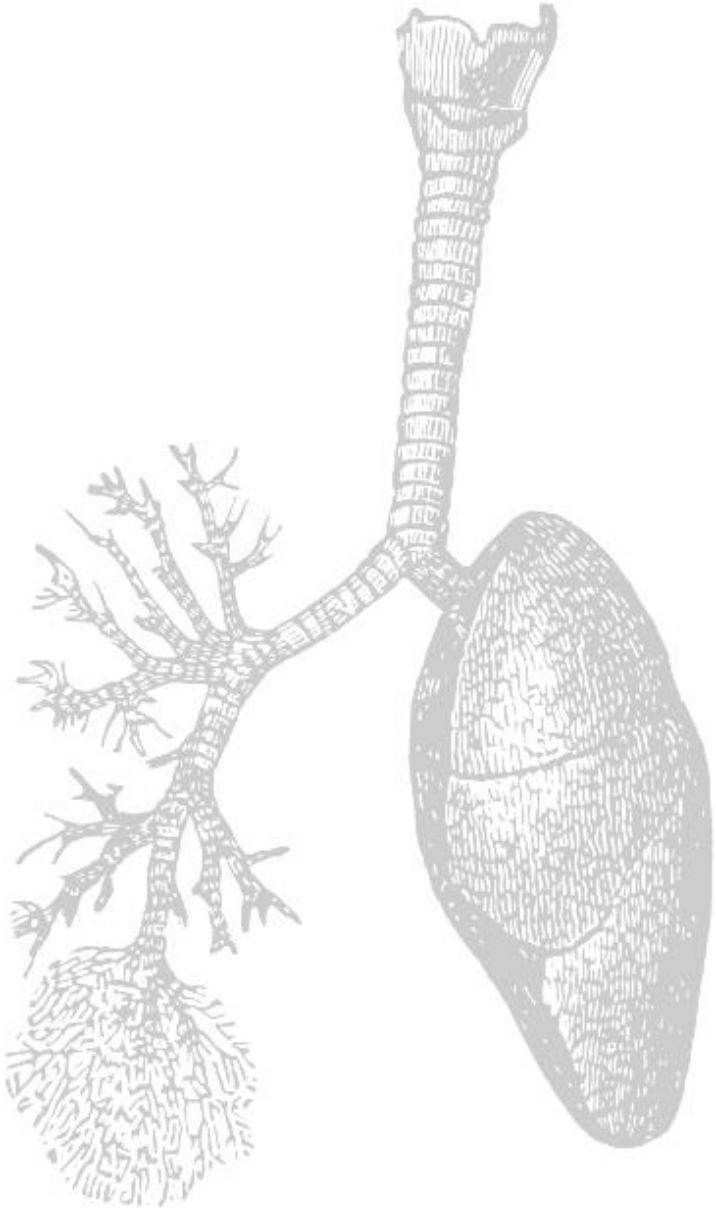
Continued on next page

Table 1 (Continued)

Cao C, 2017	• IS	LC-MS/MS	• 23 proteins increased in asthma compared to controls: A2M, ANXA1, APOA2, ELANE, GPI, S100A8/9/12, AGT, C5, FGA, FGB, APOA1, SERPINF2, CHI3L1, HPX, ITIH4, ORM1, ILRN, PRG2, B2M, CTSB, FN1.	[385]
Kasaian MT, 2018	• Serum • IS	Olink multiplex arrays	• 27 proteins with changes between controlled and uncontrolled asthma. • Serum: CCL11, CCL19, CCL25, CDCP1, FGF21, FGF23, Flt3L, IL-6, IL-10Rβ. • IS: ADA, AZU1, tPA, DNER, KLK6, MMP9, Chit1, GRN, PGLYRP1, MPO, HGF, PRTN3, RETN, PI3, Chi3L1, and OPG.	[386]

AC, affinity chromatography; AIA, aspirin-induced asthma; ATA, aspirin-tolerant asthma; BALF, bronchoalveolar lavage fluid; BB, bronchial biopsy; EBC, exhaled breath condensate; EIB, exercise-induced bronchoconstriction; IS, induced sputum; MSB, mesoporous silica beads; NB, nasal brushing; NLF, nasal lavage fluid; SRA, steroid-resistant asthma; SSA, steroid-sensitive asthma.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA



AIMS OF THE THESIS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The overall aim is to seek biomarkers related to rhinitis and different asthma phenotypes/severities. Identification of those molecules/cells is a step towards a nitid separation of asthma phenotypes, to learn about the underlying pathological mechanisms, and to develop more specific therapies intended to promote personalized medicine. Simultaneously, this thesis is also an effort to replicate and go beyond in the knowledge of the innate/adaptive immune system regarding asthma phenotypes/severities, but also an attempt to find new biomarkers through a non-target strategy based on serum proteomics. This general aim is specified in several distinctive objectives, detailed as follows:

1. To study the expression of CD14 in AA, the implications of the *CD14* (-159 C/T) SNP (rs2569190) on the levels of this molecule (mCD14/sCD14), as well as the relationship between this SNP and the susceptibility to suffer this disease (Chapter I)

2. To explore the expression pattern of CD26 in different lymphocyte subpopulations and their implications in rhinitis, AA, and NAA, as well as disease severity (Chapters II and III)

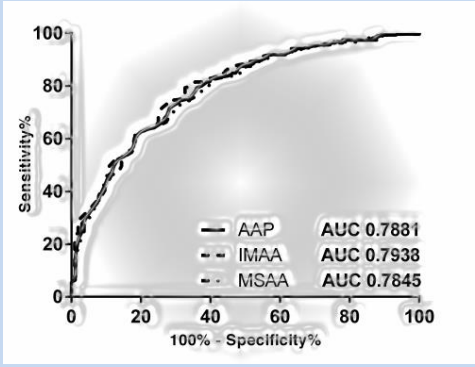
3. To develop a non-target biomarker discovery strategy to gain access to the medium-low abundant proteome of serum (Chapter IV)

4. To identify novel non-invasive biomarkers of rhinitis, AA, NAA, and different asthma severities (intermittent-mild and moderate-severe asthma) (Chapter IV)

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CHAPTER I

The CD14 (-159 C/T) SNP is associated with sCD14 levels and allergic asthma, but not with CD14 expression on monocytes.



The work presented in this chapter has been published in ***Scientific Reports*** with the following reference:

- *The CD14 (-159 C/T) SNP is associated with sCD14 levels and allergic asthma, but not with CD14 expression on monocytes. Sci Rep. 2018 Mar 7;8(1):4147. doi: 10.1038/s41598-018-20483-1.*

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

During asthma attacks allergens trigger lung epithelial cells to release cytokines (e.g., Thymic stromal lymphopoietin (TSLP), Interleukin (IL)-33, IL-25) that activate innate leukocytes and drive the differentiation of allergen-specific T helper $(TH)_2$ lymphocytes.[1] Innate defences also rely on pattern recognition receptors, such as toll-like receptors (TLRs), capable of detecting pathogen-associated molecular patterns (PAMPs). Lipopolysaccharide (LPS) is a PAMP that interacts with CD14 (monocytes, macrophages, and neutrophils),[2] a receptor whose gene has been linked to asthma/allergy.[3] CD14 transfers LPS to the TLR4-MD2 complex, which induces the secretion of a set of cytokines (IL-12/IL-18) that act as an innate-adaptive bridge and favour the TH_1 differentiation.[4] In addition, IL-12 also prevents the development of allergen-specific TH_2 cells, which ameliorates airway inflammation in allergic asthma.[4] Therefore, a functional defect in CD14 could alter TH_2 differentiation and IgE-mediated allergic diseases.[5]

Membrane-bound CD14 (mCD14) is released to the medium as soluble CD14 (sCD14),[2,6,7] which either potentiates the response to LPS in macrophages by inducing the release of proinflammatory cytokines,[8] or has a protective role by transferring LPS to lipoproteins.[9] sCD14 is released from monocytes through a process enhanced by LPS, IL-6 and IL-1β,[6] but decreased by interferon-γ (IFN-γ) and IL-4.[10] sCD14 inversely correlates with IL-4,[11] but positively with the number of sputum eosinophils.[12] Moreover, plasma sCD14 is a biomarker of ongoing or acute immune responses.[2,13] Thus, children with asthma exacerbations display augmented sCD14 levels compared to the recovery phase,[14,15] whereas adult asthmatics have higher amounts in sputum than healthy subjects.[12] An increased sCD14 concentration has also been detected in bronchoalveolar lavage

fluid (BALF) upon allergen exposure.[16] However, other works reported unaltered sCD14 levels, like for example in asthmatic children.[7,15]

Single-nucleotide polymorphisms (SNPs) also constitute modulatory agents for CD14 levels that could influence the risk factors for developing asthma or aggravate disease symptoms.[17] Baldini *et al.* found an association between *CD14* (-159 C/T) SNP (rs2569190) and sCD14 in atopic children, showing TT homozygotes higher levels than CT/CC genotypes[11] in line with the enhanced *CD14* transcriptional activity in the T allele.[6] Since those landmark studies, different works have confirmed these results,[18,19] while the C allele has been associated with high IgE levels, atopy and asthma.[15,17,20] However, this association has not been consistently replicated.[3,13,21-24] These different results may be attributed to a number of factors, like the low sample size, asthma phenotype (atopic, non-atopic or mixed asthma), age of study population (children or adults), ethnicity (African, Caucasian or Asiatic population) or gene-environment interactions (e.g., endotoxin levels).[21,25] Therefore, it seems necessary to undertake new works aimed to the simultaneous measurement of both membrane and soluble forms of CD14 as well as the *CD14* (-159 C/T) SNP in adult Caucasian subjects with allergic asthma and different degrees of severity (intermittent-mild and moderate-severe).

MATERIAL AND METHODS

Subjects

The study population was recruited from January 2009 to December 2012, at the Unit of Pneumology and Allergy of the USC University Hospital Complex of Santiago de Compostela (CHUS) and the Pontevedra Hospital Complex (CHOP). This study population

included 277 healthy controls (HC) and 277 allergic asthmatics (AA), consisting of 108 intermittent-mild allergic asthmatics (IMAA) and 169 moderate-severe allergic asthmatics (MSAA). Asthma and allergy diagnosis was confirmed according to the Global Strategy for Asthma Management and Prevention criteria (GINA 2006, http://www.seicap.es/documentos/archivos/GINA2006general.pdf).

All patients were in a stable phase for at least 4 weeks before the study initiation. Forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and the FEV1/FVC ratio, were measured. Asthma diagnosis was confirmed by a positive bronchodilator test (>12% of FEV1 change after salbutamol) or methacholine challenge. Allergic sensitization was confirmed through a skin prick test or serum IgE specific to frequent allergens. Other variables were measured: smoking, pets at home, residence (rural/urban), profession, comorbidities, the age of symptoms onset, asthma control, or number of visits to emergency units, family doctors or hospitals during the year prior to the study initiation. HC were selected from patients scheduled in the hospital for minor surgeries such as orthopedic surgery or inguinal hernia, and smoke and systemic diseases or allergies were used as exclusion criteria. The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The project was also approved by the Ethics Committee of Clinical Research of Galicia (2011/001), Spain, and all subjects signed informed consent statements.

Flow cytometry assays

Venous peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) treated tubes (BD Vacutainer K2E). To analyse the expression of CD14 on peripheral blood leukocytes, CD14-FITC (Mouse IgG2a κ; BD Biosciences) or Isotype-FITC (Mouse IgG2a; BD Biosciences) were added to 100 μL of whole blood (30 min, room

temp.). Then, red cells were lysed (BD FACS™ Lysing Solution). Finally, 10 000 events were collected and analysed by means of a BD FACSCalibur flow cytometer. Data were examined using WinMDI 2.9 software (Joseph Trotter, La Jolla, CA. USA).

Biochemical determinations

Biochemical determinations were carried out by an ADVIA®1650 analyser (SIEMENS Healthcare Diagnostics S.L., Berlin, Germany) while neuron-specific enolase (NSE) was measured with electrochemiluminescence analyser (MODULAR ANALYTICS Cobas E-601, Roche Diagnostics; Mannheim, Germany). The nucleated cell counting was performed using an ADVIA®2120 hematology counter (SIEMENS Healthcare Diagnostics S.L., Berlin, Germany).

Serum sCD14 levels were measured by means of an enzyme-linked immunosorbent assay (ELISA) with the Quantikine®Human sCD14 Immunoassay kit (R&D Systems, MN, USA). Optical densities were recorded at 450 nm and protein concentration was calculated from standard curves.

Genomic DNA purification and *CD14* (-159 C/T) promoter SNP (rs2569190) studies

Genomic DNA was purified from whole blood (200 µL) with the QIAamp® DNA Mini Kit (QIAGEN, Melbourne, Australia). The subsequent study of the rs2569190 SNP in the CD14 promoter was conducted by the CEGEN-PRB2 USC node using the iPlex® Gold chemistry and MassARRAY platform, according to manufacturer's instructions (Agena Bioscience, San Diego, CA). Genotyping assay (Polymerase chain reaction/PCR primers and single-base-extension/SBE primers) were designed using the Agena Bioscience MassARRAY Assay Designer 4.1 software. To avoid confusion in the

mass spectrum, a tag (5-ACGTTGGATG-3) was added to the 5´ end of each PCR primer. Both SBE and PCR primer sequences are shown in Supplementary Table 1. PCR reaction was set up in a 5 µL volume and contained template DNA (20 ng), 1× PCR buffer, $MgCl_2$ (2 mM), dNTPs (500 µM) and PCR enzyme (1 U/reaction). A pool of PCR primers was made at a final concentration of each primer of 100 nM (Metabion International AG, Germany). The thermal cycling conditions for the reaction consisted of an initial denaturation step at 95°C for 2 minutes, followed by 45 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute, and a final extension step of 72°C for 5 minutes. PCR products were treated with shrimp alkaline phosphatase (1.5 U) by incubation at 37°C for 40 min, followed by enzyme inactivation by heating at 85°C for 5 min to neutralize unincorporated dNTPs.

The iPLEX GOLD reactions were set up in a final 9 µL volume and contained 0.222x iPLEX buffer Plus, 0.222x iPLEX termination mix and iPLEX enzyme (1.35 U/reaction). An SBE primer mix was made to give a final concentration of each primer between 0.52 µM and 1.57 µM (Metabion International AG, Germany). The thermal cycling conditions for the reaction included an initial denaturation step at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, with an internal 5 cycles loop at 52°C for 5 seconds and 80°C for 5 seconds, followed by a final extension step of 72°C for 3 minutes. The next step is to desalt the iPLEX Gold reaction products with Clean Resin following the manufacturer's protocol. The desalted products were dispensed onto a 384 Spectrochip II using an RS1000 Nanodispenser and spectra were acquired using the MA4 mass spectrometer, followed by manual inspection of spectra by trained personnel using MassARRAY Typer software, version 4.0. All assays were performed in 384-well plates, including negative controls and a trio of Coriell samples (Na10830, Na10831 and Na12147) for

quality control. 10% of random samples were tested in duplicate and the reproducibility was 100%.

Statistics

Descriptive data are presented as either median (interquartile range; IQR1-3) or percentages. To assess the significance of changes between AA and HC, Mann–Whitney U two-tailed test or Kruskal–Wallis One Way Analysis of Variance on Ranks followed by Dunn's multiple comparison test were used. Receiver Operating Characteristic (ROC) curves and Spearman´s association test were also employed, while differences in proportions were assayed by the χ^2 test. To evaluate the association between *CD14* (-159 C/T) SNP and the risk of allergic asthma, Odd´s ratios (ORs) and 95% confidence intervals (CIs) were calculated according to different models: TT+TC vs. CC (dominant model), TT vs. TC+CC (recessive model), TT vs. CC, TC vs. CC, and T vs. C (allelic model). Hardy–Weinberg equilibrium (HWE) was calculated by using Pearson χ^2 test. All analyses and graphs were conducted using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Jose, California, USA). Data are presented in box and whisker plots, where median, 25 and 75 quartiles, 5-95 percentiles (error bars) and anomalous values are shown. The statistical signification was defined as $P < 0.05$.

RESULTS

Demographic and clinical characteristics of the study population

In this work a case-control study was performed, where adult patients with both intermittent-mild and moderate-severe allergic asthma were recruited. These patients were in a clinically stable state, had a well-

controlled disease, and the majority were non-smokers under treatment with inhaled corticosteroids (Table 1). FEV1 (%) and FEV1/FVC ratio (%) values are described in Table 1, showing decreased levels in MSAA (moderate-severe allergic asthmatics) compared to IMAA (intermittent-mild allergic asthmatics) (Table 1). Although 66.4% of AA came from rural areas, most of them had no animals or just dogs/cats; only 11% had farm animals.

Table 1. Characteristics of the study population.

	AA			HC
	ALL	**IMAA**	**MSAA**	
N	277	108	169	277
Age[a]	32 (25-42)	31 (22-38)	33 (26-46)	46 (31-60)
Sex (M/F)	119/158	42/66	77/92	105/172
Smokers (%)	21.3	15.7	24.8	0
Control:				
Good	219	98	121	-
Partial	36	8	28	-
Bad	22	2	20	-
Treatment:				
No	66	66	0	-
Inhaled Corticosteroids	196	34	162	-
Oral Corticosteroids	15	0	15	-
Antileukotrienes	63	6	57	-
Omalizumab	4	0	4	-
FEV1 (%)	97.2 (83.7-108.0)	105.0 (95.3-115.7)[#]	93 (74.5-102.2)[#]	-
FEV1/FVC (%)	78.0 (70.7-85.4)	82.9 (75.9-88.4)[#]	74.7 (67.6-81.9)[#]	-
Neutrophils (10^3 cells/µL)[a]	3.67 (2.77-4.50)	3.53 (2.99-4.27)	3.71 (2.90-4.96)	3.54 (2.77-4.50)
Lymphocytes (10^3 cells/µL)[a]	2.22 (1.85-2.71)[*]	2.20 (1.86-2.79)[&]	2.23 (1.80-2.71)[&]	2.02 (1.62-2.45)
Monocytes (10^3 cells/µL)[a]	0.47 (0.38-0.59)[*]	0.47 (0.39-0.56)[&]	0.46 (0.37-0.62)[&]	0.35 (0.28-0.43)

Continued on next page

Table 1 (Continued)				
Eosinophils (10^3 cells/µL)[a]	0.33 (0.19-0.51)[*]	0.29 (0.20-0.51)[&]	0.34 (0.18-0.51)[&]	0.16 (0.11-0.23)
Basophils ((10^3 cells/µL)[a]	0.04 (0.03-0.05)	0.04 (0.03-0.05)	0.04 (0.02-0.06)	0.04 (0.03-0.06)
IgE (IU/mL)[a]	272 (116-533)[*]	270 (111-485)[&]	276 (122-668)[&]	34 (11-102)

AA, allergic asthmatics; HC, healthy controls; IMAA, intermittent-mild allergic asthmatics; MSAA, moderate-severe allergic asthmatics.

[a] Median value (IQR1-3); [*] AA vs HC: Mann-Witney U Statistic, $P < 0.001$; [#] IMAA vs MSAA: Mann-Witney U Statistic, $P < 0.001$; [&] IMAA vs MSAA vs HC: Kruskal-Wallis One Way Analysis of Variance on Ranks ($P < 0.001$), IMAA/MSAA vs HC $P < 0.05$ Dunn's Method.

According to their allergic disease state, patients had significantly augmented levels of eosinophils and total IgE compared to HC, but there were no significant changes regarding disease severity (Table 1). IgE showed a positive correlation with eosinophil, monocyte and, to a lesser extent, lymphocyte blood count, underlying the relevance of these subsets in allergic asthma pathogenesis (Table 2). Since activation of eosinophils and macrophages has been associated with enhanced NSE levels under some pathological conditions,[26,27] we also undertook the measurement of this enzyme in serum samples. As table 2 shows, it was found a positive correlation of IgE, eosinophils and monocytes with NSE (Table 2), which prompted us to examine the utility of this parameter as an additional marker of allergic asthma. As shown in Figure 1a, NSE levels were augmented in AA compared to HC and tend to be around 14.6% higher in men than in women (Figure 1a). Moreover, the area under the curve (AUC) of the ROC plot for NSE levels was close to AUC of total IgE and higher than AUC of blood eosinophils (absolute values) (Figure 1b).

Table 2. Spearman correlation matrix of the study population.

VARIABLES	NSE	CRP	IgE	mCD14[#]	sCD14	Age
Leucocyte count	**0.163***	**0.221*****	**0.192*****	-0.021	-0.035	**-0.165*****
Neutrophil count	0.003	**0.190*****	-0.027	-0.027	-0.034	-0.052
Lymphocyte count	0.077	**0.107***	**0.143*****	-0.020	-0091	**-0.152*****
Monocyte count	**0.303*****	**0.154*****	**0.336*****	**0.234***P**	-0.021	**-0.182*****
Eosinophil count	**0.279*****	0.054	**0.459*****	**-0.199*****	-0.071	**-0.273*****
Basophil count	**0.149*****	-0.024	0.057	-0.034	-0.005	**-0.113***
FEV1%	0.099P	-0.088P	-0.057P	0.140P	-0.177P	**-0.209***P**
FEV1/FVC	0.106P	-0.071P	-0.051P	0.151	-0.146P	**-0.375***P**
TNF	-0.022	**0.179*****	-0.011	0.082	**0.119****	**0.198*****
NSE		-0.004	**0.322*****	**-0.215*****	0.087	**-0.128****
CRP			-0.033	-0.000	**0.158*****	**0.119****
IgE				**-0.264*****	-0.054	**-0.296*****
mCD14[#]					-0.079	**0.177*****
sCD14						**0.102***

CRP, C-reactive protein; IgE, immunoglobulin E; NSE, neuron specific enolase; P, patient population; TNF, tumour necrosis factor. *P<0.05, **P<0.01, ***P<0.001. [#] % of CD14[+] monocytes.

No alterations were appreciated for C-reactive protein (CRP), IgG, IgA, IgM, or tumour necrosis factor (TNF) in AA. The influence of age was also taken into consideration, with a negative correlation with FEV1%, FEV1/FVC, IgE and leukocyte subtypes (mostly eosinophils), but a small positive association with TNF, CRP, mCD14 and sCD14 (Table 2).

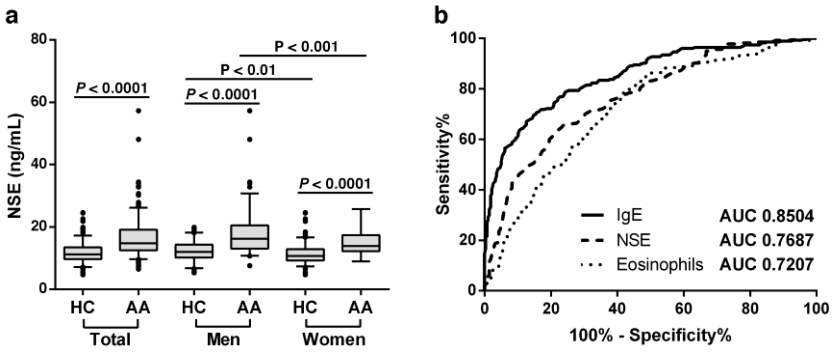


Figure 1. Analysis of NSE values in serum from AA and HC. (**a**) NSE levels in allergic asthmatics (AA) and healthy controls (HC) segregated by gender. Numbers on the graphs represent *P*-values (Mann-Whitney U test). (**b**) ROC curves for IgE, NSE and peripheral blood eosinophils. AUC values are shown for each parameter.

Allergic asthma enhances the number of peripheral blood monocytes, but causes a reduction of mCD14 in these cells

Complete blood count revealed an increased number of leukocytes in AA (HC 6.44 (5.39-7.75) x 10^3 cells/μL vs. AA 6.93 (6.00-8.23) x 10^3 cells/μL; $P < 0.001$). These differences were partially dependent on some innate subsets like eosinophils and monocytes, but not neutrophils or basophils (Table 1). Moreover, men had higher numbers of monocytes in peripheral blood than women ($P < 0.0001$), and asthma caused a slight but significant elevation of monocytes in both IMAA and MSAA (Figure 2a). mCD14 was mainly expressed by monocytes (>90% $CD14^+$) (Supplementary Figure 1), and asthma produced a decrease in the percentage of $CD14^+$ monocytes (and mean fluorescence intensity/MFI values), without changes between IMAA and MSAA (Figures 2b and c). Indeed, when a ROC curve was constructed for mCD14 values (%), the AUC gave a value of 0.7881 (whole asthmatics), which underlines the potential use of this

parameter as an allergic asthma marker (Figure 2d), even after segregating the patients in IMAA and MSAA.

Apart from monocytes, neutrophils and lymphocytes also contain $CD14^+$ cells, but the expression of this marker is much lower than in monocytes (Supplementary Figure 1). Because of this, we assessed the differences between neutrophils and lymphocytes-associated mCD14 levels in AA and HC, but they were not influenced by the presence of allergic asthma (data not shown).

Reduction of relative but not absolute sCD14 levels in allergic asthma

Despite the decreased levels of mCD14 in monocytes from AA, we did not reach significant differences in the serum concentration of sCD14 in IMAA, MSAA and HC (data not shown). Moreover, when a cut-off value of total IgE (80 IU/mL) was selected to maximize the true positive rate (70.76%) and minimize the false positive rate (15.88%) (Figure 1b), sCD14 levels remained unaltered in donors with IgE < 80 IU/mL or ≥ 80 IU/mL.

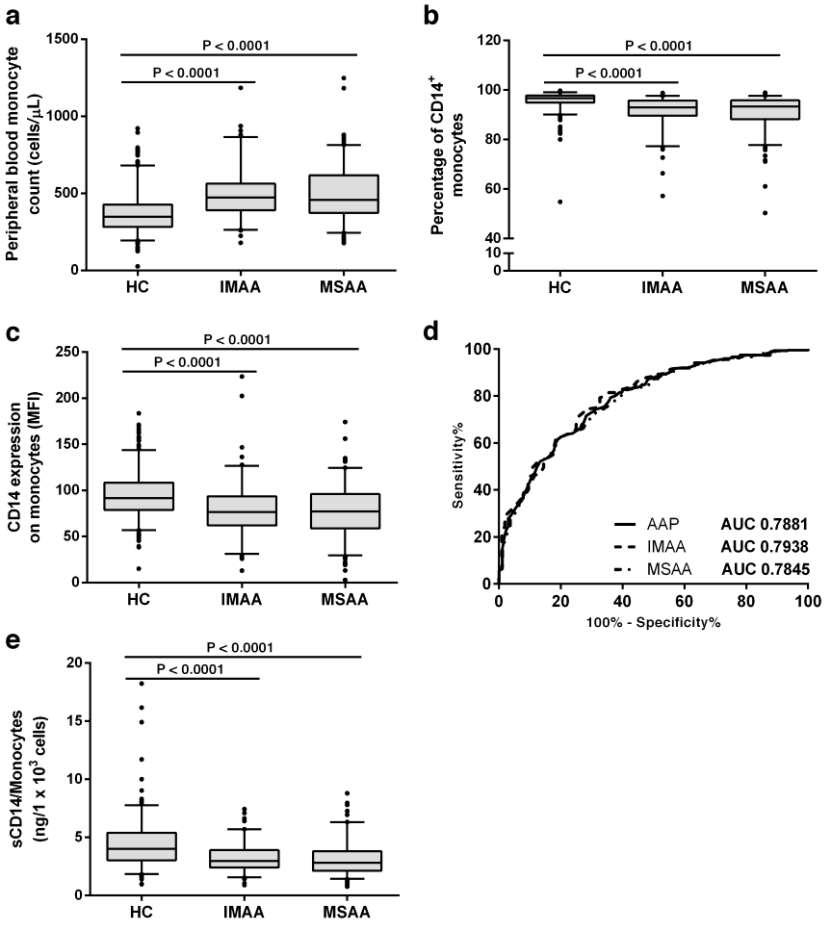


Figure 2. Monocyte counts and mCD14/sCD14 levels in AA and HC. (**a**) Peripheral blood monocytes count in IMAA, MSAA and HC. Percentage of $CD14^+$ cells (**b**) and mean fluorescence intensity (MFI) of CD14 on monocytes (**c**) in IMAA, MSAA and HC. (**d**) ROC curve for the percentage of $CD14^+$ monocytes. (**e**) Relative sCD14 levels (ng/1 x 10^3 monocytes) in serum samples from IMAA, MSAA and HC. Numbers on the graphs represent *P*-values (Mann-Whitney U test).

As our results were supporting the presence of altered numbers of monocytes in asthmatic patients, and monocytes were the most likely source of sCD14, we evaluated the correlation between the absolute count of peripheral blood monocytes and the serum concentration of sCD14, with negative results (Table 2). In spite of this, sCD14 levels could still be influenced by the combined effects of increased monocyte count and decreased expression of mCD14 on this leukocyte subset (phenotype). Accordingly, sCD14 values were normalised with respect to the absolute number of circulating monocytes to maximize the effect of cell phenotype, finding in this case a significant reduction of sCD14 in AA compared to HC even after segregating by disease severity (Figure 2e). We also detected higher levels of relative sCD14 in women compared to men (data not shown).

We next examined the mCD14-sCD14 correlation. In order to analyse the strength of this relationship, we have taken into account the absolute and normalized values of sCD14, as well as we have analysed the data as a whole or after segregating our results according to sex or donor group (healthy or diseased). However, we have found no connection between both parameters (Table 2, whole set of mCD14-sCD14 pairs), which underlines an independent regulation of both molecules. Apart from the monocyte count, alternative reasons might explain this low correlation, like other sCD14 sources (e.g. hepatocytes) or the existence of different genetic backgrounds. Regarding the first possibility, there were no differences in CRP or TNF between HC and AA ($P > 0.05$), or evidence of respiratory infection, even though there is a very small correlation between CRP or TNF and sCD14 (Table 2). Therefore, we next analysed the influence of the *CD14* (-159 C/T) SNP genotype (rs2569190) on mCD14/sCD14 levels.

Genotypic and allelic frequency distribution of *CD14* (-159 C/T) SNP in the study population

Table 3. Genotype and allele frequencies of the *CD14* (-159 C/T) SNP in the study population.

		Genotype frequencies (%)				Allele frequencies (%)		
	N	**TT**	**TC**	**CC**		**T**	**C**	
HC	277	79 (28.5)	138 (49.8)	60 (21.7)		296 (53.4)	258 (46.6)	
AA	277	59 (21.3)	137 (49.5)	81 (29.2)	**χ^2 =6.030 *P* =0.049***	255 (46.0)	299 (54.0)	**χ^2 =5.776[a] *P* =0.016***
IMAA	108	26 (24.1)	52 (48.1)	30 (27.8)	χ^2 =1.851 *P* =0.3963	104 (48.1)	112 (51.9)	χ^2 =1.531[a] *P* =0.2159
MSAA	169	33 (19.5)	85 (50.3)	51 (30.2)	**χ^2 =6.444 *P* =0.039***	151 (44.7)	187 (55.3)	**χ^2 =6.091[a] *P* =0.013***

AA, allergic asthmatics; HC, healthy controls; IMAA, intermittent-mild allergic asthmatics; MSAA, moderate-severe allergic asthmatics. [a]Yates correction. *Significant difference.

Genotype (TT, TC, CC) and allelic (C, T) frequencies were calculated in this study, and their distribution presented in Table 3. The whole population (χ^2 = 0.014, $P > 0.05$), HC (χ^2 = 0.002, $P > 0.05$), AA (χ^2 = 0.007, $P > 0.05$), IMAA (χ^2 = 0.041, $P > 0.05$), and MSAA (χ^2 = 0.012, $P > 0.05$) were in HWE. Moreover, there was

association between the (-159 C/T) SNP and the presence of asthma (Table 3), showing a decrease in the frequency of the T allele in AA (P = 0.016) and MSAA (P = 0.013), but not in IMAA compared to HC. Furthermore, the frequency of TT genotype of CD14 polymorphism were significantly lower in AA than in HC (P = 0.049), being only significant in MSAA (P = 0.039) when patients were segregated according to disease severity (Table 3).

As allelic and genotypic frequencies seem to be related to disease severity, we further divide MSAA into moderate (N=129) and severe allergic asthmatics (N=40). After this segregation, the association of the (-159 C/T) SNP was only maintained in severe asthmatics, both the allelic (T vs. C, χ^2 = 6.478, P = 0.011) and genotypic (TT vs. CC, χ^2 = 7.429, P = 0.024) frequencies, and it was lost in moderate asthmatics (T vs. C, χ^2 = 2.749, P = 0.097; TT vs. CC, χ^2 = 3.040, P = 0.219).

Association between *CD14* (-159 C/T) SNP and allergic asthma risk

A summary of allergic asthma risk according to the different genetic models is presented in Table 4. We found an association between T allele and a decreased allergic asthma risk in the overall allergic asthma population (T vs. C: OR = 0.74, 95% CI = 0.59-0.94, P = *0.0162*). Moreover, when AA were subset by disease severity (IMAA and MSAA vs. HC), these association was maintained only for MSAA (OR = 0.70, 95% CI = 0.54-0.92, P = *0.0136*), while IMAA lost the allelic association (Table 4).

Table 4. Association between *CD14* (-159 C/T) SNP and allergic asthma risk.

		OR (95% CI)	χ^2 (Yate´s correction)	*P*
AA (ALL)	**TT+TC vs CC**	0.67 (0.45-0.98)*	3.805	0.0511
	TT vs TC+CC	0.68 (0.46-1.00)	3.484	0.0620
	TT vs CC	0.55 (0.34-0.89)*	5.449	0.0196*
	TC vs CC	0.74 (0.49-1.11)	1.180	0.1704
	T vs C	0.74 (0.59-0.94)*	5.776	0.0162*
IMAA	**TT+TC vs CC**	0.72 (0.43-1.20)	1.300	0.2543
	TT vs TC+CC	0.79 (0.48-1.33)	0.566	0.4517
	TT vs CC	0.66 (0.35-1.23)	1.346	0.2460
	TC vs CC	0.75 (0.44-1.30)	0.781	0.3768
	T vs C	0.81 (0.59-1.11)	1.531	0.2159
MSAA	**TT+TC vs CC**	0.64 (0.42-0.99)*	3.630	0.0547
	TT vs TC+CC	0.61 (0.38-0.96)*	4.029	0.0442*
	TT vs CC	0.49 (0.28-0.85)*	5.767	0.0163*
	TC vs CC	0.72 (0.46-1.15)	1.572	0.2100
	T vs C	0.70 (0.54-0.92)*	6.091	0.0136*

AA, allergic asthmatics; CI, confidence interval; IMAA, intermittent-mild allergic asthmatics; MSAA, moderate-severe allergic asthmatics; OR, odds ratio; TC vs CC, heterozygote; TT vs CC, homozygote; TT vs TC+CC, recessive model; TT+TC vs CC, dominant model.
*Significant difference.

Regarding to the other genetic models, a significant association of *CD14* (-159 C/T) and allergic asthma risk was found between the homozygotes TT vs. CC (OR = 0.55, 95% CI = 0.34-0.89, *P = 0.0196*), and almost reached significance in a dominant model (TT+TC vs. CC: OR = 0.67, 95% CI = 0.45–0.98, *P = 0.0511*) (Table 4). Furthermore, after segregating by disease severity, it was found an association of allergic asthma risk with this SNP only in MSAA according to a recessive model (TT vs. TC+CC: OR = 0.61, 95% CI = 0.38-0.96, *P = 0.0442*) or TT vs. CC genotype comparisons (OR = 0.49, 95% CI = 0.28-0.85, *P = 0.0163*). Therefore, the results suggested that T allele and TT homozygote individuals have decreased risk of allergic asthma compared with C allele and CC homozygote carriers, respectively.

The influence of the *CD14* (-159 C/T) SNP on CD14 levels

Although we have shown augmented peripheral blood monocyte count and decreased levels of mCD14 (Figures 2a-c), the number of monocytes was not influenced by the *CD14* (-159 C/T) SNP genotype. We also failed to detect any change in mCD14 related to the SNP genotype (data not shown). In contrast (and regardless of whether they belong to the control group or to the asthmatic population), CC genotypes and to a lesser extent TC genotypes had lower concentrations of sCD14 (absolute values) than TT carriers, while no significant differences were observed between TC and CC subjects (Figure 3a). This association was maintained between TT and CC carriers when sCD14 levels were normalised by the absolute count of monocytes (Figure 3b). Therefore, this SNP could be partially responsible for the reduction of normalised sCD14 levels in AA and influence the severity of this disease (Figure 2e).

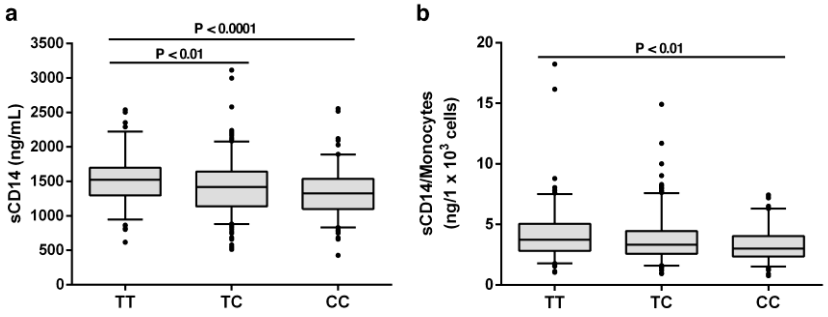


Figure 3. Impact of the *CD14* (-159 C/T) SNP (rs2569190) on sCD14 levels in serum. Absolute (ng/mL) (**a**) and relative (ng/1 x 10^3 monocytes) (**b**) sCD14 levels in serum samples from TT, TC and CC donors (healthy and asthmatics). *Kruskal-Wallis One Way Analysis of Variance on Ranks, $P < 0.001$; Dunn's Method was used for multiple comparisons (numbers on the graph represent *P*-values).

DISCUSSION

In the current study, we report data supporting an increase of NSE and monocytes and a down-modulation of their mCD14 expression in allergic asthma regardless of disease severity. In addition, we detect a decrease of normalised sCD14 values in serum samples from asthmatics, suggesting the expansion of a $CD14^{low}$ monocyte subset and the influence of the *CD14* (-159 C/T) SNP genotype. Indeed, we evidence an association of the T allele and TT genotype of *CD14* (-159 C/T) polymorphism with reduced risk of moderate-severe allergic asthma.

AA in our study have an atopic disease with eosinophilia, monocytosis and elevated IgE levels. NSE is the neuronal isomer of the glycolytic enzyme 2-phospho-D-glycerate hydrolase, and a typical biomarker of small cell lung cancer.[28] Nevertheless, changes in non-malignant inflammatory lung diseases have also been found,[27,29-32]

since this enzyme can translocate towards the cell surface upon proper stimulatory signals to enhance a proinflammatory response.[33] Our results support the presence of higher NSE levels in men, as previously reported by Collazos *et al*.,[29] but contrary to this work our asthma patient cohort displays above-normal NSE levels in serum. Monocytes/macrophages appear to be a possible source of NSE,[27,33] and increased numbers of monocytes as well as a correlation of them with NSE levels were demonstrated in our study. However, other plausible sources of NSE are eosinophils or injured epithelial cells during pulmonary infiltration,[26] while the neuronal distress or hypoxia occurring in the lung also could play a role during the disease.[29]

Apart from other cells as T and B lymphocytes, eosinophils, basophils or neutrophils, monocytes are gaining importance as regulators of inflammation in asthma and as key players in the pathogenesis.[34-36] Our results show the expansion of this subset in AA regardless of the severity of the symptoms (i.e., IMAA and MSAA), as well as a reduction of mCD14, a monocyte marker associated to asthma.[3] In contrast, some authors have described no differences in the staining for mCD14,[37,38] even though these could be the result of a low statistical sample size. Reduced levels of mCD14 or TLR4 in AA makes biological sense,[39] as signal transduction through CD14/TLR4 leads to IL-12 secretion, a powerful inducer of TH_1 polarization.[13] Therefore, attenuated mCD14 levels on antigen presenting cells (APCs) could favour TH_2-driven allergic asthma.[4,5,13,16] This diminished number of mCD14 molecules on monocytes could arise as a result of several, and not mutually exclusive, mechanisms: a) altered transcription/translation rates affecting protein abundance; b) expansion of $CD14^{low}$ monocyte subsets; c) a vesicle- or enzymatic-mediated mechanism that release mCD14 from monocytes and should also affect sCD14 concentration.

The degree of mCD14 down-modulation on monocytes suggests the preferential expansion of a small $CD14^{low}$ subset and not a globally altered transcription/translation rate. This, for example, is in line with the increased percentage of $CD14^{low/-}$ monocytes upon *in vitro* culture in the presence of TSLP, a cytokine important in allergic asthma.[40] Monocytes are heterogeneous, with major ($CD14^{high}$) and minor ($CD14^{low}$) subsets.[33] $CD14^{high}$ ("classical") monocytes display a CD16/FcγRIII^{-} phenotype, while the less frequent $CD16^{+}$ subset consists of both intermediate ($CD14^{high}CD16^{+}$) and non-classical ($CD14^{low}CD16^{+}$) subpopulations.[34] $CD16^{+}$ monocytes, particularly the intermediate subset, are expanded in inflammation, severe asthma or upon allergen challenge,[34,36] in line with their pro-inflammatory nature.[41] A major constraint of our study is that we have not analysed CD16, but our results show a significant down-modulation of mCD14 in allergic asthma, which appears to rather support the expansion of $CD14^{low}CD16^{+}$ monocytes. These cells (non-classical subset) express high levels of CD80, CD86, and CD163, suggesting a high antigen presenting capability.[42] Furthermore, non-classical monocytes are in an advanced differentiation stage and they evidence high invading ability to infiltrate and differentiate into M2-type macrophages,[43] a subset related to allergic inflammation.[44]

CD14 can be released to medium from hepatocytes as an acute phase protein.[45] Although we saw a small correlation between sCD14 and CRP or TNF, the levels of these two last molecules had no changes between AA and HC, and our patients were in a steady-state of the disease. Excluding the hepatocyte origin, monocytes are the most likely cell source of sCD14. Down-modulation of mCD14 in monocytes from AA does not fit with either its shedding[2,6,7] or the release of mCD14-enriched vesicles [http://exocarta.org/gene_summary?gene_id=12475] from these cells, because both processes should lead to a higher number of sCD14

molecules in the extracellular compartment, as happen during the acute phase.[15,16,46,47] However, patients in our study are in a chronic phase, where there is no relationship between monocyte counts and sCD14 or mCD14-sCD14 correlation.[16] Therefore, our results only make sense considering a puzzling scenery with an elevation of monocyte numbers and enhanced frequencies of both $CD14^{high}$,[34] but also $CD14^{low}$ (our results) subsets of monocytes in asthma. Indeed, in our study only normalised serum levels of sCD14 were significantly reduced in patients. In agreement, sCD14 levels have been inversely correlated with IL-4-production,[11] total IgE,[11] or asthma severity.[46] However, some authors have detected higher levels[12] or no differences[7,15] of baseline sCD14 in peripheral blood from asthmatics. Hence, we cannot rule out the contribution of many potential confounding factors that explain these different results, like gene-gene or gene-environment interactions.[11,15]

One of the most studied CD14 polymorphisms in asthma is the *CD14* (-159 C/T) SNP (rs2569190).[11] Previous studies investigating the association of this SNP with allergic asthma yielded variable results regarding the strength and direction of the association.[3,17,24] These contradictory results can be explained by differences in ethnicity, low sample size, the age of patients or gene-environment interaction.[21,25] We performed our study in a well-defined population (Caucasian, adults, allergic asthmatics and mostly non-farmers), with a high sample size (277 AA vs. 277 HC), and two different disease severity grades (IMAA and MSAA). In agreement with others,[11,17] we show an association of the frequency of the C allele and the CC genotype with allergic asthma (whole asthmatics). More interesting, this association is also related to the disease severity, as it is only maintained in MSAA and within this group, in severe asthmatics. Moreover, the risk of having moderate-severe allergic asthma (but not intermittent-mild asthma) is lower in carriers of the T allele (T vs. C)

and TT genotype, following either a recessive model (TT vs. TC+CC) or after comparing TT vs. CC homozygotes. As other works have shown,[5,11,12,18,19] we evidence augmented sCD14 levels in subjects carrying the TT genotype but no association of this polymorphism with mCD14 levels on monocytes. This suggests an adverse role for the C allele, the CC genotype and the presence of low levels of sCD14/mCD14 in allergic asthma or atopy,[11,46] especially among adult and atopic subjects exposed to low levels of endotoxin, like our cohort.[13]

In summary, our findings show an increment in the serum levels of NSE, which could be used as a novel biomarker of allergic asthma. On the other hand, we also found a decrease in the expression of CD14 on monocytes from allergic asthmatic patients, probably related to an increase of $CD14^{low}$ monocyte subset. Moreover, we evidence an association of the (-159 C/T) SNP in the CD14 promoter with allergic asthma, and a decreased risk of having moderate-severe allergic asthma in carriers of T allele and TT genotype. Furthermore, TT genotype is associated with higher levels of sCD14, pointing out a protective role for the T allele in this disease.

REFERENCES

1. Martinez, F. D. & Vercelli, D. Asthma. *Lancet* **382,** 1360-1372 (2013).
2. White, A. F. & Demchenko, A. V. Modulating LPS signal transduction at the LPS receptor complex with synthetic Lipid A analogues. *Adv. Carbohydr. Chem. Biochem.* **71,** 339-389 (2014).
3. Wang, D., Yang, Y., Xu, J., Zhou, Z. K. & Yu, H. Y. Association of CD14 -159 (-260C/T) polymorphism and

asthma risk: an updated genetic meta-analysis study. *Medicine (Baltimore)* **95,** e4959 (2016).

4. Vignali, D. A. & Kuchroo, V. K. IL-12 family cytokines: immunological playmakers. *Nat. Immunol.* **13,** 722-728 (2012).
5. Tesse, R., Pandey, R. C. & Kabesch, M. Genetic variations in toll-like receptor pathway genes influence asthma and atopy. *Allergy* **66,** 307–316 (2011).
6. Shive, C. L., Jiang, W., Anthony, D. D. & Lederman, M. M. Soluble CD14 is a nonspecific marker of monocyte activation. *AIDS* **29,** 1263-1265 (2015).
7. Marcos, V. *et al.* Expression, regulation and clinical significance of soluble and membrane CD14 receptors in pediatric inflammatory lung diseases. *Respir. Res.* **11,** 32 (2010).
8. Lévêque, M. *et al.* Soluble CD14 acts as a DAMP in human macrophages: origin and involvement in inflammatory cytokine/chemokine production. *FASEB J.* **31,** 1891-1902 (2017).
9. Kitchens, R. L., Thompson, P. A., Viriyakosol, S., O'Keefe, G. E. & Munford, R. S. Plasma CD14 decreases monocyte responses to LPS by transferring cell-bound LPS to plasma lipoproteins. *J. Clin. Invest*. **108,** 85-93 (2001).
10. Landmann, R., Fisscher, A. E. & Obrecht, J. P. Interferon-gamma and interleukin-4 down-regulate soluble CD14 release in human monocytes and macrophages. *J. Leukoc. Biol.* **52,** 323-330 (1992).
11. Baldini, M. *et al.* A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am. J. Respir. Cell Mol. Biol.* **20,** 976-983 (1999).

12. Alexis, N., Eldridge, M., Reed, W., Bromberg, P. & Peden, D. B. CD14-dependent airway neutrophil response to inhaled LPS: role of atopy. *J. Allergy Clin. Immunol.* **107,** 31-35 (2001).
13. Simpson, A. & Martinez, F. D. The role of lipopolysaccharide in the development of atopy in humans. *Clin. Exp. Allergy* **40,** 209-223 (2010).
14. Garty, B. Z., Monselise, Y. & Nitzan, M. Soluble CD14 in children with status asthmaticus. *Isr. Med. Assoc. J.* **2**, 104-107 (2000).
15. Klaassen, E. M., Thönissen, B. E., van Eys, G., Dompeling, E. & Jöbsis, Q. A systematic review of CD14 and toll-like receptors in relation to asthma in Caucasian children. *Allergy Asthma Clin. Immunol.* **9,** 10 (2013).
16. Virchow, J. C. Jr., Julius, P., Matthys, H., Kroegel, C. & Luttmann, W. CD14 expression and soluble CD14 after segmental allergen provocation in atopic asthma. *Eur. Respir. J.* **11,** 317-323 (1998).
17. Zhao, L. & Bracken, M. B. Association of CD14 -260 (-159) C>T and asthma: a systematic review and meta-analysis. *BMC Med. Genet.* **12,** 93 (2011).
18. Kabesch, M. *et al.* A promoter polymorphism in the CD14 gene is associated with elevated levels of soluble CD14 but not with IgE or atopic diseases. *Allergy* **59,** 520-525 (2004).
19. Levan, T. D. *et al.* Association between CD14 polymorphisms and serum soluble CD14 levels: effect of atopy and endotoxin inhalation. *J. Allergy Clin. Immunol.* **121,** 434-440 (2008).
20. Vercelli, D. e*t al.* CD14: a bridge between innate immunity and adaptive IgE responses. *J. Endotoxin Res.* **7,** 45-48 (2001).
21. Wang, Z. *et al.* Racial differences in the association of CD14 polymorphisms with serum total IgE levels and allergen skin test reactivity. *J. Asthma Allergy* **6,** 81-92 (2013).

22. Lau, M. Y. *et al.* CD14 polymorphisms, microbial exposure and allergic diseases: a systematic review of gene-environment interactions. *Allergy* **69,** 1440-1453 (2014).
23. Zhang, Y. N., Li, Y. J., Li, H., Zhou, H. & Shao, X. J. Association of CD14 C159T polymorphism with atopic asthma susceptibility in children from Southeastern China: a case-control study. *Genet. Mol. Res.* **14,** 4311-4317 (2015).
24. Zhang, R., Deng, R., Li, H. & Chen, H. No Association Between -159C/T Polymorphism of the CD14 Gene and Asthma Risk: a Meta-Analysis of 36 Case-Control Studies. *Inflammation* **39,** 457-466 (2016).
25. Zhang, G., Goldblatt, J. & LeSouëf, P. N. Does the relationship between IgE and the CD14 gene depend on ethnicity? *Allergy* **63,** 1411-1417 (2008).
26. Sakito, O. *et al.* Pulmonary infiltration with eosinophilia and increased serum levels of squamous cell carcinoma-related antigen and neuron specific enolase. *Intern. Med.* **33,** 550-553 (1994).
27. Nam, S. J. *et al.* Neuron-specific enolase as a novel biomarker reflecting tuberculosis activity and treatment response. *Korean J. Intern. Med.* **31,** 694-702 (2016).
28. Huang, L. *et al.* Systematic review and meta-analysis of the efficacy of serum neuron-specific enolase for early small cell lung cancer screening. *Oncotarget* **8,** 64358-64372 (2017).
29. Collazos, J., Esteban, C., Fernández, A. & Genollá, J. Measurement of the serum tumor marker neuron-specific enolase in patients with benign pulmonary diseases. *Am. J. Respir. Crit. Care Med.* **150,** 143-145 (1994).
30. Song, T. J., Choi, Y. C., Lee, K. Y. & Kim, W. J. Serum and cerebrospinal fluid neuron-specific enolase for diagnosis of tuberculous meningitis. *Yonsei Med. J.* **53,** 1068-1072 (2012).

31. Fang, S. C., Zhang, H. T., Wang, C. Y. & Zhang, Y. M. Serum CA125 and NSE: biomarkers of disease severity in patients with silicosis. *Clin. Chim. Acta* **433,** 123-127 (2014).
32. Tsybikov, N. N., Egorova, E. V., Kuznik, B. I., Fefelova, E. V. & Magen, E. Neuron-specific enolase in nasal secretions as a novel biomarker of olfactory dysfunction in chronic rhinosinusitis. *Am. J. Rhinol. Allergy* **30,** 65-69 (2016).
33. Haque, A., Ray, S. K., Cox, A. & Banik, N. L. Neuron specific enolase: a promising therapeutic target in acute spinal cord injury. *Metab. Brain Dis.* **31,** 487-495 (2016).
34. Moniuszko, M., Bodzenta-Lukaszyk, A., Kowal, K., Lenczewska, D., Dabrowska, M.. Enhanced frequencies of CD14++CD16+, but not CD14+CD16+, peripheral blood monocytes in severe asthmatic patients. *Clin. Immunol.* **130,** 338-346 (2009).
35. Khanduja, K. L. *et al.* Corticosteroids affect nitric oxide generation, total free radicals production, and nitric oxide synthase activity in monocytes of asthmatic patients. *Mol Cell Biochem.* **346,** 31-37 (2011).
36. Kowal, K., Moniuszko, M., Dabrowska, M. & Bodzenta-Lukaszyk, A. Allergen challenge differentially affects the number of circulating monocyte subsets. *Scand. J. Immunol.* **75,** 531-539 (2012).
37. Viksman, M. Y., Liu, M. C., Bickel, C. A., Schleimer, R. P. & Bochner, B. S. Phenotypic analysis of alveolar macrophages and monocytes in allergic airway inflammation. I. Evidence for activation of alveolar macrophages, but not peripheral blood monocytes, in subjects with allergic rhinitis and asthma. *Am. J. Respir. Crit. Care Med.* **155,** 858-863 (1997).
38. Yuryeva, K. *et al.* Expression of adenosine receptors in monocytes from patients with bronchial asthma. Biochem. *Biophys. Res. Commun.* **464,** 1314-1320 (2015).

39. Pace, E. *et al.* Budesonide increases TLR4 and TLR2 expression in Treg lymphocytes of allergic asthmatics. *Pulm. Pharmacol. Ther.* **32,** 93-100 (2015).
40. Hirano, R. *et al.* Human thymic stromal lymphopoietin enhances expression of CD80 in human CD14+ monocytes/macrophages. *Inflamm. Res.* **60,** 605-610 (2011).
41. Cros, J. *et al.* Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* **33,** 375-386 (2010).
42. Mukherjee, R. *et al.* Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous. *Sci. Rep.* **5,** 13886 (2015).
43. Anbazhagan, K., Duroux-Richard, I., Jorgensen, C. & Apparailly, F. Transcriptomic network support distinct roles of classical and non-classical monocytes in human. *Int. Rev. Immunol.* **33,** 470-489 (2014).
44. Jiang, Z. & Zhu, L. Update on the role of alternatively activated macrophages in asthma. *J. Asthma Allergy* **9,** 101-107 (2016).
45. Zhou, Z., Xu, M. J. & Gao, B. Hepatocytes: a key cell type for innate immunity. *Cell Mol. Immunol.* **13,** 301-315 (2016).
46. Martin, A. C. *et al.* Acute asthma in children: Relationships among CD14 and CC16 genotypes, plasma levels, and severity. *Am. J. Respir. Crit. Care Med.* **173,** 617-622 (2006).
47. Julius, P., Grosse-Thie, C., Kuepper, M., Bratke, K. & Virchow, J. C. sCD14 in bronchoalveolar lavage 18, 42 and 162 hours after segmental allergen provocation. *Scand. J. Immunol.* **71,** 304-311 (2010).

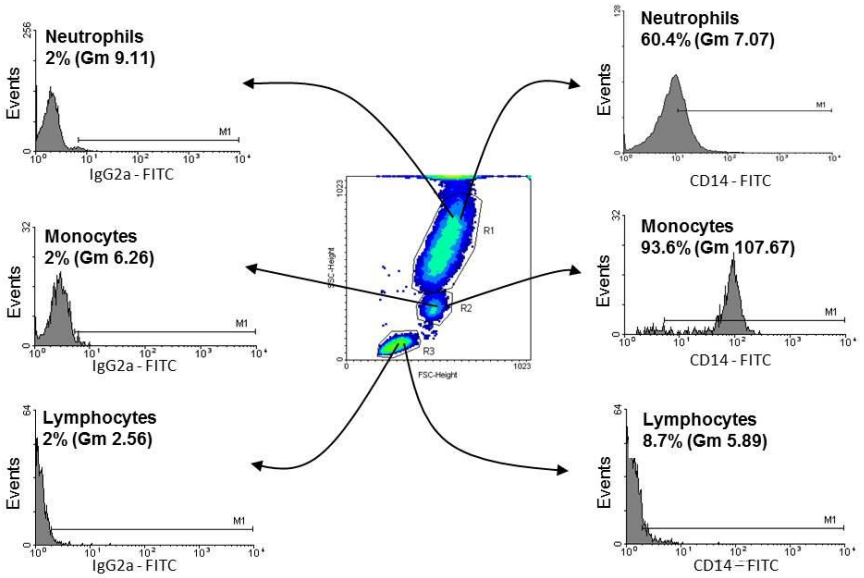
Supplementary Tables

Supplementary Table 1. PCR and SBE primers used for the iPLEX assay to assess the *CD14* (-159 C/T) polymorphism.

Second PCR Primer*	acgttggatgAGACACAGAACCCTAGATGC
First PCR Primer*	acgttggatgCAATGAAGGATGTTTCAGGG
Amplicon length (bp)	97
UEP direction	Reverse
SBE unextended primer (UEP)	AATCCTTCCTGTTACGG

*The lowercase letters in the PCR primer sequences are 5´-end tags.

Supplementary Figures

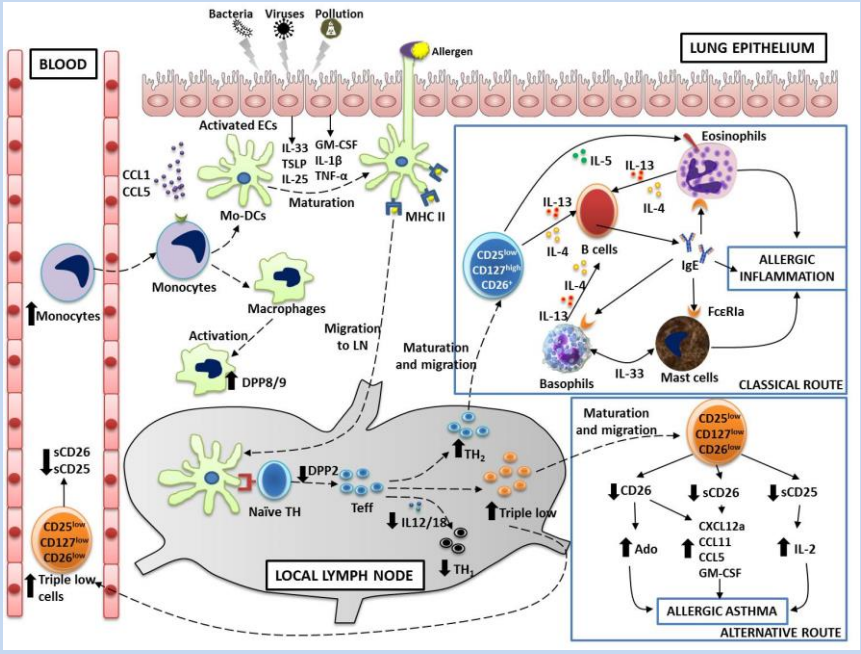


Supplementary Figure 1. CD14 expression in major peripheral blood leukocyte subpopulations. Leukocytes was marked with CD14-FITC or isotype antibody IgG2a κ-FITC. Data are shown as dot plots "forward scatter" (FSC) versus "side scatter" (SSC), where different leukocyte subpopulations are represented (neutrophils/R1, monocytes/R2 and lymphocytes/R3), and histograms, where mean fluorescence intensity (x-axis) is represented versus number of cells (y-axis) of every subpopulation (R1-R3). Left we can see the negative controls where 2% is choose to threshold value. A representative result is shown.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CHAPTER II

Expansion of a CD26low Effector TH Subset and Reduction in Circulating Levels of sCD26 in Stable Allergic Asthma in Adults



The work presented in this chapter has been published in ***Journal of Investigational Allergology and Clinical Immunology*** with the following reference:

- *Expansion of a CD26low Effector TH Subset and Reduction in Circulating Levels of sCD26 in Stable Allergic Asthma in Adults. J Investig Allergol Clin Immunol. 2018;28(2):113-125. doi: 10.18176/jiaci.0224.* "With permission from © 2018 Esmon Publicidad."

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

Asthma is influenced by genetic factors (eg, dipeptidyl peptidase 10 [*DPP10*] and ADAM metallopeptidase domain 33 [*ADAM33*]) and environmental factors [1]. Its management should be based on endotypes [1,2]. During asthma attacks, allergens trigger lung epithelial cells to release cytokines, which in turn activate innate leukocytes and drive type 2 helper T cell (T_H2) lymphocyte differentiation [1]. These cells release interleukins (IL-4, IL-5, and IL-13), stimulate IgE production and favor the activation of eosinophils, mast cells, and basophils [1]. This effector role is counteracted by regulatory T cells (Tregs) [3], whose number and/or function may be altered in asthma [4]. Both $CD4^+$ T subsets express differential levels of interleukin receptor 2 (IL-2R). Thus, CD25 (IL-2Rα) is mainly expressed by Tregs [4], while a $CD25^{-/low}$ phenotype is present in effector $CD4^+$ T lymphocytes (Teff). T-cell receptor (TCR)–triggered Teff cells release soluble CD25 (sCD25), an activation marker [5] that is elevated in serum/plasma during asthma exacerbations [6] and that correlates positively with the severity of allergic asthma [7]. In addition, Tregs are thought to be another source of sCD25 [4,8,9]. sCD25 is also increased in bronchoalveolar lavage fluid from asthma patients [8,9].

Another interesting protein in the pathogenesis of asthma is CD26/DPP4, a surface glycoprotein enriched in $CD4^+$ T cells [10]. In the form of dipeptidyl peptidase 4 (DPP4; EC 3.4.14.5), the enzyme belongs to the serine peptidase subfamily S9B, which includes an asthma susceptibility locus (*DPP10* [11]), dipeptidyl peptidase 8 (*DPP8*), dipeptidyl peptidase 9 (*DPP9*), and fibroblast activation protein alpha (*FAP*) [12-16]. Dipeptidyl peptidase 2 (*DPP2*, *DPP7*) from serine peptidase subfamily S28 also displays DPP4-like activity at acidic pH. As CD26, the glycoprotein interacts with adenosine deaminase, CD45, caveolin-1, and C-X-C chemokine receptor type 4

(CXCR4), thereby fulfilling either inhibitory or enhancing roles upon association [17]. CD26 is an activation marker known to be upregulated on lymphocytes (especially $CD4^+$) in adults with allergic asthma [18]. $CD4^+$ T cells are major actors in the pathogenesis of asthma; however, unlike CD25, Treg cells display lower CD26 levels than Teff lymphocytes. Indeed, CD26 is a negative marker of Treg cells and a marker of the remaining T_H subsets. Thus, expression of CD26 on T_H follows the order of $T_H17>>T_H1>T_H2>$Treg [19,20]. Hence, an elevated presence of CD26 on $CD4^+$ T cells in adult allergic asthma suggests an activated status [18,21] and may point to a specific T-cell phenotype. Moreover, a soluble form of CD26 (sCD26/sDPP4) is released from T cells into the bloodstream, either shed by metalloproteases [22] or secreted by $CD26^+$ vesicles (http://www.exocarta.org). In plasma/serum, sCD26 accounts for >90% of total sDPP4-like activity [23,24], and the remaining 10% is derived from the intracellular peptidases DPP2, DPP8, and DPP9. These DPP4 homologs are also involved in the pathogenesis of asthma [13] and show differential expression in leukocytes [14,15] and eosinophils [25]. Moreover, both DPP8 and DPP9 enzymes are upregulated in activated macrophages and trimmed for antigen presentation [14,15]. Besides, DPP2 is necessary for maintaining the quiescence of lymphocytes and is downmodulated upon activation [15].

CD26/DPP4 cleaves X-Pro or X-Ala amino terminal dipeptides from chemokines (eg, CXCL12a [stromal cell-derived factor-1α, SDF-1α], CCL11 [eotaxin], and CCL5 [regulated on activation, normal T cell expressed and secreted, RANTES]), thereby modulating their biological activity and immunological function, as recently reviewed [17,26,27]. Other substrates include neuropeptides and peptide hormones such as incretins [28,29], whose half-life is prolonged by the DPP4 inhibitors currently used as antidiabetic drugs

[30]. Vascular substrates of DPP4 may be cleaved by sDPP4, DPP4 expressed on leukocytes, and endothelial DPP4 [31]. Increased DPP4-like activity has been observed in bronchoalveolar lavage fluid from asthmatic rat lungs and is due mostly to sDPP4 and, to some extent, DPP8, DPP9, and DPP2 [13,32].

$CD4^+$ T lymphocytes are the main source of sCD26, as this subpopulation displays the highest percentage of $CD26^+$ cells [10,23,33]. Therefore, this molecule could be used as a "fingerprint" to test the activation status or differentiation status of $CD4^+$ T cells in asthma. However, the few studies that have been undertaken show augmented levels of sCD26 in allergic asthma that were positively correlated with eosinophils and IgE [18]. In contrast, sCD26 was inversely associated with inflammation in chronic eosinophilic pneumonia, a disease linked to asthma [34], while no changes were observed for sCD26 in children with asthma or atopy [35].

To date, few studies have monitored sCD26 in asthma, and none consider the potential roles of CD26 in the pathogenesis of asthma or take into account the possibility that abnormalities of circulating biomarkers (sCD25 and sCD26) may reflect changes in leukocyte phenotype such as $CD25^{-/low}CD26^+$ Teff cells and $CD25^{+/high}CD26^{low}$ Treg cells. Therefore, in the present study, a comprehensive analysis was carried out to assess the aforementioned immune biomarkers in patients with moderate-severe allergic asthma.

MATERIAL AND METHODS

Subjects

The study was conducted between 2009 and 2012 and included patients from hospital consultations for Pneumology and Allergy in

Galicia (Spain). Patients had confirmed diagnosis of asthma and allergy for at least one year according to Global Strategy for Asthma Management and Prevention (GINA 2006, http://www.seicap.es/documentos/archivos/GINA2006general.pdf) criteria. The research project was approved by the Ethics Committee of Clinical Research of Galicia (2011/001), Spain, and informed consent was obtained from all individual participants included in the study. A validation cohort was also recruited from 2014 to 2016 (Neumology Service, University Hospital of Santiago de Compostela, Spain). All patients were in a stable phase for at least 4 weeks before sample collection. Healthy Controls (HC) were selected from patients scheduled in the hospital for minor surgeries such as inguinal hernia or orthopedic surgery; they were non-smokers and systemic diseases or allergies were absent. None of the patients or healthy controls were on DPP4 inhibitors treatment.

Sensitization in allergic asthmatic patients (AAP) was confirmed through a skin prick test or serum IgE specific to frequent allergens. Other variables were also accounted: body mass index (BMI), rural-urban residence, profession, smoking or comorbidities. Biochemical determinations were performed using an ADVIA®1650 analyzer (SIEMENS Healthcare Diagnostics S.L., Berlin, Germany). The nucleated cells number was measured using an ADVIA®2120 hematology counter (SIEMENS Healthcare Diagnostics S.L., Berlin, Germany).

Magnetic purification of $CD4^+$ T cell subsets and in vitro culture

Buffy coats from healthy subjects were donated by "Axencia Galega de Sangue, Órganos e Tecidos" (Santiago de Compostela, Spain) and used to isolate peripheral blood mononuclear cells (PBMCs) by Ficoll® density gradients. Teff and Treg cells were prepared from PBMCs by means of the Dynabeads® Regulatory $CD4^+CD25^+$ T cell

Kit (Life-Technologies, Spain). Viability was always >90% (trypan blue exclusion).

Teff ($CD4^+CD25^-$) and Treg ($CD4^+CD25^+$) cells were cultured *in vitro* for 4 days in 96-microwell U-bottom plates with ImmunoCult™-XF T Cell Expansion Medium (StemCell, Grenoble, France) supplemented (or not) with soluble tetrameric antibody complexes (ImmunoCult™ Human CD3/CD28 T Cell Activator; StemCell). To promote a partial Teff differentiation, the following cytokines (PeproTech, London, UK) were added: 10 ng/mL IL-12 (TH_1-like), 10 ng/mL IL-4 (TH_2-like), or IL-1β (100 ng/mL), IL-6 (30 ng/ml) and IL-23 (100 ng/mL) (TH_{17}-like). In addition, 800 ng/mL of IL-2 was used for Treg cells maintenance.

Flow cytometry assays

Venous peripheral blood was collected (BD Vacutainer K2E) ir order to examine the proportion of Teff and Treg cells. Leukocytes from 100 µL of whole blood were stained (30 min, room temp.) with mouse IgG1 κ isotype antibodies (BD Biosciences) labelled with FITC, PE-Cy7, AlexaFluor-647 and PE as negative controls. Alternatively, cells were stained with mouse IgG1 κ specific antibodies (BD Biosciences) against CD4 (FITC), CD25 (PE-Cy7) or CD127 (Alexa Fluor-647) and a mouse IgG2b antibody specific for CD26 (PE; Immunostep); then, red cells were lysed (BD FACS™ Lysing Solution). The purity of Teff/Treg lymphocytes prior (or after) *in vitro* cell culture was also assayed with the same specific (CD4-FITC, CD25-PE-Cy7, CD26-PE) or isotype antibodies (Isotype-FITC, Isotype-PE-Cy7, Isotype-PE). Finally, samples were analysed (BD FACSCalibur and FACSort) and a number of 10,000-200,000 events collected. Data were examined using WinMDI 2.9 software (Joseph Trotter, La Jolla, CA. USA).

DPP4 activity measurement

Total DPP4 enzymatic activity was colourimetrically assayed by means of a flat-bottom 96-well microplate-adapted and end-point protocol. Cell culture supernatants (50 µL) were diluted with 50 µL reaction buffer (0.05 M Tris(hydroxymethyl)aminomethane (TRIS)-HCl pH 8.0 buffer) and 100 µL of 2 mM glycyl-prolyl-paranitroanilide (Gly-Pro-pNA). Plates were incubated at 37ºC and absorbance sequentially (30-120 min) recorded at 405 nm (Labsystems Multiscan MS microtiter plate reader). The concentration of pNA was calculated from a standard curve, ranging from 0 – 2000 µM. One international unit (IU) was defined as the amount of enzyme that processes 1 µmol Gly-Pro-pNA (or releases 1 µmol pNA from this substrate) per minute. Assays were performed in duplicate for each sample.

Determination of sCD25 and sCD26

Serum sCD25 levels were measured through enzyme-linked immunosorbent assay (ELISA) from R&D Systems, MN, USA (Quantikine® Human IL-2Rα Immunoassay), and sCD26 was quantified with ELISAs plates from eBioscience®, Vienna, Austria (Human sCD26 Platinum ELISA). Optical densities were recorded at 450 nm and protein concentration calculated from standard curves.

Statistics

Descriptive data are presented as either median (interquartile range; IQR1-3) or percentages. To assess the changes between AAP and HC in non-normally distributed variables we used Mann–Whitney U two-tailed test, or the Kruskal–Wallis test followed by Dunn's multiple comparison test for more than 2 groups. Spearman´s test was used to measure the association between variables. All analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, Inc., San

Jose, California, USA). The statistical significance was defined as $P < 0.05$.

RESULTS

Characteristics of the First Cohort of Allergic Asthmatic Patients

Table 1. Characteristics of the Study Population[a]

	Allergic Asthmatic Patients[b]			Healthy Controls		
	Male	Female	All	Male	Female	All
No. (%)	33 (40.7)	48 (59.3)	81 (100)	36 (37.1)	61 (62.9)	97 (100)
Age	33 (21-45)	36 (28-48)	35 (26-47)	35 (27-49)	37 (29-52)	35 (29-51)
Smokers, %[c]	45.5	18.75	29.6	0	0	0
BMI, kg/m^2	26.4 (23.8-29)	26.7 (22.6-28)	26 (23.1-28.4)	-	-	-
Asthma Severity:						
Mild	2	4	6	-	-	-
Moderate/High	31	44	75	-	-	-
Control:						
Good	23	37	60	-	-	-
Bad	10	11	21	-	-	-
Treatment:						
No	1	2	3	-	-	-
Inhaled corticosteroids	32	46	78	-	-	-
Oral corticosteroids	5	3	8	-	-	-
Antileukotrienes	6	19	25	-	-	-
Omalizumab	0	1	1	-	-	-

Continued on next page

Table 1 (Continued)

FEV_1 (%)	93 (74-101)	91.8 (73-102)	93 (74-102)	-	-	-
FEV_1/FVC (%)	74.5 (64-83)	73.5 (65-81)	74.2 (65-82)	-	-	-
Lymphocytes, cells/μL	2480 (1935-2835)	2150 (1770-2680)	2310 (1848-2765) [d]	1941.5 (1615-2372)	1892.3 (1585-1892)	1912.7 (1594-2362) [d]
Eosinophils, cells/μL	327 (154-502) [d]	326 (182-492) [d]	327 (175-494) [d]	173 (110-268) [d]	133 (97-198) [d]	152 (103-218) [d]
Monocytes, cells/μL	501 (414-658)	420 (306-518) [d]	444 (349-575) [d]	425 (350-528)	318.8 (231-399) [d]	361 (271-451) [d]
Neutrophils, cells/μL	4458 (3202-5617)	3851 (2814-4859)	4045 (3003-5232)	3286 (2645-5685)	3399 (2812-4472)	3395 (2776-4809)
IgE, IU/mL	355 (87-707) [d]	204 (89-680) [d]	241 (90-682) [d]	78 (19-195) [d]	22 (7-55) [d]	34 (10-95) [d]
CRP, mg/dL	0.09 (0.07-0.42)	0.16 (0.07-0.32)	0.15 (0.07-0.34)	0.19 (0.1-0.69)	0.16 (0.05-0.39)	0.17 (0.07-0.57)
TNF, pg/mL	9.4 (6.6-12.0)	9.5 (7.6-12.9)	9.4 (7.2-12.3)	8.6 (7.3-10.4)	10.5 (7.6-13.2)	9.7 (7.4-12.9)
Leptin, ng/mL	2.9 (1.3-7.2) [e]	18.1 (11.2-27.9) [e]	11.2 (3.1-23.9)	2.9 (1.0-8.0) [e]	13.0 (5.35-26.9) [e]	8.1 (3.3-20.0)

Abbreviations: BMI, body mass index; CRP, C-reactive protein; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; TNF, tumor necrosis factor.

[a]Values are expressed as median (IQR), unless otherwise specified.

[b]We recorded the professional activity of ~70% of patients, distributed according to the following order: students (14.8%), construction professionals (9.9%), housewives (8.6%), administrative officers (7.4%), cleaning service (6.2%), health professionals (6.2%), waiters (4.9%), educators (4.9%), farmers (2.5%), salespersons (2.5%), and textile workers (2.5%).

[c]Apart from the nonregistered (8.6%), formerly smoker (11.1%) and current smoker (29.6%) patients, they were mostly nonsmokers (50.6%).

[d]Differences between AAPs and HCs (Mann-Whitney, $P<.05$).

[e]Differences between male and female (Mann-Whitney, $P<.05$).

The characteristics of this first cohort of patients are summarized in Table 1. In AAPs, the median forced expiratory volume in the first second (FEV_1) (%) was 93 (74.5-102.2), while the FEV_1/forced vital capacity (FVC) ratio (%) was 74.2 (65.2-81.9). Asthma was mainly moderate-persistent (71.6%), and patients had an allergic disease, with positive skin prick test reactions against common allergens. In addition, most patients lived in rural areas (72%), although only a small percentage of them were farmers (Table 1). As expected, a significant group of AAPs had peripheral blood eosinophilia (45% had >350/μL) and elevated total IgE (Table 1). There was a positive correlation between eosinophils and IgE, but not between FEV_1 and serum IgE in AAPs (Table 2). Patients were under different treatments, were mostly nonsmokers, and had well-controlled asthma (Table 1).

Given the well-known association between leptin and body mass index (BMI) and the assumed correlation between the development/worsening of asthma and BMI, we also studied these parameters. First, BMI in AAPs was positively correlated with leptin and C-reactive protein (CRP), but negatively associated with IgE and both FEV_1% and FEV_1/FVC (Table 2). Second, leptin levels were generally 4 to 6–fold higher in women, although no differences were detected between HCs and AAPs (Figure S1, Table 1). These findings underline the lack of association between BMI and asthma. In contrast, some parameters were more elevated in men, including IgE, basophil counts, and sCD26 (data not shown). The influence of age was also taken into consideration, indicating a positive correlation between BMI and TNF and a negative interdependence with IgE, FEV_1%, and FEV_1/FVC; no association with age was detected for sCD26 or sCD25 (Table 2).

Table 2. Spearman Correlation Matrix of the Study Population

Variables	BMI	IgE	Leptin	sCD25	sCD26	Age
Eosinophil count	–0.004P	0.420[a]	–0.009	–0.306[b]C	0.063	–0.144
Eosinophils, %	–0.010P	0.379[a]	0.005	–0.358[b]C	0.051	–0.103
Basophil count	0.011P	0.025	–0.234[b]	–0.073	0.261[b]	–0.157
Basophils, %	–0.005P	–0.048	–0.263[a]	–0.108	0.265[b]	–0.125
Lymphocyte count	0.021P	0.178[c]	0.024	–0.327[b]C	0.091	–0.098
Lymphocytes, %	–0.043P	–0.015	–0.090	–0.341[b]C	0.201[c]	–0.032
Monocyte count	–0.004P	0.266[a]	–0.138	–0.018	0.111	–0.103
Monocytes, %	–0.015P	0.100	–0.138	–0.037	0.190[c]	–0.038
Neutrophil count	0.041P	0.101	0.057	–0.091	–0.174[c]	–0.062
Neutrophils, %	0.075P	–0.094	0.136	0.346[b]C	–0.249[b]	0.095
FEV_1,%	–0.277[c]P	–0.059P	–0.243[c]P	–0.035P	0.019P	–0.240[c]P
FEV_1/FVC	–0.252[c]P	–0.046P	–0.174P	–0.102P	–0.013P	–0.428[a]P
CRP	0.403[a]P	–0.016	0.272[a]	0.214[b]	–0.057	0.072
BMI		–0.260[c]P	0.282[c]P	0.071P	–0.072P	0.504[a]P
TNF		–0.299[b]C	0.221[b]	0.170[c]	0.040	0.252[c]C
IgE			–0.230[c]C	–0.123	–0.066	–0.275[c]P
Leptin				0.102	–0.280[c]P	0.147
sCD25					0.001	0.015
sCD26						0.003

Abbreviations: BMI, body mass index; C, control population; CRP, C-reactive protein; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; P, patient population; TNF, tumor necrosis factor.
[a]$P<.001$
[b]$P<.01$
[c]$P<.05$

sDPP4 Activity Is Highly Correlated With CD26 Expression on $CD4^+$ T Lymphocytes In Vitro

CD26 was mainly expressed by lymphocytes, especially $CD4^+$ T cells. Furthermore, CD26 expression was higher in Teff ($CD4^+CD25^{low}CD127^{high}$) than Treg cells ($CD4^+CD25^{high}CD127^{low}$)

(Figure S2). To investigate whether phenotypic differences in $CD4^+$ T cells with respect to CD26 expression could be translated into altered sCD26 levels, we performed in vitro experiments with Treg cells ($CD4^+CD25^{high}$) and Teff lymphocytes ($CD4^+CD25^-$) cultured under T_H1-, T_H2-, or T_H17-skewing conditions. Results from the determination of sDPP4 activity in cell culture supernatants (highly correlated with sCD26 levels according to the literature) and flow cytometric assays against CD26 showed a high positive correlation between sDPP4 and CD26 (MFI) (Figure 1A) and a direct association between CD26 levels and absolute lymphocyte counts (Figure 1B). Likewise, there was a positive relationship between sDPP4 and the lymphocyte count (Figure 1C). To maximize the influence of $CD4^+$ T-cell phenotype, we weighted sDPP4 for the lymphocyte count and continued to observe a strong correlation between CD26 expression (MFI) and sDPP4 (Figure 1D), thus supporting the influence of the T_H phenotype on the latter variable. Moreover, comparable results were obtained for both cell surface CD26 expression (MFI) (Figure 1E) and sDPP4 (Figure 1F), with data segregated according to in vitro culture conditions. Indeed, 2 opposite poles were observed: resting Treg and T_H cells cultured under T_H17-favoring conditions. Moreover, T_H1-like and T_H2-like cells expressed intermediate levels of CD26 on lymphocytes (Figure 1E), and this was mirrored in sDPP4 levels (Figure 1F). Therefore, at least in vitro, there was a positive correlation between the density of CD26 molecules on $CD4^+$ T cells and sDPP4 levels (ie, sCD26). Furthermore, we found differential behavior in Teff and Treg cells, with a high positive correlation between the intensity and percentage of CD26 in activated Teff cells and an opposite correlation for Tregs (Figures 1G, H).

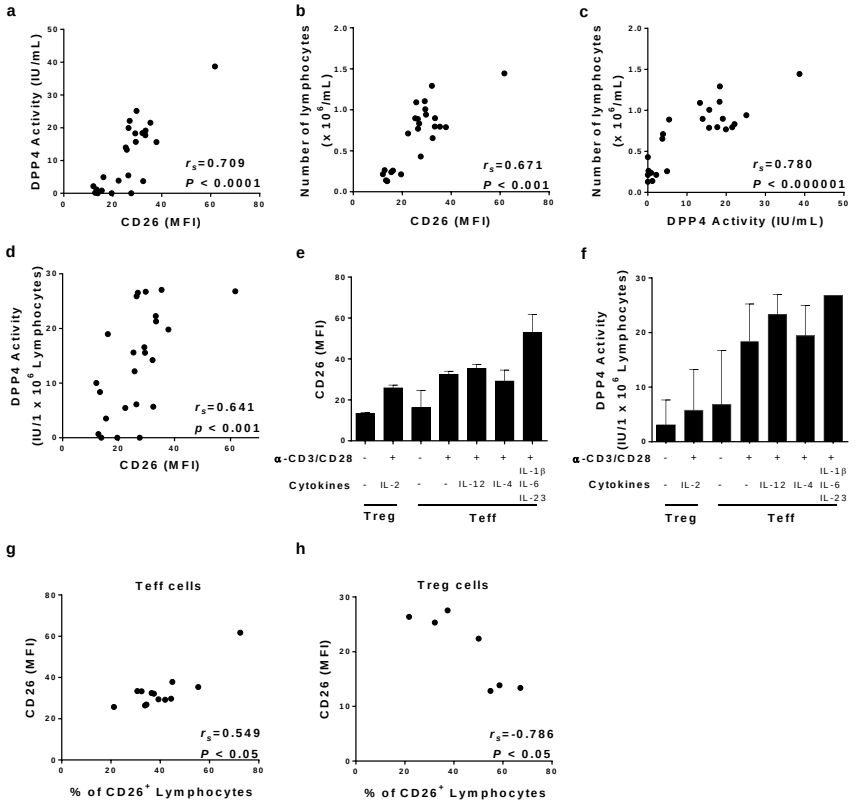


Figure 1. CD26 surface expression and DPP4 activity in culture supernatants of magnetically isolated lymphocytes. Spearman rank correlation test showing the positive relationship between the following: A, CD26 expression (MFI) in resting/activated $CD4^+$ T lymphocytes and soluble DPP4 activity (sDPP4; IU/mL) in cell culture–derived supernatants (n=24); B, CD26 expression (MFI) in resting/activated $CD4^+$ T cells and the lymphocyte count (lymphocytes/mL) at the end of the culture period (n=24); C, $CD4^+$ T lymphocyte count (lymphocytes/mL) and sDPP4 activity (IU/mL) in culture supernatants (n=24); and D, CD26 expression (MFI) in the membrane of $CD4^+$ T cells and the relative activity (IU/L $\times$ 10^6 lymphocytes) of sDPP4 in culture supernatants (n=24). Potential outliers in A-C were not eliminated, because their removal did not significantly affect r_s values. **Continued on next page.**

Figure 1 (Continued). (E-F) Histogram plots (median [IQR]) showing the alteration in membrane CD26 (MFI) in T_H cells (E) and the corresponding sDPP4 activity in cell culture supernatants (F) from resting regulatory T cells (Treg), regulatory T cells expanded with activation beads and IL-2 (Treg + B + IL-2), resting effector T cells (Teff), and activated effector T cells expanded with activation beads in the absence (Teff + B) or the presence of the cytokines required for differentiation of effector T_H subsets differentiation: T_H1 (Teff+ B + IL-12), T_H2 (Teff + B + IL-4)- and T_H17 (Teff + B + IL-1β + IL-6 + IL-23) (n=4). Figures (G) and (H) show, respectively, the positive and negative association (Spearman rank correlation test) between the fluorescence intensity (geometric mean; CD26 MFI) and the percentage of $CD26^+$ lymphocytes in activated Teff (n=13) and resting/activated Treg cells (n=7). Results with resting Teff cells were not included in figure G for clarity, since resting Teff and Treg cells are more alike than resting and activated Teff cells.

Reduction of Serum sCD26 and sCD25 and Increment of $CD4^+CD127^{low}CD25^{low}CD26^{low}$ T Cells in Allergic Asthma Patients

Despite therapy with corticosteroids, circulating lymphocytes were increased in AAPs (Table 1). Given that in vitro experiments showed a positive correlation between CD26 on $CD4^+$ T cells and sDPP4, Treg and Teff cells have a reverse phenotype ($CD25^{high}CD26^{low}$ and $CD25^{low}CD26^{high}$, respectively), and asthmatic donors were expected to have effector/regulatory disequilibrium, we evaluated both sCD26 and sCD25. Our results showed a slight positive correlation between sCD26 and basophils and a negative correlation between sCD26 and neutrophils (Table 2). However, sCD26 levels were not positively associated with BMI, and there was a slightly negative relationship with leptin (Table 2). In addition, sCD25 was directly associated with CRP and neutrophils (%), although negatively with eosinophils (Table 2). Importantly, instead of the previously reported sCD26 upregulation [18], a slight but nonsignificant decrease was observed in the absolute concentration of sCD26 in AAPs (Figure 2A), even after segregation of data by gender (data not shown). Moreover, we found a small association between sCD26 and the percentage of lymphocytes (Table 2), but not between

sCD26 and the absolute number (as shown in our experiments in vitro). Despite the latter finding, sCD26 was still divided by the lymphocyte count to maximize the effect of T-cell phenotype, revealing significantly lower concentrations in AAPs (Figure 2B). Significant downmodulation in AAPs was observed for the absolute concentrations of sCD25 (Figure 2C) and for the normalized concentrations (Figure 2D). In any case, there was no correlation between sCD26 and sCD25 levels (Table 2).

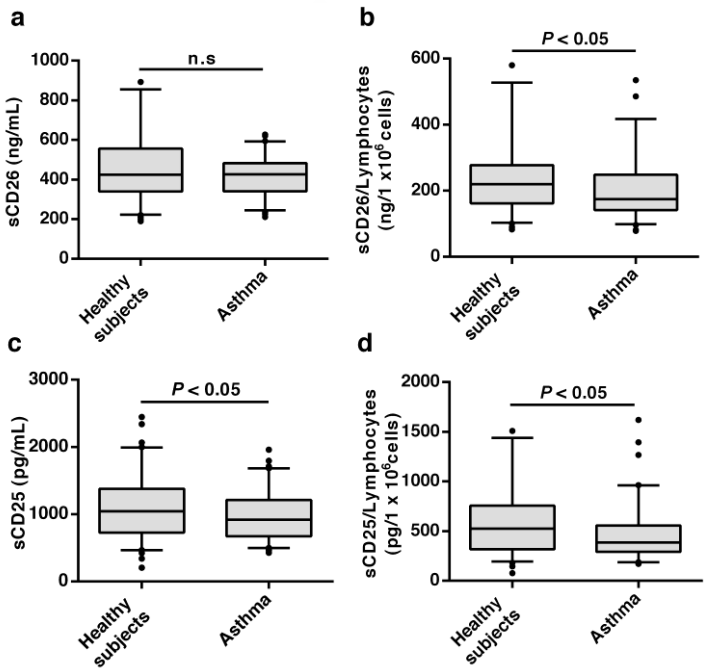


Figure 2. Concentration of sCD26 and sCD25 in serum samples from healthy and allergic asthmatic patients. Data are presented in box and whisker plots where median (IQR), 5-95 percentiles (error bars), and anomalous values are shown. Data refer to levels of sCD26 (A and B; healthy individuals, n=89; asthma patients, n=72) or sCD25 (C and D, healthy individuals, n=96; asthma patients, n=81), which were normalized (B and D) or not (A and C) for the absolute lymphocyte count in peripheral blood. The numbers on the graph represent *P* values (Mann-Whitney). NS, not significant.

According to our in vitro assays, the slight reductions in sCD26 (and sCD25) in AAPs point to expansion of a small $CD26^{low}$ subset of $CD4^{+}$ T cells. To test this hypothesis, another independent set of samples with similar characteristics (validation cohort) was obtained from patients with intermittent-mild allergic asthma (n=53) and moderate-severe allergic asthma (n=50) and HCs (n=32). First, Teff cells ($CD26^{high}$) and Treg cells ($CD26^{low}$) within circulating $CD4^{+}$ T lymphocytes were detected based on CD25 and CD127 (Figure 3A-C), but an additional $CD25^{low}CD127^{low}$ subset within Teff cells was also found in intermittent-mild asthma (Figure 3B) and moderate-severe asthma (Figure 3C). Besides, this small subset displayed the lowest levels of CD26 ($CD26^{-/low}$) among $CD4^{+}$ T cells (Figure 3D). In intermittent-mild and moderate-severe AAPs, we detected a slight (but not significant) reduction in the percentage of Teff lymphocytes ($CD25^{low}CD127^{high}CD26^{high}$) (Figure 3E), which was accompanied by a significantly increased proportion of triple-low $CD4^{+}$ T cells ($CD25^{low}CD127^{low}CD26^{low}$) (Figure 3F). In contrast, the percentage of Treg lymphocytes ($CD25^{high}CD127^{low}CD26^{low}$) remained unchanged (Figure 3G). In line with the augmented proportion of triple-low $CD4^{+}$ T cells, our data revealed significantly lower absolute levels of sCD26 in serum samples from AAPs (especially in moderate-severe asthma) than in HCs (Figure 3H). This finding was in line with the results from the first cohort. Moreover, in the second cohort, it was unnecessary to weight sCD26 levels by lymphocyte count to maximize the effect of $CD4^{+}$ T-cell phenotype. Downmodulation of sCD26 in AAPs was mainly associated with men, whose levels were higher in the reference population (HCs) than those of women. In contrast, CD26 expression in total lymphocytes was higher in women than in men (data not shown), and also in AAPs than in HCs (Figure 3I). Furthermore, even though we did not perceive abnormalities in Teff ($CD26^{high}$) or Treg ($CD26^{low}$) percentages between HCs and AAPs that could explain the reduction in sCD26

levels, women had higher and lower proportions, respectively, of Teff and Treg subsets than men (Figure 4).

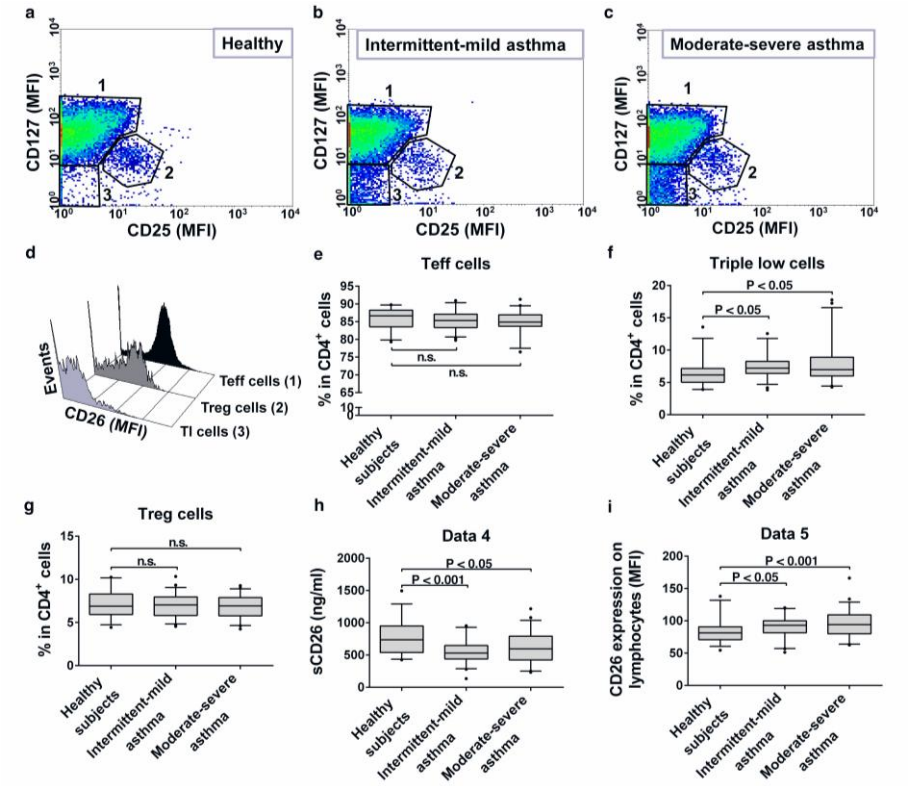


Figure 3. Four-color flow cytometry analysis of peripheral blood CD4$^+$ T subsets and evaluation of sCD26 levels in serum samples from healthy and intermittent-mild or moderate-severe allergic asthmatics. A-C, Representative dot-plots (CD25 vs CD127) of CD4-gated lymphocytes showing the 3 major cell subsets detected in peripheral blood from healthy donors (A), intermittent-mild asthmatics (B), and moderate-severe asthma patients (C) (validation cohort): Teff (region 1), Treg (region 2), and triple-low (region 3). D, CD26 expression levels (MFI) in the different CD4$^+$ T subsets observed in A-C (Teff > Treg >> "triple low" cells). E-G, Percentage of Teff (E), triple-low (F), and Treg (G) subsets among CD4$^+$ T lymphocytes in intermittent-mild asthmatics, moderate-severe asthmatics, and healthy individuals. (H), Serum sCD26 levels (ng/mL) in asthmatic patients and healthy individuals. (I), CD26 expression (MFI) in lymphocytes from healthy individuals and asthmatic patients. Kruskal-Wallis 1-way analysis of variance on ranks. All pairwise multiple comparison procedure, Dunn method. NS, non-significant.

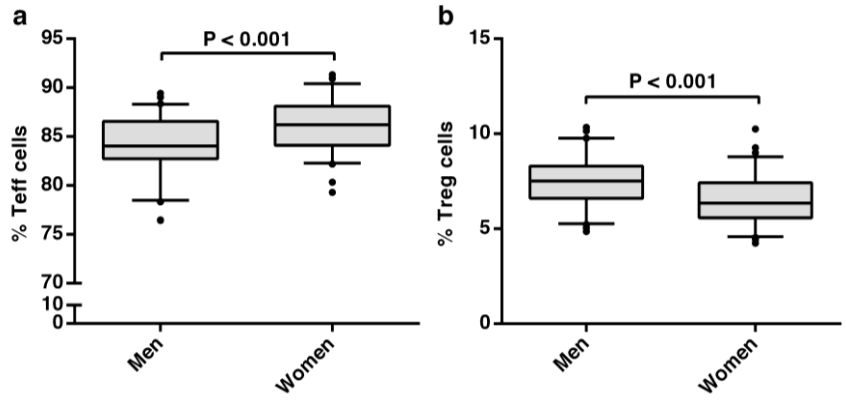


Figure 4. Percentage of Teff and Treg cells in the whole population according to gender. A, Percentage of Teff cells between men and women. B, Sex differences in the percentage of Treg cells. Mann-Whitney, *P*<.001.

DISCUSSION

In the present study, in vitro experiments provided evidence of a direct correlation between lymphocyte surface CD26 and sDPP4 activity and differential expression/secretion of CD26 related to the T_H phenotype. Although we expected increased sCD26 in patients (in line with the enhanced expression of CD26 in lymphocytes), we found lower levels of sCD26 than in healthy donors. This finding can be partially explained by the expansion of a $CD25^{low}CD127^{low}$ T_H subset, with the lowest levels of CD26 amongst $CD4^+$ T lymphocytes (triple-low). This subpopulation could have an important pathogenic role in asthma (Figure 5).

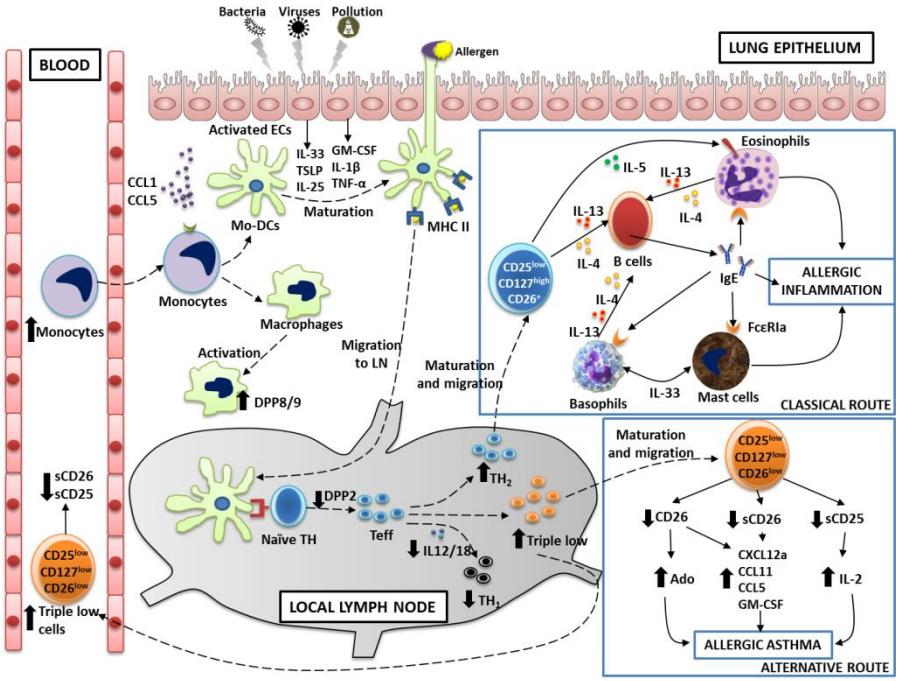


Figure 5. Major immunological pathways in the pathogenesis of allergic asthma. Epithelium damaged by pollution or infection leads to chemokine secretion by airway epithelial cells, enhancing trafficking of innate cells such as monocytes from blood to lung epithelium. Monocytes differentiate into macrophages, which increment expression of DPP8/9 upon activation, or into monocyte-derived dendritic cells (Mo-DCs), the last subset migrating to lymph nodes (LNs) upon maturation. Within the LNs, Mo-DCs present antigens to naïve T_H cells, which lost DPP2 and differentiated into $CD25^{low}CD127^{high}CD26^{+}$ T_H2 cells (classic route). On the other hand, our results also evidence the expansion of an unconventional $CD25^{low}CD127^{low}CD26^{low}$ T_H subset (triple-low) in asthma patients. Triple-low cells could have an important role in allergic asthma, as their lower levels of CD26 on plasma membrane or their diminished ability to release sCD26 could promote higher local amounts of chemokines (eg, CXCL12a/SDF-1α, CCL11/Eotaxin, or CCL5/RANTES) and adenosine (Ado) and exacerbate asthma. CCL indicates chemokine (C-C motif) ligand; TSLP, thymic stromal lymphopoietin; T_H, helper T cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; FcεRI, high-affinity IgE receptor.

CD26 is a multifunctional molecule with both stimulatory and inhibitory roles [17] and remarkable differences in expression between Treg and Teff cells [19,20], as with CD25 and CD127 [36]. IL-12 leads to T_H1 polarization [37] and potentiates the TCR-mediated upregulation of CD26 on $CD4^+$ T cells [38]. On the other hand, in 2012, Bengsch et al [20] used flow cytometry and cell sorting assays with human peripheral blood and tissue samples to show that human T_H17 cells express the highest levels of CD26, followed by T_H1, T_H2, and Treg lymphocytes [20]. Our in vitro assays reveal a similar pattern, where Teff cells undergoing T_H17-polarizing conditions (IL-1β, IL-6, IL-23) also acquire the highest levels of CD26 and sDPP4 activity ($T_H17 > T_H1 \geq T_H2 > Treg$). Although 90% of soluble DPP4 activity is derived from sCD26 [23,24], we must also consider the likely contribution to total DPP activity in cell culture supernatants of other DPP enzymes expressed by lymphocytes, such as DPP8, DPP9, and DPP2. Nevertheless, these enzymes are intracellular and only minute amounts of them reach the extracellular compartment because of cell turnover. Besides, the most likely candidate, DPP2, is a structurally unrelated aminopeptidase that is expressed in quiescent lymphocytes, is downregulated upon activation, and is active at both acidic and neutral pH, but not at the alkaline pH used in our DPP4 enzyme activity assays. Therefore, sDPP4 activity is a bona fide indicator of actual sCD26 levels in cell culture supernatants.

Stimulation by IL-4 results only in moderate upregulation of CD26 followed by $CD4^+$ T cell activation. Thus, T_H2 cell differentiation in allergic asthma should lead to cells with slightly higher levels of CD26 (although lower than T_H1 or T_H17 lymphocytes), as well as enhanced concentrations of sCD26 in serum. Accordingly, Lun et al [18] found upregulation of CD26 in $CD4^+$ T cells from adult patients with allergic asthma, which we confirmed in the second cohort. Moreover, the authors also reported increased

sCD26 in allergic asthmatics [18]. However, under pathological conditions, sCD26 has more frequently been reported to have decreased concentrations [23] than increased concentrations [39]. Indeed, we found a trend towards sCD26 downmodulation in allergic asthma. Similar sCD26 downmodulation has been reported in chronic eosinophilic pneumonia [34], while Remes et al [35] detected no differences in serum sCD26 levels in children with asthma and no association with atopy [35]. Therefore, although it is difficult to ascertain the reasons for these differences, since most studies (including ours) used the same ELISA kit (Bender MedSystems) for determination of CD26 concentration [18,35], at least in the study of Lun et al the results might be partially explained by a higher proportion of males or perhaps a more active disease status.

Contrary to findings from in vitro studies, values for circulating sCD26 in serum samples are not correlated with the number of lymphocytes, probably owing to a series of processes: (*a*) shedding of sCD26 [22] or release of $CD26^{+}$ vesicles [http://exocarta.org/gene_summary?gene_id=1803]; (*b*) changes in proportions of $CD26^{high}$ and $CD26^{low}$ subsets; and (*c*) release of sCD26 by adipose tissue or hepatocytes [40,41]. However, the third possibility is not supported by the present study owing to unaltered levels of CRP. Furthermore, the lack of variation in overall adiposity and absence of differences in leptin levels between AAPs and HCs as found in the present study indicate that adipose tissue is not a major source of sDPP4/sCD26. A negative correlation has also been reported between sCD26 (higher in men [42,43]) and leptin (higher in women [44]). No correlation was observed with BMI. Besides, CD26-specific ELISA revealed that sCD26 is downmodulated rather than augmented in asthma patients, contrary to what is expected in obesity-related asthma.

sCD25 is considered a T-cell activation marker, as it is increased during asthma exacerbations [5-8]. Surprisingly, we detected a significant reduction in sCD25 in the first asthmatic cohort and no changes in the second group. These differences are difficult to interpret, although one explanation could be the presence of "stable" disease in our cohort compared with previous studies. In addition, these results show that the behaviour of sCD26 and sCD25 is similar. Indeed, both molecules could share the same cell origin even though they are not correlated. Thus, they are carried by vesicles or released by matrix metalloproteinase-9 (MMP-9)–mediated cleavage [22]. Moreover, our data support the possibility that CD26 is mainly expressed by peripheral blood $CD4^+$ T cells and primarily secreted by Teff cells upon TCR triggering in vitro. Similarly, Teff cells upregulate and release CD25 following activation [45]. Therefore, the slight reduction in sCD26 and sCD25 in asthmatics might be attributed to the expansion of a $CD25^{low}CD26^{low}$ subset of $CD4^+$ T cells.

Treg cells are $CD25^{high}CD26^{low}$ lymphocytes [19] that tend to maintain expression of CD25 upon activation but turn into $CD25^{low}$ lymphocytes when their suppressive function is abrogated [45]. Indeed, deficient function and reduced frequency of Tregs have been described in allergic asthma [4,46,47]. However, we failed to detect a numerical alteration of Tregs, thus ruling out a link between this subset and alteration of sCD26/sCD25 levels in asthma. We also recorded increased percentages of Teff cells and reduced percentages of Tregs in women, thus explaining their higher susceptibility to asthma.

The nature of triple-low lymphocytes is unknown, but they are probably $CD25^-CD127^{high}CD26^{high}$ Teff cells that have reduced these 3 surface markers and whose expansion causes the downmodulation of sCD26 and sCD25 in AAPs. Reduction of CD26/sCD26 could be

important for T_H cells in abrogation of the inhibitory role of this molecule and the burst in cell proliferation, potentiating the activity of T_H2-chemokines (eg, CCL11/eotaxin), or favoring the proinflammatory effect of adenosine or substance P [17] (Figure 5). Thus, reduced DPP4 activity in cells or plasma could enhance the bioavailability of T_H2 (e.g., IL-4) and myeloproliferative cytokines (G-CSF, GM-CSF) [17]. Besides, CD26 down-modulation in Teff lymphocytes could be parallel to the loss of caveolin-1 (a CD26 ligand) in bronchial epithelial cells and monocytes from asthmatics [48].

Reversible competitive DPP4 activity inhibitors or "gliptins" are a novel class of small molecules for oral administration. They include the peptidomimetic and less specific vildagliptin (Galvus; EU, 2007) and saxagliptin (Onglyza; US FDA, 2010), and the nonpeptidomimetic, long-lasting, and more specific/potent sitagliptin (Januvia; US FDA, 2006), linagliptin (Tradjenta; US FDA and EU, 2011), and alogliptin (Takeda Pharmaceutical Company Limited; US FDA, 2013). These drugs have an excellent tolerability profile and have been introduced into clinical practice as second-line therapy in type 2 diabetes mellitus (T2DM) to reduce inactivation of incretins. However, several mild adverse drug reactions (ADRs) have been reported, such as skin-related disorders, infections (eg, nasopharyngitis and upper respiratory tract infections), and respiratory disorders (eg, dyspnea, coughing, wheezing) [VigiBase, WHO Programme for International Drug Monitoring; 49]. Some studies fail to detect significant differences in the incidence of upper respiratory tract infections or nasopharyngitis [50,51]. However, the mere likelihood of enhanced risk of viral infections in the upper respiratory tract deserves a careful assessment of the safety profile of gliptins, particularly in patients susceptible to airway inflammation or with an already established (sometimes unnoticed) disease (eg, allergic asthma

with reduced extracellular sCD26 levels [see above]). Indeed, viral upper respiratory infections trigger or worsen asthma symptoms (https://www.nhlbi.nih.gov/health/health-topics/topics/asthma/signs). For example, respiratory viral infections (eg, rhinovirus) or viral-derived dsRNA sensed by pattern-recognition receptors lead to asthma exacerbations in murine models of asthma induced by ovalbumin [52] or house dust mite [53], but also in children [54].

Apart from CD26/DPP4, DPP4 activity and/or structure homologue (DASH) proteins include FAP, DPP8, DPP9, DPP-like protein 1 (DPPL1), and 2 proteins linked to asthma: the soluble DPP2 (an enzyme that plays an important role in lymphocyte quiescence) [15], and the catalytically inactive and bronchi/trachea-associated DPPL2/DPP10 [15]. Some ADRs could be linked to off-target effects on some of these DASH proteins [55], but the presence of similar ADRs with different gliptins and the "moonlighting" nature of DPP4, with a large number of substrates associated with the immune system, indicate that these drugs could be enhancing T_H2-mediated responses and the asthma-dependent downmodulation of sCD26 levels. Thus, gliptins have been proposed for the treatment of the autoimmune disease type 1 diabetes by, amongst other mechanisms, upregulating T_H2 cells [56], while the highly specific and potent inhibitor sitagliptin reduces sDPP4 activity without altering CD26 levels on cells and causes a temporary proinflammatory state in T2DM patients through the reduction in Treg cell percentages [57]. In the same sense, gliptins also favor the development of allergic rhinitis [49] and a noninflammatory variant of bullous pemphigoid, diseases where eotaxins and eosinophils play important roles [58,59]. In clear contrast, sitagliptin seemed to play a beneficial role in a chronic murine model of asthma induced by ovalbumin [60]. Therefore, additional research is needed to ascertain the impact of gliptins in allergic asthma.

Taken together, our findings evidence the expansion of a $CD25^{low}CD127^{low}CD26^{low}$ subpopulation of $CD4^{+}$ T cells in allergic asthmatic patients that is coincident with a decrease in the levels of sCD26 and sCD25 in these patients. Reduction of sCD26 and CD26 levels on effector T_H lymphocytes might play an important role in improving their migratory or proliferative capabilities. Moreover, this downmodulation of CD26/sCD26 could be emulated upon the administration of certain drugs, which should be considered in the light of the clinical usage of DPP4 inhibitors [17] and humanized anti-CD26 antibodies [61] in patients with a predisposition toward developing certain allergic hypersensitivity reactions (atopy).

REFERENCES

1. Martinez FD, Vercelli D. Asthma. Lancet. 2013;382:1360-72.
2. Lötvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol. 2011;127:355-60.
3. Bayer AL, Pugliese A, Malek TR. The IL-2/IL-2R system: from basic science to therapeutic applications to enhance immune regulation. Immunol Res. 2013;57:197-209.
4. Robinson D. Regulatory T cells and asthma. Clin Exp Allergy 2009;39:1314-23.
5. Park CS, Lee SM, Uh ST, Kim HT, Chung YT, Kim YH, et al. Soluble interleukin-2 receptor and cellular profiles in bronchoalveolar lavage fluid from patients with bronchial asthma. J Allergy Clin Immunol. 1993;91:623-33.

6. Corrigan CJ, Kay AB. CD4 T-lymphocyte activation in acute severe asthma. Relationship to disease severity and atopic status. Am Rev Respir Dis. 1990;141:970-7.
7. Hoeger PH, Niggemann B, Ganschow R, Dammann C, Haeuser G. Serum levels of sCD23 and sCD25 in children with asthma and in healthy controls. Allergy. 1994;49:217-21.
8. Park CS, Lee SM, Chung SW, Uh S, Kim HT., Kim YH. Interleukin-2 and soluble interleukin-2 receptor in bronchoalveolar lavage fluid from patients with bronchial asthma. Chest. 1994;106:400-6.
9. Lindqvist CA, Christiansson LH, Simonsson B, Enblad G, Olsson-Strömberg U, Loskog AS. T regulatory cells control T-cell proliferation partly by the release of soluble CD25 in patients with B-cell malignancies. Immunology. 2010;131:371-6.
10. Lambeir AM, Durinx C, Scharpé S, De Meester I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci. 2003;40:209-94.
11. Allen M, Heinzmann A, Noguchi E, Abecasis G, Broxholme J, Ponting CP, et al. Positional cloning of a novel gene influencing asthma from chromosome 2q14. Nat Genet. 2003;35:258-63.
12. Gorrell MD. Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. Clin Sci (Lond). 2005;108:277-92.
13. Schade J, Stephan M, Schmiedl A, Wagner L, Niestroj AJ, Demuth HU, et al. Regulation of expression and function of dipeptidyl peptidase 4 (DP4), DP8/9, and DP10 in allergic responses of the lung in rats. J Histochem Cytochem. 2008;56:147-55.

14. Waumans Y, Baerts L, Kehoe K, Lambeir AM, De Meester I. The Dipeptidyl Peptidase Family, Prolyl Oligopeptidase, and Prolyl Carboxypeptidase in the Immune System and Inflammatory Disease, Including Atherosclerosis. Front Immunol. 2015;6:387.
15. Wagner L, Klemann C, Stephan M, von Hörsten S. Unravelling the immunological roles of dipeptidyl peptidase 4 (DPP4) activity and/or structure homologue (DASH) proteins. Clin Exp Immunol. 2016;184:265-83.
16. Vliegen G, Raju TK, Adriaensen D, Lambeir AM, De Meester I. The expression of proline-specific enzymes in the human lung. Ann Transl Med. 2017;5:130.
17. Nieto-Fontarigo JJ, González-Barcala FJ, San José E, Arias P, Nogueira M, Salgado FJ. CD26 and Asthma: a Comprehensive Review. Clinic Rev Allerg Immunol 2016. doi:10.1007/s12016-016-8578-z
18. Lun SW, Wong C, Ko FW, Hui DS, Lam CW. Increased expression of plasma and CD4 T lymphocyte costimulatory molecule CD26 in adult patients with allergic asthma. J Clin Immunol. 2007;27:430-7.
19. Salgado FJ, Pérez-Díaz A, Villanueva NM, Lamas O, Arias P, Nogueira M. CD26: a negative selection marker for human Treg cells. Cytometry Part A. 2012;81:843-55.
20. Bengsch B, Seigel B, Flecken T, Wolanski J, Blum HE, Thimme R. Human Th17 cells express high levels of enzymatically active dipeptidylpeptidase IV (CD26). J Immunol. 2012;188:5438-47.
21. Schade J, Schmiedl A, Kehlen A, Veres TZ, Stephan M, Pabst R, et al. Airway-specific recruitment of T cells is reduced in a CD26-deficient F344 rat substrain. Clin Exp Immunol. 2009;158:133-42.

22. Röhrborn D, Eckel J, Sell H. Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells. FEBS Lett. 2014;588:3870-7.
23. Cordero OJ, Salgado F.J, Nogueira M. On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother. 2009;58:1723-47.
24. Durinx C, Lambeir AM, Bosmans E, Falmagne JB, Berghmans R, Haemers A, et al. Molecular characterization of dipeptidyl peptidase activity in serum: soluble CD26/dipeptidyl peptidase IV is responsible for the release of X-Pro dipeptides. Eur. J Biochem. 2000;267:5608-13.
25. Forssmann U, Stoetzer C, Stephan M, Kruschinski C, Skripuletz T, Schade J, et al. Inhibition of CD26/dipeptidyl peptidase IV enhances CCL11/eotaxin-mediated recruitment of eosinophils in vivo. J Immunol. 2008;181:1120-7.
26. Metzemaekers M, Van Damme J, Mortier A, Proost P. Regulation of Chemokine Activity - A Focus on the Role of Dipeptidyl Peptidase IV/CD26. Front Immunol. 2016;7:483.
27. Mortier A, Gouwy M, Van Damme J, Proost P, Struyf S. CD26/dipeptidylpeptidase IV-chemokine interactions: double-edged regulation of inflammation and tumor biology. J Leukoc Biol. 2016;99:955-69.
28. Lambeir AM, Durinx C, Scharpé S, De Meester I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci. 2003;40:209-94.
29. Klemann C, Wagner L, Stephan M, von Hörsten S. Cut to the chase: a review of CD26/dipeptidyl peptidase-4's (DPP4) entanglement in the immune system. Clin Exp Immunol. 2016;185:1-21.

30. Röhrborn D, Wronkowitz N, Eckel J. DPP4 in Diabetes. Front Immunol. 2015;6:386.
31. Wagner L, Kaestner F, Wolf R, Stiller H, Heiser U, Manhart S, et al. Identifying neuropeptide Y (NPY) as the main stress-related substrate of dipeptidyl peptidase 4 (DPP4) in blood circulation. Neuropeptides. 2016;57:21-34.
32. Van Der Velden VH, Naber BA, Van Hal PT, Overbeek SE, Hoogsteden HC, Versnel MA. Peptidase activities in serum and bronchoalveolar lavage fluid from allergic asthmatics--comparison with healthy non-smokers and smokers and effects of inhaled glucocorticoids. Clin Exp Allergy. 1999;29:813-23.
33. Gorrell MD, Gysbers V, McCaughan GW. CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand J Immunol. 2001;54:249-64.
34. Matsuno O, Miyazaki E, Nureki S, Ueno T, Ando M, Kumamoto T. Soluble CD26 is inversely Associated with Disease Severity in Patients with Chronic Eosinophilic Pneumonia. Biomark Insights. 2007;1:201-4.
35. Remes ST, Delezuch W, Pulkki K, Pekkanen J, Korppi M, Matinlauri IH. Association of serum-soluble CD26 and CD30 levels with asthma, lung function and bronchial hyper-responsiveness at school age. Acta Paediatr. 2011;100:e106-e111.
36. Klein S, Kretz CC, Krammer PH, Kuhn A. CD127(low/-) and FoxP3(+) expression levels characterize different regulatory T-cell populations in human peripheral blood. J Invest Dermatol. 2010;130:492-9.
37. Simpson A, Martinez FD. The role of lipopolysaccharide in the development of atopy in humans. Clin Exp Allergy. 2010;40:209-23.

38. Cordero OJ, Salgado FJ, Viñuela JE, Nogueira M. Interleukin-12 enhances CD26 expression and dipeptidyl peptidase IV function on human activated lymphocytes. Immunobiology. 1997;197:522-33.
39. Mahmoudi M, Hedayat M, Aghamohammadi A, Rezaei N. Soluble CD26 and CD30 levels in patients with common variable immunodeficiency. J Investig Allergol Clin Immunol. 2013;23:120-4.
40. Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, et al. Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes. 2011;60:1917-25.
41. Itou M, Kawaguchi T, Taniguchi E, Sata M. Dipeptidyl peptidase-4: a key player in chronic liver disease. World J Gastroenterol. 2013;19:2298-306.
42. Delezuch W, Marttinen P, Kokki H, Heikkinen M, Vanamo K, Pulkki K, et al. Serum and CSF soluble CD26 and CD30 concentrations in healthy pediatric surgical outpatients. Tissue Antigens 2012;80:368-75.
43. Tejera-Alhambra M, Casrouge A, de Andrés C, Seyfferth A, Ramos-Medina R, Alonso B, et al. Plasma biomarkers discriminate clinical forms of multiple sclerosis. PLoS One. 2015;10:e0128952.
44. Lausten-Thomsen U, Christiansen M, Louise Hedley P, Esmann Fonvig C, Stjernholm T, Pedersen O, et al. Reference values for serum leptin in healthy non-obese children and adolescents. Scand J Clin Lab Invest. 2016;76:561-7.
45. Brusko TM, Wasserfall CH, Hulme MA, Cabrera R, Schatz D, Atkinson MA. Influence of membrane CD25 stability on T lymphocyte activity: implications for immunoregulation. PLoS One. 2009;4:e7980.

46. Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. J Allergy Clin Immunol. 2007;119:1258-66.
47. Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. Immunity. 2009;31:438-49.
48. Bains SN, Tourkina E, Atkinson C, Joseph K, Tholanikunnel B, Chu HW, et al. Loss of caveolin-1 from bronchial epithelial cells and monocytes in human subjects with asthma. Allergy. 2012;67:1601-4.
49. Baraniuk JN, Jamieson MJ. Rhinorrhea, cough and fatigue in patients taking sitagliptin. Allergy Asthma Clin Immunol. 2010;6:8.
50. Mikhail N. Safety of dipeptidyl peptidase 4 inhibitors for treatment of type 2 diabetes. Curr Drug Saf. 2011;6:304-9.
51. Liu X, Xiao Q, Zhang L, Yang Q, Liu X, Xu L, et al. The long-term efficacy and safety of DPP-IV inhibitors monotherapy and in combination with metformin in 18,980 patients with type-2 diabetes mellitus--a meta-analysis. Pharmacoepidemiol Drug Saf. 2014;23:687-98.
52. Mahmutovic-Persson I, Akbarshahi H, Bartlett NW, Glanville N, Johnston SL, Brandelius A, et al. Inhaled dsRNA and rhinovirus evoke neutrophilic exacerbation and lung expression of thymic stromal lymphopoietin in allergic mice with established experimental asthma. Allergy. 2014;69:348-58.
53. Mahmutovic Persson I, Akbarshahi H, Menzel M, Brandelius A, Uller L. Increased expression of upstream TH2-cytokines in a mouse model of viral-induced asthma exacerbation. J Transl Med. 2016;14:52.
54. Webley WC, Hahn DL. Infection-mediated asthma: etiology, mechanisms and treatment options, with focus on

Chlamydia pneumoniae and macrolides. Respir Res 2017;18:98.

55. Janardhan S, Sastry GN. Dipeptidyl peptidase IV inhibitors: a new paradigm in type 2 diabetes treatment. Curr Drug Targets. 2014;15:600-21.
56. Zhao Y, Yang L, Wang X, Zhou Z. The new insights from DPP-4 inhibitors: their potential immune modulatory function in autoimmune diabetes. Diabetes Metab Res Rev. 2014;30:646-53.
57. Sromova L, Busek P, Posova H, Potockova J, Skrha P, Andel M, et al. The effect of dipeptidyl peptidase-IV inhibition on circulating T cell subpopulations in patients with type 2 diabetes mellitus. Diabetes Res Clin Pract. 2016;118:183-92.
58. Günther C, Wozel G, Meurer M, Pfeiffer C. Up-regulation of CCL11 and CCL26 is associated with activated eosinophils in bullous pemphigoid. Clin Exp Immunol. 2011;166:145-53.
59. Paplińska M, Hermanowicz-Salamon J, Nejman-Gryz P, Białek-Gosk K, Rubinsztajn R, Arcimowicz M, et al. Expression of eotaxins in the material from nasal brushing in asthma, allergic rhinitis and COPD patients. Cytokine. 2012;60:393-9.
60. Nader MA. Inhibition of airway inflammation and remodeling by sitagliptin in murine chronic asthma. Int Immunopharmacol. 2015;29:761-9.
61. Angevin E, Isambert N, Trillet-Lenoir V, You B, Alexandre J, Zalcman G, et al. First-in-human phase 1 of YS110, a monoclonal antibody directed against CD26 in advanced CD26-expressing cancers. Br J Cancer. 2017;116:1126-34.

.

SUPPLEMENTARY FIGURES

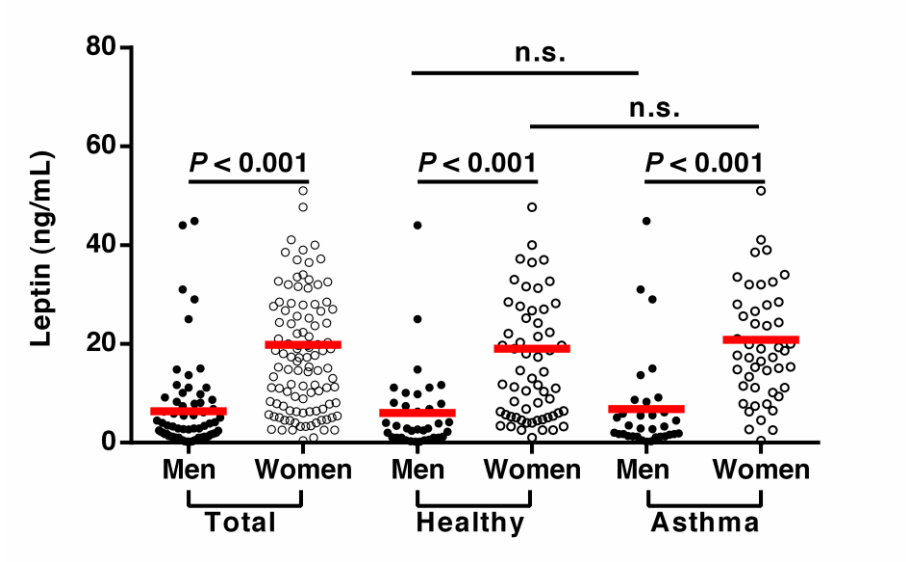


Figure S1. Serum leptin levels in allergic asthma according to gender. Leptin levels (ng/mL) according to gender in both healthy (n=97) and asthmatic (n=81) populations. Mann-Witney U statistic, numbers on the graph represent P-value.

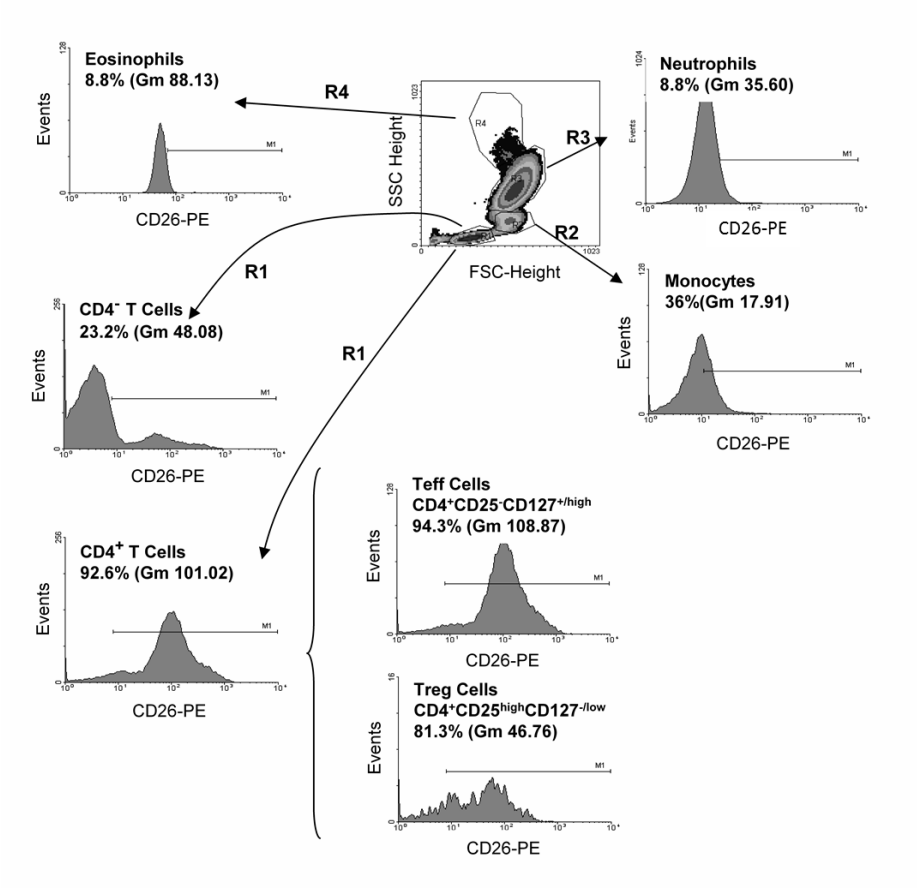
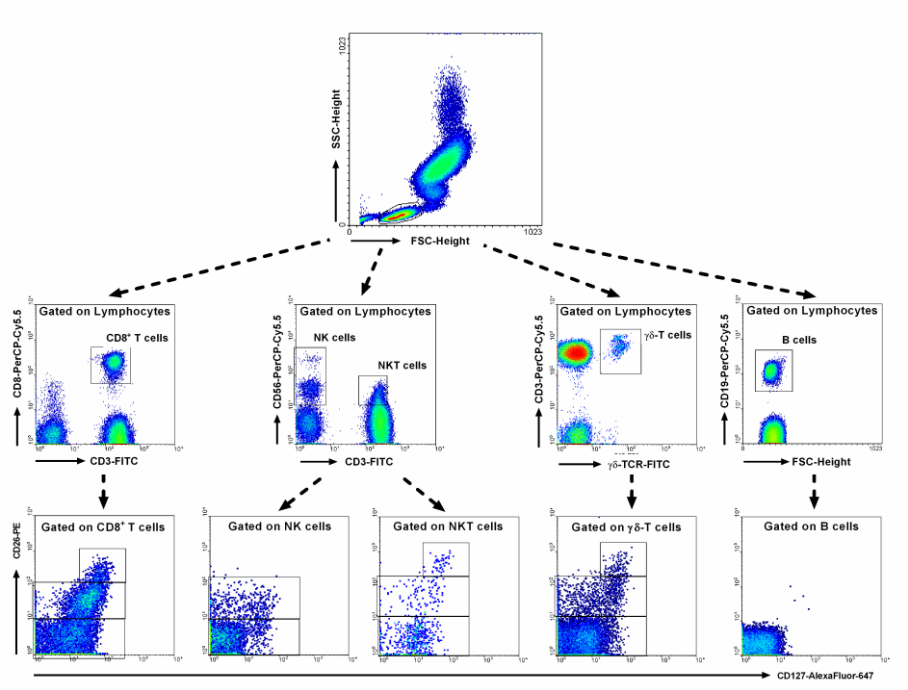


Figure S2. CD26 expression in major peripheral blood leukocyte subpopulations. Leukocytes were marked with CD4-FITC, CD25-PE-Cy7, CD127-AlexaFluor-647, CD26-PE or with isotype antibodies (IgG1κ-FITC, IgG1κ-PE-Cy7, IgGκ-Alexa 647 y IgGκ-PE). Dates are shown as dot plots "forward scatter" (FSC) versus "side scatter" (SSC), where different leukocyte subpopulations are represented (lymphocytes/R1, monocytes/R2, neutrophils/R3 and eosinophils/R4), and histograms, where mean fluorescence intensity (x-axis) is represented versus number of cells (y-axis) of every subpopulation (R1-R4). A representative result is shown.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CHAPTER III

Expansion of different subpopulations of CD26-/low T cells in allergic and non-allergic asthmatics.



The work presented in this chapter is under revision for publication in a Q1 journal.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

Asthma is a heterogeneous disease with different phenotypes (e.g., allergic and non-allergic asthma) and endotypes that remain poorly understood.[1-4] The major endotype classification of asthma is based on the predominant T helper (TH)-type inflammation.[5] On the one hand, TH_2^{high} asthma is the most common endotype, characterised by an eosinophilic and TH_2-driven inflammation and a central role for IgE.[1,6] On the other hand, TH_2^{low} asthma is more heterogeneous, including neutrophilic and paucygranulocytic inflammation and involvement of TH_1 and TH_{17} cells.[1,6] TH_{17} cells have been associated with asthma severity.[7,8] Indeed, IL-17 has been implicated in steroid resistance, airway remodelling, and the induction of neutrophilic inflammation.[9,10]

In 2012, Bengsch *et al*. highlighted the use of CD26 as a marker for human TH_{17} cells (TH_{17}>>TH_1>TH_2>regulatory T cells/Treg),[11] while our group described this molecule as a negative selection marker for Treg cells.[12] CD26/Dipeptidyl peptidase 4 (DPP4) is a "moonlighting" protein: it is an enzyme that inactivates important soluble factors (e.g., cytokines like IL-3; chemokines like CCL11/Eotaxin or CCL5/RANTES; incretins) and also a protein with proteolytic-independent roles (e.g., co-stimulation).[13-15] Furthermore, $CD26^{high}$ TH lymphocytes are considered memory[16,17] or activated cells,[18] which explains the presence of $CD26^{high}CD4^+$ T cells in allergic asthma.[19,20] A soluble version of CD26 (sCD26) has been found in the bloodstream as a free or a vesicle-associated protein [http://www.exocarta.org].[21] Our previous *in vitro* studies evidenced a positive correlation between soluble DPP4 activity (an indirect measurement of sCD26) and CD26 expression on $CD4^+$ cells.[20] Immune cells also appear to be a source of sCD26 *in vivo*.[15] However, contrary to our expectations, allergic asthmatics displayed higher membrane expression of CD26 on $CD4^+$ T cells but decreased levels

of sCD26. This finding can be explained through the expansion of a "triple low" (Tlow; $CD25^{-}CD127^{-}CD26^{-/low}$) subpopulation of effector T cells (Teff).[20]

IL-6 is an important cytokine in the differentiation of TH_{17} cells that acts via IL-6R (IL-6Rα/CD126 + gp130).[22] In this sense, this cytokine down-modulates the TGF-β-driven expression of FoxP3 and up-regulates the levels of the transcription factor that controls the development of Th17 cells: RORγt.[23,24] IL-6 signalling is also essential for the generation of functionally active memory $CD4^{+}$ T cells.[25] Like CD26, CD126 is also found in plasma as a soluble molecule: sIL-6Rα. This circulating protein binds to IL-6 and leads to the activation of $CD126^{-}gp130^{+}$ cells, a process known as *trans*-signalling.[26,27] Indeed, $CD4^{+}$ T cells down-modulate IL-6R upon inflammatory activation, but these cells retain the IL-6 response capacity through the *trans*-signalling pathway, [28] a mechanism of paramount importance for the maintenance of inflammatory diseases such as asthma.[29-32]

Most research in asthma has been focused on allergic phenotype and $CD4^{+}$ T cells. However, $CD4^{-}$ lymphocyte subsets (e.g., $CD8^{+}$ αβ-T cells or γδ-T lymphocytes) might also be relevant for this disease and its phenotypes/endotypes. Thus, γδ-T lymphocytes become activated by IL-6 *trans*-signalling,[33] and they are important inducers of allergic asthmatic responses.[34] Moreover, they are major initial producers of IL-17.[35] On the other hand, $CD8^{+}$ T cells cooperate with $CD4^{+}$ T cells to promote asthma and have been associated with poor lung function and airway obstruction.[36-39] Interestingly, the expression of both CD26 and CD126 defines diverse stages of differentiation of $CD8^{+}$ T cells,[40,41] which might be modified in asthma.[41] Given this differential expression of CD26 and CD126 between different lymphocyte subsets, we postulate that both molecules might display coordinated expression levels and help

distinguish different asthma phenotypes. Therefore, the aim of our study was to analyse the expression of CD26 and CD126 in $CD4^+$ and $CD4^-$ lymphocytes from healthy subjects and patients with allergic/non-allergic asthma or rhinitis.

Herein, we report a high correlation between the expression of CD126 and CD26 in lymphocytes. These molecules help differentiate between lymphocytes with naïve ($CD26^{intermediate}$ or $CD26^{int}$), central-memory ($CD26^{high}$), or "terminally-differentiated" (T_{EMRA})/effector-memory (T_{EM}) ($CD26^{-/low}$) phenotypes. Moreover, allergic and non-allergic asthmatics display low circulating sCD26 levels, which is consistent with the expansion of $CD26^{-/low}$ T_{EM}/T_{EMRA} lymphocytes: $CD4^+CD26^{-/low}$ T cells (previously named Tlow cells)[20] in AA and $CD4^-CD26^-$ γδ-T cells in NAA. Acceleration of the natural course of CD26 down-modulation on T lymphocytes by siRNA leads to higher *in vitro* proliferation rates, which suggests that CD26 molecules on T lymphocytes could be acting as a "brake mechanism" that prevents their proliferation and the acquisition of an effector phenotype. Finally, a decrease in the number of CD126 molecules on leukocytes correlates with higher asthma severity. Thus, our findings provide new advances in asthma immunophenotyping and on the role of CD26/CD126 in this disease.

MATERIAL AND METHODS

Subjects

Adult patients with asthma or allergic rhinitis were recruited from hospital consultations for Pneumology in Galicia (Spain) between 2014 and 2016. The diagnosis of different asthma phenotypes and allergy was confirmed according to Global Strategy for Asthma Management and Prevention (GINA 2006,

http://www.seicap.es/documentos/archivos/GINA2006general.pdf) criteria for at least one year prior to study initiation. A positive skin prick test and the presence of allergen-specific IgE were used to confirm sensitization in allergic patients. Lung function parameters (forced expiratory volume in the 1st second (FEV1), forced vital capacity (FVC), and FEV1/FVC ratio), as well as eritro-sedimentation rate (ESR), were also analysed. All asthmatics were in a stable phase of the disease (i.e., the absence of exacerbations for at least 4 weeks before sample collection). Healthy donors were subjects without allergy or systemic diseases, who were scheduled for minor surgeries (orthopaedic surgery or inguinal hernia). A second cohort of patients was also included with 12 patients with allergic asthma (M/F proportion: 6/6; age: 52.75 ± 14.12) and 12 patients with non-allergic asthma (M/F proportion: 6/6; age: 61.00 ± 10.71), recruited from hospital consultations for Pneumology in Galicia, Spain. The research project was approved by the Ethics Committee of Clinical Research of Galicia (2011/001), Spain, all subjects signed informed consent, and all research was performed in accordance with the relevant guidelines and regulations.

Biochemical determinations

Biochemical determinations and nucleated cell counting were performed by using an ADVIA®1650 analyser (SIEMENS Healthcare Diagnostics S.L., Berlin, Germany) and an ADVIA®2120 haematology counter (SIEMENS Healthcare Diagnostics S.L., Berlin, Germany), respectively. Serum TGF-β1 levels were measured using ELISA plates (Human TGF-beta1 Platinum ELISA; ref. BMS249/4TEN; eBioscience) following commercial guidelines. Optical densities were assayed at 450 nm (Labsystem Multiscan MS), and protein concentration was calculated from standard curves.

Flow cytometry assays

Venous peripheral blood from each donor was collected in EDTA tubes (BD Vacutainer K2E). Then, FITC, PE, PE-Cy7, PerCP-Cy5.5 or AlexaFlour-647-labelled mouse IgG1 κ isotype antibodies (BD Bioscience) or specific antibodies were incubated with cells for 30 min in FACS buffer (PBS, pH 7.4, 2% FBS). We used BD FACS™ Lysing Solution (15 min; room temp.) to lyse red blood cells before sample collection. Finally, 200000 events were acquired on a BD FACSort™ flow cytometer and data were analysed using WinMDI 2.9 software (Joseph Trotter, La Jolla, CA. USA). A list of antibodies is shown in Supplementary Table S1. Isotype antibodies were used to determine the non-specific binding of the antibodies and therefore to set a threshold value to identify negative and positive populations. Single stained lymphocytes were used for fluorescence compensation.

Flow cytometry data are presented as either "percentage of positive cells" or "number of antibodies per cell" (ABC) instead of mean fluorescence intensity (MFI). We use the BD Quantibrite™ Beads PE kit (Fluorescence Quantitation Kit; BD Bioscience) to estimate ABC according to manufacturer instructions. In brief, we ran a BD Quantibrite PE tube with the same instrument settings as the assay. Therefore, MFI values in FL2 were converted into the number of PE molecules bound per cell. Finally, this number of PE molecules/cell was converted to ABC values by using known ratios of PE to antibodies. We took advantage of this transformation to minimise as much as possible the inter-day variation related to the changing working conditions of the flow cytometer.

In vitro proliferation assays and CD26 mRNA silencing

PBMCs were placed in RPMI 1640 at a cell density of 10^7 cell/mL and incubated with 5 μM CFSE for 8 min at RT in the dark. Then, FBS was added to stop the reaction and cells were thoroughly washed with RPMI 1640 before cell counting. Cell cultures were set up at 0.25 × 10^6 cells/mL in 96-well microplates (round-wells). Accell delivery media (ref. B-005000-500; Dharmacon) was used to culture these cells under non-serum conditions. The Accell delivery medium was supplemented or not with 1 μg/ml PHA (± 2 ng/ml IL-12), in the presence of either DPP4-specific or non-targeting Accell siRNAs pools (Dharmacon). To achieve a partial gene silencing we used a commercial Accell SMART pool of 4 short interfering RNA (siRNA) designed to target the mRNA encoded by the human *DPP4* gene (ref. E-004181-00-0005; Dharmacon); these siRNAs were designed to minimize the off-target effects. Besides, we also used two non-target siRNAs: a) an Accell green non-targeting siRNA (ref. D-001950-01-05; Dharmacon), which is a fluorescent unspecific siRNA used for assessment of Accell siRNA passive delivery effectiveness; b) a negative control Accell non-targeting siRNA pool of four siRNAs (ref. D-001910-10-05; Dharmacon) to control the background response to siRNA. All these siRNAs were initially resuspended at 100 μM by using a 1X siRNA buffer (ref. B-002000-UB-100; Dharmacon). Strikingly, the working concentration suggested by the manufacturer (1 μM) induced high cell mortality. Therefore, we titrated down the concentration of siRNA by using the Accell green non-targeting siRNA. A final concentration of 0.02 μM was selected to carry out the final experiments. This concentration was enough to label >98% of cells and allow high cell viability (> 95%).

Upon 6 days of *in vitro* culture, CFSE fluorescence and the number of cell divisions were measured by flow cytometry (Supplementary Figure S1), as well as the amount of CD26 protein with both specific anti-CD26 and isotype antibodies. Each condition was tested several times (n=3-5 technical replicates). Unlabelled cells served as negative controls in cell proliferation assays. The calculated responder frequency (Rf) is the percentage of responder T cells that divided at least once (Supplementary Figure S1).

Statistics

Descriptive data are presented as median (interquartile range; IQR1-3). To assess the changes between asthmatic groups, rhinitis, and healthy donors for non-normally distributed variables we used the Kruskal–Wallis test followed by Dunn's multiple comparison test. Spearman´s test was used to measure the association between these variables. For CFSE proliferation studies, a two-way ANOVA followed by a Tukey´s multiple comparison tests were used. Finally, *t-test* was performed with data from figure 2c to assess changes in normally distributed variables between moderate-severe allergic asthmatics and healthy subjects. All analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, Inc., San Jose, California, USA). The statistical significance was defined as $P < .05$.

RESULTS

Characteristics of study subjects

We performed a case-control study including adult patients with different asthma phenotypes (allergic asthmatics, AA; non-allergic asthmatics, NAA), rhinitis and healthy controls (HC). The

characteristics of the donors in this study are summarized in Table 1. Pulmonary function parameters (FEV1 and FEV1/FVC) were lower in both AA (FEV1, $P = .002$; FEV1/FVC, $P < .001$) and NAA (FEV1, $P < .001$; FEV1/FVC, $P < .001$) relative to patients with rhinitis (Table 1). Haematological count revealed an increment of eosinophil numbers in asthma patients (AA, $P < .001$; NAA, $P = .001$) compared to HC (Table 1). Furthermore, AA displayed higher blood eosinophil counts than patients with rhinitis ($P = .024$) (Table 1). Levels of other leukocyte populations remained unchanged (Table 1).

Table 1. Characteristics of the study population.

	AA	NAA	R	HC
N	100	92	44	32
Age (mean (range))	36 (18-68)	52 (22-72)	35 (18-55)	43 (22-61)
Sex (M/F)	46/54	22/70	25/19	15/17
Disease control:				
Yes	83	75	44	-
No	17	17	0	-
Baseline treatment:				
ICS-LABA	79	75	0	0
ICS	16	4	0	0
OCS	1	0	0	0
Antileukotrienes	41	31	11	0
Anticholinergic	15	28	0	0
Roflumilast	0	2	0	0
Prednisone	0	5	0	0
FEV1 (%)	97.0 (88.3-107.0)[#]	97.0 (73.2-112.0)[#]	108.0 (99.0-119.0)	-
FEV1/FVC (%)	76.0 (70.5-80.4)[#]	73.7 (66.2-80.0)[#]	83.4 (79.0-87.2)	-

Continued on next page

Table 1 (Continued)

Neutrophils (10^3 cells/µL)	3.70 (3.03-4.29)	3.56 (3.08-4.30)	3.56 (2.89-3.99)	3.02 (2.28-4.05)
Lymphocytes (10^3cells/µL)	1.99 (1.67-2.29)	1.94 (1.58-2.34)	2.14 (1.78-2.58)	1.99 (1.53-2.49)
Monocytes (10^3 cells/µL)	0.40 (0.31-0.47)	0.37 (0.30-0.51)	0.39 (0.31-0.46)	0.39 (0.33-0.49)
Eosinophils (10^3 cells/µL)	0.29 (0.20-0.47)$^{\&\#}$	0.28 (0.16-0.41)$^{\&}$	0.22 (0.14-0.29)	0.13 (0.09-0.22)
Basophils (10^3 cells/µL)	0.04 (0.03-0.05)	0.03 (0.02-0.05)	0.04 (0.02-0.05)	0.03 (0.02-0.05)
ESR (1h; mm)	7.5 (4.0-15.0)*	12.0 (7.0-20.8)$^{\#\&}$	8.0 (2.0-14.7)	7.5 (2.0-10.0)
TGF-β1 (ng/mL)	14751 (11882-17299)	13712 (10118-18149)	13957 (8691-19056)	15654 (11552-18620)
IgE (IU/mL)	167.5 (72.0-301.0)*$^{\#\&}$	28.0 (10.0-60.5)$^{\#}$	66.0 (24.0-115.0)	15.0 (5.7-55.7)
IgG (mg/dL)	1075 (943-1200)	1035 (898-1160)	1010 (913-1200)	-
IgG1 (mg/dL)	659 (563-745)*	573 (461-677)	617 (530-743)	-
IgG2 (mg/dL)	346 (337-429)	337 (263-429)	315 (259-411)	-
IgG3 (mg/dL)	33 (23-49)	37 (28-56)	34 (28-48)	-
IgG4 (mg/dL)	48 (27-85)$^{\#}$	40 (19-67)	34 (21-51)	-
IgA (mg/dL)	226 (167-295)	213 (159-263)	191 (133-274)	-
IgM (mg/dL)	98.5 (72-147)	113 (75-151)	111 (69-166)	-

AA, allergic asthmatics; HC, healthy controls; NAA, non-allergic asthmatics; R, rhinitis patients.
Data are presented as median value (IQR1-3), unless otherwise expressed.
Kruskal–Wallis test followed by Dunn's multiple comparison test ($P < 0.05$). * AA vs NAA; # asthma vs R; & asthma vs HC.

As expected, AA subjects exhibited higher levels of IgE than other study groups (NAA, $P < .001$; R, $P = .019$; HC, $P < .001$; Table 1), and the same happened for rhinitis compared to NAA ($P = .01$) (Table 1). Moreover, our results evidenced a reduction of IgG1 in NAA relative to AA ($P < .001$), while IgG4 was higher in AA *vs.* R ($P = .047$) (Table 1). We also analysed the erythrocyte sedimentation rate

(ESR), which was elevated in NAA *vs.* remaining groups (AA, P = .003; R, P = .008; HC, P = .003) (Table 1).

CD26 and CD126 molecules display a highly correlated expression on lymphocytes, while the expansion of "triple low" ($CD25^{-}CD26^{-}CD127^{-}$) $CD4^{+}$ T cells accounts for the reduction of sCD26 in allergic asthma

T cells account for ~80% circulating lymphocytes and include $CD4^{-}$ and $CD4^{+}$ cells. $CD4^{+}$ lymphocytes (TH) were subdivided into "conventional" Teff ($CD25^{low}CD127^{high}$), Treg ($CD25^{high}CD127^{low}$), and Tlow cells ($CD25^{low}CD127^{low}$) (Figure 1a). TH cells displayed the highest percentage of $CD26^{+}$ cells within leukocytes (~85-90%), and this parameter evidenced a positive association with the percentage of $CD126^{+}$ cells (Figure 1b). Amongst TH lymphocytes, "conventional" Teff ($CD26^{+/high}$) and especially Tlow ($CD26^{-/low}$) cells displayed the same high CD26-CD126 correlation, but Tregs appear to lose this association (Figure 1c). Furthermore, we found a reinforced expression of CD26 (measured in Antibody Bound per Cell/ABC; see Material and Methods section) on $CD4^{+}$ T cells from AA and NAA patients (Figure 1d) compared to HC. Strikingly, CD126 showed an unaltered expression in $CD4^{+}$ T cells from the different groups of donors despite the strong correlation between CD26 and CD126 commented above.

The higher number of CD26 molecules on $CD4^{+}$ T cells from AA and NAA patients compared to HC (Figure 1d) was in line with the expected activated phenotype of these cells, but contrasted with thee decreased levels of sCD26 in asthmatics (Figure 1e). However, as most of sCD26 come from T cells,[42] the expansion of $CD26^{-/low}$ T lymphocyte populations may explain the decreased levels of sCD26 in AA. Indeed, even though Teff and Treg proportions remained

unchanged, we detected an expanded subpopulation of $CD25^{-}CD127^{-}CD26^{-/low}$ (Tlow) effector T cells in AA patients compared to HC (Figure 1f). Moreover, contrary to $CD4^{+}$ and "conventional" Teff cells ($CD26^{int/high}$ subsets) and despite the $CD26^{-/low}$ phenotype of these Tlow cells, the percentage of the last subset was negatively correlated with the percentage of $CD26^{+}$ and $CD126^{+}$ cells within the Tlow compartment (Supplementary Table S2). Interestingly, the percentage of $CD127^{+}$ Treg cells was augmented in asthmatics compared to rhinitis and HC (Supplementary Figure S2).

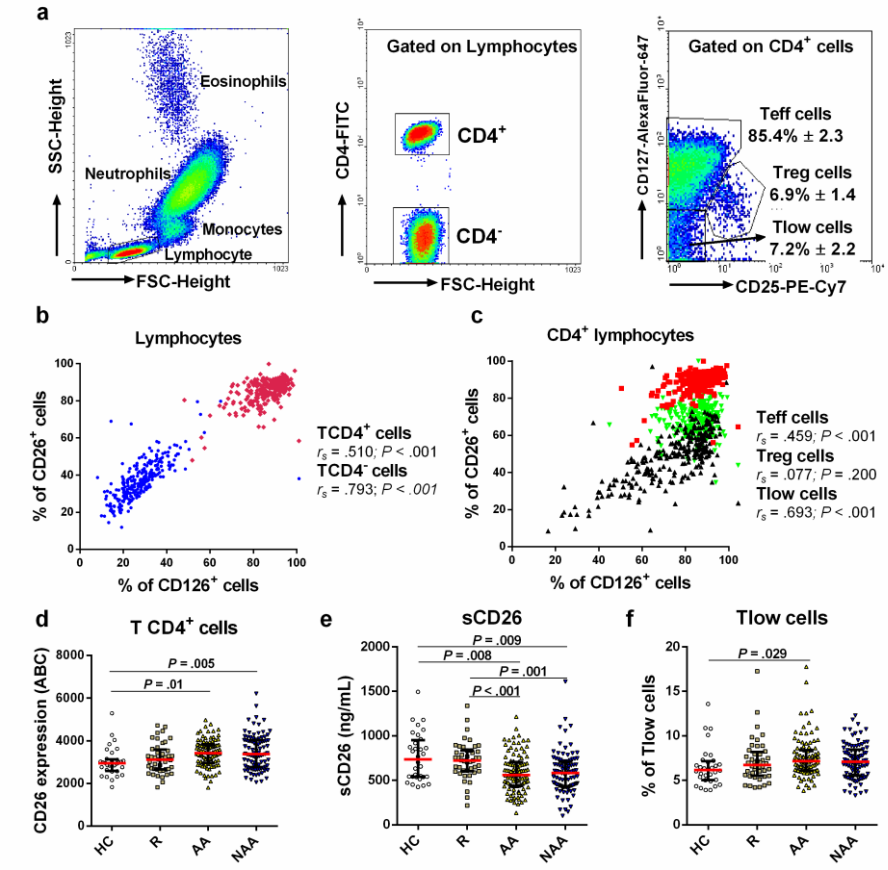


Figure 1 (Previous page). CD26 and CD126/IL-6Rα display a coordinated expression on effector CD4$^+$ T cells, but only CD26/sCD26 levels are altered in asthma. a) Gating strategy in flow cytometry assays to identify relevant CD4$^+$ T cell subsets. Lymphocytes were gated based on the forward (FSC-Height) and side-scatter (SSC-Height) parameters (left dot plot). Then, TH lymphocytes were selected according to their high CD4 expression (middle dot plot). Finally, levels of CD25 and CD127 were used to identify three different TH subsets (right dot plot): "conventional" Teff cells (Teff), regulatory T cells (Treg), and "triple low" Teff cells (Tlow). **b-c)** Correlation between the expression (% of positive cells) of CD26 and CD126 on CD4$^+$ (red) or CD4$^-$ (blue) lymphocytes **(b)** or within different CD4$^+$ subsets **(c)**: "conventional" Teff (red), Treg (green), and Tlow cells (black) (data from all study subjects; N = 268). **d)** Expression of CD26 (median ± IQR1-3) presented as number of antibody molecules bound per cell (ABC; see Material and Methods section) in healthy controls (HC; N = 32), rhinitis (R; N = 44), allergic asthma (AA; N = 100), and non-allergic asthma (NAA; N = 92). **e)** Serum levels of sCD26 (ng/mL) measured by ELISA. **f)** Percentage of Tlow cells within the CD4$^+$ T cell gate. **d-f)** Statistically significant differences between groups are indicated (Kruskal-Wallis test: $P < .05$).

The CD4$^+$CD26$^{-/low}$ T compartment from allergic asthmatics includes memory cells in an advanced stage of differentiation.

CD26 and CD127 are useful markers for identifying effector/memory T cells.[11,16,17] Based on these molecules, CD4$^+$ lymphocytes were segregated in CD26$^{-/low}$, CD26^{int}, and CD26^{high} cells (Figure 2a). As Figure 2b illustrates, CD26^{int} and CD26^{high} populations showed elevated expression of co-stimulatory (CD27, CD28) and lymph node homing (CCR7) molecules, indicating an early differentiation stage. In addition, the CD26^{high} subpopulation was mainly composed of CD45RA$^-$ cells (i.e. central-memory T cells/T_{CM}; CD45RA$^-$ CCR7$^+$CD27$^+$CD28$^+$), whereas most of naïve T lymphocytes (CD45RA$^+$CCR7$^+$CD27$^+$CD28$^+$) were included within the CD26^{int} subset (Figure 2b). In contrast, CD26$^{-/low}$ lymphocytes showed intermediate levels of CD45RA and a decreased expression of CD27, CD28, CCR7, and CD127 molecules (Figure 2b), which agrees with an advanced differentiation stage (likely T_{EM} or T_{EMRA}, CD45RA$^+$CCR7$^-$CD27$^-$CD28$^-$). Moreover, down-modulation of

CD27, CD28, and CCR7 was mainly observed in $CD26^{-/low}$ cells from patients with moderate-severe allergic asthma (Figure 2c), the group of donors where Tlow cells ($CD4^{+}CD25^{-}CD127^{-}CD26^{-/low}$) were found expanded (Figure 1f). Apart from Tlow cells, the $CD26^{-/low}$ compartment within the TH subset is also occupied by Treg lymphocytes ($CD25^{high}CD127^{low}$; Supplementary Figure S3), as Bailey *et al*. have previously reported.[17] However, 57.5 ± 7.3% of Treg lymphocytes are also found within the $CD26^{int}$ subset (Supplementary Figure S3). In summary, our results evidenced that: a) most of naïve $CD4^{+}$ T lymphocytes display intermediate expression levels of CD26; b) The $CD26^{high}$ subpopulation is mainly composed of T_{CM} cells; and c) Tlow cells expanded in AA are likely effector T lymphocytes that have lost CD26 expression to become T_{EM} or T_{EMRA} cells ($CD27^{-}CD28^{-}CCR7^{-}CD45RA^{+/-}$).

Non-allergic asthmatics display increased proportions of $CD26^{-}CD127^{-}$ and $CD126^{-}CD127^{-}$ subpopulations amongst $CD4^{-}$ lymphocytes compared to allergic patients.

Next, we also decided to analyse the population of $CD4^{-}$ lymphocytes (Figure 3a; left and middle dot plots). This cell compartment is more heterogeneous and includes γδ-T, B, NK, and NKT lymphocytes. Analogously to what happened with Tlow cells, the percentage of $CD4^{-}$ lymphocytes was negatively correlated with the percentage of $CD26^{+}/CD126^{+}$ cells in this subset (Supplementary Table S2). Moreover, along with the strong correlation between the expression levels of CD26 and CD126 on $CD4^{-}$ lymphocytes (Figure 1b), we also detected a decreased proportion of $CD4^{-}CD26^{+}$ and $CD4^{-}CD126^{+}$ cells in NAA compared to AA and R patients (Figure 3b). Additionally, both $CD4^{-}CD26^{+}$ and $CD4^{-}CD126^{+}$ cells had a negative correlation with age (r_s = -.482, P < .001; r_s = -.508, P < .001, respectively).

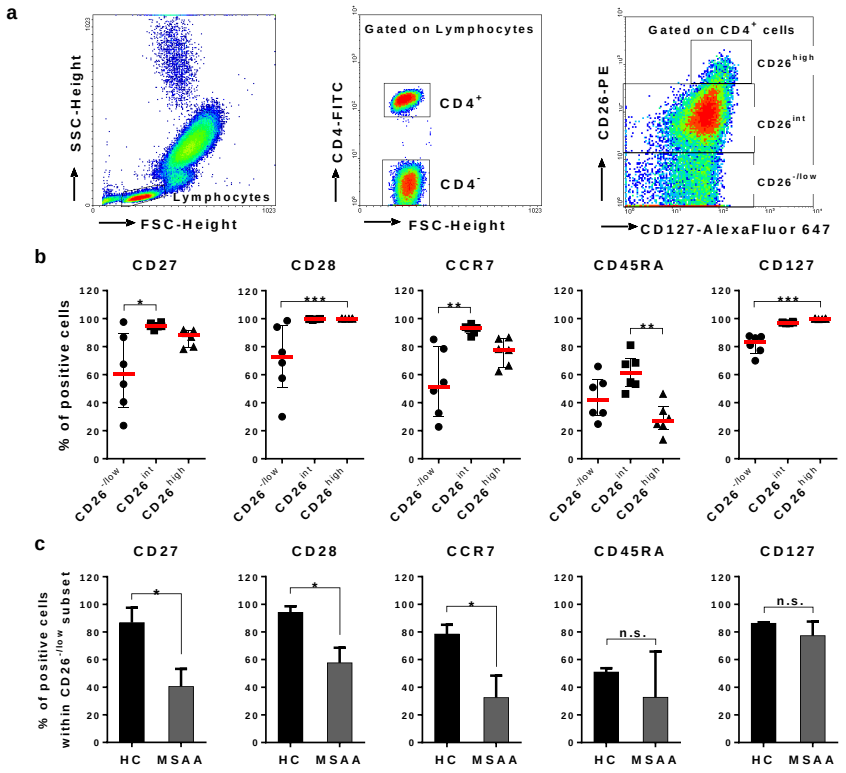


Figure 2. CD26$^{-/low}$ CD4^{+} T cells contain effector lymphocytes with an advanced differentiation stage. a) CD4^{+} T cells were identified by using the same strategy as in Figure 1a. Then, CD127 and CD26 markers were used to delimitate three CD4^{+} lymphocyte subsets (right dot plot): CD26$^{-/low}$ (including CD127^{-} and CD127^{+} cells), CD26^{int} and CD26^{high}. **b)** Phenotyping of CD26$^{-/low}$, CD26^{int}, and CD26^{high} subsets according to the surface expression of CD27, CD28, CCR7, CD45RA, and CD127. Data were obtained from 6 donors (3 healthy subjects and 3 moderate-severe allergic asthmatics) and expressed as % of positive cells for each marker (median ± IQR1-3). Kruskal-Wallis test followed by Dunn´s multiple comparison post-hoc analysis was used to assess significant changes between groups. * $P < .05$, ** $P < .01$, *** $P < .001$. **c)** Expression of each marker (Percentage of positive cells; median ± IQR1-3) in CD26$^{-/low}$ lymphocytes between healthy subjects (HC; N = 3) and moderate-severe allergic asthmatics (MSAA; N = 3). *t-test* was used to assess significant changes between HC and MSAA. *$P < .05$.

To further characterize $CD4^-$ lymphocytes, these cells were subdivided in five subpopulations according to CD26 and CD127 levels: $CD26^{int/high}CD127^-$, $CD26^-CD127^-$, $CD26^-CD127^+$, $CD26^{int}CD127^+$, and $CD26^{high}CD127^+$ cells (Figure 3a). As Figure 3c shows, we only detected an increment of double negative cells ($CD26^-CD127^-$) in NAA compared to AA, which was accompanied by a reduction of both $CD26^{int}CD127^+$ and $CD26^{high}CD127^+$ lymphocytes. These two last $CD4^-$ subsets were rather homogeneous in terms of cell composition, exhibiting a high proportion (>80-90%) of cytotoxic T cells (Tc; $CD3^+CD8^+$) (Figure 3d). In contrast, the composition of $CD4^-CD26^-CD127^-$ lymphocytes was more complex and included Tc, B ($CD19^+$) and $CD56^+$ cells (NK, NKT, or γδ-T lymphocytes) (Figure 3d).

Regarding the CD45RA and CCR7 markers, both $CD26^+$ subpopulations ($CD26^{int}CD127^+$ and $CD26^{high}CD127^+$) displayed similar percentages of positive cells (Figure 3e). Nevertheless, we noticed a decrease of CD45RA levels (mean fluorescence intensity/MFI) in $CD26^{high}$ cells (41.4 ± 4.2) compared to the $CD26^{int}$ subset (342.1 ± 198.2). This finding supports differentiation of naïve cells ($CD26^{int}$) towards a memory-like ($CD26^{high}$) phenotype, compatible with the abundance of co-stimulatory molecules (CD27 and CD28) in both subsets (Figure 3e). In contrast, only a small percentage of $CD4^-CD26^-CD127^-$ cells were $CD27^+$, $CD28^+$, or $CCR7^+$. Strikingly, they showed a $CD45RA^{high}$ phenotype (Figure 3e). Altogether, these characteristics define a T_{EMRA}-like population within $CD4^-CD26^-CD127^-$ lymphocytes. Similar results were obtained when $CD4^-$ lymphocytes were segregated based on CD126 vs CD127 levels (Supplementary Figure S4).

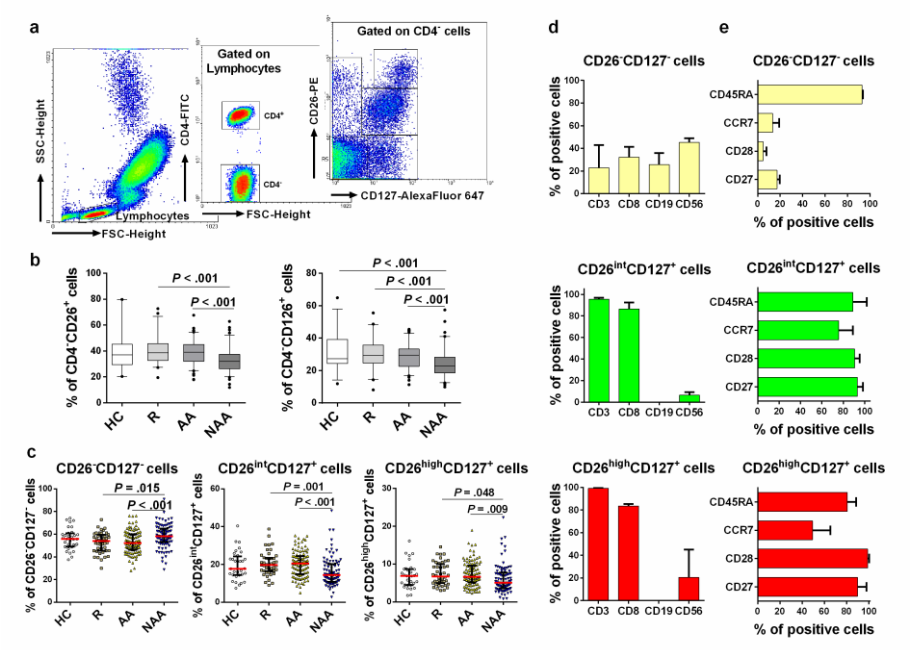


Figure 3. CD4$^-$ lymphocytes with a highly differentiated memory phenotype are expanded in non-allergic asthma. a) CD4$^-$ lymphocytes were identified by flow cytometry (left and middle dot plots). Then CD127 and CD26 were used to identify five subsets within the CD4$^-$ lymphocyte compartment (right dot plot). **b)** Percentage of CD4$^-$CD26$^+$ or CD4$^-$CD126$^+$ cells from healthy controls (HC; N = 32), rhinitis (R; N = 44), allergic asthma (AA; N = 100) and non-allergic asthma (NAA; N = 92). **c)** Percentage (median ± IQR1-3) of CD26$^-$CD127$^-$, CD26^{int}CD127$^+$, and CD26^{high}CD127$^+$ cells amongst CD4$^-$ lymphocytes in HC, R, AA, and NAA. **d)** Composition of CD4$^-$ lymphocyte subsets based on CD3 (T), CD8 (Tc), CD19 (B), and CD56 (NK, NKT) antigens (Data from 3 representative donors). **e)** Phenotypic analysis of CD4$^-$ subsets based on CD45RA, CCR7, CD127, CD28, and CD27 markers (data from 3 representative donors). Statistically significant differences between groups are indicated (Kruskal-Wallis test: $P < .05$). In order to make the figure more understandable, sections **c**, **d,** and **e** show only those CD4$^-$ subsets gated in **a** (right dot plot) where differences between groups were observed (CD26$^-$CD127$^-$, CD26^{int}CD127$^+$, and CD26^{high}CD127$^+$ cells).

The CD26$^{-/low}$ subpopulation of γδ-T lymphocytes is augmented in non-allergic compared to allergic asthmatics.

To ascertain the specific lymphocyte subset within the CD4$^-$ compartment showing altered proportions in NAA compared to AA and to limit possible confounding effects (e.g., age, gender), we analysed blood samples from a second cohort study limited to AA and NAA donors (n = 12/each) with similar age and M/F proportions.

The new analysis focused on CD8$^+$ T (CD3$^+$CD8$^+$), NK (CD3$^-$ CD56$^+$), NKT (CD3$^+$CD56$^+$), B (CD19$^+$), and γδ-T cells (TCRγδ$^+$CD3$^+$) (Supplementary Figure S5). There was no evidence for expansion of these circulating populations in NAA. However, this new study revealed an increased proportion of CD26$^{-/low}$ γδ-T lymphocytes (and an opposite pattern for CD26^{high} γδ-T cells) in NAA compared to AA (Figure 4). Once again, the percentage of γδ-T cells correlated inversely with the percentage of CD26$^+$ cells within this subset (r_s = -.460, P = .024). Interestingly, there was also a negative correlation between the percentages of γδ-T cells and B lymphocytes (r_s = -.602, P = .002).

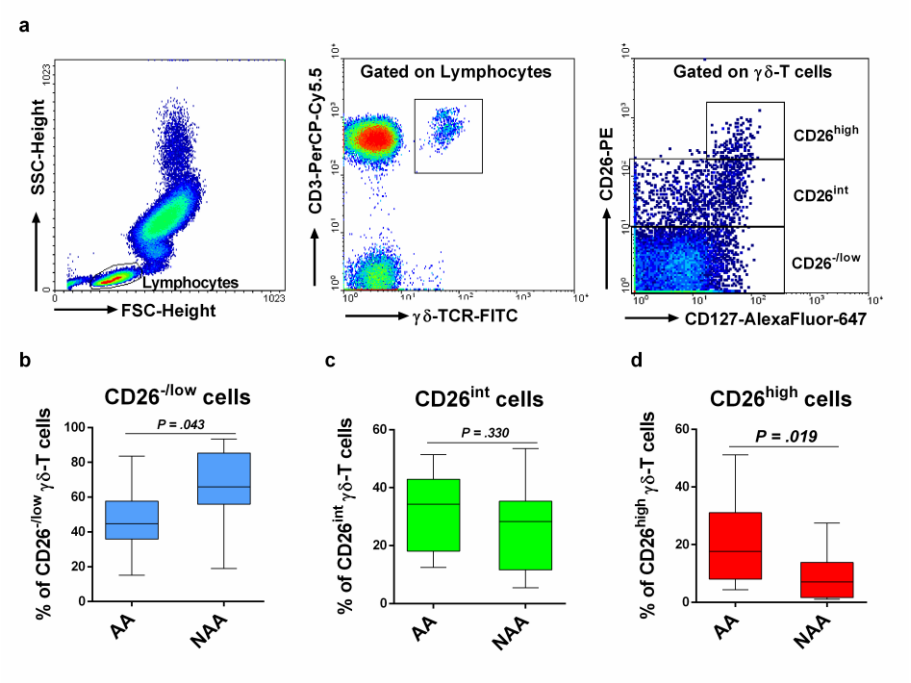


Figure 4. Non-allergic asthmatics display augmented proportions of CD26$^{-/low}$ γδ-T lymphocytes compared with allergic asthmatics. a) Gating strategy for CD3^{+}γδ-TCR^{+} lymphocytes (γδ-T cells). After gating, expression of CD127 and CD26 was used to identify three subsets (CD26$^{-/low}$CD127$^{-/+}$, CD26^{int}CD127^{+}, and CD26^{high}CD127^{+}) in γδ-T cells (right dot plot). **b-d)** Percentage (median ± IQR1-3) of CD26$^{-/low}$CD127$^{-/+}$ **(b)**, CD26^{int}CD127^{+} **(c)**, and CD26^{high}CD127^{+} cells **(d)** amongst γδ-T lymphocytes in allergic (AA; N = 12) and non-allergic (NAA; N = 12) asthmatics. Statistically significant differences between these two groups of donors are indicated (t-test: $P < .05$).

CD26 could act as a negative regulator of T-cell proliferation

CD26 down-modulation on T lymphocytes from both AA (CD4^{+} T cells) and NAA (CD4^{-} γδ-T cells) patients could be: a) a consequence of different mechanisms that simply reduce the amount of this protein

on the cell surface, like, for example, the dilution of CD26 molecules (half-life > 48h) throughout the successive cytokinesis rounds; or b) a necessary condition to initiate the proliferation/differentiation programme of naïve or memory T cells. To distinguish between both possibilities, we tested the effect of RNA interference (RNAi) on the *DPP4* gene during the proliferative response of T lymphocytes to mitogenic triggers.

Peripheral blood mononuclear cells (PBMCs) were CFSE-labelled and cultured *in vitro* with either non-target or CD26/DPP4-specific Accell siRNAs. As CD26 up-regulation during T cell activation was mainly derived from the translocation of this protein from intracellular stores toward the cell surface, T-cell division was stimulated with phytohemagglutinin P (PHA) in the presence or absence of IL-12, a cytokine that promotes CD26 mRNA translation. Furthermore, it was required to extend the *in vitro* culture incubation for 6 days to observe the inhibitory effect of the CD26-specific siRNA on protein levels. As expected, CD26-specific siRNAs down-modulated the expression of CD26, but only in IL-12-stimulated PBMCs (Figure 5a). After verification of compliance with CD26 down-modulation by RNAi, we estimated the percentage of cells that divided at least once. As Figure 5b shows, those T cells where *DPP4* gene silencing was more intense (i.e., IL-12-costimulated) were the ones showing an increase in the proliferation rate.

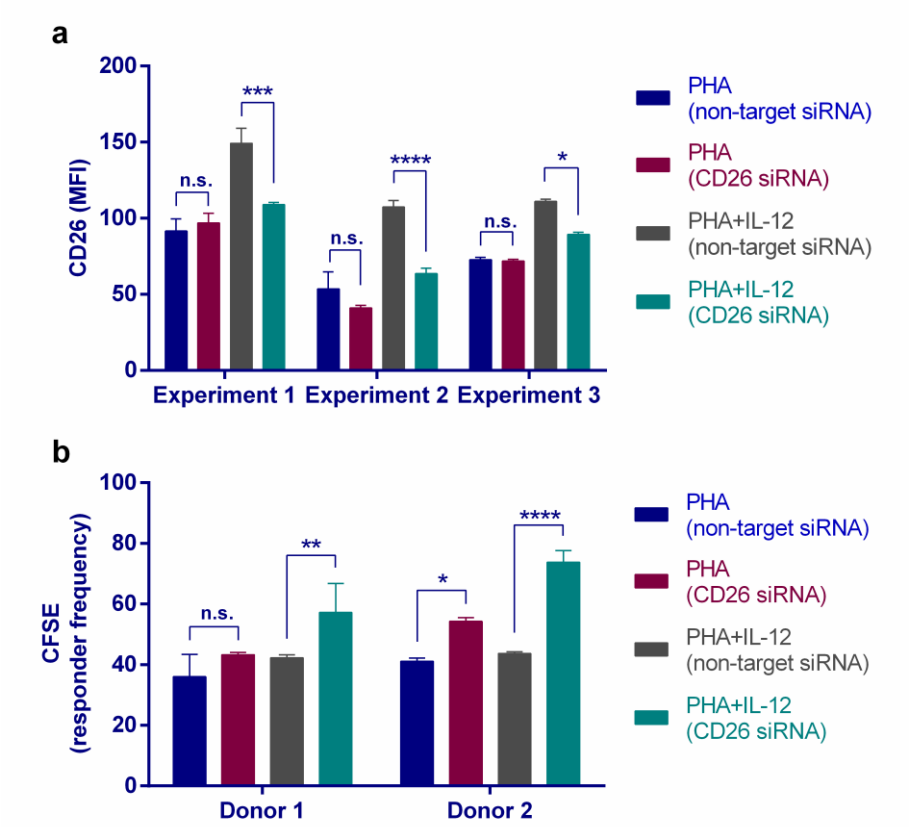


Figure 5. siRNA mediated depletion of CD26 mRNA leads to enhanced T-cell proliferation. PBMCs from healthy subjects were isolated and placed in culture for 6 days in 96 round-well plates. To promote T-cell division, Accell culture medium was supplemented with PHA ± IL-12. Besides, a CD26-specific or a non-targeting Accell siRNAs pool was also used. **a)** Expression of CD26 (MFI; mean fluorescence intensity) on PBMCs was assessed by flow cytometry. Three representative assays are shown. 2-way ANOVA with Tukey´s multiple comparison test: **P* < .05, ****P* < .001, *****P* <.0001; n.s., non-significant. **b)** PBMCs from 2 representative donors were labelled with CFSE and T-cell proliferation induced by PHA ± IL-12 was assessed by CFSE-dilution assays. Responder frequency is the percentage of T lymphocytes that divided at least once. 2-way ANOVA with Tukey´s multiple comparison test: **P* < .05, ***P* < .01, *****P* <.0001; n.s., non-significant.

CD126/IL-6Rα down-modulation in neutrophils, monocytes, and lymphocytes is associated with disease severity and asthma control.

Asthma severity could be responsible for changes in pulmonary and inflammatory parameters. To assess this, we segregated asthma patients into moderate-severe (n = 90) and intermittent-mild (n = 102) asthmatics; asthma control degree was also considered (Table 1). As expected, FEV1 and FEV1/FVC were decreased in moderate-severe and uncontrolled asthmatics (Supplementary Table S3), regardless of the phenotype. Eosinophils count was also augmented in moderate-severe and badly-controlled AA (Supplementary Table S3). In contrast, badly-controlled NAA was only characterized by an increased neutrophils count (P = .032; data not shown). Interestingly, there was a decrease in the expression of CD126 in many leukocyte subsets (monocytes, neutrophils, $CD4^-$ and $CD4^+$ lymphocytes) as the severity was higher (Figure 6). This finding extends to Teff, Treg, and Tlow lymphocytes. Similar decreased levels were obtained for CD126 on leukocytes from badly-controlled asthmatics. Finally, we did not observe changes in CD26 levels on most leukocyte subpopulations with asthma severity, although the percentage of $CD26^+$ Tregs was higher in moderate-severe NAA patients (Supplementary Figure S6). Finally, the percentage of $CD4^+$ lymphocytes was higher in badly-controlled AA (P = .006; data not shown).

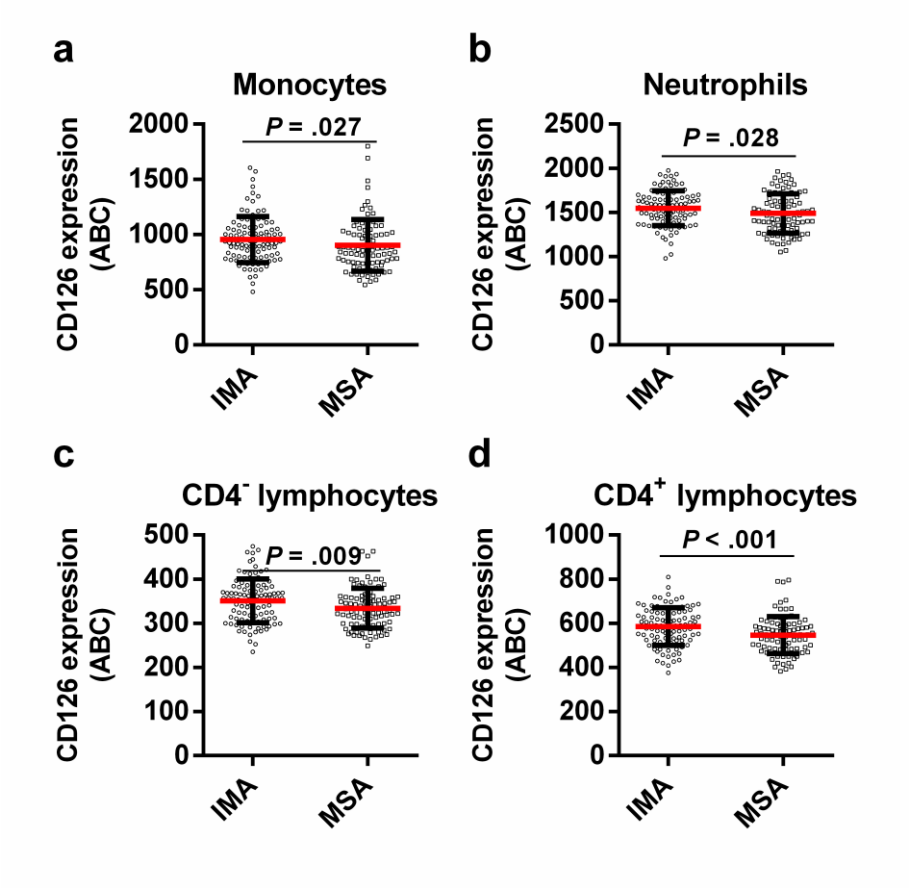


Figure 6. Generalised down-modulation of CD126 in leukocytes from patients with moderate-severe asthma. Monocytes **(a)**, neutrophils **(b)**, and lymphocytes **(c-d)** were gated on a flow cytometry FSC/SSC plot. Afterwards, lymphocytes were further subdivided into $CD4^-$ **(c)** and $CD4^+$ **(d)** cells. The number of CD126 antibodies bound per cell (ABC; median ± IQR1-3; see Materials and Methods section) was measured on every population **(a-d)** in intermittent-mild (IMA; N =102) and moderate-severe (MSA; N = 90) asthma patients. Statistically significant differences between these two groups of donors are indicated (t-test: $P < .05$).

DISCUSSION

This study shows an intense correlation between the expressions of CD26/DPP4 and CD126/IL-6Rα, two molecules that identify naïve ($CD26^{int}$ and/or $CD126^{+}$), memory ($CD26^{high}$ and/or $CD126^{+}$), and highly differentiated effector subsets (T_{EM} and T_{EMRA} cells; $CD26^{-/low}$ and/or $CD126^{-}$). Precisely, the last subset is expanded in both groups of asthma patients: AA ($CD4^{+}$ Tlow cells) and NAA (γδ-T cells). Probably as a result of this expansion, there is a reduction of sCD26 concentration in plasma that is shared by AA and NAA patients. Moreover, CD26 could be relevant to slow the rate of cell division of naïve or central-memory T lymphocytes and, conversely, its down-modulation necessary for rapid proliferation and differentiation into effector cells. Finally, down-regulation of CD126 on leukocytes may be related to asthma severity.

CD26 is an activation marker[18] that identifies TH_1 and especially TH_{17} cells.[11] Our results show an increase of "activated" $CD4^{+}$ cells ($CD26^{+}$) in asthma, in agreement with published work.[19] Despite the IL-13-dependent up-regulation of CD26/DPP4 on human bronchial epithelial cells[43] or the potential role of TH_{17} cells in NAA,[8,44] both AA and NAA patients express similar levels of CD26 on $CD4^{+}$ T cells. A possible explanation for this observation is that following antigen presentation to naïve $CD4^{+}$ T cells in lymph nodes, these lymphocytes proliferate, differentiate, and move back to the peripheral circulation, where they still have an early differentiation state ($CD26^{high}$; $TH_{17}>>TH_1>TH_2$) and an extended half-life compared to innate leukocytes ($CD26^{-/low}$; eosinophils, neutrophils) (Supplementary Table S4). Therefore, these circulating TH cells will still require the down-modulation of CD26 to become T_{EM}/T_{EMRA} cells and migrate into sites of inflammation; a similar event would occur

with IL-6Rα/CD126 on T lymphocytes to favour the proinflammatory *trans*-signalling pathway.

Three major TH subsets coexist in circulation: Treg ($CD25^{high}CD26^{low}CD127^{low}$), "conventional" Teff ($CD25^{low}CD26^{high}CD127^{high}$), and Tlow cells ($CD25^{-/low}CD26^{-/low}CD127^{-/low}$). The latter is a highly differentiated counterpart of "conventional" Teff cells. $CD4^+$ Tlow cells are expanded in AA[20] and their abundance is negatively correlated with CD26/CD126 levels, as it happens for other subpopulations (e.g., $CD26^{-/low}$ γδ-T lymphocytes). Despite the small expansion of the Tlow cell compartment, this might be relevant for AA pathogenesis considering two facts: a) total lymphocytes, instead of antigen-specific cells, have been measured; and b) patients were in stable phase (i.e., the absence of exacerbations for 4 weeks before sample collection). On the other hand, even though there is a negative association between the percentage of $CD26^+/CD126^+$ cells and the percentage of Tlow cells, this does not necessarily imply CD26-dependent causation. Therefore, we performed T cell proliferation assays after siRNA-mediated depletion of CD26 mRNA. These results show a negative regulatory role for CD26 in T cell proliferation.[15] Thus, our data agree with the study of Yan *et al.* working with ovalbumin-induced $CD26^{-/-}$ C56BL/6 animals[45], or Stephan *et al.* showing that oral administration of DPP4-inhibitors aggravates the airway inflammation in a rat model of asthma.[46]

Reduced expression of CD27, CD28, and CCR7 is the hallmark of highly-differentiated effector cells (T_{EM} or T_{EMRA}). Tlow cells fulfil these criteria; therefore, these lymphocytes might be part of a pool of "conventional" $CD4^+$ Teff cells that remains upon antigen clearance and develop into senescent lymphocytes with short telomeres, low proliferative capacity, and presence of cytotoxic

molecules.[47] Thus, in line with other works,[17,28] expression of CD26/DPP4 and CD126/IL-6Rα would identify cells with a naïve ($CD45RA^+$, $CD62L^+$, $CCR7^+$) or a central-memory ($CD45RO^+$, $CD62L^+$, $CCR7^+$) phenotype, while reduced levels would distinguish $CD4^+$ T subsets expanded in AA with a T_{EM} or T_{EMRA} phenotype. Similarly, we found augmented proportions of peripheral blood $CD4^-$ γδ-T cells with a $CD26^-CD126^-CD127^-$ phenotype (likely $V\delta2/V\gamma9^+$)[48] in NAA compared to AA. Several studies have described the presence of different circulating $V\delta2/V\gamma9^+$ T cells (naïve/$CD45RA^+CD27^+$, T_{CM}/$CD45RA^-CD27^+$, T_{EM}/$CD45RA^-CD27^-$, and T_{EMRA}/$CD45RA^+CD27^-$),[49,50] which is compatible with the existence of $CD26^-$ (T_{EM}/T_{EMRA}), $CD26^{int}$ (naïve), and $CD26^{high}$ (T_{CM}) γδ-T subsets. Thus, an enlarged population of $CD26^-$ γδ-T lymphocytes (i.e., T_{EM}/T_{EMRA}) with a preferential production of TH_{17} or TH_1 cytokines[51] in NAA patients could explain the enhanced airway inflammation and the inverse relationship between γδ-T cell and B-cell proportions. However, it has been previously reported that most of circulating γδ-T cells are $CD126^-gp130^-$ (i.e., they are not IL-6-responders),[52] which raises the question about how they can become IL-17-producers.

Another caveat is if those phenotypic changes on T lymphocytes could be mirrored in serum samples. Different circulating molecules were measured (IL-4, TGF, TNF, sCD25, sCD26), but most of them remained unaltered. We did not quantify "soluble" IL-6R/CD126 (sIL-6R/sCD126), but several authors reported higher levels in stable asthma and especially upon flare-ups due to mechanisms involving sheddases (e.g., ADAM10/17),[53-55] spliceosomes,[53,56] or vesicles.[54] In contrast, changes in sCD26 levels in asthma remain almost unexplored. Lun *et al.* reported an elevation of sCD26 in AA patients linked to the activated phenotype ($CD26^{high}$) of $CD4^+$ T lymphocytes.[19] However, reduced sCD26 levels have been

recently described in severe asthmatics[57] or a low eosinophilic TH_2^{low} severe asthma endotype.[58] Our results are in agreement with these last works and support a rather generalized (AA and NAA) sCD26 down-modulation. The underlying mechanism is likely the expansion of $CD26^-$ T subsets[20] with a T_{EM} or T_{EMRA} phenotype: $CD4^+$ T cells in AA and $CD4^-$ γδ-T cells in NAA. Reduced levels of CD26 on lymphocytes and the extracellular compartment could be concomitant with the loss of caveolin-1 (a CD26 ligand) in bronchial epithelial cells and monocytes from asthmatics.[15] Moreover, the decrease of CD26 levels may be important for the bioavailability of soluble factors (e.g., chemokines, adenosine) and to promote cell functions like proliferation, chemotaxis, and migration toward inflamed tissues.[15]

Treg cell function has been described as impaired in asthma.[59] Although we did not find deregulation of Treg numbers, they showed increased CD26 expression in asthmatic patients. This is relevant because CD39 is an ecto-enzyme expressed by $CD26^-$ Treg lymphocytes[12,60] and involved in adenosine (Ado) production.[61] Ado is an immune-regulatory molecule whose synthesis is counteracted by adenosine deaminase (ADA), an ecto-enzyme anchored to CD26.[62,63] Therefore, a $CD26^{high}$ phenotype in Tregs could decrease local Ado concentration and exacerbate disease severity.[64] Indeed, the percentage of $CD26^+$ Treg cells in NAA was higher in moderate-severe patients than intermittent-mild subjects. The percentage of $CD127^+$ Treg cells, a phenotype correlated with a diminished suppressive capacity,[65] was also augmented in asthmatics compared to rhinitis and HC. However, future studies including the assessment of Treg function in NAA and AA will be necessary.

Asthma severity is also influencing CD126 levels on $CD4^+$ T cells, neutrophils and monocytes. IL-6 acts via either IL-6R (classic-

signalling) or sIL-6R/sCD126 (*trans*-signalling).[22] Contrary to the anti-inflammatory role of the first pathway,[28] the *trans*-signalling route allows $CD126^{-}CD130^{+}$ cells to respond to IL-6[27] and is important in asthma through the maintenance of TH_{17} cells or the inhibition of T-cell apoptosis.[66] Naïve $CD4^{+}$ T cells down-modulate IL-6R upon TCR-mediated activation, probably due to protein shedding.[28] This release mechanism has been observed in asthma, and sIL-6R levels have been directly associated with IgE levels, but negatively with lung function.[67] Therefore, reduction of CD126 expression in monocytes, neutrophils, and $CD4^{+}$ cells from moderate-severe patients highlights the role of IL-6 *trans*-signalling in asthma severity.

In conclusion, our data provide evidence that both asthma phenotypes share common immune-pathologic mechanisms, with expansion of $CD26^{-/low}$ subsets in AA ($CD4^{+}$ Tlow or "highly-differentiated" Teff cells) and NAA ($CD4^{-}$ T cells; γδ-T lymphocytes) and down-modulation of additional surface molecules (IL-6Rα/CD126, CD27, CD28, IL-7Rα/CD127, CCR7) to produce differentiated effector subsets and extracellular sCD26 reduction. This CD26/sCD26 down-modulation and the potential role in T-cell proliferation should be considered in the light of clinical usage of DPP4 inhibitors and anti-CD26 antibodies.

REFERENCES

1. Wenzel, S. E. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat. Med.***18**, 716-725 (2012).
2. Chung, K. F. Defining phenotypes in asthma: a step towards personalized medicine. *Drugs* **74**, 719-728 (2014).
3. Meyers, D. A., Bleecker, E. R., Holloway, J. W. & Holgate, S. T. Asthma genetics and personalised medicine. *Lancet Respir. Med.* **2**, 405-415 (2014).
4. Canonica, G. W., Ferrando, M., Baiardini, I., Puggioni, F., Racca, F., Passalacqua, G. et al. Asthma: personalized and precision medicine. *Curr. Opin. Allergy Clin. Immunol.* **18**, 51-58 (2018).
5. Woodruff, P. G., Modrek, B., Choy, D. F., Jia, G., Abbas, A. R., Ellwanger, A. et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am. J. Respir. Crit. Care Med.* **180**, 388-395 (2009).
6. Stokes, J. R. & Casale, T. B. Characterization of asthma endotypes: implications for therapy. *Ann. Allergy Asthma Immunol.* **117**, 121-125 (2016).
7. Newcomb, D. C. & Peebles, R. S. Jr. Th17-mediated inflammation in asthma. *Curr. Opin. Immunol.* **25**, 755-760 (2013).
8. Cosmi, L., Liotta, F. & Annunziato, F. Th17 regulating lower airway disease. *Curr. Opin. Allergy Clin. Immunol.* **16**, 1-6 (2016).
9. Chesné, J., Braza, F., Mahay, G., Brouard, S., Aronica, M. & Magnan, A. IL-17 in severe asthma. Where do we stand? *Am. J. Respir. Crit. Care Med.* **190**, 1094-1101 (2014).
10. Lindén, A. & Dahlén, B. Interleukin-17 cytokine signalling in patients with asthma. *Eur. Respir. J.* **44**, 1319-1331 (2014).

11. Bengsch, B., Seigel, B., Flecken, T., Wolanski, J., Blum, H. E. & Thimme, R. Human Th17 cells express high levels of enzymatically active dipeptidyl peptidase IV (CD26). *J. Immunol.* **188**, 5438-5447 (2012).
12. Salgado, F. J., Pérez-Díaz, A., Villanueva, N. M., Lamas, O., Arias, P. & Nogueira, M. CD26: a negative selection marker for human Treg cells. *Cytometry A* **81**, 843-855 (2012).
13. Ohnuma, K., Dang, N. H. & Morimoto, C. Revisiting an old acquaintance: CD26 and its molecular mechanisms in T cell function. *Trends Immunol.* **29**, 295-301 (2008).
14. Klemann, C., Wagner, L., Stephan, M. & von Hörsten, S. Cut to the chase: a review of CD26/dipeptidyl peptidase-4's (DPP4) entanglement in the immune system. *Clin. Exp. Immunol.* **185**, 1-21 (2016).
15. Nieto-Fontarigo, J. J., González-Barcala, F. J., San José, E., Arias, P., Nogueira, M. & Salgado, F. J. CD26 and Asthma: A Comprehensive Review. *Clin. Rev. Allergy Immunol.* 10.1007/s12016-016-8578-z (2016).
16. Ibegbu, C. C., Xu, Y. X., Fillos, D., Radziewicz, H., Grakoui, A. & Kourtis, A. P. Differential expression of CD26 on virus-specific CD8(+) T cells during active, latent and resolved infection. *Immunology* **126**, 346-353 (2009).
17. Bailey, S. R., Nelson, M. H., Majchrzak, K., Bowers, J. S., Wyatt, M. M., Smith, A. S. et al. Human CD26high T cells elicit tumor immunity against multiple malignancies via enhanced migration and persistence. *Nat. Commun.* **8**:1961 (2017).
18. Ohnuma, K., Takahashi, N., Yamochi, T., Hosono, O., Dang, N. H. & Morimoto, C. Role of CD26/dipeptidyl peptidase IV in human T cell activation and function. *Front. Biosci*. **13**, 2299-2310 (2008).

19. Lun, S. W., Wong, C. K., Ko, F. W., Hui, D. S. & Lam, C. W. Increased expression of plasma and CD4+ T lymphocyte costimulatory molecule CD26 in adult patients with allergic asthma. *J. Clin. Immunol.* **27**, 430-437 (2007).
20. Nieto-Fontarigo, J. J., González-Barcala, F. J., San-José, M. E., Cruz, M. J., Linares, T., Soto-Mera, M. T. et al. Expansion of a CD26low Effector TH Subset and Reduction in Circulating Levels of sCD26 in Stable Allergic Asthma in Adults. *J. Investig. Allergol. Clin. Immunol.* **28**, 113-125 (2018).
21. Röhrborn, D., Eckel, J. & Sell, H. Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells. *FEBS Lett.* **588**, 3870-3877 (2014).
22. Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T. et al. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* **58**, 573-581 (1989).
23. Zhou, L., Ivanov, I. I., Spolski, R., Min, R., Shenderov, K., Egawa, T. et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* **8**, 967-974 (2007).
24. Maddur, M. S., Miossec, P., Kaveri, S. V. & Bayry, J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. *Am. J. Pathol.* **181**, 8-18 (2012).
25. Nish, S. A., Schenten, D., Wunderlich, F. T., Pope, S. D., Gao, Y., Hoshi, N. et al. T cell-intrinsic role of IL-6 signaling in primary and memory responses. *Elife* **3**, e01949 (2014).
26. Lust, J. A., Donovan, K. A., Kline, M. P., Greipp, P. R., Kyle, R. A. & Maihle, N. J. Isolation of an mRNA encoding a

soluble form of the human interleukin-6 receptor. *Cytokine* **4**, 96-100 (1992).

27. Rose-John, S., Scheller, J., Elson, G. & Jones, S. A. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *J. Leukoc. Biol.* **80**, 227-236 (2006).
28. Jones, G. W., McLoughlin, R. M., Hammond, V. J., Parker, C. R., Williams, J. D., Malhotra, R. et al. Loss of CD4+ T cell IL-6R expression during inflammation underlines a role for IL-6 trans signaling in the local maintenance of Th17 cells. *J. Immunol.* **184**, 2130-2139 (2010).
29. Scheller, J., Ohnesorge, N. & Rose-John, S. Interleukin-6 trans-signalling in chronic inflammation and cancer. *Scand. J. Immunol.* **63**, 321-329 (2006).
30. Chalaris, A., Garbers, C., Rabe, B., Rose-John, S. & Scheller J. The soluble Interleukin 6 receptor: generation and role in inflammation and cancer. *Eur. J. Cell Biol.* **90**, 484-494 (2011).
31. Neurath, M. F. & Finotto, S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev.* **22**, 83-89 (2011).
32. Farahi, N., Paige, E., Balla, J., Prudence, E., Ferreira, R. C., Southwood, M. et al. Neutrophil-mediated IL-6 receptor trans-signaling and the risk of chronic obstructive pulmonary disease and asthma. *Hum. Mol. Genet.* **26**, 1584-1596 (2017).
33. Ullah, M. A., Revez, J. A., Loh, Z., Simpson, J., Zhang, V., Bain, L. et al. Allergen-induced IL-6 trans-signaling activates γδ T cells to promote type 2 and type 17 airway inflammation. *J. Allergy Clin. Immunol.* **136**, 1065-1073 (2015).
34. Tamura-Yamashita, K., Endo, J., Isogai, S., Matsuoka, K., Yonekawa, H. & Yoshizawa, Y. Gamma-delta T cell is essential for allergen-induced late asthmatic response in a

murine model of asthma. *J. Med. Dent. Sci.* **55**, 113-120 (2008).
35. Chien, Y. H., Zeng, X. & Prinz, I. The natural and the inducible: interleukin (IL)-17-producing γδ T cells. *Trends Immunol.* **34**, 151-154 (2013).
36. van Rensen, E. L., Sont, J. K., Evertse, C. E., Willems, L. N., Mauad, T., Hiemstra, P. S. et al. Bronchial CD8 cell infiltrate and lung function decline in asthma. *Am. J. Respir. Crit. Care Med.* **172**, 837-841 (2005).
37. den Otter, I., Willems, L. N., van Schadewijk, A., van Wijngaarden, S., Janssen, K., de Jeu, R. C. et al. Lung function decline in asthma patients with elevated bronchial CD8, CD4 and CD3 cells. *Eur. Respir. J.* **48**, 393-402 (2016).
38. Dakhama, A., Collins, M. L., Ohnishi, H., Goleva, E., Leung, D. Y., Alam, R. et al. IL-13-producing BLT1-positive CD8 cells are increased in asthma and are associated with airway obstruction. *Allergy* **68**, 666-673 (2013).
39. Huber, M. & Lohoff, M. Change of paradigm: CD8+ T cells as important helper for CD4+ T cells during asthma and autoimmune encephalomyelitis. *Allergo. J. Int.* **24**, 8-15 (2015).
40. Hatano, R., Ohnuma, K., Yamamoto, J., Dang, N. H. & Morimoto, C. CD26-mediated co-stimulation in human CD8(+) T cells provokes effector function via pro-inflammatory cytokine production. *Immunology* **138**, 165-172 (2013).
41. Lee, N., You, S., Shin, M. S., Lee, W. W., Kang, K. S., Kim, S. H. et al. IL-6 receptor α defines effector memory CD8+ T cells producing Th2 cytokines and expanding in asthma. *Am. J. Respir. Crit. Care Med.* **190**, 1383-1394 (2014).
42. Casrouge, A., Sauer, A. V., Barreira da Silva, R., Tejera-Alhambra, M., Sánchez-Ramón, S., ICAReB. et al.

Lymphocytes are a major source of circulating soluble dipeptidyl peptidase 4. *Clin. Exp. Immunol.* **194**, 166-179 (2018).

43. Zhen, G., Park, S. W., Nguyenvu, L. T., Rodriguez, M. W., Barbeau, R., Paquet, A. C. et al. IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am. J. Respir. Cell Mol. Biol.* **36**, 244-253 (2007).
44. Zhou, T., Huang, X., Zhou, Y., Ma, J., Zhou, M., Liu, Y. et al. Associations between Th17-related inflammatory cytokines and asthma in adults: A Case-Control Study. *Sci. Rep.* **7**, 15502 (2017).
45. Yan, S., Gessner, R., Dietel, C., Schmiedek, U. & Fan, H. Enhanced ovalbumin-induced airway inflammation in CD26-/- mice. *Eur. J. Immunol.* **42**, 533-540 (2012).
46. Stephan, M., Suhling, H., Schade, J., Wittlake, M., Tasic, T., Klemann, C. et al. Effects of dipeptidyl peptidase-4 inhibition in an animal model of experimental asthma: a matter of dose, route, and time. *Physiol. Rep.* **1**, e00095 (2013).
47. Di Mitri, D., Azevedo, R. I., Henson, S. M., Libri, V., Riddell, N. E., Macaulay, R. et al. Reversible senescence in human CD4+CD45RA+CD27- memory T cells. *J. Immunol.* **187**, 2093-2100 (2011).
48. Bank I, Marcu-Malina V. Quantitative peripheral blood perturbations of γδ T cells in human disease and their clinical implications. *Clin. Rev. Allergy Immunol.* **47**, 311-333 (2014).
49. Pang DJ, Neves JF, Sumaria N, Pennington DJ. Understanding the complexity of γδ T-cell subsets in mouse and human. *Immunology* **136**, 283-290 (2012).
50. Dieli, F., Poccia, F., Lipp, M., Sireci, G., Caccamo, N., Di Sano, C. et al. Differentiation of effector/memory Vdelta2 T

cells and migratory routes in lymph nodes or inflammatory sites. *J. Exp. Med.* **198**, 391-397 (2003).

51. Caccamo, N., La Mendola, C., Orlando, V., Meraviglia, S., Todaro, M., Stassi, G. et al. Differentiation, phenotype, and function of interleukin-17-producing human Vγ9Vδ2 T cells. *Blood* **118**, 129-138 (2011).
52. Oberg, H. H., Wesch, D., Grüssel, S., Rose-John, S. & Kabelitz, D. Differential expression of CD126 and CD130 mediates different STAT-3 phosphorylation in CD4+CD25- and CD25high regulatory T cells. *Int. Immunol.* **18**, 555-563 (2006).
53. Lamas, J. R., Rodríguez-Rodríguez, L., Tornero-Esteban, P., Villafuertes, E., Hoyas, J., Abasolo, L. et al. Alternative splicing and proteolytic rupture contribute to the generation of soluble IL-6 receptors (sIL-6R) in rheumatoid arthritis. *Cytokine* **61**, 720-723 (2013).
54. Schumacher, N., Meyer, D., Mauermann, A., von der Heyde, J., Wolf, J., Schwarz, J. et al. Shedding of Endogenous Interleukin-6 Receptor (IL-6R) Is Governed by A Disintegrin and Metalloproteinase (ADAM) Proteases while a Full-length IL-6R Isoform Localizes to Circulating Microvesicles. *J. Biol. Chem.* **290**, 26059-26071 (2015).
55. Yan, I., Schwarz, J., Lücke, K., Schumacher, N., Schumacher, V., Schmidt S. et al. ADAM17 controls IL-6 signaling by cleavage of the murine IL-6Ralpha from the cell surface of leukocytes during inflammatory responses. *J. Leukoc. Biol.* **99**, 749-760 (2016).
56. Wolf, J., Waetzig, G. H., Reinheimer, T. M., Scheller, J., Rose-John, S. & Garbers, C. A soluble form of the interleukin-6 family signal transducer gp130 is dimerized via a C-terminal disulfide bridge resulting from alternative mRNA splicing. *Biochem. Biophys. Res. Commun.* **470**, 870-876 (2016).

57. Hemken, P. M., Jeanblanc, N. M., Rae, T., Brophy, S. E., Datwyler, M. J., Xu, Y. et al. Development and analytical performance of a new ARCHITECT automated dipeptidyl peptidase-4 immunoassay. *Pract. Lab. Med.* **9**, 58-68 (2017).
58. Agache, I., Strasser, D. S., Pierlot, G. M., Farine, H., Izuhara, K. & Akdis, C. A. Monitoring inflammatory heterogeneity with multiple biomarkers for multidimensional endotyping of asthma. *J. Allergy Clin. Immunol.* **141**, 442-445 (2018).
59. Hartl, D., Koller, B., Mehlhorn, A. T., Reinhardt, D., Nicolai, T., Schendel, D. J. et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J. Allergy Clin. Immunol.* **119**, 1258-1266 (2007).
60. Mandapathil, M., Szczepanski, M., Harasymczuk, M., Ren, J., Cheng, D., Jackson, E. K. et al. CD26 expression and adenosine deaminase activity in regulatory T cells (Treg) and CD4(+) T effector cells in patients with head and neck squamous cell carcinoma. *Oncoimmunology* **1**, 659-669 (2012).
61. Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A. et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* **204**, 1257-1265 (2007).
62. De Meester, I., Vanham, G., Kestens, L., Vanhoof, G., Bosmans, E., Gigase, P. et al. Binding of adenosine deaminase to the lymphocyte surface via CD26. *Eur. J. Immunol.* **24**, 566-570 (1994).
63. Moreno, E., Canet, J., Gracia, E., Lluís, C., Mallol, J., Canela, E. I. et al. Molecular Evidence of Adenosine Deaminase Linking Adenosine A2A Receptor and CD26 Proteins. *Front. Pharmacol.* **9**, 106 (2018).

64. Li, P., Gao, Y., Cao, J., Wang, W., Chen, Y., Zhang, G. et al. CD39+ regulatory T cells attenuate allergic airway inflammation. *Clin. Exp. Allergy* **45**, 1126-1137 (2015).
65. Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S. et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* **203**, 1701-1711 (2006).
66. Doganci, A., Eigenbrod, T., Krug, N., De Sanctis, G. T., Hausding, M., Erpenbeck, V. J. et al. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *J. Clin. Invest.* **115**, 313-325 (2005).
67. Wang, Y., Hu, H., Wu, J., Zhao, X., Zhen, Y., Wang, S. et al. The IL6R gene polymorphisms are associated with sIL-6R, IgE and lung function in Chinese patients with asthma. *Gene* **585**, 51-57 (2016).

SUPPLEMENTARY TABLES

Supplementary Table S1. List of flow cytometry antibodies.

Target	Clone	Fluorophore	Provider	Cat. no
CD3	UCHT1	FITC	BD Pharmigen™	555332
CD4	RPA-T4	FITC	BD Pharmigen™	555346
CD25	M-A251	PE-Cy7	BD Pharmigen™	5135905
CD127	HIL-7R-M21	AlexaFluor-647	BD Pharmigen™	558598
CD3	UCHT1	PerCP-Cy5.5	BD Pharmigen™	560835
CD8	RPA-T8	PerCP-Cy5.5	BD Pharmigen™	560662
CD19	HIB19	PerCP-Cy5.5	BD Pharmigen™	561295
CD27	M-T271	PerCP-Cy5.5	BD Pharmigen™	560612
CD28	CD28.2	PerCP-Cy5.5	BD Pharmigen™	560685
CD45RA	HI100	PerCP-Cy5.5	BD Pharmigen™	563429
CD56	B159	PerCP-Cy5.5	BD Pharmigen™	560842
CD197 (CCR7)	150503	PerCP-Cy5.5	BD Pharmigen™	561144
TCR-γ/δ	B1	FITC	Biolegend	331207
CD26	TP1/19	PE	Immunostep	PE26 100T
CD126 (IL-6Rα)	REA291	PE	MACS Miltenyi Biotec	130-104-101

Supplementary Table S2. Correlation of CD26 and CD126 expression with the % of different leukocyte subsets.

	Correlation between the % of cells and the % of CD26+ cells in each subpopulation		Correlation between the % of cells and the % of CD126+ cells in each subpopulation	
	r_s	*P*	r_s	*P*
Eosinophils	**-.181**	**.003**	**.205**	**< .001**
Monocytes	**-.206**	**< .001**	-.085	.168
Neutrophils	.041	.500	.035	.564
Lymphocytes	-.103	.094	-.057	.354
$CD4^-$ cells	**-.312**	**< .001**	**-.353**	**< .001**
$CD4^+$ cells	**.367**	**< .001**	**.211**	**< .001**
Teff cells	**.469**	**< .001**	**.247**	**< .001**
Treg cells	.023	.702	-.070	.254
Tlow cells	**-.342**	**< .001**	**-.360**	**< .001**

Supplementary Table S3. Severity and control degree of the study patients.

	IMA	MSA	CA	UA
N	102	90	158	34
Age (mean (range))	44 (20-72)	41 (18-68)	45 (18-72)	45 (20-68)
Sex (M/F)	33/69	35/55	59/99	9/25
FEV1 (%)	105.0 (95.0-113.3)***	86.5 (67.0-100.0)	99.0 (87.0-111.0)*	82.0 (60.7-100.0)
FEV1/FVC (%)	77.5 (72.5-81.2)***	70.3 (59.5-78.5)	76.4 (69.0-80.5)*	70.0 (56.4-76.8)
Neutrophils (10^3 cells/µL)	3.60 (3.09-4.23)	3.62 (2.88-4.40)	3.58 (3.07-4.23)	4.17 (2.78-5.00)
Lymphocytes (10^3cells/µL)	1.97 (1.65-2.25)	1.97 (1.64-2.54)	1.95 (1.60-2.26)	2.02 (1.86-2.74)
Monocytes (10^3 cells/µL)	0.39 (0.30-0.48)	0.39 (0.32-0.49)	0.38 (0.30-0.49)	0.41 (0.32-0.48)
Eosinophils (10^3 cells/µL)	0.26 (0.17-0.39)*	0.33 (0.21-0.48)	0.27 (0.18-0.40)*	0.38 (0.21-0.63)
Basophils (10^3 cells/µL)	0.04 (0.02-0.05)	0.04 (0.03-0.05)	0.04 (0.02-0.05)	0.04 (0.03-0.06)
IgE (IU/mL)	45.5 (15.5-205.0)	84.0 (28.2-209.5)	79.0 (20.5-200.0)	62.0 (24.7-274.0)

CA, controlled asthmatics; IMA, intermittent-mild asthmatics; MSA, moderate-severe asthmatics; UA, uncontrolled asthmatics.
Data are presented as median value (IQR1-3), unless otherwise expressed.
Mann-Whitney U test was used to compare IMA vs. MSA and CA vs. UA. * *P* < 0.05; * *P* < 0.001**

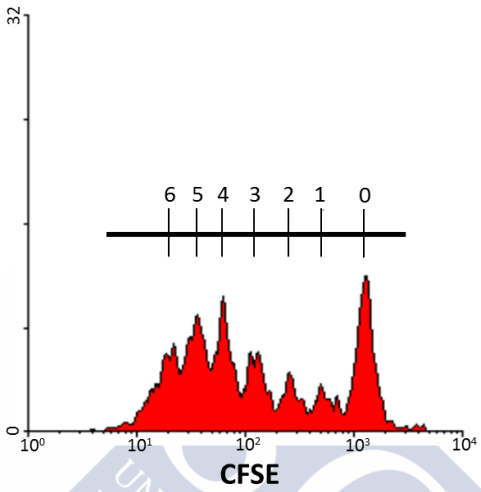
Supplementary Table S4. Average expression of CD26 and CD126 molecules in different leukocyte subsets and their half-life in peripheral circulation.

	Eosinophils	Monocytes	Neutrophils	Lymphocytes
Absolute count (x10^3 cells/mL)	0.04-0.4	0.2-0.8	2.5-7.5	1.5-3.5
% of CD126$^+$ cells*	63.4 (44.5-77.5)	78.5 (52.2-91.0)	99.5 (98.8-99.8)	55.3 (48.6-61.3)
% of CD26$^+$ cells*	2.1 (1.4-3.2)	7.4 (3.5-17.6)	11.9 (5.0-27.7)	59.2 (54.4-66.4)
Half-life in peripheral blood[#]	8-18 h	22-24 h	6 h - 5 days	days-years

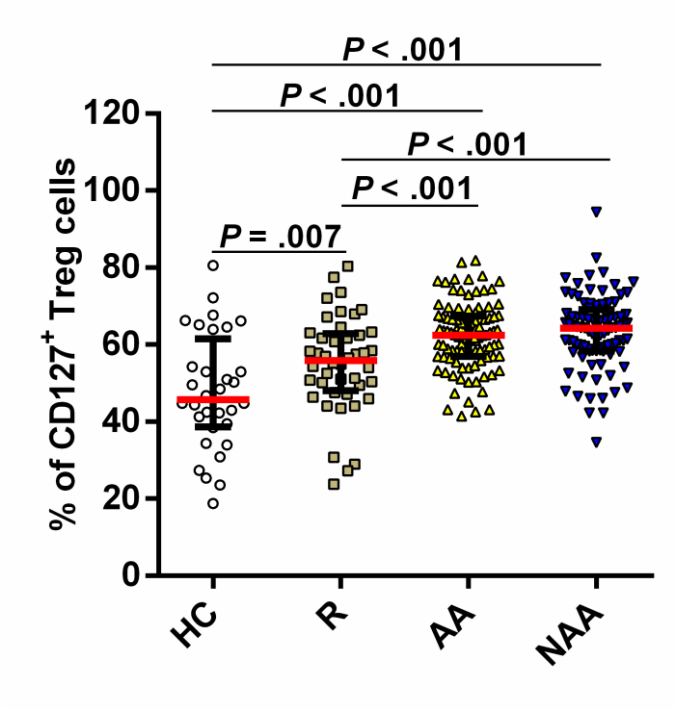
*Median value (IQR1-3).

[#]Data come from Pillay J, et al. Blood. 2010;116:625-7; Park YM, et al. Allergy Asthma Immunol Res. 2010;2:87-101; Patel AA, et al. J Exp Med. 2017;214:1913-1923; Robertson JM, et al. Immunol Rev. 2006;211:49-57.

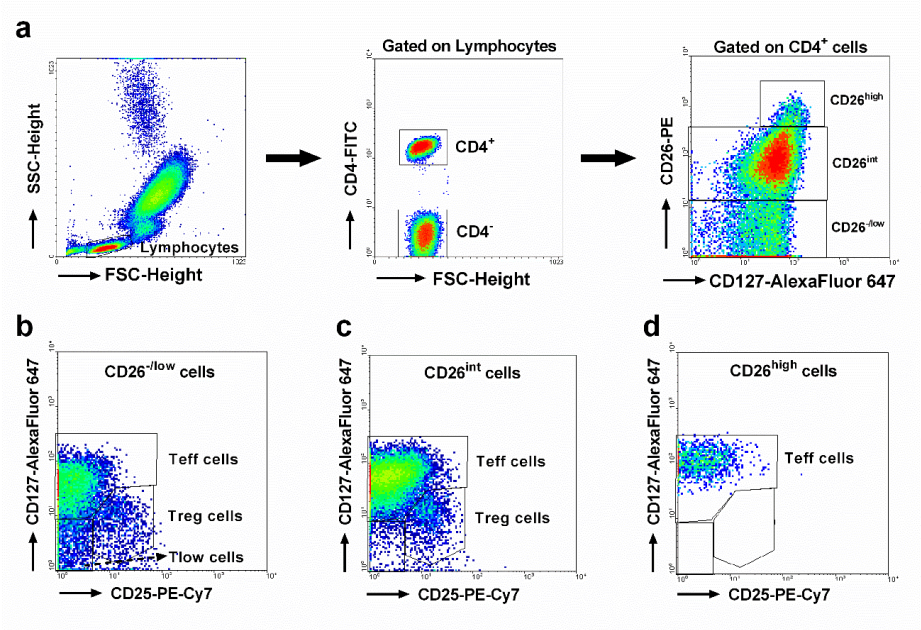
SUPPLEMENTARY FIGURES



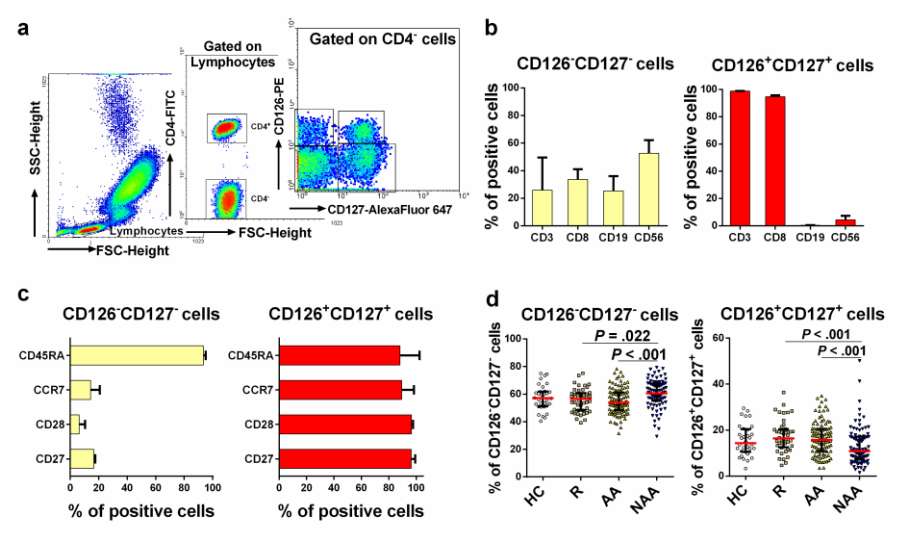
Supplementary Figure S1. Analysis of the number of cell divisions by flow cytometry. PBMCs were stained with 5 µM CFSE and cultured at 0.25 x 10^6 cells/mL in complete medium (RPMI 1640, 10% FBS, 100 µg/mL streptomycin, 100 U/mL penicillin) supplemented with 1 µg/ml PHA as a T-cell specific polyclonal stimulus. Upon 6 days of *in vitro* culture, CFSE fluorescence decay was measured by flow cytometry and allowed the calculation of the number of cell divisions. A representative histogram plot is shown, where numbers 1-6 represent the corresponding round of division, and 0 those lymphocytes that remained at a resting state. Responder frequency (Rf) was calculated as the percentage of cells that divided at least once (i.e., percentage of cells that divide from 1 to 6 times).



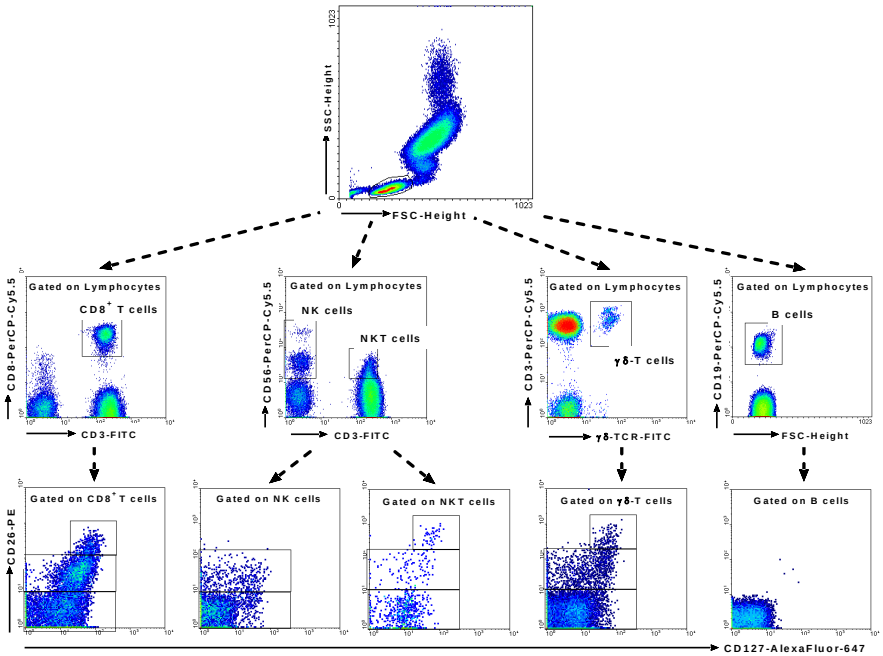
Supplementary Figure S2. Rhinitic and asthmatic patients have augmented levels of CD127$^+$ regulatory T cells. Lymphocytes from healthy controls (HC; N = 32) and patients with rhinitis (R; N = 44), allergic asthma (AA; N = 100), and non-allergic asthma (NAA; N = 92) were gated on FSC/SSC plots and further subdivided into CD4$^-$ and CD4$^+$ cells. Then, regulatory T cells (Treg) were identified amongst CD4$^+$ T lymphocytes according to CD25 and CD127 markers. Afterwards, the percentage of CD127$^+$ cells within the Treg compartment was measured. Statistically significant difference between the different groups of donors is indicated ($P < .05$).



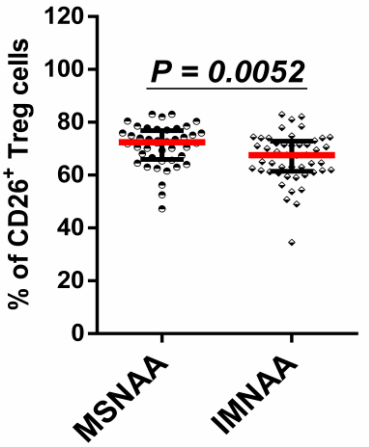
Supplementary Figure S3. Treg cells are distributed within CD26$^{-/low}$ and CD26^{int} subsets of CD4^{+} T cells. a) Lymphocytes from healthy controls (HC) and patients with rhinitis (R), allergic asthma (AA), and non-allergic asthma (NAA) were gated on FSC/SSC plots and further subdivided into CD4^{-} and CD4^{+} cells. Then, 3 subsets of CD4^{+} lymphocytes were identified based on CD26 and CD127 markers: CD26$^{-/low}$, CD26^{int} and CD26^{high}. As figures **b-d** point out, Treg cells (CD25^{high}CD127^{low}) are present in CD26$^{-/low}$ **(b)** and CD26^{int} **(c)** subsets, but not in CD26^{high} subpopulation **(d)**.



Supplementary Figure S4. CD4$^-$ lymphocytes with a CD126$^-$CD127$^-$ phenotype are increased in non-allergic asthmatics (NAA) compared to allergic asthmatics (AA). a) CD4$^-$ lymphocytes were identified through the gating strategy depicted (left and middle dot-plots), and expression of CD127 and CD126 was used to identify four subsets (right dot plot). **b)** Lymphocyte subset composition of CD4$^-$CD126$^-$CD127$^-$ and CD4$^-$CD126$^+$CD127$^+$ cells based on CD3 (T), CD8 (Tc), CD19 (B), and CD56 (NK, NKT) antigens. **c)** The phenotype of CD4$^-$CD126$^-$CD127$^-$ and CD4$^-$CD126$^+$CD127$^+$ cells based on CD45RA, CCR7, CD127, CD28, and CD27 markers. We evidenced that the former CD26^{int}CD127$^+$ and CD26^{high}CD127$^+$ subsets are combined in one single CD4$^-$ population (CD126$^+$CD127$^+$ cells), with a homogeneous naïve-memory CD3$^+$CD8$^+$ phenotype. In contrast, the CD126$^-$CD127$^-$ subset comprised T_{EMRA}-like cells (CD45RA^{high}CCR7^{low}CD27^{low}CD28^{low}), but a less-defined CD4$^-$ lymphoid origin (B, NK, NKT, or γδ-T cells). In **b)** and **c)**, data were obtained from 3 representative donors. **d)** Percentage (median ± IQR) of CD126$^-$CD127$^-$ and CD126$^+$CD127$^+$ cells in CD4$^-$ lymphocytes from healthy controls (HC; N = 32) and patients with rhinitis (R; N = 44), allergic asthma (AA; N = 100), and non-allergic asthma (NAA; N = 92). Like CD4$^-$CD26$^+$CD127$^+$ lymphocytes, CD4$^-$CD126$^+$CD127$^+$ cells were reduced in NAA compared to AA, which was associated to the expansion of the double negative (CD126$^-$CD127$^-$) compartment in NAA. Only results from those subpopulations with significant differences between donor groups are shown. Statistically significant differences between groups of donors are indicated (Kruskal-Wallis test: $P < .05$).



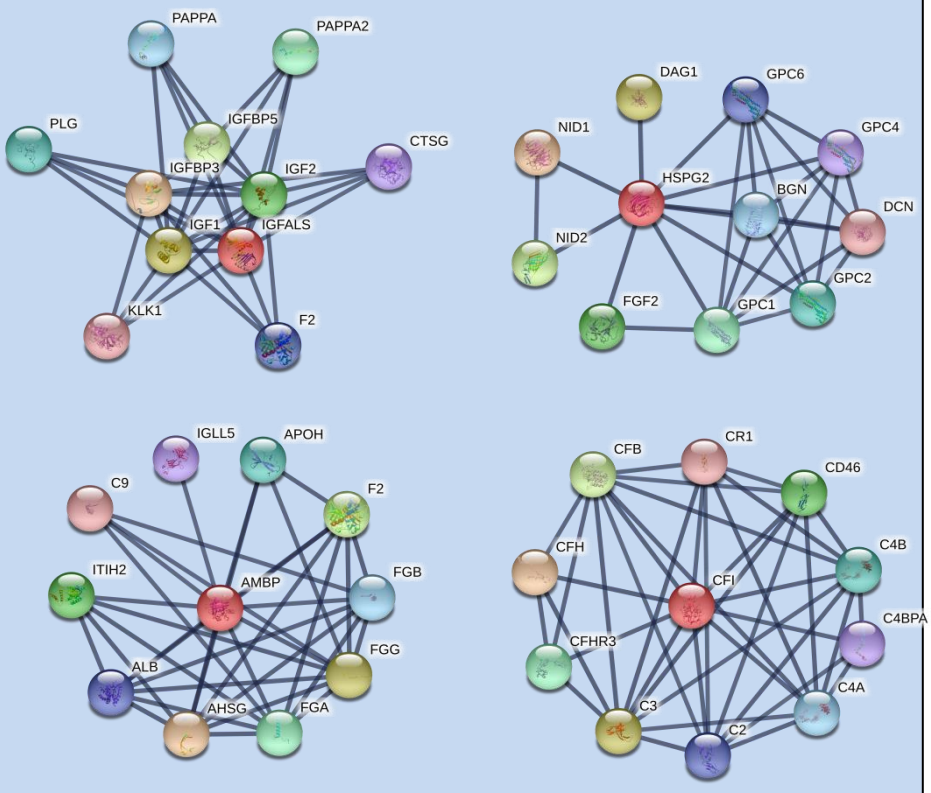
Supplementary Figure S5. Gating strategy for CD8$^+$ Tc, NK, NKT, γδ-T, and B lymphocytes. After gating, expression of CD127 and CD26 was used to identify different subsets (lower row of dot-plots). CD3$^+$ lymphocytes (Tc, NKT, γδ-T) are characterized by the presence of three subsets (CD26$^{-/low}$, CD26^{int}, and CD26^{high}), while CD3$^-$ lymphocytes display a double (CD26$^{-/low}$, CD26^{int}; NK cells) or a single (CD26$^-$: B cells) phenotype.



Supplementary Figure S6. Moderate-severe non-allergic asthmatics display higher percentage of CD26$^+$ regulatory T cells than intermittent-mild non-allergic asthmatics. Lymphocytes from either moderate-severe non-allergic (MSNAA; N = 90) or intermittent-mild non-allergic (IMNAA; N = 102) asthmatics were gated on FSC/SSC plots and further subdivided into CD4$^-$ and CD4$^+$ cells. Then, regulatory T cells (Treg) were identified amongst CD4$^+$ T lymphocytes according to CD25 and CD127 markers. Afterwards, the percentage of CD26$^+$ cells within the Treg compartment was measured. Statistically significant difference between the different groups of donors is indicated (*P*-value).

CHAPTER IV

Mining the serum proteome of rhinitis and different asthma phenotypes/severities by CPLLs and iTRAQ-coupled LC-MS/MS



USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

Asthma is a complex chronic disorder of the airways characterised by airways remodelling and hyper-responsiveness, recurrent airflow obstruction, mucosal cell hyperplasia, and increased vascular permeability, which results in shortness of breath [1-3]. There are two major clinical asthma phenotypes, named allergic/atopic asthma (AA) and non-allergic/non-atopic asthma (NAA) [4, 5], which involve several underlying pathobiological mechanisms (especially NAA): the asthma endotypes [2, 6].

The most prevalent asthma phenotype is AA, which typically develops from childhood. It is less severe than NAA [5] and mainly driven by type 2 helper T-cells (TH2) and eosinophilic airway inflammation, with enhancement of TH2 markers (IL-5, IL-13, exhaled nitric oxide fraction/FENO, blood/sputum eosinophilia, and serum periostin) [7-9]. The airway epithelium has also a key role in AA pathogenesis through IL-25, IL-33, and TSLP, a set of cytokines released in response to allergens and with a stimulatory role on DCs, basophils, and innate type 2 lymphocytes (ILC2) [10]. Subsequently, ILC2 cells boost the differentiation of TH2 and IgE-producing B-cells through the release of IL-4 and IL-13 [10]. Allergen-specific IgE has also a central role in AA leading to the activation of mast cells, which results in the release of inflammatory mediators such as histamine and leukotrienes [11]. AA patients usually present allergic rhinitis, eczema, or food and drug allergy as comorbidities [2]. Although the prevalence of rhinitis in NAA patients is similar to global rhinitis prevalence (~20-30%), the ~ 90% of AA patients suffer from allergic rhinitis. Therefore, these two pathologies could share similar immunopathogenic mechanisms and biomarkers [12, 13]. Indeed, a transition from an allergic inflammation of upper-respiratory airways in rhinitis to allergic inflammation of lower-respiratory airways in AA has been postulated [13, 14].

About 10-50% of adult patients with asthma display a difficult to diagnose NAA phenotype. This asthma variant is associated with a negative result of the skin prick test (SPT) or *in vitro* techniques like RAST or ELISA [4]. NAA subjects exhibit a more sever-persistent disease, with lower levels of FEV1 and FVC, the absence of a TH2-signature, and poorer prognosis or response to treatments like glucocorticosteroids, anti-immunoglobulin E (IgE), and anti-interleukin 5 (IL-5) [4]. This asthma phenotype is usually associated with cough, chronic rhinosinusitis (CRS), nasal polyposis (NP), and hypersensitivity to aspirin/NSAIDs [4]. Besides, NAA shows a higher prevalence amongst females, and a later age of onset (i.e., it is more typical during adulthood) [4]. NAA is a more heterogeneous phenotype, including diverse endotypes (e.g., aspirin-exacerbated respiratory disease/AERD, obesity-associated asthma, occupational asthma) and where TH17 cells, their derived cytokines (e.g., IL-17), and neutrophils appear to be key mediators [6, 15-17]. However, the underlying immunopathological mechanisms in $TH2^{low}$ asthma are not well understood. Therefore, it seems necessary to perform new studies aimed to search for new biomarkers which could a) reveal novel mechanisms of asthma pathogenesis and pathophysiology, b) make a clearer distinction between asthma phenotypes, and c) turn into novel targets for asthma treatment.

The field of Proteomics has paved the way for the discovery of potential diagnostic/prognostic biomarkers in chronic and heterogeneous diseases like asthma. Samples generally used include exhaled breath condensate, different fluids (e.g., BALF, induced sputum, serum, plasma, urine), *in vitro* lung cell cultures, or tissue biopsies/autopsies [18-20]. Blood-derived specimens (e.g., plasma, serum) are widely used in clinical studies and contain an extremely complex mixture of proteins (>10,000 unique proteins; http://www.plasmaproteomedatabase.org), which makes these

biofluids ideal for proteomic biomarker discovery studies [21]. Thus, the application of proteomic techniques to this kind of samples may allow a researcher to identify reliable and disease-relevant biomarkers or underlying pathological mechanisms associated with an asthmatic subgroup of subjects. However, several challenges must be faced in the initial steps of proteomic discovery studies, such as the poor signal-to-noise ratio obtained when sampling is carried out distal from the diseased tissue (e.g., bronchi), the protein complexity itself of serum/plasma samples, and the extremely dynamic range of protein abundances in biofluids (12 orders of magnitude) [21-23]. Several techniques have been developed in order to reduce sample complexity and allow the detection of low-abundant proteins (the so-called "deep-proteome"; nano-femtomolar range) [24], such as ultrafiltration [25, 26], the precipitation of high-molecular proteins by acetonitrile [27], the depletion of high-abundant proteins [28], or the use of combinatorial peptide ligand libraries (CPLLs) [29]. These strategies could remove as much as 99% of the initial proteins in biofluid samples. In any case, following enzymatic digestion of remaining proteins, peptides can be separated by high-throughput liquid chromatography (LC) and submitted to tandem mass spectrometry (MS/MS) to generate a highly confident list of proteins through an effective "shotgun", "bottom-up" proteomic approach [30, 31]. However, in these protocols, intense pre-fractionation of peptides by means of different chromatographic approaches [32, 33] and analysis of technical/biological replicates are necessary to reach the deep-proteome, measure protein abundances, generate an almost complete protein enumeration in samples, and consider the biological variance [34]. This means that the initial establishment of a correct protocol of analysis is a key issue in clinical proteomic studies.

Some plasma/serum proteomic studies have been applied to asthma [19, 20, 35], but only one using an LC-MS/MS approach [36].

Moreover, to our knowledge, none of them was designed to explore the differences of the serum proteome from rhinitis and allergic/non-allergic asthmatic subjects. For this reason, the aim of the present study was double: a) to develop a "shotgun / bottom-up" methodology for the quantitative analysis of the proteome of medium-low abundance in serum samples from patients with rhinitis and diverse asthmatic phenotypes; b) to perform an initial discovery/non-targeted proteomic analysis to detect candidate biomarkers associated with the presence of rhinitis or different asthma phenotypes (allergic/non-allergic) or severities (moderate-severe and intermittent-mild), which could also provide new insights into asthma pathogenesis and pathophysiology, as well as improve future therapeutic interventions.

MATERIALS AND METHODS

Please see Supplementary Information for further details.

Donors

A set of 267 adult subjects were recruited at the CHUS Pneumology Service and divided into 6 study groups: moderate-severe allergic asthma (MSAA; N = 49), intermittent-mild allergic asthma (IMAA; N = 53), rhinitis (R; N = 43), moderate-severe non-allergic asthma (MSNAA; N = 43), intermittent-mild non-allergic asthma (IMNAA; N = 47), and healthy controls (HC; N = 32). Asthmatic patients had a confirmed diagnosis of asthma for at least one year (Global Strategy for Asthma Management and Prevention criteria, GINA 2006) and were in a stable phase (>4 weeks). A positive bronchodilator test (>12% of FEV1 change after salbutamol) or methacholine challenge was used to confirm asthma diagnosis. Moreover, skin prick test and

allergen-specific IgE were used to verify the allergic phenotype. Haematology, biochemistry, spirometry, and skin sensitization studies were carried out by the clinical staff of the hospital. The research protocol was approved by the Ethics Committee of Clinical Research of Galicia (2011/001), and all the participants signed informed consent.

Serum samples preparation.

Two pools of serum samples from each group of patients (pool A and pool B) and a reference sample (pooled sample; PS) composed of all study samples were prepared. Then, delipidation was carried out by means of LRA™ (Lipid Removal Agent) resin (13358-U; Sigma, Spain). For this aim, a titration of the amount of resin needed was made (20-100 mg/mL) and a concentration of 80 mg/mL of LRA™ resin selected based on the reduction of apoAI in 1-DE gels (~ 25-30 kDa band) (Supplementary figure 1). Afterwards, serum samples were enriched in low abundance proteins by using CPLLs (ProteoMiner™ Protein Enrichment, Large-Capacity Kit, Bio-Rad, 163-3007). Finally, protein samples eluted from ProteoMiner columns were cleaned by means of the 2-D Cleanup Kit (GE Healthcare, 80-6484-51), and then diluted into 30 µL of 0.5M TEAB 6M urea for ulterior analysis.

Fifty µg of protein from each sample were placed in 20 µL of 0.5M TEAB 6M urea, reduced (TCEP), and alkylated (MMTS). Subsequently, trypsinization was performed at 37 ° C overnight. Then, all samples were lyophilized and dissolved in 30 µL of 0.5M TEAB. Finally, peptides were labelled by means of iTRAQ® Reagent-8PLEX Multiplex Kit (Sigma-Aldrich) and pooled. Reporter ions were associated with samples as follows: MSAA, 113; IMAA, 114; R, 115; MSNAA, 116; IMNAA, 117; HC, 119; PS, 121. PS sample is a pool of all the serum samples used to normalize the data between the

different MS analytical series. This procedure was performed twice, one for each biological replicate (pool A and pool B).

nanoLC/MS-MS identification and quantification of peptides

Peptide samples were reconstituted in solution A (0.1% formic acid (FA) in water) and desalted by Zip-Tips C18 (Millipore). Then, samples were analysed by LC-MS/MS by using a Proxeon EASY-nLC 1000 UHPLC (Thermo Fisher Scientific) coupled online to an LTQ-Orbitrap ELITE (Thermo Fisher Scientific). HCD-fragmentation was performed. Three technical replicates were performed from each biological replicate.

MS data analysis

Proteome Discoverer (version 2.1.1.21) was used for protein identification and iTRAQ quantification. HCD spectra were analysed using Sequest HT with Percolator validation. The intensities acquired in MS/MS were globally normalized on protein median. Then, all reporter intensities were normalized by the reporter intensity of the pooled sample (PS, 121). A 1.3 fold change (down-regulation, < 0.77; up-regulation, ≥ 1.3) in iTRAQ ratios, as well as a p-value < 0.05, was used to identify differentially abundant proteins between the different groups of study.

Validation of proteins by ELISA

Some differentially abundant proteins were selected for future validation by ELISA in 13 samples for each group. So far, only IGFALS was validated. For this aim, the Immunotag™ Human IGFALS (Insulin-Like Growth Factor Binding Protein, Acid Labile Subunit) ELISA (IT4078) from G-Biosciences was used. Optical densities were recorded at 450 nm and afterwards, protein

concentration was calculated from the standard curve. All protocols were performed according to the manufacturer´s guidelines.

Statistical and bioinformatic analysis of the data

Statistical comparisons between the groups were performed by Kruskal–Wallis one-way analysis of variance followed by Dunn's tests (GraphPad Prism 6, CA). The receiver operating characteristic (ROC) analysis was also performed with GraphPad Prism. Gene Ontology (GO) annotation, GO terms overrepresentation test, and Reactome pathway overrepresentation analyses against the plasma proteome database (Version 2015-02-02) were performed in the PANTHER Classification System (version 13.1). Protein-protein interaction analyses were also performed by means of the Search Tool for the Retrieval of Interacting Genes (STRING).

RESULTS

Clinical characteristics of the study patients

This work includes 267 adult patients with different asthma phenotypes (AA and NAA) and severities (IM and MS), a group with allergic rhinitis (R) and another group of healthy controls (HC). The characteristics of the different study groups are outlined in Table 1. As expected, patients with MS asthma had a decrease in forced expiratory volume in one second (FEV1%) and FEV1/forced vital capacity (FVC) compared with patients with IM asthma, although the reduction of FEV1% is only significant in the case of the NAA (Table 1). In addition, both FEV1% and FEV1/FVC ratio were also decreased in MS asthmatic patients (both AA and NAA) with respect to R (Table 1). The allergic asthma phenotype (MSAA, IMAA) was also characterized by higher levels of IgE than the remaining groups,

except for R patients (Table 1). On the other hand, BMI and ESR measures were slightly higher in NAA patients compared to R (Table 1). Leukocyte count was also performed, showing an increase in the number of circulating eosinophils in allergic asthma and MSNAA patients compared to HC, but also beside R in the case of MSAA (Table 1). Other biochemical or haematological parameters remained unchanged between groups (Table 1).

Table 1. Clinical and demographic characteristics of the study subjects.

	MSAA	IMAA	MSNAA	IMNAA	R	HC
N	49	53	43	47	43	32
Age (mean (range))	39 (18-68)	36 (20-66)	54 (24-68)	52 (29-72)	35 (18-55)	43 (22-61)
Sex (M/F)	23/26	25/28	13/30	8/39	24/19	15/17
Disease control:						
Yes	30	53	30	45	43	-
No	19	0	13	2	0	-
Baseline treatment:						
ICS-LABA	46	34	39	35	0	-
ICS	1	15	1	3	0	-
OCS	1	0	0	0	0	-
Antileukotrienes	25	18	21	9	10	-
Anticholinergic	13	3	25	2	0	-
Roflumilast	0	0	1	0	0	-
Prednisone	0	0	5	0	0	-
BMI (Kg/m^2)	26.9 (24.1-30.1)	25.3 (23.2-30.3)	29.2 (26.8-33.5)$^{\#}$	27.4 (255.4-30.5)$^{\#}$	25.3 (22.3-27.4)	-
FEV1 (%)	93.0 (81.5-103.0)$^{\#}$	101.0 (93.5-109.0)	75.0 (65.0-94.0)$^{\#\&}$	110.0 (97.0-116.0)	107.0 (99.0-119.0)	-
FEV1/FVC (%)	73.0 (64.1-79.2)$^{\#\&}$	79.1 (72.5-81.8)$^{\#}$	67.2 (59.5-76.6)$^{\#\&}$	76.6 (72.6-80.5)$^{\#}$	83.5 (79.0-87.4)	-

Continued on next page

Table 1 (Continued)

Neutrophils (10^3 cells/µL)	3.77 (2.75-4.50)	3.71 (3.17-4.27)	3.58 (3.03-4.49)	3.51 (2.72-4.13)	3.58 (2.93-4.00)	3.02 (2.28-4.05)
Lymphocytes (10^3cells/µL)	1.98 (1.65-2.66)	1.99 (1.67-2.24)	1.94 (1.56-2.54)	1.95 (1.58-2.25)	2.16 (1.89-2.60)	1.99 (1.53-2.49)
Monocytes (10^3 cells/µL)	0.39 (0.31-0.48)	0.41 (0.31-0.48)	0.42 (0.32-0.52)	0.35 (0.30-0.47)	0.39 (0.31-0.47)	0.39 (0.33-0.49)
Eosinophils (10^3 cells/µL)	0.36 (0.22-0.53)*#	0.26 (0.18-0.42)*	0.30 (0.18-0.45)*	0.24 (0.14-0.37)	0.22 (0.14-0.29)	0.13 (0.09-0.22)
Basophils (10^3 cells/µL)	0.04 (0.03-0.05)	0.04 (0.03-0.05)	0.03 (0.02-0.05)	0.03 (0.02-0.04)	0.03 (0.02-0.05)	0.03 (0.02-0.05)
ESR (1h; mm)	8.0 (4.5-15.0)	7.0 (2.0-14.5)	13.0 (7.0-23.0)#	12.0 (8.0-20.0)#	8.0 (2.0-15.0)	7.5 (2.0-10.0)
IgE (IU/mL)	138 (65-374)*$	163 (64-252)*$	43 (15-96)	22 (8-35)	68 (24-124)	15 (6-55)
IgG (mg/dL)	1060 (940-1150)	1080 (932-1210)	1055 (927-1255)	1020 (871-1160)	1025 (915-1208)	1015 (882-1118)
IgA (mg/dL)	213 (161-270)	243 (176-300)	220 (160-270)	206 (149-260)	187 (133-274)	291 (128-333)
IgM (mg/dL)	98 (70-150)	100 (69-143)	115 (76-160)	107 (73-149)	111 (69-163)	95 (86-114)

AA, allergic asthmatics; HC, healthy controls; NAA, non-allergic asthmatics; R, rhinitis patients.

Data are presented as median value (IQR1-3), unless otherwise expressed.

Statistical significance is show: *Disease vs HC; #Asthma vs R; $AA vs NAA; &Moderate-severe vs Intermittent-mild asthma. Kruskal-Wallys test followed by Dunn´s multiple comparison test. $p < 0.05$

Qualitative data analysis and classification of detected proteins according to GO terms

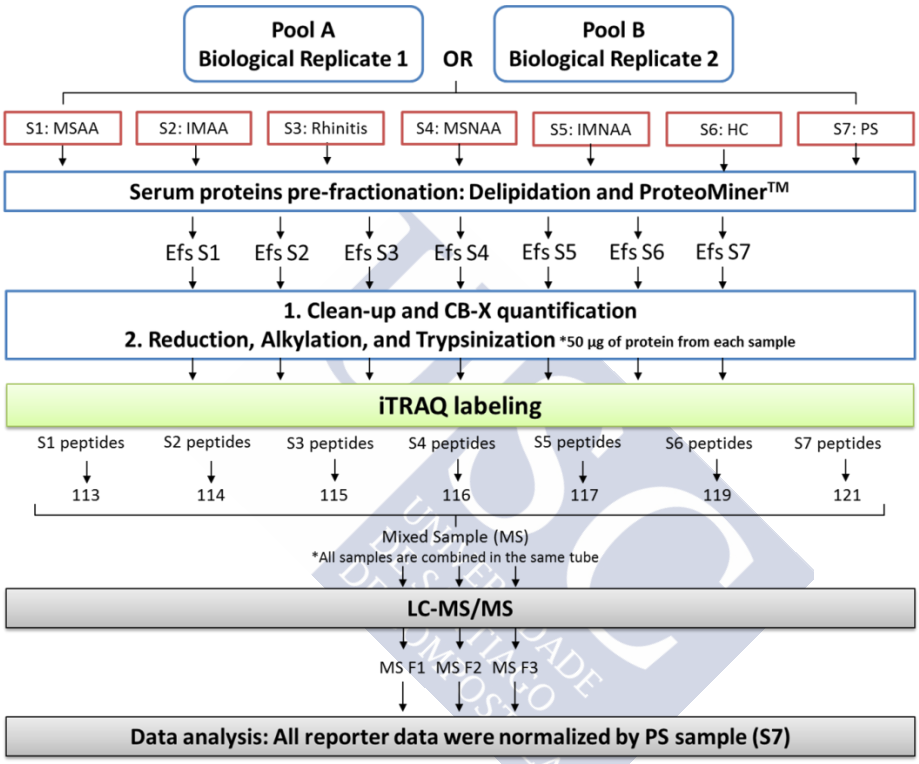


Figure 1. Experimental workflow. Samples from the different groups (S1-S6), and the pooled sample (PS, S7) were firstly delipidated. Then, low abundance proteins were enriched by means of ProteoMiner. The eluted fractions (EFs S1-S7) were then cleaned and quantified, and 50 µg of protein were reduced, alkylated and trypsinized. Tryptic peptides were labelled with different isobaric tags (113-118, 119, and 121) and afterwards, all samples were pooled in one mixed sample (MS). The MS was analysed three times by LC-MS/MS. Finally, all reporter intensities were normalized by the PS.

Six groups of subjects (MSAA, IMAA, R, MSNAA, IMNAA, and HC) were involved in this study. Serum samples from each of these groups were randomly split into 2 pooled subgroups (Pool A and B; i.e., 2 "biological replicates" per group; see Supplementary tables 1 and 2 for characteristics), which were analysed three times (3 technical replicates). Candidate biomarkers for different phenotypes and severities were identified in serum by using a high-throughput MS approach, which combined lipid removal with LRA resin, enrichment of low abundance proteins by CPLL (ProteoMiner^{TM}), and iTRAQ-LC-MS/MS analysis. The experimental design is shown in Figure 1.

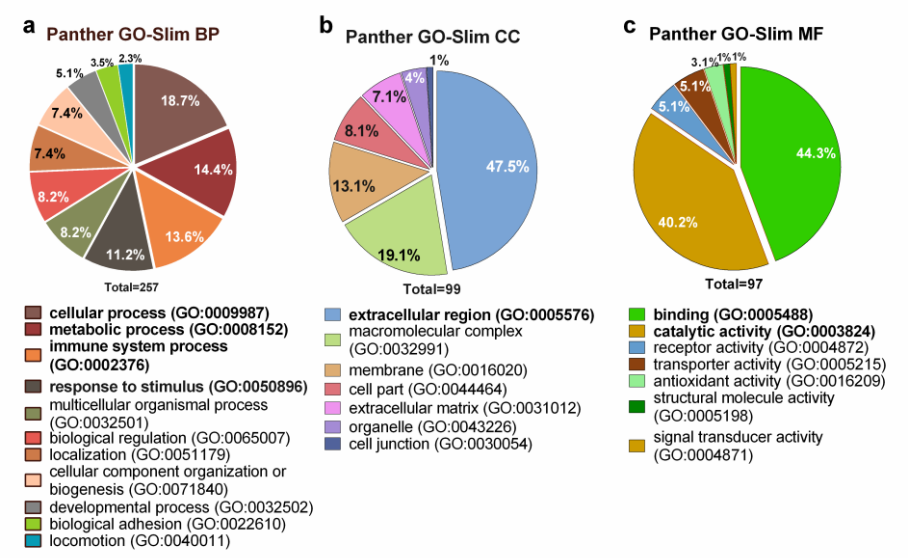


Figure 2. GO annotations of all proteins identified in this study. Two hundred and seventy-seven proteins were identified in this study and functionally classified by biological process (GO-Slim BP), cellular component (GO-Slim CC), and molecular function (GO-Slim MF). The most abundant GO terms are highlighted in bold. Percentages are displayed in graphs.

We identified a total of 9157 peptide-spectrum matches (PSMs), 1403 peptides, and 217 proteins in the serum of all study groups. Functional analysis of identified proteins was carried out by GO annotation in PANTHER (Figure 2). Moreover, GO terms overrepresentation test comparing the proteins detected in our study with reference to the plasma proteome database was also performed (Table 2).

Classification by biological process (GO-Slim BP) indicates that 4 categories, including cellular and metabolic processes, immune system process, and response to stimulus, represent more than 60% of the proteome detected (Figure 2a). Indeed, there was an enrichment of processes related to the immune system such as complement activation, B cell-mediated immunity, defence response to bacterium, response to biotic stimulus, or phagocytosis, all of them playing a key role in the pathogenesis of asthma (Table 2). As expected from a serum sample, classification by cellular component indicates that many proteins still belong to the extracellular space (47.5%) or macromolecular complexes (19.1%) (Figure 2b). The enrichment analysis also agrees with these results (Table 2). Finally, classification by molecular function (GO-Slim MF) indicates that >80% of proteins detected display binding activities (44.3%) or catalytic activity (40.2%) (Figure 2c), and the GO terms overrepresentation test yields the same results (Table 2).

Tabla 2. GO terms overrepresentation against human plasma proteome.

	Observed	Expected	Fold Enrichment	FDR
PANTHER GO-Slim: Biological Process				
cholesterol metabolic process (GO:0008203)	5	.31	15.93	6.01E-04
complement activation (GO:0006956)	9	.65	13.80	3.15E-06
B cell mediated immunity (GO:0019724)	9	.65	13.80	2.52E-06
cell recognition (GO:0008037)	10	.74	13.58	8.51E-07
defence response to bacterium (GO:0042742)	9	.80	11.29	8.45E-06
immune response (GO:0006955)	31	3.10	9.99	6.52E-19
response to biotic stimulus (GO:0009607)	9	1.01	8.87	4.20E-05
cell-matrix adhesion (GO:0007160)	4	.47	8.49	1.84E-02
phagocytosis (GO:0006909)	10	1.46	6.84	7.90E-05
steroid metabolic process (GO:0008202)	6	.93	6.45	5.87E-03
immune system process (GO:0002376)	35	5.59	6.26	7.17E-16
cell-cell adhesion (GO:0016337)	7	1.29	5.42	5.67E-03
proteolysis (GO:0006508)	13	3.69	3.52	1.82E-03
endocytosis (GO:0006897)	11	3.15	3.49	5.84E-03
response to stress (GO:0006950)	14	5.35	2.62	1.28E-02
protein metabolic process (GO:0019538)	28	12.62	2.22	1.46E-03
cellular process (GO:0009987)	48	66.83	.72	1.77E-02
phosphate-containing compound metabolic process (GO:0006796)	4	13.93	.29	2.51E-02
biosynthetic process (GO:0009058)	3	14.28	.21	6.80E-03
nitrogen compound metabolic process (GO:0006807)	3	20.90	.14	2.71E-05
nucleobase-containing compound metabolic process (GO:0006139)	3	22.98	.13	4.03E-06

Continued on next page

Table 2 (Continued)

PANTHER GO-Slim: Cellular Component				
immunoglobulin complex (GO:0019814)	10	.69	14.53	6.30E-08
extracellular space (GO:0005615)	41	4.66	8.80	8.55E-25
extracellular region (GO:0005576)	47	5.95	7.90	1.26E-26
extracellular matrix (GO:0031012)	7	1.32	5.32	3.33E-03
PANTHER GO-Slim: Molecular Function				
serine-type endopeptidase inhibitor activity (GO:0004867)	8	.51	15.77	4.46E-06
peptidase inhibitor activity (GO:0030414)	10	.70	14.28	4.29E-07
serine-type peptidase activity (GO:0008236)	11	.81	13.60	1.74E-07
antigen binding (GO:0003823)	9	.75	12.02	4.73E-06
lipid transporter activity (GO:0005319)	4	.37	10.69	1.43E-02
peptidase activity (GO:0008233)	25	3.31	7.56	2.17E-12
receptor binding (GO:0005102)	27	7.51	3.59	4.72E-07
hydrolase activity (GO:0016787)	31	15.55	1.99	4.39E-03

Quantitative data analysis of proteins identified by shotgun proteomics

iTRAQ reporter intensity values for each group were normalized by the iTRAQ reporter intensity of the pooled sample (PS). Then, volcano plot analyses were used for the detection of differentially abundant proteins across the different groups (Fold change higher than 1.3, $p < 0.05$) (Figure 3). Only proteins identified in at least two out of three technical replicates for each "biological pool" were used for quantification analysis. The list of UniProt accession numbers, protein names, gene names, fold change (ratio) and p-value of the

differentially abundant proteins is shown in Table 3. Only 26 out of 217 proteins identified showed significant changes between the different groups of donors (Table 3).

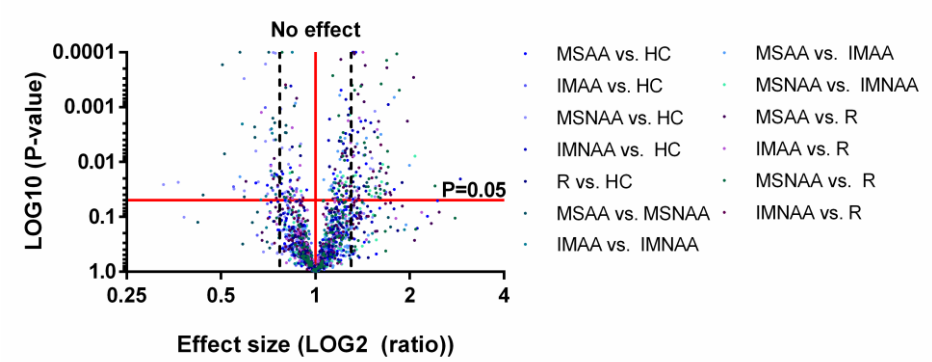


Figure 3. Volcano plot representation for all study groups. Log2 fold change (ratio) against log10 adjusted p-value showing all the differentially expressed proteins between the different groups of study (legend). The threshold value was set at 1.3 fold change (up-regulated: > 1.3; down-regulated: < 0.67) and a p-value < 0.05 was considered significant. The Volcano plot was performed by using GraphPad Prism software version 6.00 for windows.

Table 3. Proteins showing a differential abundance across the different groups of study.

Accession	Description	Gene	Fold-change	p-value
Protein changes in MSAA vs. HC				
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG)	HSPG2	**1,6987**	**0.0064**
P35858-2	Isoform 2 of Insulin-like growth factor-binding protein complex acid labile subunit (ALS)	IGFALS ALS	**1,6203**	**0.0004**
D3DNU8 (P01042)	Kininogen-1, isoform CRA_a	KNG1	**1,5808**	**0.0036**
P02763	Alpha-1-acid glycoprotein 1 (AGP 1) (Orosomucoid-1) (OMD 1)	ORM1 AGP1	**1,4812**	**0.0387**
P0C0L5	Complement C4B1a (Fragment)	C4B	**1,8544**	**0.0005**

Continued on next page

Table 3 (Continued)				
O43866	CD5 antigen-like	CD5L	**2,4099**	**0.0273**
P02760	Protein AMBP	AMBP	**1,3965**	**0.0004**
A8K2N0 (P09871)	cDNA FLJ77835, highly similar to Homo sapiens complement component 1, s subcomponent, transcript variant 2, mRNA	C1S	**1,7090**	**0.0123**
A0A0S2Z4L3 (P07225)	Protein S isoform 2 (Fragment)	PROS1	**1,6885**	**0.0001**
P00736	Complement C1r subcomponent	C1R	**1,8196**	**0.0001**
P0DJI8	Serum amyloid A-1 protein (SAA)	SAA1	**1,5553**	**0.0302**
B7Z8Q2 (P02765)	cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein (Fetuin-A)	AHSG FETUA	**1,3178**	**0.0181**
O43852-3	Isoform 3 of Calumenin (Crocalbin)	CALU	**1,3097**	**0.0003**
Protein changes in IMAA vs. HC				
D3DNU8 (P01042)	Kininogen-1, isoform CRA_a	KNG1	**1.4282**	**0.0272**
O43866	CD5 antigen-like	CD5L	**1.8921**	**0.0237**
A8K2N0 (P09871)	cDNA FLJ77835, highly similar to Homo sapiens complement component 1, s subcomponent, transcript variant 2, mRNA	C1S	**1.3782**	**0.0305**
A0A0S2Z4L3 (P07225)	Protein S isoform 2 (Fragment)	PROS1	**1.6095**	**0.0107**
P00736	Complement C1r subcomponent	C1R	**1.5430**	**0.0028**
Q15485	Ficolin-2 (Hucolin)	FCN2	**1.3191**	**0.0230**
Protein changes in MSNAA vs. HC				
A8K2N0 (P09871)	cDNA FLJ77835, highly similar to Homo sapiens complement component 1, s subcomponent, transcript variant 2, mRNA.	C1S	**1,3203**	**0.0079**
A0A0S2Z4L3 (P07225)	Protein S isoform 2 (Fragment)	PROS1	**1,383**	**0.0016**
A0A024R962 (P08603)	HCG40889, isoform CRA_b (Complement factor H)	CFH HF HF1 HF2	**1,3599**	**0.0335**
P00736	Complement C1r subcomponent	C1R	**1.5747**	**0.0025**
Q15485	Ficolin-2 (Hucolin)	FCN2	**1,4176**	**<0.0001**
Protein changes in IMNAA vs. HC				
P48740	Mannan-binding lectin serine protease 1 (MASP-1)	MASP1	**1.3017**	**0.0274**

Continued on next page

Table 3 (Continued)				
A8K2N0 (P09871)	cDNA FLJ77835, highly similar to Homo sapiens complement component 1, s subcomponent, transcript variant 2, mRNA	C1S	**1.5152**	**0.0194**
A0A0S2Z4L3 (P07225)	Protein S isoform 2 (Fragment)	PROS1	**1.6192**	**0.0048**
A0A024R962 (P08603)	HCG40889, isoform CRA_b (Complement factor H)	CFH	**1.5303**	**0.0148**
P00736	Complement C1r subcomponent	C1R	**1.6178**	**0.0129**
Protein changes in R vs. HC				
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG)	HSPG2	**1.7093**	**0.0009**
P13591	Neural cell adhesion molecule 1 (NCAM1)	NCAM1	**1.4456**	**0.0014**
O43866	CD5 antigen-like	CD5L	**2.9017**	**0.0204**
P02760	Protein AMBP	AMBP	**1.3665**	**0.0028**
P02675	Fibrinogen beta chain	FGB	**1.3742**	**0.0068**
A0A0S2Z4L3 (P07225)	Protein S isoform 2 (Fragment)	PROS1	**1.4403**	**0.0074**
P00736	Complement C1r subcomponent	C1R	**1.5021**	**0.0019**
P15169	Carboxypeptidase N catalytic chain (CPN)	CPN1	**1.3149**	**0.0027**
Protein changes in MSAA vs. R				
P35858-2	Isoform 2 of Insulin-like growth factor-binding protein complex acid labile subunit (ALS)	IGFALS	**1.36008**	**0.0046**
P0C0L5	Complement C4B1a (Fragment)	C4B	**1.6401**	**0.0111**
A8K2N0 (P09871)	cDNA FLJ77835, highly similar to Homo sapiens complement component 1, s subcomponent, transcript variant 2, mRNA	C1S	**1.4927**	**0.0332**
Q96PD5-2	Isoform 2 of N-acetylmuramoyl-L-alanine amidase	PGLYRP2	**1.3428**	**0.0175**
Protein changes in IMAA vs. R				
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG)	HSPG2	**0.7583**	**0.0280**
P13591	Neural cell adhesion molecule 1 (NCAM1)	NCAM1 NCAM	**0.6922**	**0.0010**
P02763	Alpha-1-acid glycoprotein 1 (AGP 1) (Orosomucoid-1) (OMD 1)	ORM1 AGP1	**0.5475**	**0.0281**
Q6LAM1 (P05156)	Complement factor I Heavy chain	CFI IF	**1.6860**	**0.0251**
P02675	Fibrinogen beta chain	FGB	**0.7256**	**0.0173**

Continued on next page

Table 3 (Continued)				
Protein changes in MSNAA vs. R				
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG)	HSPG2	**0.5052**	**0.0002**
P13591	Neural cell adhesion molecule 1 (NCAM1)	NCAM1 NCAM	**0.5745**	**<0.0001**
P05160	Coagulation factor XIII B chain	F13B	**0.5934**	**0.0431**
Q6LAM1 (P05156)	Complement factor I Heavy chain	CFI IF	**1.6120**	**0.0313**
O43866	CD5 antigen-like	CD5L	**0.4383**	**0.0400**
P02760	Protein AMBP	AMBP	**0.7471**	**0.0039**
P02671	Fibrinogen alpha chain	FGA	**0.5126**	**0.0071**
P02675	Fibrinogen beta chain	FGB	**0.6411**	**0.0036**
P48740	Mannan-binding lectin serine protease 1 (MASP-1)	MASP1	**1.4451**	**0.0055**
A0A024R962 (P08603)	HCG40889, isoform CRA_b (Complement factor H)	CFH	**1.3312**	**0.0283**
B2R9V7 (P08294)	Extracellular superoxide dismutase [Cu-Zn] (EC-SOD)	SOD3	**0.6405**	**0.0023**
P15169	Carboxypeptidase N catalytic chain (CPN)	CPN1	**0.7101**	**0.0039**
B7Z8Q2 (P02765)	cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein (Fetuin-A)	AHSG FETUA	**0.7414**	**0.0019**
O43852-3	Isoform 3 of Calumenin (Crocalbin)	CALU	**0.7321**	**0.0015**
Protein changes in IMNAA vs. R				
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG)	HSPG2	**0.6936**	**0.0042**
P13591	Neural cell adhesion molecule 1 (NCAM1)	NCAM1 NCAM	**0.5922**	**0.0003**
P05160	Coagulation factor XIII B chain	F13B	**0.5928**	**0.0336**
P02763	Alpha-1-acid glycoprotein 1 (AGP 1) (Orosomucoid-1) (OMD 1)	ORM1 AGP1	**0.5501**	**0.0310**
Q6LAM1 (P05156)	Complement factor I Heavy chain	CFI IF	**1.5277**	**0.0137**
O43866	CD5 antigen-like	CD5L	**0.3650**	**0.0235**
P02760	Protein AMBP	AMBP	**0.6932**	**0.0001**
P48740	Mannan-binding lectin serine protease 1 (MASP-1)	MASP1	**1.5487**	**0.0012**
A0A024R962 (P08603)	HCG40889, isoform CRA_b (Complement factor H)	CFH	**1.4979**	**0.0132**
P15169	Carboxypeptidase N catalytic chain (CPN)	CPN1	**0.7451**	**0.0001**

Continued on next page

Table 3 (Continued)				
B7Z8Q2 (P02765)	cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein (Fetuin-A)	AHSG FETUA	0.7367	0.0039
O43852-3	Isoform 3 of Calumenin (Crocalbin)	CALU	**0.7341**	**0.0001**
A6XGL1 (P02766)	Transthyretin (ATTR)	TTR	**0.7187**	**0.0025**
Protein changes in MSAA vs. IMAA				
P35858-2	Isoform 2 of Insulin-like growth factor-binding protein complex acid labile subunit (ALS)	IGFALS ALS	**1.3596**	**0.0047**
P02763	Alpha-1-acid glycoprotein 1 (AGP 1) (Orosomucoid-1) (OMD 1)	ORM1 AGP1	**1.7860**	**0.0007**
P0C0L5	Complement C4B1a (Fragment)	C4B	**1.3642**	**0.0288**
Q6LAM1 (P05156)	Complement factor I Heavy chain	CFI IF	**0.5742**	**0.0185**
Protein changes in MSNAA vs. IMNAA				
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG)	HSPG2	**0.7283**	**0.0029**
P02671	Fibrinogen alpha chain	FGA	**0.5843**	**0.0020**
B2R9V7 (P08294)	Extracellular superoxide dismutase [Cu-Zn] (EC-SOD)	SOD3	**0.7245**	**0.0025**
Protein changes in MSAA vs. MSNAA				
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG)	HSPG2	**1.9672**	**0.0018**
P13591	Neural cell adhesion molecule 1 (NCAM1)	NCAM1	**1.4436**	**0.0002**
P35858-2	Isoform 2 of Insulin-like growth factor-binding protein complex acid labile subunit (ALS)	IGFALS	**1.6919**	**0.0002**
P00734	Prothrombin (Coagulation factor II)	F2	**1.3274**	**<0.0001**
P02763	Alpha-1-acid glycoprotein 1 (AGP 1) (Orosomucoid-1) (OMD 1)	ORM1 AGP1	**1.4981**	**0.0065**
P0C0L5	Complement C4B1a (Fragment)	C4B	**2.0568**	**0.0003**
Q6LAM1 (P05156)	Complement factor I Heavy chain	CFI IF	**0.6000**	**0.0225**
P02760	Protein AMBP	AMBP	**1.3678**	**0.0006**
P02671	Fibrinogen alpha chain	FGA	**1.6837**	**0.0158**
P48740	Mannan-binding lectin serine protease 1 (MASP-1)	MASP1	**0.7578**	**0.0266**
B2R9V7 (P08294)	Extracellular superoxide dismutase [Cu-Zn] (EC-SOD)	SOD3	**1.3322**	**0.0001**

Continued on next page

Table 3 (Continued)				
B7Z8Q2 (P02765)	cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein (Fetuin-A)	AHSG FETUA	**1.4245**	**0.0024**
O43852-3	Isoform 3 of Calumenin (Crocalbin)	CALU	**1.4301**	**0.0008**
Protein changes in IMAA vs. IMNAA				
P05160	Coagulation factor XIII B chain	F13B	**1.3784**	**0.0296**
O43866	CD5 antigen-like	CD5L	**1.7863**	**0.0314**
P02760	Protein AMBP	AMBP	**1.3139**	**<0.0001**
A0A024R962 (P08603)	HCG40889, isoform CRA_b (Complement factor H)	CFH	**0.7029**	**0.0266**
B7Z8Q2 (P02765)	cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein (Fetuin-A)	AHSG FETUA	**1.3970**	**0.0018**
A6XGL1 (P02766)	Transthyretin (ATTR)	TTR	**1.3464**	**<0.0001**

To reduce data dimensionality, PCA analysis was performed with the differentially abundant proteins. Five principal components (PCs) were selected with eigenvalues higher than unity and with a cumulative variance explained of 81.6%. Each component was composed of highly correlated proteins ($r > 0.500$). PC1 explained the 29.8% of the variance and included 12 different proteins (CALU, AMBP, HSPG2, IGFALS, TTR, F2, NCAM1, AHSG, ORM1, F13B, C4B1a, and CD5L) (Table 3, Figure 4). As Figure 4a points out, 7 out of those 12 proteins in PC1 (IGFALS, F2, F13B, AHSG, AMBP, ORM1, and CALU) interacted each other with a high confidence level (0.900), while others (HSPG2 and TTR) also displayed a strong interrelation. All PC1 proteins showed an intense relationship with allergy (AA and R), especially with MSAA (Table 3). This is perfectly illustrated by IGFALS, whose serum concentration in MSAA patients is much higher than in the remaining groups of the present study (Table 3; Figure 4b). Other interesting proteins within this component were HSPG2 and AMBP, both increased in R and MSAA patients compared to NAA and HC donors (Table 3; Figure 4c, d). GO terms analyses of proteins that make up PC1 indicated that many of them were related to protein or cellular metabolic processes

(CALU, AMBP, HSPG2, IGFALS, TTR, F2, NCAM1, AHSG, F13B, and C4B1a), mostly with a role in defence/inflammatory responses or immune system processes (HSPG2, TTR, F2, NCAM1, AHSG, ORM1, C4B1a, and CD5L). In addition, PANTHER overrepresentation test against plasma proteome database highlighted an enrichment in inflammatory response process (FE, 15.12; FDR, 1.61E-02) and the Reactome pathway "*Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)"* (DE, 35.01; FDR, 8.94E-03), to which the IGFALS, F2, AHSG, and CALU belong.

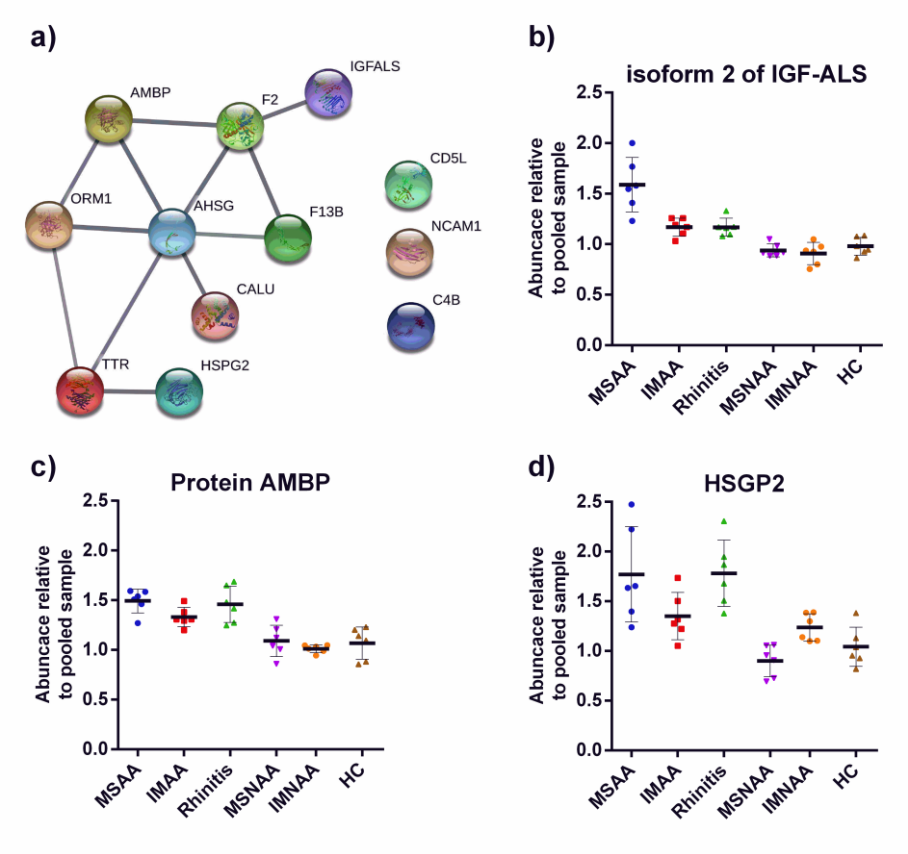


Figure 4 (Previous page). String analysis and changes in the relative abundance of proteins from PC1. a) Protein interaction network of proteins that make up PC1. **b-d)** Scatter plots show the changes of representative proteins (Abundance relative to the pooled sample) from the PC1 between the different groups of study (moderate-severe allergic asthmatics, MSAA; intermittent-mild allergic asthmatics, IMAA; rhinitis patients, moderate-severe non-allergic asthmatics, MSNAA; intermittent-mild non-allergic asthmatics, IMNAA; and healthy controls, HC).

PC2, for its part, explained the 22.01% of the variance and was composed of 8 proteins (PROS, KNG1, SAA1, C1s, C1r, CFI, CFH, and MASP) (Figure 5a). Some of the proteins associated with this component were strongly related to NAA phenotype. Thus, CFI, CFH, and MASP1 displayed an augmented concentration in serum samples compared to allergic subjects (AA and R patients) (Table 3; Figure 5b-d). Classification by biological process of PC2 proteins indicated that they were related to immune system processes (GO:0002376), either to blood coagulation (GO:0007596; PROS, KNG1, and SAA1) or complement activation (GO:0006956), including the classical (C1s, C1r, CFI), alternative (CFH, CFI), or lectin (MASP1) pathways. Indeed, this distribution is easily observed by means of the String protein interaction network (Figure 5a) and PANTHER overrepresentation test against human plasma proteome, showing the enrichment of the Reactome pathways "*Regulation of Complement cascade*" (FE, 76.58; FDR, 2.48E-06) and "*Creation of C4 and C2 activators*" (FE, 70.02; FDR, 3.97E-03).

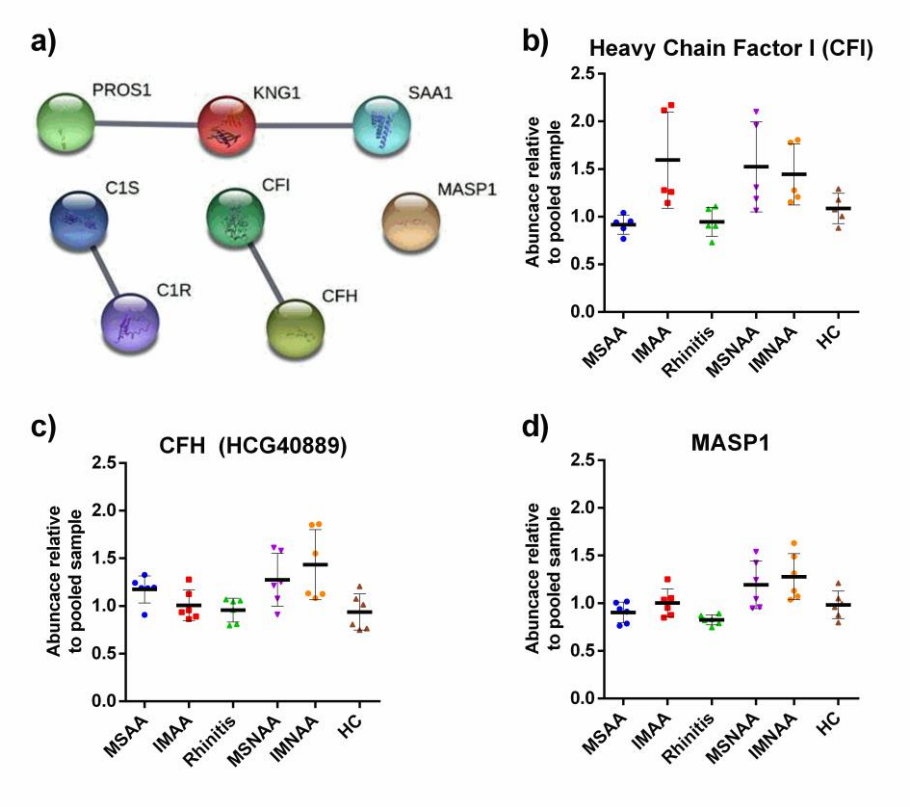


Figure 5. String analysis and changes in the relative abundance of proteins from PC2. a) Protein interaction network of proteins that make up PC2**. b-d)** Scatter plots show the changes of representative proteins (abundance relative to the pooled sample) from the PC2 between the different groups of study (moderate-severe allergic asthmatics, MSAA; intermittent-mild allergic asthmatics, IMAA; rhinitis patients, moderate-severe non-allergic asthmatics, MSNAA; intermittent-mild non-allergic asthmatics, IMNAA; and healthy controls, HC).

PC3, PC4, and PC5 explained, altogether, less than 30% of the variance. They comprised 4 (AHSG, F13B, CD5L, and FCN2), 3 (SOD3, FGA, and CPN1), and 3 (C4B, FGB, and PGLYRP2) proteins, respectively (Figure 6a-c). Three out of four proteins from PC3 (AHSG, F13B, CD5L) were also taking part in PC1, and they were related to R and MSAA. As we stated for PC1, both AHSG and F13B interact with each other with high confidence (0.900) (Figure 6a). The remaining protein (FCN2) was negatively correlated with this component (Figure 6a), being augmented in MSNAA vs. HC and in IMAA vs. HC (Table 3). PC4 proteins, for its part, were mainly increased in R patients with respect to the other groups (Table 3; Figure 6b) and no interactions were observed by String analysis (Figure 6b). Finally, PC5 proteins PGLYRP2 and C4B1a appeared to follow an increased concentration pattern in allergic subjects (R < IMAA < MSAA); in contrast, FGB displayed the opposite pattern (Figure 6c). Indeed, although FGB belongs to PC5 (with negative correlation), it became evident the positive correlation with PC4 in PCA analysis (r = 0.490).

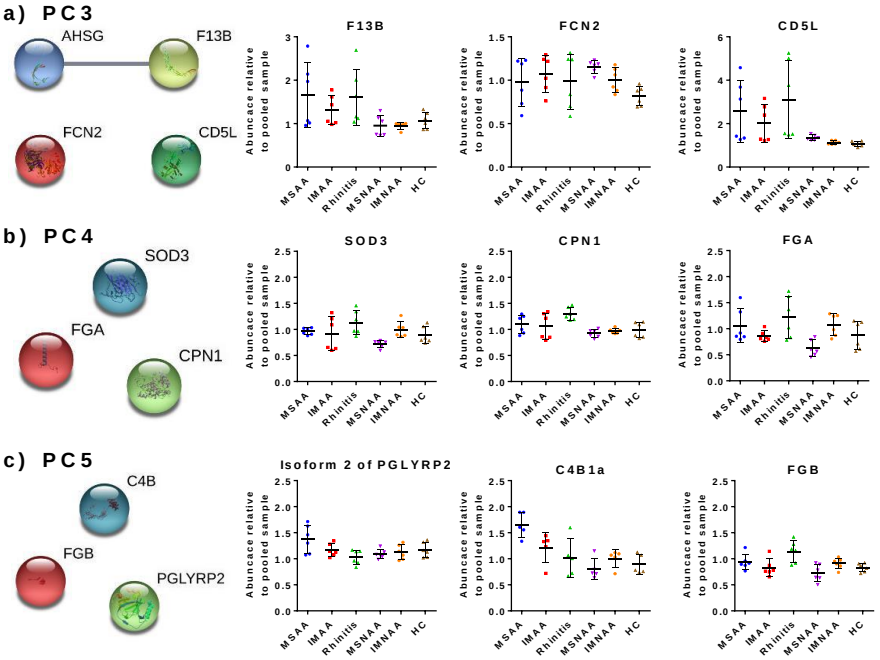


Figure 6. String analysis and changes in the relative abundance of proteins from PC3, PC4, and PC5. Figures on the left represent the protein interaction network of proteins that make up PC3 **(a)**, PC4 **(b)**, and PC5 **(c)**. Scatter plots show the changes of representative proteins (abundance relative to the pooled sample) from each PC (PC3, PC4, and PC5) between the different groups of study (moderate-severe allergic asthmatics, MSAA; intermittent-mild allergic asthmatics, IMAA; rhinitis patients, moderate-severe non-allergic asthmatics, MSNAA; intermittent-mild non-allergic asthmatics, IMNAA; and healthy controls, HC).

Validation of candidate biomarkers by means of ELISA

Three candidate biomarkers from PC1 (IGFALS, Protein AMBP, and HSPG2) and one from PC2 (CFI) were chosen for subsequent validation by ELISA. So far, only IGFALS ELISA was performed (Figure 7).

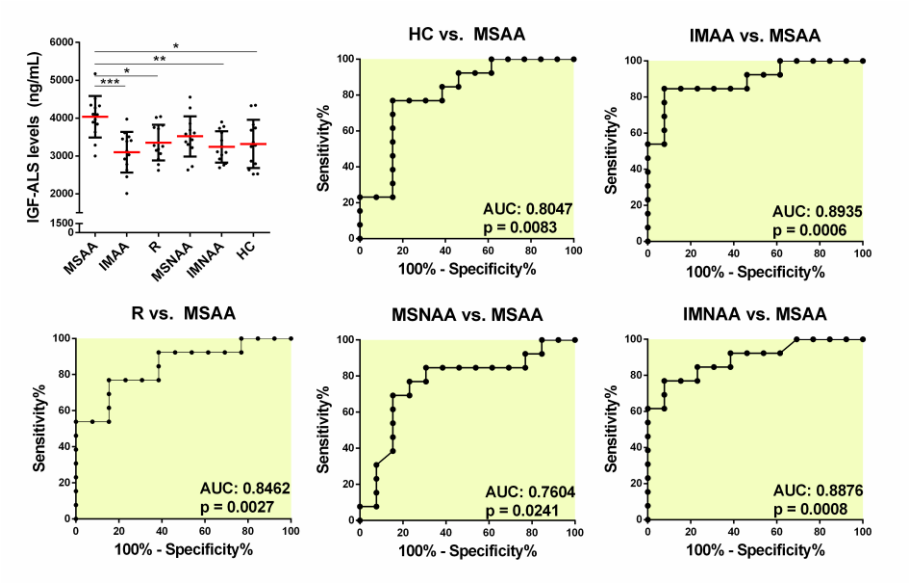


Figure 7. Validation of iTRAQ results by ELISA and ROC analysis. a) Fifteen serum samples from each donors group (HC, MSAA, IMAA, R, MSNAA, IMNAA, HC) were selected to achieve a similar average age and M/F proportion in all of them. Then, IGFALS protein was measured by ELISA. The asterisk (*) indicates the presence of a significant difference. * $P < 0.05$ ** $P< 0.01$ *** $P < 0.001$. **b)** The diagnostic ability of IGFALS to discriminate the different classes of donors was also evaluated through different receiver/relative operating characteristic (ROC) curves. An area under the ROC curve (AUC) > 0.8 was considered as a good performance of the biomarker.

Thirteen subjects from each group with similar age and M/F proportion were included for this analysis. Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn´s multiple comparison test, evidenced an increase in the IGFALS concentration

in MSAA patients compared to the remaining groups of study, with the only exception of MSNAA patients (Figure 7). Moreover, ROC analyses highlighted that IGFALS levels allow us to discriminate MSAA from the remaining groups studied with high AUCs (Figure 7). Therefore, IGFALS could be a useful biomarker of MSAA.

DISCUSSION

In the present study, a protocol for the proteomic study of serum has been optimized, in which lipoproteins have been eliminated, the dynamic range of abundances has been reduced, and quantitative analysis has been addressed (iTRAQ-LC-MS/MS). A preliminary analysis of serum samples from different donor groups (MSAA, IMAA, R, MSNAA, IMNAA, HC) demonstrates an enrichment of proteins from extracellular region which exhibit binding or catalytic activity and whose biological processes are mainly related to metabolism or immune system (e.g., complement activation, B cell immunity, defence against bacterium, or phagocytosis). In addition, this seminal study has reported a differential abundance in the serum proteomes of patients of several proteins, which could be biomarkers of either AA or NAA: IGFALS, CFI, Protein AMBP, and HSPG2.

The usefulness of the LC-MS/MS approach combined with the use of iTRAQ isobaric labels has been proven in numerous publications [37, 38]. Although several studies have been performed in asthma proteomics [19, 20, 35], only one has used a similar methodology (iTRAQ-LC-MS/MS) to ours [39], and none of them has compared rhinitis and different asthmatic phenotypes (AA vs. NAA) (Supplementary Table 2). In the present study, we have obtained a list of 217 proteins, of which 26 displayed a differential abundance across the different groups of donors. Interestingly, some of them could be

relevant as candidate biomarkers for asthma phenotypes diagnosis or assessment of asthma severity. This is the case, for example, of IGFALS, protein AMBP, and HSPG2 in the case of AA, and CFI, or CFH for NAA.

IGFALS belongs to a family of proteins previously linked to asthma pathogenesis that includes IGF1 and IGF2 [40]. IGF1 is a key factor in asthma pathogenesis, through the promotion of subepithelial fibrosis, inflammation, hyperresponsiveness, and smooth muscle cell hyperplasia in the airways [40]. Indeed, omalizumab (an anti-IgE antibody) [41], as well as oral glucocorticoids [42], decrease the levels of IGF-I. Both IGF1 and IGF2 exert their biological effects through the binding to IGF1R, but IGF2 can be also sequestrated by IGF2R/cation-independent mannose-6-phosphate receptor, a high-affinity inhibitory protein that attenuates IGF2 signalling [43]. Strikingly, this last receptor has been related to CD26/DPP4 [44, 45], a serine peptidase involved in asthma pathogenesis [46]. Therefore, there are many lines of evidence pointing to an important role of IGF family in allergic asthma.

The IGF system is completed with several binding proteins (IGFBPs: 1-6) [47], a novel IGFBP3-specific receptor (IGFBP3R) [48], and IGFALS [47, 49]. Both IGFBPs and IGFALS appear to influence free-IGF concentration in the extracellular compartment, playing a role on the bioavailability of IGFs [40]. The formation of a high molecular weight complex (IGFALS-IGF1-IGFBP) prevents the extravasation of IGF-1, the IGF-1/IGFBPs proteolysis, and the renal elimination of IGF-1 [50]. Indeed, IGFALS deficiency results in a dramatic decrease in IGF-1, IGF-2, and IGFBP3 levels [50, 51]. Apart from IGF1 [40] and IGFBP3 [40], the present results show that IGFALS is elevated in AA, especially moderate-severe forms, but not in NAA. Regarding IGFBP3, this protein controls AA inflammation through IGF-dependent and IGF-independent (IGFBPR-mediated)

mechanisms that target the HIF/VEGF axis, TGFβ1 and TH2 cytokines production, and NF-kB activation [40]. Veraldi *et al.* also described a role in AA for IGFBP-3 through the promotion of subepithelial fibrosis [52]. Regarding the role of IGFALS in asthma, this remains mostly unknown, but together with IGFs and IGFBPs this protein could be an additional therapeutic target for asthma management.

Other possible biomarkers for AA are protein AMBP and HSPG2. Although the first one has not been extensively studied in asthma or airway-related diseases, a study of serum proteome from patients with idiopathic pulmonary fibrosis (IPF) has shown decreased levels of AMBP in IPF patients compared to HC, and the same decrease was observed for AHSG, a protein with a similar pattern of changes as AMBP in our study [38]. Although the possible function of protein AMBP in AA is not known, this protein shares the same chromosomal region (9q32) as ORM1/AGP, a positive acute phase protein increased in AA (Table 3). Thus, it is probable that both proteins coordinate their levels and that AMBP participates in the acute inflammatory response. Indeed, AMBP and ORM1 levels, as well as other proteins augmented in AA and R in our study (e.g., AHSG or CD5L), have been found increased in BALF of asthmatic individuals 24 h after segmental allergen challenge [53], highlighting the possible role of these set of proteins in AA.

On the other hand, together with hyaluronan (HA) or vesicant (VCAN), HSPG2/perlecan is an extracellular matrix molecule which is deposited in the lamina reticularis and generates subepithelial fibrosis in the airways [54]. Basement membrane thickening has been reported in asthma [55-58] and even allergic rhinitis [58, 59]. Moreover, HSPG2 is an upstream regulator of genes (ACAN, COL10A1, and FGFR3) containing asthma-associated differentially methylated regions [60]. There is a negative correlation between the

levels of HSPG2 and airway hyperresponsiveness (PC_{20}) [61], and this protein has been linked to fibrosis as well. Indeed, mature fibrocytes [62] constitutively produce VCAN, HA, COL3/5/6, fibronectin, and HSPG2 [63, 64] and contribute to the subepithelial fibrosis in asthma [63]. Aligned with these results and our own data showing higher levels of HSPG2 in serum samples from AA and R patients, fibrocytes from asthmatic patients exposed to TH2- (IL-4, IL-13) but not TH17 (IL-17A) cytokines show an enhanced expression of HSPG2 and a profibrotic phenotype (i.e., elevated expression of HA, COL315, VCAN and HSPG2) [65]. Changes in HSPG2 deposition could have mechanistic effects, but also modulate the bioability of growth factors (e.g., bFGF) or cytokines (e.g., IL-4, TGF-β, or GM-CSF). Taken together, these results could explain the increase of HSPG2 and protein AMBP in our group of MSAA and R patients and their potential as atopic disease biomarkers.

Asthma and especially its NAA phenotype share certain characteristics with autoimmune disorders, like their prevalence in female the presence of autoantibodies in higher frequency than HC [66-68]. It is well known that the complement system plays an important role in autoimmune diseases [69], but also appear to have a proinflammatory role in asthma [70-72]. However, the complement factors involved remain unclear. According to the initial stimuli, three pathways (classical, alternative, and lectin) have been described for complement activation. Our results support that complement factors I and H, two regulatory proteins that control the excessive activation of the alternative complement pathway [73, 74], are upregulated in NAA. CFI is a serine protease that favours the degradation of C4b, diminishes the levels of C3-convertase (C4bC2a) and prevents inflammation arising from complement activation [75]. Complement factor H, for its part, is a cofactor for CFI [73, 76] and it is also known as adrenomedullin binding protein (AMBP-1) [77, 78].

Adrenomedullin is an anti-inflammatory peptide that suppresses TH2 inflammation and maintains tissue integrity in an OVA-induced model of asthma [79]. Moreover, Weiszhár *et al.* have found augmented levels of CFH in asthmatic sputum and a correlation with severity and the loss of lung function [80]. The increase of CFI and CFH in our group of NAA patients could be related to a mechanism to evade the immune response, as it happens with some pathogens [81-83], which could also explain the higher severity of NAA.

Additionally, the levels of the serine protease MASP1 are also increased in NAA. MASP1 participate in the lectin pathway of complement activation, but this protease has also substrates that belong to the coagulation cascade [84]; we have found some of them altered in asthma as well, such as kininogen, FXIII, or F2. Moreover, another substrate of MASP1 is the protease activation receptor 4 (PAR4) in endothelial cells, whose activation leads to the production of IL-6 and IL-8, the last one a chemotactic molecule for neutrophils [84]. This is relevant, as neutrophils have been related to some NAA endotypes [85] and higher disease severity [86, 87]. Besides, C1r and C1s proteins (classical complement pathway) are also modified in our group of asthma patients. Therefore, our results point out that all the complement activation pathways (classical, alternative, and lectin) appear to be involved in asthma, especially in the NAA phenotype.

Like other studies, the present work has also some limitations. First, not all the proteins were detected in all the groups since it is known that in LC-MS/MS assays is necessary to perform more than 3 technical replicates in order to detect all proteins in a sample (completeness of analysis issue) [88]. A second problem is related to the "pooled sample" analysed with the isobaric label 121. This sample has been introduced in all analytical series to allow the normalization of the signals and the adjustment of the interserial variability. However, the use of this type of reference samples makes difficult to

detect low abundance proteins, which in addition makes the normalization process difficult. Third, another issue is the whole number of proteins detected, especially low abundance species, despite the use of lipoprotein-depletion, CPLLs-based enrichment, and reverse phase LC. Therefore, an additional step of peptides-fractionation using strong cation exchange (SCX) chromatography before LC-MS/MS analysis would be of benefit. Finally, some of our results are still preliminary, especially those referring to the proteins detected with differential abundance by LS-MS/MS which have not been validated by ELISA yet.

In conclusion, a shotgun/bottom-up/non-targeted methodology has been developed for the quantitative analysis of the proteome of medium-low abundance in serum samples from patients with rhinitis and various asthmatic phenotypes. This protocol is based on the elimination of lipoproteins by the LRA resin, protein enrichment of medium and low abundance by CPLLs application, labelling with iTRAQ 8plex reagents, and analysis by LC-MS/MS. Our approach detected several differentially abundant proteins, and therefore potential non-invasive biomarkers of asthma phenotypes (e.g., IGFALS, Protein AMBP, HSPG2 for AA, and CFI for NAA) or severities (e.g., IGFALS for MSAA). In any case, future studies will be necessary to get a better understanding of asthma pathophysiology, to evaluate the diagnostic performance of these new biomarkers, and to translate this knowledge into the clinic to get a better therapeutic response and prognosis.

REFERENCES

1. Holgate ST. Innate and adaptive immune responses in asthma. Nat Med. 2012;18(5):673-83.
2. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med. 2012;18(5):716-25.
3. Reddel HK, Levy ML. The GINA asthma strategy report: what's new for primary care? NPJ Prim Care Respir Med. 2015;25:15050.
4. Peters SP. Asthma phenotypes: nonallergic (intrinsic) asthma. J Allergy Clin Immunol Pract. 2014;2(6):650-2.
5. Schatz M, Rosenwasser L. The allergic asthma phenotype. J Allergy Clin Immunol Pract. 2014;2(6):645-8.
6. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med. 2009;180(5):388-95.
7. Fajt ML, Wenzel SE. Asthma phenotypes and the use of biologic medications in asthma and allergic disease: the next steps toward personalized care. J Allergy Clin Immunol. 2015;135(2):299-310.
8. Tiotiu A. Biomarkers in asthma: state of the art. Asthma Res Pract. 2018;4:10.
9. Pavlidis S, Takahashi K, Ng Kee Kwong F, Xie J, Hoda U, Sun K, et al. "T2-high" in severe asthma related to blood eosinophil, exhaled nitric oxide and serum periostin. Eur Respir J. 2019;53(1). pii: 1800938.
10. Mitchell PD, O'Byrne PM. Epithelial-Derived Cytokines in Asthma. Chest. 2017;151(6):1338-44.
11. Navinés-Ferrer A, Serrano-Candelas E, Molina-Molina GJ, Martín M. IgE-Related Chronic Diseases and Anti-IgE-Based Treatments. J Immunol Res. 2016;2016:8163803.

12. Linneberg A, Henrik Nielsen N, Frølund L, Madsen F, Dirksen A, Jørgensen T. The link between allergic rhinitis and allergic asthma: a prospective population-based study. The Copenhagen Allergy Study. Allergy. 2002;57(11):1048-52.
13. Kim H, Bouchard J, Renzi PM. The link between allergic rhinitis and asthma: a role for antileukotrienes? Can Respir J. 2008;15(2):91-8
14. Haccuria A, Van Muylem A, Malinovschi A, Doan V, Michils A. Small airways dysfunction: the link between allergic rhinitis and allergic asthma. Eur Respir J. 2018;51(2). pii: 1701749.
15. Miranda C, Busacker A, Balzar S, Trudeau J, Wenzel SE. Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. J Allergy Clin Immunol. 2004;113(1):101-8.
16. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. Am J Respir Crit Care Med. 2010;181(4):315-23.
17. Wu W, Bleecker E, Moore W, Busse WW, Castro M, Chung KF, et al. Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. J Allergy Clin Immunol. 2014;133(5):1280-8.
18. Pelaia G, Terracciano R, Vatrella A, Gallelli L, Busceti MT, Calabrese C, et al. Application of proteomics and peptidomics to COPD. Biomed Res Int. 2014;2014:764581.
19. Rossi R, De Palma A, Benazzi L, Riccio AM, Canonica GW, Mauri P. Biomarker discovery in asthma and COPD by proteomic approaches. Proteomics Clin Appl. 2014 8(11-12):901-15.
20. Fujii K, Nakamura H, Nishimura T. Recent mass spectrometry-based proteomics for biomarker discovery in lung cancer,

COPD, and asthma. Expert Rev Proteomics. 2017;14(4):373-386.

21. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics. 2002;1(11):845-67.
22. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics. 2003;2(10):1096-103.
23. De Bock M, de Seny D, Meuwis MA, Servais AC, Minh TQ, Closset J, et al. Comparison of three methods for fractionation and enrichment of low molecular weight proteins for SELDI-TOF-MS differential analysis. Talanta. 2010;82(1):245-54.
24. Righetti PG, Castagna A, Antonioli P, Boschetti E. Prefractionation techniques in proteome analysis: the mining tools of the third millennium. Electrophoresis. 2005;26(2):297-319.
25. Greening DW, Simpson RJ. A centrifugal ultrafiltration strategy for isolating the low-molecular weight (<or=25K) component of human plasma proteome. J Proteomics. 2010;73(3):637-48.
26. Greening DW, Simpson RJ. Characterization of the Low-Molecular-Weight Human Plasma Peptidome. Methods Mol Biol. 2017;1619:63-79.
27. Kay R, Barton C, Ratcliffe L, Matharoo-Ball B, Brown P, Roberts J, et al. Enrichment of low molecular weight serum proteins using acetonitrile precipitation for mass spectrometry based proteomic analysis. Rapid Commun Mass Spectrom. 2008;22(20):3255-60.
28. Kim B, Araujo R, Howard M, Magni R, Liotta LA, Luchini A. Affinity enrichment for mass spectrometry: improving the yield of low abundance biomarkers. Expert Rev Proteomics. 2018;15(4):353-366.

29. Meng R, Gormley M, Bhat VB, Rosenberg A, Quong AA. Low abundance protein enrichment for discovery of candidate plasma protein biomarkers for early detection of breast cancer. J Proteomics. 2011;75(2):366-74.
30. Cox J, Mann M. Quantitative, high-resolution proteomics for data-driven systems biology. Annu Rev Biochem. 2011;80:273-99.
31. Zhang Y, Fonslow BR, Shan B, Baek MC, Yates JR 3rd. Protein analysis by shotgun/bottom-up proteomics. Chem Rev. 2013;113(4):2343-94.
32. Yoshida T. Peptide separation by Hydrophilic-Interaction Chromatography: a review. J Biochem Biophys Methods. 2004;60(3):265-80.
33. Zhu MZ, Li N, Wang YT, Liu N, Guo MQ, Sun BQ, et al. Acid/Salt/pH Gradient Improved Resolution and Sensitivity in Proteomics Study Using 2D SCX-RP LC-MS. J Proteome Res. 2017;16(9):3470-5.
34. Durr E, Yu J, Krasinska KM, Carver LA, Yates JR, Testa JE, et al. Direct proteomic mapping of the lung microvascular endothelial cell surface in vivo and in cell culture. Nat Biotechnol. 2004;22(8):985-92.
35. Terracciano R, Pelaia G, Preianò M, Savino R. Asthma and COPD proteomics: current approaches and future directions. Proteomics Clin Appl. 2015;9(1-2):203-20.
36. Izbicka E, Streeper RT, Michalek JE, Louden CL, Diaz A 3rd, Campos DR. Plasma biomarkers distinguish non-small cell lung cancer from asthma and differ in men and women. Cancer Genomics Proteomics. 2012;9(1):27-35.
37. Tan HT, Ling LH, Dolor-Torres MC, Yip JW, Richards AM, Chung MC. Proteomics discovery of biomarkers for mitral regurgitation caused by mitral valve prolapse. J Proteomics. 2013;94:337-45.

38. Niu R, Liu Y, Zhang Y, Zhang Y, Wang H, Wang Y, et al. iTRAQ-Based Proteomics Reveals Novel Biomarkers for Idiopathic Pulmonary Fibrosis. PLoS One. 2017;12(1):e0170741.
39. O'Neil SE, Sitkauskiene B, Babusyte A, Krisiukeniene A, Stravinskaite-Bieksiene K, Sakalauskas R, et al. Network analysis of quantitative proteomics on asthmatic bronchi: effects of inhaled glucocorticoid treatment. Respir Res. 2011;12:124.
40. Lee H, Kim SR, Oh Y, Cho SH, Schleimer RP, Lee YC. Targeting insulin-like growth factor-I and insulin-like growth factor-binding protein-3 signaling pathways. A novel therapeutic approach for asthma. Am J Respir Cell Mol Biol. 2014;50(4):667-77.
41. Bulut I, Ozseker ZF, Coskun A, Serteser M, Unsal I. Pregnancy-associated plasma protein-A (PAPP-A) levels in patients with severe allergic asthma are reduced by omalizumab. J Asthma. 2017;55(10):1116-21.
42. Frystyk J, Schou AJ, Heuck C, Vorum H, Lyngholm M, Flyvbjerg A, et al. Prednisolone reduces the ability of serum to activate the IGF1 receptor in vitro without affecting circulating total or free IGF1. Eur J Endocrinol. 2012;168(1):1-8.
43. Brown J, Jones EY, Forbes BE. Interactions of IGF-II with the IGF2R/cation-independent mannose-6-phosphate receptor mechanism and biological outcomes. Vitam Horm. 2009;80:699-719.
44. Ikushima H, Munakata Y, Ishii T, Iwata S, Terashima M, Tanaka H, et al. Internalization of CD26 by mannose 6-phosphate/insulin-like growth factor II receptor contributes to T cell activation. Proc Natl Acad Sci U S A. 2000;97(15):8439-44.
45. Ikushima H, Munakata Y, Iwata S, Ohnuma K, Kobayashi S, Dang NH, et al. Soluble CD26/dipeptidyl peptidase IV enhances transendothelial migration via its interaction with mannose 6-

phosphate/insulin-like growth factor II receptor. Cell Immunol. 2002;215(1):106-10.

46. Nieto-Fontarigo JJ, González-Barcala FJ, San José E, Arias P, Nogueira M, Salgado FJ. CD26 and Asthma: A Comprehensive Review. Clin Rev Allergy Immunol. 2016. [Epub ahead of print]
47. Pozo J, Martos-Moreno GA, Barrios V, Argente J. The IGF system in childhood: physiology and clinical implications. J Endocrinol Invest. 2005;28(5 Suppl):38-42.
48. Lee YC, Jogie-Brahim S, Lee DY, Han J, Harada A, Murphy LJ, et al. Insulin-like growth factor-binding protein-3 (IGFBP-3) blocks the effects of asthma by negatively regulating NF-κB signaling through IGFBP-3R-mediated activation of caspases. J Biol Chem. 2011;286(20):17898-909.
49. Boisclair YR, Rhoads RP, Ueki I, Wang J, Ooi GT. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. J Endocrinol. 2001;170(1):63-70.
50. Domené HM, Hwa V, Argente J, Wit JM, Camacho-Hübner C, Jasper HG, et al. Human acid-labile subunit deficiency: clinical, endocrine and metabolic consequences. Horm Res. 2009;72(3):129-41.
51. Domené HM, Hwa V, Jasper HG, Rosenfeld RG. Acid-labile subunit (ALS) deficiency. Best Pract Res Clin Endocrinol Metab. 2011;25(1):101-13.
52. Veraldi KL, Gibson BT, Yasuoka H, Myerburg MM, Kelly EA, Balzar S, et al. Role of insulin-like growth factor binding protein-3 in allergic airway remodeling. Am J Respir Crit Care Med. 2009;180(7):611-7.
53. Wu J, Kobayashi M, Sousa EA, Liu W, Cai J, Goldman SJ, et al. Differential proteomic analysis of bronchoalveolar lavage fluid in asthmatics following segmental antigen challenge. Mol Cell Proteomics. 2005;4(9):1251-64

54. Bergeron C, Tulic MK, Hamid Q. Airway remodelling in asthma: from benchside to clinical practice. Can Respir J. 2010;17(4):e85-93.
55. Ward C, Pais M, Bish R, Reid D, Feltis B, Johns D, et al. Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma. Thorax. 2002;57(4):309-16.
56. Bonato M, Bazzan E, Snijders D, Tinè M, Biondini D, Turato G, et al. Clinical and Pathologic Factors Predicting Future Asthma in Wheezing Children. A Longitudinal Study. Am J Respir Cell Mol Biol. 2018;59(4):458-66.
57. Elliot JG, Noble PB, Mauad T, Bai TR, Abramson MJ, McKay KO, et al. Inflammation-dependent and independent airway remodelling in asthma. Respirology. 2018;23(12):1138-45.
58. Samitas K, Carter A, Kariyawasam HH, Xanthou G. Upper and lower airway remodelling mechanisms in asthma, allergic rhinitis and chronic rhinosinusitis: The one airway concept revisited. Allergy. 2018;73(5):993-1002.
59. Kim TH, Lee JY, Lee HM, Lee SH, Cho WS, Ju YH, et al. Remodelling of nasal mucosa in mild and severe persistent allergic rhinitis with special reference to the distribution of collagen, proteoglycans, and lymphatic vessels. Clin Exp Allergy. 2010;40(12):1742-54.
60. DeVries A, Wlasiuk G, Miller SJ, Bosco A, Stern DA, Lohman IC, et al. Epigenome-wide analysis links SMAD3 methylation at birth to asthma in children of asthmatic mothers. J Allergy Clin Immunol. 2017;140(2):534-42.
61. Westergren-Thorsson G, Chakir J, Lafrenière-Allard MJ, Boulet LP, Tremblay GM. Correlation between airway responsiveness and proteoglycan production by bronchial fibroblasts from normal and asthmatic subjects. Int J Biochem Cell Biol. 2002;34(10):1256-67.

62. Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest. 2007;87(9):858-70.
63. Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. J Immunol. 2003;171(1):380-9.
64. Bianchetti L, Barczyk M, Cardoso J, Schmidt M, Bellini A, Mattoli S. Extracellular matrix remodelling properties of human fibrocytes. J Cell Mol Med. 2012;16(3):483-95.
65. Bellini A, Marini MA, Bianchetti L, Barczyk M, Schmidt M, Mattoli S. Interleukin (IL)-4, IL-13, and IL-17A differentially affect the profibrotic and proinflammatory functions of fibrocytes from asthmatic patients. Mucosal Immunol. 2012;5(2):140-9.
66. Comi AL, Tedeschi A, Lorini M, Miadonna A. Novel clinical and serological aspects in non-allergic asthma. Respir Med. 2007;101(12):2526-33.
67. Tedeschi A, Asero R. Asthma and autoimmunity: a complex but intriguing relation. Expert Rev Clin Immunol. 2008;4(6):767-76.
68. Sin E, Anand P, Frieri M. A link: Allergic rhinitis, Asthma & Systemic Lupus Erythematosus. Autoimmun Rev. 2016;15(5):487-91.
69. Vignesh P, Rawat A, Sharma M, Singh S. Complement in autoimmune diseases. Clin Chim Acta. 2017;465:123-30.
70. Gerard NP, Gerard C. Complement in allergy and asthma. Curr Opin Immunol. 2002;14(6):705-8.
71. Bohács A, Bikov A, Ivancsó I, Czaller I, Böcskei R, Müller V, et al. Relationship of Circulating C5a and Complement Factor H Levels With Disease Control in Pregnant Women With Asthma. Respir Care. 2016;61(4):502-9.

72. Laumonnier Y, Wiese AV, Figge J, Karsten C. Regulation and function of anaphylatoxins and their receptors in allergic asthma. Mol Immunol. 2017;84:51-6.
73. Nilsson SC, Sim RB, Lea SM, Fremeaux-Bacchi V, Blom AM. Complement factor I in health and disease. Mol Immunol. 2011;48(14):1611-20.
74. Blaum BS. The lectin self of complement factor H. Curr Opin Struct Biol. 2017;44:111-8.
75. Rezende SM, Simmonds RE, Lane DA. Coagulation, inflammation, and apoptosis: different roles for protein S and the protein S-C4b binding protein complex. Blood. 2004;103(4):1192-201.
76. Xue X, Wu J, Ricklin D, Forneris F, Di Crescenzio P, Schmidt CQ, et al. Nat Struct Mol Biol. 2017;24(8):643-51.
77. Pio R, Martinez A, Unsworth EJ, Kowalak JA, Bengoechea JA, Zipfel PF, et al. Complement factor H is a serum-binding protein for adrenomedullin, and the resulting complex modulates the bioactivities of both partners. J Biol Chem. 2001;276(15):12292-300
78. Sim RB, Ferluga J, Al-Rashidi H, Abbow H, Schwaeble W, Kishore U. Complement factor H in its alternative identity as adrenomedullin-binding protein 1. Mol Immunol. 2015;68(1):45-8.
79. Hagner S, Welz H, Kicic A, Alrifai M, Marsh LM, Sutanto EN, et al. Suppression of adrenomedullin contributes to vascular leakage and altered epithelial repair during asthma. Allergy. 2012;67(8):998-1006.
80. Weiszhár Z, Bikov A, Gálffy G, Tamási L, Ungvári I, Szalai C, et al. Elevated complement factor H levels in asthmatic sputa. J Clin Immunol. 2013;33(2):496-505.

81. Hallström T, Zipfel PF, Blom AM, Lauer N, Forsgren A, Riesbeck K. Haemophilus influenzae interacts with the human complement inhibitor factor H. J Immunol. 2008;181(1):537-45.
82. Vogl G, Lesiak I, Jensen DB, Perkhofer S, Eck R, Speth C, et al. Immune evasion by acquisition of complement inhibitors: the mould Aspergillus binds both factor H and C4b binding protein. Mol Immunol. 2008;45(5):1485-93.
83. Malm S, Jusko M, Eick S, Potempa J, Riesbeck K, Blom AM. Acquisition of complement inhibitor serine protease factor I and its cofactors C4b-binding protein and factor H by Prevotella intermedia. PLoS One. 2012;7(4):e34852.
84. Dobó J, Schroeder V, Jenny L, Cervenak L, Závodszky P, Gál P. Multiple roles of complement MASP-1 at the interface of innate immune response and coagulation. Mol Immunol. 2014;61(2):69-78.
85. Chang HS, Lee TH, Jun JA, Baek AR, Park JS, Koo SM, et al. Neutrophilic inflammation in asthma: mechanisms and therapeutic considerations. Expert Rev Respir Med. 2017;11(1):29-40.
86. Ray A, Kolls JK. Neutrophilic Inflammation in Asthma and Association with Disease Severity. Trends Immunol. 2017;38(12):942-54.
87. Panettieri RA Jr. The Role of Neutrophils in Asthma. Immunol Allergy Clin North Am. 2018;38(4):629-38.
88. Deutsch EW, Lam H, Aebersold R. Data analysis and bioinformatics tools for tandem mass spectrometry in proteomics. Physiol Genomics. 2008;33(1):18-25.

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATERIALS AND METHODS

Blood and serum samples

To obtain serum samples, BD Vacutainer SST II Advance tubes were used. After 30 minutes at room temperature (RT) and centrifuge at 3,000 xg (15 min at 4 °C), the serum was collected in 1 mL aliquots and frozen at -80 ° C. For proteomic experiments, 2 pools of each group of patients (pool A and pool B) and a reference sample (pooled sample; PS) composed of all study samples were prepared.

In order to eliminate serum lipoproteins, delipidation was carried out by means of a resin called LRA ™ (Lipid Removal Agent) (13358-U; Sigma, Spain). Firstly, a titration of the amount of resin needed was made. Thus, initial centrifugation was performed at 3,000 xg for 30 min at 4 ° C, and then several concentrations of resin (20-100 mg/mL) were added to 2.5 mL of serum samples. The mixture was incubated overnight (4 °C, in rotation) and subsequently centrifuged at 2,500 xg (10 min, 4 ° C). Next, the delipidated serum samples were collected for protein quantification. Afterwards, 1D SDS-PAGE with a high percentage of monomer (15% T) was performed in the Mini-Protean III system (Bio-Rad). 30 µg of each sample and 10 uL of molecular weight markers (NZYColour Protein Marker II; Nzytech) were loaded. During the electrophoresis, the voltage was set at 200 V (Powerpac 300, Bio-Rad). Finally, the gels were stained with Coomassie R-250 and scanned for analysis. A final concentration of 80 mg/mL of resin was selected for subsequent analysis.

Enrichment of low abundance proteins: Proteominer™.

To enrich samples in low abundance proteins, ProteoMiner™ Protein Enrichment Large-Capacity Kit (Bio-Rad, cat163-3007) was used following commercial guidelines. In brief, 1 mL from each serum sample was loaded in each column and subsequently incubated under stirring conditions (2 h, RT). After incubation, the samples were washed 4 times and then eluted by using a buffer provided by the kit. All centrifugations were performed at 1000 xg, 1 min, RT. Three fractions were collected and mixed in a single sample. Then, in order to eliminate contaminants such as the salts of the Proteominer elution buffer, the 2-D Cleanup Kit (GE Healthcare, Cat No. 80-6484-51) was used also following commercial guidelines. Finally, the pellet obtained was diluted into 30 µL of 0.5M TEAB 6M urea for ulterior analysis.

Protein quantification

Two methodologies were used according to the manufacturer's protocol. The first one, Pierce™ BCA Protein Assay Kit (Thermo Fisher, # 23225), was used for protein quantification after the delipidation process. A calibration line was generated using different concentrations of bovine serum albumin (BSA) in phosphate buffer (10 mM, pH 7.0): 0-2 mg/mL. The test samples were also diluted in phosphate buffer (1:60 dilution). In a 96-well plate, 25 µL of the blank (PBS), samples and standards were added. Each condition was analysed at least in duplicate. Subsequently, 200 µL of working reagent (50 A: 1 B) was added and incubated at 37 °C, 20 min. Finally, the reading was made at 550 nm in a plate reader (model 680, Bio-Rad).

The quantification after Clean-up required using the CB-X kit (786-12X, G-Biosciences). Firstly, to precipitate proteins from

samples, 1 mL of previously cooled (-20 ° C) CB-X ™ reagent was added to 50 µL of each sample (diluted 1:10 in TEAB buffer) and mixed by vortexing. The samples were then centrifuged at 16,000 xg for 5 min, the supernatant was removed, and 50 µL of CB-X ™ I and 50 µL CB-X ™ II solubilization buffers were sequentially added. The proteins were dissolved by vortex, and 1 mL/sample of CB-X ™ test dye was added. After vortexing and incubation for 5 min at RT, the samples were transferred to 96-well plates and the absorbance was read at 595 nm on a microplate reader (Labsystems Multiskan MS). A standard line with BSA (0.2-1 mg/mL) diluted in 0.5M TEAB buffer containing 6M urea was used to determine the protein concentration.

Reduction, alkylation, and trypsinization

Fifty µg of protein from each sample in 20 µL of 0.5M TEAB 6M urea were reduced and alkylated. For this purpose, 2 µL of reducing agent (TCEP) were added to each tube, followed by 1 h incubation at RT. Next, the samples were alkylated by adding 1 µL/sample of MMTS, followed by 10 min incubation at RT. Subsequently, 123 µL of 1M TEAB were added to reduce the urea concentration, and trypsinization was performed (1 µg/µL trypsin; 4326682, Sciex) at 37 ° C overnight. To stop the reaction (pH < 6.0), 4 µL of acetic acid was added. Finally, all samples were lyophilized.

iTRAQ labelling

The iTRAQ® Reagent-8PLEX Multiplex Kit (Sigma-Aldrich) was used according to the manufacturer's protocol. First of all, the samples were dissolved in 30 µL of 0.5M TEAB. Half of the contents of each vial (10 µL) were diluted in 25 µL of isopropanol, and then added to each peptide samples with the following labels: MSAA, 113; IMAA, 114; R, 115; MSNAA, 116; IMNAA, 117; HC, 119; PS, 121. PS sample is a pool of all the serum samples used to normalize the data

between the different MS analytical series. After 2h incubation, the seven labelled peptide samples were collected in a single tube and lyophilized. This procedure was performed twice, one for each biological replicate (pool A and pool B).

nanoLC/MS-MS identification and quantification of peptides

The samples were analysed in the Structural, Proteomic and Genomic Determination Service, Cacti Mass Spectrometry Unit, University of Vigo. Firstly, the peptide samples were reconstituted in solution A (0.1% formic acid (FA) in water) and desalted by Zip-Tips C18 (Millipore). Secondly, peptides were dispensed to nano-reverse phase EASY-Spray Columns (PepMap® RSLC, C18, 2µm, 100 Å, 75µm x 500mm, Thermo Fisher Scientific) mounted in the Proxeon EASY-nLC 1000 UHPLC (Thermo Fisher Scientific), and were eluted with a gradient of solution B (ACN) of 5-30% (240 min), 30%-90% (10min), and 90%-5% (17min). The nanoLC system is coupled online to an LTQ-Orbitrap ELITE (Thermo Fisher Fisher) used in a data dependent and positive ion mode. A full MS scan was carried out from 380−1600 m/z with a resolution at 12000. HCD-fragmentation was used and the MS/MS scan was accomplished with top 15, at 28% normalized collision energy, with a resolution at 30000, a dynamic exclusion time at 30 s, a minimum signal required at 1000, and an isolation width at 1.50 Da. Three technical replicates were performed from each biological replicate.

MS data analysis

Proteome Discoverer (version 2.1.1.21) was used for protein identification and iTRAQ quantification. HCD spectra were analysed using Sequest HT with Percolator validation. Spectra were searched against the latest UniProtKB Release and common contaminant sequences (e.g., trypsin, or keratins). The peptide mass tolerance was

10 ppm and the fragment mass tolerance 0.020 Da. As static modifications, the iTRAQ 8-plex N-terminus, and carbamidomethylation of cysteine were specified, and as variable modifications, the oxidation of methionine, the iTRAQ 8-plex of tyrosine and lysine, the carbamylation of lysine, and the N-terminus acetylation. The intensities acquired in MS/MS were globally normalized on protein median. Then, all reporter intensities were normalized by the reporter intensity of the pooled sample (PS, 121). The abundance of each protein was calculated as an average abundance of all its detected peptides. A 1.3 fold change (down-regulation, < 0.77; up-regulation, ≥1.3) in iTRAQ ratios, as well as a p-value less than 0.05, was used to identify differentially expressed proteins between the different groups of study.

Statistical and bioinformatic analysis of the data

Statistical data analyses were carried out using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Quantitative data comparisons between the different sample groups were performed by Kruskal–Wallis one-way analysis of variance followed by Dunn's multiple comparison tests. The receiver operating characteristic (ROC) analysis was also performed with GraphPad Prism. A $p < 0.05$ was considered of statistical significance. Bioinformatic analysis: GO annotation, GO terms overrepresentation test, and Reactome pathway overrepresentation analyses against the plasma proteome database (Version 2015-02-02) (www.plasmaproteomedatabase.org/) were performed in PANTHER Classification System (PANTHER version 13.1. Released 2018-02-03). A prospective study of all the potential physical or functional protein-protein interactions was also performed by means of the Search Tool for the Retrieval of Interacting Genes (STRING) (Version: 11.0, www.string-db.org)

SUPPLEMENTARY TABLES

Supplementary Table 1. Characteristics of the subjects from pool A.

	MSAA	IMAA	MSNAA	IMNAA	R	HC
N	25	26	22	24	22	16
Age (mean (range))	39 (21-64)	37 (20-64)	53 (24-68)	53 (29-72)	31 (18-46)	45 (22-58)
Sex (M/F)	11/14	15/11	6/16	2/22	12/10	5/11
Disease control:						
Yes	18	26	13	22	22	-
No	7	0	9	2	0	-
Baseline treatment:						
ICS-LABA	24	16	18	17	0	-
ICS	0	7	1	0	0	-
OCS	0	0	0	0	0	-
Antileukotrienes	14	8	10	5	3	-
Anticholinergic	5	3	13	2	0	-
Roflumilast	0	0	0	0	0	-
Prednisone	0	0	2	0	0	-
FEV1 (%)	97.0 (85.5-111.5)$	102.5 (94.5-110.5)	70.6 (60.0-88.8)#&	112.0 (98.0-120.5)	109.5 (99.5-121.5)	-
FEV1/FVC (%)	76.5 (70.7-79.6)#	79.6 (72.6-82.7)	67.6 (58.1-77.1)#	76.6 (73.7-80.6)#	84.6 (79.1-88.3)	-
Neutrophils (10^3 cells/µL)	3.14 (2.28-4.19)	3.94 (3.21-4.45)	3.61 (2.98-4.77)	3.16 (2.49-3.53)	3.73 (2.86-4.90)	3.68 (2.25-4.34)
Lymphocytes (10^3cells/µL)	1.89 (1.37-2.12)	1.98 (1.74-2.21)	2.07 (1.64-2.50)	1.87 (1.49-2.16)	2.20 (1.95-2.71)	2.04 (1.48-2.45)

Continued on next page

Supplementary Table 1 (Continued)						
Monocytes (10^3 cells/µL)	0.33 (0.29-0.42)	0.41 (0.31-0.46)	0.37 (0.32-0.50)	0.32 (0.29-0.43)	0.40 (0.34-0.50)	0.42 (0.33-0.62)
Eosinophils (10^3 cells/µL)	0.36 (0.22-0.55)*	0.24 (0.16-0.34)	0.31 (0.18-0.46)	0.25 (0.13-0.37)	0.22 (0.13-0.38)	0.20 (0.09-0.30)
Basophils (10^3 cells/µL)	0.04 (0.03-0.05)	0.04 (0.03-0.05)	0.03 (0.02-0.05)	0.03 (0.02-0.04)	0.03 (0.02-0.06)	0.03 (0.02-0.05)
ESR (1h; mm)	10.0 (6.5-17.5)	5.5 (2.0-17.2)	15.0 (9.7-25.5)*#	12.0 (7.2-20.5)	4.5 (2.0-14.8)	10.0 (7.0-11.0)
IgE (IU/mL)	122.0 (62.5-316.0)*$	174 (79-241)*$	38 (10-96)	17 (5-32)	76 (24-283)	11 (3-45)
IgG (mg/dL)	1100 (951-1260)	1075 (952-1195)	1045 (951-1313)	1030 (794-1140)	1005 (943-1288)	980 (842-1090)
IgG1 (mg/dL)	630 (548-807)	681 (594-732)	658 (495-761)	550 (447-605)#	673 (546-775)	-
IgG2 (mg/dL)	374 (285-448)	365 (276-469)	293 (213-459)	338 (246-426)	337 (268-405)	-
IgG3 (mg/dL)	32 (22-45)	32 (21-48)	42 (22-55)	33 (28-57)	39 (29-59)	-
IgG4 (mg/dL)	50 (27-87)	47 (23-96)	65 (39-125)	28 (10-60)	23 (14-51)	-
IgA (mg/dL)	223 (159-270)	226 (176-295)	204 (147-262)	209 (162-251)	166 (100-262)	250 (121-333)
IgM (mg/dL)	113 (76-166)	97 (64-153)	115 (93-172)	101 (70-133)	109 (67-171)	88 (71-118)

HC, healthy controls; IMAA, intermittent-mild allergic asthmatics; IMNAA, intermittent-mild non-allergic asthmatics;MSAA, moderate-severe allergic asthmatics; MSNAA, moderate-severe non-allergic asthmatics; R, rhinitis patients.

Data are presented as median value (IQR1-3), unless otherwise expressed.

Statistical significance is shown: *Disease vs HC; #Asthma vs R; $AA vs NAA; &Moderate-severe vs Intermittent-mild asthma. Kruskal-Wallys test followed by Dunn´s multiple comparison test. $p < 0.05$

Supplementary Table 2. Characteristics of the subjects from pool B.

	MSAA	IMAA	MSNAA	IMNAA	R	HC
N	24	27	21	23	21	16
Age (mean (range))	39 (18-68)	36 (21-66)	54 (28-67)	51 (34-72)	39 (24-55)	41 (27-61)
Sex (M/F)	12/12	10/17	7/14	6/17	8/13	9/7
Disease control:						
Yes	12	27	17	23	21	-
No	12	0	4	0	0	-
Baseline treatment:						
ICS-LABA	23	17	21	18	18	-
ICS	1	8	0	3	0	-
OCS	1	0	0	0	0	-
Antileukotrienes	11	10	10	4	7	-
Anticholinergic	8	0	12	0	0	-
Roflumilast	0	0	1	0	0	-
Prednisone	0	0	3	0	0	-
FEV1 (%)	89.5 (65.7-99.2)[#]	100.0 (92.0-108.0)	77.0 (66.0-96.0)[#&]	105.0 (97.0-113.0)	102.5 (96.2-115.0)	-
FEV1/FVC (%)	69.8 (55.9-78.6)[#]	78.9 (72.3-80.7)	67.2 (61.0-76.9)[#]	77.0 (72.5-80.1)	83.2 (78.1-86.1)	-
Neutrophils (10^3 cells/µL)	3.90 (2.93-4.61)	3.55 (3.14-4.06)	3.56 (3.31-4.31)	3.78 (3.19-4.53)	3.54 (2.97-3.86)	3.02 (2.40-3.69)
Lymphocytes (10^3cells/µL)	1.94 (1.81-2.81)	2.02 (1.59-2.29)	1.83 (1.51-2.67)	1.96 (1.67-2.28)	2.13 (1.65-2.48)	1.95 (1.53-2.57)
Monocytes (10^3 cells/µL)	0.31 (0.30-0.47)	0.31 (0.40-0.50)	0.43 (0.32-0.52)	0.37 (0.30-0.51)	0.34 (0.28-0.46)	0.39 (0.32-0.47)

Continued on next page

Supplementary Table 2 (Continued)

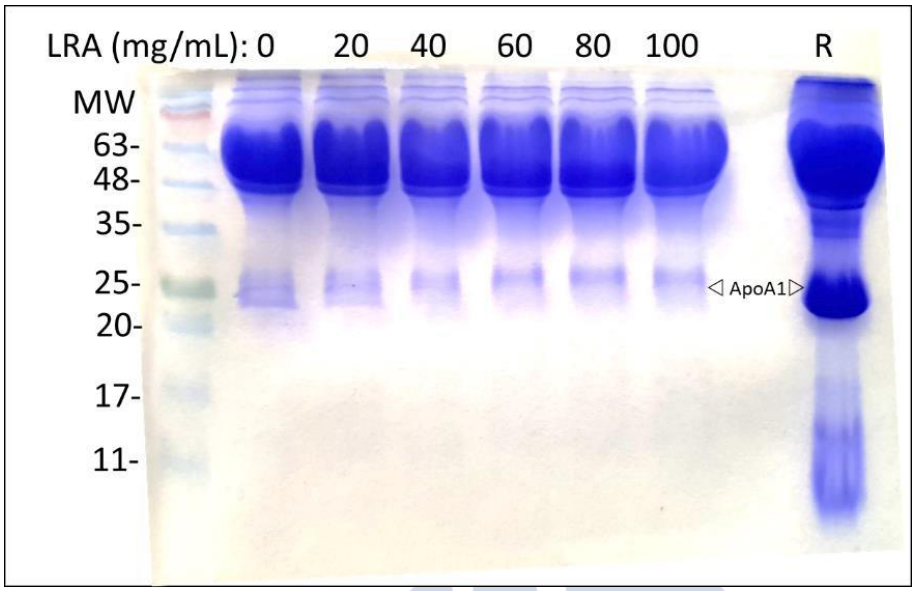
Eosinophils (10^3 cells/µL)	0.35 (0.22-0.50)*	0.29 (0.20-0.45)*	0.28 (0.15-0.42)*	0.24 (0.17-0.35)	0.22 (0.14-0.28)	0.12 (0.10-0.22)
Basophils (10^3 cells/µL)	0.04 (0.03-0.06)	0.04 (0.03-0.05)	0.04 (0.02-0.06)	0.03 (0.02-0.05)	0.04 (0.02-0.05)	0.04 (0.03-0.05)
ESR (1h; mm)	7.0 (4.0-13.7)	7.0 (4.0-12.0)	9.0 (4.0-20.0)	11.0 (8.0-20.0)*	11.0 (2.5-15.5)	3.0 (2.0-8.5)
IgE (IU/mL)	209.0 (72.5-523.3)*$	122 (50-304)$	44 (16-104)	25 (11-43)	59 (27-99)	15 (12-144)
IgG (mg/dL)	1030 (898-1100)	1100 (923-1250)	1065 (915-1203)	1010 (880-1175)	1045 (879-1180)	1080 (904-1290)
IgG1 (mg/dL)	644 (555-725)	651 (538-768)	607 (474-733)	549 (455-654)	603 (521-692)	-
IgG2 (mg/dL)	333 (257-383)	331 (279-409)	345 (318-436)	347 (273-393)	312 (258-426)	-
IgG3 (mg/dL)	30 (20-43)	39 (31-56)	36 (27-64)	36 (32-62)	31 (26-38)	-
IgG4 (mg/dL)	52 (38-86)	47 (23-68)	37 (21-64)	32 (17-67)	41 (29-54)	-
IgA (mg/dL)	200 (170-279)	254 (172-315)	238 (170-296)	197 (147-270)	214 (135-275)	332 (148-375)
IgM (mg/dL)	93 (65-143)	106 (85-133)	111 (72-149)	123 (79-154)	112 (78-160)	102 (86-103)

HC, healthy controls; IMAA, intermittent-mild allergic asthmatics; IMNAA, intermittent-mild non-allergic asthmatics;MSAA, moderate-severe allergic asthmatics; MSNAA, moderate-severe non-allergic asthmatics; R, rhinitis patients.

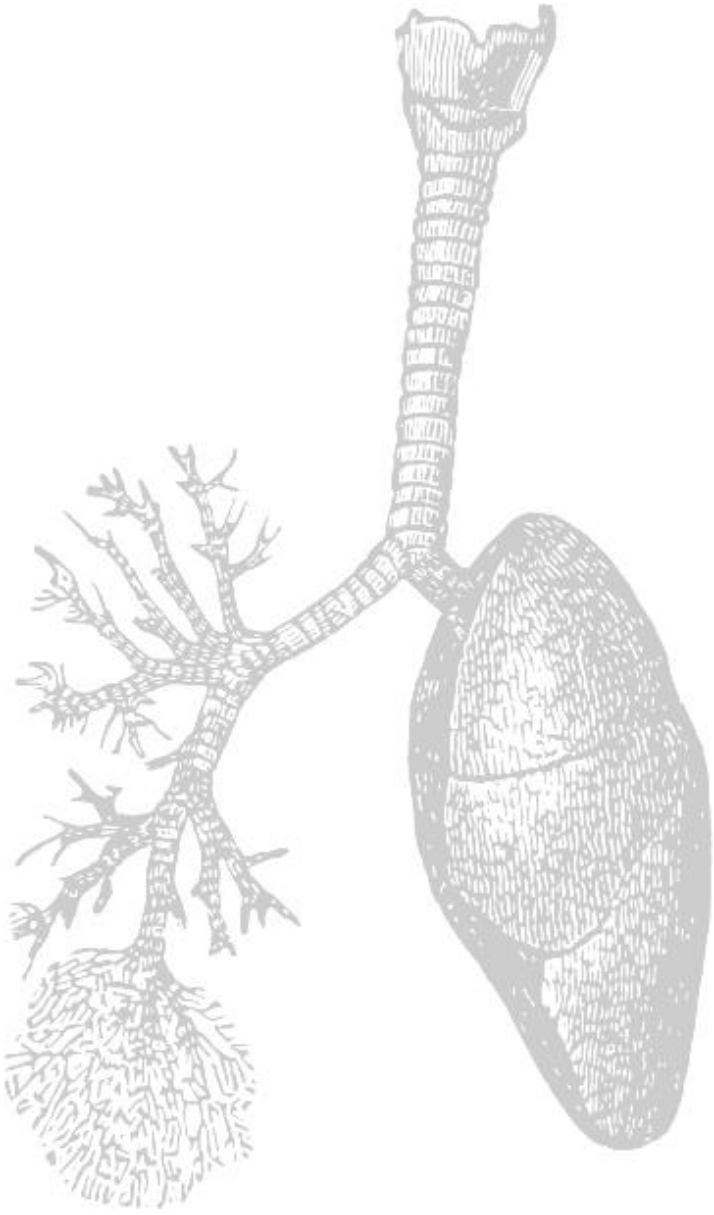
Data are presented as median value (IQR1-3), unless otherwise expressed.

Statistical significance is shown: *Disease vs HC; #Asthma vs R; $AA vs NAA; &Moderate-severe vs Intermittent-mild asthma. Kruskal-Wallys test followed by Dunn´s multiple comparison test. $p < 0.05$

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Treatment with LRA resin reduces serum lipoprotein levels. A volume of 300 µL of serum was treated with different concentrations of LRA resin (0-100 mg / mL) overnight at 4 ° C in rotation. An SDS-PAGE (15% T separating gel) is shown in which 30 µg of protein belonging to the fraction not retained by the resin (channels 2-7) has been loaded. Channel 1 corresponds to molecular weight markers, and channel 9 to the retained fraction (R).



GENERAL DISCUSSION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The overall aim of the present thesis was a search for potential biomarkers of different asthma phenotypes and rhinitis. Due to the key role of the immune system in asthma, we decided to deepen the study of certain molecules previously related to asthma pathogenesis, but with many open questions. Furthermore, these molecules were selected as representatives of the innate (CD14; Chapter I) and the acquired (CD26 and CD126; Chapter II and III) arms of the immune system. Finally, there was a lack in the literature of comparative serum proteomic studies including, at the same time, allergic and non-allergic asthmatics, as well as rhinitis patients. This significant absence of information prompted us to approach this problem through a non-target biomarker discovery strategy based on affinity chromatography (CPLLs) and iTRAQ LC–MS/MS (Chapter IV).

1. INNATE IMMUNE SYSTEM BIOMARKERS OF ALLERGIC ASTHMA (AA) (Chapter I)

The specific aim of this work was to simultaneously study, in a well-defined population of allergic asthmatics, both the levels of mCD14, a monocyte marker associated to asthma [87], and its soluble counterpart, sCD14. In addition, since the existing studies have yielded conflicting results, we wanted to study the association with AA of one SNP polymorphism (rs2569190; -159 C/T) in the *CD14* gene, which is known by regulating both the activity of *CD14* promotor and the sCD14 levels [36].

Monocytes have been described as important modulators of inflammation and key players in asthma pathogenesis [396-398]. Indeed, an expansion of this leukocyte subset in severe allergic asthmatics was found in the present study, and this result has not been

reported so often in previous works. In contrast, the expression of mCD14 on the surface of these circulating monocytes was found decreased in allergic asthmatics compared to healthy donors. Monocytes/macrophages may modulate T_N cells differentiation towards a TH2-phenotype through a transduction pathway that starts with the binding of LPS to CD14, the co-receptor of TLR4. CD14 transfers LPS to the MD2/TLR4 complex, triggering the production of IL12/IL18 by monocytes/macrophages and thus promoting a TH1-differentiation [113, 399]. Therefore, attenuated mCD14 levels on APCs could favour TH2-driven AA [36, 37, 103, 113]. Thus, the reduction of mCD14 observed in our group of AA donors makes biological sense, as a receptor implicated in a pathway leading to TH1-differentiation should be reduced in allergic asthmatics, a disease mainly driven by TH2 cells.

The diminished number of mCD14 molecules on monocytes could be the result of: **a)** an altered transcription/translation rate (or any other process) affecting the abundance of this protein; **b)** the release of CD14 molecules into the bloodstream from monocytes, leading to higher sCD14 levels; or **c)** the preferential expansion of $CD14^{low}$ monocyte subsets. The first option is dismissed because the alteration of the transcription/translation rate of CD14 should induce an important and widespread down-modulation of mCD14 on monocytes, and not the small changes in the percentage of $CD14^{+}$ monocytes that we have observed in asthmatics. As well, the second option is disregarded because serum sCD14 levels are not augmented in our cohort of patients, quite the contrary. Therefore, despite we have not measured CD16, our results are suggesting the preferential expansion of a small $CD14^{low}$ monocyte subset, which fits with the $CD14^{low}CD16^{+}$ subset of "non-classical" monocytes [396]. There are three major monocyte subtypes, the classical monocytes ($CD14^{++}CD16^{-}$), which represent more than 90% of circulating

monocytes, non-classical monocytes ($CD14^{low}CD16^{+}$), and an intermediate subset ($CD14^{+}CD16^{+}$) that has been considered as a transitional stage between the classical and non-classical subpopulations [75]. The last population of monocytes has been found expanded in inflammation, severe asthma, and upon allergen challenge [396, 398]. Together with intermediate monocytes ($CD14^{+}CD16^{+}$), non-classical monocytes express high levels of CD80, CD86, and CD163, suggesting a high antigen presenting capability [77]. These non-classical monocytes are characterised by an advanced differentiation stage and evidence a high invading ability to infiltrate and differentiate into M2-type macrophages [400]. This last subset has been related to allergic inflammation [80]. Furthermore, circulating monocytes recruited into the lung to be transformed into inflammatory macrophages, but not the tissue-resident counterparts, promote allergic inflammation in murine models of asthma [401].

Simultaneous measurement of sCD14 was also undertaken in this first study. This soluble isoform of CD14 probably appears in serum samples due to different mechanisms, including enzymatic-shedding [38, 95, 402] and/or vesicle-release (for example, $CD14^{+}$ exosomes or ectosomes; http://exocarta.org/gene_summary?gene_id=12475) from monocytes. Indeed, two sCD14 isoforms of 49-51 (α) and 55-57 (β) kDa can be detected in human plasma [403]. Although absolute sCD14 levels remained unchanged, we found in this first paper a decrease in the relative amount of sCD14 (sCD14/number of monocytes) in serum samples from allergic asthmatics. This is in line with the inverse correlation between IL-4, total IgE, or asthma severity with sCD14 [107]. As well, with the selective expansion of a small $CD14^{low}(CD16^{+})$ "non-classical" monocyte subset in AA that could leave its imprint in serum proteome. However, other studies have

found no differences [95, 101] or even higher levels [102] of sCD14 in asthmatics.

The above-mentioned differences regarding sCD14 levels could also be explained by the presence of potential confounding factors, such as gene-gene or gene-environment interactions [101, 106]. For example, the decreased levels of sCD14 in allergic asthmatic subjects may arise from changes in the allelic and genotypic frequencies of the *CD14* (-159 C/T) SNP (rs2569190) in the asthmatic subset. The -159 C/T SNP of the promoter of CD14 is one of the most studied polymorphisms in asthma, yielding variable results regarding the strength and the direction of the association [87, 112, 115]. These different results can be explained by several factors, such as low sample size, age, ethnicity, or level of endotoxin exposure during childhood (gene-environment interaction) [41, 116]. Therefore, we performed our study in a well-defined population of Caucasian adults with AA, mostly non-farmers, and with a relatively high sample size (N = 554 subjects; 277 AA patients and 277 healthy subjects). In agreement with others [106, 112], our results evidence a drop in the frequency of the T allele and the TT genotype in allergic asthmatic patients, especially as the severity was higher (moderate-severe allergic asthmatics). Moreover, T allele is associated with a decreased risk of AA, and the same was shown for the TT genotype following different genetic models (TT vs. CC, or the dominant model / TT vs. TC+CC). Additionally, sCD14 levels were increased in carriers of the T allele or the TT genotype compared to C carriers, as other works previously showed [36, 102, 106, 109, 110]. This is in line with the higher transcriptional activity of the *CD14* promoter containing the T allele [36]. Therefore, our results suggest an adverse role for the C allele, the CC genotype, and the presence of low levels of sCD14/mCD14 in AA [102, 107] especially among adult subjects exposed to low levels of environmental endotoxin [113].

Finally, a small part of this work was devoted to the study of neuron-specific enolase (NSE) in serum samples from AA patients. This analysis is justified because NSE levels have been shown to increase upon activation of eosinophils and macrophages under some pathological conditions [404, 405]. NSE is the neuronal isomer of the glycolytic enzyme 2-phospho-D-glycerate hydrolase and a biomarker for diagnosis of small cell lung cancer [406]. Nevertheless, some groups have also detected changes in circulating NSE levels associated with non-malignant inflammatory lung diseases, such as tuberculosis or silicosis [405, 407-410]. These changes are explained because this enzyme can translocate towards the cell surface to enhance a pro-inflammatory response upon proper stimulatory signals [411]. However, this molecule has not been extensively studied in asthma [407]. As our study points out, NSE levels in serum are positively correlated with total IgE and could be a useful biomarker for AA, with AUC (area under the curve) values in ROC (Receiver Operating Characteristic) curve similar to those found for IgE and eosinophils. Monocytes/macrophages, as well as eosinophils, appear to be a possible source of NSE [404, 405, 411]. Indeed, we found a positive correlation between NSE levels and the absolute count of these two leukocyte subsets. In addition, the neuronal distress or hypoxia taking place in the lung could be also responsible for the changes in NSE levels with asthma [407].

2. CD26, ADAPTIVE IMMUNE CELLS, AND ASTHMA PHENOTYPES (Chapters II and III)

In the same way that innate immunity, where there are monocyte subsets with opposing functions and variable levels of a lineage-specific marker (CD14) with a role in asthma, there is certain parallelism with another marker in the adaptive system: CD26. Thus,

like mCD14, this glycoprotein presents a membrane and a soluble (sCD26) isoform. Moreover, even though CD26 does not anchor to the plasma membrane through a glycophosphatidylinositol linkage as CD14 does, this molecule presents an extremely short cytoplasmic region of 6 residues that requires the participation of other molecules (e.g., CARMA1) to downstream signal transduction [252]. Likewise, CD26 expression is rather confined to $CD4^+$ T lymphocytes in a manner comparable to CD14 and monocytes. Finally, CD26/DPP4 belongs to a subfamily of serine proteases (the S9B) with several members showing an association with asthma: CD26/DPP4 itself and DPP10. Based on all these points, Chapters II and III were aimed to study the expression pattern of CD26 in different lymphocyte subpopulations and their implications in both AA and NAA, as well as disease severity. Because of the complexity of this issue, we carried out a comprehensive review (see appendix I) aimed to provide a structured overview of the numerous functions of CD26 and its implications in asthma pathogenesis and progression. Up until that time, there was only one review paper specifically covering the relationship between CD26, T cells, and asthma [412].

CD26 is a prolyl oligopeptidase that belongs to the serine protease family (S9B) [243]. CD26 is widely distributed over several cell types and tissues. However, expression of CD26 is especially associated with immune cells, such as granulocytes, monocytes, B cells, and T lymphocytes (particularly $CD4^+$ T cells) [251]. Within $CD4^+$ T lymphocytes, its expression is higher in Teff cells compared to Treg cells [258]. In addition, Bengsch *et al.* used flow cytometry data and FACS sorting to shown that the expression of CD26 amongst Teff cells is quite variable, according to the following order: TH17>> TH1> TH2 [259, 413, 414]. These results have been confirmed *in vitro* in Chapter II, where $CD4^+CD25^-$ Teff cells were activated and differentiated towards a TH1-like (IL-12), TH2-like (IL-4), and TH17-

like (IL-1β, IL-6, and IL-23) phenotypes. Our results show the same differential expression for CD26 in Teff cells (TH17>> TH1> TH2) that Bengsch *et al.* described previously.

As above commented, CD26 can also be found in the extracellular space (e.g., serum/plasma, BALF, CSF) as sCD26 [286]. This soluble version of CD26 is released by $CD26^+$ cells through enzymatic-shedding [291] or production of $CD26^+$ vesicles (http://www.exocarta.com). It has been shown that visceral fat release sCD26 as an adipokine [415], but $CD4^+$ T cells remain as the most likely source [286, 293, 294]. Indeed, our *in vitro* assays (Chapter II) reveal a strong positive correlation between the expression of CD26 (mean fluorescent intensity/MFI) on different TH lymphocyte subsets (TH17>>TH1>TH2>Treg) and the soluble DPP4 activity (a *bona fide* indicator of sCD26 levels) in culture supernatants. Therefore, if this was happening *in vitro*, it was expected that serum sCD26 levels were influenced by the number of $CD4^+$ T cells or mirror the predominant subpopulation of $CD4^+$ T cells (e.g., TH1, TH2, TH17) in the different asthma phenotypes/endotypes. In other words, we were expecting a coordinated and variable elevation of both CD26 and sCD26 in asthma, higher or lower depending on the disease phenotype and the prevailing TH subset.

With this in mind, we undertook a systematic analysis of the expression of CD26 on TH lymphocytes from AA, NAA, rhinitis, and healthy individuals, as well as an evaluation of sCD26 levels in serum samples (Chapters II and III). Firstly, as Lun and coworkers [246] previously showed in AA (and as expected from an activation marker) [252, 416, 417], there was an increase in the expression of CD26 on $CD4^+$ lymphocytes from AA an NAA patients (Chapters II and III). However, inconsistently with the higher sCD26 levels detected by Lun *et al.* in AA [246], in the first study (Chapter II) it was shown a diminished concentration of sCD26 in serum samples from two

cohorts of AA patients compared to healthy subjects. Moreover, the same happened for sCD25 (Chapter II), another soluble isoform of an activation marker: CD25/IL-2Rα [220-224]. A similar down-modulation of sCD26 was found in NAA patients (Chapter III) and eosinophilic pneumonia [418], whereas other authors did not detect any difference in sCD26 levels in children with asthma [303]. The different results reported by Lun and coworkers [246] regarding sCD26 levels in AA as compared with our findings in Chapter II may have several explanations. On the one hand, the different male/female proportions between AA patients and controls in the paper of Samantha Lun, as sCD26 concentration is higher in males. On the other hand, the more active disease status of patients in these studies compared to our cohort of AA patients, which were in a stable phase.

Our i*n vitro* experiments (Chapter II) support that $CD4^{+}CD25^{-}$ Teff cells primarily secrete CD26 upon TCR triggering. In the same line, Teff cells up-regulate and release CD25 after being activated [419]. Therefore, the small reduction detected for both soluble markers (sCD26 and sCD25) in serum samples from AA patients may be indicative of the expansion of a $CD25^{-/low}CD26^{-/low}$ TH subpopulation in this disease. Indeed, in Chapter II we describe, for AA patients, a significant increase in the percentage of a small population of $CD4^{+}CD25^{-/low}CD26^{-/low}CD127^{-/low}$ TH cells; we have called this subset "triple low" or Tlow cells. These results have been confirmed in Chapter III, showing that $CD26^{-/low}$ TH cells belong to a group of $CD4^{+}$ T lymphocytes that have lost the expression of several markers: CD27, CD28, CCR7, and CD127. This fact highlights the advanced differentiation stage (T_{EM} or T_{EMRA}) of this subset. Thus, in line with other works [262], the number of CD26 molecules on TH cells allows describing lymphocytes with a T_N ($CD26^{int}$; $CD45RA^{+}CCR7^{+}CD28^{+}$), a T_{CM} ($CD26^{high}$; $CD45RA^{-}CCR7^{+}CD28^{+}$), or a highly differentiated phenotype ($CD26^{-/low}$; $CD45RA^{+/-}CCR7^{-}$

$CD28^-$): i.e., T_{EM} or T_{EMRA}. Perhaps some of these cells stop recirculating between blood/lymphatic system and non-lymphoid tissues and enter the lung tissues to become tissue-resident memory T cells [420, 421].

Research in asthma is mostly focused on $CD4^+$ T cells and the AA phenotype. However, $CD4^-$ lymphocytes (i.e., B cells, $CD8^+$ T cells, NKT, γδ-T lymphocytes, and NK cells) could have an active role in asthma pathogenesis and be relevant for asthma immune-phenotyping. Along with this line of reasoning, the work shown in Chapter III has focused on the possibility that $CD4^-$ subpopulations may be altered amongst patients with rhinitis or different asthma phenotypes (AA and NAA) or severities. In this way, that study draws a parallel between $CD4^+$ T cells in AA and $CD4^-$ lymphocytes in NAA, as it was also found an expansion of $CD26^{-/low}$ subpopulations in $CD4^-$ lymphocytes from NAA patients that explains why serum sCD26 levels are also reduced in this phenotype. Moreover, in Chapter III it was also shown that this expansion in NAA (compared to AA) can be mostly ascribed to $CD26^-CD4^-$ γδ-T lymphocytes.

Most of circulating γδ-T cells are $V\delta2/V\gamma9^+$ cells [119], which display a similar distribution of naïve-memory populations as $CD4^+$ αβ T cells: T_N ($CD45RA^+CD27^+$), T_{CM} ($CD45RA^-CD27^+$), T_{EM} ($CD45RA^-CD27^-$), and T_{EMRA} ($CD45RA^+CD27^-$) [122, 135]. Therefore, these findings are compatible with the description in Chapter III of $CD26^-$ (T_{EM}/T_{EMRA}), $CD26^{int}$ (T_N), and $CD26^{high}$ (T_{CM}) γδ-T subsets. Furthermore, γδ-T cells are the major producers of early IL-17 [134]. Therefore, the increase of a population of $CD26^-$ γδ-T lymphocytes with a T_{EM}/T_{EMRA} phenotype in NAA (Chapter III) with preferential production of TH17 or TH1 cytokines [422] might explain the inverse relationship between γδ-T cells and B cells (Chapter III) and the enhanced airway inflammation in NAA [309]. The number of peripheral blood γδ-T cells was previously found decreased in AA

[119, 133, 423], whereas no changes or even an increase was found in BALF or bronchial biopsies from AA patients [142, 143]. We did not measure the number of γδ-T cells in healthy subjects, but we found in AA a slightly lower percentage (2.9%) than the one previously described in the literature for healthy subjects (4.1%) [119]. In any case, more studies are needed to validate these findings because, to our knowledge, this is the first work addressing the levels of CD26 in γδ-T cells in patients with NAA.

To summarize, data from the studies shown in Chapter II and III provide evidence that both asthma phenotypes share common immunopathological mechanisms, with expansion of $CD26^{-/low}$ subsets in AA ($CD4^{+}$ Tlow) and NAA ($CD4^{-}$ T cells; γδ-T lymphocytes) and down-modulation of additional surface molecules (CD27, CD28, IL-7Rα/CD127, CCR7) to produce differentiated effector subsets and extracellular sCD26 reduction. Decline in sCD26 and CD26 expression in different lymphocyte populations must be considered in the light of different findings such as the reduction of caveolin-1 (a CD26 ligand) in monocytes and bronchial epithelial cells from asthmatics [424], or the role of CD26 controlling the bioavailability of soluble factors such as cytokines (e.g., IL-3, GM-CSF) [425] and chemokines (e.g., eotaxin 1/CCL11, RANTES/CCL5, MDC/CCL22) [274, 286]. Thus, the cleavage by CD26/DPP4 of chemokines that are potent chemoattractant for eosinophils and TH cells such as RANTES [282] and eotaxin [275, 277, 278] plays a key role in asthma pathogenesis. For example, the treatment with CD26 inhibitors or the use of $CD26^{-/-}$ animals results in higher levels of eosinophils, higher infiltration of these cells into the airways, and higher disease severity [275, 281]. In the same way, CD26 mediated cleavage of SDF-1α/CXCL12 [426] or IFN-γ induced chemokines (CXCL9-11) [279, 427, 428] reduce the chemotaxis of TH1 cells towards the epithelial barrier. Therefore, CD26 forms part of a homeostatic mechanism

aimed to the down-modulation of airways inflammation. Thus, the immunomodulatory potential of CD26/DPP4 should be considered in light of the clinical usage of CD26 inhibitors (gliptins) (see appendix I).

Another area of attention in Chapter III was the CD126/IL-6Rα molecule. Together with gp130, CD126 composes the receptor of IL-6, a molecule with a key role in the differentiation of T_N lymphocytes towards a TH17 (i.e., $CD26^{high}$) phenotype [176]. IL-6 signalling is also essential for the generation of functionally active memory $CD4^+$ T cells [429], while CD126 expression on $CD8^+$ T cells defines diverse naïve-memory differentiation stages in these cells [152], in the same way that CD26 [261]. Indeed, we report a highly positive correlation between the levels of these two markers on circulating lymphocytes, but no differences in CD126 expression between the different groups of patients (healthy controls, rhinitis, AA, NAA). As well, this paper describes that $CD4^-$ lymphocytes can be segregated in different subpopulations based on the expression of CD126. Therefore, this molecule could be useful for naïve-memory characterization as well. Moreover, $CD126^-CD4^-$ lymphocytes (augmented in NAA vs. AA) downmodulate the levels of CCR7, CD28, and CD27, but retain a high abundance of CD45RA molecules, highlighting their advanced differentiation stage (T_{EMRA}-like).

As CD26, CD126 can be released from the membrane of $CD126^+$ cells as sIL-6Rα. This soluble receptor retains the capacity to bind IL-6, leading to the activation of $CD126^-$ $gp130^+$ cells. This process is known as *trans*-signalling [179, 180], and might be important for asthma through the maintenance of TH17 cells or the inhibition of T cell apoptosis [430]. For example, it has been described that CD126 is down-modulated upon inflammation on $CD4^+$ T cells, but these cells retain the IL-6 response capacity through *trans*-signalling events [431]. Indeed, the release of sIL6Rα has been

described in asthma, and sIL-6Rα levels have been directly associated with IgE levels. Furthermore, sIL-6Rα in serum is negatively correlated with lung function [432]. Therefore, the down-modulation of the number of CD126 molecules on monocytes, neutrophils, and $CD4^+$ cells from moderate-severe patients reported in Chapter III highlights the potential role of IL-6 *trans*-signalling in asthma severity.

The effector function of human TH lymphocytes is counteracted by the immunosuppressive branch of this subset, called Treg cells [214]. Therefore, a defect in their biological function or a reduction in their number could result in higher inflammation levels in asthma. Although some authors have described a reduction of the frequency of Treg cells in asthmatics, in Chapter II and III we fail to detect such a numerical alteration. Nevertheless, women did show an increased Teff/Treg ratio compared to men (Chapter II), and this could be behind the greater susceptibility of this gender to adult asthma.

Our results also suggest an impaired function of Treg cells in asthma (Chapter III). Thus, it appears that Treg lymphocytes from asthmatic patients display higher levels of CD26 than those from healthy subjects. Moreover, the percentage of $CD26^+$ Treg cells was higher in moderate-severe patients compare to intermittent-mild non-allergic asthmatics. Why are CD26 levels so important for Treg function? CD26 is a glycoprotein that anchors ADA to the cell-surface, and ADA is an ecto-enzyme involved in the catabolism of adenosine [264]. Moreover, CD39 is another ecto-enzyme expressed by $CD26^-$ Treg lymphocytes involved in adenosine production [258, 433]. Therefore, a $CD26^{high}$ phenotype in Tregs from asthmatics could cause higher asthma severity through a decreased local concentration of adenosine, a purine nucleoside with immunomodulatory functions [434]. On the other hand, the percentage of $CD127^+$ Treg cells was increased in our asthmatic cohort compared to healthy and rhinitis, a

parameter negatively correlated with the suppressive capacity of these cells according to others [435]. Therefore, future studies including the assessment of Treg function in NAA and AA will be necessary.

3. SEARCHING FOR BIOMARKERS IN SERUM SAMPLES FROM PATIENTS WITH RHINITIS OR DIFFERENT ASTHMA PHENOTYPES/SEVERITIES (Chapter IV)

Because of the absence of studies of this kind, the last chapter of the present thesis is devoted to the search of serum biomarkers associated to asthma phenotypes (AA vs. NAA) or severities (intermittent-mild and moderate-severe) by means of modern proteomic techniques. The first serum/plasma proteomic studies were only able to "scratch" the first layer of the proteome (i.e., the high-abundance proteins) as a result of the high dynamic range of protein concentrations in serum. Since then, several techniques have been developed to deal with this problem, such as ultrafiltration, targeted protein-depletion, or low abundant protein enrichment. At the same time, analytical methods have evolved from classical procedures with a low sensitivity, precision, and throughput (e.g. 2-DE), to more modern and high-performance technologies (e.g., LC-MS/MS). However, despite these refinements, most of the current proteomic approaches are not yet capable to reach with ease the medium-low abundant proteome (the so-called "deep proteome"). Therefore, the first aim of the work accomplished in Chapter IV was to develop a protocol for the quantitative study of the medium-low abundance proteome of serum samples, which combines lipoproteins-depletion, CPLL-based enrichment of low abundant proteins (ProteoMiner®), and iTRAQ-LC-MS/MS analysis. The second aim was to apply this protocol to different serum samples from rhinitis, allergic asthmatics, non-allergic asthmatics, and healthy subjects, in order to discover novel biomarkers capable of predicting different asthma phenotypes or severities.

In the last years, a high number of proteomic studies have been carried out in asthma (summarized in Table 1 from introduction). In all these pieces of work, different types of samples (e.g., BALF, EBC, NLF, BB, NB, IS, serum/plasma) were used, and several types of biomarkers pursued (e.g., diagnosis, exacerbation, treatment response) [337, 436-438]. However, none of them was aimed to analyse the serum proteome of different asthma phenotypes (AA vs. NAA) or severities, as above commented. Additionally, most of the studies in serum samples used 2-DE, and just a handful was carried out by LC-MS/MS [391]. Moreover, one single proteomic study used iTRAQ-LC-MS/MS to evaluate bronchial brushing samples from asthmatics [379]. Therefore, the first part of Chapter IV addresses the development of a new quantitative proteomic approach based on lipoproteins-depletion, low abundant protein enrichment (ProteoMiner), iTRAQ peptides labelling, and subsequent LC-MS/MS and iTRAQ quantification, with applications in a diverse set of pathologies.

On the other hand, the second part of the work accomplished in Chapter IV undertakes the identification of the serum proteome signature in different groups of donors: moderate-severe AA, intermittent-mild AA, rhinitis, moderate-severe NAA, intermittent-mild NAA, and healthy controls (HC; reference group). Due to a large number of serum specimens and the ability of iTRAQ reagents to label up to 8 biological samples, each group of patients was split into two subgroups to generate two biological replicates. In turn, these biological samples were analysed in triplicate (technical replicates), together with a pooled serum sample formed by all the samples (internal pool; 121 reporter ion), which was used for normalization. After LC-MS/MS analysis, a set of 217 proteins was identified with high confidence, of which 26 presented changes between the groups of study. Principal Component Analysis of the differentially abundant

proteins revealed the presence of 5 principal components (PCs) explaining 81.6% of the variation. PC1 is made up of proteins related to allergy (AA and rhinitis), most of them implicated in metabolic (e.g., protein AMBP/alpha-1-microglobulin/bikunin precursor; HSPG2/Heparan sulphate proteoglycan 2/Perlecan; IGFALS/Insulin-like growth factor binding protein, acid labile subunit; or AHSG/alpha-2-HS-glycoprotein) or immune system processes (e.g., HSPG2, AHSG, ORM1/AGP1/Alpha-1-acid glycoprotein 1, or CD5L). Moreover, PC1 has an enrichment of the Reactome pathway "Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)" to which calumenin, IGFALS, AHSG, and prothrombin belong. Amongst proteins in PC1, IGFALS, protein AMBP, and HSPG2 were chosen for further confirmation by Enzyme-Linked ImmunoSorbent Assays (ELISAs). So far, only IGFALS ELISA was performed with identical results.

IGFALS is a protein produced by hepatocytes, but also expressed in other tissues, including thymus and lungs [439]. This protein can be found in plasma or as a free molecule or forming a complex with IGF1-IGFBP (3 or 5 proteins) [440]. IGFALS has an important role on the bioavailability of IGFs [441], preventing the extravasation, proteolysis or renal elimination of IGF-1 [442]. For this reason, IGFALS deficiency results in a dramatic decrease of IGF-1 levels, but also affects the IGF-2 and IGFBP3 proteins [442, 443]. IGF1 is important for asthma pathogenesis through the promotion of inflammation, sub-epithelial fibrosis, hyperresponsiveness, and smooth muscle cell hyperplasia in the lungs [441]. Apart from the previously reported elevation of IGF1 [441] and IGFBP3 [441] in AA, the work presented in Chapter IV shows that IGFALS is also augmented in this kind of patients, especially those displaying a high disease severity. IGFBP3 controls AA inflammation through

mechanisms involving the HIF/VEGF axis, TGF-β1 and TH2 cytokines production, and NF-kB activation [441], but a deleterious role of this protein in AA by promoting sub-epithelial fibrosis has also been reported [444]. Finally, although the role of IGFALS in asthma remains unknown, this protein could be an additional therapeutic target for asthma management together with IGFs and IGFBPs.

Protein AMBP has not been extensively studied in asthma, but a reduction of serum levels has been described in idiopathic pulmonary fibrosis compared to HC [445]. However, a strong up-regulation of this protein was shown in BALF from patients with lung cancer [446]. Although the possible role of protein AMBP in AA is not known, it shares the same chromosomal region (9q32) with ORM1/AGP1, an acute phase protein whose plasma concentration is increased in this disease. Indeed, protein AMBP and ORM1 levels have been found increased in BALF of asthmatic individuals 24 h after segmental allergen challenge [375]. Thus, it is likely that both genes display a coordinated expression and that protein AMBP participate in the acute inflammatory response.

On the other hand, HSPG2/perlecan is an extracellular matrix molecule whose increase in lamina reticularis could be responsible for sub-epithelial fibrosis in the airway [447, 448]. This molecule is constitutively produced by mature fibrocytes [447, 449, 450], which can contribute to the sub-epithelial fibrosis in asthma [447]. Furthermore, the expression of HSPG2 in asthmatic fibrocytes is increased after stimulation with IL-4 or IL-13, but not with IL-17A [451], highlighting the possible role of this molecule in AA. In addition, HSPG2 has been related to AHR [452] and is an upstream regulator of genes (ACAN, COL10A1, and FGFR3) containing asthma associated differentially methylated regions [453]. Changes in HSPG2 deposition could have mechanistic effects, but also modulate the bioability of growth factors such as bFGF [454], a molecule

increased in BALF from atopic asthmatics and after allergen exposure [455]. Taken together, these results could explain the increase of IGFALS, HSPG2, and protein AMBP in AA (Chapter IV), and highlight the potential of these molecules as AA biomarkers.

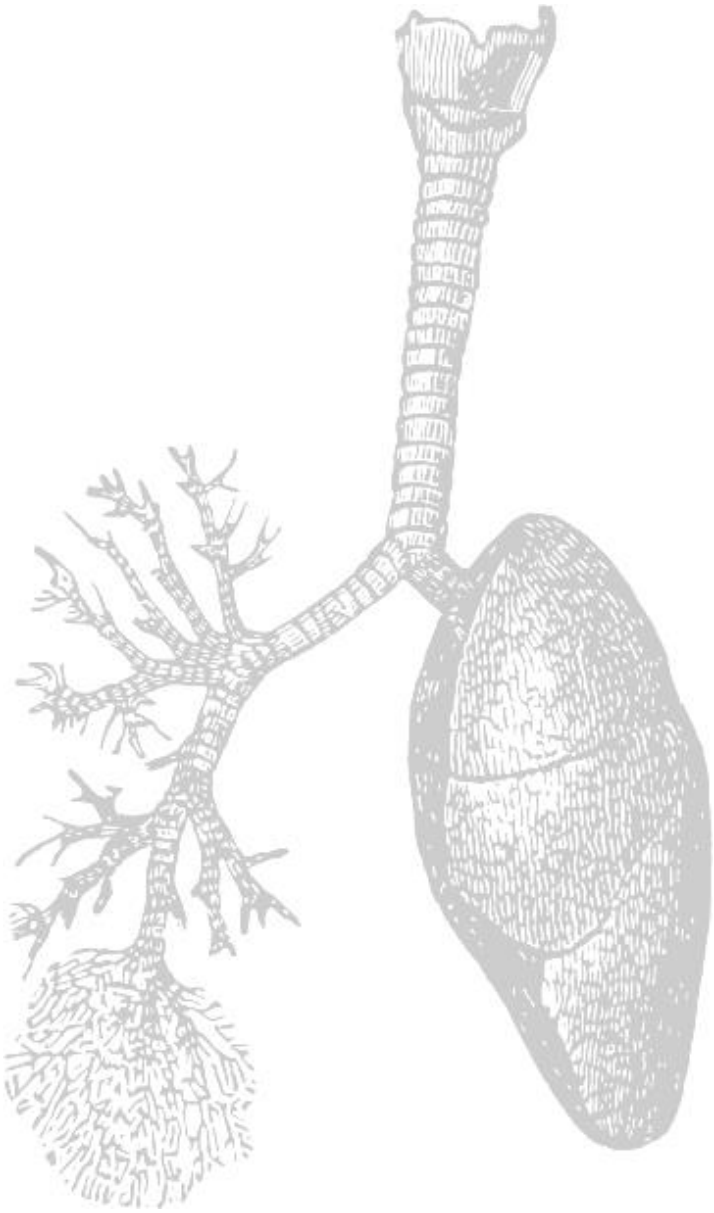
PC2, for its part, comprises a set of proteins related to blood coagulation and complement activation, some of them strongly related to NAA. Indeed, our results in Chapter IV support the upregulation of certain complement factors in NAA. On the one hand, complement factors I (CFI) and H (CFH), which are regulatory proteins that control the excessive activation of the alternative pathway [456, 457]. On the other hand, mannose-associated serine protease 1 (MASP1), which is a lectin pathway molecule. Of these, only CFI was chosen for future validation by ELISA.

CFI prevents the formation of C3-convertase (C4bC2a) through the degradation of C4b, and therefore prevents inflammation due to complement activation [12907438]. In order to carry out this function, CFI needs the participation of CFH as a cofactor [456, 458]. CFH is also known as adrenomedullin binding protein (AMBP-1) [459], a molecule that binds to adrenomedullin in plasma. The last molecule is known to downmodulate TH2 inflammation in an OVA-induced model of asthma [460]. CFH levels were found increased in asthmatic sputum and correlated with asthma severity [461]. Moreover, the increase of CFI and CFH in NAA patients (Chapter IV) could be related to a mechanism to evade the immune response, as it happens with some pathogens [462, 463], which could also explain the higher severity in NAA.

On the other hand, in absence of further validation, MASP1 levels are also increased in NAA. MASP1 participate in the lectin pathway of complement activation, but this protease acts on substrates that belong to the coagulation cascade [464], such as kininogen,

Coagulation factor XIII, or prothrombin, molecules that we also found altered in asthma. More interesting, MASP1 processes the protease activation receptor 4 in endothelial cells to induce the production of IL-6 and IL-8 [464]. The last cytokine is a molecule essential for neutrophils chemotaxis, a leukocyte subset involved in NAA [465] or severe asthma [325, 466]. Besides, complement factor 1 r (C1r) and 1s (C1s), two molecules that belong to the classical pathway of complement activation, were altered in asthmatics. Therefore, our results in Chapter IV point out that all the complement activation pathways (classical, alternative, and lectin) appear to be involved in asthma, especially in the non-allergic phenotype.

To sum up all the results in Chapter IV, a non-targeted proteomic approach for the analysis of medium-low abundance serum proteins has been developed. This protocol included the depletion of lipoproteins (LRA resin), the enrichment of medium/low abundance proteins by means of random peptides libraries (CPLLs), LC-MS/MS analysis, and iTRAQ-based quantification. The use of this approach in serum samples from different asthma phenotypes, rhinitis and healthy subjects allowed us to detect several differentially abundant proteins, which could be useful in the future as non-invasive biomarkers of asthma phenotypes (e.g., IGFALS, Protein AMBP, HSPG2, or CFI) or severities (e.g., IGFALS). However, future studies are needed to get a better understanding of asthma pathophysiology, to evaluate the level of reproducibility and specificity of these new biomarkers, and to translate this knowledge into the clinic in order to get a better therapeutic response and prognosis.



CONCLUSIONS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter I

1. The levels of mCD14 on monocytes and normalised sCD14 in serum are decreased in AA compared to HC, even though both mCD14 and sCD14 show a differential regulation.
2. Part of the reduction of mCD14/sCD14 levels in AA can be explained by the expansion of $CD14^{low}$ cells, probably a "non-classical" $CD14^{-}CD16^{++}$ subset of monocytes with a high ability to infiltrate and differentiate into M2-type macrophages.
3. In addition, sCD14 is also regulated by the (-159 C/T) SNP in the CD14 promoter (rs2569190), since both T allele and TT genotype are associated with higher levels of sCD14 (but not mCD14) and with a lower risk of MSAA in adult caucasian populations exposed to low endotoxin levels.
4. NSE levels are augmented in AA and correlated with IgE, eosinophils and monocytes (two possible cell sources of NSE). Therefore, NSE could be used as a biomarker of AA.

Chapters II and III

1. There is a strong positive correlation between the expression of CD26 (MFI) on TH cells differentiated *in vitro* to several effector subsets (TH17>> TH1> TH2) and the activity of soluble DPP4 (comparable to sCD26 levels) in cell culture supernatants.
2. The expression of CD26 on T lymphocytes discriminates cells with a T_{CM} ($CD26^{high}$; $CD45RA^{-}CCR7^{+}CD28^{+}$), T_N ($CD26^{int}$; $CD45RA^{+}CCR7^{+}CD28^{+}$), or a T_{EM}/T_{EMRA} ($CD26^{-/low}$; $CD45RA^{+/-}CCR7^{-}CD28^{-}$) phenotype.
3. There is an expansion of $CD26^{-/low}$ subpopulations of lymphocytes in asthma. These populations have lost the expression of CD27, CD28, CCR7, and CD127, and therefore

resemble memory/effector cells with an advanced differentiation stage: T_{EM} or T_{EMRA} lymphocytes.

4. The T_{EM}/T_{EMRA} subsets expanded in asthma patients belong to different lymphocyte lineages: $CD4^+$ TH cells in AA and $CD4^-$ γδ-T lymphocytes in NAA.
5. Serum sCD26 levels are decreased in both AA and NAA patients; therefore, they probably reflect the "fingerprint" in serum proteome of the expansion of $CD26^{-/low}CD4^+$ TH in AA and $CD26^{-/low}CD4^-$ γδ-T subsets in NAA.
6. The reduction of circulating sCD26 levels as well as CD26 expression on effector TH lymphocytes from asthmatics might be important to improve their migratory and proliferative capabilities. This finding should be considered in light of the current clinical usage of DPP4 inhibitors and anti-CD26 antibodies.
7. There is a strong correlation between the expression of CD26 and CD126/IL-6Rα on Teff lymphocytes, particularly those lacking the CD4 marker. Therefore, the T_{EM}/T_{EMRA} subsets expanded in asthma (especially amongst the NAA patients) are also $CD126^{-/low}$ cells.
8. The down-modulation of CD126/IL-6Rα in moderate-severe patients compared to intermittent-mild asthmatics is a widespread event, taking place on monocytes, neutrophils, and $CD4^+$ cells. This highlights the potential role of IL-6 *trans*-signalling in asthma severity.
9. Although the number of Treg cells remains unchanged in asthma, their suppressive capacity could be impaired according to their higher CD26 levels compared to healthy controls.

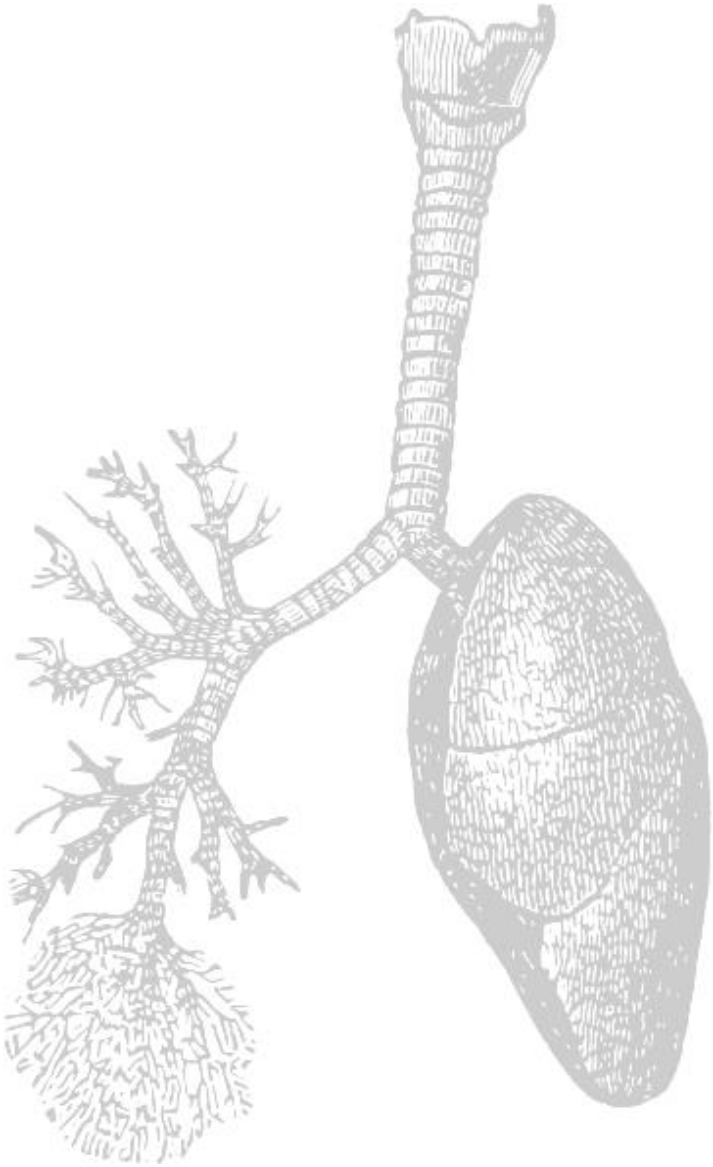
Chapter IV

1. A "shotgun" proteomics methodology has been developed for the quantitative analysis of medium-low abundance serum

proteins. This protocol is based on dynamic range compression with CPLLs, peptides-labelling with iTRAQ reagents, and LC-MS/MS analysis.

2. Twenty-six out of two hundred and seventeen proteins display abundance changes associated with different groups of donors (HC, R, MSAA, IMAA, MSNAA, and IMNAA). Some of these serum proteins could be useful biomarkers of asthma phenotypes (e.g., IGFALS, Protein AMBP, and HSPG2 for AA; CFI for NAA) or severities (e.g., IGFALS in MSAA).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA



REFERENCES

(Introduction and General Discussion)

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. Holgate ST. Innate and adaptive immune responses in asthma. Nat Med. 2012;18(5):673-83.
2. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med. 2012;18(5):716-25.
3. Reddel HK, Levy ML; Global Initiative for Asthma Scientific Committee and Dissemination and Implementation Committee. The GINA asthma strategy report: what's new for primary care? NPJ Prim Care Respir Med. 2015;25:15050.
4. GINA. [Accessed 2019-02-15], 2018 GINA Report, Global Strategy for Asthma Management and Prevention. https://ginasthma.org/wp-content/uploads/2018/04/wms-GINA-2018-report-V1.3-002.pdf.
5. GBD 2015 Chronic Respiratory Disease Collaborators. Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Respir Med. 2017;5(9):691-706.
6. Mallol J, Crane J, von Mutius E, Odhiambo J, Keil U, Stewart A; ISAAC Phase Three Study Group. The International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three: a global synthesis. Allergol Immunopathol (Madr). 2013;41(2):73-85.
7. Almqvist C, Worm M, Leynaert B; working group of GA2LEN WP 2.5 Gender. Impact of gender on asthma in childhood and adolescence: a GA2LEN review. Allergy. 2008;63(1):47-57.
8. Zein JG, Erzurum SC. Asthma is Different in Women. Curr Allergy Asthma Rep. 2015;15(6):28.
9. de Marco R, Locatelli F, Sunyer J, Burney P. Differences in incidence of reported asthma related to age in men and women. A retrospective analysis of the data of the European

Respiratory Health Survey. Am J Respir Crit Care Med. 2000;162(1):68-74.

10. CDC. [Accessed 2019-02-15], National Health Interview Survey (NHIS) data. https://www.cdc.gov/asthma/nhis/2016/data.htm.
11. ERS. [Accessed 2019-02-15], The European lung white book 2013. https://www.erswhitebook.org/.
12. Beasley R, Semprini A, Mitchell EA. Risk factors for asthma: is prevention possible? Lancet. 2015;386(9998):1075-85.
13. Moorman JE, Akinbami LJ, Bailey CM, Zahran HS, King ME, Johnson CA, et al. National surveillance of asthma: United States, 2001-2010. Vital Health Stat 3. 2012;(35):1-58.
14. DeWan AT, Triche EW, Xu X, Hsu LI, Zhao C, Belanger K, et al. PDE11A associations with asthma: results of a genome-wide association scan. J Allergy Clin Immunol. 2010;126(4):871-873.e9.
15. Mathias RA, Grant AV, Rafaels N, Hand T, Gao L, Vergara C, et al. A genome-wide association study on African-ancestry populations for asthma. J Allergy Clin Immunol. 2010;125(2):336-346.e4.
16. Binia A, Van Stiphout N, Liang L, Michel S, Bhavsar PK, Fan Chung K, et al. A polymorphism affecting MYB binding within the promoter of the PDCD4 gene is associated with severe asthma in children. Hum Mutat. 2013;34(8):1131-9.
17. Martinez FD, Vercelli D. Asthma. Lancet. 2013;382(9901):1360-72.
18. Portelli M, Sayers I. Genetic basis for personalized medicine in asthma. Expert Rev Respir Med. 2012;6(2):223-36.
19. He Y, Peng S, Xiong W, Xu Y, Liu J. Association between polymorphism of interleukin-1 beta and interleukin-1

receptor antagonist gene and asthma risk: a meta-analysis. ScientificWorldJournal. 2015;2015:685684.

20. Sobkowiak P, Wojsyk-Banaszak I, Kowalewska M, Wasilewska E, Langwiński W, Kycler Z, et al. Interleukin 1β polymorphism and serum level are associated with pediatric asthma. Pediatr Pulmonol. 2017;52(12):1565-71.
21. Zhu M, Wang T, Chen R, Wang C, Liu S, Ji Y. Association between interleukin-17a gene polymorphisms and asthma risk: a meta-analysis. Asian Pac J Allergy Immunol. 2016;34(2):115-23.
22. Liu M, Zhu W, Wang J, Zhang J, Guo X, Wang J, et al. Interleukin-23 receptor genetic polymorphisms and ulcerative colitis susceptibility: A meta-analysis. Clin Res Hepatol Gastroenterol. 2015;39(4):516-25.
23. Beghé B, Hall IP, Parker SG, Moffatt MF, Wardlaw A, Connolly MJ, et al. Polymorphisms in IL13 pathway genes in asthma and chronic obstructive pulmonary disease. Allergy. 2010;65(4):474-81.
24. Zhang S, Li Y, Liu Y. Interleukin-4 -589C/T Polymorphism is Associated with Increased Pediatric Asthma Risk: A Meta-Analysis. Inflammation. 2015;38(3):1207-12.
25. Nabih ES, Kamel HF, Kamel TB. Association Between CD14 Polymorphism (-1145G/A) and Childhood Bronchial Asthma. Biochem Genet. 2016;54(1):50-60.
26. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrländer C, et al. Exposure to environmental microorganisms and childhood asthma. N Engl J Med. 2011;364(8):701-9.
27. Illi S, Depner M, Genuneit J, Horak E, Loss G, Strunz-Lehner C, et al. Protection from childhood asthma and allergy in Alpine farm environments-the GABRIEL Advanced Studies. J Allergy Clin Immunol. 2012;129(6):1470-7.e6.

28. Lodge CJ, Tan DJ, Lau MX, Dai X, Tham R, Lowe AJ, et al. Breastfeeding and asthma and allergies: a systematic review and meta-analysis. Acta Paediatr. 2015;104(467):38-53.
29. Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. N Engl J Med. 2000;343(8):538-43.
30. Chen CM, Gehring U, Wickman M, Hoek G, Giovannangelo M, Nordling E, et al. Domestic cat allergen and allergic sensitisation in young children. Int J Hyg Environ Health. 2008;211(3-4):337-44.
31. Douwes J, Cheng S, Travier N, Cohet C, Niesink A, McKenzie J, et al. Farm exposure in utero may protect against asthma, hay fever and eczema. Eur Respir J. 2008;32(3):603-11.
32. Loss G, Bitter S, Wohlgensinger J, Frei R, Roduit C, Genuneit J, et al. Prenatal and early-life exposures alter expression of innate immunity genes: the PASTURE cohort study. J Allergy Clin Immunol. 2012;130(2):523-30.e9.
33. Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, et al. Disordered microbial communities in asthmatic airways. PLoS One. 2010;5(1):e8578.
34. von Mutius E. The microbial environment and its influence on asthma prevention in early life. J Allergy Clin Immunol. 2016;137(3):680-9.
35. Michel O. Role of lipopolysaccharide (LPS) in asthma and other pulmonary conditions. J Endotoxin Res. 2003;9(5):293-300.
36. Tesse R, Pandey RC, Kabesch M. Genetic variations in toll-like receptor pathway genes influence asthma and atopy. Allergy. 2011;66(3):307-16.

37. Vignali DA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. Nat Immunol. 2012;13(8):722-8.
38. Shive CL, Jiang W, Anthony DD, Lederman MM. Soluble CD14 is a nonspecific marker of monocyte activation. AIDS. 2015;29(10):1263-5.
39. van den Biggelaar AH, van Ree R, Rodrigues LC, Lell B, Deelder AM, Kremsner PG, et al. Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10. Lancet. 2000;356(9243):1723-7.
40. Arnold IC, Dehzad N, Reuter S, Martin H, Becher B, Taube C, et al. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. J Clin Invest. 2011;121(8):3088-93.
41. Lau MY, Dharmage SC, Burgess JA, Lowe AJ, Lodge CJ, Campbell B, et al. CD14 polymorphisms, microbial exposure and allergic diseases: a systematic review of gene-environment interactions. Allergy. 2014;69(11):1440-53.
42. Davies DE. Epithelial barrier function and immunity in asthma. Ann Am Thorac Soc. 2014;11 Suppl 5:S244-51.
43. Mitchell PD, O'Byrne PM. Epithelial-Derived Cytokines in Asthma. Chest. 2017;151(6):1338-1344.
44. Bønnelykke K, Sleiman P, Nielsen K, Kreiner-Møller E, Mercader JM, Belgrave D, et al. A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. Nat Genet. 2014;46(1):51-5.
45. Meyers DA, Bleecker ER, Holloway JW, Holgate ST. Asthma genetics and personalised medicine. Lancet Respir Med. 2014;2(5):405-15.

46. Martin NT, Martin MU. Interleukin 33 is a guardian of barriers and a local alarmin. Nat Immunol. 2016;17(2):122-31.
47. Barlow JL, Peel S, Fox J, Panova V, Hardman CS, Camelo A, et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. J Allergy Clin Immunol. 2013;132(4):933-41.
48. Hardman CS, Panova V, McKenzie AN. IL-33 citrine reporter mice reveal the temporal and spatial expression of IL-33 during allergic lung inflammation. Eur J Immunol. 2013;43(2):488-98.
49. Préfontaine D, Lajoie-Kadoch S, Foley S, Audusseau S, Olivenstein R, Halayko AJ, et al. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. J Immunol. 2009;183(8):5094-103.
50. Christianson CA, Goplen NP, Zafar I, Irvin C, Good JT Jr, Rollins DR, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. J Allergy Clin Immunol. 2015;136(1):59-68.e14.
51. Makrinioti H, Toussaint M, Jackson DJ, Walton RP, Johnston SL. Role of interleukin 33 in respiratory allergy and asthma. Lancet Respir Med. 2014;2(3):226-37.
52. Cayrol C, Girard JP. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. Curr Opin Immunol. 2014;31:31-7.
53. Van Dyken SJ, Mohapatra A, Nussbaum JC, Molofsky AB, Thornton EE, Ziegler SF, et al. Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and γδ T cells. Immunity. 2014;40(3):414-24.
54. Allakhverdi Z, Smith DE, Comeau MR, Delespesse G. Cutting edge: The ST2 ligand IL-33 potently activates and

drives maturation of human mast cells. J Immunol. 2007;179(4):2051-4.

55. Lloyd CM, Saglani S. Epithelial cytokines and pulmonary allergic inflammation. Curr Opin Immunol. 2015;34:52-8.
56. Tang W, Smith SG, Beaudin S, Dua B, Howie K, Gauvreau G, et al. IL-25 and IL-25 receptor expression on eosinophils from subjects with allergic asthma. Int Arch Allergy Immunol. 2014;163(1):5-10.
57. Yao XJ, Liu XF, Wang XD. Potential Role of Interleukin-25/Interleukin-33/Thymic Stromal Lymphopoietin-Fibrocyte Axis in the Pathogenesis of Allergic Airway Diseases. Chin Med J (Engl). 2018;131(16):1983-9.
58. Lei L, Zhang Y, Yao W, Kaplan MH, Zhou B. Thymic stromal lymphopoietin interferes with airway tolerance by suppressing the generation of antigen-specific regulatory T cells. J Immunol. 2011;186(4):2254-61.
59. Yao W, Zhang Y, Jabeen R, Nguyen ET, Wilkes DS, Tepper RS, et al. Interleukin-9 is required for allergic airway inflammation mediated by the cytokine TSLP. Immunity. 2013;38(2):360-72.
60. Salter BM, Oliveria JP, Nusca G, Smith SG, Watson RM, Comeau M, et al. Thymic stromal lymphopoietin activation of basophils in patients with allergic asthma is IL-3 dependent. J Allergy Clin Immunol. 2015;136(6):1636-1644.
61. Mahmutovic-Persson I, Akbarshahi H, Bartlett NW, Glanville N, Johnston SL, Brandelius A, et al. Inhaled dsRNA and rhinovirus evoke neutrophilic exacerbation and lung expression of thymic stromal lymphopoietin in allergic mice with established experimental asthma. Allergy. 2014;69(3):348-58.
62. Klein Wolterink RG, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, et al. Pulmonary innate lymphoid

cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. Eur J Immunol. 2012;42(5):1106-16.

63. Kabata H, Moro K, Koyasu S, Asano K. Group 2 innate lymphoid cells and asthma. Allergol Int. 2015;64(3):227-34.
64. Fajt ML, Gelhaus SL, Freeman B, Uvalle CE, Trudeau JB, Holguin F, et al. Prostaglandin D_2 pathway upregulation: relation to asthma severity, control, and TH2 inflammation. J Allergy Clin Immunol. 2013;131(6):1504-12.
65. Chang JE, Doherty TA, Baum R, Broide D. Prostaglandin D2 regulates human type 2 innate lymphoid cell chemotaxis. J Allergy Clin Immunol. 2014;133(3):899-901.e3.
66. Xue L, Salimi M, Panse I, Mjösberg JM, McKenzie AN, Spits H, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells. J Allergy Clin Immunol. 2014;133(4):1184-94.
67. Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. Sci Transl Med. 2013;5(174):174ra26.
68. Jonckheere AC, Bullens DMA, Seys SF. Innate lymphoid cells in asthma: pathophysiological insights from murine models to human asthma phenotypes. Curr Opin Allergy Clin Immunol. 2019;19(1):53-60.
69. Kim HY, Lee HJ, Chang YJ, Pichavant M, Shore SA, Fitzgerald KA, et al. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. Nat Med. 2014;20(1):54-61.
70. Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front Immunol. 2014;5:514.

71. Tashiro H, Takahashi K, Hayashi S, Kato G, Kurata K, Kimura S, et al. Interleukin-33 from Monocytes Recruited to the Lung Contributes to House Dust Mite-Induced Airway Inflammation in a Mouse Model. PLoS One. 2016;11(6):e0157571.
72. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Blood. 1989;74(7):2527-34.
73. Ziegler-Heitbrock HW, Fingerle G, Ströbel M, Schraut W, Stelter F, Schütt C, et al. The novel subset of CD14+/CD16+ blood monocytes exhibits features of tissue macrophages. Eur J Immunol. 1993;23(9):2053-8.
74. Yona S, Jung S. Monocytes: subsets, origins, fates and functions. Curr Opin Hematol. 2010;17(1):53-9.
75. Wong KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood. 2011;118(5):e16-31.
76. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity. 2010;33(3):375-86.
77. Mukherjee R, Kanti Barman P, Kumar Thatoi P, Tripathy R, Kumar Das B, Ravindran B. Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous. Sci Rep. 2015;5:13886.
78. Anbazhagan K, Duroux-Richard I, Jorgensen C, Apparailly F. Transcriptomic network support distinct roles of classical and non-classical monocytes in human. Int Rev Immunol. 2014;33(6):470-89.
79. Robbe P, Draijer C, Borg TR, Luinge M, Timens W, Wouters IM, et al. Distinct macrophage phenotypes in allergic and

nonallergic lung inflammation. Am J Physiol Lung Cell Mol Physiol. 2015;308(4):L358-67.

80. Jiang Z, Zhu L. Update on the role of alternatively activated macrophages in asthma. J Asthma Allergy. 2016;9:101-7.
81. Byers DE, Holtzman MJ. Alternatively activated macrophages and airway disease. Chest. 2011;140(3):768-774.
82. Nabe T, Wakamori H, Yano C, Nishiguchi A, Yuasa R, Kido H, et al. Production of interleukin (IL)-33 in the lungs during multiple antigen challenge-induced airway inflammation in mice, and its modulation by a glucocorticoid. Eur J Pharmacol. 2015;757:34-41.
83. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, Fantino E. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. Am J Respir Cell Mol Biol. 2015;53(5):676-88.
84. Takeda K, Akira S. TLR signaling pathways. Semin Immunol. 2004;16(1):3-9.
85. Cochet F, Peri F. The Role of Carbohydrates in the Lipopolysaccharide (LPS)/Toll-Like Receptor 4 (TLR4) Signalling. Int J Mol Sci. 2017;18(11). pii: E2318.
86. Bouzigon E, Forabosco P, Koppelman GH, Cookson WO, Dizier MH, Duffy DL, et al. Meta-analysis of 20 genome-wide linkage studies evidenced new regions linked to asthma and atopy. Eur J Hum Genet. 2010;18(6):700-6.
87. Wang D, Yang Y, Xu J, Zhou ZK, Yu HY. Association of CD14 -159 (-260C/T) polymorphism and asthma risk: an updated genetic meta-analysis study. Medicine (Baltimore). 2016;95(39):e4959.
88. Teghanemt A, Prohinar P, Gioannini TL, Weiss JP. Transfer of monomeric endotoxin from MD-2 to CD14:

characterization and functional consequences. J Biol Chem. 2007;282(50):36250-6.

89. Deguine J, Barton GM. MyD88: a central player in innate immune signaling. F1000Prime Rep. 2014;6:97.
90. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev. 2009;22(2):240-73.
91. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11(5):373-84.
92. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637-50.
93. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol. 2001;2(10):947-50.
94. Anas A, van der Poll T, de Vos AF. Role of CD14 in lung inflammation and infection. Crit Care. 2010;14(2):209.
95. Marcos V, Latzin P, Hector A, Sonanini S, Hoffmann F, Lacher M, et al. Expression, regulation and clinical significance of soluble and membrane CD14 receptors in pediatric inflammatory lung diseases. Respir Res. 2010;11:32.
96. Lévêque M, Simonin-Le Jeune K, Jouneau S, Moulis S, Desrues B, Belleguic C, et al. Soluble CD14 acts as a DAMP in human macrophages: origin and involvement in inflammatory cytokine/chemokine production. FASEB J. 2017;31(5):1891-1902.
97. Pugin J, Schürer-Maly CC, Leturcq D, Moriarty A, Ulevitch RJ, Tobias PS. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by

lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci U S A. 1993;90(7):2744-8.

98. Kitchens RL, Thompson PA, Viriyakosol S, O'Keefe GE, Munford RS. Plasma CD14 decreases monocyte responses to LPS by transferring cell-bound LPS to plasma lipoproteins. J Clin Invest. 2001;108(3):485-93.
99. Zou Q, Wen W, Zhang XC. Presepsin as a novel sepsis biomarker. World J Emerg Med. 2014;5(1):16-9.
100. Garty BZ, Monselise Y, Nitzan M. Soluble CD14 in children with status asthmaticus. Isr Med Assoc J. 2000;2(2):104-7.
101. Klaassen EM, Thönissen BE, van Eys G, Dompeling E, Jöbsis Q. A systematic review of CD14 and toll-like receptors in relation to asthma in Caucasian children. Allergy Asthma Clin Immunol. 2013;9(1):10.
102. Alexis N, Eldridge M, Reed W, Bromberg P, Peden DB. CD14-dependent airway neutrophil response to inhaled LPS: role of atopy. J Allergy Clin Immunol. 2001;107(1):31-5.
103. Virchow JC Jr, Julius P, Matthys H, Kroegel C, Luttmann W. CD14 expression and soluble CD14 after segmental allergen provocation in atopic asthma. Eur Respir J. 1998;11(2):317-23.
104. Landmann R, Fisscher AE, Obrecht JP. Interferon-gamma and interleukin-4 down-regulate soluble CD14 release in human monocytes and macrophages. J Leukoc Biol. 1992;52(3):323-30.
105. Cosentino G, Soprana E, Thienes CP, Siccardi AG, Viale G, Vercelli D. IL-13 down-regulates CD14 expression and TNF-alpha secretion in normal human monocytes. J Immunol. 1995;155(6):3145-51.
106. Baldini M, Lohman IC, Halonen M, Erickson RP, Holt PG, Martinez FD. A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14

levels and with total serum immunoglobulin E. Am J Respir Cell Mol Biol. 1999;20(5):976-83.

107. Martin AC, Laing IA, Khoo SK, Zhang G, Rueter K, Teoh L, et al. Acute asthma in children: Relationships among CD14 and CC16 genotypes, plasma levels, and severity. Am J Respir Crit Care Med. 2006;173(6):617-22.
108. Wang Z, Sundy JS, Foss CM, Barnhart HX, Palmer SM, Allgood SD, et al. Racial differences in the association of CD14 polymorphisms with serum total IgE levels and allergen skin test reactivity. J Asthma Allergy. 2013;6:81-92.
109. Kabesch M, Hasemann K, Schickinger V, Tzotcheva I, Bohnert A, Carr D, et al. A promoter polymorphism in the CD14 gene is associated with elevated levels of soluble CD14 but not with IgE or atopic diseases. Allergy. 2004;59(5):520-5.
110. Levan TD, Michel O, Dentener M, Thorn J, Vertongen F, Beijer L, et al. Association between CD14 polymorphisms and serum soluble CD14 levels: effect of atopy and endotoxin inhalation. J Allergy Clin Immunol. 2008;121(2):434-440.e1.
111. Vercelli D, Baldini M, Stern D, Lohman IC, Halonen M, Martinez F. CD14: a bridge between innate immunity and adaptive IgE responses. J Endotoxin Res. 2001;7(1):45-8.
112. Zhao L, Bracken MB. Association of CD14 -260 (-159) C>T and asthma: a systematic review and meta-analysis. BMC Med Genet. 2011;12:93.
113. Simpson A, Martinez FD. The role of lipopolysaccharide in the development of atopy in humans. Clin Exp Allergy. 2010;40(2):209-23.
114. Zhang YN, Li YJ, Li H, Zhou H, Shao XJ. Association of CD14 C159T polymorphism with atopic asthma susceptibility in children from Southeastern China: a case-control study. Genet Mol Res. 2015;14(2):4311-7.

115. Zhang R, Deng R, Li H, Chen H. No Association Between -159C/T Polymorphism of the CD14 Gene and Asthma Risk: a Meta-Analysis of 36 Case-Control Studies. Inflammation. 2016;39(1):457-66.
116. Zhang G, Goldblatt J, LeSouëf PN. Does the relationship between IgE and the CD14 gene depend on ethnicity? Allergy. 2008;63(11):1411-7.
117. Munthe-Kaas MC, Torjussen TM, Gervin K, Lødrup Carlsen KC, Carlsen KH, Granum B, et al. CD14 polymorphisms and serum CD14 levels through childhood: a role for gene methylation? J Allergy Clin Immunol. 2010;125(6):1361-8.
118. Hussein YM, Shalaby SM, Zidan HE, Sabbah NA, Karam NA, Alzahrani SS. CD14 tobacco gene-environment interaction in atopic children. Cell Immunol. 2013;285(1-2):31-7.
119. Bank I, Marcu-Malina V. Quantitative peripheral blood perturbations of γδ T cells in human disease and their clinical implications. Clin Rev Allergy Immunol. 2014;47(3):311-33.
120. Holtmeier W, Kabelitz D. gammadelta T cells link innate and adaptive immune responses. Chem Immunol Allergy. 2005;86:151-83.
121. Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol. 2000;18:975-1026.
122. Pang DJ, Neves JF, Sumaria N, Pennington DJ. Understanding the complexity of γδ T-cell subsets in mouse and human. Immunology. 2012;136(3):283-90.
123. Morita CT, Mariuzza RA, Brenner MB. Antigen recognition by human gamma delta T cells: pattern recognition by the adaptive immune system. Springer Semin Immunopathol. 2000;22(3):191-217.

124. Deusch K, Lüling F, Reich K, Classen M, Wagner H, Pfeffer K. A major fraction of human intraepithelial lymphocytes simultaneously expresses the gamma/delta T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment. Eur J Immunol. 1991;21(4):1053-9.
125. Khairallah C, Chu TH, Sheridan BS. Tissue Adaptations of Memory and Tissue-Resident Gamma Delta T Cells. Front Immunol. 2018;9:2636.
126. Wu Y, Wu W, Wong WM, Ward E, Thrasher AJ, Goldblatt D, et al. Human gamma delta T cells: a lymphoid lineage cell capable of professional phagocytosis. J Immunol. 2009;183(9):5622-9.
127. Moser B, Eberl M. γδ T-APCs: a novel tool for immunotherapy? Cell Mol Life Sci. 2011;68(14):2443-52.
128. Porcelli SA, Morita CT, Modlin RL. T-cell recognition of non-peptide antigens. Curr Opin Immunol. 1996;8(4):510-6.
129. Kaufmann SH, Schaible UE. Antigen presentation and recognition in bacterial infections. Curr Opin Immunol. 2005;17(1):79-87.
130. Wesch D, Glatzel A, Kabelitz D. Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype. Cell Immunol. 2001;212(2):110-7.
131. Lockhart E, Green AM, Flynn JL. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol. 2006;177(7):4662-9.
132. Scotet E, Nedellec S, Devilder MC, Allain S, Bonneville M. Bridging innate and adaptive immunity through gammadelta T-dendritic cell crosstalk. Front Biosci. 2008;13:6872-85.
133. Zhao Y, Yang J, Gao YD. Altered expressions of helper T cell (Th)1, Th2, and Th17 cytokines in CD8(+) and γδ T cells

in patients with allergic asthma. J Asthma. 2011;48(5):429-36.

134. Chien YH, Zeng X, Prinz I. The natural and the inducible: interleukin (IL)-17-producing γδ T cells. Trends Immunol. 2013;34(4):151-4.
135. Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Di Sano C, et al. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med. 2003;198(3):391-7.
136. Kabelitz D, He W. The multifunctionality of human Vγ9Vδ2 γδ T cells: clonal plasticity or distinct subsets? Scand J Immunol. 2012;76(3):213-22.
137. Battistini L, Caccamo N, Borsellino G, Meraviglia S, Angelini DF, Dieli F, et al. Homing and memory patterns of human gammadelta T cells in physiopathological situations. Microbes Infect. 2005;7(3):510-7.
138. Tamura-Yamashita K, Endo J, Isogai S, Matsuoka K, Yonekawa H, Yoshizawa Y. Gamma-delta T cell is essential for allergen-induced late asthmatic response in a murine model of asthma. J Med Dent Sci. 2008;55(1):113-20.
139. Schauer U, Dippel E, Gieler U, Bräuer J, Jung T, Heymanns J, et al. T cell receptor gamma delta bearing cells are decreased in the peripheral blood of patients with atopic diseases. Clin Exp Immunol. 1991;86(3):440-3.
140. Chen KS, Miller KH, Hengehold D. Diminution of T cells with gamma delta receptor in the peripheral blood of allergic asthmatic individuals. Clin Exp Allergy. 1996;26(3):295-302.
141. Krejsek J, Král B, Vokurková D, Derner V, Tousková M, Paráková Z, et al. Decreased peripheral blood gamma delta T cells in patients with bronchial asthma. Allergy. 1998;53(1):73-7.

142. Spinozzi F, Agea E, Bistoni O, Forenza N, Monaco A, Bassotti G, et al. Increased allergen-specific, steroid-sensitive gamma delta T cells in bronchoalveolar lavage fluid from patients with asthma. Ann Intern Med. 1996;124(2):223-7.
143. Hamzaoui A, Kahan A, Ayed K, Hamzaoui K. T cells expressing the gammadelta receptor are essential for Th2-mediated inflammation in patients with acute exacerbation of asthma. Mediators Inflamm. 2002;11(2):113-9.
144. Walker C, Kaegi MK, Braun P, Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. J Allergy Clin Immunol. 1991;88(6):935-42.
145. Fajac I, Roisman GL, Lacronique J, Polla BS, Dusser DJ. Bronchial gamma delta T-lymphocytes and expression of heat shock proteins in mild asthma. Eur Respir J. 1997;10(3):633-8.
146. Annunziato F, Romagnani C, Romagnani S. The 3 major types of innate and adaptive cell-mediated effector immunity. J Allergy Clin Immunol. 2015;135(3):626-35.
147. Dullaers M, De Bruyne R, Ramadani F, Gould HJ, Gevaert P, Lambrecht BN. The who, where, and when of IgE in allergic airway disease. J Allergy Clin Immunol. 2012;129(3):635-45.
148. Wu LC, Zarrin AA. The production and regulation of IgE by the immune system. Nat Rev Immunol. 2014;14(4):247-59.
149. Tsitsiou E, Williams AE, Moschos SA, Patel K, Rossios C, Jiang X, et al. Transcriptome analysis shows activation of circulating CD8+ T cells in patients with severe asthma. J Allergy Clin Immunol. 2012;129(1):95-103.
150. Ying S, Humbert M, Barkans J, Corrigan CJ, Pfister R, Menz G, et al. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and

nonatopic (intrinsic) asthmatics. J Immunol. 1997;158(7):3539-44.

151. Cho SH, Stanciu LA, Holgate ST, Johnston SL. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. Am J Respir Crit Care Med. 2005;171(3):224-30.
152. Lee N, You S, Shin MS, Lee WW, Kang KS, Kim SH, et al. IL-6 receptor α defines effector memory CD8+ T cells producing Th2 cytokines and expanding in asthma. Am J Respir Crit Care Med. 2014;190(12):1383-94.
153. Zhu J. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. Cytokine. 2015;75(1):14-24.
154. Junttila IS. Tuning the Cytokine Responses: An Update on Interleukin (IL)-4 and IL-13 Receptor Complexes. Front Immunol. 2018;9:888.
155. Gandhi NA, Pirozzi G, Graham NMH. Commonality of the IL-4/IL-13 pathway in atopic diseases. Expert Rev Clin Immunol. 2017;13(5):425-437.
156. Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J, Foster PS. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. J Immunol. 2000;165(1):108-13.
157. Park KS, Korfhagen TR, Bruno MD, Kitzmiller JA, Wan H, Wert SE, et al. SPDEF regulates goblet cell hyperplasia in the airway epithelium. J Clin Invest. 2007;117(4):978-88.
158. Chen G, Korfhagen TR, Xu Y, Kitzmiller J, Wert SE, Maeda Y, et al. SPDEF is required for mouse pulmonary goblet cell differentiation and regulates a network of genes associated with mucus production. J Clin Invest. 2009;119(10):2914-24.

159. Laoukili J, Perret E, Willems T, Minty A, Parthoens E, Houcine O, et al IL-13 alters mucociliary differentiation and ciliary beating of human respiratory epithelial cells. J Clin Invest. 2001;108(12):1817-24.
160. Xiang YY, Wang S, Liu M, Hirota JA, Li J, Ju W, et al. A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. Nat Med. 2007;13(7):862-7.
161. Izuhara K, Ohta S, Shiraishi H, Suzuki S, Taniguchi K, Toda S, et al. The mechanism of mucus production in bronchial asthma. Curr Med Chem. 2009;16(22):2867-75.
162. Lin J, Jiang Y, Li L, Liu Y, Tang H, Jiang D. TMEM16A mediates the hypersecretion of mucus induced by Interleukin-13. Exp Cell Res. 2015;334(2):260-9.
163. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. Nat Med. 2002;8(8):885-9.
164. Perkins C, Yanase N, Smulian G, Gildea L, Orekov T, Potter C, et al. Selective stimulation of IL-4 receptor on smooth muscle induces airway hyperresponsiveness in mice. J Exp Med. 2011;208(4):853-67.
165. Yang M, Rangasamy D, Matthaei KI, Frew AJ, Zimmmermann N, Mahalingam S, et al. Inhibition of arginase I activity by RNA interference attenuates IL-13-induced airways hyperresponsiveness. J Immunol. 2006;177(8):5595-603.
166. Evans CM, Raclawska DS, Ttofali F, Liptzin DR, Fletcher AA, Harper DN, et al. The polymeric mucin Muc5ac is required for allergic airway hyperreactivity. Nat Commun. 2015;6:6281.

167. Gour N, Wills-Karp M. IL-4 and IL-13 signaling in allergic airway disease. Cytokine. 2015;75(1):68-78.
168. Yanagibashi T, Satoh M, Nagai Y, Koike M, Takatsu K. Allergic diseases: From bench to clinic - Contribution of the discovery of interleukin-5. Cytokine. 2017;98:59-70.
169. Dent LA, Strath M, Mellor AL, Sanderson CJ. Eosinophilia in transgenic mice expressing interleukin 5. J Exp Med. 1990;172(5):1425-31.
170. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med. 1996;183(1):195-201.
171. Rothenberg ME, Hogan SP. The eosinophil. Annu Rev Immunol. 2006;24:147-74.
172. Kouro T, Takatsu K. IL-5- and eosinophil-mediated inflammation: from discovery to therapy. Int Immunol. 2009;21(12):1303-9.
173. Muehling LM, Lawrence MG, Woodfolk JA. Pathogenic CD4+ T cells in patients with asthma. J Allergy Clin Immunol. 2017;140(6):1523-1540.
174. Raundhal M, Morse C, Khare A, Oriss TB, Milosevic J, Trudeau J, Huff R, et al. High IFN-γ and low SLPI mark severe asthma in mice and humans. J Clin Invest. 2015;125(8):3037-50.
175. Guglani L, Khader SA. Th17 cytokines in mucosal immunity and inflammation. Curr Opin HIV AIDS. 2010;5(2):120-7.
176. Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, et al. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell. 1989;58(3):573-81.
177. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by

promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol. 2007;8(9):967-74.

178. Maddur MS, Miossec P, Kaveri SV, Bayry J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. Am J Pathol. 2012;181(1):8-18.
179. Lust JA, Donovan KA, Kline MP, Greipp PR, Kyle RA, Maihle NJ. Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. Cytokine. 1992;4(2):96-100.
180. Rose-John S, Scheller J, Elson G, Jones SA. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol. 2006;80(2):227-36.
181. Scheller J, Ohnesorge N, Rose-John S. Interleukin-6 trans-signalling in chronic inflammation and cancer. Scand J Immunol. 2006;63(5):321-9.
182. Chalaris A, Garbers C, Rabe B, Rose-John S, Scheller J. The soluble Interleukin 6 receptor: generation and role in inflammation and cancer. Eur J Cell Biol. 2011;90(6-7):484-94.
183. Neurath MF, Finotto S. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. Cytokine Growth Factor Rev. 2011;22(2):83-9.
184. Farahi N, Paige E, Balla J, Prudence E, Ferreira RC, Southwood M, et al. Neutrophil-mediated IL-6 receptor trans-signaling and the risk of chronic obstructive pulmonary disease and asthma. Hum Mol Genet. 2017;26(8):1584-1596.
185. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance

expression of antimicrobial peptides. J Exp Med. 2006;203(10):2271-9.

186. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol. 2007;25:821-52.
187. Jin Y, Deng Z, Cao C, Li L. IL-17 polymorphisms and asthma risk: a meta-analysis of 11 single nucleotide polymorphisms. J Asthma. 2015;52(10):981-8.
188. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Pagé N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol. 2001;108(3):430-8.
189. Rahman MS, Yang J, Shan LY, Unruh H, Yang X, Halayko AJ, et al. IL-17R activation of human airway smooth muscle cells induces CXCL-8 production via a transcriptional-dependent mechanism. Clin Immunol. 2005;115(3):268-76.
190. Ota K, Kawaguchi M, Matsukura S, Kurokawa M, Kokubu F, Fujita J, et al. Potential involvement of IL-17F in asthma. J Immunol Res. 2014;2014:602846.
191. Laan M, Cui ZH, Hoshino H, Lötvall J, Sjöstrand M, Gruenert DC, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. J Immunol. 1999;162(4):2347-52.
192. Lindén A. Role of interleukin-17 and the neutrophil in asthma. Int Arch Allergy Immunol. 2001;126(3):179-84.
193. Chang Y, Al-Alwan L, Risse PA, Halayko AJ, Martin JG, Baglole CJ, et al. Th17-associated cytokines promote human airway smooth muscle cell proliferation. FASEB J. 2012;26(12):5152-60.
194. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. Respir Med. 2010;104(8):1131-7.

195. Chenuet P, Fauconnier L, Madouri F, Marchiol T, Rouxel N, Ledru A, et al. Neutralization of either IL-17A or IL-17F is sufficient to inhibit house dust mite induced allergic asthma in mice. Clin Sci (Lond). 2017;131(20):2533-2548.
196. Chen JH, Qin L, Shi YY, Feng JT, Zheng YL, Wan YF, et al. IL-17 protein levels in both induced sputum and plasma are increased in stable but not acute asthma individuals with obesity. Respir Med. 2016;121:48-58.
197. Zhao Y, Yang J, Gao YD, Guo W. Th17 immunity in patients with allergic asthma. Int Arch Allergy Immunol. 2010;151(4):297-307.
198. Al-Ramli W, Préfontaine D, Chouiali F, Martin JG, Olivenstein R, Lemière C, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. J Allergy Clin Immunol. 2009;123(5):1185-7.
199. McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. J Immunol. 2008;181(6):4089-97.
200. Krishna S, Low IC, Pervaiz S. Regulation of mitochondrial metabolism: yet another facet in the biology of the oncoprotein Bcl-2. Biochem J. 2011;435(3):545-51.
201. Jing D, Bhadri VA, Beck D, Thoms JA, Yakob NA, Wong JW, et al. Opposing regulation of BIM and BCL2 controls glucocorticoid-induced apoptosis of pediatric acute lymphoblastic leukemia cells. Blood. 2015;125(2):273-83.
202. Banuelos J, Shin S, Cao Y, Bochner BS, Morales-Nebreda L, Budinger GR, et al. BCL-2 protects human and mouse Th17 cells from glucocorticoid-induced apoptosis. Allergy. 2016;71(5):640-50.

203. Koch S, Sopel N, Finotto S. Th9 and other IL-9-producing cells in allergic asthma. Semin Immunopathol. 2017;39(1):55-68.
204. Wiener Z, Falus A, Toth S. IL-9 increases the expression of several cytokines in activated mast cells, while the IL-9-induced IL-9 production is inhibited in mast cells of histamine-free transgenic mice. Cytokine. 2004;26(3):122-30.
205. Saeki M, Kaminuma O, Nishimura T, Kitamura N, Mori A, Hiroi T. Th9 cells induce steroid-resistant bronchial hyperresponsiveness in mice. Allergol Int. 2017;66S:S35-S40.
206. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. Nat Immunol. 2009;10(8):864-71.
207. Tamasauskiene L, Sitkauskiene B. Role of Th22 and IL-22 in pathogenesis of allergic airway diseases: Pro-inflammatory or anti-inflammatory effect? Pediatr Neonatol. 2018;59(4):339-344.
208. Farfariello V, Amantini C, Nabissi M, Morelli MB, Aperio C, Caprodossi S, et al. IL-22 mRNA in peripheral blood mononuclear cells from allergic rhinitic and asthmatic pediatric patients. Pediatr Allergy Immunol. 2011;22(4):419-23.
209. Sherkat R, Yazdani R, Ganjalikhani Hakemi M, Homayouni V, Farahani R, Hosseini M, et al. Innate lymphoid cells and cytokines of the novel subtypes of helper T cells in asthma. Asia Pac Allergy. 2014;4(4):212-21.
210. Linterman MA, Vinuesa CG. T follicular helper cells during immunity and tolerance. Prog Mol Biol Transl Sci. 2010;92:207-48.

211. Ma X, Sun Z, Zhai P, Yu W, Wang T, Li F, et al. Effect of follicular helper T cells on the pathogenesis of asthma. Exp Ther Med. 2017;14(2):967-72.
212. Havenar-Daughton C, Lindqvist M, Heit A, Wu JE, Reiss SM, Kendric K, et al. CXCL13 is a plasma biomarker of germinal center activity. Proc Natl Acad Sci U S A. 2016;113(10):2702-7.
213. Liu X, Yan X, Zhong B, Nurieva RI, Wang A, Wang X, et al. Bcl6 expression specifies the T follicular helper cell program in vivo. J Exp Med. 2012;209(10):1841-52, S1-24.
214. Robinson DS. Regulatory T cells and asthma. Clin Exp Allergy. 2009;39(9):1314-23.
215. Plitas G, Rudensky AY. Regulatory T Cells: Differentiation and Function. Cancer Immunol Res. 2016;4(9):721-5.
216. Miyara M, Sakaguchi S. Human FoxP3(+)CD4(+) regulatory T cells: their knowns and unknowns. Immunol Cell Biol. 2011;89(3):346-51.
217. Palomares O, Martín-Fontecha M, Lauener R, Traidl-Hoffmann C, Cavkaytar O, Akdis M, et al. Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-β. Genes Immun. 2014;15(8):511-20.
218. Josefowicz SZ, Niec RE, Kim HY, Treuting P, Chinen T, Zheng Y, et al. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature. 2012;482(7385):395-9.
219. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med. 2006;203(7):1693-700.
220. Park CS, Lee SM, Uh ST, Kim HT, Chung YT, Kim YH, et al. Soluble interleukin-2 receptor and cellular profiles in

bronchoalveolar lavage fluid from patients with bronchial asthma. J Allergy Clin Immunol. 1993;91(2):623-33.

221. Park CS, Lee SM, Chung SW, Uh S, Kim HT, Kim YH. Interleukin-2 and soluble interleukin-2 receptor in bronchoalveolar lavage fluid from patients with bronchial asthma. Chest. 1994;106(2):400-6.
222. Lindqvist CA, Christiansson LH, Simonsson B, Enblad G, Olsson-Strömberg U, Loskog AS. T regulatory cells control T-cell proliferation partly by the release of soluble CD25 in patients with B-cell malignancies. Immunology. 2010;131(3):371-6.
223. Hoeger PH, Niggemann B, Ganschow R, Dammann C, Haeuser G. Serum levels of sCD23 and sCD25 in children with asthma and in healthy controls. Allergy. 1994;49(4):217-21.
224. Corrigan CJ, Kay AB. CD4 T-lymphocyte activation in acute severe asthma. Relationship to disease severity and atopic status. Am Rev Respir Dis. 1990;141(4 Pt 1):970-7.
225. Carrette F, Surh CD. IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis. Semin Immunol. 2012;24(3):209-17.
226. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med. 2004;199(11):1567-75.
227. Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. J Allergy Clin Immunol. 2007;119(5):1258-66.
228. Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of

innate immune memory in health and disease. Science. 2016;352(6284):aaf1098.

229. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: human memory T-cell subsets. Eur J Immunol. 2013;43(11):2797-809.
230. Sallusto F, Lanzavecchia A. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. Eur J Immunol. 2009;39(8):2076-82.
231. Sallusto F, Zielinski CE, Lanzavecchia A. Human Th17 subsets. Eur J Immunol. 2012;42(9):2215-20.
232. Shin H, Iwasaki A. Tissue-resident memory T cells. Immunol Rev. 2013;255(1):165-81.
233. Mojtabavi N, Dekan G, Stingl G, Epstein MM. Long-lived Th2 memory in experimental allergic asthma. J Immunol. 2002;169(9):4788-96.
234. Lloyd CM, Hessel EM. Functions of T cells in asthma: more than just T(H)2 cells. Nat Rev Immunol. 2010;10(12):838-48.
235. Wang YH, Voo KS, Liu B, Chen CY, Uygungil B, Spoede W, et al. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. J Exp Med. 2010;207(11):2479-91.
236. Abdulamir AS, Hafidh RR, Abubakar F, Abbas KA. Changing survival, memory cell compartment, and T-helper balance of lymphocytes between severe and mild asthma. BMC Immunol. 2008;9:73.
237. Machura E, Mazur B, Pieniazek W, Karczewska K. Expression of naive/memory (CD45RA/CD45RO) markers by peripheral blood CD4+ and CD8 + T cells in children with asthma. Arch Immunol Ther Exp (Warsz). 2008;56(1):55-62.

238. Wiest M, Upchurch K, Yin W, Ellis J, Xue Y, Lanier B, et al. Clinical implications of CD4+ T cell subsets in adult atopic asthma patients. Allergy Asthma Clin Immunol. 2018;14:7.
239. Zhou H, Hong X, Jiang S, Dong H, Xu X, Xu X. Analyses of associations between three positionally cloned asthma candidate genes and asthma or asthma-related phenotypes in a Chinese population. BMC Med Genet. 2009;10:123.
240. Kim SH, Choi H, Yoon MG, Ye YM, Park HS. Dipeptidyl-peptidase 10 as a genetic biomarker for the aspirin-exacerbated respiratory disease phenotype. Ann Allergy Asthma Immunol. 2015;114(3):208-13.
241. Waumans Y, Baerts L, Kehoe K, Lambeir AM, De Meester I. The Dipeptidyl Peptidase Family, Prolyl Oligopeptidase, and Prolyl Carboxypeptidase in the Immune System and Inflammatory Disease, Including Atherosclerosis. Front Immunol. 2015;6:387.
242. Schade J, Stephan M, Schmiedl A, Wagner L, Niestroj AJ, Demuth HU, et al. Regulation of expression and function of dipeptidyl peptidase 4 (DP4), DP8/9, and DP10 in allergic responses of the lung in rats. J Histochem Cytochem. 2008;56(2):147-55.
243. Wagner L, Klemann C, Stephan M, von Hörsten S. Unravelling the immunological roles of dipeptidyl peptidase 4 (DPP4) activity and/or structure homologue (DASH) proteins. Clin Exp Immunol. 2016;184(3):265-83.
244. Zagha E, Ozaita A, Chang SY, Nadal MS, Lin U, Saganich MJ, et al. DPP10 modulates Kv4-mediated A-type potassium channels. J Biol Chem. 2005;280(19):18853-61.
245. Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, et al. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature. 2013;500(7461):227-31.

246. Lun SW, Wong CK, Ko FW, Hui DS, Lam CW. Increased expression of plasma and CD4+ T lymphocyte costimulatory molecule CD26 in adult patients with allergic asthma. J Clin Immunol. 2007;27(4):430-7.
247. Schade J, Schmiedl A, Kehlen A, Veres TZ, Stephan M, Pabst R, et al. Airway-specific recruitment of T cells is reduced in a CD26-deficient F344 rat substrain. Clin Exp Immunol. 2009;158(1):133-42.
248. Boonacker E, Van Noorden CJ. The multifunctional or moonlighting protein CD26/DPPIV. Eur J Cell Biol. 2003;82(2):53-73.
249. Zhen G, Park SW, Nguyenvu LT, Rodriguez MW, Barbeau R, Paquet AC, et al. IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. Am J Respir Cell Mol Biol. 2007;36(2):244-53.
250. De Meester I, Vanhoof G, Hendriks D, Demuth HU, Yaron A, Scharpé S. Characterization of dipeptidyl peptidase IV (CD26) from human lymphocytes. Clin Chim Acta. 1992;210(1-2):23-34.
251. Klemann C, Wagner L, Stephan M, von Hörsten S. Cut to the chase: a review of CD26/dipeptidyl peptidase-4's (DPP4) entanglement in the immune system. Clin Exp Immunol. 2016;185(1):1-21.
252. Ohnuma K, Dang NH, Morimoto C. Revisiting an old acquaintance: CD26 and its molecular mechanisms in T cell function. Trends Immunol. 2008;29(6):295-301.
253. Cordero OJ, Salgado FJ, Viñuela JE, Nogueira M. Interleukin-12 enhances CD26 expression and dipeptidyl peptidase IV function on human activated lymphocytes. Immunobiology. 1997;197(5):522-33.

254. Cordero OJ, Salgado FJ, Viñuela JE, Nogueira M. Interleukin-12-dependent activation of human lymphocyte subsets. Immunol Lett. 1998;61(1):7-13.
255. Bauvois B, De Meester I, Dumont J, Rouillard D, Zhao HX, Bosmans E. Constitutive expression of CD26/dipeptidylpeptidase IV on peripheral blood B lymphocytes of patients with B chronic lymphocytic leukaemia. Br J Cancer. 1999;79(7-8):1042-8.
256. Arndt M, Lendeckel U, Spiess A, Faust J, Neubert K, Reinhold D, et al. Dipeptidyl peptidase IV (DP IV/CD26) mRNA expression in PWM-stimulated T-cells is suppressed by specific DP IV inhibition, an effect mediated by TGF-beta(1). Biochem Biophys Res Commun. 2000 2;274(2):410-4.
257. Tan EY, Richard CL, Zhang H, Hoskin DW, Blay J. Adenosine downregulates DPPIV on HT-29 colon cancer cells by stimulating protein tyrosine phosphatase(s) and reducing ERK1/2 activity via a novel pathway. Am J Physiol Cell Physiol. 2006;291(3):C433-44.
258. Salgado FJ, Pérez-Díaz A, Villanueva NM, Lamas O, Arias P, Nogueira M. CD26: a negative selection marker for human Treg cells. Cytometry A. 2012;81(10):843-55.
259. Bengsch B, Seigel B, Flecken T, Wolanski J, Blum HE, Thimme R. Human Th17 cells express high levels of enzymatically active dipeptidylpeptidase IV (CD26). J Immunol. 2012;188(11):5438-47.
260. Esmaeili B, Mansouri P, Meysamie A, Izad M. Evaluation of IL-17 Producing Memory Regulatory and Effector T Cells Expressing CD26 Molecule in Patients with Psoriasis. Iran J Allergy Asthma Immunol. 2018;17(5):453-463.
261. Hatano R, Ohnuma K, Yamamoto J, Dang NH, Morimoto C. CD26-mediated co-stimulation in human CD8(+) T cells

provokes effector function via pro-inflammatory cytokine production. Immunology. 2013;138(2):165-72.

262. Bailey SR, Nelson MH, Majchrzak K, Bowers JS, Wyatt MM, Smith AS, et al. Human CD26high T cells elicit tumor immunity against multiple malignancies via enhanced migration and persistence. Nat Commun. 2017;8(1):1961.
263. Ishii T, Ohnuma K, Murakami A, Takasawa N, Kobayashi S, Dang NH, et al. CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO. Proc Natl Acad Sci U S A. 2001;98(21):12138-43.
264. Moreno E, Canet J, Gracia E, Lluís C, Mallol J, Canela EI, et al. Molecular Evidence of Adenosine Deaminase Linking Adenosine A2A Receptor and CD26 Proteins. Front Pharmacol. 2018;9:106.
265. Herrera C, Morimoto C, Blanco J, Mallol J, Arenzana F, Lluis C, et al. Comodulation of CXCR4 and CD26 in human lymphocytes. J Biol Chem. 2001;276(22):19532-9.
266. Ohnuma K, Yamochi T, Uchiyama M, Nishibashi K, Yoshikawa N, Shimizu N, et al. CD26 up-regulates expression of CD86 on antigen-presenting cells by means of caveolin-1. Proc Natl Acad Sci U S A. 2004;101(39):14186-91.
267. Gonzalez-Gronow M, Grenett HE, Weber MR, Gawdi G, Pizzo SV. Interaction of plasminogen with dipeptidyl peptidase IV initiates a signal transduction mechanism which regulates expression of matrix metalloproteinase-9 by prostate cancer cells. Biochem J. 2001;355(Pt 2):397-407.
268. Löster K, Zeilinger K, Schuppan D, Reutter W. The cysteine-rich region of dipeptidyl peptidase IV (CD 26) is the collagen-binding site. Biochem Biophys Res Commun. 1995;217(1):341-8.

269. Cheng HC, Abdel-Ghany M, Pauli BU. A novel consensus motif in fibronectin mediates dipeptidyl peptidase IV adhesion and metastasis. J Biol Chem. 2003;278(27):24600-7.
270. Davoodi J, Kelly J, Gendron NH, MacKenzie AE. The Simpson-Golabi-Behmel syndrome causative glypican-3, binds to and inhibits the dipeptidyl peptidase activity of CD26. Proteomics. 2007;7(13):2300-10.
271. Salgado FJ, Lojo J, Alonso-Lebrero JL, Lluis C, Franco R, Cordero OJ, et al. A role for interleukin-12 in the regulation of T cell plasma membrane compartmentation. J Biol Chem. 2003;278(27):24849-57.
272. Ohnuma K, Yamochi T, Uchiyama M, Nishibashi K, Iwata S, Hosono O, et al. CD26 mediates dissociation of Tollip and IRAK-1 from caveolin-1 and induces upregulation of CD86 on antigen-presenting cells. Mol Cell Biol. 2005;25(17):7743-57.
273. Ohnuma K, Uchiyama M, Yamochi T, Nishibashi K, Hosono O, Takahashi N, et al. Caveolin-1 triggers T-cell activation via CD26 in association with CARMA1. J Biol Chem. 2007;282(13):10117-31.
274. Yu DM, Yao TW, Chowdhury S, Nadvi NA, Osborne B, Church WB, et al. The dipeptidyl peptidase IV family in cancer and cell biology. FEBS J. 2010;277(5):1126-44.
275. Struyf S, Proost P, Schols D, De Clercq E, Opdenakker G, Lenaerts JP, et al. CD26/dipeptidyl-peptidase IV down-regulates the eosinophil chemotactic potency, but not the anti-HIV activity of human eotaxin by affecting its interaction with CC chemokine receptor 3. J Immunol. 1999;162(8):4903-9.
276. Romagnani S. Cytokines and chemoattractants in allergic inflammation. Mol Immunol. 2002;38(12-13):881-5.

277. Sehmi R, Dorman S, Baatjes A, Watson R, Foley R, Ying S, et al. Allergen-induced fluctuation in CC chemokine receptor 3 expression on bone marrow CD34+ cells from asthmatic subjects: significance for mobilization of haemopoietic progenitor cells in allergic inflammation. Immunology. 2003;109(4):536-46.
278. Manns J, Rieder S, Escher S, Eilers B, Forssmann WG, Elsner J, et al. The allergy-associated chemokine receptors CCR3 and CCR5 can be inactivated by the modified chemokine NNY-CCL11. Allergy. 2007;62(1):17-24.
279. Lambeir AM, Proost P, Durinx C, Bal G, Senten K, Augustyns K, et al. Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. J Biol Chem. 2001;276(32):29839-45.
280. Forssmann U, Stoetzer C, Stephan M, Kruschinski C, Skripuletz T, Schade J, et al. Inhibition of CD26/dipeptidyl peptidase IV enhances CCL11/eotaxin-mediated recruitment of eosinophils in vivo. J Immunol. 2008;181(2):1120-7.
281. Yan S, Gessner R, Dietel C, Schmiedek U, Fan H. Enhanced ovalbumin-induced airway inflammation in CD26-/- mice. Eur J Immunol. 2012;42(2):533-40.
282. Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. Trends Immunol. 2001;22(2):83-7.
283. Iwata S, Yamaguchi N, Munakata Y, Ikushima H, Lee JF, Hosono O, et al. CD26/dipeptidyl peptidase IV differentially regulates the chemotaxis of T cells and monocytes toward RANTES: possible mechanism for the switch from innate to acquired immune response. Int Immunol. 1999;11(3):417-26.
284. Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR. The HIV coreceptors CXCR4 and CCR5 are differentially

expressed and regulated on human T lymphocytes. Proc Natl Acad Sci U S A. 1997;94(5):1925-30.

285. Lim JK, Burns JM, Lu W, DeVico AL. Multiple pathways of amino terminal processing produce two truncated variants of RANTES/CCL5. J Leukoc Biol. 2005;78(2):442-52.
286. Cordero OJ, Salgado FJ, Nogueira M. On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother. 2009;58(11):1723-47.
287. Duke-Cohan JS, Morimoto C, Rocker JA, Schlossman SF. A novel form of dipeptidylpeptidase IV found in human serum. Isolation, characterization, and comparison with T lymphocyte membrane dipeptidylpeptidase IV (CD26). J Biol Chem. 1995;270(23):14107-14.
288. Duke-Cohan JS, Morimoto C, Rocker JA, Schlossman SF. Serum high molecular weight dipeptidyl peptidase IV (CD26) is similar to a novel antigen DPPT-L released from activated T cells. J Immunol. 1996;156(5):1714-21.
289. Kobayashi H, Hosono O, Mimori T, Kawasaki H, Dang NH, Tanaka H, et al. Reduction of serum soluble CD26/dipeptidyl peptidase IV enzyme activity and its correlation with disease activity in systemic lupus erythematosus. J Rheumatol. 2002;29(9):1858-66.
290. Tejera-Alhambra M, Casrouge A, de Andrés C, Ramos-Medina R, Alonso B, Vega J, et al. Low DPP4 expression and activity in multiple sclerosis. Clin Immunol. 2014;150(2):170-83.
291. Röhrborn D, Eckel J, Sell H. Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells. FEBS Lett. 2014;588(21):3870-7.
292. Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, et al. Dipeptidyl peptidase 4 is a novel

adipokine potentially linking obesity to the metabolic syndrome. Diabetes. 2011;60(7):1917-25.

293. Gorrell MD, Gysbers V, McCaughan GW. CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand J Immunol. 2001;54(3):249-64.
294. Lambeir AM, Durinx C, Scharpé S, De Meester I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci. 2003;40(3):209-94.
295. Uematsu T, Urade M, Yamaoka M. Decreased expression and release of dipeptidyl peptidase IV (CD26) in cultured peripheral blood T lymphocytes of oral cancer patients. J Oral Pathol Med. 1998;27(3):106-10.
296. Kruschinski C, Skripuletz T, Bedoui S, Tschernig T, Pabst R, Nassenstein C, et al. CD26 (dipeptidyl-peptidase IV)-dependent recruitment of T cells in a rat asthma model. Clin Exp Immunol. 2005;139(1):17-24.
297. Schmiedl A, Krainski J, Schwichtenhövel F, Schade J, Klemann C, Raber KA, et al. Reduced airway inflammation in CD26/DPP4-deficient F344 rats is associated with altered recruitment patterns of regulatory T cells and expression of pulmonary surfactant proteins. Clin Exp Allergy. 2010;40(12):1794-808.
298. Stephens LA, Barclay AN, Mason D. Phenotypic characterization of regulatory CD4+CD25+ T cells in rats. Int Immunol. 2004;16(2):365-75.
299. Skripuletz T, Schmiedl A, Schade J, Bedoui S, Glaab T, Pabst R, et al. Dose-dependent recruitment of CD25+ and CD26+ T cells in a novel F344 rat model of asthma. Am J Physiol Lung Cell Mol Physiol. 2007;292(6):L1564-71.
300. Tasic T, Stephan M, von Hörsten S, Pabst R, Schmiedl A. Differential OVA-induced pulmonary inflammation and

unspecific reaction in Dark Agouti (DA) rats contingent on CD26/DPPIV deficiency. Immunobiology. 2014;219(11):888-900.

301. Stephan M, Suhling H, Schade J, Wittlake M, Tasic T, Klemann C, et al. Effects of dipeptidyl peptidase-4 inhibition in an animal model of experimental asthma: a matter of dose, route, and time. Physiol Rep. 2013;1(5):e00095.
302. Shiobara T, Chibana K, Watanabe T, Arai R, Horigane Y, Nakamura Y, et al. Dipeptidyl peptidase-4 is highly expressed in bronchial epithelial cells of untreated asthma and it increases cell proliferation along with fibronectin production in airway constitutive cells. Respir Res. 2016;17:28.
303. Remes ST, Delezuch W, Pulkki K, Pekkanen J, Korppi M, Matinlauri IH. Association of serum-soluble CD26 and CD30 levels with asthma, lung function and bronchial hyper-responsiveness at school age. Acta Paediatr. 2011;100(9):e106-11.
304. Delezuch W, Marttinen P, Kokki H, Heikkinen M, Vanamo K, Pulkki K, et al. Serum and CSF soluble CD26 and CD30 concentrations in healthy pediatric surgical outpatients. Tissue Antigens. 2012;80(4):368-75.
305. Chung KF. Defining phenotypes in asthma: a step towards personalized medicine. Drugs. 2014;74(7):719-28.
306. Canonica GW, Ferrando M, Baiardini I, Puggioni F, Racca F, Passalacqua G, et al. Asthma: personalized and precision medicine. Curr Opin Allergy Clin Immunol. 2018;18(1):51-58.
307. Kim H, Ellis AK, Fischer D, Noseworthy M, Olivenstein R, Chapman KR, et al. Asthma biomarkers in the age of biologics. Allergy Asthma Clin Immunol. 2017;13:48.

308. Schatz M, Rosenwasser L. The allergic asthma phenotype. J Allergy Clin Immunol Pract. 2014;2(6):645-8; quiz 649.
309. Peters SP. Asthma phenotypes: nonallergic (intrinsic) asthma. J Allergy Clin Immunol Pract. 2014;2(6):650-2.
310. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med. 2009;180(5):388-95.
311. Fajt ML, Wenzel SE. Asthma phenotypes and the use of biologic medications in asthma and allergic disease: the next steps toward personalized care. J Allergy Clin Immunol. 2015;135(2):299-310; quiz 311.
312. Stokes JR, Casale TB. Characterization of asthma endotypes: implications for therapy. Ann Allergy Asthma Immunol. 2016;117(2):121-5.
313. Tiotiu A. Biomarkers in asthma: state of the art. Asthma Res Pract. 2018;4:10.
314. Pavlidis S, Takahashi K, Ng Kee Kwong F, Xie J, Hoda U, Sun K, et al. "T2-high" in severe asthma related to blood eosinophil, exhaled nitric oxide and serum periostin. Eur Respir J. 2019;53(1). pii: 1800938.
315. Djukanović R, Wilson SJ, Kraft M, Jarjour NN, Steel M, Chung KF, et al. Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma. Am J Respir Crit Care Med. 2004;170(6):583-93.
316. Humbert M, Beasley R, Ayres J, Slavin R, Hébert J, Bousquet J, et al. Benefits of omalizumab as add-on therapy in patients with severe persistent asthma who are inadequately controlled despite best available therapy (GINA 2002 step 4 treatment): INNOVATE. Allergy. 2005;60(3):309-16.

317. Normansell R, Walker S, Milan SJ, Walters EH, Nair P. Omalizumab for asthma in adults and children. Cochrane Database Syst Rev. 2014;(1):CD003559.
318. Chung KF. Asthma phenotyping: a necessity for improved therapeutic precision and new targeted therapies. J Intern Med. 2016;279(2):192-204.
319. Miranda C, Busacker A, Balzar S, Trudeau J, Wenzel SE. Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. J Allergy Clin Immunol. 2004;113(1):101-8.
320. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. Am J Respir Crit Care Med. 2010;181(4):315-23.
321. Wu W, Bleecker E, Moore W, Busse WW, Castro M, Chung KF, et al. Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. J Allergy Clin Immunol. 2014;133(5):1280-8.
322. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, et al. Cluster analysis and clinical asthma phenotypes. Am J Respir Crit Care Med. 2008;178(3):218-224.
323. Barczyk A, Pierzchala W, Sozañska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respir Med. 2003;97(6):726-33.
324. Chien JW, Lin CY, Yang KD, Lin CH, Kao JK, Tsai YG. Clin Exp Allergy. 2013;43(9):1018-26.
325. Ray A, Kolls JK. Neutrophilic Inflammation in Asthma and Association with Disease Severity. Trends Immunol. 2017;38(12):942-954.
326. Greco V, Pieragostino D, Piras C, Aebersold R, Wiltfang J, Caltagirone C, et al. Direct analytical sample quality

assessment for biomarker investigation: qualifying cerebrospinal fluid samples. Proteomics. 2014;14(17-18):1954-62.

327. Pelaia G, Terracciano R, Vatrella A, Gallelli L, Busceti MT, Calabrese C, et al. Application of proteomics and peptidomics to COPD. Biomed Res Int. 2014;2014:764581.
328. De Bock M, de Seny D, Meuwis MA, Servais AC, Minh TQ, Closset J, et al. Comparison of three methods for fractionation and enrichment of low molecular weight proteins for SELDI-TOF-MS differential analysis. Talanta. 2010;82(1):245-54.
329. Cai T, Yang F. Strategies for Characterization of Low-Abundant Intact or Truncated Low-Molecular-Weight Proteins From Human Plasma. Enzymes. 2017;42:105-123.
330. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics. 2002;1(11):845-67.
331. Hu S, Loo JA, Wong DT. Human body fluid proteome analysis. Proteomics. 2006;6(23):6326-53.
332. Liotta LA, Ferrari M, Petricoin E. Clinical proteomics: written in blood. Nature. 2003;425(6961):905.
333. Petricoin EF, Belluco C, Araujo RP, Liotta LA. The blood peptidome: a higher dimension of information content for cancer biomarker discovery. Nat Rev Cancer. 2006;6(12):961-7.
334. Dolci A, Panteghini M. The exciting story of cardiac biomarkers: from retrospective detection to gold diagnostic standard for acute myocardial infarction and more. Clin Chim Acta. 2006;369(2):179-87.
335. Gravett MG, Novy MJ, Rosenfeld RG, Reddy AP, Jacob T, Turner M, et al. Diagnosis of intra-amniotic infection by

proteomic profiling and identification of novel biomarkers. JAMA. 2004;292(4):462-9.

336. Ohlmeier S, Vuolanto M, Toljamo T, Vuopala K, Salmenkivi K, Myllärniemi M, et al. Proteomics of human lung tissue identifies surfactant protein A as a marker of chronic obstructive pulmonary disease. J Proteome Res. 2008;7(12):5125-32.
337. Terracciano R, Pelaia G, Preianò M, Savino R. Asthma and COPD proteomics: current approaches and future directions. Proteomics Clin Appl. 2015;9(1-2):203-20.
338. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics. 2003;2(10):1096-103.
339. Righetti PG, Castagna A, Antonioli P, Boschetti E. Prefractionation techniques in proteome analysis: the mining tools of the third millennium. Electrophoresis. 2005;26(2):297-319.
340. Greening DW, Simpson RJ. Characterization of the Low-Molecular-Weight Human Plasma Peptidome. Methods Mol Biol. 2017;1619:63-79.
341. Kim B, Araujo R, Howard M, Magni R, Liotta LA, Luchini A. Affinity enrichment for mass spectrometry: improving the yield of low abundance biomarkers. Expert Rev Proteomics. 2018;15(4):353-66.
342. Gianazza E, Arnaud P. A general method for fractionation of plasma proteins. Dye-ligand affinity chromatography on immobilized Cibacron blue F3-GA. Biochem J. 1982 201(1):129-36.
343. Adkins JN, Varnum SM, Auberry KJ, Moore RJ, Angell NH, Smith RD, et al. Toward a human blood serum proteome:

analysis by multidimensional separation coupled with mass spectrometry. Mol Cell Proteomics. 2002;1(12):947-55.

344. Qiu F, Hou T, Huang D, Xue Z, Liang D, Li Q, et al. Evaluation of two high-abundance protein depletion kits and optimization of downstream isoelectric focusing. Mol Med Rep. 2015;12(5):7749-55.
345. Merrell K, Southwick K, Graves SW, Esplin MS, Lewis NE, Thulin CD. Analysis of low-abundance, low-molecular-weight serum proteins using mass spectrometry. J Biomol Tech. 2004;15(4):238-48.
346. Lowenthal MS, Mehta AI, Frogale K, Bandle RW, Araujo RP, Hood BL, et al. Analysis of albumin-associated peptides and proteins from ovarian cancer patients. Clin Chem. 2005;51(10):1933-45.
347. Anderson NL, Jackson A, Smith D, Hardie D, Borchers C, Pearson TW. SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device. Mol Cell Proteomics. 2009;8(5):995-1005.
348. Khoury LR, Goldbart R, Traitel T, Enden G, Kost J. Harvesting Low Molecular Weight Biomarkers Using Gold Nanoparticles. ACS Nano. 2015;9(6):5750-9.
349. Desrosiers RR, Beaulieu E, Buchanan M, Béliveau R. Proteomic analysis of human plasma proteins by two-dimensional gel electrophoresis and by antibody arrays following depletion of high-abundance proteins. Cell Biochem Biophys. 2007;49(3):182-95.
350. Meng R, Gormley M, Bhat VB, Rosenberg A, Quong AA. Low abundance protein enrichment for discovery of candidate plasma protein biomarkers for early detection of breast cancer. J Proteomics. 2011;75(2):366-74.

351. Siuti N, Kelleher NL. Decoding protein modifications using top-down mass spectrometry. Nat Methods. 2007;4(10):817-21.
352. Cox J, Mann M. Quantitative, high-resolution proteomics for data-driven systems biology. Annu Rev Biochem. 2011;80:273-99.
353. Zhang Y, Fonslow BR, Shan B, Baek MC, Yates JR 3rd. Protein analysis by shotgun/bottom-up proteomics. Chem Rev. 2013;113(4):2343-94.
354. Müller SA, Kohajda T, Findeiss S, Stadler PF, Washietl S, Kellis M, et al. Optimization of parameters for coverage of low molecular weight proteins. Anal Bioanal Chem. 2010;398(7-8):2867-81.
355. Rabilloud T, Chevallet M, Luche S, Lelong C. Two-dimensional gel electrophoresis in proteomics: Past, present and future. J Proteomics. 2010;73(11):2064-77.
356. Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, et al. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics. 2001;1(3):377-96.
357. Mauri P, Riccio AM, Rossi R, Di Silvestre D, Benazzi L, De Ferrari L, et al. Proteomics of bronchial biopsies: galectin-3 as a predictive biomarker of airway remodelling modulation in omalizumab-treated severe asthma patients. Immunol Lett. 2014;162(1 Pt A):2-10.
358. Jiang H, Chi X, Zhang X, Wang J. Increased serum VDBP as a risk predictor for steroid resistance in asthma patients. Respir Med. 2016;114:111-6.
359. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422(6928):198-207.

360. Lundby A, Olsen JV. GeLCMS for in-depth protein characterization and advanced analysis of proteomes. Methods Mol Biol. 2011;753:143-55.
361. Chen M, Ying W, Song Y, Liu X, Yang B, Wu S, et al. Analysis of human liver proteome using replicate shotgun strategy. Proteomics. 2007;7(14):2479-88.
362. Betancourt LH, De Bock PJ, Staes A, Timmerman E, Perez-Riverol Y, Sanchez A, et al. SCX charge state selective separation of tryptic peptides combined with 2D-RP-HPLC allows for detailed proteome mapping. J Proteomics. 2013;91:164-71.
363. Yoshida T. Peptide separation by Hydrophilic-Interaction Chromatography: a review. J Biochem Biophys Methods. 2004;60(3):265-80.
364. Zhu S, Zhang X, Gao M, Hong G, Yan G, Zhang X. Developing a strong anion exchange/RP (SAX/RP) 2D LC system for high-abundance proteins depletion in human plasma. Proteomics. 2012;12(23-24):3451-63.
365. Yates JR, Ruse CI, Nakorchevsky A. Proteomics by mass spectrometry: approaches, advances, and applications. Annu Rev Biomed Eng. 2009;11:49-79.
366. Haag AM. Mass Analyzers and Mass Spectrometers. Adv Exp Med Biol. 2016;919:157-69.
367. Makarov A, Denisov E, Kholomeev A, Balschun W, Lange O, Strupat K, et al. Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer. Anal Chem. 2006;78(7):2113-20.
368. Deracinois B, Flahaut C, Duban-Deweer S, Karamanos Y. Comparative and Quantitative Global Proteomics Approaches: An Overview. Proteomes. 2013;1(3):180-218.
369. Bondarenko PV, Chelius D, Shaler TA. Identification and relative quantitation of protein mixtures by enzymatic

digestion followed by capillary reversed-phase liquid chromatography-tandem mass spectrometry. Anal Chem. 2002;74(18):4741-9.

370. Arike L, Peil L. Spectral counting label-free proteomics. Methods Mol Biol. 2014;1156:213-22.
371. Lange V, Malmström JA, Didion J, King NL, Johansson BP, Schäfer J, et al. Targeted quantitative analysis of Streptococcus pyogenes virulence factors by multiple reaction monitoring. Mol Cell Proteomics. 2008;7(8):1489-500.
372. Shi T, Fillmore TL, Sun X, Zhao R, Schepmoes AA, Hossain M, et al. Antibody-free, targeted mass-spectrometric approach for quantification of proteins at low picogram per milliliter levels in human plasma/serum. Proc Natl Acad Sci U S A. 2012;109(38):15395-400.
373. Wang H, Alvarez S, Hicks LM. Comprehensive comparison of iTRAQ and label-free LC-based quantitative proteomics approaches using two Chlamydomonas reinhardtii strains of interest for biofuels engineering. J Proteome Res. 2012;11(1):487-501.
374. Lindahl M, Ståhlbom B, Tagesson C. Newly identified proteins in human nasal and bronchoalveolar lavage fluids: potential biomedical and clinical applications. Electrophoresis. 1999;20(18):3670-6.
375. Wu J, Kobayashi M, Sousa EA, Liu W, Cai J, Goldman SJ, et al. Differential proteomic analysis of bronchoalveolar lavage fluid in asthmatics following segmental antigen challenge. Mol Cell Proteomics. 2005;4(9):1251-64.
376. Cederfur C, Malmström J, Nihlberg K, Block M, Breimer ME, Bjermer L, et al. Glycoproteomic identification of galectin-3 and -8 ligands in bronchoalveolar lavage of mild

asthmatics and healthy subjects. Biochim Biophys Acta. 2012;1820(9):1429-36.

377. Choi GS, Kim JH, Shin YS, Ye YM, Kim SH, Park HS. Eosinophil activation and novel mediators in the aspirin-induced nasal response in AERD. Clin Exp Allergy. 2013;43(7):730-40.
378. Suojalehto H, Kinaret P, Kilpeläinen M, Toskala E, Ahonen N, Wolff H, et al. Level of Fatty Acid Binding Protein 5 (FABP5) Is Increased in Sputum of Allergic Asthmatics and Links to Airway Remodeling and Inflammation. PLoS One. 2015;10(5):e0127003.
379. O'Neil SE, Sitkauskiene B, Babusyte A, Krisiukeniene A, Stravinskaite-Bieksiene K, Sakalauskas R, et al. Network analysis of quantitative proteomics on asthmatic bronchi: effects of inhaled glucocorticoid treatment. Respir Res. 2011;12:124.
380. Gomes-Alves P, Imrie M, Gray RD, Nogueira P, Ciordia S, Pacheco P, et al. SELDI-TOF biomarker signatures for cystic fibrosis, asthma and chronic obstructive pulmonary disease. Clin Biochem. 2010;43(1-2):168-77.
381. Gray RD, MacGregor G, Noble D, Imrie M, Dewar M, Boyd AC, et al. Sputum proteomics in inflammatory and suppurative respiratory diseases. Am J Respir Crit Care Med. 2008;178(5):444-52.
382. Gharib SA, Nguyen EV, Lai Y, Plampin JD, Goodlett DR, Hallstrand TS. Induced sputum proteome in healthy subjects and asthmatic patients. J Allergy Clin Immunol. 2011;128(6):1176-1184.e6.
383. Terracciano R, Preianò M, Palladino GP, Carpagnano GE, Barbaro MP, Pelaia G, et al. Peptidome profiling of induced sputum by mesoporous silica beads and MALDI-TOF MS for

non-invasive biomarker discovery of chronic inflammatory lung diseases. Proteomics. 2011;11(16):3402-14.

384. Lee TH, Jang AS, Park JS, Kim TH, Choi YS, Shin HR, et al. Elevation of S100 calcium binding protein A9 in sputum of neutrophilic inflammation in severe uncontrolled asthma. Ann Allergy Asthma Immunol. 2013;111(4):268-275.e1.
385. Cao C, Li W, Hua W, Yan F, Zhang H, Huang H, et al. Proteomic analysis of sputum reveals novel biomarkers for various presentations of asthma. J Transl Med. 2017;15(1):171.
386. Kasaian MT, Lee J, Brennan A, Danto SI, Black KE, Fitz L, et al. Proteomic analysis of serum and sputum analytes distinguishes controlled and poorly controlled asthmatics. Clin Exp Allergy. 2018;48(7):814-24.
387. Lee SH, Rhim T, Choi YS, Min JW, Kim SH, Cho SY, et al. Complement C3a and C4a increased in plasma of patients with aspirin-induced asthma. Am J Respir Crit Care Med. 2006;173(4):370-8.
388. Nishioka T, Uchida K, Meno K, Ishii T, Aoki T, Imada Y, et al. Alpha-1-antitrypsin and complement component C7 are involved in asthma exacerbation. Proteomics Clin Appl. 2008 Jan;2(1):46-54. doi: 10.1002/prca.200780065.
389. Rhim T, Choi YS, Nam BY, Uh ST, Park JS, Kim YH, et al. Plasma protein profiles in early asthmatic responses to inhalation allergen challenge. Allergy. 2009;64(1):47-54.
390. Verrills NM, Irwin JA, He XY, Wood LG, Powell H, Simpson JL, et al. Identification of novel diagnostic biomarkers for asthma and chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2011;183(12):1633-43.
391. Izbicka E, Streeper RT, Michalek JE, Louden CL, Diaz A 3rd, Campos DR. Plasma biomarkers distinguish non-small

cell lung cancer from asthma and differ in men and women. Cancer Genomics Proteomics. 2012;9(1):27-35.

392. Hamsten C, Häggmark A, Grundström J, Mikus M, Lindskog C, Konradsen JR, et al. Protein profiles of CCL5, HPGDS, and NPSR1 in plasma reveal association with childhood asthma. Allergy. 2016;71(9):1357-61.
393. Xu H, Radabaugh T, Lu Z, Galligan M, Billheimer D, Vercelli D, et al. Exploration of early-life candidate biomarkers for childhood asthma using antibody arrays. Pediatr Allergy Immunol. 2016;27(7):696-701.
394. Ko YC, Hsu WH, Chung JG, Dai MP, Ou CC, Wu WP. Proteomic analysis of CD4+ T-lymphocytes in patients with asthma between typical therapy (controlled) and no typical therapy (uncontrolled) level. Hum Exp Toxicol. 2011;30(7):541-9.
395. Bloemen K, Van Den Heuvel R, Govarts E, Hooyberghs J, Nelen V, Witters E, et al. A new approach to study exhaled proteins as potential biomarkers for asthma. Clin Exp Allergy. 2011;41(3):346-56.
396. Moniuszko M, Bodzenta-Lukaszyk A, Kowal K, Lenczewska D, Dabrowska M. Enhanced frequencies of CD14++CD16+, but not CD14+CD16+, peripheral blood monocytes in severe asthmatic patients. Clin Immunol. 2009;130(3):338-46.
397. Khanduja KL, Kaushik G, Khanduja S, Pathak CM, Laldinpuii J, Behera D. Corticosteroids affect nitric oxide generation, total free radicals production, and nitric oxide synthase activity in monocytes of asthmatic patients. Mol Cell Biochem. 2011;346(1-2):31-7.
398. Kowal K, Moniuszko M, Dabrowska M, Bodzenta-Lukaszyk A. Allergen challenge differentially affects the number of circulating monocyte subsets. Scand J Immunol. 2012;75(5):531-9.

399. Behzadi P, Behzadi E, Ranjbar R. IL-12 Family Cytokines: General Characteristics, Pathogenic Microorganisms, Receptors, and Signalling Pathways. Acta Microbiol Immunol Hung. 2016;63(1):1-25.
400. Anbazhagan K, Duroux-Richard I, Jorgensen C, Apparailly F. Transcriptomic network support distinct roles of classical and non-classical monocytes in human. Int Rev Immunol. 2014;33(6):470-89.
401. Zasłona Z, Przybranowski S, Wilke C, van Rooijen N, Teitz-Tennenbaum S, Osterholzer JJ, et al. Resident alveolar macrophages suppress, whereas recruited monocytes promote, allergic lung inflammation in murine models of asthma. J Immunol. 2014;193(8):4245-53.
402. White AF, Demchenko AV. Modulating LPS signal transduction at the LPS receptor complex with synthetic Lipid A analogues. Adv Carbohydr Chem Biochem. 2014;71:339-89.
403. Durieux JJ, Vita N, Popescu O, Guette F, Calzada-Wack J, Munker R, et al. The two soluble forms of the lipopolysaccharide receptor, CD14: characterization and release by normal human monocytes. Eur J Immunol. 1994;24(9):2006-12.
404. Sakito O, Kadota J, Kohno S, Itoh N, Takahara O, Hara K. Pulmonary infiltration with eosinophilia and increased serum levels of squamous cell carcinoma-related antigen and neuron specific enolase. Intern Med. 1994;33(9):550-3.
405. Nam SJ, Jeong JY, Jang TW, Jung MH, Chun BK, Cha HJ, et al. Neuron-specific enolase as a novel biomarker reflecting tuberculosis activity and treatment response. Korean J Intern Med. 2016;31(4):694-702.
406. Huang L, Zhou JG, Yao WX, Tian X, Lv SP, Zhang TY, et al. Systematic review and meta-analysis of the efficacy of

serum neuron-specific enolase for early small cell lung cancer screening. Oncotarget. 2017;8(38):64358-64372.

407. Collazos J, Esteban C, Fernández A, Genollá J. Measurement of the serum tumor marker neuron-specific enolase in patients with benign pulmonary diseases. Am J Respir Crit Care Med. 1994;150(1):143-5.
408. Song TJ, Choi YC, Lee KY, Kim WJ. Serum and cerebrospinal fluid neuron-specific enolase for diagnosis of tuberculous meningitis. Yonsei Med J. 2012;53(6):1068-72.
409. Fang SC, Zhang HT, Wang CY, Zhang YM. Serum CA125 and NSE: biomarkers of disease severity in patients with silicosis. Clin Chim Acta. 2014;433:123-7.
410. Tsybikov NN, Egorova EV, Kuznik BI, Fefelova EV, Magen E. Neuron-specific enolase in nasal secretions as a novel biomarker of olfactory dysfunction in chronic rhinosinusitis. Am J Rhinol Allergy. 2016;30(1):65-9.
411. Haque A, Ray SK, Cox A, Banik NL. Neuron specific enolase: a promising therapeutic target in acute spinal cord injury. Metab Brain Dis. 2016;31(3):487-95.
412. Ohnuma K, Yamochi T, Hosono O, Morimoto C. CD26 T cells in the pathogenesis of asthma. Clin Exp Immunol. 2005;139(1):13-6.
413. Willheim M, Ebner C, Baier K, Kern W, Schrattbauer K, Thien R, et al. Cell surface characterization of T lymphocytes and allergen-specific T cell clones: correlation of CD26 expression with T(H1) subsets. J Allergy Clin Immunol. 1997;100(3):348-55.
414. Krakauer M1, Sorensen PS, Sellebjerg F. CD4(+) memory T cells with high CD26 surface expression are enriched for Th1 markers and correlate with clinical severity of multiple sclerosis. J Neuroimmunol. 2006;181(1-2):157-64.

415. Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, et al. Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome.
416. von Bonin A, Hühn J, Fleischer B. Dipeptidyl-peptidase IV/CD26 on T cells: analysis of an alternative T-cell activation pathway. Immunol Rev. 1998;161:43-53.
417. De Meester I, Korom S, Van Damme J, Scharpé S. CD26, let it cut or cut it down. Immunol Today. 1999;20(8):367-75.
418. Matsuno O, Miyazaki E, Nureki S, Ueno T, Ando M, Kumamoto T. Soluble CD26 is inversely Associated with Disease Severity in Patients with Chronic Eosinophilic Pneumonia. Biomark Insights. 2007;1:201-4.
419. Brusko TM, Wasserfall CH, Hulme MA, Cabrera R, Schatz D, Atkinson MA. Influence of membrane CD25 stability on T lymphocyte activity: implications for immunoregulation. PLoS One. 2009;4(11):e7980.
420. Caccamo N, Joosten SA, Ottenhoff THM, Dieli F. Atypical Human Effector/Memory CD4+ T Cells With a Naive-Like Phenotype. Front Immunol. 2018;9:2832.
421. Sun H, Sun C, Xiao W, Sun R. Tissue-resident lymphocytes: from adaptive to innate immunity. Cell Mol Immunol. 2019. doi: 10.1038/s41423-018-0192-y. [Epub ahead of print]
422. Caccamo N, La Mendola C, Orlando V, Meraviglia S, Todaro M, Stassi G, et al. Differentiation, phenotype, and function of interleukin-17-producing human Vγ9Vδ2 T cells. Blood. 2011;118(1):129-38.
423. Krejsek J, Král B, Vokurková D, Derner V, Tousková M, Paráková Z, et al. Decreased peripheral blood gamma delta T cells in patients with bronchial asthma. Allergy. 1998;53(1):73-7.

424. Bains SN, Tourkina E, Atkinson C, Joseph K, Tholanikunnel B, Chu HW, et al. Loss of caveolin-1 from bronchial epithelial cells and monocytes in human subjects with asthma. Allergy. 2012;67(12):1601-4.
425. Broxmeyer HE, Hoggatt J, O'Leary HA, Mantel C, Chitteti BR, Cooper S, et al. Dipeptidylpeptidase 4 negatively regulates colony-stimulating factor activity and stress hematopoiesis. Nat Med. 2012;18(12):1786-96.
426. Havre PA, Abe M, Urasaki Y, Ohnuma K, Morimoto C, Dang NH. CD26 expression on T cell lines increases SDF-1-alpha-mediated invasion. Br J Cancer. 2009;101(6):983-91.
427. Ludwig A, Schiemann F, Mentlein R, Lindner B, Brandt E. Dipeptidyl peptidase IV (CD26) on T cells cleaves the CXC chemokine CXCL11 (I-TAC) and abolishes the stimulating but not the desensitizing potential of the chemokine. J Leukoc Biol. 2002;72(1):183-91.
428. Casrouge A, Bisiaux A, Stephen L, Schmolz M, Mapes J, Pfister C, et al. Discrimination of agonist and antagonist forms of CXCL10 in biological samples. Clin Exp Immunol. 2012;167(1):137-48.
429. Nish SA, Schenten D, Wunderlich FT, Pope SD, Gao Y, Hoshi N, et al. T cell-intrinsic role of IL-6 signaling in primary and memory responses. Elife. 2014;3:e01949.
430. Doganci A, Eigenbrod T, Krug N, De Sanctis GT, Hausding M, Erpenbeck VJ, et al. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. J Clin Invest. 2005;115(2):313-25.
431. Jones GW, McLoughlin RM, Hammond VJ, Parker CR, Williams JD, Malhotra R, et al. Loss of CD4+ T cell IL-6R expression during inflammation underlines a role for IL-6

trans signaling in the local maintenance of Th17 cells. J Immunol. 2010;184(4):2130-9.

432. Wang Y, Hu H, Wu J, Zhao X, Zhen Y, Wang S, et al. The IL6R gene polymorphisms are associated with sIL-6R, IgE and lung function in Chinese patients with asthma. Gene. 2016;585(1):51-57.
433. Mandapathil M, Szczepanski M, Harasymczuk M, Ren J, Cheng D, Jackson EK, et al. CD26 expression and adenosine deaminase activity in regulatory T cells (Treg) and CD4(+) T effector cells in patients with head and neck squamous cell carcinoma. Oncoimmunology. 2012;1(5):659-669.
434. Li P, Gao Y, Cao J, Wang W, Chen Y, Zhang G, et al. CD39+ regulatory T cells attenuate allergic airway inflammation. Clin Exp Allergy. 2015;45(6):1126-37.
435. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med. 2006;203(7):1701-11.
436. Rossi R, De Palma A, Benazzi L, Riccio AM, Canonica GW, Mauri P. Biomarker discovery in asthma and COPD by proteomic approaches. Proteomics Clin Appl. 2014;8(11-12):901-15.
437. Wiktorowicz JE, Jamaluddin M. Proteomic analysis of the asthmatic airway. Adv Exp Med Biol. 2014;795:221-32.
438. Fujii K, Nakamura H, Nishimura T. Recent mass spectrometry-based proteomics for biomarker discovery in lung cancer, COPD, and asthma. Expert Rev Proteomics. 2017;14(4):373-86.
439. Boisclair YR, Rhoads RP, Ueki I, Wang J, Ooi GT. The acid-labile subunit (ALS) of the 150 kDa IGF-binding protein complex: an important but forgotten component of the circulating IGF system. J Endocrinol. 2001;170(1):63-70.

440. David A, Kelley LA, Sternberg MJ. A new structural model of the acid-labile subunit: pathogenetic mechanisms of short stature-causing mutations. J Mol Endocrinol. 2012;49(3):213-20.
441. Lee H, Kim SR, Oh Y, Cho SH, Schleimer RP, Lee YC. Targeting insulin-like growth factor-I and insulin-like growth factor-binding protein-3 signaling pathways. A novel therapeutic approach for asthma. Am J Respir Cell Mol Biol. 2014;50(4):667-77.
442. Domené HM, Hwa V, Argente J, Wit JM, Camacho-Hübner C, Jasper HG, et al. Human acid-labile subunit deficiency: clinical, endocrine and metabolic consequences. Horm Res. 2009;72(3):129-41.
443. Domené HM, Hwa V, Jasper HG, Rosenfeld RG. Acid-labile subunit (ALS) deficiency. Best Pract Res Clin Endocrinol Metab. 2011;25(1):101-13.
444. Veraldi KL, Gibson BT, Yasuoka H, Myerburg MM, Kelly EA, Balzar S, et al. Role of insulin-like growth factor binding protein-3 in allergic airway remodeling. Am J Respir Crit Care Med. 2009;180(7):611-7.
445. Niu R, Liu Y, Zhang Y, Zhang Y, Wang H, Wang Y, et al. iTRAQ-Based Proteomics Reveals Novel Biomarkers for Idiopathic Pulmonary Fibrosis. PLoS One. 2017;12(1):e0170741.
446. Uribarri M, Hormaeche I, Zalacain R, Lopez-Vivanco G, Martinez A, Nagore D, et al. A new biomarker panel in bronchoalveolar lavage for an improved lung cancer diagnosis. J Thorac Oncol. 2014;9(10):1504-12.
447. Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. J Immunol. 2003;171(1):380-9.

448. Bergeron C, Tulic MK, Hamid Q. Airway remodelling in asthma: from benchside to clinical practice. Can Respir J. 2010;17(4):e85-93.
449. Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest. 2007;87(9):858-70.
450. Bianchetti L, Barczyk M, Cardoso J, Schmidt M, Bellini A, Mattoli S. Extracellular matrix remodelling properties of human fibrocytes. J Cell Mol Med. 2012;16(3):483-95.
451. Bellini A, Marini MA, Bianchetti L, Barczyk M, Schmidt M, Mattoli S. Interleukin (IL)-4, IL-13, and IL-17A differentially affect the profibrotic and proinflammatory functions of fibrocytes from asthmatic patients. Mucosal Immunol. 2012;5(2):140-9.
452. Westergren-Thorsson G, Chakir J, Lafrenière-Allard MJ, Boulet LP, Tremblay GM. Correlation between airway responsiveness and proteoglycan production by bronchial fibroblasts from normal and asthmatic subjects. Int J Biochem Cell Biol. 2002;34(10):1256-67.
453. DeVries A, Wlasiuk G, Miller SJ, Bosco A, Stern DA, Lohman IC, et al. Epigenome-wide analysis links SMAD3 methylation at birth to asthma in children of asthmatic mothers. J Allergy Clin Immunol. 2017;140(2):534-542.
454. Yamashita Y, Nakada S, Yoshihara T, Nara T, Furuya N, Miida T, et al. Perlecan, a heparan sulfate proteoglycan, regulates systemic metabolism with dynamic changes in adipose tissue and skeletal muscle. Sci Rep. 2018;8(1):7766.
455. Redington AE, Roche WR, Madden J, Frew AJ, Djukanovic R, Holgate ST, Howarth PH. Basic fibroblast growth factor in asthma: measurement in bronchoalveolar lavage fluid basally and following allergen challenge. J Allergy Clin Immunol. 2001;107(2):384-7.

456. Nilsson SC, Sim RB, Lea SM, Fremeaux-Bacchi V, Blom AM. Complement factor I in health and disease. Mol Immunol. 2011;48(14):1611-20.
457. Blaum BS. The lectin self of complement factor H. Curr Opin Struct Biol. 2017;44:111-118.
458. Xue X, Wu J, Ricklin D, Forneris F, Di Crescenzio P, Schmidt CQ, et al. Regulator-dependent mechanisms of C3b processing by factor I allow differentiation of immune responses. Nat Struct Mol Biol. 2017;24(8):643-651.
459. Sim RB, Ferluga J, Al-Rashidi H, Abbow H, Schwaeble W, Kishore U. Complement factor H in its alternative identity as adrenomedullin-binding protein 1. Mol Immunol. 2015;68(1):45-8.
460. Hagner S, Welz H, Kicic A, Alrifai M, Marsh LM, Sutanto EN, et al. Suppression of adrenomedullin contributes to vascular leakage and altered epithelial repair during asthma. Allergy. 2012;67(8):998-1006.
461. Weiszhár Z, Bikov A, Gálffy G, Tamási L, Ungvári I, Szalai C, et al. Elevated complement factor H levels in asthmatic sputa. J Clin Immunol. 2013;33(2):496-505.
462. Hallström T, Zipfel PF, Blom AM, Lauer N, Forsgren A, Riesbeck K. Haemophilus influenzae interacts with the human complement inhibitor factor H. J Immunol. 2008;181(1):537-45.
463. Malm S, Jusko M, Eick S, Potempa J, Riesbeck K, Blom AM. Acquisition of complement inhibitor serine protease factor I and its cofactors C4b-binding protein and factor H by Prevotella intermedia. PLoS One. 2012;7(4):e34852.
464. Dobó J, Schroeder V, Jenny L, Cervenak L, Závodszky P, Gál P. Multiple roles of complement MASP-1 at the interface of innate immune response and coagulation. Mol Immunol. 2014;61(2):69-78.

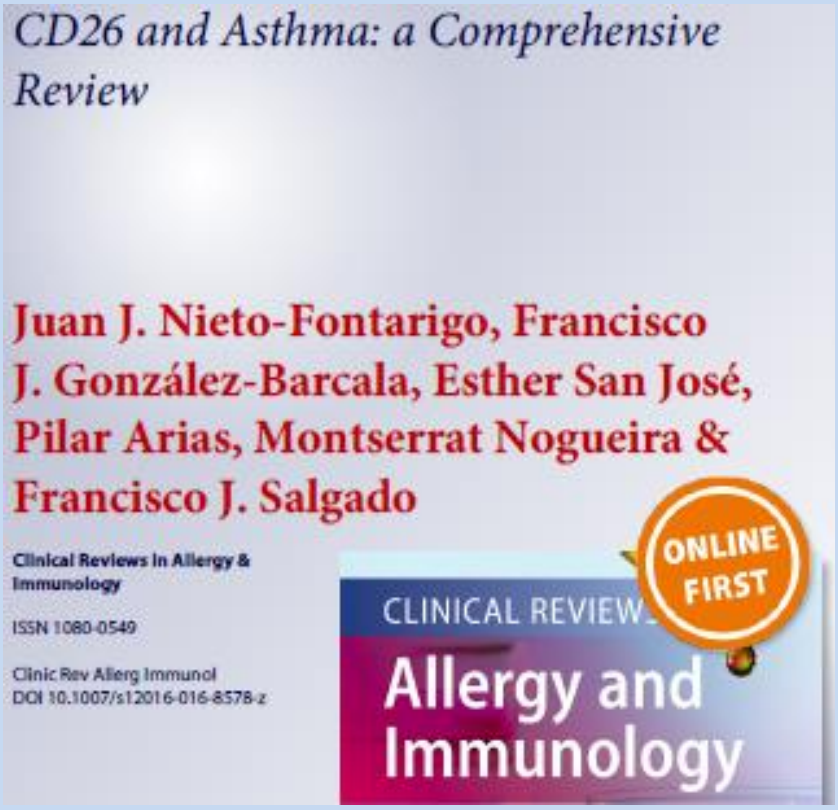
465. Chang HS, Lee TH, Jun JA, Baek AR, Park JS, Koo SM, et al. Neutrophilic inflammation in asthma: mechanisms and therapeutic considerations. Expert Rev Respir Med. 2017;11(1):29-40.
466. Panettieri RA Jr. The Role of Neutrophils in Asthma. Immunol Allergy Clin North Am. 2018;38(4):629-38.

APPENDIX I

CD26 and asthma: A Comprehensive Review.

Nieto-Fontarigo JJ, González-Barcala FJ, San José E, Arias P, Nogueira M, Salgado FJ.

Clin Rev Allergy Immunol. 2016 Aug 25. [Epub ahead of print] Review. https://doi.org/10.1007/s12016-016-8578-z



USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CD26 and asthma: A Comprehensive Review.

Authors:

Juan J. Nieto-Fontarigo[1], Francisco J. González-Barcala[2], Esther San José[3], Pilar Arias[1], Montserrat Nogueira[1], Francisco J. Salgado[1].

Affiliations:

[1]Department of Biochemistry and Molecular Biology, Faculty of Biology-Biological Research Centre (CIBUS), University of Santiago de Compostela, Santiago de Compostela, Spain.

[2]Pneumology Service, USC University Hospital Complex (CHUS), Santiago de Compostela, Spain; Interdisciplinary Group of Investigation in Pneumology, Santiago de Compostela Health Research Institute (IDIS), Santiago de Compostela, Spain.

[3]Clinical Analysis Service, USC University Hospital Complex (CHUS), Santiago de Compostela, Spain.

Correspondence:

Francisco Javier Salgado Castro, Department of Biochemistry and Molecular Biology, Faculty of Biology-Biological Research Centre (CIBUS) , University of Santiago de Compostela, Santiago de Compostela, 15782, A Coruña, Spain. E-mail: franciscojavier.salgado@usc.es. Phone: +34 881816941.

Acknowledgments

This project was funded by *Instituto de Salud Carlos III, Ministerio de Economía y Competitividad* (PI13/02046) and by *Sociedad Española de Neumología y Cirugía Torácica, SEPAR* (121/2012).

ABSTRACT

Asthma is a heterogeneous and chronic inflammatory family of disorders of the airways with an increasing prevalence that results in recurrent and reversible bronchial obstruction and expiratory airflow limitation. These diseases arise from the interaction between environmental and genetic factors, which collaborate to cause increased susceptibility and severity. Many asthma susceptibility genes are linked to the immune system or encode enzymes like metalloproteases (e.g., ADAM-33) or serine proteases. The S9 family of serine proteases (prolyl oligopeptidases) is capable to process peptide bonds adjacent to proline, a kind of cleavage-resistant peptide bonds present in many growth factors, chemokines or cytokines that are important for asthma. Curiously, two serine proteases within the S9 family encoded by genes located on chromosome 2 appear to have a role in asthma: CD26/dipeptidyl peptidase 4 (DPP4) and DPP10. The aim of this review is to summarize the current knowledge about CD26, and to provide a structured overview of the numerous functions and implications that this versatile enzyme could have in this disease, especially after the detection of some secondary effects (e.g., viral nasopharyngitis) in type II diabetes mellitus patients (a subset with a certain risk of developing obesity-related asthma) upon CD26 inhibitory therapy.

KEYWORDS:

CD26, DPP4, asthma, cytokines and chemokines, sCD26, CD26 inhibitors.

1.- DEFINITION AND GENETIC FACTORS IMPLICATED IN ASTHMA

Asthma is a heterogeneous and chronic disease characterized by reversible expiratory airflow limitation, bronchial hyperresponsiveness, mucous cell hyperplasia, higher vasculature permeability, airway remodelling with fibrosis and inflammatory cell infiltration [1, 2]. Asthma is a major concern, with 334 million people worldwide affected, increasing prevalence [3] and a high economic charge [4]. It is more frequent and severe in boys until the age of 13 years, but both prevalence and severity of asthma rise in women after puberty, becoming even more prevalent in women [3, 5, 6]. 5-10% of cases display a highly severe and treatment-refractory disease, suffering from recurrent exacerbations that threaten patient´s life and increase the health care costs. This is a complex pathology, with both genetic and environmental factors causing increased susceptibility and severity. Great efforts have been made to discover the genetic bases (mostly GWAS), revealing the influence of several genes encoding proteins with an important role in the immune system (HLA-DQ, HLA-G, IL1RL1, IL18R, TSLP, PDE, IL-33, LRRC32, SMAD3, IL2RB, IL6R, IL13) and also proteases (ADAM33, and DPP10) [7-9].

2.- ASTHMA AND THE PROLYL OLIGOPEPTIDASE FAMILY OF PROTEASES: DIPEPTIDYL PEPTIDASE 10 (DPP10) AND CD26/DPP4

As commented above, proteases are involved in many physiological and pathological processes, including chronic respiratory conditions like asthma. Amongst all proteases, there are only a few proline-specific enzymes, as peptide bonds adjacent to proline (present in some growth factors, chemokines or cytokines) are resistant to

cleavage [10]. These enzymes include serine proteases, with a serine residue in their catalytic region (sequence consensus Gly-Xaa-**Ser**-Xaa-Gly) [11] and covering different families (S1-S81) and subfamilies. Thus, the S9 family (prolyl oligopeptidases) includes from S9A to S9D, all with the catalytic triad Ser, Asp, His but with slightly different sequence consensus around the catalytic Ser. For example, most of members of the S9B subfamily (EC 3.4.14.5; CD26/DPP4, DPP8, DPP9, and fibroblast activation protein/FAP/Seprase) (http://merops.sanger.ac.uk/) present the sequence Gly-Trp-**Ser**-Tyr-Gly-Gly, while the other additional two members, DPP6 (DPPX; Gly-Lys-**Asp**-Tyr-Gly-Gly) and DPP10 (Gly-Lys-**Gly**-Tyr-Gly-Gly), do not possess the catalytic serine and, therefore, DPP4 activity [12]. Curiously, four of these peptidases (CD26, FAP, DPP6, and DPP10) are type II membrane proteins released to the extracellular medium, three are encoded by genes located on chromosome 2 (CD26, FAP and DPP10), and only two (CD26, DPP10) appear to have a role in asthma [13-16]. For example, DPP10 have been linked to asthma susceptibility in different populations [7, 13, 15], and this protein (as well as DPP8 and DPP9) has been primarily located in the trachea and the bronchi of the airways in rats [17]. For its part, the protease CD26 is the major member of the S9B family and also a receptor for the Middle-East respiratory syndrome coronavirus (MERS-CoV) [18, 19], a new coronavirus that causes severe lower respiratory tract infections that could lead to asthma exacerbations. CD26 has been found elevated in both plasma samples (sCD26) and the surface of peripheral $CD4^+$ T cells from adult patients with allergic asthma [20, 21], and different animal models [14, 16, 22] suggest a role of this peptidase in the pathogenesis of this disease.

3.- STRUCTURE AND DISTRIBUTION OF CD26

3.1.- Structure of the CD26 molecule

CD26 is a single-pass type II integral membrane glycoprotein of 105-110 kDa [23]. Only the homodimeric form of CD26 is biologically active [24-27]. Homodimerization takes place in both the Golgi apparatus [28] and the endoplasmic reticulum [29]. Each monomer displays a highly conserved and short (6 amino acids) cytoplasmic tail at the N-terminus, a 22 amino acid hydrophobic transmembrane region and a long extracellular domain of 738 amino acids. The heavily N-glycosylated extracellular domain can be subdivided, in turn, in several regions. The closest to the amino-terminal part starts with a flexible stalk region and contains 8 out of 10 possible N-glycosylation sites, while the intermediate region is highly enriched in cysteines (9 out of 12). Finally, the active centre is located towards the carboxyl-terminal end and is another highly conserved region [30-34].

3.2.- Tissue and cell distributions of CD26

Human CD26 is broadly distributed in a variety of cell types, tissues, and organs [35-39]. Lungs display the second highest CD26 activity amongst organs, especially in lung parenchyma [17, 22]. Contrary to DPP8/9 and DPP10, bronchi almost no express CD26 [17], but can be found in the apical membrane of epithelial cells, capillary endothelial cells, fibroblasts and serosal submucosal glands of human bronchi [40-42]. In most of these places, CD26 levels are constitutive and highly correlated with the mRNA content (http://www.proteinatlas.org/ENSG00000197635-DPP4/tissue) [43]. However, IL-13 causes a strong proinflammatory upregulation of CD26 in the airway epithelial cells [44]. Moreover, both DPP4 activity and CD26 protein (but not mRNA) increase after allergen

exposure in lung parenchyma in a rat model of allergic airway inflammation [17], suggesting that CD26 on lung epithelium could be important in asthma pathogenesis [45].

CD26 is also expressed in the Immune System, being detected in medullar thymocytes, T cell areas of spleen and lymph nodes, peripheral blood T cells and, at a lesser extent, B cells, NK cells, monocytes/macrophages and granulocytes [24, 25, 30, 41, 46, 47]. CD26 density in these cells is heavily controlled and augments upon cell activation and acquisition of a memory phenotype, especially in the T cell lineage [33, 41, 48]. Human T lymphocytes display variable basal expression of CD26 ($CD4^+$ T >> $CD8^+$ T cells), which depends on the individual and the mAb used. Despite not all resting T cells are $CD26^+$, most of them contain CD26 mRNA and display CD26 molecules on the surface 4-8 hours after stimulation [49-51]. However, only a certain regulation on CD26 mRNA levels has been detected by either northern blot [36, 50, 52-54] or gene arrays [55] upon activation. Additionally, CD26 is upregulated by IFNγ on renal epithelial cells [56] and B-CLL [57] through the modification of mRNA levels.

Despite the above-described adjustment of CD26 expression through mRNA levels, several pieces of evidence support the presence of upstream control levels. Thus, CD26 is strongly regulated at protein level by several soluble factors. For example, the T_{H1} cytokine IL-12 (and to a lesser extent IL-2) enhances the expression of CD26 on both activated T cells [58, 59] and NK cells (together with IL-15) [47, 60]. On the contrary, IFNγ (another T_{H1} cytokine) has no effect on CD26 levels in these lymphocytes [47, 58]. Regarding T_{H2} cytokines, IL-4 promotes the expression of CD26 on human B lymphocytes activated with *Staphylococcus aureus cowan I* [61, 62], while this cytokine moves from a lack of effect [63] or a certain downmodulation of CD26 levels in T cells at high concentrations [unpublished results]. In

addition, *in vitro* exposure to regulatory T cells (Treg)-derived soluble factors like TGFβ1 [64, 65] or Ado (adenosine) [66] leads to diminished CD26 levels.

Most of peripheral blood Treg lymphocytes display an "activated-like" (e.g., $CD25^{high}$), "memory" ($CD45RO^{+}$) and "anergic/apoptosis-prone" phenotype [67] and were expected to express other activation/memory markers such as CD26. This seems to be the case for rats, where $CD25^{+}$ (Treg) and $CD25^{-}$ (effector T cells or Teff) subsets of peripheral $CD4^{+}$ T cells show equivalent CD26 expression [68]. In contrast, human Treg cells ($CD4^{+}CD25^{high}$ or $CD4^{+}FoxP3^{high}$) display lower levels of CD26 compared to Teff lymphocytes ($CD4^{+}CD25^{-/low}$ or $CD4^{+}FoxP3^{-/low}$) [69-72]. This likely explains why cytokines that favour "effector" responses (e.g., IL-12) cause a strong upregulation of CD26 on "bulk" T_H cell cultures, while others important for the Treg homeostasis (e.g., IL-2, IL-15) only induce a slight increase on CD26 levels [58, 73]. It also gives a clue on why CD26 is downmodulated upon exposure of T cells to TGF-β1 [64, 65].

CD26 expression amongst Teff lymphocytes is also variable, and T_{H17} cells appear to display the highest levels of CD26 according to the following order: $T_{H17}>>T_{H1}>T_{H2}$ [72, 74-77]. Furthermore, two subsets were reported amongst T_{H17} cells on the base of CCR4 expression [78], but only the $CCR4^{-}$ T_{H17} subset (and not the TGF-β-secreting $CCR4^{+}$ T_{H17} subpopulation) has a $CD26^{high}$ phenotype [79]. This finding likely indicates that the degree of phenotypic diversity found in Teff cells for CD26 levels is also probably present in Tregs and reflects the presence of functionally different subsets that mirror the corresponding Teff subsets [80].

The different expression levels of CD26 in Treg and Teff lymphocytes could depend on a variation in CD26 mRNA levels as it happens with "regulatory-like" $CD4^{+}$ T cells in classical Hodgkin´s

lymphoma [81]. However, it is worth to mention that, apart from regulating this set of CD26 mRNA molecules, there is an intracellular pool of CD26 protein maintained by continuous translation in human T cells (regardless their $CD26^+$ or $CD26^-$ phenotype) [50] that can be mobilized towards the plasma membrane [54] or released into the extracellular space. Therefore, there could be a number of additional mechanisms leading to a $CD26^{-/low}$ phenotype in human Treg cells.

3.3.- Body fluid compartments and distribution of soluble CD26

CD26 can be found in the extracellular space, with autocrine, paracrine or endocrine effects. The soluble version of CD26 (sCD26) has been described in many biological fluids (e.g., serum, plasma, synovial fluid, cerebrospinal fluid) from different organisms [82]. Bronchoalveolar lavage contains DPP4 enzymatic activity in rats [17] and humans [83]. In serum or plasma at least 90% of this activity is associated to a heavily glycosylated 110 kDa CD26 isoform [84, 85], whose concentration displays a normal distribution [86] and high biological variability [87, 88] that seems to depend on pre-analytical variables, like age or gender. Thus, serum sCD26 concentration increased in up to 10-12 years in children, after which the values start decreasing [89]. In adults, it has been also detected a slight decrease of sCD26 or DPP4 activity in serum with age [87, 88], but other studies did not describe such correlation [86, 90]. In addition, no differences were detected regarding gender in some studies [90], while different authors did find a higher sCD26 concentration in serum/plasma samples [86-89, 91] but a lower CD26 expression on $CD4^+$ T cells (our unpublished results) from males.

Several studies have found a positive correlation between sDPP4 activity and sCD26 in humans [91-93], but others have reported a low correlation instead, with different explanations like the

presence of hypersialylated CD26 isoforms or alternative proteins with DPP4 activity [82]. Thus, plasma samples contain a soluble glycoprotein named DPPT-L or attractin without homology with CD26 but with DPP4 activity [94-96], although this activity has been questioned more recently [97]. Attractin is accumulated in the plasma membrane upon T cell receptor (TCR) triggering (~24h) [95, 96], especially in $CD26^{high}$ Teff cells (our unpublished results), and released into the plasma at 48-72h [95, 96]. Curiously, linkage disequilibrium studies show an association of asthma with a region including the attractin (ATRN) gene, which is upstream of the gene encoding the secretase/sheddase ADAM-33 (an asthma susceptibility gene) [97].

Release of CD26 could be accomplished by a classical or non-classical pathway, and either by a constitutive or induced mechanism. The classical pathway requires vesicular traffic from the endoplasmic reticulum/Golgi towards the plasma membrane. Intravesicular proteins are liberated in the extracellular medium after membranes merging (exocytosis), while transmembrane proteins appear in the secretome through proteolysis mediated by “secretases”/“sheddases”. This last process ("shedding") delivers growth factors, cytokines, receptors and probably molecules like CD26 [98]. This is additionally supported by the lack of posttranscriptional splicing in CD26 mRNA, the absence of cytoplasmic and transmembrane domains in sCD26, or results from pulse chase and transfection experiments [25, 82]. However, CD26 has been detected in microvesicles and exosomes from lymphocytes (http://www.exocarta.org). Therefore, constitutive or induced release of $CD26^{+}$ vesicles (exosomes, microvesicles or apoptotic bodies) into the medium could also contribute to the pool of sCD26 in the circulation and reflect the cell subset of origin (“cell lineage fingerprint").

The cell source of sCD26 is still controversial [25, 82]. Visceral fat, at least in obese patients, overexpresses CD26 and releases this "adipokine" into the circulation [88], and there are data supporting the presence of a positive correlation between fasting sCD26 levels and body mass indexes/BMI >25 [88, 99]. sCD26 may also originate from other sources, such as endothelial cells (e.g. lung) or epithelial cells from liver (bile canaliculi) or kidney. However, immune cells are also a likely source [25, 82, 100]. Thus, a transplantation model in rats has determined that, under healthy conditions, bone marrow-derived cells represent a significant source for sCD26 [101]. Therefore, the concentration of sCD26 could also be influenced by the number of lymphocytes [102] or mirror the predominant phenotype of circulating $CD4^+$ T lymphocytes in a pathological situation.

4.- CD26 AND ASTHMA

4.1.- Asthma phenotypes, disease severity and CD26 levels in $CD4^+$ T cells.

$CD4^+$ T_H cells play a central role in adaptive immune responses and the induction/persistence of asthma and other allergic/atopic diseases. They are plastic and heterogeneous lymphocytes, with four main lineages (T_{H1}, T_{H2}, T_{H17} and Treg cells) and different roles. For example, T_{H2} (GATA-3^+) cells infiltrate airways in allergic asthma and produce a set of cytokines (IL-4, IL-5, IL-13) important for airway remodelling, leucocytosis, eosinophilia, macrophages/mast cells activation, and B-cell dependent IgE elevation [103, 104]. In the same way that T_H lymphocytes are heterogeneous, there are also a number of asthma phenotypes as a likely reflection of the T_H cells heterogeneity itself, with both T_{H2}^{high} and T_{H2}^{low} phenotypes sharing

different clinical signs: a) the *early allergic asthma*, with a strong familial background and mediated by T_{H2} cytokines (T_{H2}^{high}) and allergen-specific IgE (atopy); b) the less-allergic *late-onset asthma* (T_{H2}^{high}), which is characterized by eosinophilic inflammation, absence of specific IgE (i.e., non-atopic) and worse response to corticosteroids; c) *neutrophilic asthma*, a T_{H2}^{low} phenotype refractory to corticosteroids and linked to T_{H17}-responses, sputum neutrophilia, and augmented levels of IL-8 and IL-17; d) and the *obesity-related asthma*, a T_{H2}^{low} phenotype predominant in obese women, with higher levels of TNFα, IL-6 and leptin but low numbers of eosinophils [1] (Table 1). Therefore, alternative $CD26^{+/high}$ effector T_H subpopulations are gaining importance in asthma, particularly as the disease becomes chronic and refractory to treatment. In this context, even though it was initially described that $CD26^{+}$ T_{H1} cells were protective ("hygiene hypothesis"), now it is considered that these cells could be favouring the inflammation of the airways [105]. In addition, proinflammatory $CD26^{high}$ T_{H17} cells have been detected in biopsies from asthma patients, and their cytokines (e.g., IL-17, IL-6, IL-21, IL-22) involved in eosinophilia/neutrophilia and higher severity during acute attacks [106]. On the other hand, Treg cells ($FoxP3^{+}$, $CD26^{-/low}$) also seem to play a relevant role in controlling exaggerated T_{H2} responses and asthma development [9, 107, 108]. Treg cells exert their suppressor activity by using both soluble (e.g., adenosine, TGFβ, IL-10) and membrane-associated (e.g., TGFβ, CTLA-4) molecules [67], and a number of GWAS in asthma have identified some genes (e.g., IL2RB, SMAD3, GARP/LRRC32) linked to this suppressor activity. Thus, the lower or higher prevalence of different Teff subsets and/or the distortion of the Teff/Treg balance could also alter the phenotype and severity of asthma [104, 109, 110], Moreover, depending on the asthma phenotype or the severity of this disease, the levels and functions of CD26 on the surface of immune cells and biofluids could be rather different and be a factor influencing disease development.

Therefore, it is time to ask what the biological functions of CD26 are and what their connection with asthma is.

Table 1. Asthma molecular and clinical classification

	Symptomatology and pathophysiology	Pathobiology	Therapy response
Th2-High Phenotype			
Early onset ***Allergic***	Allergic and rhinitis symptoms. Moderate to severe.	Elevated levels of IL4, IL5 and IL13 (Th2-related chemokines), specific IgE and thicker sub-epithelial basement membrane.	Corticosteroid response and Th2-related targets
Late onset ***Eosinophilic***	Presence of sinusitis and less allergic. Normally severe.	Eosinophillia and IL5 elevation (Th2-related chemokine)	Against IL5-Ab response and to cysteinyl leukotriene modifiers. Refractory to corticosteroids.
Th2-Low Phenotype			
Obesity-related	Mostly women, very symptomatic, epithelial hiperresponsivenes.	Loss Th2-markers and oxidative stress.	Response to weight loss, to antioxidants and to hormonal therapy.
Late onset ***Neutrophilic***	Low FEV1.	Sputum Neutrophilia, Th17 and IL8 ways.	Refractory to corticosteroids and to other asthma medicines. Possibly response to macrolide antibiotics.

IL5 = Interleucin 5, IL8 = Interleucin 8, FEV1 = Force expiratory volum in 1 s, Ab= Antibody.

Modified table from Martínez *et al.* [9].

4.2.- Enhancing functions of both membrane and soluble CD26 on asthma

Functionally speaking, CD26 is a complex molecule with both harmful and beneficial activities in relation to asthma. With regard to the first ones, CD26 has been traditionally linked to both the amplification of TCR-mediated stimulatory signals and cell adhesion/migration. These functions could be dependent on production of certain biomolecules, the enzymatic activity of CD26 or, the extracellular association with other proteins: adenosine deaminase (ADA) [111], CD45 [112], caveolin-1 [113], CXCR4 [114], collagen [115], plasminogen 2 [116], glypican-3 [117] or fibronectin III [118, 119] (Fig. 1). For example, CD26-mediated costimulation plays a role in the migration of mesothelioma cells through upregulated production of a current biomarker of "T_{H2}^{high}" asthma: periostin [120, 121]. CD26 location could also be important for this harmful function of CD26 in asthma. As a raft resident protein [112, 122], CD26 improves the immunological synapse formation between antigen-presenting cells (APC) and T cells [112, 113], important for T_H cells proliferation/differentiation and to empower these lymphocytes to proportionate help to B cells and produce immunoglobulins like IgE [30] (Fig. 1). This raft-dependent costimulatory function of CD26 could be mediated by other proteins like CD45, a tyrosine phosphatase (PTPase) essential for TCR signalling [123]. Crosslinking with anti-CD26 antibodies in T lymphocytes drives the recruitment to rafts and the interaction of both CD26 and CD45RO [112], which probably causes CD45 dimers dissociation [112], increased PTPase activity [124] and a signal transduction cascade that both overlaps with and boost the TCR-pathway [112, 125-128] (Fig. 1).

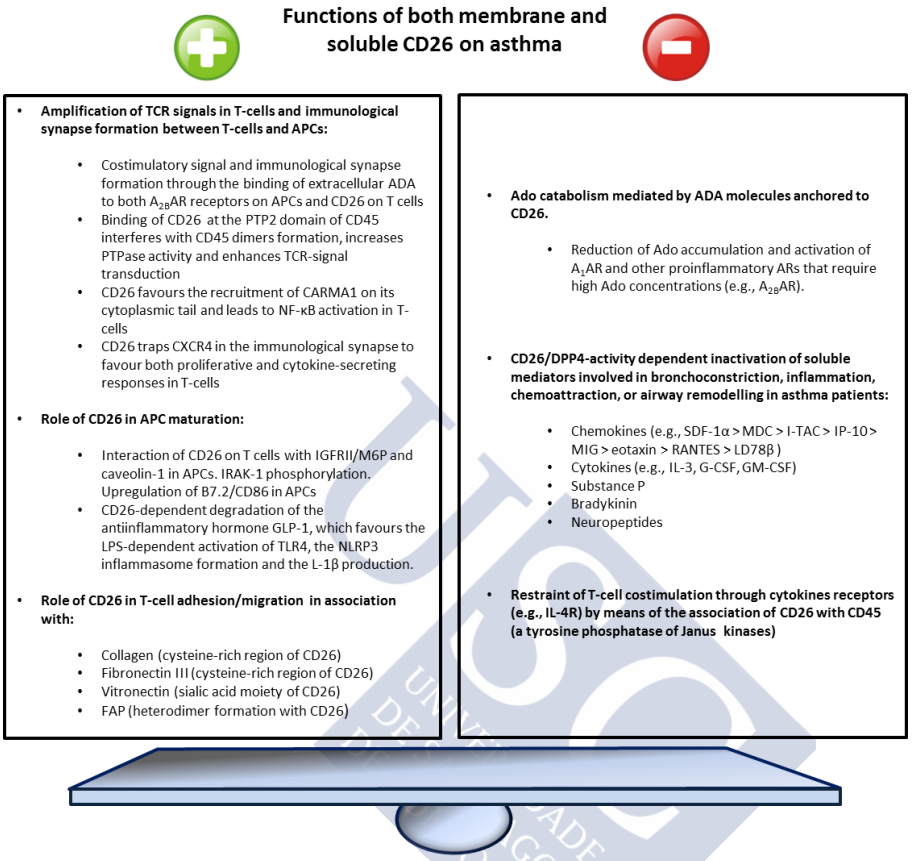


Fig 1. Functions of both membrane and soluble CD26 on asthma. This figure summarizes both positive (i.e., those that favour asthma development or exacerbation) and negative (i.e., those that prevent asthma development or exacerbation) functions of CD26 on this disease.

This potentially detrimental function of CD26 to the lungs of a person with asthma seems to involve the active centre [112, 129], but not necessarily the DPP4 activity. Thus, CD26 on T_H cells recognizes caveolin-1 and induces the upregulation of the proinflammatory B7.2/CD86 molecule in APCs [113, 129]. In T cells, CD26-caveolin-1 interaction induces the coalescence of lipid rafts, recruitment of

CARMA1 and activation/nuclear translocation of the proinflammatory and "rapid-acting" transcription factor NF-κB [130] (Fig. 1). Therefore, the $CD26^{+/high}$ phenotype of Teff cells may favour allergen-induced inflammation in lungs [131], while the $CD26^{-/low}$ phenotype of Treg cells may contribute to keep low levels of CD86 on APCs and trigger anergy/apoptosis in allergen-specific naïve T cells. However, all these findings of the costimulatory role of CD26 need further clarification, as either a partial or complete genetic deletion of CD26 or the use of CD26 inhibitors do not impact this function [132, 133].

The costimulatory actions of CD26 also could depend on the interaction with another enzyme: ADA [111, 134-136]. Most of ADA molecules in lymphocytes are cytosolic, but a small proportion (10%) are associated with either CD26 [111, 134, 135] or adenosine (Ado) receptors (ARs; A_1AR and A_{2B}AR) on the T cell membrane (ecto-ADA) [114, 135-138]. Binding of ADA to CD26 takes place in human (but not murine) lymphocytes [25, 63] and this interaction could potentially trigger a costimulatory signal that favour lymphocytes proliferation and T_{H1} cytokines and chemokines production [139-141] (Fig. 1). In turn, activation through the TCR/CD3 complex and costimulation with T_{H1} (but not T_{H2}) cytokines increase the number of ecto-ADA molecules [63, 139] and the formation of the ADA-CXCR4-CD26 triad [114], which might help to trap the chemokine receptor CXCR4 at the immunological synapse to make lymphocytes insensitive to the chemokine SDF1α (stromal cell-derived factor 1 /CXCL12) and trigger a proliferative and cytokine-secreting response [142] (Fig. 1).

Costimulatory effects could also come from sCD26, which is capable of enhancing the T-cell mediated reaction against recall antigens (e.g., tetanus toxoid) or suboptimal amounts of polyclonal stimuli (PHA or anti-CD3) [143, 144]. Contrary to initial results [143], the most recent data point out that this proinflammatory effect

of sCD26 is not dependent on CD26 or ADA-binding activities [145] and is likely triggered upon interaction with proteins like caveolin-1 [113, 129, 130] or insulin-like growth factor II/mannose-6-phosphate (IGF-II/M6P) receptor [146, 147] in APCs or protease-activated receptor 2 (PAR2) in smooth muscle cells [148]. sCD26 promotes CD86 upregulation in APCs [113, 129, 146] and the generation of reactive oxygen species (ROS) and Toll-like receptors [149] (Fig. 1).

CD26 is the major member of the prolyl oligopeptidase family, and cleaves X-proline or X-alanine dipeptides from the N-terminus of different polypeptides, even though other amino acids are accepted with lower efficiency: Pro>Ala>Hyp>>Ser>Gly>Val>Leu [25]. Apart from the primary sequence, the 3D structure, size and substrate ionization (optimum pH 7.5-8.5) significantly influence the catalytic efficiency of CD26 [10, 94, 100, 150, 151]. Peptides containing X-proline or X-alanine at the N-terminus are not easy to process by most of the proteases [152], which means that CD26 has a key biological role. Indeed, DPP4 activity has been linked to the activation of T lymphocytes in response to extracellular stimuli. For example, Jurkat cells transfected with CD26 display greater activation than those expressing DPP4-deficient CD26 molecules [153]. DPP4 activity is also necessary for sCD26 molecules to boost the tetanus toxoid dependent proliferation [146]. CD26 inhibitors yield similar results *in vitro*, with suppression of T cell proliferation and secretion of proinflammatory cytokines but enhanced release of TGFβ1 [154-157]. *In vivo*, the use of CD26 inhibitors in murine models of human diseases like rheumatoid arthritis [158], multiple sclerosis [159], or asthma (aerosolized simultaneously with the allergen) [45] also describes a proinflammatory role of CD26. Moreover, CD26 inhibitors also support the positive role of CD26 in NLRP3 inflammasome formation [160]. However, enzyme activity is required, but not in absolute terms, during the costimulatory function of CD26,

since this biological activity is retained after deleting part of the hydrolase domain [132] (Fig. 1).

4.3.- Inhibitory functions of both membrane and soluble CD26 on asthma

Apart from its costimulatory and asthma-detrimental role, there is also evidence that CD26 exhibits other asthma-preventive activities. This protective function of CD26 is linked to the indirect or direct control of different soluble mediators like Ado, bradykinin or neuropeptides that are involved in bronchoconstriction, inflammation, chemoattraction, or airway remodelling in asthma patients [161]. For instance, the nucleoside Ado is the substrate of ADA, an enzyme that catalyses its irreversible deamination to inosine [139, 140]. As already mentioned, ADA has been found anchored to CD26 (Fig. 1) in many cells but two frequently regarded proinflammatory ARs (A_1AR and A_{2B}AR) also interact with this enzyme, which seems important for their efficient ligand-dependent signalling [111, 134-138]. ARs are novel targets for the treatment of human asthma, and some antagonists and agonists have entered the clinical phase with a not totally clear efficacy [161, 162]. Indeed, there is an elevated expression of asthma-favouring and ADA-interacting receptors A_1AR and A_{2B}AR in bronchial tissue [161, 162], but probably reduced amounts of asthma-protective A_{2A}AR [161]. In addition, there is an augmented Ado concentration in the extracellular compartment in asthma. This nucleoside plays a detrimental role in this disease by causing bronchoconstriction, airway inflammation (e.g., mast cells degranulation, airway accumulation of eosinophils), airway remodelling, edema, or mucus production [161, 162]. These harmful effects also depend on the specific engaged type I purinergic receptor and the particular cells expressing the ARs in airways (e.g., APCs,

lymphocytes, eosinophils, neutrophils, endothelial and mast, epithelial, endothelial, and smooth muscle cells) [161, 162]. This role of Ado in asthma is affected by binding affinity, receptor density and local levels of this nucleoside, which are influenced by a delicate balance where different mechanisms are involved (e.g., extracellular/intracellular Ado metabolism) [161, 162]. Amongst these mechanisms, the Ado catabolism mediated by ADA molecules anchored to CD26 is the major regulated pathway that influences the local concentration and biological effects of Ado. In this respect, relevant T_{H2} cytokines in allergic asthma (IL-4, IL-13) reduce ADA activity in the lung [161] and ecto-ADA levels on T_H lymphocytes [63]. These findings suggest that low levels of CD26/ecto-ADA on the T_{H2} subset in T_{H2}^{high} (allergic/atopic) asthma could favour the local accumulation of Ado, the activation of A_1AR, and the participation of proinflammatory ARs that require Ado concentrations over the physiological levels (e.g., the low-affinity $A_{2B}AR$) in order to neutralize the off-signals provided by the engagement of high-affinity and asthma-protective $A_{2A}ARs$. Therefore, it is likely that ARs-targeted therapy is more effective in T_{H2}^{high} asthma than in T_{H2}^{low} or severe asthma, wherein CD26/ecto-ADA^{high} T_{H1} and T_{H17} cells are more frequent. However, additional studies are needed to clarify what could be exactly the role of Treg cells in asthma, a lymphocyte subset with a CD26/ecto-ADA^{low} phenotype that should favour the high production of Ado [70]. On the other hand, CD45 has been involved in Janus kinases dephosphorylation [163]. Therefore, the CD26-CD45 association could have a negative and DPP4-independent role important to restrain the signal transduction of cytokine receptors [122] (Fig. 1). In this sense, the low PTPase activity in human naïve $CD4^+CD45RA^+CD26^{-/low}$ T cells makes them susceptible to small amounts of IL-4, as mentioned an important cytokine in allergic asthma, while memory/effector $CD4^+CD45RO^+CD26^{+/high}$ T cells secrete this soluble factor upon activation, but are less sensitive to its

effects [164]. Indeed, high levels of CD26 in memory/effector T_H lymphocytes might act as a "brake" for the proliferative responses to cytokines (for example, IL-12; our unpublished results).

The protective function of CD26 in asthma could also be linked to the DPP4 activity (Fig. 1). Some CD26 substrates fall outside the scope of the immune system and are beyond the scope of this review, like incretins (GIP/glucose-dependent insulinotropic peptide, GLP-1/glucagon-like peptide-1, GLP-2/ glucagon-like peptide-2) [10, 165, 166], glucagon, PACAP (pituitary adenylate cyclase-activating polypeptide), GRP (gastrin-releasing peptide), peptide YY, vasoactive peptides (bradykinin and VIP/vasoactive intestinal peptide), natriuretic peptides (BNP/B-type natriuretic peptide), and neuropeptides (NPY/neuropeptide Y, beta-casomorphins, endomorphins, substance P) [41, 82, 166, 167] (Table 2). However, some others have an important immunomodulatory role, such as cytokines (e.g., IL-3, G-CSF, GM-CSF) [168] or chemokines (e.g., RANTES/CCL5, eotaxin/CCL11, MDC/CCL22) [82, 166]. CD26 enzymatic activity down-modulates the biological function of most of these chemokines and cytokines (Table 3). Consistent with this observation, it has been found that CD26 inhibitors can enhance some *in vivo* immune responses depending on the dose, application route, timing or predominant Teff subset [45, 169], which could be explained by the presence of off-targets effects as well [25, 170]. However, animal models of rheumatoid arthritis [93], multiple sclerosis [171], and inflammatory bowel disease [172] in CD26 KO mice do support an immunosuppressive role of both CD26 and DPP4 activity. This immunosuppressive function has also been observed for sCD26 during strong *in vitro* proliferative responses of immune cells [143, 145]. Moreover, CD26 has been even regarded as a tumour suppressor gene in melanomas or neuroblastomas [173, 174]. For this

reason, we will focus this part of this review on the CD26 substrates with a direct connection with the immune system like chemokines.

Table 2. Known substrates processed by CD26/DPP4 out of the immune system

Substrate name and family	N-terminal sequence	Effect of DPP4 processing	*In vitro* / *in vivo* evidence	Reference
β-casomorphins (neuropeptide)	Tyr-Pro▼Phe	Inhibitory	Yes/Yes	[10] [82] [156]
BNP (natriuretic peptide)	Ser-Pro▼Lys	Inhibitory Change in receptor preference	Yes/Yes	[82] [233]
Bradykinin (vasoactive)	Arg-Pro▼Pro	Inhibitory Change in receptor preference	Yes/Yes	[10] [82] [156]
Endormorphins (neuropeptide)	Tyr-Pro▼Phe	Inhibitory Change in receptor preference	Yes/Yes	[10] [41] [82] [156]
Enterostatin (grastointestinal hormone)	Val-Pro▼Asp	Inhibitory	Yes/Yes	[10] [41] [156]
GIP (incretin)	Tyr-Ala▼Glu	Inhibitory	Yes/Yes	[10] [41] [82] [154] [156]
GLP (GLP-1 [7-36]amide, GLP-1 [7-37][&] and GLP-2) (incretin)	His-Ala▼Glu (GLP-1) His-Ala▼Asp (GLP-2)	Inhibitory	Yes/Yes	[10] [41] [82] [154] [156]
Glucagon (gastrointestinal hormone)[*]	His-Ser▼Gln	Inhibitory	Yes/Yes	[82] [156]
GRH (GRH[1-29] and GRH[1-44] (hypothalamic hormone)	Tyr-Ala▼Asp	Inhibitory	Yes/No	[10]

Continued on next page

Table 1 (Continued)

GRP (hormone bombesin family)	Val-Pro▼Leu	Not known	Yes/Yes	[82]
NPY (neuropeptide)	Tyr-Pro▼Ser	Receptor Y1 Inactivation Change in receptor preference	Yes/Yes	[10] [41] [82] [156]
PACAP (PACAP27 and PACAP38)*	His-Ser▼Asp	Probably inhibitory	Yes/Yes	[82] [234]
Peptide YY (Pancreatic peptide)	Tyr-Pro▼Ile	Receptor Y1 Inactivation Change in receptor preference	Yes/Yes	[10] [41] [82] [156]
Substance P (neuropeptide)	Arg-Pro▼Lys	Inhibitory Questionable	Yes/Yes	[10] [41] [82] [156]
VIP peptides (VIP, PHV42, PHM27) (vasoactive)*	His-Ala▼Asp (PHV, PHM) His-Ser▼Asp (VIP)	Probably inhibitory	Yes/Yes	[10] [41] [82] [154] [156]

BNP: B-type natriuretic peptide; GIP: glucagon inhibitory peptide; GLP: glucagon like peptide; GRH: growth hormone-releasing hormone; GRP: gastrin releasing peptide; NPY: neuropeptide Y; PCAP: pituitary adenylate cyclase-activating polypeptide; VIP: vasoactive intestinal peptide; PHV42: peptide histidine valine 42; PHM27: peptide histidine methionine 27 *: Glucagon, PACAP and VIP have a Ser in position 2 and, with less efficiency that Pro or Ala, also can be DPP4 potential substrate; [&]: GLP-1 [7-36] amide and [7-37] are the active forms of GLP-1 and substrates of DPP4.

4.3.1.- CD26, T_{H2}-related chemokines and T_{H2}^{high} asthma.

Even though cytokines like IL-1β or IL-2 contain an appropriate N-terminal sequence, the molecular weight precludes their N-terminal clipping, with exceptions like IL-3, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), or Epo (erythropoietin) [168]. In contrast, CD26-dependent processing of chemokines (8-10 kDa) like RANTES (regulated on activation, normal T cell expressed and secreted) is one

of the most interesting aspects of CD26 biology [175]. Moreover, this function is mostly dependent on CD26 levels, which is influenced by the T cell subset or disease, as we have just seen.

Chemokines are proinflammatory cytokines with a role in leukocyte activation and migration. They are classified according to the position of two N-terminal cysteine residues (CC, CXC, C and CX3C) [176], but can also be divided as a function of the cells they attract or the receptor they recognize. As table 3 shows, the major branches of effector (T_{H1}, T_{H2}, and T_{H17}) and regulatory T cells express characteristic (but not totally selective) chemokine receptors [76, 103]. For example, T_{H2} lymphocytes and other leukocytes important in asthma (basophils, mast cells, and eosinophils) express CCR3, CCR4, CCR8, CXCR4 and, in humans, CRTH2 [103]. CCR3, CCR4, and CXCR4 interact with CD26-substrates, but only CCR3 and CCR4 are actually specific for T_{H2} cells. Regarding CXCR4, T_{H2} cells seem to display a slight overexpression compared to T_{H1} lymphocytes [177], but other authors describe CXCR4 as a merely trafficking marker [178-180].

Ligation of CCR3 with eotaxin/CCL11, RANTES/CCL5, and MCP-1/CCL2, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13 participates in the recruitment of basophils, eosinophils and mast cells [103, 181, 182] (Table 3). Eotaxin is an important chemokine in allergic asthma produced by endothelial cells and monocytes in response to IFNγ and TNFα, respectively. This chemokine is recognized by CCR3 (eosinophils, basophils, mast cells and T_{H2}) and a lesser extent by CCR5, a putative T_{H1}-marker (see later) [183]**.** Eotaxin is a chemoattractant for eosinophils that facilitates their mobilization from bone marrow [184]. Truncation of eotaxin by CD26 is characterized by an intermediate-low efficiency (k_{cat}/K_m: SDF-1α > MDC > I-TAC > IP-10 > MIG > eotaxin > RANTES > LD78β) [185]. Despite this, N-terminal clipping by CD26 results in an isoform (3-74)

with a reduced chemotactic activity that causes CCR3-desensitization [183, 186, 187]**.** Indeed, administration of eotaxin to F344 rats leads to a mobilization of eosinophils, an effect enhanced in CD26-deficient animals or with CD26 inhibitors [187]. Additionally, $CD26^{-/-}$ mice challenged with ovalbumin/OVA show higher levels of CCR3 and CCR3-ligands (eotaxin, RANTES) and stronger eosinophils infiltration in lungs [16]. Another ligand of CCR3 is CCL14 (Table 3), a chemokine processed by plasmin and urokinase plasminogen activator (UPA) to yield CCL14 (9-74). This isoform binds to CCR3 (and also CCR1 and CCR5) to efficiently attract eosinophils, monocytes and T_{H2} cells, a process also dampened by CD26 [188]. Therefore, a $CD26^{-/low}$ phenotype in both eosinophils and T_{H2} cells ($CCR3^{+}$) seems to favour the inflammatory response in T_{H2}^{high}-asthma.

CXCR4 is found in monocytes and B/T (preferentially T_{H2}) cells [177, 189] and promotes the recruitment of T-cells in the lungs during allergic airway diseases [178, 190]. CXCR4 recognizes macrophage migration inhibitory factor (MIF) and stromal cell-derived factor 1 (SDF1/CXCL12) [189] (Table 3), the last one a small chemokine synthesized by endothelial cells and fibroblasts that induces T/B cells activation [142] and regulates the traffic of lymphocytes, monocytes and dendritic cells toward inflamed epithelia [179, 189, 191, 192]. Amongst the several SDF1 isoforms, at least two (SDF1α and SDF1β) are efficiently processed by CD26 *in vitro* [185, 193-195] and *in vivo* [93]. The SDF-1α (3-67) isoform is unable to activate CXCR4 and displays antagonistic activity [185, 193-198]. Moreover, both CD26 and CXCR4 have similar expression kinetics upon activation [58, 199] and interact in lymphocytes [114]. Binding of SDF1α to CXCR4 initiates the CXCR4-CD26 complex endocytosis [114], a process disrupted by N-terminal clipping of SDF1α [195]. This finding could not explain however the preferential expression of CXCR4 in $CD26^{low}$ cells (e.g., T_{H2} or naïve T_H) [76, 199], or why

TGFβ (a cytokine that induces CD26 downmodulation) leads to augmented expression of CXCR4 and a potentiated SDF1α-CXCR4 axis [200-202]. This $CD26^{-/low}CXCR4^{+}$ phenotype also facilitates a vigorous response to SDF1α in the recruitment of T cells and eosinophils in a mouse model of lung allergic inflammation [178]. On the other hand, CD26 has also been positively correlated with the *in vitro* invasive capacity of T cell lines in response to SDF1α, and this positive effect is mediated by CD45 [203], a tyrosine phosphatase that enhances cell migration in response to SDF-1α [204].

CCR4 is present on monocytes, dendritic cells, NK cells and T_H cells (T_{H2}, T_{H17}, Treg). CCR4 recognizes both CCL17/TARC and CCL22/MDC (Table 3), the last chemokine produced by macrophages, dendritic cells, NK cells, and B/T lymphocytes [205]. TARC and MDC are expressed by epithelial cells in the airways, and their levels are upregulated after allergen challenge [103]. T_{H2} cytokines (e.g., IL-4 and IL-13), LPS, IL-1 and TNFα stimulate the secretion of MDC, whereas T_{H1} cytokines (e.g., IFNα, IL-12) inhibit MDC production [205, 206]. Moreover, MDC generates and amplifies T_{H2} responses and recruits T_{H2} lymphocytes [205-207], being important in diseases with a T_{H2}-cytokine profile (e.g., asthma) or equivalent models in mice [207]. In these models, CCR4-MDC has a dominant role in later and chronic stages of the disease as compared with CCR3-Eotaxin [181]. Furthermore, CD26 processes very efficiently MDC (half-life: 2-5 min) to generate MDC(3-69) and, subsequently, MDC(5–69) [185]. This last isoform preserves the attractant power for monocytes, but displays reduced chemotactic activity for lymphocytes and dendritic cells [150], two subsets important in asthma. As Tregs, T_{H2} and $CCR4^{+}$ T_{H17} cells are $CD26^{-/low}$ cells, while $CCR4^{-}$ T_{H17} cells are $CD26^{high}$ [72, 79], the $CD26^{-/low}$ phenotype of these $CCR4^{+}$ subsets should apparently facilitates their recruitment to inflammatory sites.

Table 3. Chemokines processed by CD26/DPP4

T_H subset	Chemokine receptor expressed:	Common ligands of these receptors	Substrates of DPP4 activity	Effect of clipping on chemokine activity or receptor preference	N-terminal cleavage	M_r (Da) Expasy	Half-life (min)	K_{CAT}/K_M ($10^{-6}M^{-1}S^{-1}$)	References
Treg (FoxP3, $CD26^{-/low}$)	CCR4	CCL17/TARC (n.d.)	No						
		CCL22/MDC	**Yes**	**↓, RP**	**Gly-Pro▼Tyr**	**8090.47**	**1.6**	**4±1**	**[10] [41] [82] [155] [156] [184]**
	CCR6	CCL20/MIP-3α (n.d.)	No						
T_{H2} (GATA3, CRTH2, CD30, $CD26^{+}$)	CCR3	**CCL5/RANTES**	**Yes**	**RP**	**Ser-Pro▼Tyr**	**7851.01**	**400**	**0.04±0.01**	**[10] [41] [82] [156] [184]**
		CCL11/Eotaxin 1	**Yes**	**↓**	**Gly-Pro▼Ala**	**8364.90**	**30**	**0.08±0.01**	**[10] [41] [82] [155] [156] [184]**
		CCL24/Eotaxin 2 (n.d.)	No						
		CCL26/Eotaxin 3 (n.d.)	No						
		CCL8/MCP2 (n.d.)	No						
		CCL7/MCP3 (n.d.)	No						
		CCL13/MCP4	No						
		CCL14a [9-74]	**Yes**	**↓**	**Gly-Pro▼Tyr**	**7800.83**	**n.d.**	**n.d.**	**[185]**
		CCL15/MIP-5	No						
		CCL28/MEC	No						
	CCR4	CCL17/TARC	No						
		CCL22/MDC	**Yes**	**↓, RP**	**Gly-Pro▼Tyr**	**8090.47**	**1.6**	**4±1**	**[10] [41] [82] [155] [156] [184]**
	CCR8	CCL1	No						
		CCL17/TARC	No						
	CXCR4	**CXCL12/SDF-1α**	**Yes**	**↓, CXCR4 antagonist**	**Lys-Pro▼Val**	**7609.97**	**<1**	**5±2**	**[10] [41] [82] [155] [156] [184]**

Continued on next page

Table 3 (Continued)

T_{H1} (t-bet, $CD26^{++}$)	CCR5	CCL3L1/LD78β	Yes	↑, RP	Ala-Pro▼Lys	7797.74	6000	0.003±0.002	[82] [155] [156] [184]
		CCL4/MIP-1β	No						
		CCL5/RANTES	Yes	RP	Ser-Pro▼Tyr	7851.01	400	0.04±0.01	[10] [41] [82] [156] [184]
		CCL8/MCP2	No						
		CCL7/MCP3	No						
		CCL13/MCP4	No						
		CCL11/Eotaxin	Yes	↓	Gly-Pro▼Ala	8364.90	30	0.08±0.01	[10] [41] [82] [155] [156] [184]
		CCL14a/HCC-1[9-74]	Yes	↓	Gly-Pro▼Tyr	7800.83	n.d.	n.d.	[185]
		CCL16	No						
		CCL23/MPIF-1	No						
	CXCR3A	CXCL9/Mig	Yes	↓, CXCR3 antagonist	Thr-Pro▼Val	11724.81	24	>0.4±0.2	[82] [155] [156] [184]
		CXCL10/IP-10	Yes	↓, CXCR3 antagonist	Val-Pro▼Leu	8646.30	4	0.5±1	[10] [41] [82] [155] [156] [184]
		CXCL11/I-TAC	Yes	↓, CXCR3 antagonist	Phe-Pro▼Met	8307.01	2	1.2±0.1	[82] [155] [156] [184]
T_{H17} (RORγT, $CD26^{+++}$)	CCR4	CCL17/TARC	No						
		CCL22/MDC	Yes	↓, RP	Gly-Pro▼Tyr	8090.47	1.6	4±1	[10] [41] [82] [155] [156] [184]
	CCR6	CCL20/MIP-3α	No						

CCR: C-C motif chemokine receptor; CXCR: C-X-C motif chemokine receptor; CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; TARC: thymus and activation regulated chemokine; MDC: macrophage-derived chemokine; MIP-3α: macrophage inflammatory protein-3 α; RANTES: regulated on activation, normal T cell expressed and secreted; MCP(2, 3 and 4): monocyte chemotactic protein; MIP5: macrophage inflammatory protein 5; MEC: mucosae-associated epithelial chemokine; SDF-1α: stromal cell-derived factor 1 α; MIP-1β: macrophage inflammatory protein-1β; MPIF-1: myeloid progenitor inhibitory factor 1; Mig: monokine induced by γ-interferon; IP-10: 10 kDa interferon γ-induced protein; I-TAC: interferon-inducible T-cell α chemoattractant; RP: Change in receptor preference; ↓↑: Change in activity/function; n.d.: no determined.

4.3.2.- CD26, T_{H1}/T_{H17}-related chemokines and T_{H2}^{low} asthma

Most of asthma patients present a T_{H2}^{high} disease, but there are other two types (obesity-associated and neutrophilic asthma) linked to a T_{H2}^{low} (i.e., T_{H1}/T_{H17}) profile [1]. The $CD26^{high}$ T cell subset displays a $CD45RO^{+}CCR7^{low}$ effector-memory phenotype and produces T_{H1}/T_{H17} cytokines [72, 77, 208]. T_{H1} cells are $CCR5^{+}CXCR3A^{+}$ lymphocytes [72, 208], are essential for the production of IgM, IgG, and IgA (but not IgE) by B-cells, and orchestrate the response against intracellular microbes [103]. A few CCR5 ligands and all the CXCR3A-specific chemokines are CD26 substrates (Table 3). For example, CCL5/RANTES is produced by endothelial and epithelial cells, platelets, macrophages, eosinophils and T cells [209]. RANTES activates CCR1, CCR3 (T_{H2}) and preferentially CCR5 (T_{H1}), thereby attracting monocytes, eosinophils and T cells to inflammatory sites [209]. This chemokine is cleaved with low efficiency (half-life of 400 min) by CD26 [100, 185], leading to RANTES(3-68), a chemokine that loses its capacity to bind CCR1 and CCR3 (monocytes, eosinophils and T_{H2} cells), but not CCR5 [197]. As CCR5 is expressed on T_{H1} and $CD45RA^{-}CD45R0^{+}CCR7^{low}$ effector-memory $CD4^{+}$ T cells (both $CD26^{+/high}$) [76, 103, 199], this means that RANTES(3-68) favours their attraction toward inflammatory sites [175, 197, 198, 210].

Another T_{H1}-chemokine and CD26-substrate that binds CCR5 is LD78β/CCL3L1 [211]**.** Macrophage inflammatory protein–1α (MIP-1α) is encoded by 2 different loci: LD78α and LD78β [212]. Both chemokines are agonists of CCR1, CCR3 and CCR5, but LD78β binds with high affinity to CCR5 [213], regulating the traffic/activation of macrophages/monocytes, NK cells, eosinophils, basophils, immature dendritic cells, and T lymphocytes. Strikingly, the inefficient (half-life ~ 5 h) [185, 211] clipping of LD78β by CD26 generates LD78β(3-70) enhances the chemotactic activity for T_{H1} cells

(CCR5) and monocytes/neutrophils (CCR1) (Table 3). This fact, together with the $CCR1^{low}$ phenotype in eosinophils (<20%) and the reduced affinity of LD78β (3-70) for CCR3 (T_{H2}), proves once again that this posttranslational modification tends to favour T_{H1}/T_{H17} responses [211, 214].

CXCR3A is overrepresented on T_{H1} cells [208] and is recognized by IFNγ-induced chemokines (CXCL9/Mig, CXCL10/IP-10, CXCL11/I-TAC) secreted by endothelial cells, hepatocytes, fibroblasts, keratinocytes and leucocytes in response to infections and several diseases including asthma. These chemokines are important to bring T_{H1} cells into epithelial barriers [103]. Moreover, after being N-terminally processed by CD26 with intermediate-high efficiency [185] (Table 3), they become less active T_{H1}-chemoattractants. In addition, this modification also makes IP-10 a CXCR3-antagonist and induces receptor desensitization, being part of a negative feedback mechanism important to downmodulate inflammation [215-217].

T_{H17} lymphocytes play a major role in tissue inflammation, are essential to activate macrophages and recruit neutrophils, and drive the defensive activities against extracellular bacteria and fungi. Human T_{H17} cells are associated with the expression of CCR4, CCR6, and CXCR6 [72, 103, 218]. Skin-homing addressins (e.g., CCR4, CCR6) are also shared by $CD26^{-/low}$ Treg cells and T_{H2} cells [73]. However, T_{H17} cells are rare at sites of inflammation, maybe due to homeostatic mechanisms that avoid their expansion, a high plasticity to shift to T_{H1} cells [103], or the heterogeneous expression of CCR4, with a proinflammatory $CCR4^{-}CCR6^{+}CD26^{high}$ phenotype in most of T_{H17} cells and a small subset of immunosuppressive $CCR4^{+}CCR6^{+}CD26^{low}$ [79, 81]. This heterogeneity for CCR4 is also detected in T_{H22} cells, a lineage that produces IL-22, expresses CLA, CCR6 and CCR10, and plays an anti-inflammatory and tissue-protective role in asthma [219]. Curiously, $CCR4^{-}$ T_{H22} cells are

$CD26^-$, whereas $CCR4^+$ T_{H22} cells are constituted by either $CD26^-$ or $CD26^+$ lymphocytes [79].

In summary, taking into consideration the CD26 expression gradient in the major T_H subsets (T_{H17}>>T_{H1}>T_{H2}>>T_{reg}), the small proportion of chemokines affecting T_{reg}/T_{H2}/T_{H17} cells that are processed by CD26 as compared with those recruiting T_{H1} cells, and that CD26-dependent clipping produce a drop in the biological functions of most of them, it is evident that CD26 forms part of a homeostatic mechanism to downmodulate airways inflammation mediated by T_{H2} and especially T_{H17}/T_{H1} cells (e.g., obesity linked asthma).

5.- CD26 AND ASTHMA: ANIMAL MODELS, STUDIES IN HUMAN SYSTEM AND CLINICAL IMPLICATIONS

Despite the reported evidence on the role of CD26 in T_{H1}-related diseases, it is not yet fully understood the involvement of both the peptidase-dependent and peptidase-independent functions of CD26 in the pathophysiology of asthma. In this section, we summarize some results obtained in animal models and the human system.

5.1.- Rat models

Rat models using inhaled allergens such as OVA have been used to evaluate the role of CD26 in bronchial asthma. Thus, the severity of the airway inflammation decrease as the endogenous CD26 level of the strain becomes lower [220], likely due to the costimulatory role of CD26. One of the models more extensively used is the F344 strain. Challenge of F344 rats with OVA generates bronchoconstriction and higher CD26 expression on lymphocytes and epithelial cells of lung

parenchyma [14, 17], enhanced levels of DPP8/9 and DPP10 in bronchi [17], and dose-dependent recruitment to the lungs of dendritic cells, eosinophils, and $CD4^+CD25^+CD26^+$ T cells [14]. More specifically, OVA causes increased recruitment of T cells to bronchi (but not lung parenchyma) [22] and enhanced sDPP4 activity in bronchoalveolar lavage fluid (BALF) [17], the last finding likely related with the augmented presence of $CD26^-$ T cells in bronchi. Interestingly, there is an F344 substrain with an active-site mutation in the *Dpp4* gene that causes protein retention/degradation in the endoplasmic reticulum and lower CD26 levels [221]. This F344 substrain also displays the early bronchoconstriction in response to OVA-challenge [222], but the late inflammatory response is milder regarding OVA-specific serum IgE, eosinophilia and recruitment of T cells in both bronchi and BALF [14, 22, 220]. In contrast, the entrance of T cells in bronchoalveolar lymphoid tissue (BALT) is enhanced, no matter the $CD26^+$ or $CD26^-$ phenotype of transferred T cells [223].

Because bronchi are $DPP4^-$ (but $DPP8/9/10^+$) compartments [17], $CD4^+CD25^+$ T cells recruited in the airways of OVA-challenged F344 rats are either T_H cells with a $CD26^{low}$ phenotype or they downmodulate CD26 during peribronchial infiltration. Thus, a potential higher response of $CD4^+CD25^+CD26^+$ T cells to SDF1α [203] could allow their recruitment to bronchi ($CD26^-$ $SDF\text{-}1^{high}$ environment) of wild type rats [22] with a concomitant SDF1-driven downmodulation of CD26, as it happens during skin homing of Sézari cells [224]. In contrast, $CD4^+CD25^+CD26^-$ Teff cells in CD26-deficient rats would not be attracted to the bronchi as a result from either an anomalous/aberrant response to SDF1α [203] or a reduce chemokine gradient [22], resulting in milder asthma-like disease. However, despite SDF-1 is augmented in the bronchi of asthmatic patients [225], OVA-challenge does not increase the number of SDF-1 transcripts in the large airways of F344-rats [22]. Curiously, the

amount of SDF-1 is increased in the BALT of CD26-deficient rats, likely supporting the above commented higher recruitment of T cells to this lymphoid compartment after OVA-challenge [223].

CD26-deficient F344 rats display a parallel increased influx of Tregs into the lungs, with a concomitant rise in the IL-10 production that explains the diminished inflammation [222]. However, this may not happen in the same way in humans, where Treg cells display a $CD26^{-/low}$ phenotype [69-72] in contrast to rats [14, 68]. Moreover, it is difficult to conciliate a milder asthmatic-like inflammation in OVA-challenged CD26-deficient F344 rats with either a lower truncation rate of T_{H2} chemokines (e.g., eotaxin) or the *in vivo* enhanced eotaxin-mediated recruitment of eosinophils caused by a CD26 inhibitor [187]. Perhaps the different genetic background [226] or the administration route might play a significant role [45]. For example, oral administrated inhibitors seem to enhance allergic inflammation of the airways, whereas topic inhibition (aerosolization) has a rather protective effect in line with that observed in CD26-deficient F344 rats [45]. Moreover, dual deficiency of enzymatic and extra enzymatic activities of CD26 in murine asthma models may generate results different from the observed effects of CD26 inhibitors, which only inhibit enzymatic functions [166].

5.2.- Mice models

CD26 KO mice display a healthy phenotype, with increased glucose clearance, resistance to obesity, and small changes in the percentages of NK, NKT and $CD4^+$ T cells [227, 228]. In addition, *in vitro* PWM-activated splenocytes from $CD26^{-/-}$ C56BL/6 mice show a decreased IL-4 production, while IgE and IL-4 are significantly reduced in sera from $CD26^{-/-}$ animals upon PWM-treatment [228]. In 2012, Yan *et al*. reported unchanged IgE concentration but enhanced eosinophilia and

T_{H2} cytokines (IL-4, IL-5, IL-13) in BALF from OVA-induced $CD26^{-/-}$ C56BL/6 animals, as well as higher mRNA and protein levels of CD26 substrates (eotaxin and RANTES) and T_{H2} chemokine receptors (CCR3 and CCR5) [16]. These partially contradictory results probably depend on the type, administration route, and strength of the polyclonal stimulus (PWM vs. OVA) [16]. They also point out that induced and targeted (cell-type specific) KO models could be necessary to truly dissect the role of CD26 in asthma [16].

5.3.- Human system

In line with the small differences in CD26 levels observed between T_{H1} and T_{H2} cells [72], this marker is not helpful to differentiate T_{H1}- from T_{H2}-driven responses in patients with atopic asthma [229]. However, augmented CD26 levels are actually detected on total lymphocytes, $CD4^+$ T cells and iNKT (but not $CD8^+$ T lymphocytes, monocytes, or B-cells) in adult allergic asthma, reflecting the existence of an activated status [20]. This last work also published an elevation of plasma sCD26 in patients, which is associated with eosinophil counts and IgE concentration. Additionally, this group reported lower production of T_{H1}-chemokines (IP-10, MIG), and higher presence of other chemokines (RANTES, MDC) and their receptors (CCR3, CCR4), in both cases attributable to a T_{H2}-response predominance [20]. In clear contrast, Matsuno *et al.* found that sCD26 is inversely correlated with the inflammation level in chronic eosinophilic pneumonia, a disease frequently preceded or accompanied by asthma [230]. More recently, no differences were found in serum sCD26 levels in children with asthma, or association of this variable with either asthma or atopy (e.g., skin prick test, IgE, eosinophil cationic protein) [86].

From the analysis of all these studies is obvious the need to expand them to different asthmatic phenotypes (or endotypes) and levels of severity/chronicity. In addition, it is also clear that other variables (sample size, lymphocytes counts, age, sex) should also be taken into account, especially knowing: a) the influence of sex on CD26 expression (our unpublished results) and sCD26 levels [86-89, 91]; b) the augmented prevalence of asthma in boys at early ages and the higher prevalence of asthma in women after puberty [5, 6], especially in the case of certain T_{H2}^{low} phenotypes [1]. For example, sCD26 is apparently increased in the blood of both atopic dermatitis (a T_{H2}-like disease) [231] and adult asthma patients [20], but in the last study, these results might be partially explained by a higher proportion of males in the group of patients. In contrast, Remes did not show altered sCD26 levels in asthmatic children and took into consideration certain pre-analytical variables (gender, age) [86]. Therefore, more studies are needed to reveal the potential correlation between CD26/sCD26 and the severity and phenotype of the asthmatic pathology.

5.4.- Clinical implications

High body-mass index is a factor with a positive association with asthma development and severity [232,233, 234]. This association of asthma and obesity appears to be stronger in women than in men, with a role for both non-inflammatory and subclinical/systemic chronic inflammatory pathways, including the release of proinflammatory (e.g., leptin) or anti-inflammatory (e.g., adiponectin) adipokines that regulate de survival of eosinophils and their recruitment to the lungs [233, 234]. As mentioned above, visceral fat in obese patients releases the "adipokine" sCD26 into the circulation [88], but as we have

already been highlighting throughout this review, with proinflammatory or anti-inflammatory effects depending on the processed substrate: incretins or chemokines/substance P, respectively.

Obese persons are at risk of a number of co-morbidities like type 2 diabetes mellitus (T2DM), a disease characterized by impair production of incretins, with subsequently hyperglycaemia. Incretins (GIP, GLP-1, GLP-2) are well-known CD26 substrates that lose their function when they are processed. Inhibition of CD26 enzymatic activity avoids the degradation of these hormones, enhances the incretin effect, improves both the insulin secretion and the glucose uptake and lowers the glycosylated haemoglobin A1c levels. To achieve these goals, a new group of anti-hyperglycaemic drugs (CD26 inhibitors or gliptins) have come on the market, with sitagliptin, (Januvia®; Merck Sharp & Dohme Ltd) and vildagliptin (Galvus®; Novartis Europharm Ltd) (both approved by European Medicines Agency/EMA in 2007) as the first outpost (Table 4). Currently, there are several orally administered CD26 inhibitors that have been approved by agencies like the Food and Drug Administration (FDA) or EMA and are being used with satisfactory results as a second or third line medication in combination with other oral antidiabetic drugs. Commercially available gliptins include the above-mentioned sitagliptin (Januvia®, Ristaben®), but also saxagliptin (Onglyza®), linagliptin (Trajenta®), alogliptin (Vipidia®), and vildagliptin (Galvus®, Jalra®, Xiliarx®) (Table 4). However, there is a controversial debate about the secondary effects on co-morbidities (e.g., cardiovascular outcome, renal impairment, acute pancreatitis) that the long-term treatment with these long half-life inhibitors may have. These concerns are as a result of several issues related to the great number of CD26 substrates linked or not to the immune system. Thus, the SAVOR TIMI-53 (saxagliptin) [235] trial detected a

significantly increased rate for hospitalization due to heart failure, and a more recent study based on FAERS (US-FDA Adverse Even Reporting System) [236] is also consistent with this finding. In clear contrast, the TECOS trial (sitagliptin) [237] did not find an increased risk of heart failure.

Table 4. DPP4 inhibitors registered for clinical use in EMA and adverse drugs reactions identified during both the clinical and post-marketing surveillance stage

Active substance used as monotherapy	Proprietary Name (Company; Date of EMA authorization)	Immune system & Respiratory disorders, infections (Frequency)**	Skin and subcutaneous tissue disorders (Frequency)**
Sitagliptin	Januvia® (Merck Sharp & Dohme Ltd; 2007) Ristaben® (Merck Sharp & Dohme Ltd; 2010)	• Hypersensitivity, including anaphylactic responses (not known)* • Interstitial lung disease (not known)	• Pruritus (uncommon)* • Angioedema (not known) * • Rash (not known) * • Urticaria (not known) * • Cutaneous vasculitis (not known) * • Exfoliative skin conditions including Stevens-Johnson syndrome (not known) * • Bullous pemphigoid (not known) *
Vildagliptin	Galvus® (Novartis Europharm Ltd; 2007) Jalra® (Novartis Europharm Ltd; 2008) Xiliarx® (Novartis Europharm Ltd; 2008)	• Upper respiratory infection (very rare) • Nasopharyngitis (very rare)	• Urticaria (not known) * • Bullous pemphigoid (not known)* • Exfoliative skin conditions (not known)*

Continued on next page

Table 4 (Continued)			
Saxagliptin	Onglyza® (AstraZeneca AB; 2009)	• Small decrease in the absolute count of peripheral blood lymphocytes • Upper respiratory infection (common) • Sinusitis (common)	• Pruritus (uncommon) * • Angioedema (rare) * • Rash (common) * • Urticaria (uncommon) * • Dermatitis (uncommon)
Linagliptin	Trajenta® (Boehringer Ingelheim International GmbH; 2011)	• Nasopharyngitis (uncommon) • Hypersensitivity (e.g. bronchial hyperreactivity) (not known) • Cough (uncommon)	• Angioedema (rare) * • Rash (uncommon) * • Urticaria (rare)* • Bullous pemphigoid (not known)*
Alogliptin	Vipidia® (Takeda Pharma A/S; 2013)	• Upper respiratory infection (common) • Nasopharyngitis (common) • Hypersensitivity reactions (not known) *	• Pruritus (common) • Rash (common) • Exfoliative skin conditions, including Stevens-Johnson syndrome, Erythema multiforme, Angioedema and Urticaria (not known) *

*Adverse drug reactions (ADRs) identified based on post-marketing surveillance **Absolute frequencies of ADRs; very common (≥ 1/10), common (≥ 1/100 to < 1/10), uncommon (≥ 1/1,000 to 1/100), rare (≥ 1/10,000 to 1/1,000), very rare (< 1/10,000), or not known (i.e., not estimated).

More in accordance with the scope of this review, CD26 reversible inhibitors seem to increase the frequency of risk factors for asthma development/exacerbation like atopic sensitization, rhinitis, or rhinovirus infection in T2DM patients [232]. Thus, a rapid review of clinical and data published on the EMA web page

(http://www.ema.europa.eu) supports increased risk of nonserious upper respiratory tract infections (e.g., viral nasopharyngitis, rhinitis, sinusitis) (Table 4), which would be in line with the costimulatory role of CD26. As Willemen and co-authors sustain, this increased risk of infections with CD26 inhibitors (~3% cases) cannot be compared with the magnitude of the effects seen with biological agents like tumour necrosis factor inhibitors [238]. However, this effect should not be underestimated and must be added to the immune impairment caused by T2DM itself to increase the overall asthma development/exacerbation risk in these patients.

Other adverse drug reactions (ADRs) reported in association with gliptins and more associated with an inhibitory role of CD26 would be interstitial lung disease, hypersensitivity reactions (including anaphylactic responses or bronchial hyperreactivity), and skin and subcutaneous tissue disorders such as pruritus, angioedema, rash, urticaria, cutaneous vasculitis, or more severe and rare skin conditions (Table 4). For example, Bullous pemphigoid [187, 239-241] is an autoimmune subepidermal disease with overproduction of eotaxin and eosinophilia that affects skin and mucosae. Therefore, the association between gliptins and Bullous pemphigoid is in tune with *in vivo* studies in F344 rats showing that CD26 limits the eotaxin-mediated recruitment of eosinophils [187], or with the finding that oral administration of CD26 inhibitors promotes the allergic inflammation of the airways [45]. This means that CD26 could be part of a homeostatic mechanism to downmodulate airways inflammation mediated by T_{H2} and especially T_{H17}/T_{H1} cells important in some T_{H2}^{low} asthma phenotypes such as obesity-related asthma. Nevertheless, it should be noted that besides chemokines there are other CD26 substrates with implications on obesity and asthma-like the substance P. Inhaled substance P, but not bradykinin enhances the airway response to bronchoconstricting agents in guinea-pigs [242].

Substance P also favours allergen sensitization and bronchial inflammation, as observed in a mouse model of diet-induced obesity sensitized and challenged with OVA and treated with an antagonist of the substance P receptor (NK1-R) [243]. Therefore, expanded half-life in substance P caused by treatment with CD26 inhibitors in T2DM patients could increase certain obesity-related co-morbidities such as asthma. In the same way, administration of sitagliptin in T2DM generates a temporary decrease in the percentage of peripheral blood Tregs that also could favour asthma prevalence in these patients [244]. In clear contrast, GLP-1-based therapies have anti-inflammatory effects in chronic inflammatory diseases including T2DM and asthma [245]. For example, *in vitro* studies have described that CD26 inhibitors induce a decrease of NLRP3 inflammasome, toll-like receptor 4 (TLR4) signalling and IL-1β in macrophages via GLP-1 receptor [160]. Therefore, more studies are needed to clarify whether gliptins have an overall positive or negative role in T2DM patients with obesity-related asthma.

6.- CONCLUDING REMARKS

CD26 is a relevant molecule in asthma for several reasons. The first one because of its potential utility together with periostin to test the efficacy of the treatment with interleukin-13 neutralising monoclonal antibodies (e.g., Tralokinumab) in patients with severe uncontrolled asthma [246, 247]. Secondly, because of its multifunctionality nature, with both costimulatory and inhibitory roles in the immune system, apart from the high (but variable) CD26 levels in a subset of lymphocytes central to asthma: the $CD4^+$ T cells. Different authors have highlighted the inhibitory functions of this molecule relative to signalling mediated by cytokines and chemokines. A handful of chemokines important for $CD26^+$ T_{H2} cells during early-onset allergic

or late-onset eosinophilic asthma includes chemotactic factors whose biological activity is reduced with low-intermediate (eotaxin) or high (MDC, SDF-1α) efficiency, whereas important chemokines for $CD26^{+}$ T_{H1} (I-TAC, IP-10, Mig) and $CD26^{high}$ T_{H17} cells (MDC), two Teff subsets relevant in obesity-related asthma, late-onset neutrophilic asthma or severe asthma, are inactivated with intermediate-high effectiveness. Therefore, CD26 could act as a "biological brake" by reducing the attraction of Teff cells at sites of inflammation (e.g., the bronchi). This means that CD26 inhibitors used in T2DM patients could lead to a higher persistence of unprocessed substance P or chemokines and exacerbation of T_{H2}^{high} and especially T_{H2}^{low} asthma. In conclusion, it is necessary to previously take into consideration the many existing questions regarding the biological functions of this enzyme before the therapeutic targeting of this molecule [248], as CD26 inhibitors could enhance the frequency of hospital admission among people with certain asthma phenotypes.

REFERENCES

[1] Wenzel SE (2012) Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med 18:716-725

[2] Holgate ST (2012) Innate and adaptive immune responses in asthma. Nat Med 18:673-683

[3] Beasley R, Semprini A, Mitchell EA (2015) Risk factors for asthma: is prevention possible? Lancet 386:1075-1085

[4] Moorman JE, Akinbami LJ, Bailey CM, Zahran HS, King ME, Johnson CA, Liu X (2012) National surveillance of asthma: United States, 2001-2010.

National Center for Health Statistics. Vital Health Stat 3(35)

[5] de Marco R, Locatelli F, Sunyer J, Burney P (2000) Differences in incidence of reported asthma related to age in men and women. A retrospective analysis of the data of the European Respiratory Health Survey. Am J Respir Crit Care Med 162:68–74

[6] Tan DJ, Walters EH, Perret JL, Lodge CJ, Lowe AJ, Matheson MC, Dharmage SC (2015) Age-of-asthma onset as a determinant of different asthma phenotypes in adults: a systematic review and meta-analysis of the literature. Expert Rev Respir Med 9:109-123

[7] Mathias RA, Grant AV, Rafaels N, Hand T, Gao L, Vergara C, Tsai YJ, Yang M, Campbell M, Foster C, Gao P, Togias A, Hansel NN, Diette G, Adkinson NF, Liu MC, Faruque M, Dunston GM, Watson HR, Bracken MB, Hoh J, Maul P, Maul T, Jedlicka AE, Murray T, Hetmanski JB, Ashworth R, Ongaco CM, Hetrick KN, Doheny KF, Pugh EW, Rotimi CN, Ford J, Eng C, Burchard EG, Sleiman PM, Hakonarson H, Forno E, Raby BA, Weiss ST, Scott AF, Kabesch M, Liang L, Abecasis G, Moffatt MF, Cookson WO, Ruczinski I, Beaty TH, Barnes KC (2010) A genome-wide association study on African-ancestry populations for asthma. J Allergy Clin Immunol 125:336-346

[8] Portelli M, Sayers I (2012) Genetic basis for personalized medicine in asthma. Expert Rev Respir Med 6:223-236

[9] Martinez FD, Vercelli D (2013) Asthma. Lancet 382:1360-1372

[10] Mentlein R (1999) Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides. Regul Pept 85:9-24

[11] Heutinck KM, ten Berge IJ, Hack CE, Hamann J, Rowshani AT (2010) Serine proteases of the human immune system in health and disease. Mol Immunol 47:1943-1955

[12] Qi SY, Riviere PJ, Trojnar J, Junien JL, Akinsanya KO (2003) Cloning and characterization of dipeptidyl peptidase 10, a new member of an emerging subgroup of serine proteases. Biochem J 373:179-189

[13] Allen M, Heinzmann A, Noguchi E, Abecasis G, Broxholme J, Ponting CP, Bhattacharyya S, Tinsley J, Zhang Y, Holt R, Jones EY, Lench N, Carey A, Jones H, Dickens NJ, Dimon C, Nicholls R, Baker C, Xue L, Townsend E, Kabesch M, Weiland SK, Carr D, von Mutius E, Adcock IM, Barnes PJ, Lathrop GM, Edwards M, Moffatt MF, Cookson WO (2003) Positional cloning of a novel gene influencing asthma from chromosome 2q14. Nat Genet 35:258-263

[14] Skripuletz T, Schmiedl A, Schade J, Bedoui S, Glaab T, Pabst R, von Hörsten S, Stephan M (2007) Dose-dependent recruitment of CD25+ and CD26+ T cells in a novel F344 rat model of asthma. Am J Physiol Lung Cell Mol Physiol 292:L1564-1571

[15] Zhou H, Hong X, Jiang S, Dong H, Xu X, Xu X (2009) Analyses of associations between three positionally cloned asthma candidate genes and asthma or asthma-related phenotypes in a Chinese population. BMC Med Genet 10:123-131

[16] Yan S, Gessner R, Dietel C, Schmiedek U, Fan H (2012) Enhanced ovalbumin-induced airway

inflammation in CD26-/- mice. Eur J Immunol 42:533-540

[17] Schade J, Stephan M, Schmiedl A, Wagner L, Niestroj AJ, Demuth HU, Frerker N, Klemann C, Raber KA, Pabst R, von Hörsten S (2008) Regulation of expression and function of dipeptidyl peptidase 4 (DP4), DP8/9, and DP10 in allergic responses of the lung in rats. J Histochem Cytochem 56:147-155

[18] Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, Zhang Y, Zhang W, Yuan Y, Bao J, Zhang B, Shi Y, Yan J, Gao GF (2013) Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature 500:227-231

[19] Bønnelykke K, Vissing NH, Sevelsted A, Johnston SL, Bisgaard H (2015) Association between respiratory infections in early life and later asthma is independent of virus type. J Allergy Clin Immunol 136:81-86

[20] Lun SW, Wong CK, Ko FW, Hui DS, Lam CW (2007) Increased expression of plasma and CD4+ T lymphocyte costimulatory molecule CD26 in adult patients with allergic asthma. J Clin Immunol 27:430-437

[21] Rojas-Ramos E, Garfias Y, Jiménez-Martínez Mdel C, Martínez-Jiménez N, Zenteno E, Gorocica P, Lascurain R (2007) Increased expression of CD30 and CD57 molecules on CD4(+) T cells from children with atopic asthma: a preliminary report. Allergy Asthma Proc 28:659–666

[22] Schade J, Schmiedl A, Kehlen A, Veres TZ, Stephan M, Pabst R, von Hörsten S (2009) Airway-specific recruitment of T cells is reduced in a CD26-deficient F344 rat substrain. Clin Exp Immunol 158:133–142

[23] Torimoto Y, Dang NH, Tanaka T, Prado C, Schlossman SF, Morimoto C (1992) Biochemical characterization of CD26 (dipeptidyl peptidase IV): functional comparison of distinct epitopes recognized by various anti-CD26 monoclonal antibodies. Mol Immunol 29:183-192

[24] De Meester I, Vanhoof G, Hendriks D, Demuth HU, Yaron A, Scharpé S (1992) Characterization of dipeptidyl peptidase IV (CD26) from human lymphocytes. Clin Chim Acta 210:23-34

[25] Gorrell MD, Gysbers V, McCaughan GW (2001) CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand J Immunol 54:249-264

[26] Ajami K, Abbott CA, Obradovic M, Gysbers V, Kähne T, McCaughan GW, Gorrell MD (2003) Structural requirements for catalysis, expression, and dimerization in the CD26/DPIV gene family. Biochemistry 42:694-701

[27] Chien CH, Tsai CH, Lin CH, Chou CY, Chen X (2006) Identification of hydrophobic residues critical for DPP-IV dimerization. Biochemistry 45:7006-7012

[28] Jascur T, Matter K, Hauri HP (1991) CD26 dimerization in the Golgi apparatus Oligomerization and intracellular protein transport: dimerization of intestinal dipeptidylpeptidase IV occurs in the Golgi apparatus. Biochemistry 30:1908-1915

[29] Danielsen EM (1994) Dimeric assembly of enterocyte brush border enzymes. Biochemistry 33:1599-1605

[30] Fleischer B (1994) CD26: a surface protease involved in T-cell activation. Immunol Today 15:180-184

[31] Morimoto C, Schlossman SF (1998) The structure and function of CD26 in the T-cell immune response. Immunol Rev 161:55-70

[32] Dobers J, Grams S, Reutter W, Fan H (2000) Roles of cysteines in rat dipeptidyl peptidase IV/CD26 in processing and proteolytic activity. Eur J Biochem 267:5093-5100

[33] Ohnuma K, Dang NH, Morimoto C (2008) Revisiting an old acquaintance: CD26 and its molecular mechanisms in T cell function. Trends Immunol 29:295-301

[34] Chung KM, Huang CH, Cheng JH, Tsai CH, Suen CS, Hwang MJ, Chen X (2011) Proline in transmembrane domain of type II protein DPP-IV governs its translocation behavior through endoplasmic reticulum. Biochemistry 50:7909-7918

[35] Dinjens WN, Ten Kate J, Kirch JA, Tanke HJ, Van der Linden EP, Van den Ingh HF, Van Steenbrugge GJ, Meera Khan P, Bosman FT (1990) Adenosine deaminase complexing protein (ADCP) expression and metastatic potential in prostatic adenocarcinomas. J Pathol 160:195-201

[36] Abbott CA, Baker E, Sutherland GR, McCaughan GW (1994) Genomic organization, exact localization, and tissue expression of the human CD26 (dipeptidyl peptidase IV) gene. Immunogenetics 40:331-338

[37] Wesley UV, Albino AP, Tiwari S, Houghton AN (1999) A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. J Exp Med 190:311-322

[38] Wesley UV, Tiwari S, Houghton AN (2004) Role for dipeptidyl peptidase IV in tumor suppression of human

non small cell lung carcinoma cells. Int J Cancer 109:855-866

[39] Wesley UV, McGroarty M, Homoyouni A (2005) Dipeptidyl peptidase inhibits malignant phenotype of prostate cancer cells by blocking basic fibroblast growth factor signaling pathway. Cancer Res 65:1325-1334

[40] van der Velden VH, Wierenga-Wolf AF, Adriaansen-Soeting PW, Overbeek SE, Möller GM, Hoogsteden HC, Versnel MA (1998) Expression of aminopeptidase N and dipeptidyl peptidase IV in the healthy and asthmatic bronchus. Clin Exp Allergy 28:110-120

[41] De Meester I, Korom S, Van Damme J, Scharpé S (1999) CD26, let it cut or cut it down. Immunol Today 20:367-375

[42] van der Velden VH, Hulsmann AR (1999) Peptidases: structure, function and modulation of peptide-mediated effects in the human lung. Clin Exp Allergy 29:445-456

[43] Hong WJ, Petell JK, Swank D, Sanford J, Hixson DC, Doyle D (1989) Expression of dipeptidyl peptidase IV in rat tissues is mainly regulated at the mRNA levels. Exp Cell Res 182:256-266

[44] Zhen G, Park SW, Nguyenvu LT, Rodriguez MW, Barbeau R, Paquet AC, Erle DJ (2007) IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. Am J Respir Cell Mol Biol 36:244-253

[45] Stephan M, Suhling H, Schade J, Wittlake M, Tasic T, Klemann C, Pabst R, Jurawitz MC, Raber KA, Hoymann HG, Braun A, Glaab T, Hoffmann T, Schmiedl A, von Hörsten S (2013) Effects of

dipeptidyl peptidase-4 inhibition in an animal model of experimental asthma: a matter of dose, route, and time. Physiol Rep 1:e00095

[46] Micouin A, Bauvois B (1997) Expression of dipeptidylpeptidase IV (DPP IV/CD26) activity on human myeloid and B lineage cells, and cell growth suppression by the inhibition of DPP IV activity. Adv Exp Med Biol 421:201-205

[47] Yamabe T, Takakura K, Sugie K, Kitaoka Y, Takeda S, Okubo Y, Teshigawara K, Yodoi J, Hori T (1997) Induction of the 2B9 antigen/dipeptidyl peptidase IV/CD26 on human natural killer cells by IL-2, IL-12 or IL-15. Immunology 91:151-158

[48] von Bonin A, Hühn J, Fleischer B (1998) Dipeptidyl-peptidase IV/CD26 on T cells: analysis of an alternative T-cell activation pathway. Immunol Rev 161:43-53

[49] Dang NH, Torimoto Y, Sugita K, Daley JF, Schow P, Prado C, Schlossman SF, Morimoto C (1990) Cell surface modulation of CD26 by anti-1F7 monoclonal antibody. Analysis of surface expression and human T cell activation. J Immunol 145:3963-3971

[50] Mattern T, Reich C, Duchrow M, Ansorge S, Ulmer AJ, Flad HD (1995) Antibody-induced modulation of CD26 surface expression. Immunology 84:595-600

[51] Kähne T, Kröning H, Thiel U, Ulmer AJ, Flad HD, Ansorge S (1996) Alterations in structure and cellular localization of molecular forms of DP IV/CD26 during T cell activation. Cell Immunol 170:63-70

[52] Darmoul D, Baricault L, Sapin C, Chantret I, Trugnan G, Rousset M (1991) Decrease of mRNA levels and biosynthesis of sucrase-isomaltase but not

dipeptidylpeptidase IV in forskolin or monensin-treated Caco-2 cells. Experientia 47:1211-1215

[53] Kahne T, Lendeckel U, Wrenger S, Neubert K, Ansorge S, Reinhold D (1999) Dipeptidyl peptidase IV: a cell surface peptidase involved in regulating T cell growth. Int J Mol Med 4:3-15

[54] Salgado FJ, Vela E, Martín M, Franco R, Nogueira M, Cordero OJ (2000) Mechanisms of CD26/dipeptidyl peptidase IV cytokine-dependent regulation on human activated lymphocytes. Cytokine 12:1136-1141

[55] Rogge L, Bianchi E, Biffi M, Bono E, Chang SY, Alexander H, Santini C, Ferrari G, Sinigaglia L, Seiler M, Neeb M, Mous J, Sinigaglia F, Certa U (2000) Transcript imaging of the development of human T helper cells using oligonucleotide arrays. Nat Genet 25:96-101

[56] Stefanovic V, Ardaillou N, Vlahovic P, Placier S, Ronco P, Ardaillou R (1993) Interferon-gamma induces dipeptidylpeptidase IV expression in human glomerular epithelial cells. Immunology 80:465-470

[57] Bauvois B, Djavaheri-Mergny M, Rouillard D, Dumont J, Wietzerbin J (2000) Regulation of CD26/DPPIV gene expression by interferons and retinoic acid in tumor B cells. Oncogene 19:265-272

[58] Cordero OJ, Salgado FJ, Viñuela JE, Nogueira M (1997) Interleukin-12 enhances CD26 expression and dipeptidyl peptidase IV function on human activated lymphocytes. Immunobiology 197:522-533

[59] Cordero OJ, Salgado FJ, Viñuela JE, Nogueira M (1998) Interleukin-12-dependent activation of human lymphocyte subsets. Immunol Lett 61:7-13

[60] Bühling F, Kunz D, Reinhold D, Ulmer AJ, Ernst M, Flad HD, Ansorge S (1994) Expression and functional role of dipeptidyl peptidase IV (CD26) on human natural killer cells. Nat Immun 13:270-279

[61] Bühling F, Junker U, Reinhold D, Neubert K, Jäger L, Ansörge S (1995) Functional role of CD26 on human B lymphocytes. Immunol Lett 45:47-51

[62] Bauvois B, De Meester I, Dumont J, Rouillard D, Zhao HX, Bosmans E (1999) Constitutive expression of CD26/dipeptidylpeptidase IV on peripheral blood B lymphocytes of patients with B chronic lymphocytic leukaemia. Br J Cancer 79:1042-1048

[63] Cordero OJ, Salgado FJ, Fernández-Alonso CM, Herrera C, Lluis C, Franco R, Nogueira M (2001) Cytokines regulate membrane adenosine deaminase on human activated lymphocytes. J Leukoc Biol 70:920-930

[64] Arndt M, Lendeckel U, Spiess A, Faust J, Neubert K, Reinhold D, Ansorge S (2000) Dipeptidyl peptidase IV (DP IV/CD26) mRNA expression in PWM-stimulated T-cells is suppressed by specific DP IV inhibition, an effect mediated by TGF-beta(1). Biochem Biophys Res Commun 274:410-414

[65] Uematsu T, Tanaka H, Yamaoka M, Furusawa K (2004) Effects of oral squamous cell carcinoma-derived TGF-beta1 on CD26/DPPIV expression in T cells. Anticancer Res 24:619-624

[66] Tan EY, Richard CL, Zhang H, Hoskin DW, Blay J (2006) Adenosine downregulates DPPIV on HT-29 colon cancer cells by stimulating protein tyrosine phosphatase(s) and reducing ERK1/2 activity via a

novel pathway. Am J Physiol Cell Physiol 29:C433-444

[67] Sakaguchi S, Miyara M, Costantino CM, Hafler DA (2010) FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol 10:490-500

[68] Stephens LA, Barclay AN, Mason D (2004) Phenotypic characterization of regulatory CD4+CD25+ T cells in rats. Int Immunol 16:365-375

[69] Kattah MG, Coller J, Cheung RK, Oshidary N, Utz PJ (2008) HIT: a versatile proteomics platform for multianalyte phenotyping of cytokines, intracellular proteins and surface molecules. Nat Med 14:1284-1289

[70] Mandapathil M, Hilldorfer B, Szczepanski MJ, Czystowska M, Szajnik M, Ren J, Lang S, Jackson EK, Gorelik E, Whiteside TL (2010) Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. J Biol Chem 285:7176-7186

[71] Salgado FJ, Pérez-Díaz A, Villanueva NM, Lamas O, Arias P, Nogueira M (2012) CD26: a negative selection marker for human Treg cells. Cytometry A 81:843-855

[72] Bengsch B, Seigel B, Flecken T, Wolanski J, Blum HE, Thimme R (2012) Human Th17 cells express high levels of enzymatically active dipeptidylpeptidase IV (CD26). J Immunol 188:5438-5447

[73] Clark RA, Kupper TS (2007) IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. Blood 109:194-202

[74] Romagnani S, Manetti R, Annunziato F, Maggi E (1995) Role of IL12 in the development of human Th1 type cells. Res Immunol 146:452-460

[75] Willheim M, Ebner C, Baier K, Kern W, Schrattbauer K, Thien R, Kraft D, Breiteneder H, Reinisch W, Scheiner O (1997) Cell surface characterization of T lymphocytes and allergen-specific T cell clones: correlation of CD26 expression with T(H1) subsets. J Allergy Clin Immunol 100:348-355

[76] Annunziato F, Galli G, Cosmi L, Romagnani P, Manetti R, Maggi E, Romagnani S (1998) Molecules associated with human Th1 or Th2 cells. Eur Cytokine Netw 9:12-16

[77] Krakauer M, Sorensen PS, Sellebjerg F (2006) CD4(+) memory T cells with high CD26 surface expression are enriched for Th1 markers and correlate with clinical severity of multiple sclerosis. J Neuroimmunol 181:157-164

[78] Zhao F, Hoechst B, Gamrekelashvili J, Ormandy LA, Voigtländer T, Wedemeyer H, Ylaya K, Wang XW, Hewitt SM, Manns MP, Korangy F, Greten TF (2012) Human CCR4+ CCR6+ Th17 cells suppress autologous CD8+ T cell responses. J Immunol 188:6055-6062

[79] Cordero OJ, Varela-Calviño R, López-González T, Calviño-Sampedro C, Viñuela JE, Mouriño C, Hernández-Rodríguez Í, Rodríguez-López M, Aspe de la Iglesia B, Pego JM (2015) CD26 Expression on T Helper Populations and sCD26 Serum Levels in Patients with Rheumatoid Arthritis. PLoS One 10:e0131992

[80] Duhen T, Duhen R, Lanzavecchia A, Sallusto F, Campbell DJ (2012) Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. Blood 119:4430-4440

[81] Ma Y, Visser L, Blokzijl T, Harms G, Atayar C, Poppema S, van den Berg A (2008) The CD4+CD26- T-cell population in classical Hodgkin's lymphoma displays a distinctive regulatory T-cell profile. Lab Invest 88:482-490

[82] Cordero OJ, Salgado FJ, Nogueira M (2009) On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother 58:1723-1747

[83] Van Der Velden VH, Naber BA, Van Hal PT, Overbeek SE, Hoogsteden HC, Versnel MA (1999) Peptidase activities in serum and bronchoalveolar lavage fluid from allergic asthmatics--comparison with healthy non-smokers and smokers and effects of inhaled glucocorticoids. Clin Exp Allergy 29:813-823

[84] Durinx C, Lambeir AM, Bosmans E, Falmagne JB, Berghmans R, Haemers A, Scharpé S, De Meester I (2000) Molecular characterization of dipeptidyl peptidase activity in serum: soluble CD26/dipeptidyl peptidase IV is responsible for the release of X-Pro dipeptides. Eur J Biochem 267:5608-5613

[85] Iwaki-Egawa S, Watanabe Y, Kikuya Y, Fujimoto Y (1998) Dipeptidyl peptidase IV from human serum: purification, characterization, and N-terminal amino acid sequence. J Biochem 124:428-433

[86] Remes ST, Delezuch W, Pulkki K, Pekkanen J, Korppi M, Matinlauri IH (2011) Association of serum-soluble CD26 and CD30 levels with asthma, lung function and bronchial hyper-responsiveness at school age. Acta Paediatr 100:e106-11

[87] Durinx C, Neels H, Van der Auwera JC, Naelaerts K, Scharpe S, De Meester I (2001) Reference values for

plasma dipeptidyl-peptidase IV activity and their association with other laboratory parameters. Clin Chem Lab Med 39:155-159

[88] Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, Ouwens DM, Eckardt K, Kaufman JM, Ryden M, Müller S, Hanisch FG, Ruige J, Arner P, Sell H, Eckel J (2011) Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes 60:1917-1925

[89] Delezuch W, Marttinen P, Kokki H, Heikkinen M, Vanamo K, Pulkki K, Matinlauri I (2012) Serum and CSF soluble CD26 and CD30 concentrations in healthy pediatric surgical outpatients. Tissue Antigens 80:368-375

[90] De Chiara L, Rodríguez-Piñeiro AM, Cordero OJ, Rodríguez-Berrocal FJ, Ayude D, Rivas-Hervada And FJ, de la Cadena MP (2009) Soluble CD26 levels and its association to epidemiologic parameters in a sample population. Dis Markers 27:311-316

[91] Tejera-Alhambra M, Casrouge A, de Andrés C, Ramos-Medina R, Alonso B, Vega J, Albert ML, Sánchez-Ramón S (2014) Low DPP4 expression and activity in multiple sclerosis. Clin Immunol 150:170-183

[92] Kobayashi H, Hosono O, Mimori T, Kawasaki H, Dang NH, Tanaka H, Morimoto C (2002) Reduction of serum soluble CD26/dipeptidyl peptidase IV enzyme activity and its correlation with disease activity in systemic lupus erythematosus. J Rheumatol 29:1858-1866

[93] Busso N, Wagtmann N, Herling C, Chobaz-Péclat V, Bischof-Delaloye A, So A, Grouzmann E (2005)

Circulating CD26 is negatively associated with inflammation in human and experimental arthritis. Am J Pathol 166:433-442

[94] Duke-Cohan JS, Morimoto C, Rocker JA, Schlossman SF (1995) A novel form of dipeptidylpeptidase IV found in human serum. Isolation, characterization, and comparison with T lymphocyte membrane dipeptidylpeptidase IV (CD26). J Biol Chem 270:14107-14114

[95] Duke-Cohan JS, Morimoto C, Rocker JA, Schlossman SF (1996) Serum high molecular weight dipeptidyl peptidase IV (CD26) is similar to a novel antigen DPPT-L released from activated T cells. J Immunol 156:1714-1721

[96] Duke-Cohan JS, Gu J, McLaughlin DF, Xu Y, Freeman GJ, Schlossman SF (1998) Attractin (DPPT-L), a member of the CUB family of cell adhesion and guidance proteins, is secreted by activated human T lymphocytes and modulates immune cell interactions. Proc Natl Acad Sci U S A 95:11336-11341

[97] Wjst M (2007) Public data mining shows extended linkage disequilibrium around ADAM33. Allergy 62:444-446

[98] Röhrborn D, Eckel J, Sell H (2014) Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells. FEBS Lett 588:3870-3877

[99] Aso Y, Terasawa T, Kato K, Jojima T, Suzuki K, Iijima T, Kawagoe Y, Mikami S, Kubota Y, Inukai T, Kasai K (2013) The serum level of soluble CD26/dipeptidyl peptidase 4 increases in response to acute hyperglycemia after an oral glucose load in

healthy subjects: association with high-molecular weight adiponectin and hepatic enzymes. Transl Res 162:309-316

[100] Lambeir AM, Durinx C, Scharpé S, De Meester I (2003) Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci 40:209-294

[101] Wang Z, Grigo C, Steinbeck J, von Hörsten S, Amann K, Daniel C (2014) Soluble DPP4 originates in part from bone marrow cells and not from the kidney. Peptides 57:109-117

[102] Uematsu T, Urade M, Yamaoka M (1998) Decreased expression and release of dipeptidyl peptidase IV (CD26) in cultured peripheral blood T lymphocytes of oral cancer patients. J Oral Pathol Med 27:106-110

[103] Annunziato F, Romagnani C, Romagnani S (2015) The 3 major types of innate and adaptive cell-mediated effector immunity. J Allergy Clin Immunol 135:626-635

[104] Robinson DS (2010) The role of the T cell in asthma. J Allergy Clin Immunol 126:1081-1091

[105] Raundhal M, Morse C, Khare A, Oriss TB, Milosevic J, Trudeau J, Huff R, Pilewski J, Holguin F, Kolls J, Wenzel S, Ray P, Ray A (2015) High IFN-γ and low SLPI mark severe asthma in mice and humans. J Clin Invest 125:3037-3050

[106] Nakajima H, Hirose K (2010) Role of IL-23 and Th17 Cells in Airway Inflammation in Asthma. Immune Netw 10:1-4

[107] Zennaro D, Scala E, Pomponi D, Caprini E, Arcelli D, Gambineri E, Russo G, Mari A (2012) Proteomics plus

genomics approaches in primary immunodeficiency: the case of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. Clin Exp Immunol 167:120-128

[108] Ohshima M, Futamura M, Kamachi Y, Ito K, Sakamoto T (2009) Allergic bronchopulmonary aspergillosis in a 2-year-old asthmatic boy with immune dysregulation, polyendocrinopathy, enteropathy, X-linked. Pediatr Pulmonol 44:297-299

[109] Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, Thunberg S, Deniz G, Valenta R, Fiebig H, Kegel C, Disch R, Schmidt-Weber CB, Blaser K, Akdis CA (2004) Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 199:1567-1575

[110] Runyon RS, Cachola LM, Rajeshuni N, Hunter T, Garcia M, Ahn R, Lurmann F, Krasnow R, Jack LM, Miller RL, Swan GE, Kohli A, Jacobson AC, Nadeau KC (2012) Asthma discordance in twins is linked to epigenetic modifications of T cells. PLoS One 7:e48796

[111] De Meester I, Vanham G, Kestens L, Vanhoof G, Bosmans E, Gigase P, Scharpé S (1994) Binding of adenosine deaminase to the lymphocyte surface via CD26. Eur J Immunol 24:566-570

[112] Ishii T, Ohnuma K, Murakami A, Takasawa N, Kobayashi S, Dang NH, Schlossman SF, Morimoto C (2001) CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO. Proc Natl Acad Sci U S A 98:12138-12143

[113] Ohnuma K, Yamochi T, Uchiyama M, Nishibashi K, Yoshikawa N, Shimizu N, Iwata S, Tanaka H, Dang NH, Morimoto C (2004) CD26 up-regulates expression of CD86 on antigen-presenting cells by means of caveolin-1. Proc Natl Acad Sci U S A 101:14186-14191

[114] Herrera C, Morimoto C, Blanco J, Mallol J, Arenzana F, Lluis C, and Franco R (2001) Comodulation of CXCR4 and CD26 in Human Lymphocytes. J Biol Chem 276:19532-19539

[115] Löster K, Zeilinger K, Schuppan D, Reutter W (1995) The cysteine-rich region of dipeptidyl peptidase IV (CD 26) is the collagen-binding site. Biochem Biophys Res Commun 217:341-348

[116] Gonzalez-Gronow M, Grenett HE, Weber MR, Gawdi G, Pizzo SV (2001) Interaction of plasminogen with dipeptidyl peptidase IV initiates a signal transduction mechanism which regulates expression of matrix metalloproteinase-9 by prostate cancer cells. Biochem J 355:397-407

[117] Davoodi J, Kelly J, Gendron NH, MacKenzie AE (2007) The Simpson-Golabi-Behmel syndrome causative glypican-3, binds to and inhibits the dipeptidyl peptidase activity of CD26. Proteomics 7:2300-2310

[118] Piazza GA, Callanan HM, Mowery J, Hixson DC (1989) Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix. Biochem J 262:327-334

[119] Cheng HC, Abdel-Ghany M, Pauli BU (2003) A novel consensus motif in fibronectin mediates dipeptidyl

peptidase IV adhesion and metastasis. J Biol Chem 278:24600-24607

[120] Komiya E, Ohnuma K, Yamazaki H, Hatano R, Iwata S, Okamoto T, Dang NH, Yamada T, Morimoto C (2014) CD26-mediated regulation of periostin expression contributes to migration and invasion of malignant pleural mesothelioma cells. Biochem Biophys Res Commun 447:609-615

[121] Bobolea I, Barranco P, Del Pozo V, Romero D, Sanz V, López-Carrasco V, Canabal J, Villasante C, Quirce S (2015) Sputum periostin in patients with different severe asthma phenotypes. Allergy 70:540-546

[122] Salgado FJ, Lojo J, Alonso-Lebrero JL, Lluis C, Franco R, Cordero OJ, Nogueira M (2003) A role for interleukin-12 in the regulation of T cell plasma membrane compartmentation. J Biol Chem 278:24849-24857

[123] Saunders AE, Johnson P (2010) Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45. Cell Signal 22:339-348

[124] Hermiston ML1, Xu Z, Weiss A (2003) CD45: a critical regulator of signaling thresholds in immune cells. Annu Rev Immunol 21:107-137

[125] Torimoto Y, Dang NH, Vivier E, Tanaka T, Schlossman SF, Morimoto C (1991) Coassociation of CD26 (dipeptidyl peptidase IV) with CD45 on the surface of human T lymphocytes. J Immunol 147:2514-2517

[126] Hegen M, Kameoka J, Dong RP, Schlossman SF, Morimoto C (1997) Cross-linking of CD26 by antibody induces tyrosine phosphorylation and activation of

mitogen-activated protein kinase. Immunology 90:257-264

[127] Kähne T, Neubert K, Faust J, Ansorge S (1998) Early phosphorylation events induced by DPIV/CD26-specific inhibitors. Cell Immunol 189:60-66

[128] Kobayashi S, Ohnuma K, Uchiyama M, Iino K, Iwata S, Dang NH, Morimoto C (2004) Association of CD26 with CD45RA outside lipid rafts attenuates cord blood T-cell activation. Blood 103:1002-1010

[129] Ohnuma K, Yamochi T, Uchiyama M, Nishibashi K, Iwata S, Hosono O, Kawasaki H, Tanaka H, Dang NH, Morimoto C (2005) CD26 mediates dissociation of Tollip and IRAK-1 from caveolin-1 and induces upregulation of CD86 on antigen-presenting cells. Mol Cell Biol 25:7743-7757

[130] Ohnuma K, Uchiyama M, Yamochi T, Nishibashi K, Hosono O, Takahashi N, Kina S, Tanaka H, Lin X, Dang NH, Morimoto C (2007) Caveolin-1 triggers T-cell activation via CD26 in association with CARMA1. J Biol Chem 282:10117-10131

[131] Ohnuma K, Yamochi T, Hosono O, Morimoto C (2005) CD26 T cells in the pathogenesis of asthma. Clin Exp Immunol 139:13-16

[132] Hühn J, Ehrlich S, Fleischer B, von Bonin A (2000) Molecular analysis of CD26-mediated signal transduction in T cells. Immunol Lett 72:127-132

[133] Vora KA, Porter G, Peng R, Cui Y, Pryor K, Eiermann G, Zaller DM (2009) Genetic ablation or pharmacological blockade of dipeptidyl peptidase IV does not impact T cell-dependent immune responses. BMC Immunol 10:19-29

[134] Kameoka J, Tanaka T, Nojima Y, Schlossman SF, Morimoto C (1993) Direct association of adenosine deaminase with a T cell activation antigen, CD26. Science 261:466-469

[135] Gracia E, Farré D, Cortés A, Ferrer-Costa C, Orozco M, Mallol J, Lluís C, Canela EI, McCormick PJ, Franco R, Fanelli F, Casadó V (2013) The catalytic site structural gate of adenosine deaminase allosterically modulates ligand binding to adenosine receptors. FASEB J 27:1048-1061

[136] Blackburn MR, Thompson LF (2012) Adenosine deaminase deficiency: unanticipated benefits from the study of a rare immunodeficiency. J Immunol 188:933-935

[137] Ciruela F, Saura C, Canela EI, Mallol J, Lluis C, Franco R (1996) Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. FEBS Lett 380:219-223

[138] Gracia E, Pérez-Capote K, Moreno E, Barkešová J, Mallol J, Lluís C, Franco R, Cortés A, Casadó V, Canela EI (2011) A2A adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase. Biochem J 435:701-709

[139] Martín M, Huguet J, Centelles JJ, Franco R (1995) Expression of ecto-adenosine deaminase and CD26 in human T cells triggered by the TCR-CD3 complex. Possible role of adenosine deaminase as costimulatory molecule. J Immunol 155:4630-4643

[140] Pacheco R, Martinez-Navio JM, Lejeune M, Climent N, Oliva H, Gatell JM, Gallart T, Mallol J, Lluis C, Franco R (2005) CD26, adenosine deaminase, and adenosine receptors mediate costimulatory signals in

the immunological synapse. Proc Natl Acad Sci U S A 102:9583-9588

[141] Naval-Macabuhay I, Casanova V, Navarro G, García F, León A, Miralles L, Rovira C, Martinez-Navio JM, Gallart T, Mallol J, Gatell JM1, Lluís C, Franco R, McCormick PJ, Climent N (2016) Adenosine deaminase regulates Treg expression in autologous T cell-dendritic cell cocultures from patients infected with HIV-1. J Leukoc Biol 99:349-359

[142] Molon B, Gri G, Bettella M, Gómez-Moutón C, Lanzavecchia A, Martínez-A C, Mañes S, Viola A (2005) T cell costimulation by chemokine receptors. Nat Immunol 6:465-471

[143] Tanaka T, Duke-Cohan JS, Kameoka J, Yaron A, Lee I, Schlossman SF, Morimoto C (1994) Enhancement of antigen-induced T-cell proliferation by soluble CD26/dipeptidyl peptidase IV. Proc Natl Acad Sci U S A 91 :3082-3086

[144] Schmitz T, Underwood R, Khiroya R, Bachovchin WW, Huber BT (1996) Potentiation of the immune response in HIV-1+ individuals. J Clin Invest 97:1545-1549

[145] Yu DM, Slaitini L, Gysbers V, Riekhoff AG, Kähne T, Knott HM, De Meester I, Abbott CA, McCaughan GW, Gorrell MD (2011) Soluble CD26/dipeptidyl peptidase IV enhances human lymphocyte proliferation in vitro independent of dipeptidyl peptidase enzyme activity and adenosine deaminase binding. Scand J Immunol 73:102-111

[146] Ohnuma K, Munakata Y, Ishii T, Iwata S, Kobayashi S, Hosono O, Kawasaki H, Dang NH, Morimoto C (2001) Soluble CD26/dipeptidyl peptidase IV induces

T cell proliferation through CD86 up-regulation on APCs. J Immunol 167:6745-6755

[147] Ikushima H, Munakata Y, Ishii T, Iwata S, Terashima M, Tanaka H, Schlossman SF, Morimoto C (2000) Internalization of CD26 by mannose 6-phosphate/insulin-like growth factor II receptor contributes to T cell activation. Proc Natl Acad Sci U S A 97:8439-8444

[148] Wronkowitz N, Görgens SW, Romacho T, Villalobos LA, Sánchez-Ferrer CF, Peiró C, Sell H, Eckel J (2014) Soluble DPP4 induces inflammation and proliferation of human smooth muscle cells via protease-activated receptor 2. Biochim Biophys Acta 1842:1613-1621

[149] Lee DS, Lee ES, Alam MM, Jang JH, Lee HS, Oh H, Kim YC, Manzoor Z, Koh YS, Kang DG, Lee DH (2016) Soluble DPP-4 up-regulates toll-like receptors and augments inflammatory reactions, which are ameliorated by vildagliptin or mannose-6-phosphate. Metabolism 65:89-101

[150] Proost P, Struyf S, Schols D, Opdenakker G, Sozzani S, Allavena P, Mantovani A, Augustyns K, Bal G, Haemers A, Lambeir AM, Scharpé S, Van Damme J, De Meester I (1999) Truncation of macrophage-derived chemokine by CD26/ dipeptidyl-peptidase IV beyond its predicted cleavage site affects chemotactic activity and CC chemokine receptor 4 interaction. J Biol Chem 274:3988-3993

[151] Engel M, Hoffmann T, Manhart S, Heiser U, Chambre S, Huber R, Demuth HU, Bode W (2006) Rigidity and flexibility of dipeptidyl peptidase IV: crystal structures of and docking experiments with DPIV. J Mol Biol 354:768-783

[152] Vanhoof G, Goossens F, De Meester I, Hendriks D, Scharpé S (1995) Proline motifs in peptides and their biological processing. FASEB J 9:736-744

[153] Tanaka T, Kameoka J, Yaron A, Schlossman SF, Morimoto C (1993) The costimulatory activity of the CD26 antigen requires dipeptidyl peptidase IV enzymatic activity. Proc Natl Acad Sci U S A 90:4586-4590

[154] Schön E, Mansfeld HW, Demuth HU, Barth A, Ansorge S. (1985) The dipeptidyl peptidase IV, a membrane enzyme involved in the proliferation of T lymphocytes. Biomed Biochim Acta 44:K9-15

[155] Reinhold D, Bank U, Bühling F, Lendeckel U, Faust J, Neubert K, Ansorge S (1997) Inhibitors of dipeptidyl peptidase IV induce secretion of transforming growth factor-beta 1 in PWM-stimulated PBMC and T cells. Immunology 91:354-360

[156] Reinhold D, Bank U, Bühling F, Täger M, Born I, Faust J, Neubert K, Ansorge S (1997) Inhibitors of dipeptidyl peptidase IV (DP IV, CD26) induces secretion of transforming growth factor-beta 1 (TGF-beta 1) in stimulated mouse splenocytes and thymocytes. Immunol Lett 58:29-35

[157] Reinhold D, Hemmer B, Gran B, Born I, Faust J, Neubert K, McFarland HF, Martin R, Ansorge S (1998) Inhibitors of dipeptidyl peptidase IV/CD26 suppress activation of human MBP-specific CD4+ T cell clones. J Neuroimmunol 87:203-209

[158] Tanaka S, Murakami T, Horikawa H, Sugiura M, Kawashima K, Sugita T (1997) Suppression of arthritis by the inhibitors of dipeptidyl peptidase IV. Int J Immunopharmacol 19:15-24

[159] Steinbrecher A, Reinhold D, Quigley L, Gado A, Tresser N, Izikson L, Born I, Faust J, Neubert K, Martin R, Ansorge S, Brocke S (2000) Dipeptidyl peptidase IV in inflammatory CNS disease. Adv Exp Med Biol 477:145-153

[160] Dai Y, Dai D, Wang X, Ding Z, Mehta JL (2014) DPP-4 inhibitors repress NLRP3 inflammasome and interleukin-1beta via GLP-1 receptor in macrophages through protein kinase C pathway. Cardiovasc Drugs Ther 28:425-432

[161] Wilson CN, Nadeem A, Spina D, Brown R, Page CP, Mustafa SJ (2009) Adenosine receptors and asthma. Handb Exp Pharmacol 329-362

[162] Polosa R, Blackburn MR (2009) Adenosine receptors as targets for therapeutic intervention in asthma and chronic obstructive pulmonary disease. Trends Pharmacol Sci 30:528-535

[163] Irie-Sasaki J, Sasaki T, Matsumoto W, Opavsky A, Cheng M, Welstead G, Griffiths E, Krawczyk C, Richardson CD, Aitken K, Iscove N, Koretzky G, Johnson P, Liu P, Rothstein DM, Penninger JM (2001) CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. Nature 409:349-354

[164] Stütz A, Graf P, Beinhauer B, Hammerschmid F, Neumann C, Woisetschläger M and Jung T (2005) CD45 isoform expression is associated with different susceptibilities of human naive and effector CD4+ T cells to respond to IL-4. Eur J Immunol 35:575–583

[165] Mentlein R, Gallwitz B, Schmidt WE (1993) Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their

degradation in human serum. Eur J Biochem 214:829-835

[166] Yu DM, Yao TW, Chowdhury S, Nadvi NA, Osborne B, Church WB, McCaughan GW, Gorrell MD (2010) The dipeptidyl peptidase IV family in cancer and cell biology. FEBS J 277:1126-1144

[167] Boonacker E, Van Noorden CJ (2003) The multifunctional or moonlighting protein CD26/DPPIV. Eur J Cell Biol 82:53-73

[168] Broxmeyer HE, Hoggatt J, O'Leary HA, Mantel C, Chitteti BR, Cooper S, Messina-Graham S, Hangoc G, Farag S, Rohrabaugh SL, Ou X, Speth J, Pelus LM, Srour EF, Campbell TB (2012) Dipeptidylpeptidase 4 negatively regulates colony-stimulating factor activity and stress hematopoiesis. Nat Med 18:1786-1796

[169] Tasic T, Bäumer W, Schmiedl A, Schwichtenhövel F, Pabst R, Raap U, von Hörsten S, Stephan M (2011) Dipeptidyl peptidase IV (DPP4) deficiency increases Th1-driven allergic contact dermatitis. Clin Exp Allergy 41:1098-1107

[170] Lankas GR, Leiting B, Roy RS, Eiermann GJ, Beconi MG, Biftu T, Chan CC, Edmondson S, Feeney WP, He H, Ippolito DE, Kim D, Lyons KA, Ok HO, Patel RA, Petrov AN, Pryor KA, Qian X, Reigle L, Woods A, Wu JK, Zaller D, Zhang X, Zhu L, Weber AE, Thornberry NA (2005) Dipeptidyl peptidase IV inhibition for the treatment of type 2 diabetes: potential importance of selectivity over dipeptidyl peptidases 8 and 9. Diabetes 54:2988-2994

[171] Preller V, Gerber A, Wrenger S, Togni M, Marguet D, Tadje J, Lendeckel U, Röcken C, Faust J, Neubert K, Schraven B, Martin R, Ansorge S, Brocke S, Reinhold

D (2007) TGF-beta1-mediated control of central nervous system inflammation and autoimmunity through the inhibitory receptor CD26. J Immunol 178:4632-4640

[172] Baticic L, Detel D, Kucic N, Buljevic S, Pugel EP, Varljen J (2011) Neuroimmunomodulative properties of dipeptidyl peptidase IV/CD26 in a TNBS-induced model of colitis in mice. J Cell Biochem 112:3322-3333

[173] McGuinness C, Wesley UV (2008) Dipeptidyl peptidase IV (DPPIV), a candidate tumor suppressor gene in melanomas is silenced by promoter methylation. Front Biosci 13:2435-2443

[174] Arscott WT, LaBauve AE, May V, Wesley UV (2009) Suppression of neuroblastoma growth by dipeptidyl peptidase IV: relevance of chemokine regulation and caspase activation. Oncogene 28:479-491

[175] Oravecz T, Pall M, Roderiquez G, Gorrell MD, Ditto M, Nguyen NY, Boykins R, Unsworth E, Norcross MA (1997) Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage. J Exp Med 186:1865-1872

[176] Kufareva I, Salanga CL, Handel TM (2015) Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies. Immunol Cell Biol 93:372-383

[177] Wang J, Harada A, Matsushita S, Matsumi S, Zhang Y, Shioda T, Nagai Y, Matsushima K (1998) IL-4 and a glucocorticoid up-regulate CXCR4 expression on

human CD4+ T lymphocytes and enhance HIV-1 replication. J Leukoc Biol 64:642-649

[178] Gonzalo JA, Lloyd CM, Peled A, Delaney T, Coyle AJ, Gutierrez-Ramos JC (2000) Critical involvement of the chemotactic axis CXCR4/stromal cell-derived factor-1 alpha in the inflammatory component of allergic airway disease. J Immunol 165:499-508

[179] Bai Z, Hayasaka H, Kobayashi M, Li W, Guo Z, Jang MH, Kondo A, Choi BI, Iwakura Y, Miyasaka M (2009) CXC chemokine ligand 12 promotes CCR7-dependent naive T cell trafficking to lymph nodes and Peyer's patches. J Immunol 182:1287-1295

[180] Werner L, Guzner-Gur H, Dotan I (2013) Involvement of CXCR4/CXCR7/CXCL12 Interactions in Inflammatory bowel disease. Theranostics 3:40-46

[181] Romagnani S (2002) Cytokines and chemoattractants in allergic inflammation Mol Immunol 38:881-885

[182] Islam SA, Luster AD (2012) T cell homing to epithelial barriers in allergic disease. Nat Med 18:705-715

[183] Manns J, Rieder S, Escher S, Eilers B, Forssmann WG, Elsner J, Forssmann U (2007) The allergy-associated chemokine receptors CCR3 and CCR5 can be inactivated by the modified chemokine NNY-CCL11. Allergy 62:17-24

[184] Sehmi R, Dorman S, Baatjes A, Watson R, Foley R, Ying S, Robinson DS, Kay AB, O'Byrne PM, Denburg JA (2003) Allergen-induced fluctuation in CC chemokine receptor 3 expression on bone marrow CD34+ cells from asthmatic subjects: significance for mobilization of haemopoietic progenitor cells in allergic inflammation. Immunology 109:536-546

[185] Lambeir AM, Proost P, Durinx C, Bal G, Senten K, Augustyns K, Scharpé S, Van Damme J, De Meester I (2001) Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. J Biol Chem 276:29839-29845

[186] Struyf S, Proost P, Schols D, De Clercq E, Opdenakker G, Lenaerts JP, Detheux M, Parmentier M, De Meester I, Scharpé S, Van Damme J (1999) CD26/dipeptidyl-peptidase IV down-regulates the eosinophil chemotactic potency, but not the anti-HIV activity of human eotaxin by affecting its interaction with CC chemokine receptor 3. J Immunol 162:4903-4909

[187] Forssmann U, Stoetzer C, Stephan M, Kruschinski C, Skripuletz T, Schade J, Schmiedl A, Pabst R, Wagner L, Hoffmann T, Kehlen A, Escher SE, Forssmann WG, Elsner J, von Hörsten S (2008) Inhibition of CD26/dipeptidyl peptidase IV enhances CCL11/eotaxin-mediated recruitment of eosinophils in vivo. J Immunol 181:1120-1127

[188] Savino B, Borroni EM, Torres NM, Proost P, Struyf S, Mortier A, Mantovani A, Locati M, Bonecchi R (2009) Recognition versus adaptive up-regulation and degradation of CC chemokines by the chemokine decoy receptor D6 are determined by their N-terminal sequence. J Biol Chem 284:26207-26215

[189] Pawig L, Klasen C, Weber C, Bernhagen J, Noels H (2015) Diversity and Inter-Connections in the CXCR4 Chemokine Receptor/Ligand Family: Molecular Perspectives. Front Immunol 6:429

[190] Lukacs NW, Berlin A, Schols D, Skerlj RT, Bridger GJ (2002) AMD3100, a CxCR4 antagonist, attenuates

allergic lung inflammation and airway hyperreactivity. Am J Pathol 160:1353-1360

[191] Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA (1996) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med 184:1101-1109

[192] Nanki T, Hayashida K, El-Gabalawy HS, Suson S, Shi K, Girschick HJ, Yavuz S, Lipsky PE (2000) Stromal cell-derived factor-1-CXC chemokine receptor 4 interactions play a central role in CD4+ T cell accumulation in rheumatoid arthritis synovium. J Immunol 165:6590- 6598

[193] Ohtsuki T, Hosono O, Kobayashi H, Munakata Y, Souta A, Shioda T, Morimoto C (1998) Negative regulation of the anti-human immunodeficiency virus and chemotactic activity of human stromal cell-derived factor 1alpha by CD26/dipeptidyl peptidase IV. FEBS Lett 431:236-240

[194] Proost P, Struyf S, Schols D, Durinx C, Wuyts A, Lenaerts JP, De Clercq E, De Meester I, Van Damme J (1998) Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity of stromal-cell-derived factor-1alpha. FEBS Lett 432:73-76

[195] De La Luz Sierra M, Yang F, Narazaki M, Salvucci O, Davis D, Yarchoan R, Zhang HH, Fales H, Tosato G (2004) Differential processing of stromal-derived factor-1alpha and stromal-derived factor-1beta explains functional diversity. Blood 103:2452-2459

[196] Christopherson KW 2nd, Hangoc G, Broxmeyer HE (2002) Cell surface peptidase CD26/dipeptidylpeptidase IV regulates

CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34+ progenitor cells. J Immunol 169:7000-7008

[197] Iwata S, Yamaguchi N, Munakata Y, Ikushima H, Lee JF, Hosono O, Schlossman SF, Morimoto C (1999) CD26/dipeptidyl peptidase IV differentially regulates the chemotaxis of T cells and monocytes toward RANTES: possible mechanism for the switch from innate to acquired immune response. Int Immunol 11:417-426

[198] Ohtsuki T, Tsuda H, Morimoto C (2000) Good or evil: CD26 and HIV infection. J Dermatol Sci 22:152-160

[199] Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR (1997) The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc Natl Acad Sci U S A 94:1925-1930

[200] Franitza S, Kollet O, Brill A, Vaday GG, Petit I, Lapidot T, Alon R, Lider O (2002) TGF-beta1 enhances SDF-1alpha-induced chemotaxis and homing of naive T cells by up-regulating CXCR4 expression and downstream cytoskeletal effector molecules. Eur J Immunol 32:193-202

[201] Post S, Smits AM, van den Broek AJ, Sluijter JP, Hoefer IE, Janssen BJ, Snijder RJ, Mager JJ, Pasterkamp G, Mummery CL, Doevendans PA, Goumans MJ (2010) Impaired recruitment of HHT-1 mononuclear cells to the ischaemic heart is due to an altered CXCR4/CD26 balance. Cardiovasc Res 85:494-502

[202] Post S, van den Broek AJ, Rensing BJ, Pasterkamp G, Goumans MJ, Doevendans PA (2012) Reduced CD26 expression is associated with improved cardiac

function after acute myocardial infarction: insights from the REPERATOR study. J Mol Cell Cardiol 53:899-905

[203] Havre PA, Abe M, Urasaki Y, Ohnuma K, Morimoto C, Dang NH (2009) CD26 expression on T cell lines increases SDF-1-alpha-mediated invasion. Br J Cancer 101:983-991

[204] Fernandis AZ, Cherla RP, Ganju RK (2003) Differential regulation of CXCR4-mediated T-cell chemotaxis and mitogen-activated protein kinase activation by the membrane tyrosine phosphatase, CD45. J Biol Chem 278:9536-9543

[205] Mantovani A, Gray PA, Van Damme J, Sozzani S (2000) Macrophage-derived chemokine (MDC). J Leukoc Biol 68:400-404

[206] Yamashita U, Kuroda E (2002) Regulation of macrophage-derived chemokine (MDC, CCL22) production. Crit Rev Immunol 22:105-114

[207] Yanai M, Sato K, Aoki N, Takiyama Y, Oikawa K, Kobayashi H, Kimura S, Harabuchi Y, Tateno M (2007) The role of CCL22/macrophage-derived chemokine in allergic rhinitis. Clin Immunol 125:291-298

[208] Kim CH, Rott L, Kunkel EJ, Genovese MC, Andrew DP, Wu L, Butcher EC (2001) Rules of chemokine receptor association with T cell polarization in vivo. J Clin Invest 108:1331-1339

[209] Appay V, Rowland-Jones SL (2001) RANTES: a versatile and controversial chemokine. Trends Immunol 22:83-87

[210] Lim JK, Burns JM, Lu W, DeVico AL (2005) Multiple pathways of amino terminal processing produce two

truncated variants of RANTES/CCL5. J Leukoc Biol 78:442-452

[211] Proost P, Menten P, Struyf S, Schutyser E, De Meester I, Van Damme J (2000) Cleavage by CD26/dipeptidyl peptidase IV converts the chemokine LD78beta into a most efficient monocyte attractant and CCR1 agonist. Blood 96:1674-1680

[212] Nakao M, Nomiyama H, Shimada K (1990) Structures of human genes coding for cytokine LD78 and their expression. Mol Cell Biol 10:3646-3658

[213] Menten P, Struyf S, Schutyser E, Wuyts A, De Clercq E, Schols D, Proost P, Van Damme J (1999) The LD78beta isoform of MIP-1alpha is the most potent CCR5 agonist and HIV-1-inhibiting chemokine. J Clin Invest 104:R1-5

[214] Struyf S, Menten P, Lenaerts JP, Put W, D'Haese A, De Clercq E, Schols D, Proost P, Van Damme J (2001) Diverging binding capacities of natural LD78beta isoforms of macrophage inflammatory protein-1alpha to the CC chemokine receptors 1, 3 and 5 affect their anti-HIV-1 activity and chemotactic potencies for neutrophils and eosinophils. Eur J Immunol 31:2170-2178

[215] Ludwig A, Schiemann F, Mentlein R, Lindner B, Brandt E (2002) Dipeptidyl peptidase IV (CD26) on T cells cleaves the CXC chemokine CXCL11 (I-TAC) and abolishes the stimulating but not the desensitizing potential of the chemokine. J Leukoc Biol 72:183-191

[216] Proost P, Mortier A, Loos T, Vandercappellen J, Gouwy M, Ronsse I, Schutyser E, Put W, Parmentier M, Struyf S, Van Damme J (2007) Proteolytic processing of CXCL11 by CD13/aminopeptidase N

impairs CXCR3 and CXCR7 binding and signaling and reduces lymphocyte and endothelial cell migration. Blood 110:37-44

[217] Casrouge A, Bisiaux A, Stephen L, Schmolz M, Mapes J, Pfister C, Pol S, Mallet V, Albert ML (2012) Discrimination of agonist and antagonist forms of CXCL10 in biological samples. Clin Exp Immunol 167:137-148

[218] Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, Sallusto F, Napolitani G (2007) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 8:639-646

[219] Pennino D, Bhavsar PK, Effner R, Avitabile S, Venn P, Quaranta M, Marzaioli V, Cifuentes L, Durham SR, Cavani A, Eyerich K, Chung KF, Schmidt-Weber CB, Eyerich S (2013) IL-22 suppresses IFN-γ-mediated lung inflammation in asthmatic patients. J Allergy Clin Immunol 131:562-570

[220] Kruschinski C, Skripuletz T, Bedoui S, Tschernig T, Pabst R, Nassenstein C, Braun A, von Hörsten S (2005) CD26 (dipeptidyl-peptidase IV)-dependent recruitment of T cells in a rat asthma model. Clin Exp Immunol 139:17-24

[221] Tsuji E, Misumi Y, Fujiwara T, Takami N, Ogata S, Ikehara Y (1992) An active-site mutation (Gly633→Arg) of dipeptidyl peptidase IV causes its retention and rapid degradation in the endoplasmic reticulum. Biochemistry 31:11921-11927

[222] Schmiedl A, Krainski J, Schwichtenhövel F, Schade J, Klemann C, Raber KA, Zscheppang K, Beekmann T, Acevedo C, Glaab T, Wedekind D, Pabst R, von

Hörsten S, Stephan M (2010) Reduced airway inflammation in CD26/DPP4-deficient F344 rats is associated with altered recruitment patterns of regulatory T cells and expression of pulmonary surfactant proteins. Clin Exp Allergy 40:1794-1808

[223] Schade J, Schmiedl A, Stephan M, Pabst R, von Hörsten S (2010) Transferred T cells preferentially adhere in the BALT of CD26-deficient recipient lungs during asthma. Immunobiology 215:321-331

[224] Narducci MG, Scala E, Bresin A, Caprini E, Picchio MC, Remotti D, Ragone G, Nasorri F, Frontani M, Arcelli D, Volinia S, Lombardo GA, Baliva G, Napolitano M, Russo G (2006) Skin homing of Sézary cells involves SDF-1-CXCR4 signaling and down-regulation of CD26/dipeptidylpeptidase IV. Blood 107:1108-1115

[225] Hoshino M, Aoike N, Takahashi M, Nakamura Y, Nakagawa T (2003) Increased immunoreactivity of stromal cell-derived factor-1 and angiogenesis in asthma. Eur Respir J 21:804-809

[226] Tasic T, Stephan M, von Hörsten S, Pabst R, Schmiedl A (2014) Differential OVA-induced pulmonary inflammation and unspecific reaction in Dark Agouti (DA) rats contingent on CD26/DPPIV deficiency. Immunobiology 219:888-900

[227] Conarello SL, Li Z, Ronan J, Roy RS, Zhu L, Jiang G, Liu F, Woods J, Zycband E, Moller DE, Thornberry NA, Zhang BB (2003) Mice lacking dipeptidyl peptidase IV are protected against obesity and insulin resistance. Proc Natl Acad Sci U S A 100:6825-6830

[228] Yan S, Marguet D, Dobers J, Reutter W, Fan H (2003) Deficiency of CD26 results in a change of cytokine and

immunoglobulin secretion after stimulation by pokeweed mitogen. Eur J Immunol 33:1519-1527

[229] Rogala B, Glück J, Mazur B (2002) Do the molecules CD26 and lymphocytes activation gene-3 differentiate between type 1 and 2 T cell response? J Investig Allergol Clin Immunol 12:198-203

[230] Matsuno O, Miyazaki E, Nureki S, Ueno T, Ando M, Kumamoto T (2007) Soluble CD26 is inversely Associated with Disease Severity in Patients with Chronic Eosinophilic Pneumonia. Biomark Insights 1:201-204

[231] Katoh N, Hirano S, Suehiro M, Ikenaga K, Yamashita T, Sugawara N, Yasuno H (2000) Soluble CD30 is more relevant to disease activity of atopic dermatitis than soluble CD26. Clin Exp Immunol 121:187-192

[232] Beasley R, Semprini A, Mitchell EA (2015) Risk factors for asthma: is prevention possible? Lancet 386:1075-1085

[233] Leiria LO, Martins MA, Saad MJ (2015) Obesity and asthma: beyond T(H)2 inflammation. Metabolism 64:172-181

[234] Kim SH, Sutherland ER, Gelfand EW (2014) Is there a link between obesity and asthma? Allergy Asthma Immunol Res 6:189-195

[235] Scirica BM, Bhatt DL, Braunwald E, Steg PG, Davidson J, Hirshberg B, Ohman P, Frederich R, Wiviott SD, Hoffman EB, Cavender MA, Udell JA, Desai NR, Mosenzon O, McGuire DK, Ray KK, Leiter LA, Raz I (2013) Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus. N Engl J Med 369:1317-1326

[236] Raschi E, Poluzzi E, Koci A, Antonazzo IC, Marchesini G, De Ponti F (2016) Dipeptidyl peptidase-4 inhibitors and heart failure: Analysis of spontaneous reports submitted to the FDA Adverse Event Reporting System. Nutr Metab Cardiovasc Dis 26:380-386

[237] Green JB, Bethel MA, Armstrong PW, Buse JB, Engel SS, Garg J, Josse R, Kaufman KD, Koglin J, Korn S, Lachin JM, McGuire DK, Pencina MJ, Standl E, Stein PP, Suryawanshi S, Van de Werf F, Peterson ED, Holman RR (2015) Effect of Sitagliptin on Cardiovascular Outcomes in Type 2 Diabetes. N Engl J Med 373:232-242

[238] Willemen MJ, Mantel-Teeuwisse AK, Straus SM, Meyboom RH, Egberts TC, Leufkens HG (2011) Use of dipeptidyl peptidase-4 inhibitors and the reporting of infections: a disproportionality analysis in the World Health Organization VigiBase. Diabetes Care 34:369-374

[239] Amori RE, Lau J, Pittas AG (2007) Efficacy and safety of incretin therapy in type 2 diabetes: systematic review and meta-analysis. JAMA 298:194-206

[240] Monami M, Iacomelli I, Marchionni N, Mannucci E (2010) Dipeptydil peptidase-4 inhibitors in type 2 diabetes: a meta-analysis of randomized clinical trials. Nutr Metab Cardiovasc Dis 20:224-235

[241] Béné J, Jacobsoone A, Coupe P, Auffret M, Babai S, Hillaire-Buys D, Jean-Pastor MJ, Vonarx M, Vermersch A, Tronquoy AF, Gautier S (2015) Bullous pemphigoid induced by vildagliptin: a report of three cases. Fundam Clin Pharmacol 29:112-114

[242] El-Hashim AZ, Amine SA (2005) The role of substance P and bradykinin in the cough reflex and

bronchoconstriction in guinea-pigs. Eur J Pharmacol 513:125-133

[243] Ramalho R, Almeida J, Beltrão M, Pirraco A, Costa R, Sokhatska O, Guardão L, Palmares C, Guimarães JT, Delgado L, Moreira A, Soares R (2012) Substance P antagonist improves both obesity and asthma in a mouse model. Allergy 68:48-54

[244] Sromova L, Busek P, Posova H, Potockova J, Skrha P, Andel M, Sedo A (2016) The effect of dipeptidyl peptidase-IV inhibition on circulating T cell subpopulations in patients with type 2 diabetes mellitus. Diabetes Res Clin Pract 118:183-192

[245] Lee YS, Jun HS (2016) Anti-Inflammatory Effects of GLP-1-Based Therapies beyond Glucose Control. Mediators Inflamm 2016:3094642

[246] Brightling CE, Chanez P, Leigh R, O'Byrne PM, Korn S, She D, May RD, Streicher K, Ranade K, Piper E (2015) Efficacy and safety of tralokinumab in patients with severe uncontrolled asthma: a randomised, double-blind, placebo-controlled, phase 2b trial. Lancet Respir Med 3:692-701

[247] Shiobara T, Chibana K, Watanabe T, Arai R, Horigane Y, Nakamura Y, Hayashi Y, Shimizu Y, Takemasa A, Ishii Y (2016) Dipeptidyl peptidase-4 is highly expressed in bronchial epithelial cells of untreated asthma and it increases cell proliferation along with fibronectin production in airway constitutive cells. Respir Res 17:28

[248] Matteucci E, Giampietro O (2009) Dipeptidyl peptidase-4 (CD26): knowing the function before inhibiting the enzyme. Curr Med Chem 16:2943-2951

[249] Vanderheyden M, Bartunek J, Goethals M, Verstreken S, Lambeir AM, De Meester I, Scharpé S (2009) Dipeptidyl-peptidase IV and B-type natriuretic peptide. From bench to bedside. Clin Chem Lab Med 47:248-252

[250] Zhu L, Tamvakopoulos C, Xie D, Dragovic J, Shen X, Fenyk-Melody JE, Schmidt K, Bagchi A, Griffin PR, Thornberry NA, Sinha Roy R (2003) The role of dipeptidyl peptidase IV in the cleavage of glucagon family peptides: in vivo metabolism of pituitary adenylate cyclase activating polypeptide-(1-38). J Biol Chem 278:22418-22423

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA