DOCTORAL THESIS

NANOCARRIERS FOR ENHANCING THE PERFORMANCE OF IMMUNOMODULATORY MOLECULES

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TESE DE DOUTORAMENTO

NANOVEHÍCULOS PARA MELLORAR A EFICACIA DE MOLÉCULAS INMUNOMODULADORAS

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Nanovehículos para mejorar la eficacia de moléculas inmunomoduladoras

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Á miña familia

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"*O verdadeiro heroísmo consiste en convertir os soños en realidades e as ideas en feitos*"

Alfonso Rodríguez Castelao

"*Science and everyday life cannot and should not be separated*"

Rosalind Franklin

"Hakuna matata"

El Rey León

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Abstract / Resumen

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Abstract

Despite the great potential of the immunomodulatory molecule selected in this thesis to prevent some of the most prevalent diseases of our time, several limitations have hindered its clinical use. For instance, the off-target toxicity associated to the high doses required to achieve its immunomodulatory effect and the high fluctuation in its oral absorption are critical drawbacks. In this sense, nanotechnology offers the potential to improve the biodistribution of this molecule by targeting it to the immune cell populations. Furthermore, the capacity of nanocarriers for overcoming biological barriers opens the possibility to explore administration routes alternative to the oral one for its supplementation. Based on this background, the main objective of this thesis was to develop nanotechnology-based formulations to improve the immunomodulatory effect of the selected molecule and to explore the dermal route as an alternative modality of administration for its supplementation.

The first chapter, which contains part of a recently published review entitled "*Modulating the immune system through nanotechnology*", provides an overview of how nanocarriers can be designed for its preferential access to immune cells pursuing the generation of a tolerogenic response. Furthermore, the chapter discusses specific formulation strategies developed for the treatment and prevention of autoimmune diseases.

The second chapter describes the development and evaluation of polymeric nanocarriers encapsulating the immunomodulatory molecule selected in this thesis as a preventive treatment for type 1 diabetes. The results obtained showed the promotion of a tolerogenic phenotype *in vitro* in human dendritic cells and the delay of diabetes onset in a murine model of autoimmune diabetes.

In chapter 3, polymeric nanocarriers of a small size and variable zeta potential (positive and negative) were studied for the capacity to enhance the transport of the immunomodulatory molecule across the skin. The results obtained *ex vivo,* using human skin, showed that anionic nanocarriers enhanced the accumulation of this molecule in the viable epidermis in a greater extent than cationic nanocarriers. Furthermore, we found that this effect was mediated by the interaction with hair follicles.

Overall, the results of this thesis show the potential of polymeric nanocarriers for the delivery of the selected immunomodulatory molecule in different contexts, enhancing its inmunomodulatory potential and contributing to overcome biological barriers such as the skin.

Resumen

A pesar del potencial de la molécula inmunomoduladora seleccionada en esta tesis para prevenir algunas de las patologías más prevalentes de nuestra época, sus limitaciones han obstaculizado su uso clínico. Por ejemplo, la toxicidad asociada a la administración de dosis altas necesarias para producir un efecto inmunomodulador o la alta variabilidad de su absorción oral son sus mayores inconvenientes. En este sentido, la nanotecnología ofrece la posibilidad de mejorar la biodistribución de dicha molécula promoviendo su interacción preferente con células del sistema inmune. Además, la capacidad de los nanovehículos para superar barreras biológicas abre la posibilidad de explorar vías de administración alternativas para la suplementación de dicha molécula. En este contexto, el principal objetivo de esta tesis se ha centrado en el desarrollo de formulaciones basadas en la nanotecnología para mejorar el efecto inmunomodulador de la molécula seleccionada y para explorar la administración transdérmica como una alternativa para su suplementación.

El primer capítulo, que contiene parte de una revisión recientemente publicada titulada "*Modulación del sistema inmune a través de la nanotecnología*", proporciona una visión general del diseño de nanovehículos para su acceso preferente a células del sistema inmune con el objetivo de generar una respuesta tolerogénica. Además, en él se discuten estrategias de formulación específicas que han sido desarrolladas para el tratamiento y prevención de enfermedades autoinmunes.

El segundo capítulo describe el desarrollo y evaluación de nanovehículos poliméricos conteniendo la molécula inmunomoduladora como tratamiento preventivo de diabetes tipo 1. Los resultados obtenidos muestran la estimulación *in vitro* células dendríticas humanas hacia un fenotipo tolerogénico y un retraso en la aparición de síntomas en un modelo murino de diabetes tipo 1.

En el capítulo 3, se estudian nanovehículos poliméricos de pequeño tamaño y carga superficial diferente (positivos y negativos) para evaluar su capacidad de mejora del transporte de la molécula inmunomoduladora seleccionada a través de la piel. Los resultados obtenidos *ex vivo,* en piel humana, muestran una mayor acumulación de dicha molécula en la epidermis

viva promovida por los nanovehículos aniónicos. Además, se comprobó que este efecto está mediado por la interacción con los folículos pilosos.

En resumen, los resultados de esta tesis muestran el potencial de los nanovehículos poliméricos para la administración de la molécula inmunomoduladora en diferentes contextos, mejorando su efecto inmunomodulador y contribuyendo a superar barreras biológicas como la piel.

Resumen *in extenso*

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Los compuestos naturales se utilizan de manera habitual en la medicina moderna para tratar y prevenir algunas de las enfermedades más prevalentes de nuestros días, como el cáncer o las infecciones microbianas. Entre ellos, la molécula seleccionada en esta tesis es uno de los compuestos que ha sido objeto de numerosos estudios debido a su influencia en distintos procesos fisiológicos. Su papel en dichos procesos, como apoptosis, respuesta inmune o proliferación ha puesto de manifiesto su potencial para intervenir en el tratamiento y prevención de patologías como el cáncer o las enfermedades autoinmunes entre otras. También se ha propuesto la suplementación de este compuesto como una buena alternativa para la prevención de esas patologías.

Sin embargo, la molécula seleccionada presenta varios problemas que limitan su uso clínico. Por un lado, requiere de altas dosis para conseguir un efecto terapéutico debido a su distribución sistémica no dirigida, produciendo efectos adversos. Por otro lado, su suplementación oral se ha asociado con distintos problemas, como sus escasas fuentes en la dieta o la fluctuación de su biodisponibilidad oral. Además, todas las sus formas derivadas muestran alta inestabilidad tanto en almacenamiento como tras su administración *in vivo*, lo que conlleva la necesidad de una formulación efectiva que mantenga su estabilidad en ambas situaciones. Como resultado de todo esto, la eficacia de la dicha molécula para prevención y tratamiento de enfermedades no ha llegado a ser confirmada en ensayos clínicos.

En este sentido, la nanotecnología es una herramienta que permite modificar la biodistribución de este tipo de compuestos, facilitando la interacción preferente con diferentes poblaciones celulares. Gracias a esta administración dirigida, se pueden evitar efectos adversos relacionados con una biodistribución no selectiva. Además, los nanovehículos pueden ser adaptados para su uso en diferentes vías de administración que podrían ser convenientes para la suplementación de esta molécula, como puede ser la vía transdérmica. Asimismo, estas nanoformulaciones pueden proteger de forma eficaz a los diferentes derivados de la molécula seleccionada mediante su encapsulación o la co-encapsulación con agentes antioxidantes. Por tanto, todo ello hace de la nanotecnología una buena alternativa para mejorar tanto el potencial terapéutico como preventivo de la molécula que hemos seleccionado en esta tesis.

Los nanovehículos presentan características que adecuadas para la administración de moléculas altamente lipofílicas como son los derivados de la molécula seleccionada. Su estructura permite la encapsulación de compuestos lipofílicos. Por otro lado, la versatilidad de sus componentes facilita la modulación de propiedades fisicoquímicas críticas para su biodistribución, como son el tamaño de partícula y la carga superficial [1,2]. En este contexto, nuestro grupo de investigación ha contribuido significativamente a la generación de conocimiento relativo a la influencia de las propiedades fisicoquímicas de los nanovehículos poliméricos en su biodistribución e interacción con diferentes poblaciones celulares [2–9].

Teniendo en cuenta estos antecedentes, el objetivo de esta tesis ha sido el desarrollo de nanovehículos poliméricos para mejorar la eficacia de la molécula inmunomoduladora seleccionada y aportar una nueva vía de administración para su suplementación. Por un lado, se ha evaluado el potencial de los nanovehículos poliméricos encapsulando la molécula inmunomoduladora para la prevención de diabetes tipo 1 (T1D). Por otro lado, se ha evaluado la capacidad de este tipo de nanovehículos para mejorar la penetración de dicha molécula a través de la piel.

1. Nanovehículos tolerogénicos para la prevención de enfermedades autoinmunes

Las células dendríticas (DCs) tienen un papel central en el mantenimiento de tolerancia periférica y, por tanto, en la respuesta frente a autoantígenos desarrollada en las enfermedades autoinmunes, como la T1D [10]. En este contexto, la modulación de las DCs hacia un fenotipo tolerogénico se ha considerado una buena alternativa para controlar la respuesta inmune frente a autoantígenos y evitar la aparición de los síntomas [11]. En esta tesis, hemos propuesto el desarrollo de nanovehículos poliméricos (NVs) encapsulando una molécula inmunomoduladora (IMM) como tratamiento preventivo en T1D. Estos NVs permitirían la administración dirigida de IMM a las células del sistema inmune, potenciando su efecto inmunomodulador sin efectos adversos en otros tejidos. Además, su co-encapsulación con un autoantígeno peptídico de T1D podría mejorar su efecto preventivo promoviendo una terapia antígeno-específica.

Nanovehículo	Tamaño de partícula (nm)	PDI	Carga superficial (mV)	PPI B_{10-18} EE (%)
NV blancas	158 ± 11	0,1	-50 ± 4	n/a
IMM-NV	146 ± 25	0,2	-54 ± 5	n/a
NVC blancas	224 ± 4	0,1	+55 ± 2	n/a
IMM-NVC	221 ± 4	0,2	+49 ± 1	n/a
(PPI B_{10-18} + IMM)-NVC	136 ± 12	0,1	+61 ± 7	34

Fig. 1 – **Propiedades fisicoquímicas de los nanovehículos desarrollados**. Tabla con las propiedades fisicoquímicas de todos los nanovehículos. Los valores representan la media ± desviación estándar de al menos 3 replicados. EE, eficacia de encapsulación; n/a, no aplica; NV, nanovehículo; IMM, molécula inmunomoduladora; PPI B_{10-18}, péptido antigénico; NVC, nanovehículo catiónico.

La interacción entre nanovehículos y células del sistema inmune depende en gran medida de su composición y características fisicoquímicas [12]. Se ha descrito que parámetros como el tamaño de partícula o la carga superficial influyen significativamente en la interacción entre nanovehículos y las células inmunes. En este sentido, se ha demostrado que los nanovehículos catiónicos tienen mayor interacción con las DCs [13–16]. Por esa razón, hemos decidido utilizar un polímero catiónico para dotar a nuestros NVs de una carga superficial positiva (Fig. 1A). Además, se han formulado NVs control sin polímero siguiendo el mismo protocolo para su uso como control en los ensayos *in vitro* e *in vivo*. Ambos prototipos presentaron una estrecha distribución de tamaño de partícula y se mantuvieron estables en condiciones de cultivo celular durante 24 horas. La encapsulación de IMM y del péptido antigénico seleccionado por nuestros colaboradores (un fragmento de preproinsulina, PPI B_{10-18}) en los NVs catiónicos (NVC) se pudo llevar a cabo, sin que se produjesen grandes alteraciones en sus características fisicoquímicas (Fig. 1B).

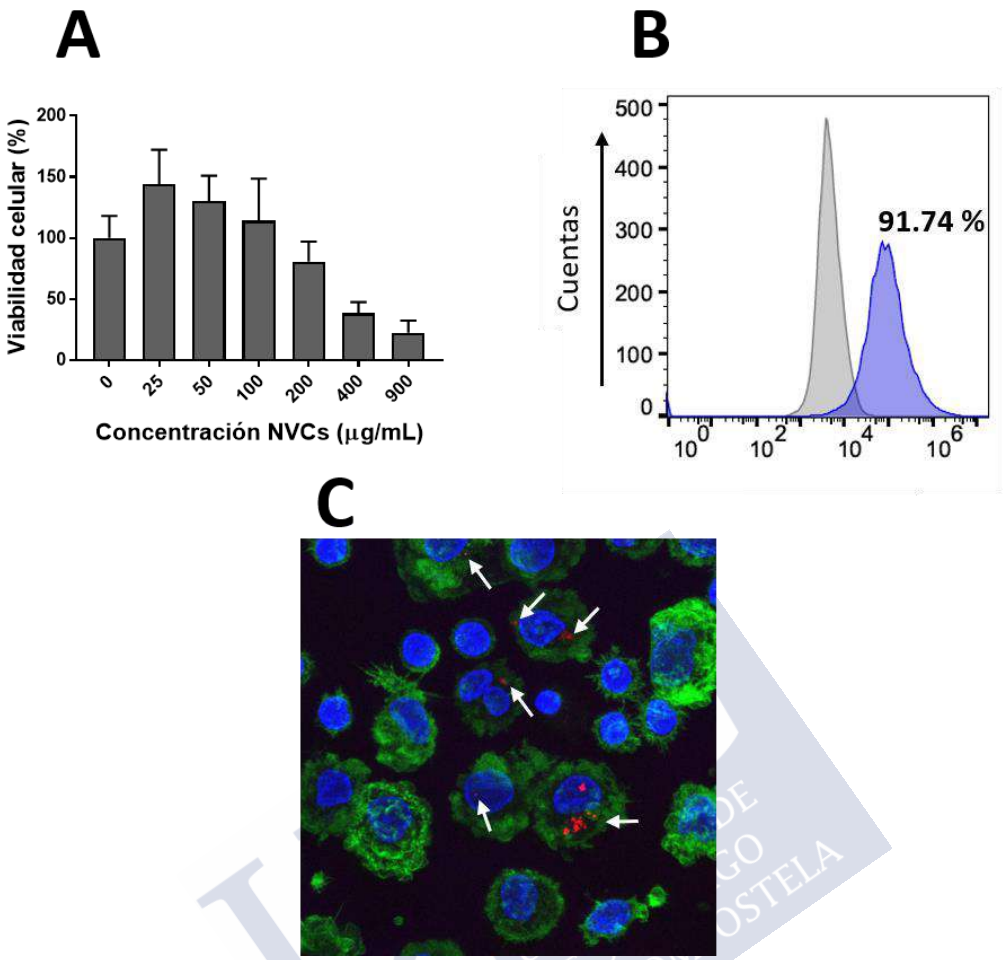

Fig. 2 – **Viabilidad celular (A) e interacción (B-C) de NVCs tras su incubación con hDCs**. La toxicidad en células dendríticas humanas (hDCs) se determinó tras su incubación con diferentes concentraciones de NVCs durante 24 horas **(A)**. Los resultados se expresan como porcentaje de viabilidad celular. Los valores representan la media ± desviación estándar de al menos 2 replicados. Histograma de citometría de flujo representativo que muestra la interacción de los NVCs marcados con DiD con hDCs tras una incubación de 1 hora a una concentración de 100 µg/mL **(B)**. Se muestran en gris las hDCs no tratadas, y se indica el porcentaje de células positivas sobre la población de hDCs tratadas. Imagen de microscopía confocal representativa (plano XY, 63x) de hDCs incubadas con NVCs marcados con DiD bajo las mismas condiciones **(C)**. Las flechas blancas señalan los puntos rojos que se corresponden con las NVCs marcadas con DiD. Canal rojo: NVCs marcados con DiD, canal verde: citoesqueleto, canal azul: núcleo celular.

Las DCs tienen un papel clave en los procesos autoinmunes, y por ello en este estudio se utilizaron células dendríticas humanas (hDCs) para llevar a cabo los ensayos *in vitro*. Se ha evaluado el potencial de nos NVCs conteniendo IMM (IMM-NVCs) para inducir un fenotipo tolerogénico en las hDCs, el cual podría traducirse en un efecto de prevención de la T1D *in vivo*. En primer lugar, se evaluó la citotoxicidad de los NVCs blancos, que mostraron una

toxicidad dosis-dependiente (Fig. 2A). Posteriormente, la interacción entre las NVCs y las hDCs se analizó mediante citometría de flujo y microscopía confocal tras su incubación con una concentración no tóxica de NVCs marcados con DiD (DiD-NVCs). Los resultados indicaron la existencia de una elevada interacción entre NVCs y hDCs, con alrededor del 92% de las células positivas (Fig. 2B). Además, las imágenes de microscopía confocal confirmaron la internalización de las DiD-NVCs (Fig. 2C). Estos resultados son consistentes con estudios previos que describían la internalización preferente en hDCs de nanovehículos catiónicos con un tamaño de partícula alrededor de 200 nm [7,9].

Para evaluar la influencia de los IMM-NVCs sobre el fenotipo de las hDCs, se determinaron diferentes parámetros, como la expresión de marcadores de superficie, la secreción de citocinas y la actividad de la indoleamina 2,3-dioxigenasa (IDO). En primer lugar, se evaluó la expresión de marcadores de superficie que se han relacionado con un fenotipo tolerogénico en las hDCs. Concretamente, en distintos estudios se ha asociado una elevada expresión de marcadores como ILT3, TLR2 o CD209, entre otros, con un fenotipo tolerogénico en hDCs y la promoción de anergia en linfocitos T [17–21]. En nuestro caso, los resultados muestran un aumento en la expresión de ILT3 y TLR2 en hDCs incubadas con IMM-NVCs similar al observado en el control de hDCs tolerogénicas (tolDC), sin alcanzar significatividad estadística (Fig. 3A-B). En el caso de CD209, los niveles de expresión fueron similares para todas las condiciones probadas (Fig. 3C).

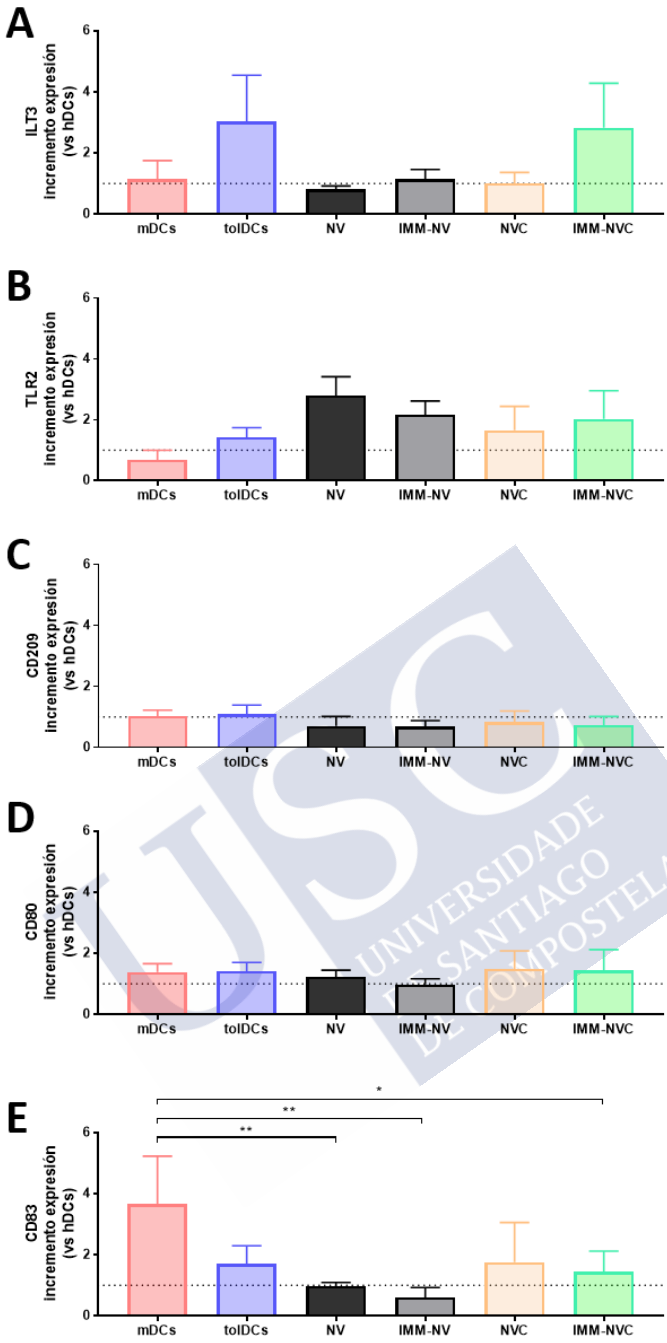

Fig. 3 – **Expresión de los marcadores de superficie ILT3 (A), TLR2 (B), CD209 (C), CD80 (D) and CD83 (E) en hDCs tras su incubación con los diferentes nanovehículos**. La expresión de marcadores de superficie se determinó por citometría de flujo tras la incubación de hDCs con las formulaciones durante 2 horas. Los resultados se muestran como el incremento de expresión con respecto a iDCs no tratadas. La línea de puntos representa el nivel de expresión de hDCs no tratadas. Los valores representan la media ± desviación estándar de al menos 4 replicados. El análisis estadístico se llevó a cabo utilizando el ANOVA unidireccional seguido de una prueba de Tukey. Los niveles de significación corresponden a * $p < 0.05$ y ** $p < 0.01$. La comparación estadística se hizo entre IMM-NVC y el resto de grupos. mDCs, células dendríticas maduras; tolDCs, células dendríticas tolerogénicas; NV, nanovehículo; NVC, nanovehículo catiónico; IMM, molécula inmunomoduladora.

Por otro lado, se evaluó la expresión de marcadores de superficie relacionados con un fenotipo proinflamatorio en hDCs. En este contexto, se ha descrito que el aumento de la expresión de receptores de co-estimulación, como CD80 o CD83, es necesario para la activación de linfocitos T [22,23]. En el caso de la expresión de CD83, tanto VIMM-NVCs como IMM-NVs promueven una disminución significativa de su expresión en comparación con hDCs maduras (mDCs) (Fig.3E). Además, los niveles de expresión de CD83 promovidos por los estos nanovehículos fueron similares a los niveles alcanzados por tolDCs (Fig. 3E). Sin embargo, en el caso de CD80, los niveles de expresión fueron similares para todas las condiciones probadas (Fig. 3D). Con respecto a la expresión de marcadores de superficie, estos resultados muestran una tendencia hacia la promoción de un fenotipo tolerogénico en hDCs tras su incubación con IMM-NVCs. Además, la actividad *in vitro* de IMM es similar tanto si se utiliza en forma soluble para generar tolDCs como incorporada en NVCs, demostrando que no se ve afectada por su encapsulación.

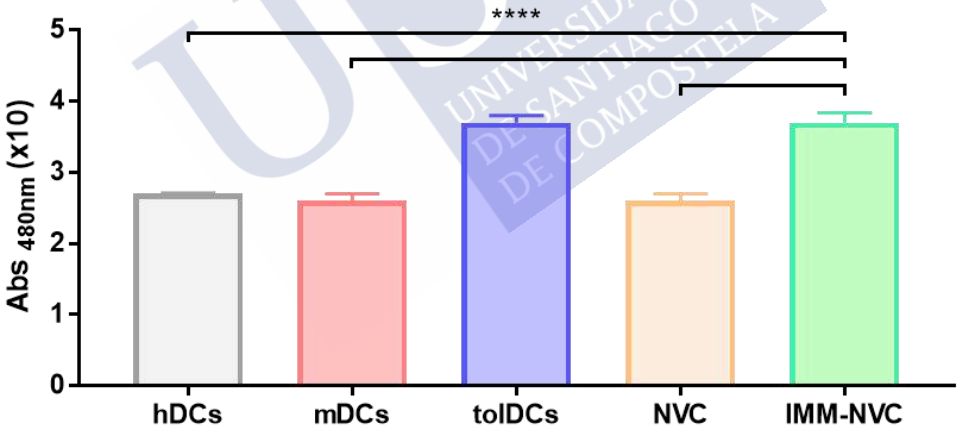

Fig. 4 – **Actividad IDO de iDCs incubadas con NVCs**. Se determinaron los niveles de kineurina en el medio celular tras la incubación de iDCs con NVCs blancas y IMM-NVCs durante 24 horas. Los resultados se muestran como absorbancia a 490 nm. Los valores representan la media ± desviación estándar (n=4). El análisis estadístico se llevó a cabo utilizando el ANOVA unidireccional seguido de una prueba de Tukey. Los niveles de significación corresponden a **** $p < 0.0001$. La comparación estadística se hizo entre IMM-NVC y el resto de grupos. hDCs, células dendríticas no tratadas; mDCs, células dendríticas maduras; tolDCs, células dendríticas tolerogénicas; NVC, nanovehículo catiónico; IMM, molécula inmunomoduladora.

A continuación, se determinó la actividad enzimática de IDO. La actividad de esta enzima, implicada en el catabolismo del triptófano, se ha relacionado con la promoción de la tolerancia en hDCs [24]. En el caso específico del modelo de ratón diabético no obeso (NOD), el desarrollo de la respuesta autoinmune se ha relacionado con un defecto en el catabolismo del triptófano relacionado con una disminución de la actividad IDO [25]. Como se representa en la Fig. 4, la incubación de hDCs con IMM-NVCs aumenta significativamente la actividad IDO en comparación con hDCs sin tratar, mDCs o la incubación con NVCs blancos.

Finalmente, se determinó la secreción de citocinas por hDCs tras su incubación con NVCs tanto blancas como cargadas con IMM. Como se puede observar en la Fig. 5, la maduración de las hDCs promueve un aumento general de la secreción de citocinas. En ese contexto, se observa un incremento generalizado de citocinas proinflamatorias como IL-12p70, IL-8, TNF-α e IFN-γ (Fig. 5A-C-D-E). Sorprendentemente, la expresión de IL-10, una citocina antiinflamatoria conocida, también se vio aumentada (Fig. 5B). En el caso de tolDCs, los resultados mostraron una disminución generalizada en la secreción de citoquinas. Esto también se observó en el caso de hDCs incubadas con NVCs blancos o cargadas con IMM. Con respecto a la expresión de citocinas, estos resultados muestran que los NVCs no promueven un fenotipo proinflamatorio en hDCs, ya que los niveles de expresión de hDCs incubadas con NVCs blancos son similares a los de hDCs sin tratar. Además, estos niveles de expresión son similares a los mostrados por tolDCs, indicando que la incubación con NVCs mantiene un estado inmaduro considerado tolerogénico en hDCs.

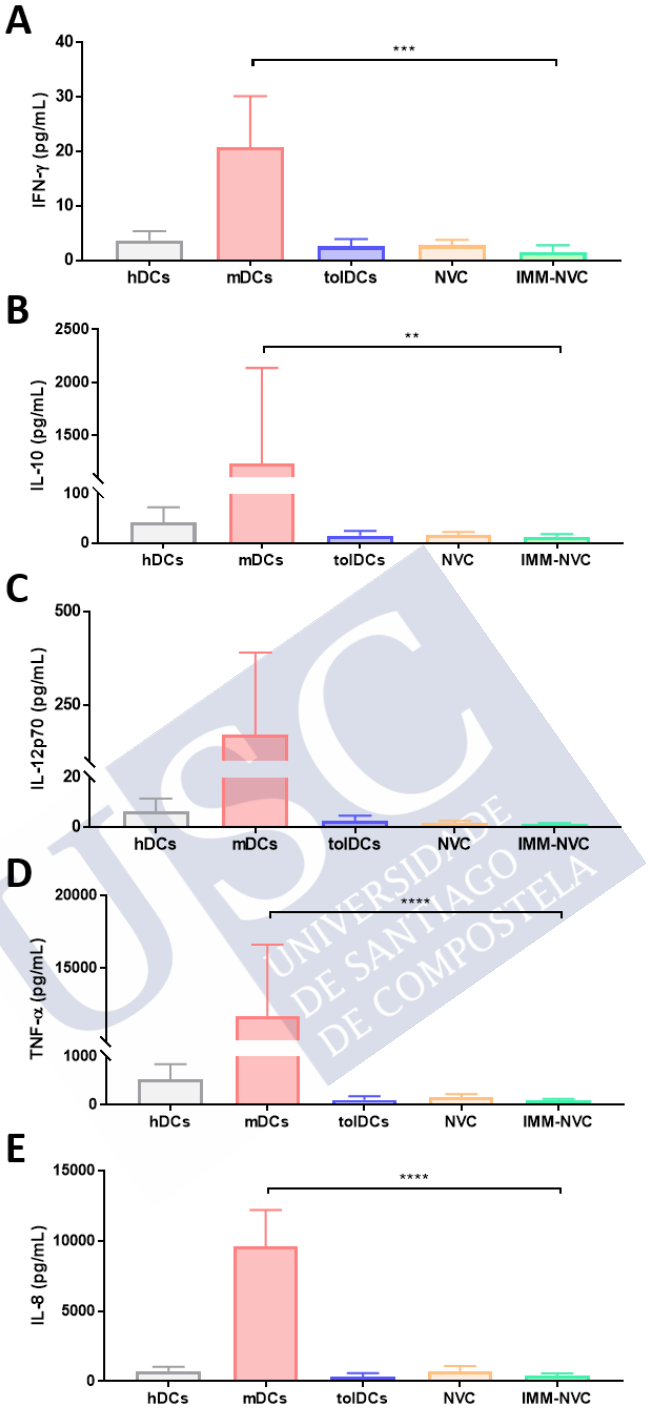

Fig. 5 – **Expresión de citocinas en hDCs incubadas con NVCs**. Se determinaron los niveles de citocinas en el medio de cultivo celular luego de la incubación de hDCs con NVCs blancos y IMM-NVCs durante 24 horas. Los valores representan la media ± desviación estándar (n=4). El análisis estadístico se llevó a cabo utilizando el ANOVA unidireccional seguido de una prueba de Tukey. Los niveles de significación corresponden a ** $p < 0.01$, *** $p < 0.001$ y **** $p < 0.0001$. La comparación estadística se hizo entre IMM-NVC y el resto de grupos. hDCs, células dendríticas no tratadas; mDCs, células dendríticas maduras; tolDCs, células dendríticas tolerogénicas; NVC, nanovehículo catiónico; IMM, molécula inmunomoduladora.

En resumen, gracias a estos estudios *in vitro* se puede concluir que los IMM-NVCs promueve un fenotipo tolerogénico en hDCs. Este efecto es similar al producido por la IMM libre, lo que sugiere que la encapsulación de IMM en NVCs no afecta a sus propiedades inmunológicas.

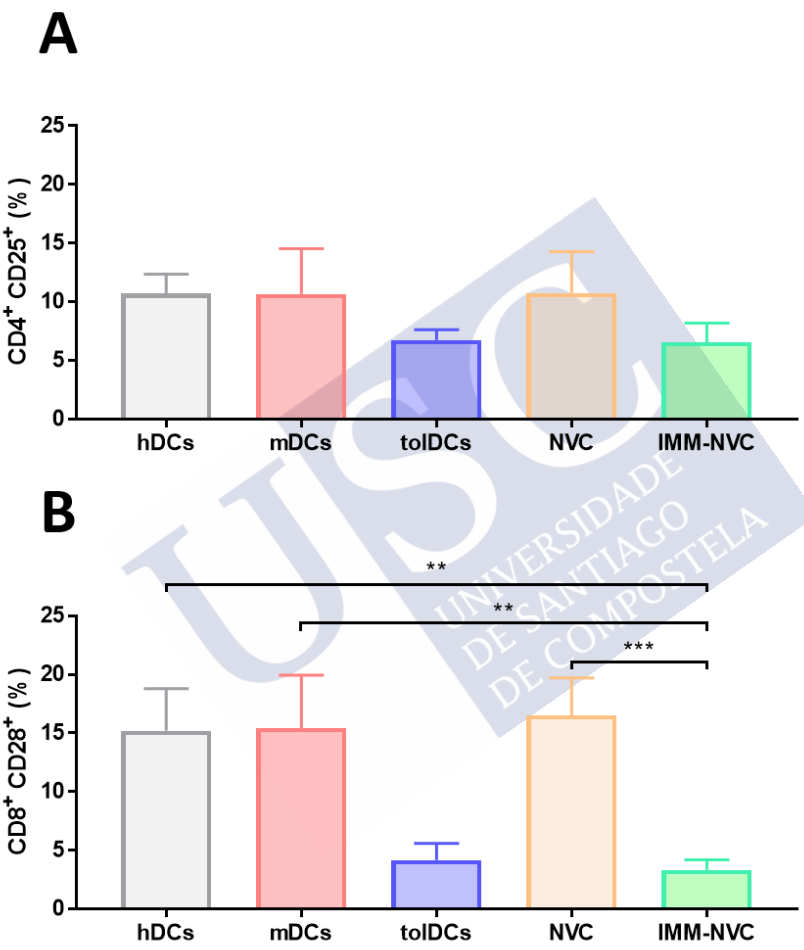

Fig. 6 – **Capacidad de activación de linfocitos T de hDCs pre-incubadas en cultivos alogénicos**. El porcentaje de linfocitos T $CD4^+$ $CD25^+$ **(A)** y linfocitos T $CD8^+$ $CD28^+$ **(B)** activos se determinó mediante citometría de flujo tras el co-cultivo de hDCs pretratadas con linfocitos alogénicos de sangre periférica. Los valores representan la media ± desviación estándar de al menos 3 replicados. El análisis estadístico se llevó a cabo utilizando el ANOVA unidireccional seguido de una prueba de Tukey. Los niveles de significación corresponden a ** $p < 0.01$ y *** $p < 0.001$. La comparación estadística se hizo entre IMM-NVC y el resto de grupos. hDCs, células dendríticas no tratadas; mDCs, células dendríticas maduras; tolDCs, células dendríticas tolerogénicas; NVC, nanovehículo catiónico; IMM, molécula inmunomoduladora.

Una vez confirmada la promoción de un fenotipo tolerogénico en hDCs mediada por los IMM-NVCs, el siguiente paso fue determinar si este efecto se traduce en una disminución de la activación de los linfocitos T. Para ello, se evaluó mediante citometría de flujo la activación de linfocitos T alogénicos tras su co-cultivo con hDCs previamente incubadas con IMM-NVCs. Como se muestra en la Fig. 6A, la pre-incubación de hDCs con IMM-NVCs disminuyó ligeramente el porcentaje de linfocitos T $CD4^+$ activados en comparación con linfocitos co-cultivados con hDCs, mDCs o hDCs pre-incubadas con NVCs blancos, aunque sin alcanzar significatividad estadística. En el caso de linfocitos T $CD8^+$, se observó una disminución significativa de su activación mediada por hDCs pre-incubadas con IMM-NVCs en comparación con hDCs, mDCs o hDCs pre-incubadas con NVCs blancos (Fig. 6B). En ambos casos, este efecto de disminución de la activación fue similar al promovido por la IMM soluble, puesto de manifiesto en el co-cultivo con tolDCs.

Por último, se realizaron estudios en animales para determinar si el potencial inmunomodulador de los IMM-NVCs observado *in vitro* se traduce en un retraso en la aparición de T1D *in vivo*. Aunque se ha descrito previamente el potencial de la IMM para la prevención de T1D, los efectos secundarios derivados de su distribución sistémica han limitado su uso en terapia. En esta tesis, nuestro trabajo se ha dirigido a validar la hipótesis de que la inclusión de IMM en un nanovehículo facilitaría su direccionamiento hacia las células del sistema inmune evitando su distribución a tejidos no diana y, por tanto, disminuyendo sus efectos tóxicos. Por otro lado, como hipótesis adicional se ha planteado que la co-encapsulación del autoantígeno PPI $B_{10\text{-}18}$ y IMM podría mejorar la prevención de T1D. Esta hipótesis adicional se basa en diferentes estudios que muestran que el uso de dosis altas de antígenos solubles promueve la generación de tolerancia mediada por la inhibición de la proliferación de linfocitos T o por su eliminación selectiva [26,27]. Además, el uso de autoantígenos podría promover la generación de respuestas tolerogénicas selectivas manteniendo la capacidad de generar inmunidad frente a otros antígenos.

Para la realización de los estudios *in vivo* se seleccionó el modelo de ratón NOD.B6-Tg(HLA-A2.1)Enge/DvSJ (NOD-HHD). En general, los islotes pancreáticos del modelo de ratón NOD presentan una infiltración temprana de células inmunes, como DCs, macrófagos o neutrófilos [28–31]. Asimismo, la especificidad antigénica de los linfocitos T en ratones NOD es similar a

la de los humanos, con antígenos comunes reconocidos [32–36]. Por último, además de las características citadas, los ratones NOD-HHD son transgénicos para el alelo HLA-A*02:02 que codifica para el antígeno leucocitario humano A de clase I. En relación a esta molécula, se ha descrito que es capaz de seleccionar linfocitos T $CD8^+$ autorreactivos que aceleran significativamente el desarrollo de la enfermedad en comparación con otras cepas de ratones NOD [37,38]. En este caso, se utilizaron ratones NOD-HHD hembras de 4 semanas, que fueron tratadas por vía intraperitoneal siguiendo la pauta de administración descrita en la Fig. 7A. Las dosis de IMM y PPI B_{10-18} utilizadas fueron de 5 µg/kg y 4 mg/kg por administración, respectivamente. Se consideraron diabéticos los ratones con glucosuria ≥ 500 mg/dL durante dos semanas consecutivas.

Como cabía esperar, los grupos tratados con solución salina, IMM o PPI B_{10-18} mostraron una incidencia de diabetes similar (Fig. 7B-C-D). Por el contrario, el tratamiento con IMM-NVCs mostraron una incidencia de diabetes significativamente menor que todos los grupos de control (Fig. 7B-C-D). Por ello, se pudo concluir de forma directa que, siguiendo el protocolo experimental descrito, solo la IMM encapsulada es eficaz en cuanto a la prevención de T1D. Por otro lado, los resultados presentados en la Fig. 7F indican que el control del IMM-NV no tuvo un efecto preventivo sobre la aparición de T1D. En consecuencia, estos resultados indican que el efecto preventivo de las IMM-NVCs proviene no solo de las propiedades inmunomoduladoras de la IMM, sino también de la carga superficial de los NVs. Esto podría estar relacionado con la captura preferencial de los nanovehículos catiónicos por parte de las DCs [13–16]. Sin embargo, serían necesarios estudios de biodistribución *in vivo* para comprender el mecanismo que se encuentra detrás del efecto de prevención mediado por los NVCs diseñados en esta tesis.

Contrariamente a lo esperado, la co-encapsulación del péptido antigénico PPI B_{10-18} e IMM en NVCs no mostró un efecto sinérgico sobre la incidencia de diabetes (Fig. 7G). Esto indica que el efecto principal sobre la prevención de la diabetes mostrado por los NVCs proviene de la actividad inmunomoduladora de la IMM y no de su sinergia con este autoantígeno específico. En este sentido, se ha descrito que la selección del autoantígeno, la vía y la pauta de administración, así como la dosis utilizada, son aspectos clave para la inducción de tolerancia y la prevención de enfermedades autoinmunes [39–42]. Por lo tanto, ajustar la pauta de

administración y la dosis del autoantígeno seleccionado, así como incluir diferentes autoantígenos, podría mejorar el efecto de la co-encapsulación de ambas moléculas en NVCs.

En el caso de formulaciones basadas en nanotecnología, la inclusión de antígenos para generar un efecto preventivo en T1D ha reportado resultados controvertidos. Parece que el efecto promovido por el antígeno depende del tipo de aproximación utilizada para la generación de tolerancia. Se ha mostrado que la encapsulación del antígeno en ausencia de moléculas inmunomoduladoras no promueve una respuesta tolerogénica [43]. Sin embargo, cuando se incluyen señales de apoptosis, como la fosfatidilserina, la combinación del antígeno con estas moléculas mejora la respuesta tolerogénica en comparación con el uso de la molécula señalizadora solamente [44]. Por otro lado, se ha mostrado que la combinación de autoantígenos y moléculas inmunomoduladoras en nanovehículos promueve una respuesta tolerogénica antígeno-específica, manteniendo la capacidad de generar una respuesta inmune frente a otros antígenos [45]. Teniendo esto en cuenta, sería necesaria la realización de estudios para determinar la especificidad de la respuesta tolerogénica desarrollada por los NVCs para entender el efecto del antígeno sobre la misma.

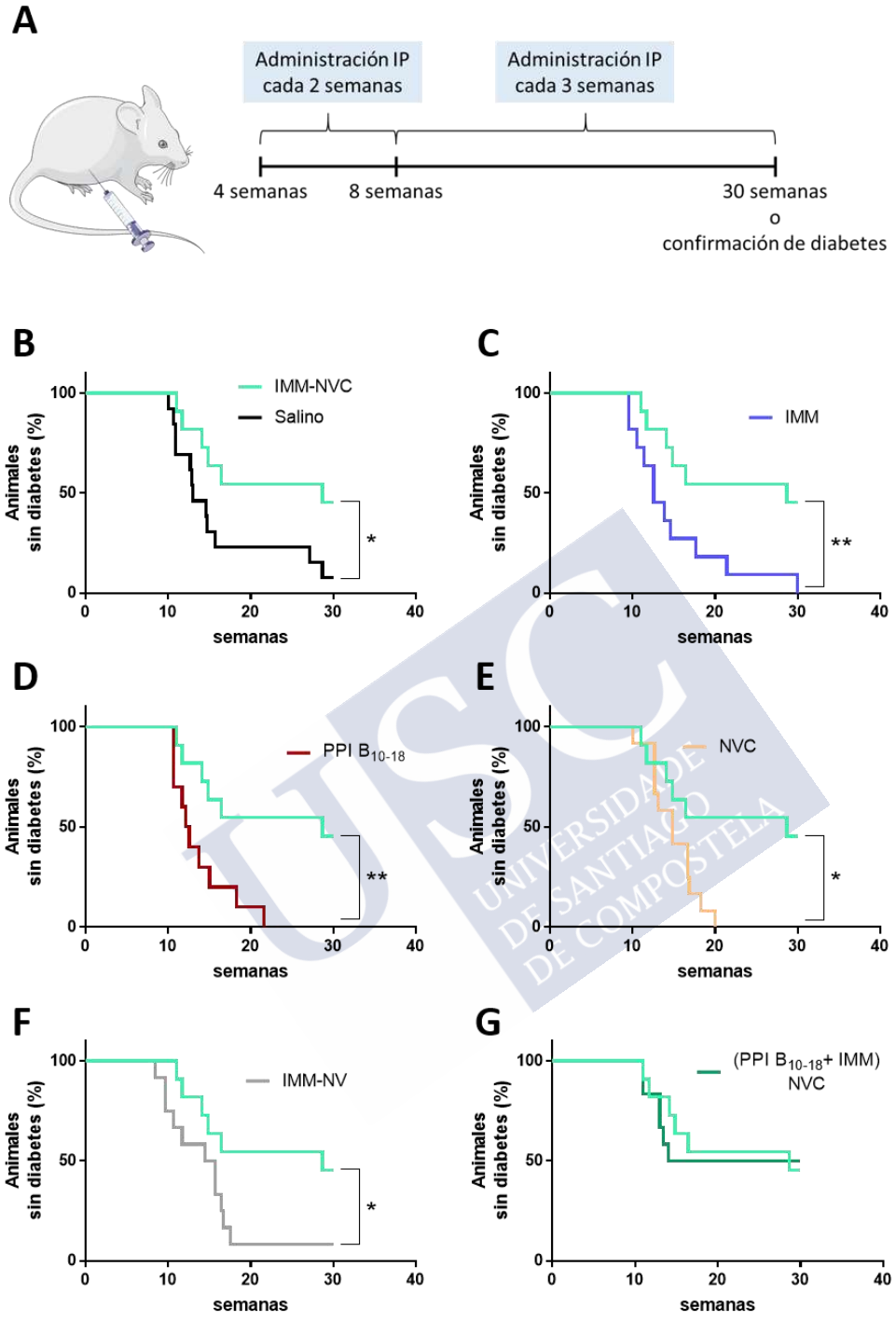

Fig. 7 – **Efecto preventivo de la aparición de diabetes mediado por las IMM-NVCs**. Pauta de administración **(A)** e incidencia de diabetes en ratones NOD-HHD hembra tratados mediante administración intraperitoneal con IMM-NVCs en comparación con varios grupos: salino **(B)**, IMM soluble **(C)**, PPI B_{10-18} soluble **(D)**, NVCs blancos **(E),** IMM-NV **(F)** y (PPI B_{10-18} + IMM)-NVC **(G)** (n de al menos 10 animales por grupo). El análisis estadístico de las curvas de supervivencia se llevó a cabo utilizando el análisis log-rank de Kaplan-Meier. Los niveles de significación corresponden a * $p < 0.05$ y ** $p < 0.01$. IP, intraperitoneal; IMM, molécula inmunomoduladora; PPI B_{10-18}, péptido antigénico; NVC, nanovehículo catiónico; NV, nanovehículo.

En resumen, estos estudios preliminares indican que el uso de IMM-NVCs podría ser una buena estrategia para retrasar la aparición de T1D. La simplicidad de nuestros IMM-NVCs podría ser una notable ventaja competitiva para su traslado al uso clínico.

2. Suplementación de compuestos naturales por vía dérmica utilizando nanovehículos

Tomando en consideración los NVs poliméricos desarrolladas previamente en esta tesis para mejorar las propiedades inmunomoduladoras de la molécula seleccionada, nos hemos planteado la hipótesis de que su optimización podría mejorar su suplementación por vía transdérmica. La difusión de IMM a través del *estrato córneo* (StC) es un proceso limitado por su alta lipofilia. En este contexto, la encapsulación de IMM en un nanovehículo ofrece la posibilidad de mejorar su penetración transdérmica sin alterar las propiedades de la piel. Además, la versatilidad de la composición de los NVs poliméricos permite la modulación de propiedades fisicoquímicas como el tamaño de partícula o la carga superficial, que influyen en su interacción con la piel. De hecho, se ha descrito que la penetración a través de la piel de los nanovehículos de pequeño tamaño se ve favorecida por una mayor interacción con los folículos pilosos [46–48]. Por ejemplo, se ha reportado que nanopartículas de PLGA de 70 nm son capaces de atravesar capar superficiales tanto en piel sana como inflamada [49]. Teniendo en cuenta estos antecedentes, el primer paso en este trabajo fue la optimización del tamaño de partícula de los NVCs previamente desarrollados en nuestro laboratorio. Además, se desarrollaron NVs aniónicos (NVAs) para determinar la influencia de la carga superficial en la penetración del IMM a través de la piel.

Nanovehículo	Tamaño de partícula (nm)	PDI	Carga superficial (mV)	EE (%)
NVC blancos	85 ± 4	0,2	+58 ± 5	n/a
NVC cargados	80 ± 1	0,1	+61 ± 9	98
NVA blancos	77 ± 1	0,1	-44 ± 4	n/a
NVA cargados	86 ± 6	0,2	-20 ± 5	103

Fig. 8 – **Propiedades fisicoquímicas de los NVs poliméricos de pequeño tamaño**. Tabla con las características fisicoquímicas de todos los nanovehículos. Los valores representan la media ± desviación estándar de al menos 3 replicados. EE, eficacia de encapsulación; n/a, no aplica; NVC, nanovehículo catiónico; NVA, nanovehículo aniónico; PDI, índice de polidispersión.

Nuestro grupo de investigación ha realizado estudios previos con el objetivo de determinar qué parámetros de formulación afectan al tamaño de partícula en los NVs preparados utilizando la técnica de desplazamiento de solvente [50,51]. Teniendo en cuenta esta información, hemos modificado las variables de formulación de NVCs para disminuir su tamaño de partícula. En ese sentido, se consiguió disminuir el tamaño de los NVCs de 224 nm a 88 nm. En el caso de los NVAs, el ajuste de estas variables permitió obtener un tamaño de partícula de aproximadamente 90 nm. Como se refleja en los resultados obtenidos, ambas formulaciones encapsularon IMM de manera muy eficaz (Fig. 8B).

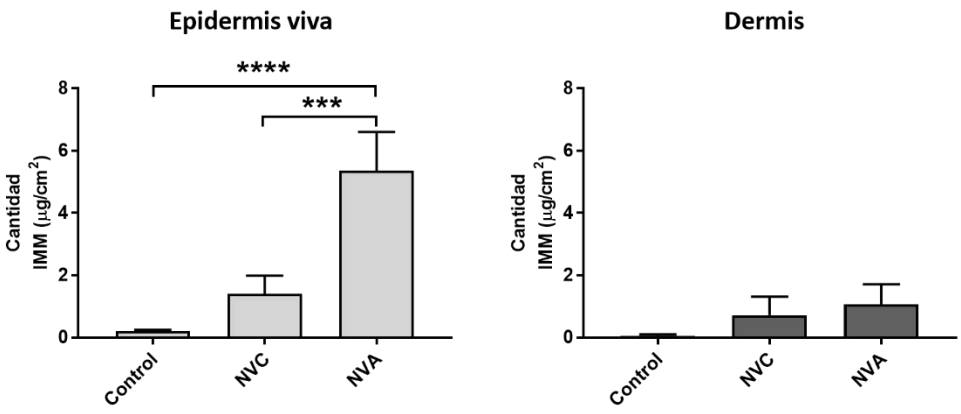

Fig. 9 – **Penetración de IMM en distintas capas de piel humana tras la incubación durante 24 horas con una dosis finita (≈ 10 µL/cm^2) de NVs conteniendo IMM**. Como control se utilizó una solución de IMM en 0.25% aceite a la misma concentración que la usada en los NVs. Los valores representan la media ± desviación estándar (n=4). El análisis estadístico se llevó a cabo utilizando el ANOVA unidireccional seguido de una prueba de Tukey. Los niveles de significación corresponden a *** $p < 0.001$ y **** $p < 0.0001$. IMM, molécula inmunomoduladora; NVC, nanovehículo catiónico; NVA, nanovehículo aniónico.

Una vez desarrolladas las formulaciones, el siguiente paso fue determinar la penetración del IMM a través de piel humana. Como se muestra en la Fig. 9, el transporte de IMM libre hacia la epidermis viable y la dermis fue despreciable (0.26 ± 0.84 µg/cm^2), en consonancia con su alta lipofilia. De hecho, debido a su carácter lipofílico, se espera que IMM libre interactúe con los lípidos del StC y permanezca retenido en esa capa de piel. Por el contrario, la penetración de IMM encapsulado fue mucho mayor y dependiente de la composición de los NVs. Los resultados muestran que los NVAs son la formulación más eficaz para favorecer el acceso del IMM a la epidermis viva (Fig. 9). Sin embargo, este aumento del transporte hacia la epidermis viva, en comparación a el de IMM no encapsulado, no se tradujo en una mayor cantidad detectada en dermis. Esa cantidad acumulada en la epidermis viva podría actuar como un depósito que permitiría la liberación controlada de IMM. Por otro lado, los NVCs lograron mejorar la penetración de IMM en la epidermis viva, aunque la penetración mediada por NVAs fue significativamente mayor. En consonancia con nuestros resultados, otros autores han demostrado una mayor penetración a través de las diferentes capas de la piel en el caso de nanovehículos aniónicos [52–55]. La hipótesis que explicaría este comportamiento se basa en

una interacción más estrecha entre los nanovehículos catiónicos y la superficie de la piel debido a su carga neta negativa, lo cual promueve su retención en las capas superficiales de la piel.

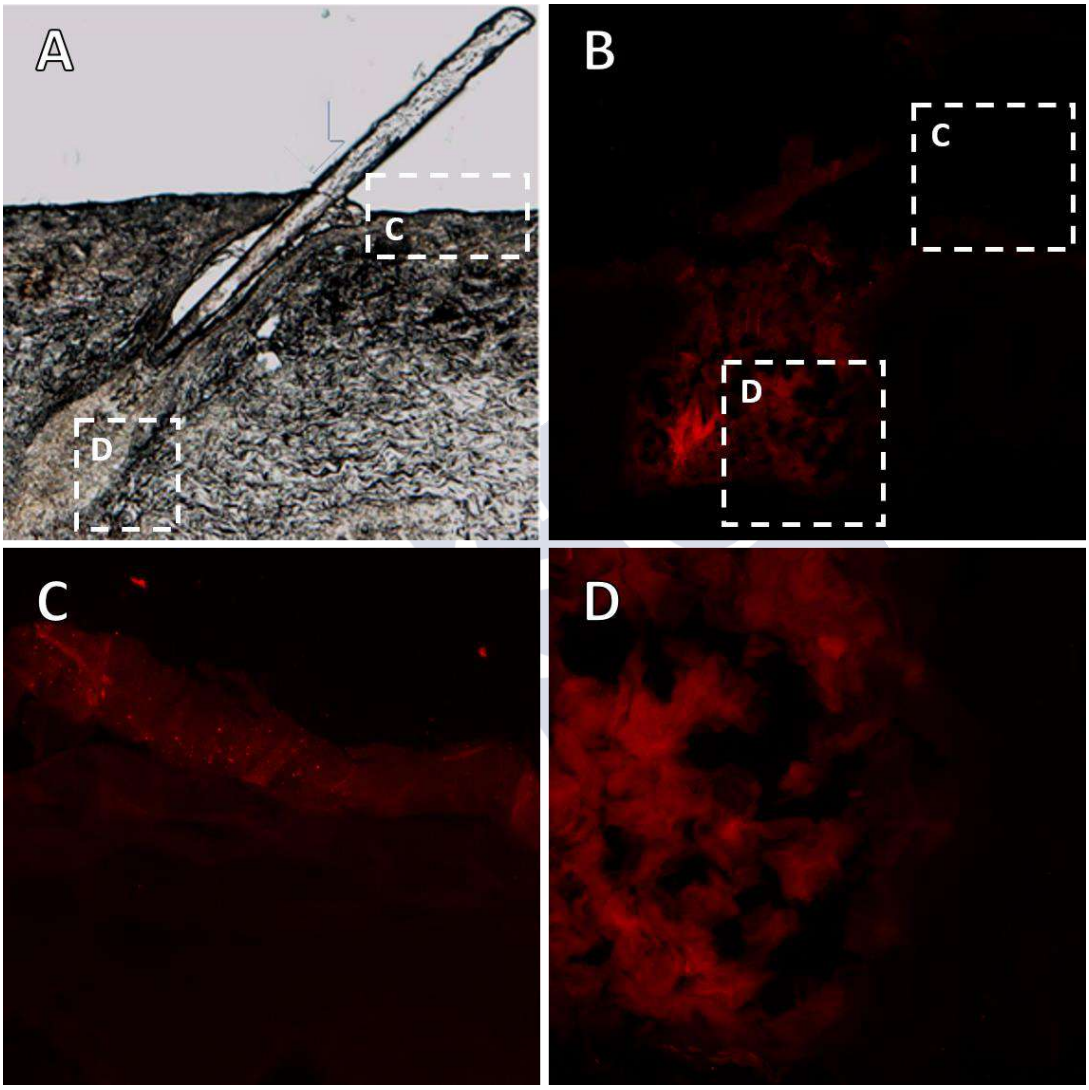

Fig. 10 – **Visualización el folículo piloso tras la incubación de piel humana con Cy5-NVAs**. Imagen de microscopía electrónica de un folículo piloso adaptada con permiso de [56] **(A)**; imagen de microscopía confocal (plano X-Z, 20x) de un folículo piloso de piel humana incubada con Cy5-NVAs (en rojo) **(B)**; imagen de microscopía confocal (plano X-Z, 63x) de la superficie de la piel de la misma muestra **(C)**; imagen de microscopía confocal (plano X-Z, 63x) de capas profundas del folículo piloso de la misma muestra **(D)**.

Para comprender el mecanismo que podría explicar la mejora del acceso a la epidermis viva que logran los NVAs, se evaluó su interacción con piel humana mediante microscopía confocal. Como se muestra en la Fig. 10B, los NVAs marcados con Cy5 presentan una distribución uniforme alrededor del pelo desde la superficie de la piel hasta su raíz, en el folículo piloso. Aunque se observa que la formulación queda retenida en la superficie de la piel (Fig. 10C), su movimiento a través del folículo piloso le permite acceder a capas más profundas de la piel (Fig. 10D). Estos resultados están en consonancia con estudios recientes que muestran la importancia de la vía transfolicular en la penetración transdérmica de nanovehículos [48,57–59].

En resumen, estos resultados preliminares sugieren que el IMM encapsulado en los NVAs podría ser una buena alternativa para la administración segura y efectiva de la molécula inmunomoduladora seleccionada por vía transdérmica. Sin embargo, serían necesarios estudios de prueba de concepto *in vivo* para determinar si la administración transdérmica utilizando NVAs se traduce en un aumento en los niveles sanguíneos de dicha molécula.

Como conclusión, en esta tesis hemos demostrado el potencial de los nanovehículos poliméricos para la administración de derivados de una molécula inmunomoduladora en diferentes contextos. La versatilidad de su composición y la posibilidad de modular sus propiedades fisicoquímicas nos ha permitido demostrar su interacción favorable con las células inmunes y su penetración transdérmica. Como resultado, la actividad inmunomoduladora de IMM se tradujo en una menor incidencia de diabetes *in vivo*, y por otro lado, se mejoró su penetración a través de la piel.

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Introduction

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Since ancient times, natural compounds have been incorporated into our medicine. Many marketed products include natural molecules as their active ingredients. Ingredients like curcumin, essential oils or many more phytochemicals have shown potential for both preventing and treating several diseases such as cancer or microbial infections [1–3]. In this thesis, we focus on a natural molecule with known immunomodulatory activity and beneficial effect in human's health. This molecule is implicated in several physiological processes such as apoptosis, differentiation and proliferation among others. Furthermore, its immunomodulatory activity makes it a good candidate to improve the prevention and treatment of autoimmune diseases due to its effect promoting a tolerogenic phenotype in dendritic cells. In addition, its supplementation has been suggested as an alternative for prevention of diseases such as cancer and type 1 diabetes.

However, the selected molecule, as many of the natural compounds mentioned before, have several drawbacks that limit their use in the clinics. Usually, high doses are required to achieve a therapeutic effect due to their non-targeted biodistribution, thereby leading to off-site toxicity. Furthermore, these molecules have shown high instability both, during storage and during their *in vivo* fate, illustrating the need of an effective formulation to assure their stability in both situations. In the specific case of the selected molecule, its oral supplementation has been associated with several drawbacks, such as intestinal absorption fluctuations and limited sources.

In this sense, nanotechnology have shown great potential to improve biodistribution and efficacy of compounds like the one selected in this thesis. The development of nanocarriers that specific deliver active molecules to targeted tissues can enhance their efficacy and avoid off-target toxicity. Furthermore, these nanocarriers can efficiently protect these natural compounds by themselves or by co-encapsulating antioxidant agents, preserving their activity for longer time. In addition, nanocarriers can load multiple cargo molecules, offering the possibility of combining different drugs with natural compounds for enhancing synergic effects. All these characteristics make nanotechnology a great approach to enhance the therapeutic efficacy of natural compounds.

Considering the specific case of the molecule selected in this thesis, nanotechnology can improve it immunomodulatory potential by targeting the immune cells while avoiding off-

target toxicity. In this context, our group has generated significant knowledge regarding the modulation of nanocarriers' properties and their influence in their biodistribution and interaction with different cells subsets [4–11]. Further information about how nanotechnology can enhance the interaction with immune cells and promote a tolerogenic effect is discussed in Chapter 1. In addition, in the context of supplementation, nanocarriers can be adapted to different administration routes that could be convenient, such as the dermal route.

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Chapter 1

Tolerance generation by modulating the immune system using nanotechnology

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Tolerance generation by modulating the immune system using nanotechnology

This chapter has been partially adapted/extracted from a published review entitled "Modulating the immune system through nanotechnology" [1].

Abstract

Nowadays, nanotechnology-based modulation of the immune system is a cutting-edge strategy, expected to lead to significant improvements in the treatment of severe diseases. In particular, important efforts have been focused on the development of nanotechnology approaches for the generation of tolerance. In this chapter, we highlight how nanocarriers can be designed to have a preferential access to the immune cells and promote a tolerogenic response. In addition, here we discuss specific formulation strategies than have been adopted for the development of new treatments for autoimmune diseases, as well as for the prevention of the formation of antibodies against biologicals.

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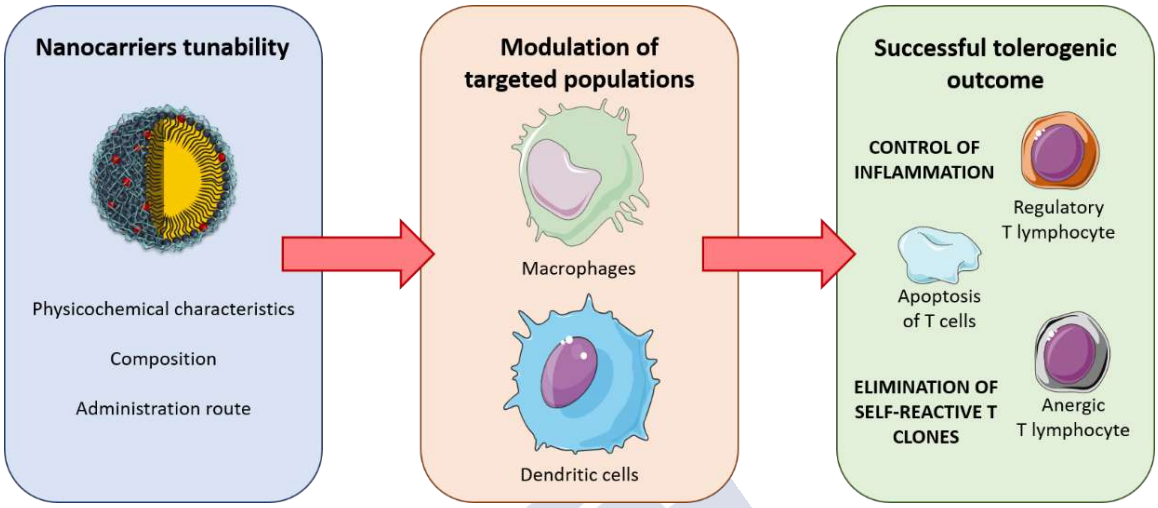
Graphical abstract



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1. Introduction

The immune system plays a critical role in the most severe diseases of our time, such as cancer, microbial infections or autoimmune diseases. The increasing understanding of the immune network opens a wide range of possibilities for its modulation and has set the foundations for new promising therapies [2]. Considering the autoimmunity scenario, these approaches are mainly focused on modulating antigen presenting cells (APCs) due to their central role in maintaining peripheral tolerance [3]. In particular, dendritic cells (DCs) are key for the activation or suppression of adaptative immunity. The mechanisms underlying the generation of immune tolerogenic responses by DCs are summarized in Figure 1. Enhancing these pathways for specific antigens can help to resolve exacerbated immune responses underlying autoimmune diseases.

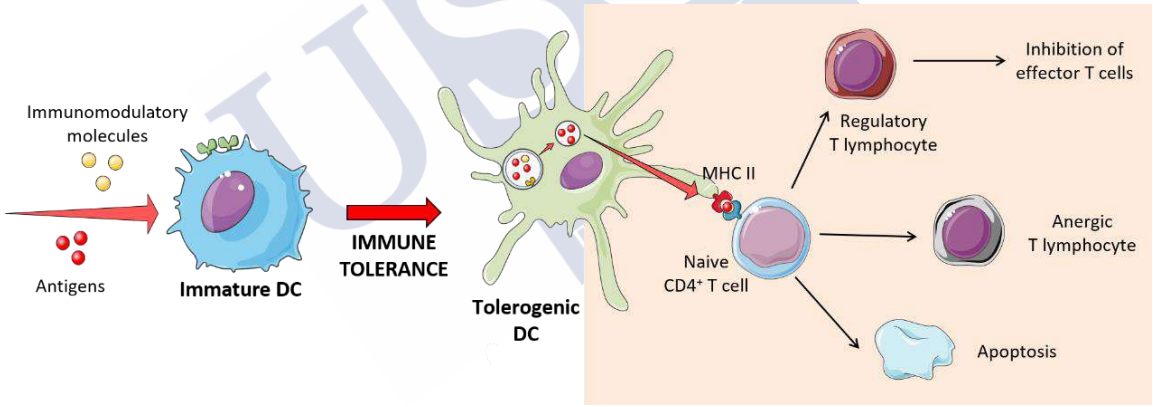

Fig. 1 – Schematic overview of the generation of immune responses by tolerogenic dendritic cells. In the case of antigens presented in the absence of co-stimulatory molecules, or in the presence of immunomodulatory molecules, dendritic cells are driven to a state of immune tolerance. In this state, dendritic cells can inhibit T cell activation by different mechanisms. Different stimuli, such as IL-10 or PD-L1 can cause T regulatory cells proliferation that, at the same time, can inhibit effector T cells. Furthermore, the absence of co-stimulatory surface molecules can lead to an unresponsive state in T cells known as anergy. Finally, co-stimulatory Fas-signaling in the immune synapsis can lead to T cell apoptosis and deletion. Adapted with permissions from [1]

In this scenario, the use of nanotechnology for modulating the immune system is growing exponentially due to the ability of nanocarriers to interacts with APCs [4]. Mimicking the interaction mechanisms between APCs and apoptotic bodies and/or pathogens, nanocarriers have been used to enhance antigen-specific immune tolerance in different diseases [5].

In this chapter, we will describe critical parameters regarding nanocarriers design to achieve a tolerogenic response. Furthermore, we will discuss different approaches based on nanotechnology that have been developed for the treatment of autoimmune diseases. Finally, recent achievements to fight antidrug antibodies are summarized.

2. Considerations for the design of tolerogenic nanocarriers

The interaction between immune cells and nanocarriers and the final immune outcome depend on numerous factors. This includes both the physicochemical characteristics or composition of the nanocarriers, and the administration route. Depending on these features, different biodistribution patterns may be observed and preferential interaction with specific cell subsets could be promoted. The final immune outcome could be modulated and vary from activation to tolerance generation. In order to modulate the immune response to the desired outcome, nanocarriers need to be carefully designed. Herein, we describe different parameters that influence the crosstalk between immune cells and nanocarriers for the promotion of immune tolerance.

2.1. Physicochemical properties

The physicochemical properties of the nanocarriers are determinants of their biodistribution and interaction with different cell subsets. Parameters such as size, shape, hydrophobicity and surface charge can influence the preferential access of the nanocarriers to certain cell populations. In this section, we summarize the influence of these parameters in the uptake of nanocarriers by the APCs for the subsequent development of a tolerogenic response.

Size is one of the most studied parameters regarding nanocarriers' biodistribution and uptake by APCs. In this sense, it was reported that DCs preferentially internalized small virus-like particles (< 500 nm) compared to the preferential uptake of particles in the µm range by macrophages [6,7]. However, recent *in vitro* and *in vivo* studies have underlined the preferential uptake of small nanocapsules (NCs) (100 nm) compared to medium-size NCs (200 nm) by both, DCs and macrophages [8,9]. In addition, it has been shown that the small size (less than 100 nm) facilitates the lymphatic drainage of nanocarriers and their subsequent interaction with large APCs populations [7,10–12].

Another important parameter regarding particle uptake is **shape**. It has been described in the literature that spherical particles have more favorable uptake by APCs than rod-shaped nanoparticles (NPs) [13]. However, Roberts *et al.* observed that phosphatidylserine-loaded polylactic-co-glycolic acid (PLGA) NPs displaying nanorod shape induced more efficient tolerogenic responses than spherical NPs [14]. This highlights the importance of the interplay between both composition and shape to modulate the immune response. In addition, further studies revealed that particle orientation also have a major role in the successful internalization of non-spherical particles by macrophages [15].

The influence of nanocarriers' **surface charge** on their uptake and biodistribution has also been widely described. It has been shown that cationic nanocarriers have preferential interaction with DCs [16–20]. In the case of macrophages, contradictory results have been shown. While some studies showed higher uptake of cationic nanocarriers by macrophages compared to anionic ones [16,21–24], other studies have shown the opposite behavior [17,25–28]. In addition to uptake, biodistribution, and hence the access to APCs population, is also conditioned by surface charge. In the case of subcutaneous administration, it has been found that positive nanocarriers tend to form a depot while negative nanocarriers had favored lymphatic drainage [29–32]. However, it has been described that small cationic nanocarriers can effectively drain to the closest lymph nodes [33,34]. Therefore, with the data currently available, it could be deduced that particle size and, probably composition may be more important determinants on the final output of nanocarriers.

In addition, **hydrophobicity** of nanomaterials is also considered a feature influencing recognition by APCs and modulation of the immune response. Hydrophobicity is considered a

nonspecific danger-associated molecular pattern (DAMP), which is involved in danger signal pathways of the innate immune system [35–37]. It has been described that increasing hydrophobicity in polystyrene NPs increases their uptake by DCs [38]. However, hydrophobicity hampers lymphatic drainage. Therefore, upon subcutaneous administration it is possible that hydrophobic particles are more retained at the injection size and taken up by macrophages, whereas hydrophilic small particles may have facilitated diffusion to the lymphatic system, where, eventually, may interact with the DCs population. Beyond this speculation, we also need to take into account the interaction with proteins that may occurs upon injection. Specially after intravenous administration, but also reported for subcutaneous administration, nanocarriers interact in a lower o higher extent with the proteins present in plasma or ECM [39]. This protein corona may play a major role in phagocytosis by APCs. In particular, complement proteins or apolipoproteins are highly present in protein corona of hydrophobic nanocarriers [39,40].

Considering all this information, the interplay between all these parameters and both particle biodistribution and uptake by APCs needs to be balanced to maximize the interaction between the nanocarriers and their targeted population.

2.2. Active targeting

In addition to enhancing passive targeting by adjusting nanocarriers' physicochemical properties, the use of targeting ligands has shown to be effective for the specific uptake by APCs in order to achieve a tolerogenic outcome. For example, authors have taken advantage of the specific expression of folate receptor β in activated macrophages in inflamed joints for rheumatoid arthritis (RA) treatment, showing an increased joint accumulation of folate-functionalized dendrimers after intravenous (IV) injection in collagen-induced arthritis (CIA) mice model [41,42]. Similarly, the use of hyaluronic acid (HA) coating on NPs has been proposed as a way to specifically interact with the CD44 receptor, found in macrophages, among other cells [43,44]. In this sense, it has been described that high molecular weight (Mw) HA enhance the interaction with immune cells compared with medium and low Mw HA [45]. Furthermore, it has been demonstrated that mannosylation of nanocarriers can enhance their

interaction with C-type lectin receptors and hence their internalization by DCs [46–48]. Indeed, the combination of this strategy with HA-coating produces a synergistic effect enhancing the uptake of NPs by DCs [49]. Moreover, direct targeting to scavenger receptors present in APCs, such as DEC-205 or MARCO, have shown to be successful increasing NPs' interaction and uptake by these cell types. For instance, the specific recognition of dextran by scavenger receptors was explored to develop an anti-inflammatory therapy. Namely, dextran NPs containing dexamethasone were used to target pro-inflammatory macrophages from obese patients [50]. Also, experiments show that MARCO-targeted polystyrene microparticles (MPs) follow the debris elimination route, and help to present the antigens loaded in a non-inflammatory way [51]. In addition, the attachment of antibodies recognizing DEC-205 surface receptor on the NP's surface has been shown to enhance NPs uptake by DCs [52,53].

In parallel to the direct modification of NPs to achieve an active targeting, the modulation of protein corona formation is an emerging strategy to enhance NPs uptake by APCs. In this sense, NPs surface could be decorated with molecules that promote binding of specific proteins present in plasma, such as active complement CD3 or immunoglobulins, and hence increasing their interaction with APCs through natural opsonization pathways. For example, liposomes have been modified with orthopyridyl disulfide to increase active CD3 surface binding and thus enhancing their possibility to interact with DCs [54].

2.3. Administration route

It has been described in the literature that the biodistribution of nanocarriers depends on the interplay between their physicochemical characteristics and the administration route. Ultimately, the tissue and cell distribution determine the final immunological outcome. Accordingly, it has been reported that the administration route may determine the achievement of a tolerogenic effect [55]. For example, in the case of the IV administration, the possibility to generate a tolerogenic effect by antigen-loaded polystyrene MPs and PLGA NPs has been related to their accumulation in the liver [51,56]. Liver Kupffer cells and DCs are essential for the elimination of apoptotic cells and other debris from the blood, a mechanism that is associated with the maintenance of peripheral tolerance [57]. Indeed, upon exposure

to PLGA NPs, Kupffer cells and liver DCs increase the expression of PD-L1 in their surface, thereby contributing to enhancing tolerance [56]. In another study, it was proposed that the intraperitoneal route would be of interest for the treatment of type 1 diabetes (T1D), as this route provides easy access of the nanocarriers to pancreatic lymph nodes [58].

Subcutaneous (SC), intramuscular (IM) or intradermal (ID) administration, considered more conventional routes for vaccination, have also been used to generate tolerogenic responses in T1D or multiple sclerosis (MS) [59–67]. Nanocarriers administered by these routes are exposed to a highly immune environment and can drain to lymph nodes to promote immune responses. To enhance tolerogenic responses by these administration pathways, nanocarriers' composition usually includes immunomodulatory molecules to promote a tolerogenic phenotype in immune cells.

Non-parenteral modalities of administration have also been explored. For example, in the case of the oral route, the M cells present in the gut-associated lymphoid tissue (GALT) have a key role not only in responding against pathogens but also in maintaining peripheral tolerance [68]. It has been described that the effectiveness of oral tolerance depends on several factors such as antigen dose, frequency and uptake in the gastrointestinal tract. In the case of nanocarriers, it has been demonstrated that oral administration of PLGA NPs loaded with autoantigens enhanced the rate of IL-4 and IL-10 secreting T cells in Peyer patches [69]. Therefore, the oral administration of antigen-loaded nanocarriers could be an alternative for tolerance generation.

2.4. Delivery of immunomodulatory molecules

In addition to the design of nanocarriers with adequate properties and the selection of the administration route, the loading of immunomodulatory molecules has been extensively used to enhance the generation of tolerogenic responses. In this sense, several approaches have been followed (Fig. 2)

The debris produced during apoptosis, a process of programmed cell death, are eliminated by APCs. The APCs present the processed antigens within a tolerogenic environment, without

activating immune responses [70]. Mimicking this environment, nanocarriers can follow debris elimination routes and take advantage of this process to generate tolerance. For the uptake of apoptotic debris, scavenger receptors play the main role in apoptotic signal recognition and debris endocytosis [71]. The incorporation of these apoptosis signaling molecules, such as phosphatidylserine (PS), in the nanocarrier's composition may enhance its uptake in APCs and allow for a tolerogenic antigen presentation. For example, in an experimental approach, 50 % of mice treated with antigen-loaded PS liposomes could be prevented from acquiring T1D [58]. Interestingly, not only the presence of PS, but also its geometrical surface disposition was found to play a role in tolerance induction. For example, Roberts *et al.* observed that PLGA NPs displaying a nanorod-presentation were more efficient at inducing tolerogenic responses than the spherical ones [14].

Furthermore, co-encapsulation of molecules such as rapamycin or dexamethasone with antigens inside nanocarriers can promote its presentation in a tolerant environment in APCs [62,63,72]. Moreover, the delivery of nucleic acids coding for modulatory cytokines has been explored with the goal of inducing tolerogenic profiles in immune cells [73,74].

Finally, the association of antigens attached to the major histocompatibility complex (pMHC) in the surface iron oxide NPs has been shown to expand autoregulatory T cell memory in different animal models. Indeed, Tsai *et al.* showed that pMHC class I-coated NPs triggered massive expansions of autoregulatory $CD8^+$ T cells, and these cells were able to suppress polyclonal autoimmune responses by selectively targeting autoantigen-loaded APCs in the target tissue and draining lymph nodes [75]. On the other hand, another report showed that the use of pMHC class II-coated NPs expanded disease-specific regulatory $CD4^+$ T cells [76].

Overall, this information underlines the fact that a careful design of the nanocarriers needs to be addressed in order to achieve the desired outcome. In this sense, the simultaneous influence of the parameters summarized herein needs to be considered to enhance tolerogenic immune responses.

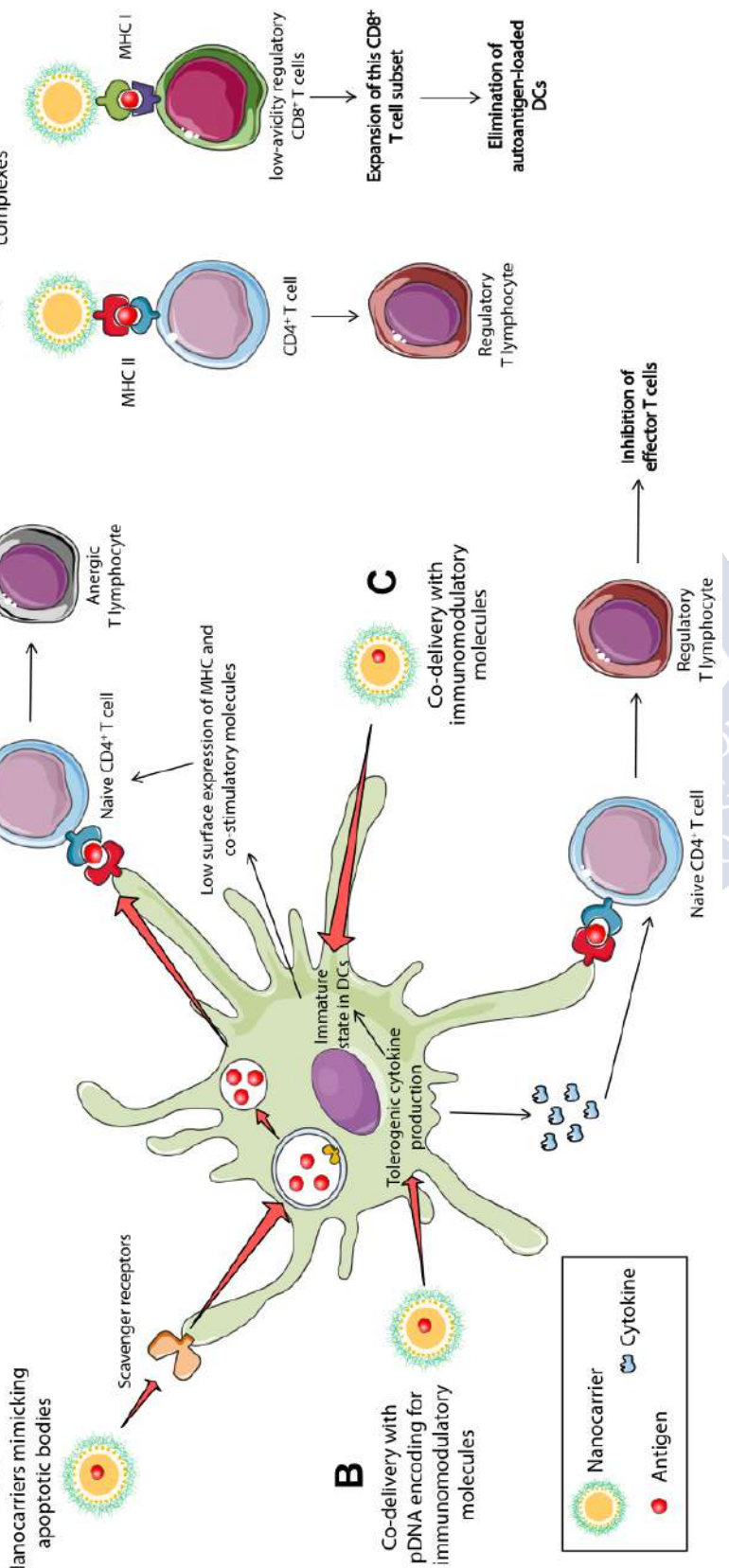

Fig. 2 – Nanotechnology-based antigen-specific approaches for tolerance generation. Strategies for antigen-specific tolerance mediated by dendritic cells (DCs) modulation through nanotechnology. **(A)** Nanocarriers mimicking apoptotic bodies may follow the debris elimination process, where self-antigen presentation induces regulatory T cells. **(B)** The co-delivery with pDNA encoding for tolerogenic cytokines, i.e. IL-10, may enhance its expression and induce regulatory T cells and anergy. **(C)** Using immunomodulatory molecules may promote the maintenance of immature state of DCs, while presentation of antigens with low surface density of major histocompatibility complexes (MHC) and costimulatory molecules may promote T cell anergy. **(D)** The delivery of peptide-MHC (pMHC) complexes to T cells may expand memory T cells with regulatory capacity. Adapted with permissions from [1]

3. The potential of nanotechnology for immunomodulation of autoimmune diseases

Immunomodulation is a desirable strategy to avoid exacerbated immune responses against ubiquitous molecules, such as self-proteins and, hence, it is of particular interest for the treatment of autoimmune diseases. In autoimmune diseases, autologous proteins are recognized as non-self-antigens by the immune system, leading to the generation of autoreactive T and B cell clones. Currently, the treatment of this kind of diseases is symptomatic and relies on the use of classical anti-inflammatory drugs as well as immunosuppressive therapies. Unfortunately, these therapies are unspecific and lead to significant side effects (Table 1). Due to the complex regulatory network of the immune processes, moving from these therapies to targeted and specific treatments have been found to be an important challenge in biomedical research. In that sense, nanotechnology offers the possibility of the specific delivery of the drug/antigen to the desired cell population, as well as the co-delivery of the targeted drugs with adequate immunomodulatory molecules. Furthermore, nanotechnology offers the possibility to protect the drug from degradation, increasing its half-time life.

In this section, we discuss recent advances in nanotechnology regarding immunomodulation to fight against autoimmunity. First, we present the role of the nanocarriers used to enhance the response of immunosuppressant drugs. Next, we focus on more specific approaches evaluating the potential of nanotechnology for antigen-specific therapies in autoimmune diseases with known self-antigens. From the delivery point of view, the common feature of these strategies is that the target cells are the immunocompetent cells.

Table 1. Current and most used treatments for selected autoimmune diseases

Disease	Treatment	Adm. route	Mechanism of Action
Multiple sclerosis	IFNβ	SC / IM	Balances the expression of pro- and anti-inflammatory agents in the brain Reduces the number of inflammatory cells that cross the blood brain barrier
	Glatiramer acetate	SC	Strong promiscuous binding to MHC molecules and consequent competition with myelin antigens for their presentation to T cells
	Natalizumab	IV	Blockade of α4 integrin and consequent inhibition of immune cells extravasation
	Immunosuppresive agents	Oral / IV	Blockade of immune response at different levels
Type 1 diabetes	Insulin injections	SC	Decrease of glucose levels
Rheumatoid arthritis	NSAIDs	Oral	Inhibition of the synthesis of prostaglandins and thromboxanes
	Corticosteroids	Oral / intra-articular	Regulation of genes related with inflammation and suppression of immune response
	TNFα antagonists	SC / IV	Blockade of either TNFα or its receptor
	Disease-modifying anti-rheumatic drugs (DMARDs)	Oral / SC / IV	Slow down disease progression by different mechanisms
Inflammatory bowel disease	Aminosalicylates	Oral	Modulation of gene expression and consequently inhibition of cyclooxygenase and NF-κβ and its downstream signals
	Corticosteroids	Oral	Regulation of genes related with inflammation and suppression of immune response
	Immunosuppressive agents	SC / IV	Blockade of immune response at different levels
	TNFα antagonists	SC / IV	Blockade of either TNFα or its receptor
	Antibiotics	Oral	Decreasing concentrations of bacteria in the gut lumen Altering the composition of intestinal microbiota
Systemic lupus erythematosus	NSAIDs	Oral	Inhibition of the synthesis of prostaglandins and thromboxanes
	Antimalarial drugs	Oral	Altering lysosome stability Suppressing antigen presentation Inhibiting prostaglandin and cytokine synthesis Influencing both TLR signaling and leukocyte activation
	Corticosteroids	Oral	Regulation of genes related with inflammation and suppression of immune response
	Immunosuppressive agents	SC / IV	Blockade of immune response at different levels

Adm, administration; IM: intramuscular; NSAID: nonsteroidal anti-inflammatory drug; MHC: major histocompatibility complexes; SC: subcutaneous; TLR: toll-like receptor; NF-κβ, nuclear factor kappa-light-chain-enhancer of activated B cells.

3.1. Nanomedicines for the treatment of inflammatory diseases

Inflammation is a common immune process that helps the body to eliminate injury related debris, such as microbes, toxins, and necrotic cells. This mechanism is triggered by extracellular signaling factors that attract plasma proteins, immune cells and phagocytes. This inflammatory response could be either acute or chronic. Chronic inflammation usually lasts longer and leads to complications due to tissue degeneration [77]. The chronic inflammatory diseases include autoimmune diseases and auto-inflammatory diseases. In the case of autoimmune diseases, such as inflammatory bowel disease, rheumatoid arthritis, type 1 diabetes, lupus or multiple sclerosis, T cells are thought to be the main triggers of the disease process. Different cytokines, such as tumor necrosis factor α (TNF-α), play a role in maintaining these autoreactive T cells. On the other hand, auto-inflammatory diseases, such as sepsis, gout or type II diabetes, are mainly mediated by innate immune system effectors, such as macrophages, the complement cascade, and cytokines such as interleukin 1β (IL-1β) [78,79]. In these chronic diseases, the targeted treatment of inflammatory conditions could be considered as an immunomodulatory approach, slowing down disease progression and ameliorating the symptoms by changing the immune response, both directly (using immunosuppressant drugs) or indirectly (using anti-inflammatory drugs). This section focuses on different nanotechnology-based therapies developed for the treatment of inflammation in autoimmune diseases.

Immunosuppressant molecules are frequently used for the treatment of chronic inflammation. The many drugs available on the market for the treatment of inflammatory conditions have shown limited success in controlling disease symptoms due to their non-targeted biodistribution. Moreover, the immunosuppressant therapy is normally associated to off-target organ side effects and systemic toxicity, exacerbated by frequent and long-term dosing. Nanoencapsulation of immunosuppressive agents has been shown to increase the therapeutic success of those drugs based on the principle of passive or active targeting. The targeted delivery of these molecules, mainly to macrophages in the inflammation site, has led to the reduction of their side effects and also to improve their action on the inflammatory signaling routes mediated by immune cells, which can be consider also as immunomodulation.

This has been widely reviewed in the literature for pathologies as inflammatory bowel disease, rheumatoid arthritis, or systemic lupus erythematous [80–82] (Fig 3).

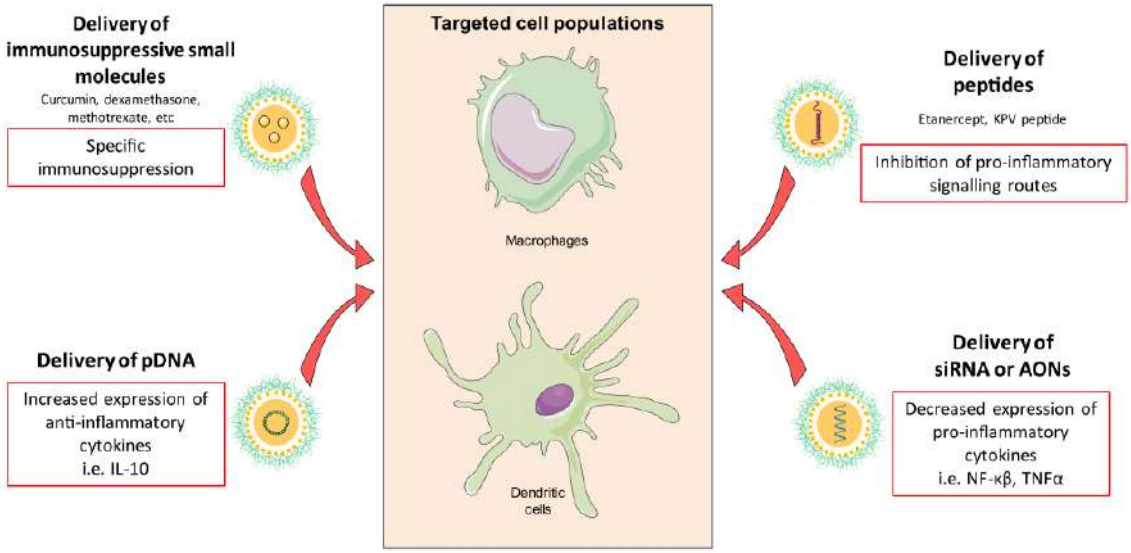

Figure 3. Main strategies for nanotechnology-based anti-inflammatory treatment of autoimmune diseases. There are four approaches of nanotechnology-based treatments depending on their cargo. First, the delivery of small immunomodulatory molecules has been extensively explored in the suppression of the inflammatory activity of macrophages and dendritic cells (DCs). Second, the delivery of anti-inflammatory peptides has been used in different pathologies. Finally, gene therapy strategies include: plasmid DNA (pDNA) delivery for the expression of anti-inflammatory cytokines, and small interfering RNA (siRNA) or antisense oligonucleotide (AONs) delivery for the downregulation of pro-inflammatory molecules expression.

3.1.1. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the digestive tract, including ulcerative colitis (UC) and Crohn's disease (CD). UC is confined to the colon, whereas CD can affect any region of the gastrointestinal tract, being the terminal ileum and the colon the most commonly affected areas. Recent research has shown that genetic susceptibility, external environment, intestinal microbial flora and immunological profile are all involved in the pathogenesis of IBD, but the specific causes remain unknown [83]. Current treatments are

symptomatic for the induction of remission in acute episodes and avoiding relapsing events. Conventional drugs, including 5-aminosalicylic acid (5-ASA), corticosteroids, immunosuppressant drugs, and anti-TNFα agents are the main treatments today. Depending on localization and activity of the inflammation, these drugs are administered topically, systemically or in combination.

In the case of IBD, colon targeted delivery of immunosuppressive agents is desirable to avoid side effects. For the delivery of small immunosuppressive molecules, polymeric NPs have been widely explored and reviewed in the literature [81,84]. Apart from immunosuppressive drugs, nanotechnology-based small interfering RNA (siRNA) delivery directed to APCs is another approach that has been explored for resolving inflammation in IBD [85,86]. For example, chitosan and its derivatives have been investigated for the siRNA delivery in the colonic region due to its mucoadhesive properties. In one case, chitosan-PLGA NPs were tested orally for the delivery of an antisense oligonucleotide to block nuclear factor κβ (NF-κβ) in an induced-colitis model. The results showed that chitosan-PLGA NPs were selectively accumulated in inflamed tissue and improved the clinical scoring [87]. Similarly, galactosylated trimethylchitosan NPs loaded with a siRNA against mitogen-activated protein kinase (MAPK) showed good *in vivo* efficacy in induced-colitis mice model after oral administration [88]. Finally, the local delivery of anti-inflammatory peptides or protein antagonists of immune receptors in the inflammation site is a promising approach for the *in situ* modulation of immune effector cells. For example, the colonic delivery of an alginate-chitosan hydrogel (double oral gavage procedure for *in situ* gelation) containing KPV peptide-loaded PLGA NPs to an induced-colitis mice model, resulted in a marked amelioration of the inflammatory symptoms. In fact, a considerably lower dose of peptide (12,000-fold) compared to the free peptide, led to a similar therapeutic efficacy. This effect was explained taking into account the better access of the peptide-loaded NPs to the target epithelial and immune cells [89].

3.1.2. Rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune disorder that primarily affects joints. RA is characterized by synovial inflammation and swelling, autoantibody production as well as

cartilage and bone destruction [90]. It has been proposed that the course of the RA development follows a three-step process. Autoimmunity starts to develop in genetic-susceptible individuals, with the presentation of autoantibodies in serum [91]. In a second step, there is an expansion of reactive immune cells that leads the infiltration of inflammatory cells in the joints as a prelude of the chronic inflammatory response. Finally, the patient presents a chronic joint inflammation promoted mainly by macrophages, which constitutes the major hallmark of the third phase of the disease [92]. The systemic delivery of immunosuppressant molecules, both classic small drugs and anti-TNFα antibodies are the main current treatments (Table 1) [90].

The design of nanotechnology-based approaches in RA is focused on increasing the retention time of small immunosuppressive drugs in the joint [80]. For that purpose, a wide variety of nanocarriers have been tested and extensively reviewed in the literature, including polymeric NPs, liposomes, solid-lipid NPs and polymeric micelles [80,93]. Moreover, nanotechnology-based gene therapy has also been explored for the treatment of RA. As in IBD, this therapy is focused in siRNA knockdown of TNFα [85]. Also, the encapsulation of plasmid DNA (pDNA) encoding for IL-10 was widely explored. As an example, Jain *et al.* showed effective macrophage repolarization from M1 to M2 phenotype in adjuvant-induced arthritis (AIA) mice model after intraperitoneal administration of IL-10-encoding pDNA-loaded alginate NPs [94]. Regarding protein delivery, different anti-inflammatory proteins have been explored. This is the case of self-assembled NPs composed of metracrylate-based copolymers loaded with an IL-1 receptor antagonist (a protein implicated in blocking pro-inflammatory signals). This system was able to maintain the biological activity of IL-1 receptor antagonist *in vitro* and prolong its retention in rat stifle joint following intra-articular administration in healthy rats [95]. Another example is the nanocomplex of etanercept with succinylated pullulan-g-oligo (L-lactide) polymer. After two months of fortnightly subcutaneous injection of this nanocomplex to a CIA rat model, no cartilage erosion and a depletion of the synovial inflammation were observed [96].

3.1.3. Systemic lupus erythematosus

Systemic lupus erythematous (SLE) is a chronic autoimmune disease characterized by loss of tolerance to self-antigens and production of numerous autoantibodies, due to its heterogenic and non-organ specific origin [97]. The most common treatment strategies are nonsteroidal anti-inflammatory drugs (NSAIDs), antimalarial drugs and oral glucocorticoids. Immunosuppressive medications are used to control serious lupus activity that affects major organs (Table 1).

Nanotechnology-based therapies for the treatment of SLE have been reviewed recently [82]. In the following lines, we highlight some of the most significant works in this field. Look *et al.* developed a liposomal system with a gel-like core containing cyclodextrins surrounded by a lipid bilayer for the delivery of anti-inflammatory agents. Following intraperitoneal administration of this system loaded with mycophenolic acid in a murine lupus model, it was found an increased 2 - 3 months the mean survival time, and this was attributed to the preferential accumulation of the system in DCs [98]. The same group also found that the DCs immunosuppression achieved with this new system was more significant than for the one observed for PLGA NPs loaded with the same drug [99]. In another example, methylprednisolone-loaded liposomes were administered subcutaneously in a murine lupus model and the results showed a reduced the mortality for this group of mice, as compared to that of the group treated with the free drug [100]. Attempts have also been made to treat lupus with gene therapy approaches. For example, following intraperitoneal administration of siRNA anti-MAPK1 (a protein implicated in the pro-inflammatory signaling cascade) loaded into PEG-poly(L-lysine) NPs, in a murine model of lupus nephritis, a significant amelioration of the renal damage was observed [101].

To summarize, different nanotechnology approaches were developed for the treatment of the inflammation in autoimmune diseases (Fig 3). This offers the possibility of controlled and targeted release of immunosupressive drugs, which would avoid the systemic effects of the drugs currently on the market.

3.2. Nanovaccines for the treatment of autoimmune diseases

Apart from the symptomatic treatment using anti-inflammatory and immunosuppressive drugs, nanotechnology can contribute with more specific treatments for autoimmune diseases. In this sense, antigen-specific therapies seem a good option to prevent self-antigen recognition that would lead to the activation of auto-reactive T or B cell clones.

The best-known disease-specific self-antigens are: myelin in MS, insulin in T1D, and collagen in RA. Loss of tolerance towards self-antigens is often thought to be the result of both genetic and environmental risk factors, including exposure to infection by particular pathogens, molecular mimicry of endogenous antigens, or bystander activation [102]. However, the molecular mechanisms behind the autoimmune process are not well understood yet. Furthermore, in most of the cases, the self-antigens involved in the physiopathology of the disease remain unknown, limiting these therapies to illnesses with known self-antigens. In a healthy situation, T lymphocytes can distinguish between different antigens with high specificity; however, they cannot discriminate between self or non-self-antigens. Central tolerance process occurs in the thymus during the first years of life. During this process, thymic epithelial cells expose in their surface a great variety of self-antigens to T cells. Normally, the T cells that recognize those antigens are eliminated to prevent self-reactivity [103,104]. Besides central tolerance process, peripheral mechanisms regulate these self-reactive T cells if they reach the bloodstream. However, in the case of patients with autoimmune disorders, these peripheral mechanisms fail and the self-reactive T cells stay and cause damage [105].

Different mechanisms for maintenance of peripheral self-tolerance have been proposed. Most of them include DCs and regulatory T cells as the main modulators of self-reactive T cell response [106,107]. The molecular signals in the microenvironment drive DCs homeostasis and function, especially regarding cytokines production and surface expression of co-stimulatory molecules. Differences in the microenvironment can lead to phenotypical changes in DCs, promoting T cell anergy, T cell depletion and regulatory T cell proliferation after immune synapsis formation and antigen recognition [108]. This regulatory T cell expansion promotes the suppression of specific self-reactive T cell clones by different mechanism [107]. Within this context, the “holy grail” of immunotherapy in autoimmune diseases would be the

development of antigen-specific treatments targeted to dendritic cells. This approach could maintain the functionality of the immune system whereas specifically blocking the self-reactive T cells which are pathogenic in autoimmune diseases. For this purpose, different protocols where developed during the last decades for the induction of specific tolerance [109].

Trying to simulate the process elicited in allergy treatment, high doses of soluble antigen were injected in order to induce anergy or activation-induced cell death after T cell re-stimulation in autoimmune diseases [110,111]. Unfortunately, although promising result based on this strategy were obtained [112,113], in others, a hyper-sensitivity reaction was observed after the administration of the soluble antigen [114,115]. These contradictory results can be explained by the fact that soluble peptides can induce specific tolerance, but cannot block polyspecific responses in the case of epitope spreading, which is the situation that exists in autoimmune diseases [116].

Based on the high amount of foreign antigens present in food and the general lack of immune reaction against them (except in the case of food allergies), the mucosal administration of soluble antigens has also been explored to induce tolerance. This is thought to happen by different mechanisms dependent on antigen dose. Low-dose of self-antigen is processed by antigen presenting cells in the gastrointestinal tract, promoting the activation of regulatory T cells. On the other hand, high doses of antigens seem to cross the gastrointestinal barrier and promote anergy once in systemic circulation [117]. Studies in animal models led to promising results in terms of blocking disease progression [118–120], however, so far, these results did not translate to a clinical set-up [121].

Nanotechnology is a promising approach to improve vaccination strategies to treat autoimmune diseases. It offers the ability of specific targeting and association of multiple antigens capable of inducing tolerance before epitope spreading happens. Most of the nanotechnology-based strategies are focused on the delivery of self-antigens to DCs, taking advantage of natural peripheral tolerance mechanisms mediated by this cell type (Fig. 2). In the next lines, we will summarize and discuss the latest and most relevant nanotechnology-based approaches in antigen-specific therapy against different autoimmune pathologies (Table 2).

3.2.1. Multiple sclerosis

Multiple sclerosis affects around 2.3 million people worldwide and is the second most common cause of disability in young adults. MS is a central nervous system disorder of autoimmune origin, in which encephalitogenic T cells are involved in damaging the myelin, promoting inflammation, and triggering neuronal and axonal damage [122]. Some self-antigens are known to be related with the pathology, including myelin basic protein (MBP), myelin oligodendrocyte protein (MOG), and proteolipid protein (PLP) [123,124]. The most common treatments for MS are interferon β (IFN-β), glatiramer acetate (GA), and the monoclonal antibody natalizumab, known as disease-modifying therapies (DMT). These treatments are unspecific for MS and often have serious side effects, such as opportunistic infections and tumors [125–127]. As previously indicated, the ideal treatment should be antigen-specific and DCs-targeted, to avoid systemic immunosuppression. In addition, the co-administration of antigen and immunomodulatory molecules using nanotechnology is now emerging as a new therapeutic option for the treatment of MS.

Most of the systems developed for MS treatment are based on PLGA and designed for the co-delivery of the antigen and immunomodulatory molecules such as rapamycin or IL-10. Following subcutaneous or intra-nodal administration in experimental allergic encephalomyelitis (EAE) mice model, it was found that these systems were able to successfully inhibit the progression of the disease [65,72,128]. In another report it was described that a new antigen-coupled PLGA formulation induced liver-dependent tolerance in a relapsing-remitting EAE mice model after IV administration [129]. Similarly, Carambia *et al*. showed that antigen-coupled poly(maleic anhydride-alt-1-octadecene) polymeric NPs induced also liver-dependent tolerance in an EAE mice model, providing effective control of the disease with a single IV administration due to the efficient induction of regulatory T cells [130].

The ionic complexation of antigenic peptides, or their DNA encoding sequences, and immunomodulatory molecules is nowadays presented as a new nanotechnology approach to induce tolerance. For example, Yuan *et al.* developed self-assembled NPs using a plasmid encoding for the co-inhibitory receptor B and T lymphocyte attenuator (BTLA) as immunomodulatory signal and MOG antigen modified with the cell penetrating peptide Tat49-

57. When DCs pretreated with these NPs were administrated intraperitoneally to an EAE mice model, a decrease in the spinal cord inflammation and inhibition of specific T cell proliferation were observed [131]. A similar approach involved the complexation of arginine-modified MOG antigen and GpG oligonucleotide, an antagonist of TLR9. Studies in EAE mice model showed an improvement in the progression, severity, and incidence of the disease [59,60].

Recently, PS liposomes were also tested as peptide carriers for MS therapy. Liposomes loaded with MOG peptide were administered intraperitoneally (2 boosts) in EAE mice model and the result of this treatment was a decrease in the clinical score and the incidence of the disease [132].

3.2.2. Type 1 diabetes

Nowadays, 415 million people worldwide have diabetes [133]. Diabetes mellitus is a pandemic group of disorders where insulin metabolism is altered. Within this group, T1D is considered a chronic autoimmune disease caused by the destruction of β-cells located in the Langerhans islets by the immune system, causing the loss of insulin production in pancreas [134]. Human and murine models have been extensively used to study the pathophysiology of the disease. Results from these studies have shown that the destruction of β cells occurs in a cell-mediated manner, requiring both $CD4^+$ and $CD8^+$ T cells and macrophages [135,136]. Most well-known antigens recognized by T cells in T1D are preproinsulin, glutamic acid decarboxylase and islet-cell antigen-2 [137]. The standard treatment for T1D is the subcutaneous injection of insulin to maintain normoglucemia. As an alternative, antigen-specific treatments aim to avoid the underlying autoimmune response, treating the disease at its origin [109]. The main barriers for the design of antigen-specific approaches are: the complexity of T1D autoantigens map, and the specific targeting to the immune cells involved in disease onset and progression. Nanotechnology offers the possibility of specific targeting and loading multiple antigens at the same time, with or without immunomodulatory molecules. The recently developed nanotechnology-based treatments for T1D are summarized below.

Liposomes containing PS were also developed for the delivery of insulin antigens. As mentioned above, PS was selected as it works as an “eat me” signal in apoptotic cells that can

promote the presentation of the antigen with the secretion of tolerogenic cytokines such as prostaglandin E_2 (PGE_2). According to the results, around 50% of NOD mice did not develop diabetes after intraperitoneal liposomes administration [58].

3.2.3. Rheumatoid arthritis

As we stated previously, RA is a long-term autoimmune disorder that primarily affects the joints. Currently, RA treatment is focused on easing the symptoms, or slowing the course of the disease by using immunosuppressant drugs such as corticosteroids or anti-TNF antibodies [90] (Table 5). Although most of the novel approaches for RA treatment are focused on the targeted delivery of immunosuppressant drugs or tissue regeneration, antigen-specific approaches are also being explored as a promising treatment at the onset of the disease through the downregulation of the underlying autoimmune processes, although the number is still limited [69,138,139]. Self-antigens, such as collagen derived peptides, were found to be RA triggers in different animal models [90], and mucosal administration of collagen peptides were found to ameliorate the progression of the disease in patients [140].

Among the different nanotechnology approaches to treat RA, there is the attempt described by Kim *et al.* based on the oral administration of PLGA NPs loaded with both whole type II collagen (CII) and CII derived peptides. The results showed a reduction of the severity of arthritis after a single oral administration to CIA mice and this positive effect was associated to the accumulation of the CII-loaded PLGA NPs in the Peyer's patches [139]. Similarly, using CII-derived peptides modified with PEG, Lee *et al.* developed peptide-loaded PLGA NPs for oral administration. They found that a single administration of the encapsulated PEG-conjugated peptides to healthy DBA/1 mice was able to increase both the rate of $IL\text{-}4^+$ $CD4^+$ cells and of $IL\text{-}10^+$ $CD4^+$ cells, which could be a promising approach for inducing tolerogenic phenotypes by the oral route [69]. Moreover, liposomes were also explored in antigen-specific therapy for RA by Capini *et al*. In a methylated BSA-induced arthritis model, they showed that, after SC administration of methylated BSA and lipophilic NF-κβ inhibitors (Bay11-7082, curcumin, or quercetin) co-encapsulated in liposomes, all of the combined formulations diminished the score of disease symptoms, compared with untreated mice [138].

Table 2. Most representative polymeric and lipidic nanocarriers for antigen-specific tolerance generation in autoimmunity

Nanocarrier type	Loaded molecule	Administration characteristics	Animal model	*In vivo* results	Ref.
PLGA NPs	IL-10 or MOG antigen	SC, co-administration of both systems	EAE	Inhibited disease development No vaccination delayed disease onset	[65]
	Rapamycin and PLP antigen	SC and IV	R-EAE	Delay in disease onset Complete inhibition of relapse episodes (IV)	[72]
	PLP antigen covalently linked	IV	R-EAE	Prevention of disease onset Complete inhibition of relapse episodes	[56,129]
	CII	Oral	CIA	Peyer's patches accumulation for longer time Reduced plasma levels of CII-antibodies Reduced incidence of arthritis	[139]
	PEG-CII derived peptides	Oral	Healthy	Expansion of IL-4$^+$ and IL-10$^+$ CD4$^+$ T cells	[69]
PLGA MPs	Rapamycin and MOG antigen	Intra-nodal	EAE	Permanently reduction of disease onset and severity	[128]
Liposomes	PS and insulin peptides	IP	NOD	Reduced incidence of T1D Delay in disease onset	[58]
	PS and MOG peptide	IP	EAE	Reduced clinical score Delay in disease onset	[132]
	Methylated BSA and NF-κβ inhibitors	SC	AIA	Reduction in joint swelling severity scores	[138]
Nano-complexes	pDNA encoding for BTLA and MOG antigen	IP injection of pre-treated DCs	EAE	Delay in disease onset Reduction of disease severity	[131]
	GpG and arginine-modified MOG antigen	SC	EAE	Reduced clinical score In some cases mice remained asymptomatic for the duration of the study (24 days)	[59,60]

AIA: adjuvant-induced arthritis ; BSA: bovine serum albumin; BTLA: B and T lymphocyte attenuator; CIA: collagen-induced arthritis; CII: type II collagen; DC: dendritic cell; EAE: experimental allergic encephalomyelitis; MOG: myelin oligodendrocyte protein; NOD: non-obese diabetic; PEG: pegylated; PLGA: poly(lactic-co-glycolic) acid; PLP: proteolipid protein; PS: phosphatidylserine; IP: intra-peritoneal; R-EAE: relapsing-EAE; SC: subcutaneous

In summary, nanotechnology-based antigen-specific approaches are promising for autoimmune diseases therapy and offer the possibility of controlled and targeted release of self-antigens. In addition, the possibility of loading immunomodulatory agents gives nanosystems the ability of enhancing tolerance generation. However, more research is needed for antigen identification and for a better understanding of what causes an autoimmune disease. These studies could help us elucidate the multiple factors that are involved in both epitope spreading and autoimmune response processes. Therefore, multiple antigen approaches could be the best option for efficiently blocking disease progression. To this end, nanotechnology could be a very valuable tool in combining multifactor therapy.

4. The next challenges of immunomodulation

4.1. Overcoming antidrug antibodies

We are in the era of biologicals, also named as biodrugs or biotherapeutics. The development of the recombinant DNA technology starting in the early 70s and the introduction of recombinant insulin in the market have laid the foundations for the use of biomolecules as therapeutic agents [141,142]. Nowadays, biomolecule-based therapies for a huge variety of diseases are already being used clinically, or are in trials, which shows the great potential of biotherapeutics [143,144]. Linked to this development of biodrugs, one of the major safety concerns that needs to be assessed during preclinical and clinical trials is undesired immunogenicity [145].

One of the first approaches to address unwanted immunogenicity is to measure the formation of antidrug antibodies (ADAs). ADAs recognize different epitopes in a recombinant molecule and bind to them, causing different outcomes in the pharmacological activity of the drug, depending on their neutralizing potential. ADAs formation and their effect on biotherapeutics have been extensively reviewed due to its direct relation with immunogenicity and treatment efficacy [146]. In the case of replacement therapies, ADAs formation could result in cross reactivity with endogenous proteins and, thus, cause severe adverse effects. One highlighted example is erythropoietin, a hormone required for red blood cell development which is used as treatment for anemia in patients with chronic kidney disease [147]. A few clinical subjects

were found to develop pure red cell aplasia after erythropoietin infusion, due to ADAs formation and subsequent endogenous erythropoietin recognition [148]. In this case, several factors affected the protein immunogenicity including those depending on product-formulation (leakage of polysorbate 80 from the rubber stoppers of the syringes) and administration (change from IV to SC administration route) [149–151].

There are multiple factors that influence the immunogenicity of biodrugs and formation of ADAs. Originally, a bacterial or fungi origin of the recombinant proteins could cause immunogenicity, due to the differences in sequence and structure of biomolecules between species. Although nowadays the use of humanized or fully-human biodrugs has greatly contributed to reduce this risk, immunogenicity associated to the aggregation of biodrug molecules and other factors is still a major concern for the optimum exploitation of these modern drugs [152,153]. The association between biodrug molecules upon injection has been thought to be a natural way to enhance antigen processing and presentation in the cells [154,155]. On the other hand, the presence of impurities could also be part of the problem. Finally, the administration route [156,157] and patient-related issues, such as genetic predisposition to ADAs formation or cytokine pattern, could impact immunogenicity [158,159].

Today, there is not a single standard therapy available for ADAs formation. The most frequent therapy is the administration of a prolonged immunosuppressive regimen as in the case of “Pompe disease”, a lisosomal storage disorder [160,161]. Nevertheless, this approach could enhance opportunistic infections and other complications due to systemic immunosuppression. The ideal treatment to avoid ADAs effects would be drug-specific: achieving tolerance to the delivered biotherapeutic and maintaining its safety and efficacy without systemic immunosuppression. In this field, nanotechnology is emerging as a new approach where a biotherapeutic agent can be specifically delivered together with an immunosuppressive drug, avoiding systemic immunogenic effects and increasing the treatment efficacy. For this purpose, DCs are the usual target, due to their important role in antigens presentation and also because of their relevance in the fate of T cells [106,162]. Indeed, as indicated above, it has been described how the uptake of rapamycin by DCs promotes the differentiation of T cells towards a regulatory phenotype [163]. Furthermore,

rapamycin encapsulation, both in PLGA NPs and MPs, enhances the tolerogenic activity of DCs [164,165].

Within this context, it is worthwhile to mention the formulation developed by Selecta Biosciences, in terms of inducing immunotolerance through the use of nanotechnology. They co-administered the coagulation factor VIII together with the immunosuppressive agent rapamycin loaded into PLGA NPs and observed promising *in vivo* results in terms of maintaining the efficacy of the biologic entity in a hemophilia A mice model [72]. In their search for a more universal approach, they explored the interest of co-administering rapamycin-loaded PLGA NPs together with different proteins (OVA, pegsiticase, adalimumab) and the results showed durable inhibition of ADAs formation [166]. Nowadays, a phase II clinical trial is ongoing for the evaluation of the co-administration of rapamycin-loaded PLGA NPs with free pegsiticase: a pegylated uricase enzyme implicated in the metabolization of uric acid, that is currently administered for the treatment of chronic gout [167].

Despite this original work there is still a limited understanding of the processes that underlie the immunogenicity of biotherapeutics and further research is needed to determine how nanocarriers could modulate immune mechanism to promote tolerance. All this knowledge will help us come up with a rational design of nanotherapeutic agents with better performance for tolerance generation.

4.2. Modulating exacerbated immune responses to viral infections

During the last decades, great efforts have been made to develop vaccines capable of generating protective immune responses against bacterial and viral infections. The immunity generated can act in early stages of the infection and resolve it without further complications. However, specific viral infections have also been associated with exacerbated immune responses that cause several complications, ending up in severe clinical prognosis [168]. Recently, the SARS-CoV-2 pandemic has brought this issue to the spotlight [169]. Accumulated evidence suggests that patients with severe prognosis of coronavirus disease 2019 (COVID-19) develop mild or severe cytokine storm syndrome [170]. Therefore, in these patients, treating this syndrome has become an important part to achieve a successful outcome. In this sense,

specific cytokine blockade has emerged as an approach for the treatment of COVID-19. Drugs such as tocilizumab, an antibody against IL-6 used for the treatment of RA, have shown promising results in patients with severe COVID-19 [171,172]. Nevertheless, the doses used to treat COVID-19 patients are far greater than the ones used for treating RA. Although the adverse effects of these doses are still not known, the urgency of the situation encouraged its use to decrease mortality. Within this context, nanotechnology-based formulations could emerge as new formulation approaches to improve safety and efficacy of these treatments. Further studies regarding the physiopathology of COVID-19 and the underlying immune mechanisms will be useful for the rational design of new therapeutic approaches.

5. Conclusions

During the last years, nanotechnology has shown a great potential as a tool for immunotherapy. The modulation of a broad variety of immune processes can be achieved with nanotechnology, showing promising results for the treatment of autoimmune diseases and prevention of ADAs. The interplay between nanocarrier composition, physicochemical properties and administration route drives the final immune outcome of the nanosystems. A deeper knowledge from the immunological point of view will help to rationally design and engineer new nanosystems that are expected to contribute to find a cure for some of the most threatening illnesses of our time.

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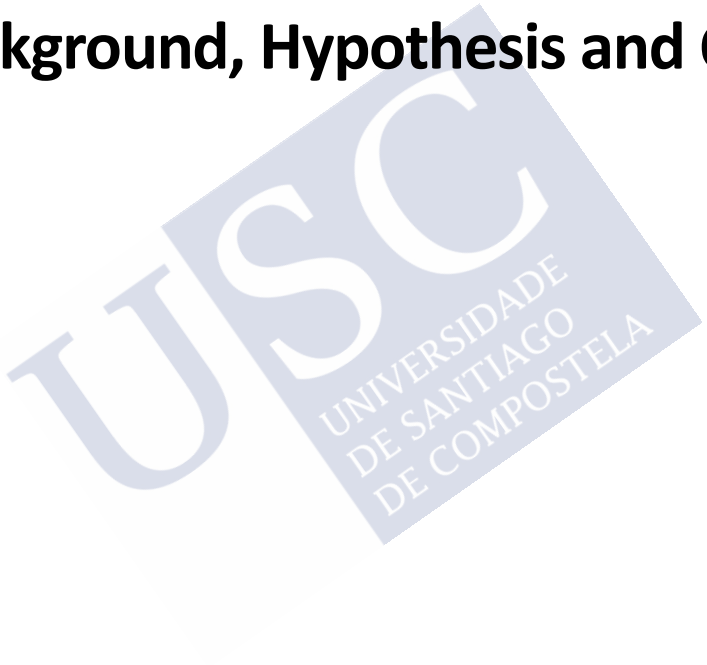
Background, Hypothesis and Objectives



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Background

The immunomodulatory molecule selected in this thesis, coded as IMM, has been linked with some of the most prevalent pathologies of our time, such as cancer, autoimmune diseases and microbial infections. Although its immunomodulatory activity suggested its potential for the treatment of autoimmune diseases, limitations inherent to its absorption and biodistribution have restricted the full exploitation of its therapeutic effects. Indeed, the high doses of its active form required to achieve a therapeutic effect led to off-site toxicity. Moreover, its oral administration has been associated with an erratic bioavailability. Besides, this molecule has shown high instability under storage and after its administration *in vivo*. All these limitations illustrate the need of an effective formulation that would expand its clinical use.

In this sense, nanotechnology may be a good tool to develop new formulations. Nanocarriers offer the possibility to improve biodistribution of drugs by targeting them to different cell populations. In addition, nanotechnology-based formulations can co-encapsulate multiple cargos, such as antigenic peptides, to enhance the immunomodulatory properties of the selected molecule. Moreover, nanocarriers can be adapted for different administration routes that could be convenient for natural compounds supplementation, such as the dermal route. Furthermore, these nanovehicles can efficiently protect drugs by themselves or by co-encapsulating antioxidant agents. Therefore, these characteristics make nanotechnology a great approach to enhance both therapeutic and preventive potential of the selected immunomodulatory molecule.

Among other nanocarriers, polymeric nanocarriers present features that make them suitable for the delivery of highly lipophilic compounds. Their structure allows the encapsulation of lipophilic compounds. Furthermore, their tunable composition facilitates the modulation of physicochemical properties critical for their biodistribution, such as size or surface charge [1,2]. In this context, our group has generated significant knowledge regarding the modulation of polymeric nanocarriers properties and their influence in their biodistribution and interaction with different cells subsets [2–9].

Hypothesis

1. The encapsulation of an immunomodulatory molecule in polymeric nanocarriers can allow its targeted delivery to immune cells while avoiding off-target toxicity.

2. The co-encapsulation of an immunomodulatory molecule and an autoantigenic peptide can enhance their preventive effect in type 1 diabetes.

3. The inclusion of the immunomodulatory molecule selected in polymeric nanocarriers of around 100 nm can enhance its penetration across the skin layers.

Objectives

Considering the background and the hypothesis previously outlined, the main objectives of this work were oriented in two directions:

A. To design and develop a prototype consisting of polymeric nanocarriers for the delivery of the selected immunomodulatory molecule and to investigate their potential for the prevention of type 1 diabetes.

B. To design and develop small polymeric nanocarriers in order to enhance the transport of this molecule across the skin.

To address these objectives, the following experimental activities were undertaken:

1. Development of polymeric nanocarriers encapsulating the selected immunomodulatory molecule and evaluation of their potential for the prevention of type 1 diabetes. This activity has involved:
    a. Development, physicochemical and morphological characterization of polymeric nanocarriers suitable for the immunomodulatory molecule encapsulation.
    b. Study of polymeric nanocarriers' stability, cytotoxicity and interaction with immune cells.
    c. Evaluation of the capacity of immunomodulatory molecule-loaded nanocarriers to promote a tolerogenic phenotype in human dendritic cells *in vitro*.
    d. Assessment of the effect of immunomodulatory molecule-loaded nanocarriers on diabetes onset in a mice model of type 1 diabetes.

    The results corresponding to this objective are presented in Chapter 2 "Tolerogenic nanocarriers for the prevention of type 1 diabetes".

2. Development of small polymeric nanocarriers for enhancing the transport of IMM across the skin. This activity has involved:

a. Optimization of the formulation parameters in order to produce small nanocarriers with different surface charge.
b. Physicochemical and morphological characterization of loaded polymeric nanocarriers.
c. *Ex vivo* evaluation of polymeric nanocarriers capacity to enhance penetration in human skin of the selected molecule.

The results corresponding to this objective are presented in Chapter 3 “Small polymeric nanocarriers as a dermal delivery system for supplementation”.

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Chapter 2

Tolerogenic nanocarriers for the prevention of type 1 diabetes

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Chapter 2

Tolerogenic nanocarriers for the prevention of type 1 diabetes

This work was done in collaboration with the Department of Biochemistry and Molecular Biology, School of Pharmacy, Universidade de Santiago de Compostela

Abstract

Type 1 diabetes (T1D) is a chronic autoimmune disease caused by the destruction of the Langerhans islets β-cells by the immune system, which leads to the loss of insulin production in pancreas. Within this context, specific treatments to prevent the immune reaction against β-cells are desirable. Nanotechnology offers the possibility of targeting immunomodulatory compounds to the immunocompetent cells, thereby developing a tolerogenic profile while avoiding off-target effects. In this chapter, we evaluated the potential of cationic nanocarriers (NVCs) containing an immunomodulatory molecule (IMM) and a T1D autoantigen (PPI B_{10-18}) as a preventive treatment for T1D. The NVCs showed a particle size around 200 nm and a surface charge around +50 mV. The results of the *in vitro* cell culture studies indicated that NVCs containing IMM were effective in terms of inducing a tolerogenic profile in hDCs, this being associated to the inhibition of T cell activation. Finally, the results of the *in vivo* studies performed in a NOD-HHD mice model indicated that both, IMM and (PPI B_{10-18} + IMM)-loaded NVCs were able to induce a significant delay in diabetes onset as compared to non-encapsulated IMM. These results highlighted the importance of IMM encapsulation for its targeting to the immune system and subsequent improvement of its inherent tolerogenic effect. Overall, these data show the promising potential of IMM-loaded NVCs for T1D prevention.

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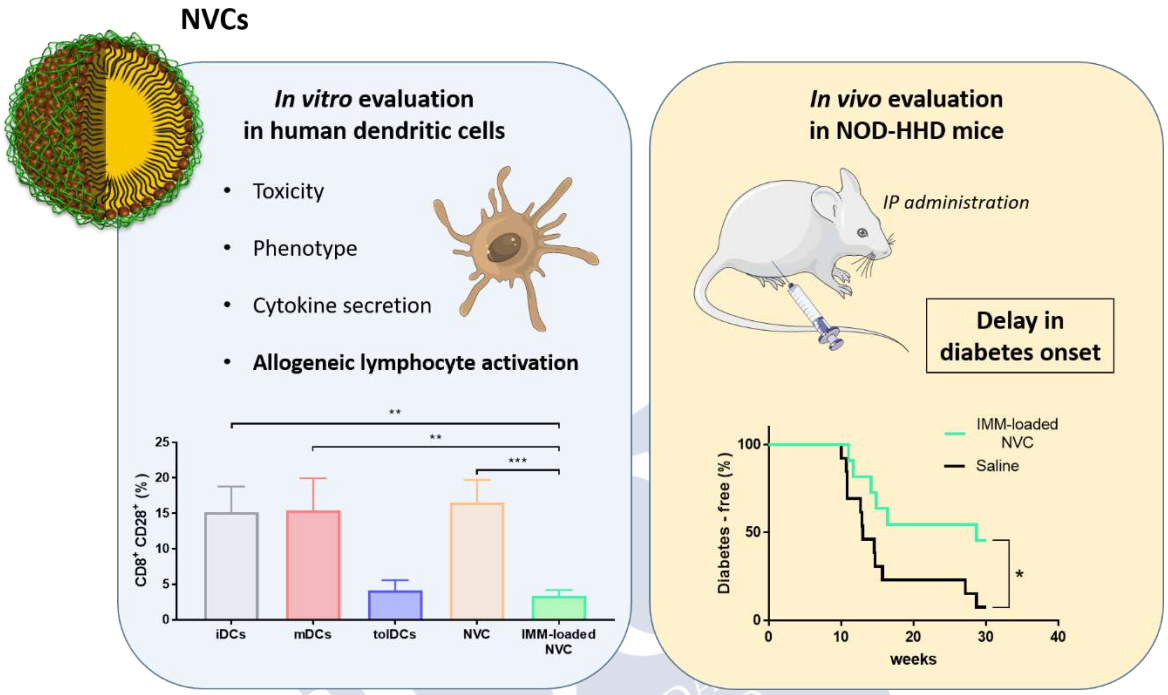
Graphical abstract



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1. Introduction

In 2019, around 130,000 new cases of type 1 diabetes (T1D) were diagnosed in people under 20 years [1]. These numbers are estimated to grow 3% per year, increasing health expenditure globally. T1D is considered an autoimmune disease that is characterized by the breakdown of self-tolerance and, hence, the development of an immune response against autologous antigens, such as proteins or peptides [2]. In the case of T1D, disease symptoms are caused by the destruction of β cells by autoreactive T lymphocytes that infiltrate in pancreatic Langerhans islets [3]. The result of this pathological condition is a lack of insulin production and a subsequent dysregulation of glucose metabolism. Nowadays, the main treatment of T1D is a subcutaneous injection of insulin. However, the suboptimal managing of glucose levels often leads to major complications, such as diabetic retinopathy or ulcers among others [4,5]. This highlights the need of new treatments focused on the prevention of T1D through immune modulation in early stages of the autoimmune process.

The central role of dendritic cells (DCs) in autoimmunity and the maintenance of peripheral self-tolerance has been described in the literature [6]. DCs are professional antigen presenting cells (APCs) responsible of capturing and presenting antigens to T lymphocytes [7]. The subsequent immune response can be biased depending on DCs phenotype. In this sense, the presence/absence of different co-stimulatory signals and secretion of cytokines are mechanisms used by DCs to modulate the effector response after antigen presentation [8]. In the autoimmunity scenario, the modulation of DCs towards a tolerogenic phenotype has been considered the best way to control the immune response against autoantigens [9]. The increasing understanding of immune signaling pathways has led to the discovery of different immunomodulatory molecules, such as rapamycin or dexamethasone, that can tune the DCs phenotype towards a tolerogenic profile [10]. Unfortunately, the systemic administration of these drugs has led to undesired off-target effects such as systemic immunosuppression and toxicity [11–13]. In this context, the controlled delivery of immunomodulatory molecules would be necessary for their use in clinics.

Nanotechnology offers the possibility for targeting and controlling the delivery of drugs to the immune system. Indeed, there are multiple examples of nanocarriers that have resulted in

positive out-comes with regard to their use for the treatment of autoimmune diseases [14–16]. For example, acetalated dextran microparticles loaded with dexamethasone and an antigenic peptide succeeded in diminishing the clinical score in a mice model of multiple sclerosis [14]. Similarly, Yeste *et al.* developed gold nanoparticles for the co-delivery of an antigen and the immunomodulatory molecule 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester in multiple sclerosis, resulting in suppression of multiple sclerosis symptoms in mice with experimental autoimmune encephalomyelitis [16]. In addition, it is known that the composition and properties of the nanocarriers may influence the type of immune response achieved [17,18]. In this context, our research group has generated significant knowledge regarding the modulation of the nanocarriers biodistribution and interaction with the immune system [19–22].

Considering this background, the objective of this work was to develop a new preventive strategy for T1D based on the use of cationic nanocarriers (NVCs) for the delivery of an immunomodulatory molecule (IMM) and a peptide antigen candidate. The cationic surface charge was selected based on the known avidity of macrophages and DCs for positively charged surfaces [23,24]. The antigen selected was a fragment of human preproinsulin (PPI B_{10-18}), a known autoantigen in T1D. The potential of NVCs to interact and modulate immune cells *in vitro* was assessed in human dendritic cells (hDCs). Furthermore, the capacity of our NVCs to delay diabetes onset *in vivo* was validated using a non-obese diabetic (NOD) mice model.

2. Materials and methods

2.1. Materials

Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin-streptomycin-glutamine (PSG), phosphate buffer saline (PBS) and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen Corporation, Life Technologies, UK). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) was acquired from Invitrogen (CA, US). Bovine serum albumin (BSA), bacterial lipopolysaccharide (LPS) and triton X-100 were purchased from Sigma-Aldrich (ST Louis, MO, US). PPI B_{10-18} (HLVEALYLV) peptide was

obtained from China Peptides (Shanghai, China). Ficoll-Paque™ Plus (density 1.077 g/mL) was purchased from GE Healthcare LifeSciences (Pasching, Austria). Human granulocyte-macrophage colony-stimulating factor (GM-CSF) and human interleukin 4 (IL-4) were obtained from Miltenyi Biotech (Bergisch Gladbach, Germany). Human interferon γ (IFN-γ) was acquired from PeProtech (London, UK). CytoPainter Phalloidin-iFluor 488 was purchased from Abcam (Cambridge, UK). L-tryptophan was obtained from Thermo Fisher Scientific (Waltham, MA, US). CellTiter 96® AQueous non-radioactive cell proliferation assay used to perform MTS assays was supplied by Promega (Madison, WI, US). Antibodies for flow cytometry studies were acquired from Miltenyi Biotech (Bergisch Gladbach, Germany) excluding anti-CD25 that was purchased from BD Biosciences (Franklin Lakes, NJ, US). Organic solvents used were of high-performance liquid chromatography (HPLC) grade and all other products used were of high purity or reagent grade. Endotoxin free water and sterile/autoclaved materials were used for *in vitro* and *in vivo* experiments.

2.2. Physicochemical characterization

Particle size and polydispersity index (PDI) of the formulations were measured by photon correlation spectroscopy (PCS) using a Zetasizer® (Nano ZS, ZEN 3600, Malvern Instruments, Worcestershire, UK). Samples were previously diluted 10 times in ultrapure water and measurements were done at room temperature (RT). Zeta potential measurements were done by Laser-Doppler Anemometry (LDA) with the same instrument and experimental conditions.

For additional characterization, particle size of NVCs was analyzed by Nanoparticle Tracking Analysis (NTA) using NanoSight NS3000 equipment (Malvern Instruments, Worcestershire, UK). Samples were diluted 1000 times in ultrapure water and all the measurements were done in triplicate, with 5 videos of each sample captured over 60 seconds at RT.

Furthermore, NVCs morphology was examined by field emission scanning electron microscopy (FESEM) (ZEISS, ULTRA Plus, Germany). Samples were diluted in ultrapure water 1:1000 and mixed with the same volume of 2% (w/v) phosphotungstic acid solution. Then, 1 µL of the mixture was placed on cooper grids with carbon films and left to dry in the open air. Finally,

the grids were washed dropwise with 1 mL of ultrapure water and left to dry overnight for its final observation under the microscope using both scanning emission electron microscopy (STEM) and InLens detectors.

2.3. Colloidal stability

The stability of the isolated blank nanocarriers under storage conditions (4°C) was evaluated through monthly measurements of their colloidal properties (particle size, PDI and zeta potential) as previously described in section 2.3.

To ensure the colloidal stability of the formulations in the cell culture medium to be used in the *in vitro* cell culture studies, isolated prototypes were incubated at an appropriate dilution (1/20) with complete RPMI medium containing 10% FBS and 1% PSG (R10 medium), during 48 hours under horizontal shaking (300 rpm, Heidolph Titramax/Inkubator 1000, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). At different time points, particle size and PDI were evaluated as previously described.

2.4. Fluorescent labelling of NVCs and characterization of the labelled nanosystem

The preparation of the fluorescent labelled NVCs was done by adding DiD for a final concentration of 25 or 75 µg/mL. Besides physicochemical characterization, DiD-loaded NVCs were also characterized in terms of fluorophore encapsulation efficiency, stability of the labelling in storage and release profile. For the evaluation of the encapsulation efficiency, DiD-loaded NVCs were isolated by ultracentrifugation and fluorescence intensity in the undernatant containing the non-encapsulated fluorophore was measured. Regarding the stability of the labelling under storage conditions (4°C), DiD leakage during time was determined using isolated DiD-loaded NVCs. Samples were re-isolated as described previously at different time points and DiD leakage was measured in the undernatant by spectrofluorimetry. For the study of the release profile, isolated DiD-loaded NVCs were diluted 5 times with R10 medium and incubated at 37°C during 24 hours under horizontal shaking (300 rpm, Heidolph Titramax/Inkubator 1000, Heidolph Instruments GmbH & Co. KG,

Schwabach, Germany). At different time points, samples were isolated as previously described and fluorescence measurements were done for determining the free fluorophore present in the undernatant. All fluorescence measurements were performed at the excitation (640 nm) and emission (675 nm) wavelengths using Synergy 2 H1M Microplate reader (Biotek, Winooski, VT, US).

2.5. Preparation of nanocarriers loaded with an immunomodulatory molecule and an autoantigen

2.5.1. IMM and PPI B_{10-18} loaded nanocarriers

For *in vivo* experiments, a fragment of human preproinsulin (PPI B_{10-18}) was included in IMM-loaded NVCs as the autoantigenic epitope to generate specific immune tolerance (Fig. 1).

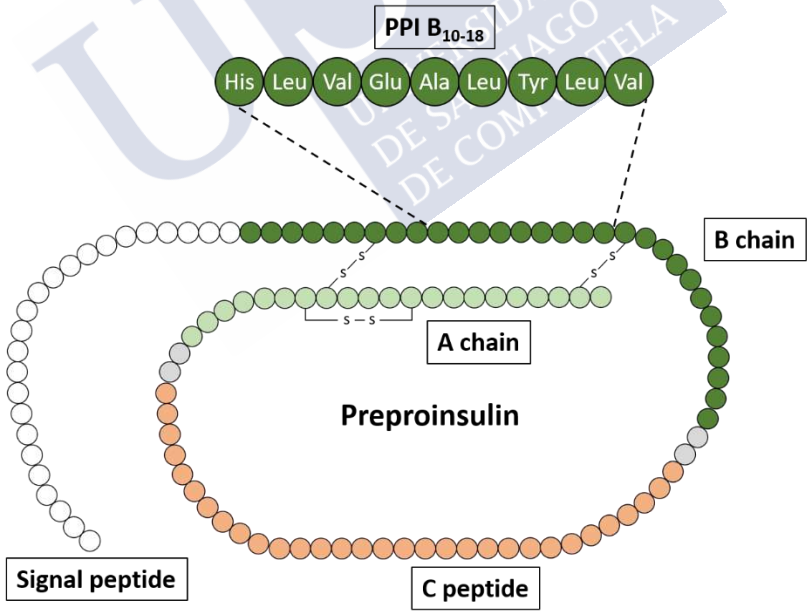

Fig. 1 – **PPI B_{10-18} structure**. Preproinsulin structure contains a signal peptide (white), insulin B (dark green) and A (light green) chains and the C peptide (orange). C-peptide cleavage sites are represented in grey. The antigen used, PPI B_{10-18}, is a fragment of preproinsulin B chain containing the amino acids from 10 to 18.

2.5.1.1. PPI B_{10-18} quantification and EE%

The amount of PPI B_{10-18} was directly determined from non-isolated NVCs dispersion, undernatant and isolated NVCs dispersion, all of them previously disrupted for PPI B_{10-18} extraction. For digestion, each fraction was diluted with a combination of acetonitrile and ultrapure water using a 3:1 proportion and then vortexed (1/20 non-isolated dispersion, 1/20 isolated dispersion, 1/10 undernatant). Then, each fraction was analyzed by HPLC (Agilent 1100 Series HPLC, Agilent, CA, US). Gradient elution was performed with a C18 column as stationary phase (SunFire® C18 100 Å, 5 µm 4.6 × 250 mm, Waters, Spain) at 35°C. Flow rate was settle in 1 mL/min, injection volume of samples was 60 µL and absorbance was measured at 220 nm. The detection method was optimized using as mobile phases 0.1% trifluoroacetic acid (TFA) in ultrapure water (v/v, solvent A) and 0.1% TFA in acetonitrile (v/v, solvent B) in the conditions detailed in table 1.

Table 1 – **HPLC analytical method for PPI B_{10-18} quantification**

Time	% solvent A	% solvent B
0 min	100	0
1 min	100	0
2 min	75	25
3 min	75	25
19 min	48	52
20 min	100	0

The EE (%) of PPI B_{10-18} was calculated according the following equation:

$$EE\ (\%) = \frac{PPI\ B_{10-18}\ in\ the\ disrupted\ NVCs}{total\ PPI\ B_{10-18}}\ X\ 100$$

Where *PPI B_{10-18} in the disrupted NVCs* is the antigen concentration determined by HPLC after treating the isolated NVCs dispersion for its disruption, and *total PPI B_{10-18}* is the theoretical total PPI B_{10-18} amount in the formulation. Analysis was done in triplicate.

2.6. Human dendritic cell generation

hDCs were generated from adherent monocytes isolated from human blood. Briefly, heparinized blood was diluted 1:1 in PBS and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using Ficoll-Paque™ Plus (400 × g, 30 minutes without brake, RT). PBMCs were washed 2 times with 50 mL of PBS (300 × g, 10 minutes, RT) and resuspended to a final concentration of 6×10^6 cells/mL using R2 medium (complete RMPI with 2% FBS and 1% PSG). Then, 10 mL of PBMCs were incubated during 2 hours at 37°C in T75 BioLite flask (ThermoFisher Scientific) for isolation of adherent monocytes. On one hand, medium containing non-adherent cells (peripheral blood lymphocytes, PBLs) was recovered after the incubation period and PBLs were washed with PBS and stored in liquid nitrogen for its use in allogenic response experiments (see below). On the other hand, adherent monocytes in T75 flask were washed 3 times with pre-heated R2 medium and cultured for 6 days in R10 medium. For obtaining immature hDCs (iDCs), 100 ng/mL of both IL-4 and GM-CSF were added to R10 from the beginning of the incubation and half of the medium was renewed after 3 days.

Both mature and tolerogenic dendritic cells were obtained from iDCs for their use as controls in all *in vitro* experiments. Mature hDCs (mDCs) were obtained by incubation of iDCs with R10 containing 10 ng/mL of LPS and 100 U/mL of IFN-γ during 48 hours. Tolerogenic hDCs (tolDCs) were obtained from iDCs adding 50 nM of IMM in the same conditions indicated for mDCs.

2.7. Cell viability in iDCs after incubation with both nanosystems

iDCs were incubated for 24 hours with the different blank nanosystems at increasing concentrations (25, 50, 100, 200, 400 and 900 µg/mL). After the incubation period, cells were harvested, washed 3 times with PBS and resuspended in R10 at a concentration of 1×10^4 cell/mL. Then, cells were cultured during 20h in 96 well plate (100 µL/well), and after the incubation time, 20 µL/well of MTS reactive (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added. The conversion of MTS to soluble formazan is proportional to the number of living cells and was measured by absorbance at 490 nm after a 4-hours incubation period using a microplate reader (model 680, Bio-Rad, CA, US) and it is proportional to the number of living cells.

2.8. Interaction of DiD-loaded NVCs with hDCs

NVCs interaction with hDCs was evaluated by flow cytometry and confocal microscopy. For these studies, iDCs were plated into a 24-well plate with 0.5 mL of R10 medium (5×10^5 cells/well) for incubation with DiD-loaded NVCs.

2.8.1. Flow cytometry

To evaluate by flow cytometry the percentage of NVCs that interacts with iDCs, DiD-loaded NVCs (25 µg/mL DiD) were added to the previously plated cells at a concentration of 100 µg/mL. After 1-hour incubation, cells were washed 3 times with PBS and fixed with 200 µL of 1% paraformaldehyde (PFA) in PBS in a flow cytometry tube. For acquisition by flow cytometry, 500 µL of PBS were added to the samples and cell suspension was analyzed using BD Accuri C6 cytometer (BD Biosciences, Franklin Lakes, NJ, US).

2.8.2. Confocal laser scanning microscopy (CLSM)

To evaluate by CLSM the internalization of NVCs by iDCs, DiD-loaded NVCs (75 µg/mL DiD) were added to the previously plated cells at a concentration of 100 µg/mL. After 1-hour incubation, cells were washed 3 times with PBS, resuspended in PBS at a concentration of 1×10^6 cells/mL, and plated into a 24-well plate (0.5 mL/well) with a round cover glass in the bottom of each well. After 30 minutes of incubation under RT, PBS was removed and cells were incubated during 10 minutes with 0.5 mL of 4% PFA in PBS. Then, cells were permeabilized with 0.5 mL of 0.5% Triton X-100 during 10 minutes. After this, each well was incubated with 0.5 mL of phalloidin solution in PBS for 30 minutes. Finally, each round cover glass containing cell sample was mounted on microscope slides and examined by CLSM using LEICA AOBS-SP5X microscope (Leica Microsystems, Mannheim, Germany). All the incubations were done in dark conditions at RT and there was a 3 times PBS washing step between all the steps of this protocol.

2.9. Effect of nanocarriers on hDCs phenotype

For the analysis of the effect of nanocarriers on hDCs phenotype, iDCs were cultured in two-steps. First, iDCs were incubated with the different prototypes during 2 hours (R10 medium containing 200 µg/mL of formulation, 50 nM IMM) and second, cultured with R10 medium for 46 hours. To determine the phenotype of iDCs, surface markers expression was measured by flow cytometry. Briefly, cells were washed 3 times with 0.1% BSA in PBS and stained with optimal amounts of different antibodies for 30 minutes (in the dark, 4°C). Levels of immunoglobulin-like transcript 3 (ILT3), toll-like receptor 2 (TLR2), CD209, CD80, CD83 were quantified by flow cytometry. CD14 and CD11c were included as DC markers to verify correct monocyte differentiation. Vital marker eFluor™660 was used to determine cell viability. Then, cells were washed 3 times with PBS and fixed with 200 µL of 1% PFA in PBS in a flow cytometry tube. For acquisition, 500 µL of PBS were added to the samples and cell suspension was analyzed using BD FACSCalibur™ cytometer (BD Biosciences, Franklin Lakes, NJ, US). Data was analyzed using the Flowing Software (Cell Imaging Core, Turku Centre for Biotechnology, Finland).

2.10. Indoleamine 2,3-dioxygenase (IDO) activity in iDCs incubated with NVCs

For the determination of IDO activity, iDCs were cultured in two-steps as described in section 2.9, but incubating 24 hours with the different prototypes and 24 hours with R10 medium. Using a previously described method, IDO activity was determined by the quantification of kineurin in culture medium obtained from iDCs incubated with our prototypes [25]. Briefly, 4 hours before the end of the second culture step, L-tryptophan was added in a final concentration of 100 µM. After this period, 100 µL of culture medium were mixed with 50 µL of 30% TFA in water (v/v) for proteins and cell debris precipitation and centrifuged at 10000 × g during 5 minutes at RT. Finally, 75 µL of supernatant were mixed with 75 µL of Ehrlich Reagent and absorbance at 490 nm was determined immediately using Synergy 2 H1M Microplate reader (Biotek, Winooski, VT, US).

2.11. Gene expression of iDCS incubated with NVCs

For the analysis of gene expression, iDCs were cultured in two steps as previously described in section 2.10. Then, cells were harvested and total RNA was isolated and concentrated using commercial kits following manufacturer`s instructions (SV Total RNA Isolation System and RNeasy® MinElute® Cleanup, Promega, Madison, WI, US). Afterwards, cDNA was synthetized from total RNA using qScript™ cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, US) following manufacturer's protocol. The expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4), lactate dehydrogenase A (LDHA) and c-MYC transcription factor was analyzed by qPCR using cDNA previously synthetized and using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene. qPCR analysis was done in an iCycler CFX9™ thermal cycler (Bio-Rad, CA, US) using iQ SYBR Green Supermix (Bio-Rad, CA, US). Sequences of primers were 5'-CAACATCGTGCAAGTGAACTG-3' (forward) and 5'-GACTCGTAGGAGTTCTCATAGCA-3' (reverse) for PFKFB4; 5'-ATGGCAACTCTAAAGGATCABC-3' (forward) and 5'-CCAACCCCAACAACTGTAATCT-3' (reverse) for LDHA; 5'-GTCAAGAGGCGAACACAAC-3' (forward) and 5'-TTGGACGGACAGGATGTATGT-3' (reverse) for c-MYC; 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-ATGGTGGTGAAGACGCCAGT-3' (reverse) for GAPDH. The target gene expression level was calculated as the number of fold increase over the reference gene using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method [26,27].

2.12. Lymphocyte activation capacity of iDCs pre-incubated with NVCs

The capacity to activate $CD8^+$ and $CD4^+$ T lymphocytes of iDCs was determined by flow cytometry. First, iDCs were incubated with NVCs by the two steps method described in section 2.10. Afterwards, pre-incubated iDCs were harvested, washed with PBS and plated with allogeneic PBLs (non-adherent cells from human blood obtained after washing adherent monocytes, described previously) at a ratio 1:10 (iDCs:PBLs) for 7 to 10 days. Next, culture medium was used for quantification of cytokines secretion (see section 2.12) and cells were harvested, washed, stained with optimal quantities of CD4, CD8, CD25 and CD28 antibodies and analyzed by flow cytometry as previously described in section 2.10.

2.13. Cytokine secretion by iDCs incubated with NVCs

Cytokine secretion was determined by measuring cytokines levels in culture medium from iDCs incubated with NVCs using the two-steps culture conditions described previously, and from pre-incubated iDCs co-cultured with allogeneic PBLs. For quantification of cytokines present in culture medium, MILLIPLEX®MAP kits were used. These personalized kits contained microparticles to capture IL-10, IL-15, IL-1β, IL-4, IL-27, IL-23, IL-2, IL-12p70, IL-5, IL-8, TNF-α, TNF-β and IFN-γ present in the medium. The assay was done following the manufacturer protocol (Millipore). MAGPIX® analyzer (Merck Millipore, Darmstadt, Germany) was used for reading the plates (flow cytometry facilities, Biomedical Research Centre, Universidade de Vigo, Spain). Different calibration curves were used for the determination of cytokines concentration, and those were calculated as pg/mL.

2.14. Evaluation *in vivo* of T1D prevention

2.14.1. Animals

NOD.B6-Tg(HLA-A2.1)Enge/DvSJ mice (NOD-HHD) were obtained from Jackson Laboratories and bred in-house. Mice were housed in the specific pathogen-free animal facility of "Centro de Investigación en Medicina Molecular y Enfermedades Crónicas" (CiMUS, Universidade de Santiago de Compostela) in ventilated cages. All experimental procedures were in accordance with the national guidelines (RD 53/2013) and were approved by the "Comité Ético para la Experimentación Animal" from the Universidade de Santiago de Compostela (project number 15010/16/002).

2.14.2. Evaluation of diabetes onset

Female NOD-HHD mice at 4 weeks of age and normoglycemic were used. Mice were treated with intraperitoneal (IP) injections (100 µL) following the regimen described in Fig. 2. The formulations tested were: blank NVCs, IMM-loaded NVCs, (PPI B_{10-18} + IMM)-loaded NVCs and IMM-loaded NV. Control groups of saline and free IMM or PPI B_{10-18} were also included in the study. The tested doses were 4 mg/kg for PPI B_{10-18} dose and 5 µg/kg for IMM. A minimum of

10 animals per group were analyzed. Weekly measurements of urine glucose were performed from week 9 of age using Medi-Test Glucose Strips (BHR Pharmaceuticals, UK). Mice with glycosuria were confirmed diabetic when urine glucose was ≥ 500 mg/dL after two consecutive weeks.

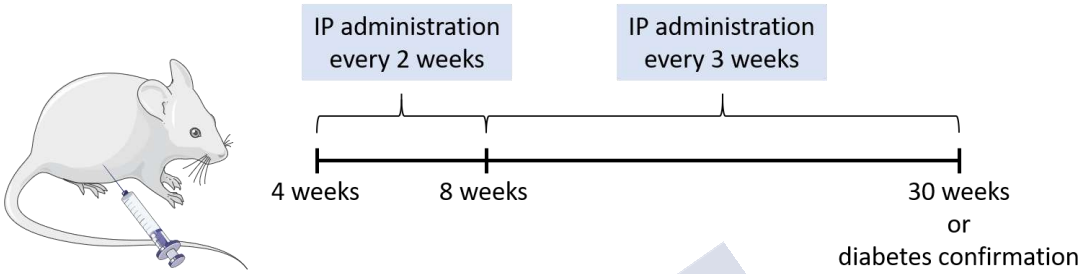

Fig. 2 – **Schematic representation of *in vivo* administration schedule in female NOD-HHD mice.**

2.15. Statistical analysis

All the statistical analyses were carried out with GraphPad Prism Version 7.04 software. Unless otherwise indicated, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied. Kaplan-Meier log-rank analysis was used for comparison between survival curves. The differences were considered significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. Results and discussion

New immunological therapies could change the paradigm in T1D treatment and prevention. In particular, the modulation of the immune response to avoid β-cell destruction could be a way to improve patient outcome and prevent T1D onset. Our hypothesis has been that a change in the phenotype of DCs would be a straight-forward strategy to avoid autoreactive T lymphocyte activation [9]. Although small immunomodulatory molecules, such as rapamycin or dexamethasone are known to induce a tolerogenic phenotype in DCs, their systemic

administration at relatively high doses has been associated to major off-target effects such as systemic immunosuppression and toxicity. Hence, our approach to overcome this hurdle has relied on the use of nanotechnology, which not only offers the capacity for the controlled delivery of immunomodulatory molecules but also the possibility of loading multiple drugs. Combining autoantigens with drugs with tolerogenic effect in the context of a nanostructure opens the possibility for developing antigen-specific therapies for T1D and other autoimmune diseases [28]. Having this information in mind, our objective in this work was the development of NVCs loaded with an immunomodulatory molecule and an antigenic peptide and the *in vitro* and *in vivo* evaluation of the potential of this technological approach for the promotion of a tolerogenic effect in hDCs to delay the diabetes onset in NOD-HDD mice.

3.1. NVCs characterization

The physicochemical characteristics of nanocarriers are key factors in their biodistribution and interaction with cells and tissues. Parameters such as size and surface charge are known to significantly influence the interaction of nanocarriers with immune cells [29]. It has been described that providing nanovehicles with a positive surface charge increases their chances for interaction with DCs [30–33]. Hence, our election was to use NVCs as a carrier for targeting DCs. NVCs and control nanocarriers (NV) were formulated. Different techniques were used for the characterization of blank NVCs and colloidal stability was assessed to assure stability during *in vitro* studies. Furthermore, different active molecules were included in NVCs for the evaluation of their potential in terms of enhancing a tolerogenic response, both *in vitro* and *in vivo*.

3.1.1. Physicochemical and morphological characterization of blank NVCs

The physicochemical characterization of blank NVCs is presented in Table 2. We also formulated a control NV following the same protocol without the addition of a cationic polymer, as a control for *in vitro* and *in vivo* studies. NVCs exhibited an average size of around 220 nm and a positive ζ potential of + 55 mV.

Table 2 – **Physicochemical properties of blank nanocarriers.** Values represent the mean ± SD of at least 3 replicates.

Nanosystem	Particle Size (nm)	PDI	ζ-potential (mV)
Blank NV	158 ± 11	0.1	-50 ± 4
Blank NVC	224 ± 4	0.1	+55 ± 2

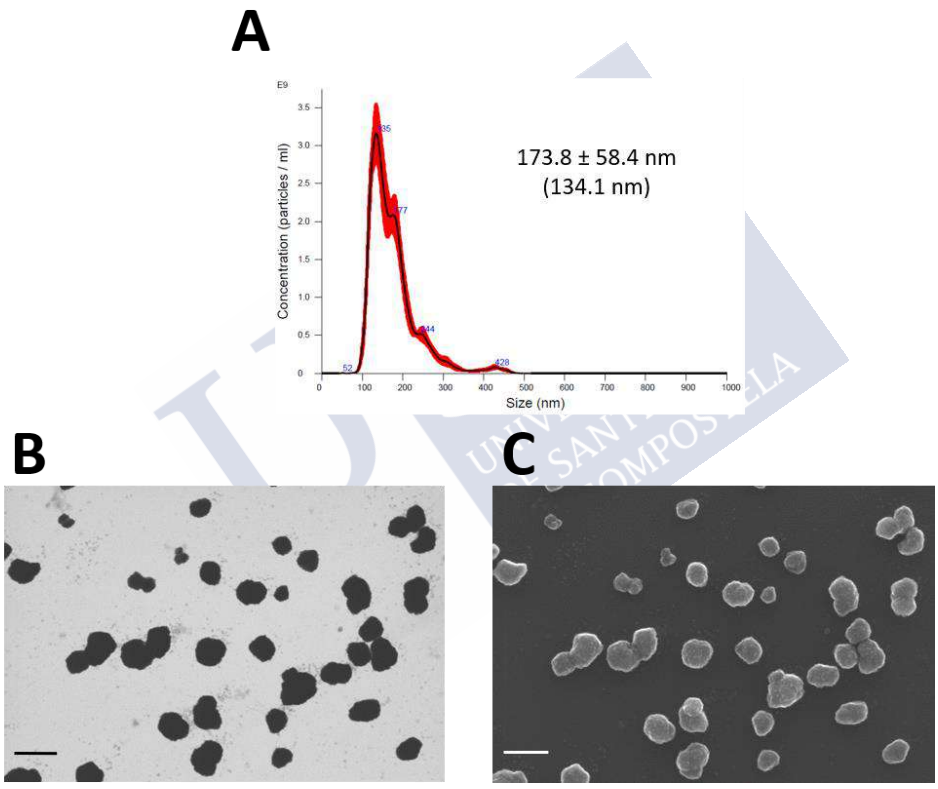

Fig. 3 – **NTA (A) and FESEM characterization (B, C) of blank NVCs**. A representative image of the particle size distribution obtained for each system by NTA is shown **(B).** The mean particle size with the standard deviation and the mode (between brackets) are indicated in the figure (n=3). FESEM images, using InLens **(C)** and STEM **(D)** detectors. All scale bars = 200nm.

For a rigorous characterization, we used different techniques to analyze particle size and morphology of NVCs (Fig. 3). Complementary to DLS measurements, NTA was used to

determine particle size. Higher resolution of this technique may discriminate between populations even with narrow particle size distributions. In the case of NVCs, we observed populations ranging from 100 to 260 nm, in accordance with DLS data (Fig. 3B). Moreover, round-shape of NVCs was shown in both InLens and STEM images (Fig. 3C-D).

3.1.2. Colloidal stability

Colloidal stability of nanocarriers is a key parameter for their final performance *in vivo*. Size, among other parameters, is determinant of the biodistribution of the nanocarriers and their subsequent interaction with specific cells and tissues [20]. It has been shown that DCs preferentially ingest virus-like particles in the nanometric range [34,35]. Pursuing passive targeting to DCs, maintaining colloidal stability of NVCs in biorelevant media would enhance their interaction with DCs. Hence, we tested the colloidal stability of both blank control NV and NVCs, in the R10 culture medium to be used for *in vitro* evaluation of the nanocarriers. The results showed that the size of the control NV was maintained and the PDI was below 0.3 up to 48 hours incubation (Fig. 4A). In the case of NVCs, their particle size was maintained for up to 24 hours of incubation (Fig. 4B). Therefore, based on these results, we could argue that both formulations were suitable for their use *in vitro*.

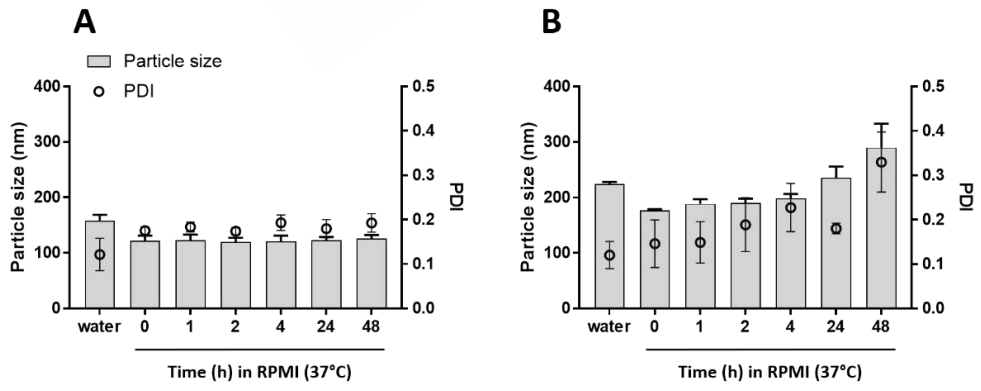

Fig. 4 – **Stability of control NV (A) or blank NVCs (B) upon incubation with cell culture media** (37°C). Control was done incubating the nanocarriers in water under the same conditions. Values represent the mean ± SD (n=3).

Furthermore, colloidal stability of blank NVCs was assessed under storage conditions (4°C), showing stable size and ζ potential up to 8 months (Fig. S1).

3.1.3. Preparation of NVCs loaded with fluorescent or immunomodulatory molecules

To assess the interaction of our nanocarriers with iDCs, NVCs were labelled with the fluorescent dye DiD. To achieve optimal conditions for *in vitro* detection of the loaded NVCs, two concentrations of DiD were used. In both cases, the encapsulation efficiency was high, and the physicochemical characteristics of the formulation were maintained (Table 3).

Table 3 – **Physicochemical properties of DiD-loaded NVCs.** Values represent the mean ± SD of at least 3 replicates.

Nanosystem	Theoretical DiD concentration	Particle Size (nm)	PDI	ζ-potential (mV)	EE (%)
DiD-loaded NVCs	25 µg/mL	222 ± 5	0.1	+54 ± 2	91
	75 µg/mL	211 ± 8	0.1	+42 ± 5	97

Furthermore, the leakage of DiD from NVCs during storage was less than 10% after 3 days (Fig. S2A). In addition, DiD release in cell culture conditions was also determined and resulted in less than 10% after 4 hours of incubation with R10 for both concentrations of the dye (Fig. S2B). Based on these results, DiD-loaded NVCs were considered suitable for *in vitro* evaluation of their interaction with iDCs.

To promote a tolerogenic phenotype in DCs, we decided to include IMM as an immunomodulatory molecule. Our hypothesis was that the inclusion of soluble IMM in a nanocarrier could control its delivery avoiding off-target toxicity. As it is shown in Table 4, both prototypes maintained their physicochemical properties when IMM was included.

Table 4 – **Physicochemical properties of nanocarriers loaded with IMM or/and PPI B_{10-18}.** Values represent the mean ± SD of at least 3 replicates.

Nanosystem	Particle Size (nm)	PDI	ζ-potential (mV)	PPI B_{10-18} EE (%)
IMM-loaded NV	146 ± 25	0.2	-54 ± 5	n/a
IMM-loaded NVCs	221 ± 4	0.2	+49 ± 1	n/a
(PPI B_{10-18} + IMM)-loaded NVCs	136 ± 12	0.1	+61 ± 7	34

Pursuing an antigen-specific treatment, we decided to include an epitope derived from an autoantigen in T1D within our NVCs. The goal of these therapies is to achieve specific tolerance for the autoantigens avoiding unspecific immunosuppression. We selected PPI B_{10-18} (HLVEALYLV) an epitope derived from pre-proinsulin that has been reported to be recognized by $CD8^+$ T lymphocytes in T1D patients [36,37]. Our collaborators have shown that PPI B_{10-18} has high binding affinity to HLA A2.1 molecule and high cytotoxic response in NOD-HHD mice (data not published).

The formulation containing the PPI B_{10-18} peptide exhibited an encapsulation efficiency of 34% and a final concentration of 1 mg/mL. As it is shown in Table 4, NVCs loaded with both, PPI B_{10-18} and IMM, had a decrease in size compared to blank NVCs. The reason of this difference could be dependent on the higher volume of solvents necessary to prepare the loaded formulation. In this sense, our group has previously described the influence of solvent volume in final size of nanocarriers [38].

3.2. *In vitro* evaluation of the interaction and effect of NVCs on iDCs

DCs play a central role in self-tolerance maintenance. Different DCs subtypes can promote lymphocyte tolerance by different mechanisms (chapter 1). Furthermore, in the autoimmunity scenario, DCs can modulate T cell response to avoid exacerbated immune responses. Tolerogenic phenotype can be induced in DCs by different factors, such as drugs and other

agents. Here we evaluate *in vitro* the potential of IMM-loaded NVCs to induce a tolerogenic phenotype in DCs that could be translated in a preventive effect *in vivo* for T1D.

3.2.1. Cytotoxicity

The cytotoxicity of blank nanosystems, NV and NVC, was tested in primary iDCs differentiated from adherent monocytes as described in section 2.7. These cells were incubated with increasing concentrations of the blank NV and NVCs for 24 hours and then metabolic activity was tested by MTS assay. As depicted in Fig. 5, the NV showed no toxicity at any of the concentrations tested, while NVCs showed dose-dependent toxicity with higher toxicity at the higher dose tested. As previously reported by our research group, positive charged particles normally show a higher toxicity than the neutral and negatively charged ones in different cell types [22]. Based on these results, 200 µg/mL was selected as the maximum concentration to be used in further studies.

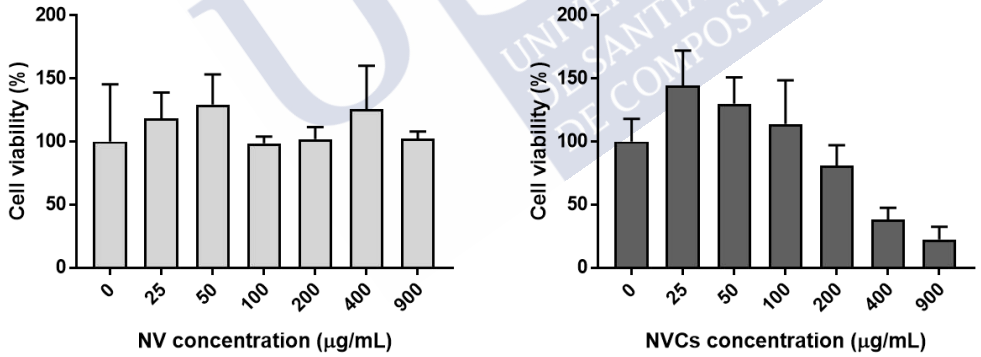

Fig. 5 – **Cell viability of blank nanocarriers upon incubation with iDCs**. Cells were incubated with either NV **(A)** of NVCs **(B)** at different concentration for 24 h. Results are shown as the percentage of cell viability. Values represent mean ± SD of at least 2 replicates.

3.2.2. NVCs interaction with iDCs

The capacity of NVCs to interact with hDCs was assessed by incubation of DiD-loaded NVCs with iDCs for 1 hour. The analysis by flow cytometry showed high interaction between NVCs

and iDCs, with around 92% of positive cells (Fig. 6A). Confocal images showed the internalization of DiD-loaded NVCs by iDCs, confirmed by the XZ plane (Fig. 6B-C). This result is consistent with previously described studies that have shown the internalization of particles with positive surface charge by DCs [19].

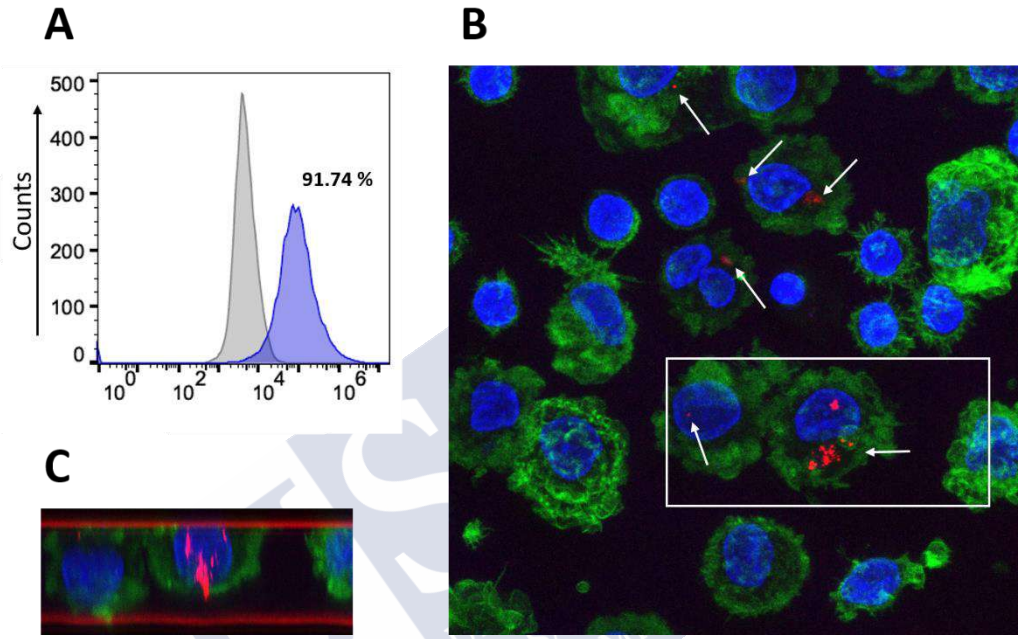

Fig. 6 – Interaction of DiD-loaded NVCs with iDCs. Representative flow cytometry histogram showing the interaction of DiD-loaded NVCs at 100 µg/mL after 1-hour incubation with iDCs is shown in **(A)**. Non-treated cells are represented in grey and percentage of positive cells is indicated. Representative CSLM image (XY plane, 63x) of iDCs incubated with DiD-loaded NVCs under the same conditions **(B)**. White arrows point out red dots corresponding to DiD-loaded NVCs. CSLM image (XZ plane, 63x) from the field enclosed in the white rectangle **(C)**. Blue channel: nuclei, green channel: actin, red channel: DiD-loaded NVCs

3.2.3. Effect on iDCs phenotype

For the evaluation of the effect of NV and NVCs on iDCs phenotype, different cell surface markers were analyzed by flow cytometry after the incubation with both blank and IMM-loaded prototypes. Controls of iDCs, mDCs and tolDCs were also evaluated. Cell viability was determined during the study, showing no cell toxicity at the concentration tested (Fig. S3C).

First, surface markers related with DCs differentiation were evaluated (Fig. S3A-B). The surface markers selected were CD11c, an integrin, and CD14, an LPS co-receptor, both highly expressed in DCs. In the case of CD11a, the levels were similar in all the conditions tested, indicating the maintenance of DCs phenotype. With regard to the expression of CD14, an increase was observed, although it was not statistically significant, upon treatment with IMM-loaded nanosystems.

Then, we evaluated the expression of several surface markers known to be upregulated in tolerogenic DCs. It has been described that DCs with a high expression of ILT3 and ILT4 are associated with a tolerogenic phenotype and T cell anergy promotion by inhibition of their proliferative capacity [39,40]. In addition, high TLR2 levels have also been linked with a tolerogenic profile in DCs [41]. The activation of its signaling pathway promotes IL-10 expression, while decreases IL-6, TNF-α and IL-12 [42]. Furthermore, it has been shown that TLR2 activation can induce regulatory T cell differentiation and protection in an experimental allergic encephalomyelitis mice model [43]. Besides, the expression of C-type lectin receptors, such as CD209, with high affinity to high-mannose type N-glycans is also found in DCs with a tolerogenic phenotype [44]. Our results showed a slight, not statistically significant, increase in ILT3 and TLR2 expression in DCs incubated with IMM-loaded NVCs similar to the one observed in tolDCs (Fig 7A-B). In the case of CD209, similar expression levels were found for all the conditions tested (Fig. 7C).

Finally, the expression of surface markers related with a pro-inflammatory phenotype of DCs was evaluated. In this context, it has been described that the upregulation of co-stimulation receptors, such as CD80 or CD83, is necessary to activate T lymphocytes [45,46]. Our results showed a significant decrease in the expression of CD83 upon exposure to IMM-loaded nanosystems, reaching levels similar to those observed in tolDCs (Fig. 7E). However, in the case of CD80, no changes in the expression levels were found for all the conditions tested (Fig. 7D).

Considering the surface markers expression, these results highlight a tendency in promoting a tolerogenic phenotype in DCs after the incubation with IMM-loaded NVCs. Furthermore, the results indicate that the *in vitro* activity of IMM was maintained once incorporated into the nanosystems.

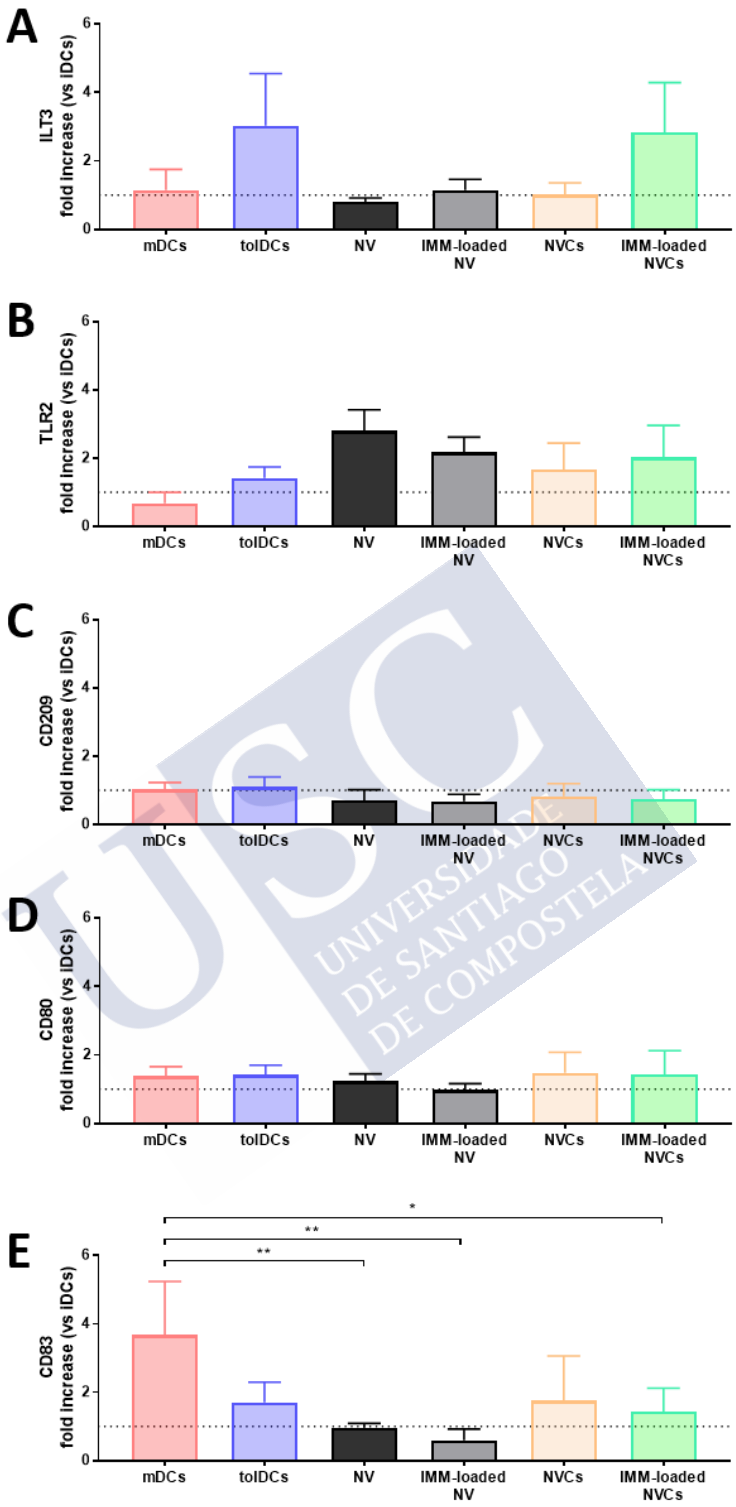

Fig. 7 – Expression of surface markers ILT3 (A), TLR2 (B), CD209 (C), CD80 (D) and CD83 (E) in iDCs after incubation with the different nanosystems. Surface markers expression was determined by flow cytometry after incubation of iDCs with the nanosystems for 2 h. Data is shown as expression fold increase compared to untreated iDCs. Dotted line represents iDCs expression levels. Values represent mean ± SD of at least 4 replicates.

For further characterization of the effect of NVCs on iDCs phenotype, gene expression and IDO activity were determined. The activity of this enzyme, implicated in the tryptophan catabolism, has been linked to tolerance promotion by DCs [47]. In the specific case of NOD mice, the development of autoreactivity has been linked to a defect in tryptophan catabolism related to impaired IDO activity [48]. As depicted in Fig. 8, incubation of iDCs with IMM-loaded NVCs significantly increases IDO activity compared to untreated iDCs, mDCs and the incubation with blank NVCs.

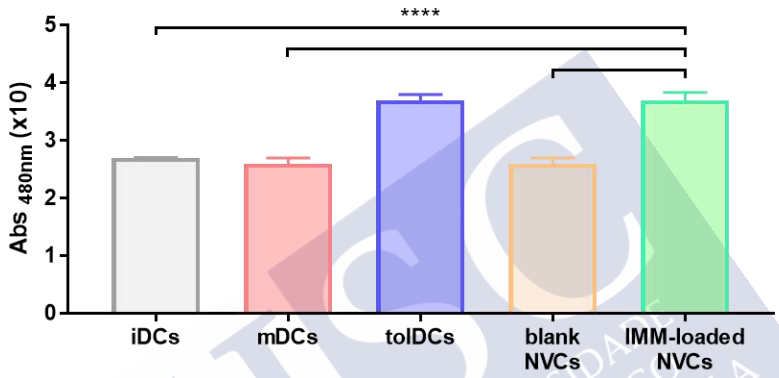

Fig. 8 – **IDO activity of iDCs incubated with NVCs.** Determination of kineurin in the culture medium was done after incubation of iDCs with blank and IMM-loaded NVCs for 24 h. Data is shown as the absorbance at 490 nm. Values represent mean ± SD (n=4). Statistical comparison was done between IMM-loaded NVCs against the other groups.

The expression levels of different genes in iDCs incubated with NVCs was evaluated by qPCR. We decided to evaluate the expression of some genes with increased expression in tolerogenic DCs, such as PFKFB4, LDHA and C-MYC. After 24-hour incubation, iDCs treated with IMM-loaded NVCs showed higher expression levels of both PFKFB4 and C-MYC compared to mDCs and iDCs incubated with blank NVCs, but without reaching statistical significance (Fig. 9B-C). On the contrary, this tendency was not shown for LDHA expression (Fig. 9A).

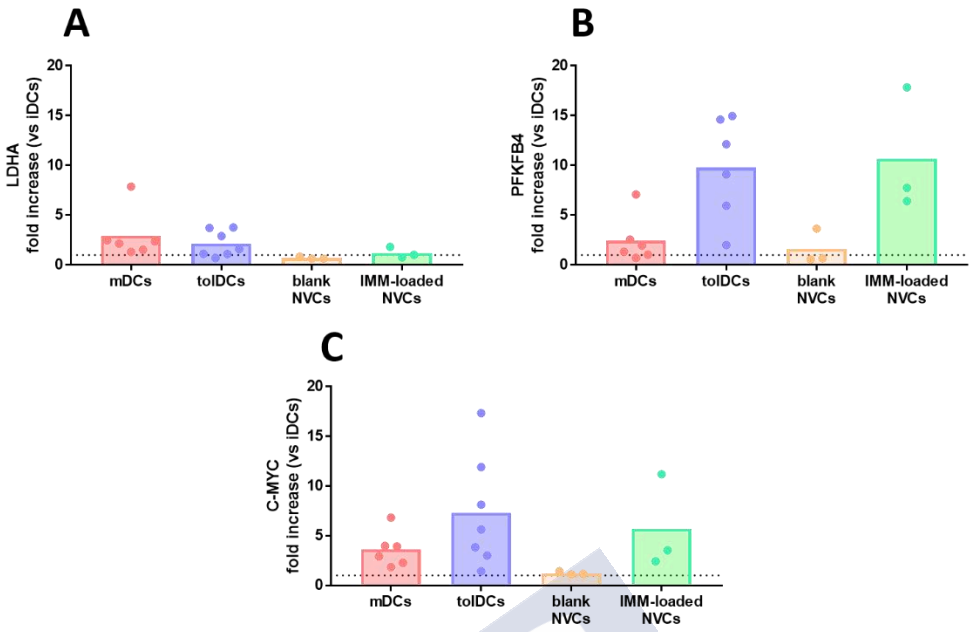

Fig. 9 – Expression of LDHA (A), PFKFB4 (B) and C-MYC (C) in iDCs incubated with NVCs. Data is shown as expression fold increase compared to untreated iDCs. Dotted line represents iDCs expression levels. Values represent mean ± SD of at least 3 replicates. No significant differences in gene expression were found between iDCs incubated with IMM-loaded NVCs compared to the rest of the groups.

Overall, from these *in vitro* studies we could conclude that IMM-loaded NVCs were able to promote a tolerogenic phenotype on iDCs. This effect is similar to that observed for soluble IMM, used to differentiate iDCs to tolDCs, suggesting that the loading of IMM in NVCs does not affect its immunological properties.

3.2.4. Stimulation of T lymphocytes

The capacity of iDCs pre-incubated with both blank and IMM-loaded NVCs to activate allogeneic T lymphocytes was evaluated by flow cytometry. In the case of $CD4^+$ T lymphocytes, CD25 was used to discriminate activated lymphocytes since its upregulation is related with activation of these cells [49]. Simultaneously, CD28 was used for the discrimination of active $CD8^+$ T lymphocytes as its expression is necessary for the generation of cytotoxic T lymphocytes [50].

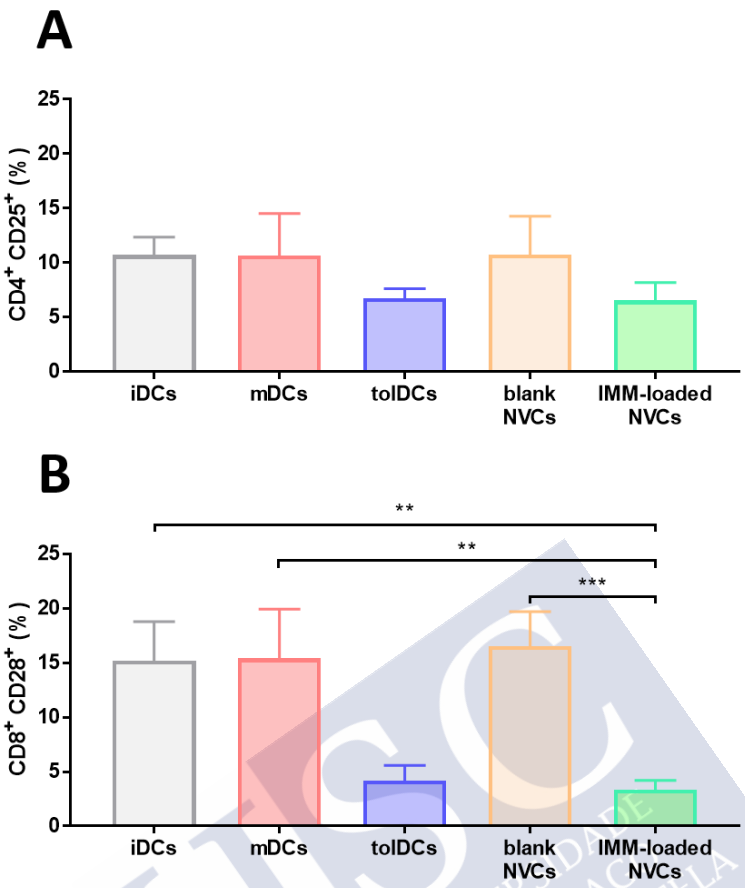

Fig. 10 – T lymphocyte activation capacity of pre-incubated iDCs in allogeneic culture. The percentage of activated $CD4^+$ $CD25^+$ T lymphocytes **(A)** and $CD8^+$ $CD28^+$ T lymphocytes **(B)** was determined by flow cytometry after incubation of pre-treated iDCs with allogeneic PBLs. Values represent mean ± SD of at least 3 replicates. Statistical comparison was done between IMM-loaded NVCs against the other groups.

Preincubation of iDCs with IMM-loaded NVCs slightly decreased the percentage of activated $CD4^+$ T lymphocytes compared to untreated iDCs, mDCs and iDCs pre-treated with blank NVCs, although without statistical significance (Fig. 10A). This reduction in lymphocyte activation by iDCs pre-treated with IMM-loaded NVCs was greatly accentuated in $CD8^+$ T lymphocytes, showing a significant decrease compared to iDCs, mDCs and blank NVCs (Fig. 10B). In both cases, this behavior was similar to the one promoted by soluble IMM, epitomized by tolDCs.

3.2.5. Cytokine secretion

Cytokine secretion of iDCs after incubation with both, blank and IMM-loaded NVCs is shown in Fig. 11. Although levels of IL-1β, IL-2, IL-4, IL-5, IL-15, IL-23, IL-27 and TNF-β were below the detection limit; IL-8, IL-10, IL12p70, TNF-α and IFN-γ levels were detectable. As we can observe in Fig. 11, maturation of DCs promotes a general increase of cytokine secretion. Notably, the expression of pro-inflammatory cytokines such as IL-12p70, IL-8, TNF-α and IFN-γ was significantly increased greatly (Fig. 11A-C-D-E). Surprisingly, the expression of IL-10, a known anti-inflammatory cytokine, was also increased (Fig. 11B). In the case of tolDCs, the results showed a generalized decrease in cytokine secretion. This generalized decrease in cytokine secretion was also observed upon incubation of DCs with either blank or IMM-loaded NVCs. Regarding cytokine expression, the results showed that NVCs do not promote a pro-inflammatory state in DCs. Furthermore, cytokine expression levels of iDCs incubated with blank or IMM-loaded NVCs are similar to tolDCs, thus indicating that iDCs exposed to NVCs maintained an immature tolerogenic state.

Cytokine secretion was also measured in co-cultures of pre-incubated iDCs and allogeneic PBLs. In this case, just IL-10, IL15, TNF-α and IFN-γ levels were detectable. As shown in Fig. 12A, IFN-γ secretion decreases significantly when allogeneic PBLs were cocultured with iDCs pre-incubated with IMM-loaded NVCs or blank NVCs. Similar results were observed in the case of TNF-α secretion (Fig. 12B). These cytokines are characteristic of Th1 lymphocytic responses, known to be implicated in T1D pathogenesis [51]. Indeed, it has been shown that IFN-γ secreted by pancreatic cells is a trigger for the induction of diabetes and its neutralization using antibodies protects from diabetes development [52,53]. On the other hand, the secretion levels of anti-inflammatory cytokines, such as IL-10 and IL-15 were not increased when allogeneic PBLs were cocultured with iDCs pre-incubated with IMM-loaded NVCs (Fig. 12C-D).

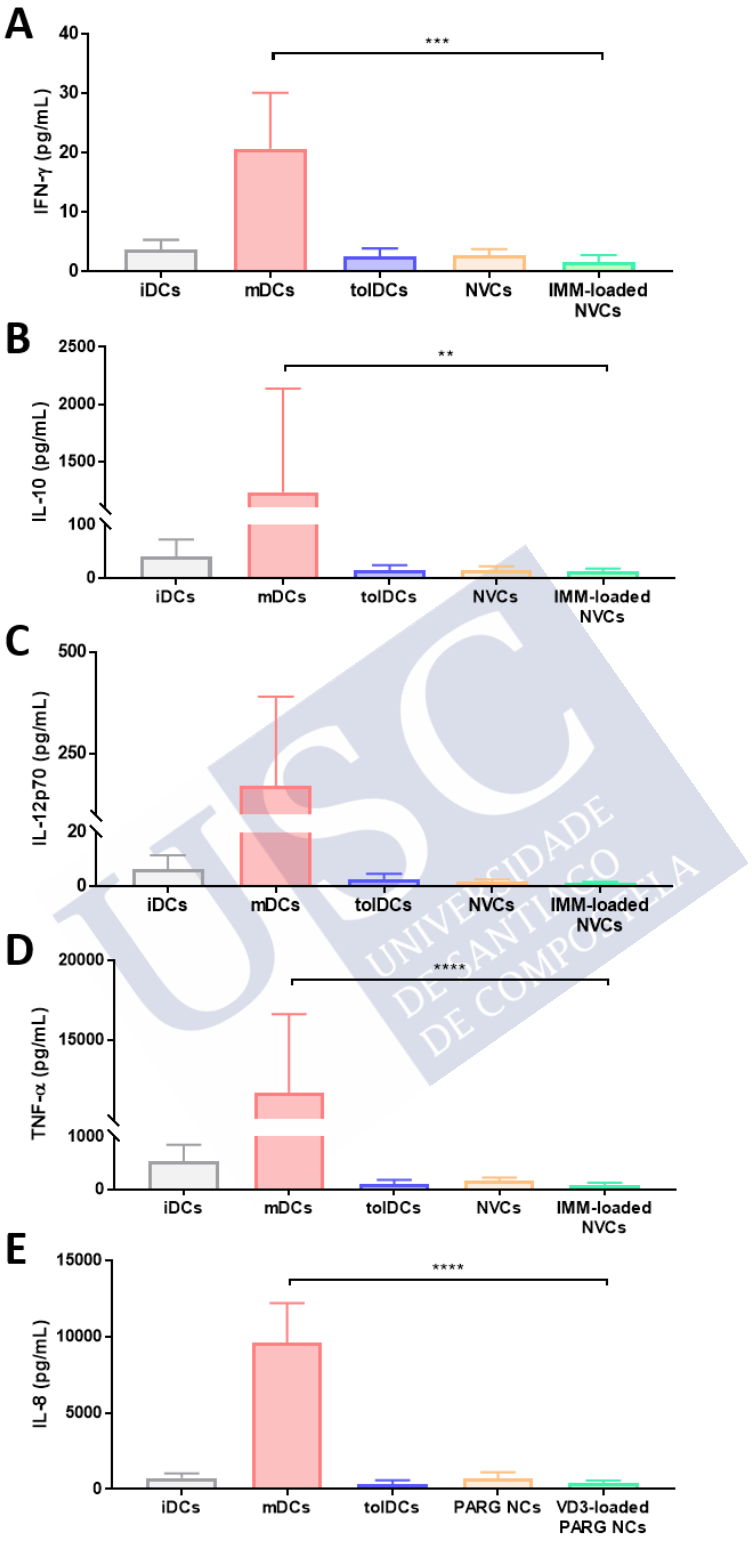

Fig. 11 – **Cytokine expression by iDCs incubated with NVCs.** Determination of cytokines in the culture medium was done after incubation of iDCs with blank and IMM-loaded NVCs for 24h. Values represent mean ± SD (n=4). Statistical comparison was done between IMM-loaded NVCs against the other groups.

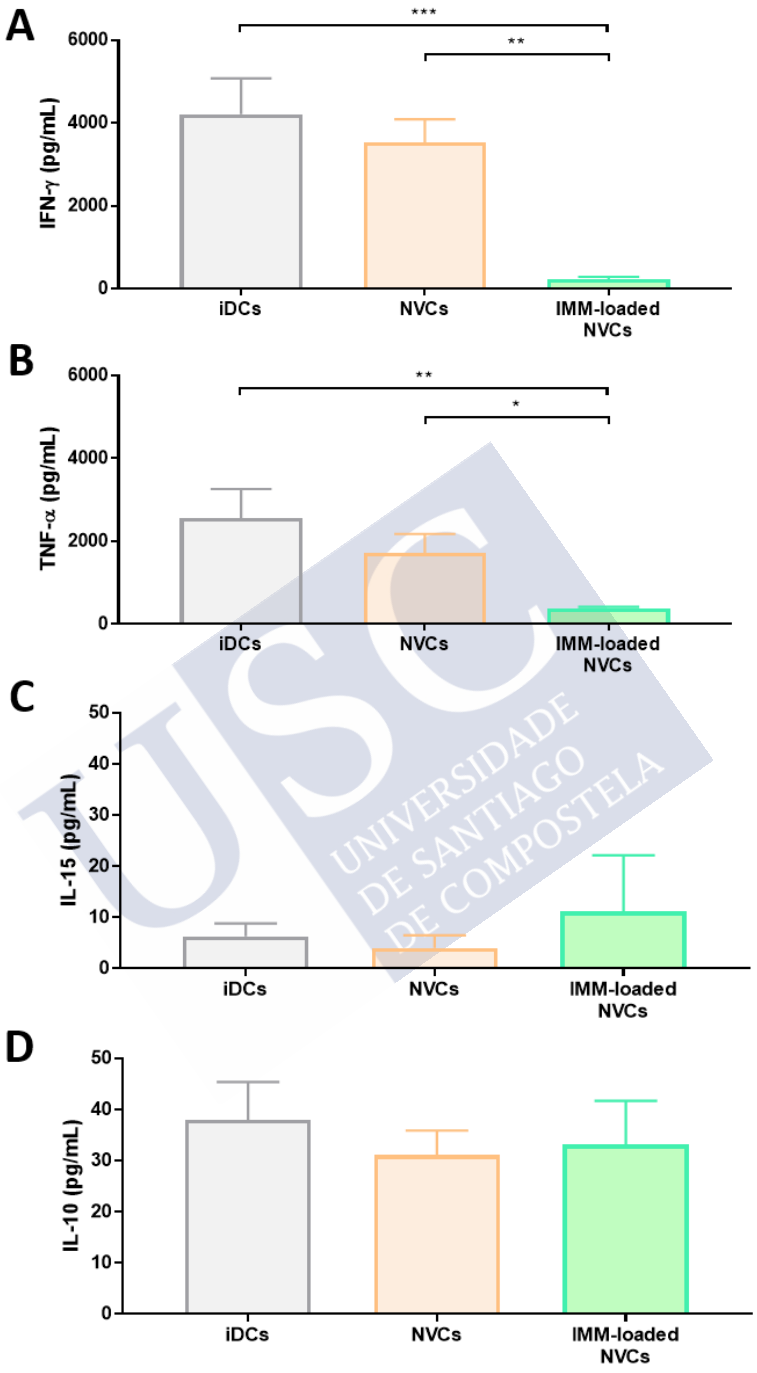

Fig. 12 – Cytokine expression by pre-incubated iDCs co-cultured with allogeneic PBLs. Determination of cytokines in the culture medium was done after incubation of pre-incubated iDCs with allogeneic PBLs for 7 days. Values represent mean ± SD (n=4). Statistical comparison was done between IMM-loaded NVCs against the other groups.

3.3. *In vivo* evaluation of diabetes prevention in NOD-HHD mice

The potential of NVCs loaded with IMM and the PPI $B_{10\text{-}18}$ antigen in terms of preventing autoimmune diseases was evaluated *in vivo*, using the NOD-HHD mice model. This animal model mimics several features observed in human T1D patients. In general, NOD mice have early infiltration of immune cells in pancreatic islets, such as DCs, macrophages or neutrophils [54–56]. This infiltration has been shown to be key for the progression of the disease [57]. The phenotype of immune cells has also been related to disease onset in this animal model, showing a reduction in tolerogenic DCs in pancreatic lymph nodes [58]. Furthermore, antigenic specificity of T lymphocytes in NOD mice is similar to humans [59,60]. In this sense, preproinsulin has been reported as an antigen with multiple epitopes that can be recognized by lymphocytes [61–63]. In addition to NOD mice characteristics and genetic background, NOD-HHD mice are transgenic for HLA-A*02:02 (allele coding for the HLA-A human protein of the major histocompatibility complex class I). This molecule is capable of selecting autoreactive T $CD8^+$ lymphocytes accelerating significantly disease development compared to NOD/Lt no-transgenic strain [64,65].

Our hypothesis in this work has been that the encapsulation of IMM into NVCs would enable its targeted delivery to the immune cells and reduce its systemic exposure. On the other hand, the use of high doses of soluble antigens have shown tolerance induction by inhibiting T lymphocytes proliferation or promoting their selective depletion [66,67]. Therefore, our additional hypothesis has been that the co-encapsulation of IMM and PPI $B_{10\text{-}18}$ antigen would be a good strategy for the prevention of T1D. Furthermore, the use of an autoantigen in T1D could help to generate selective tolerance, maintaining the capacity of DCs to elicit immune responses against other antigens.

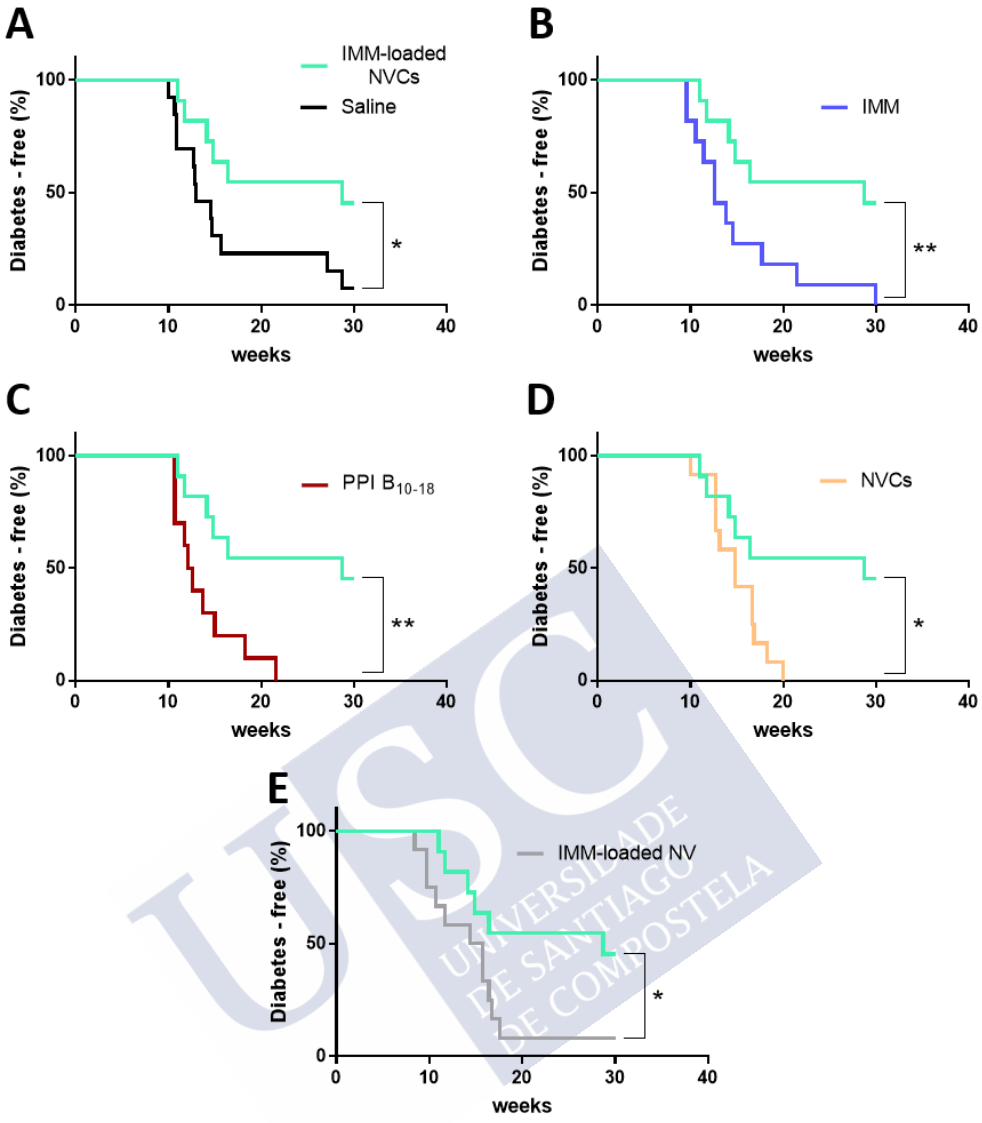

Fig. 13 – **Preventive effect of IMM-loaded NVCs in diabetes onset.** Diabetes incidence in female NOD-HHD mice treated intraperitoneally with IMM-loaded NVCs compared with several groups: saline **(A)**, soluble IMM **(B)**, soluble PPI B_{10-18} **(C)**, blank NVCs **(D)** and IMM-loaded NV **(E)** (n of at least 10 animals per group).

Female NOD-HHD mice were treated intraperitoneally starting during the prediabetic period (4 weeks old) and following the administration schedule described in Fig. 1. The doses of IMM and PPI B_{10-18} per administration were 5 µg/kg and 4 mg/kg respectively. As expected, saline-control group developed diabetes from the age of 10 weeks with a final incidence of 92.3% (Fig. 13A). Similar incidence was shown by groups treated with soluble IMM or PPI B_{10-18} (Fig. 13B-C). No significant differences were found between all control groups. In contrast, IMM-

loaded NVCs showed a T1D incidence significantly lower than all control groups (Fig. 13 A-B-C). Therefore, a straight-forward conclusion was that under the experimental protocol described, only encapsulated IMM was efficient in terms of preventing T1D. It is important to note that although *in vitro* results indicated a similar behavior between soluble and encapsulated IMM, in respect to the tolerance induction mechanism, the differences attributed to the encapsulation of IMM in NVCs are clearly shown *in vivo* in the prevention of T1D onset. On the other hand, the results presented in Fig. 13E, indicate that a control IMM-loaded NV was not efficient at preventing T1D. Therefore, these results indicate not only that the preventive effect of IMM-loaded NVCs comes from IMM immunomodulatory properties but also that the cationic surface charge of the NVCs is necessary to promote this effect. This could be related to the preferential uptake of cationic nanocarriers showed by DCs [30–33]. However, specific *in vivo* biodistribution studies would be necessary to understand the mechanistic behavior of NVCs.

Contrary to what was expected, the antigenic peptide PPI $B_{10\text{-}18}$ did not enhance the protective response. In fact, no significative differences were found when comparing the response to IMM-loaded NVCs containing antigen or not (Fig. 14). This indicates that the main effect in diabetes prevention observed for NVCs comes from the IMM immunomodulatory activity and not from its synergy with this specific autoantigen.

Although the use of high doses of soluble antigens have shown tolerance induction [66,67], it has also been described that the dose and the administration route, may influence the final response [68–71]. Therefore, adjusting the administration schedule and dose of the selected autoantigen or including different ones might be a way to improve the performance of (PPI $B_{10\text{-}18}$ + IMM) loaded NVCs.

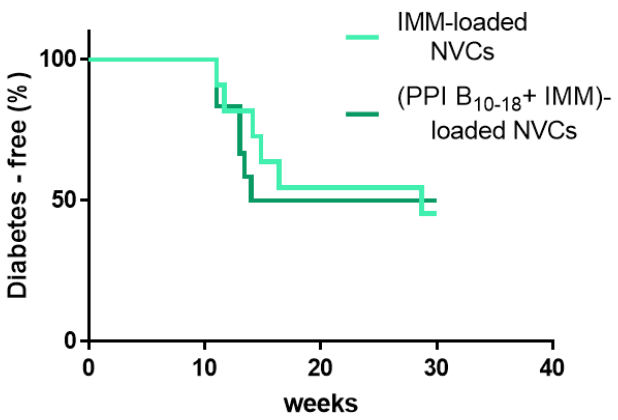

Fig. 14 – Comparison of preventive effect of (PPI B_{10-18}+ IMM)-loaded NVCs and IMM-loaded NVCs in diabetes onset. Diabetes incidence in female NOD-HHD mice treated intraperitoneally with (PPI B_{10-18} + IMM) loaded NVCs compared with IMM-loaded NVCs group (n of at least 10 animals per group).

Nevertheless, the inclusion of antigens in nanotechnology-based formulations for T1D prevention has shown controversial results (Table 5). In agreement with our results, some studies have reported that the use of immunomodulatory molecules to promote tolerogenic responses could lead to substantial tolerogenic responses irrespective of the presence of an antigen [72]. However, in other studies the use of the antigen alone was sufficient to achieve a preventive effect. For example, Pujol-Autonell *et al.* showed that phosphatidylserine-based liposomes loaded with both insulin chains were efficient at delaying the diabetes onset in NOD mice after single intraperitoneal administration at the age of 8 weeks [73]. This effect was attributed to the specific internalization route promoted by phosphatidylserine [74]. In a different study, Prasad *et al.* used antigen-coupled poly(lactic-co-glycolic acid) nanoparticles (NPs) in a NOD mice model with T cells that specifically recognize the antigen used. They showed that after intravenous administration, these NPs maintained normal blood glucose levels up to 50 days. They demonstrated that this effect was mediated by the retention of antigen-specific T cells in the spleen and the expansion of specific T regulatory lymphocytes, causing the reduction of T cell infiltration in the pancreas [75].

Table 5 – Nanotechnology-based formulation including antigens for the prevention of T1D

Nanocarrier	Antigen	Dose	Admin.	Animal model	Key results	Ref
PS liposomes	InsA and InsB	1 mg/kg InsA 2.1 mg/kg InsB	IP single dose	NOD mice	Empty liposomes did not have an impact on disease while loaded ones prevent disease onset in 50% of the animals	[73]
ITE loaded gold NPs	Proinsulin	unknown	IM weekly	CAD mice	Ag-loaded NPs did not have an impact on disease while NPs loaded with both compounds showed disease prevention	[72]
PLGA NPs	BDC2.5 mimetope 1040-31 (covalently attached)	unknown	IV single dose	NOD.SCID mice transferred with $CD4^+$ BDC2.5 Tg T cells specific for the antigen	Maintenance of normal blood glucose levels up to 50 days	[75]

Admin, administration route; PS, phosphatidylserine; InsA, insulin A chain; InsB, insulin B chain; IP, intraperitoneal; NOD, non obese diabetic; ITE, 2-(1'*H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester; IM, intramuscular; Ag, antigen; CAD, cyclophosphamide-accelerated model of diabetes; PLGA, poly(lactic-co-glycolic acid); NPs, nanoparticles; IV, intravenous; NOD.SCID, immunodeficient NOD mice

Considering this information, it seems that the molecular mechanisms behind the tolerance promotion of nanocarriers, either the impact of immunomodulatory molecules or the internalization through scavenger receptors used in apoptosis, play a key role in the effect mediated by the antigen. Furthermore, the antigenic specificity of the animal model used also increases the chances to observe an effect mediated by the antigen. Moreover, it has been described that the use of an antigen could provide the specificity needed to promote a tolerogenic response specific for the selected antigen while maintaining the recognition and response against other antigens [76]. In this sense, further studies for evaluation the specificity of the tolerogenic response generated by NVCs would be necessary to understand the effect of the selected antigen.

To sum up, the simplicity of IMM-loaded NVCs would be a remarkable competitive advantage for its translation to the clinic. In order to understand the mechanism of action of NVCs, a biodistribution study together with the assessment of DCs and lymphocyte phenotype and the evaluation of the specificity of the generated response would need to be performed.

4. Conclusions

Here we report the development of nanoformulations intended for the prevention of T1D. NVCs were selected as carriers for IMM, based on its immunomodulatory properties, and an autogenic peptide pursuing an antigen-specific effect. *In vitro*, IMM-loaded NVCs exhibited the capacity to promote a tolerogenic phenotype in hDCs in terms of cytokine expression, surface markers expression and IDO activity. Furthermore, pre-incubated hDCs showed a reduced capacity for lymphocyte activation in allogeneic culture. Most importantly, the prevention of diabetes was observed in NOD-HHD mice treated with IMM-loaded NVCs intraperitoneally. Similar results were shown when both molecules were loaded, suggesting a leading role of IMM in this effect. Overall, these results suggest that IMM-loaded NVCs might be a simple alternative for prevention of T1D. Further *in vivo* studies would be necessary to assess the mechanism of action.

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Supplementary information

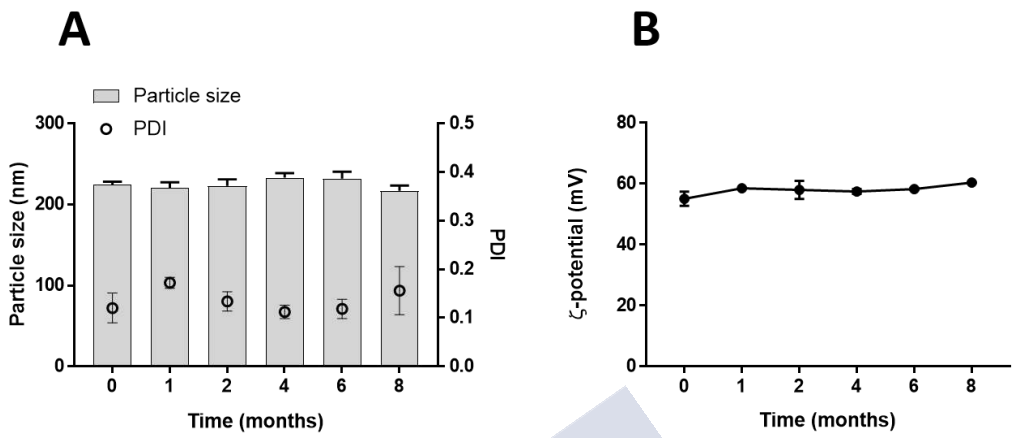

Fig. S1 – Colloidal stability of blank NVCs under storage conditions (4°C). Values represent the mean ± SD (n=3).

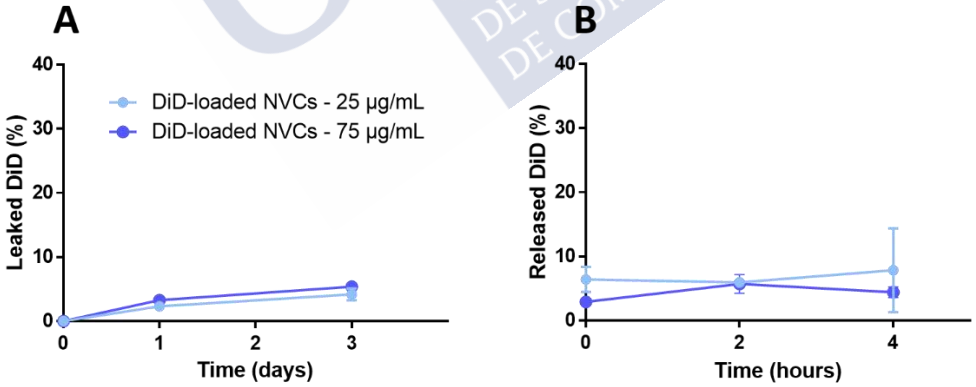

Fig. S2 – Leakage of DiD under storage conditions (A) and release of DiD in culture medium (B) of DiD-loaded NVCs. Values represent the mean ± SD (n=3).

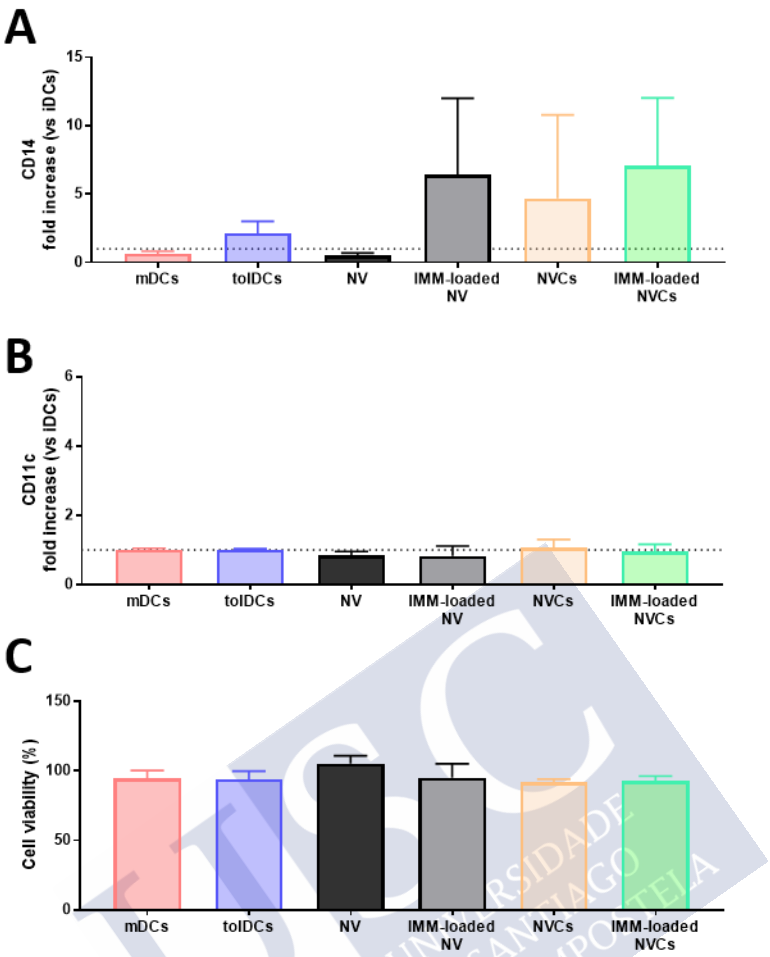

Fig. S3 – Expression of surface markers CD14 (A), CD11c (B) and cell viability (C) of iDCs incubated with both nanosystems. Dotted line represents iDCs expression levels. Values represent the mean ± SD (n=4).

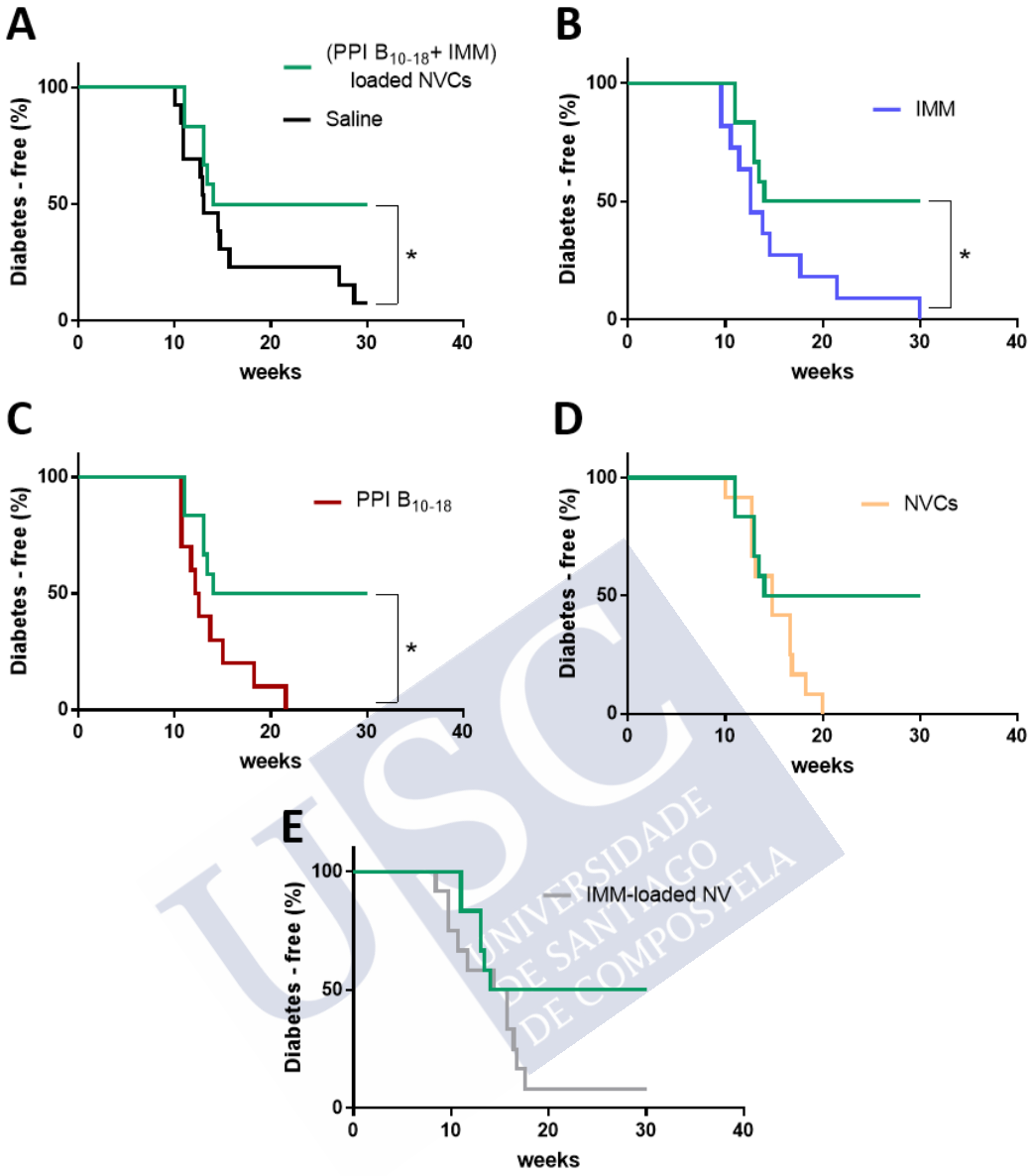

Fig. S4 – **Preventive effect of (PPI B_{10-18}+ IMM)-loaded NVCs in diabetes onset.** Diabetes incidence in female NOD-HHD mice treated intraperitoneally with (PPI B_{10-18} + IMM) loaded NVCs compared with several groups: saline **(A)**, soluble IMM **(B)**, soluble PPI B_{10-18} **(C)**, blank NVCs **(D)** and IMM-loaded NV **(E)** (n of at least 10 animals per group).

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Chapter 3

Small polymeric nanocarriers as a dermal delivery system for supplementation

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Chapter 3

Small polymeric nanocarriers as a dermal delivery system for supplementation

Abstract

Transdermal or dermal delivery of natural compounds would be an interesting alternative for patients with intestinal fat malabsorption or for the treatment of topical diseases, respectively. Unfortunately, this modality of administration has not been implemented so far due to the fact that the high lipophilicity of some of these compounds limits its percutaneous penetration. Our hypothesis is that the use of a nanotechnology-based formulations could facilitate the penetration of the immunomodulatory molecule used in Chapter 2 (IMM) across the epidermis. Hence, the objective of this work was the development of polymeric nanocarriers (NVs) for the dermal delivery of IMM. Two prototypes of NVs, with positive (NVCs) or negative surface charge (NVAs) were developed and characterized. The NVs exhibited a size of around 100 nm, a narrow size distribution, an anionic (NVA) or cationic surface charge (NVC) and a high capacity to encapsulate IMM. The results found in an *ex vivo* assay using human skin indicated that NVAs enhanced the penetration of IMM across the epidermis in a higher extent than NVCs. This enhanced penetration was found to be mediated by the access of NVAs to hair follicles. Overall, these promising data show the potential of IMM-loaded NVAs for the administration of natural compounds by the dermal route.

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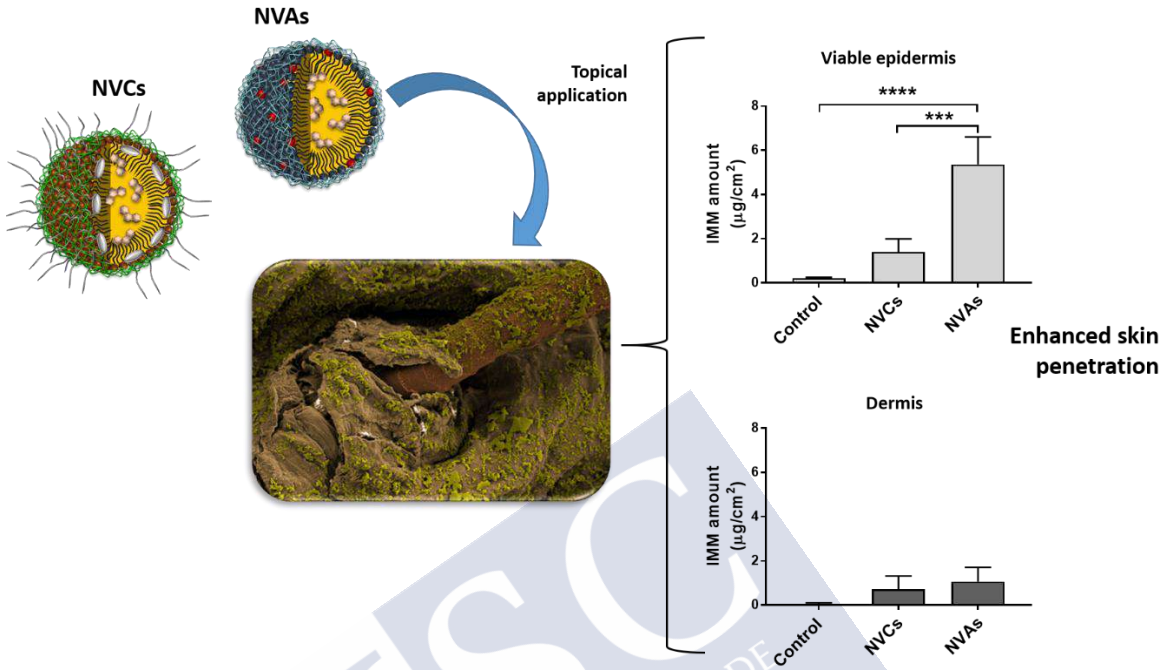
Graphical abstract



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1. Introduction

As we discussed previously in Chapter 2, the molecule selected has immunomodulatory activity that has been associated with the prevention of several pathologies, such as cancer or autoimmune diseases. Currently a number of derivatives of the immunomodulatory molecules are administered orally. However, this modality of administration is known to lead to important intestinal absorption fluctuations between individuals and in different disease scenarios.

The delivery of the selected immunomodulatory molecule (IMM) across the skin could be an alternative route for its administration. Furthermore, epidermal delivery of IMM would be useful for the treatment skin pathologies where the immune systems has an important role, such as psoriasis or cancer.

Overcoming biological barriers for drug transport is not an easy task. In the case of the skin, the brick-and-mortar structure of *stratum corneum* (StC) and its complex lipid matrix represent the principal resistance to drug transport [1]. Penetration across this layer depends on the physicochemical properties of the drug, limited by molecular weight (up to 500 Da), lipophilicity (logP around 1 – 3), melting point and pharmacological potency. In this sense, there is an unmet need for an efficient technique to enhance penetration through the viable skin layers of highly lipophilic drugs such as IMM.

The dermal administration of drugs using different approaches to overcome the skin barrier function, such as permeation enhancers, electroporation or ultrasound among others, have been extendedly studied [2–5]. As an alternative to these methods, nanotechnology offers the potential for enhancing skin penetration without disturbing its natural barrier properties. Nanocarriers could facilitate drug delivery by its interaction with skin appendages. This interaction is highly dependent on nanocarriers' physicochemical properties such as size, deformability and surface charge [6]. Furthermore, nanocarriers allow controlled release and loading of multiple cargos [7,8].

Based on this background information, the main objective of this work was to develop small polymeric nanocarriers (NVs) for enhancing the transport of IMM across the skin. These NVs present features that make them suitable for the delivery of highly lipophilic drugs across the

skin. Their structure allows the encapsulation of lipophilic compounds. Moreover, their tunable composition facilitates the modulation of physicochemical properties critical for transdermal penetration, such as size or surface charge [9]. For the definition of their composition, we have taken advantage of all the knowledge generated by our group in the development of polymeric nanocarriers as drug delivery systems for different applications [10–13]. Two prototypes were developed and optimized in terms of size and colloidal stability. Both polymeric NVs were studied *ex vivo* in human skin to assess their potential for the delivery of IMM across the skin.

2. Materials and methods

2.1. Materials

Optimal Cutting Temperature (OCT) medium was received from VWR International (France). Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Gibco (Invitrogen Corporation, Life Technologies, UK). Phosphate buffer saline (PBS) tablets was provided by Sigma Aldrich (St Louis, MO, US). N-(3-dimetrylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Merck Millipore (Billerica, MA, US). Cyanine5-amine (Cy5) was obtained from Lumiprobe (Hunt Valley, MD, US). Organic solvents used were of HPLC grade and all other products used were of high purity or reagent grade.

2.2. Preparation of small size nanocarriers

2.2.1. Cationic nanocarriers (NVCs)

NVCs from chapter 2 were optimized for dermal delivery. In order to decrease NVCs size, we included few modifications to the formulation method previously reported by our group [9]. First, we injected the solvent with a syringe. Second, we increased the volume of solvent. Third, we increased the volume of water.

2.2.2. Anionic nanocarriers (NVAs)

NVAs were prepared adopting the size-reduction strategy described for NVCs.

2.3. Physicochemical characterization

Particle size and polydispersity index (PDI) of NVs dispersions were measured by dynamic light scattering (DLS) using a Zetasizer® (Nano ZS, ZEN 3600, Malvern Instruments, Worcestershire, UK) at 25°C after 10 times dilution in ultrapure water. Zeta potentials were measured by Lased-Doppler Anemometry (LDA) with the same instrument and experimental conditions.

Additional particle size characterization was performed by Nanoparticle Tracking Analysis (NTA). The measurements were conducted after particle dilution in ultrapure water (1:1000 NVCs and 1:2000 NVAs) using NanoSight NS3000 equipment (Malvern Instruments, Worcestershire, UK). All the measurements were performed in triplicate, with 5 videos of each sample captured over 60s at 25°C.

NVs morphology was examined by field emission scanning electron microscopy (FESEM) (ZEISS, ULTRA Plus, Germany). For this study, the nanocarriers were diluted in water 1:10000 and mixed 1:1 (v:v) with 2% (w/v) phosphotungstic acid solution. Then, 1 µL of this mixture was placed on cooper grids with carbon films. These grids were left to dry in the open air, then washed dropwise with 1 mL of water and left to dry again overnight. Finally, they were observed in the microscope using both scanning transmission electron microscopy (STEM) and InLens detectors.

2.4. UPLC method for IMM quantification

The amount of IMM encapsulated in NVs dispersions was determined by ultra-performance liquid chromatography (Acquity UPLC, Waters, Spain). Isocratic separation was performed with a C18 column as stationary phase (Kinetex® C18 100 Å, 1.7 µm 2.1 × 50 mm, Phenomenex, US) at 30°C. Briefly, the method used a mixture of ACN:MeOH (66:34, v/v) as mobile phase, pumped at a flow rate of 0.3 mL/min. Injection volume of samples was 2 µL and absorbance was measured at 265 nm.

For the analysis of skin distribution samples, the above method was optimized. In this case, it was used a mixture of acetonitrile:methanol:water (66:24:10, v/v) as mobile phase and injection volume of samples was 7 µL. This UPLC-UV method was validated according to ICH guidelines [14]. Lower limits of detection (LOD) and quantification (LOQ) were 7 and 15 ng/mL, respectively.

2.5. Encapsulation efficiency (EE %) of IMM-loaded NVs

The NVs formulations (0.5 mL) were isolated by ultrafiltration using Amicon® ultra centrifugal filter devices, 100 kDa pore size (Merck Millipore, Germany) at 14000 × g 5 minutes at 15°C a Hettich Universal 32R centrifuge (Tuttlingen, Germany). Non-encapsulated IMM was considered to cross the filter and could be quantified in the filtrate. The volume of the NVs dispersion that remained onto the filter was recovered by centrifugation at 1000 × g 2 minutes at 15°C.

The amount of IMM was directly determined from non-filtered NVs dispersion, filtrate and filtrated NVs dispersion. For digestion, each fraction was diluted 50 times with a combination of methanol and acetonitrile using a 2:8 proportion and then vortexed. Then, each fraction was analyzed by the UPLC method above-described.

The EE (%) of IMM was calculated according the following equation:

$$EE\ (\%) = \frac{IMM\ in\ the\ disrupted\ NVs}{total\ IMM}\ X\ 100$$

Where *IMM in the disrupted NVs* is the IMM concentration determined by UPLC after treating the filtrated NVs dispersion for its disruption, and *total IMM* is the theoretical total IMM amount in the formulation. Analysis was done in triplicate.

2.6. Cy5-labelled NVAs

In order to investigate the permeation of the NVAs across the skin, fluorescent NVAs were prepared with Cy5-labelled anionic polymer. Briefly, anionic polymer (1 mol) was dissolved in ultrapure water at a concentration of 5 mg/ml and 1.5 mol of EDC and 0.25 mol of NHS were

added to this solution and stirred during 1 hour. Then, 0.25 mol of Cy5-amine were added to the solution and maintained under magnetic stirring for 48 hours at RT. The final product was purified by dialysis using SnakeSkin (3.5K MWCO, Thermo Fisher Scientific, Spain), first against 25 mM NaCl and, then, against ultrapure water (3 cycles each), freeze-dried and stored at -20°C.

2.7. Skin penetration study of IMM and fluorescent NVs

2.7.1. Skin preparation

Human skin samples were collected immediately after surgery from the Department of Plastic, Aesthetic & Reconstructive Surgery (Complexo Hospitalario Universitario de Santiago, Universidade de Santiago de Compostela). Skin was provided already microtomed at a thickness of about 500 µm and was kept stored at 16°C no longer than 7 days. All experimental procedures were in accordance with the national guidelines (RD 1716/2011 and Law 14/2007) and were approved by the "Comité de Ética de la Investigación" from Xunta de Galicia (project number 2017/103).

2.7.2. Infinite or finite dose skin penetration study

Intact human skin samples were mounted in standard Franz diffusion cells with the StC facing upward. Donor compartment was filled with an infinite dosing (≈ 200 µL/cm^2) or a finite dose (≈ 10 µL/cm^2) of IMM-loaded NVs dispersion or IMM solution in 0.25% of oil. The receptor compartment was filled with 4 mL of 0.1% Tween 80 in PBS, thermostated at 37°C and stirred. At the end of the experiment (24 hours), samples were taken from the receptor compartment, diffusion cells were dismantled, and the residual formulation was removed from the surface. Skin samples were, then, frozen at -80°C and horizontally sectioned in 3 different fractions: 0 – 25 µm considered to be the StC, 25 – 150 µm considered to be the viable epidermis and > 150 µm considered to be the dermis. Quantitative evaluation of the data consisted in the evaluation of the amounts of drug in each fraction. For IMM extraction, each skin layer was vortexed together with 1 mL of water:acetonitrile (2:8, v/v). Then, samples were centrifuged at 10,000 rpm for 15 with a Hettich Universal 32R centrifuge (Tuttlingen, Germany) and the

supernatant was analyzed by UPLC-UV. Drug extraction procedure was validated by extracting IMM from skin samples previously spiked with the drug (99% efficiency, n=3).

2.7.3. Electron microscopy imaging of NVAs interaction with the skin

Intact human skin sample was dehydrated using increasing dilutions of ethanol until reaching 100%. Then, epidermis was put in contact with an infinite dose of IMM-loaded NVAs (≈ 200 µL/cm^2) and let it evaporate at RT during 24 hours. Skin sample was placed in silicon wafers and, then, examined by FESEM (ZEISS, Ultra Plus Germany) using STEM detectors.

2.7.4. Confocal laser scanning microscopy (CLSM) visualization of NVAs skin penetration

Intact skin samples were mounted in standard Franz diffusion cells with the StC facing upward. The donor compartment was filled with an infinite dose (≈ 200 µL/cm^2) of Cy5-labelled NVAs dispersion. The receptor compartment was filled with 0.1% Tween 80 in PBS, thermostated at 37°C and stirred. At the end of the experiment (24 hours), diffusion cells were dismantled, the residual formulation was removed from the skin surface, and the skin samples were frozen at -80°C. Then, the skin samples were embedded in OCT medium, vertically sectioned using a Leica CM1850 UV Clinical Cryostat (Leica Microsystems, Mannheim, Germany), mounted on microscope slides and examined with a confocal microscope LEICA AOBS-SP5X (Leica Microsystems, Mannheim, Germany).

2.8. Statistical analysis

Results were compared statistically using analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The differences were considered significant for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. All the statistical analyses were carried out with Graph-Pad Prism Version 7.04 software.

3. Results and discussion

Topical administration of IMM could be considered as a promising modality of administration. Some nanotechnology-based approaches have been proposed for the delivery of drugs across the skin. However, these nanocarriers were tested mainly in barrier-impaired skin showing epidermal accumulation when StC was removed before application of the formulation. In this context, polymeric NVs could provide additional mechanisms to enhance the penetration of IMM across the skin. Moreover, the versatile composition of polymeric NVs allows the modulation of their physicochemical properties, such as size or surface charge, which may influence their performance of nanocarriers in terms of their interaction with the skin. Taking all this information together, herein we describe the development of small polymeric NVs, with either, a cationic or an anionic surface charge, for the topical administration of IMM.

3.1. Development and characterization of small polymeric NVs

It has been reported in the literature that the penetration of nanocarriers across the skin is mainly associated to their interaction with hair follicles and subsequent transfollicular permeation, and that this process is dependent on the nanocarriers' particle size [15–17]. Namely, small nanoparticles of around 70 nm and composed of PLGA have shown an enhanced skin permeation in both, healthy and inflamed skin [18]. Based on this knowledge, the first step in this work was the reduction of the size of previously developed NVCs (Chapter 2) and NVAs.

3.1.1. Evaluation of the optimal formulation parameters

The formulation parameters affecting the particle size of NVs have been previously investigated by our group [9,19]. The results showed that the mixing process is a key parameter influencing their size. For example, differences were found when the solvent was added to the water phase dropwise, by pouring or by injection. The injection mode was the one that led to the formation of the smallest nanocarriers. This effect has been attributed to the increased shear force at the injection site, causing higher dispersion degree of the solvent

[20]. On the other hand, the decrease in components' concentration resulted in a reduction of the particle size [20]. In the case of NVCs (Fig. 1), we selected the injection mode of the solvent (condition 1). Then, keeping this condition, we reduced the concentration of the components in the organic phase by increasing the volume of solvent (condition 2). Keeping this condition, we increased the volume of water (condition 3). By adjusting all these parameters, the particle size of NVCs could be reduced from 224 to 88 nm.

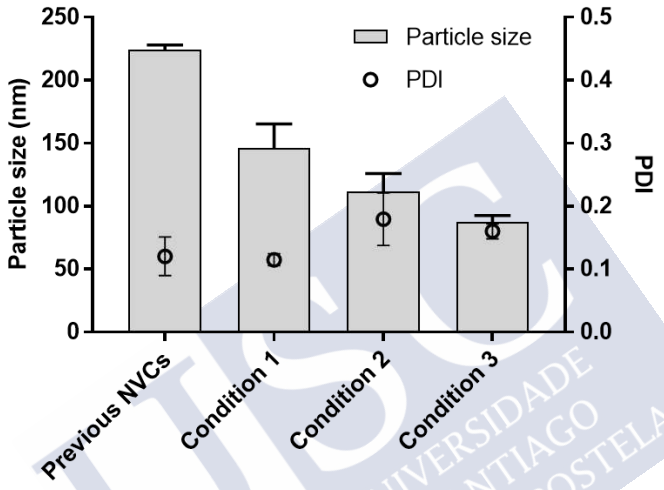

Fig. 1 – **Impact of different formulation parameters on NVCs size.** Condition 1: injection of solvent, Condition 2: injection of solvent and increasing the volume of solvent, Condition 3: injection of solvent, increasing the volume of both, solvent and water. Values represent the mean ± SD (n=3).

For the preparation of NVAs, we applied the optimized formulation parameters selected for NVCs. This resulted in a formulation of about 90 nm size. This particle size was considered adequate for comparison with NVCs in terms of their interaction with the skin.

3.1.2. Physicochemical and morphological characterization of IMM-loaded NVs

The physicochemical characterization of blank and IMM-loaded prototypes is presented in Table 1. The results indicate that the particle size and PDI were not affected by the encapsulation of IMM, being the size in all cases below 100 nm. With regard to surface charge

(ζ-potential), a significant reduction was observed for NVAs upon drug encapsulation. As shown in Table 1, both prototypes resulted in around 100% of EE%.

Table 1 – Physicochemical properties and IMM encapsulation efficiency (EE) of selected NVs prototypes. Values represent the mean ± SD (n=3). n/a not applicable

Nanosystem	Particle Size (nm)	PDI	ζ-potential (mV)	EE (%)
Blank NVCs	85 ± 4	0.2	+58 ± 5	n/a
Loaded NVCs	80 ± 1	0.1	+61 ± 9	98
Blank NVAs	77 ± 1	0.1	-44 ± 4	n/a
Loaded NVAs	86 ± 6	0.2	-20 ± 5	103

In order to further characterize the loaded NVs, we used different techniques for their morphological analysis (Fig. 2). As shown in both, InLens and STEM images, all nanocarriers have round-shape with similar particle size. Furthermore, NTA measurements corroborated DLS data observed for both prototypes, showing particle a particle size of around 100 nm and narrow size distribution.

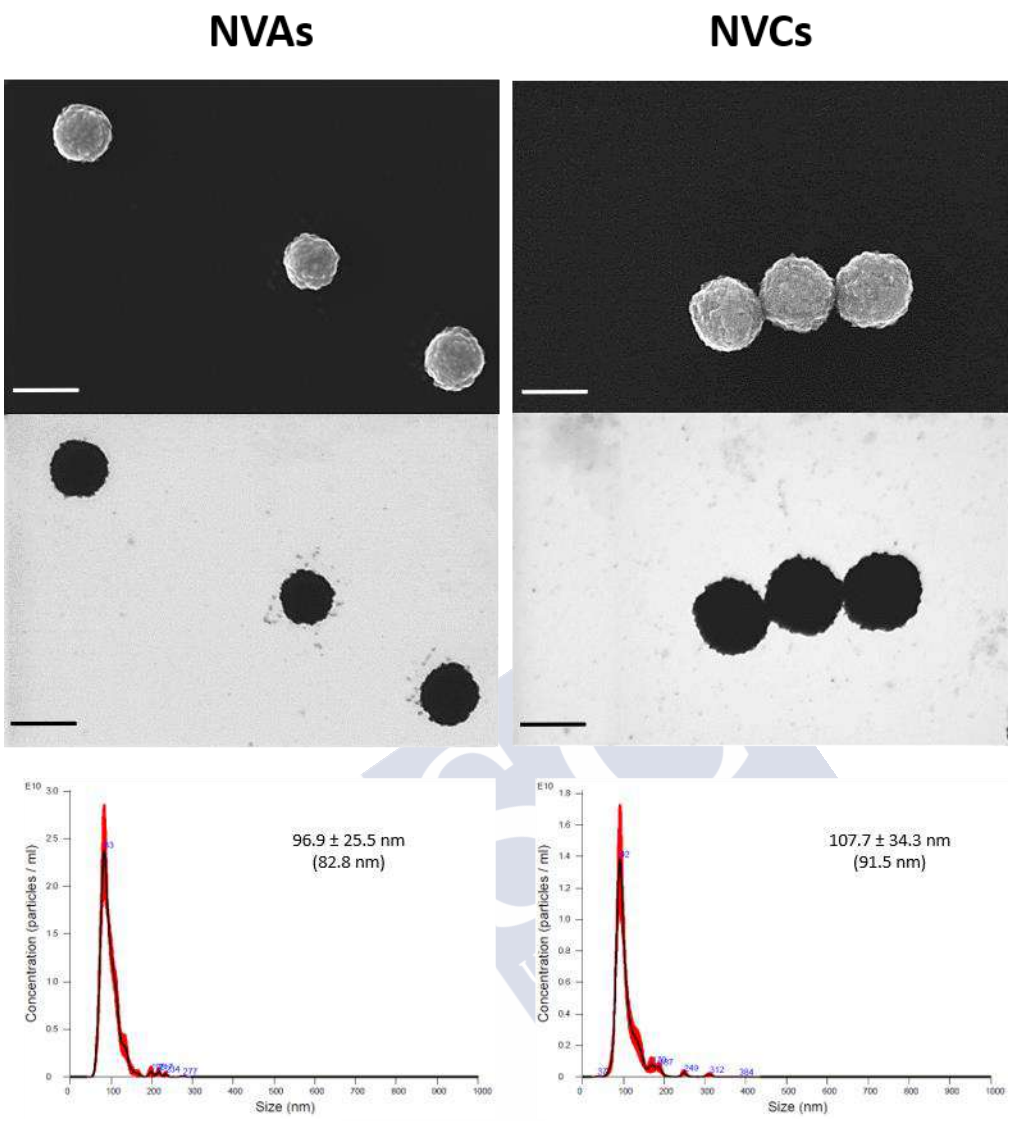

Fig. 2 – **FESEM and NTA characterization of IMM-loaded NVAs and NVCs**. FESEM images, using InLens (second row) and STEM (third row) detectors. All scale bars = 200nm. In the fourth row, a representative image of the particle size distribution obtained for each system by NTA is shown. The mean particle size with the standard deviation and the mode (between brackets) are indicated in the figure (n=3).

3.2. Transport of IMM and fluorescent NVs across the skin

Following topical administration, IMM is not able to reach the viable epidermis. Hence, a number of approaches including the use of penetration enhancers and nanocarriers has been investigated as a way to supply the skin with the necessary amount of this molecule. Interestingly, so far penetration enhancers have shown a limited success. In the case of

nanotechnology-based formulations, several nanocarriers were developed for dermal or transdermal delivery of IMM. However, most of them were designed to enhance dermal accumulation of its derivatives for psoriasis treatment without systemic distribution. In this work we have hypothesized that the encapsulation of IMM into polymeric NVs will help its transport across the skin without the need of skin disruption. Herein we disclose the assessment of the capacity of NVCs and NVAs for enhancing skin penetration of IMM.

3.2.1. Penetration of IMM across the skin

IMM-loaded NVAs and NVCs were incubated with human skin at different concentrations and IMM was quantified in the different skin layers by UPLC. The amount of IMM retained in the skin was quantified in the viable epidermis (25 – 150 µm) and dermis (deeper than 150 µm). The StC layer (0 – 25 µm) was not considered for this study because of the impossibility to differentiate between surface adsorbed amount and deposited amount inside the StC. We considered the epidermis as the target layer for IMM delivery. Further penetration to the dermis would be also desirable to enhance its access to the systemic circulation. We tested both formulations under infinite dose conditions (≈ 200 µL/cm2) for a preliminary evaluation. The results in Fig. 3 indicate that the amounts of IMM retained in the epidermis and dermis are similar for both prototypes.

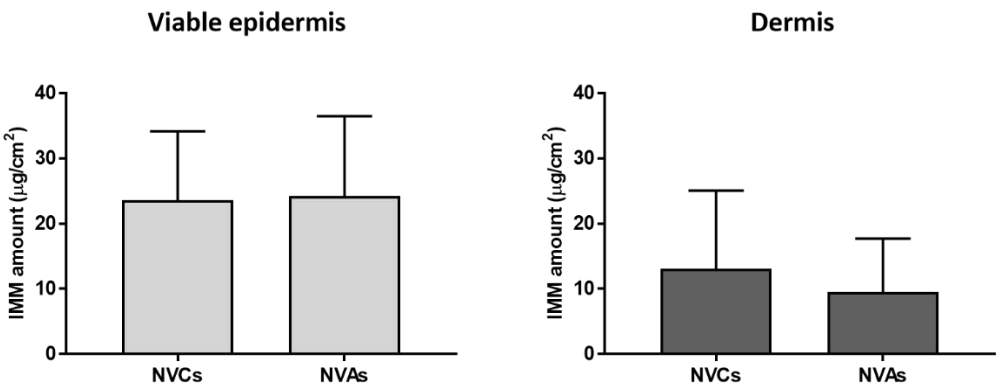

Fig. 3 – **Penetration of IMM into different skin layers after 24 hours of incubation with infinite dose (≈ 200 μL/cm²) of loaded NVs**. Values represent mean ± SD (n=4).

In a subsequent step, we tested a finite dose (≈ 10 μL/cm2) simulating real conditions of a topical administration (Fig. 4) [21]. In this case, we used as a control for IMM penetration a solution in 0.25% oil. The results in Fig. 4 indicate that the transport of free IMM to the viable epidermis and dermis was negligible (0.26 ± 0.84 μg/cm2), a result that is in agreement with its high lipophilicity (logP ≈ 7.5). Indeed, free IMM is expected to interact with the lipids in the StC and remain retained in this skin layer. On the contrary, the permeation of IMM administered in the encapsulated form was much higher and dependent on the NVs composition.

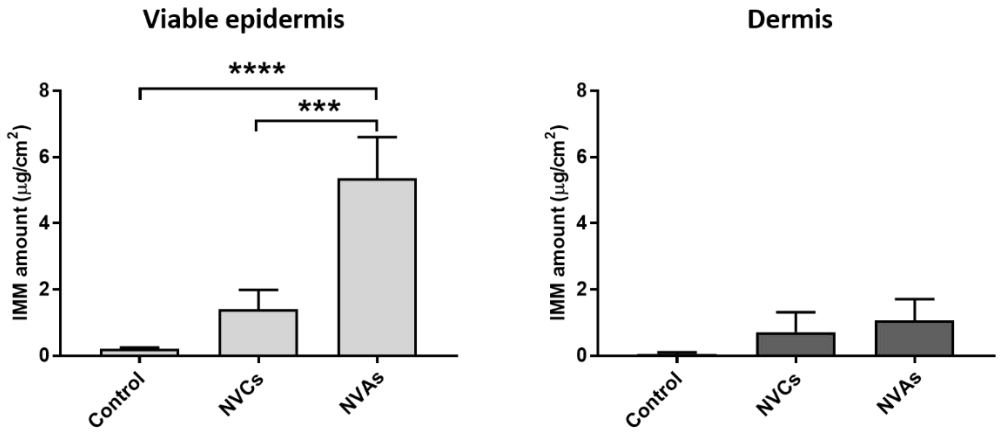

Fig. 4 – **Penetration of IMM into different skin layers after 24 hours of incubation with finite dose (≈ 10 µL/cm²) of loaded NVs**. Control was done with a solution of IMM in 0.25% oil at the same concentration as the NVs. Values represent mean ± SD (n=4).

NVAs were found to be the most efficient vehicle to deliver IMM to epidermis (Fig. 4). However, this increased accumulation in the epidermis was not translated into a greater accumulation in the dermis layer. Our hypothesis is that in the *ex vivo* conditions, this transport mechanism might be saturated, limiting the accumulation in the dermis and promoting epidermal accumulation of IMM. In an *in vivo* scenario, the epidermal accumulation shown by NVAs might be beneficial given that the transporter may provide a prolonged delivery of IMM from the epidermis to the systemic circulation.

Although NVCs succeeded in enhancing drug penetration to the viable epidermis, the amount permeated from NVAs was significantly higher. These differences between formulations could be explained by their different composition. The entrapment of IMM inside NVAs enhanced 3.8 fold its penetration in the viable epidermis compared to NVCs. In accordance with our findings, other authors have shown the importance of surface charge for skin penetration for different nanocarriers [22–25]. Anionic liposomes, dendrimers or gold nanorods have shown increased skin penetration compared to the same cationic nanocarriers. Considering the net negative charge of both skin surface and cell membranes, the authors hypothesized that cationic nanocarriers might have a closer interaction with cell surface and get retained in superficial skin layers compared to anionic nanocarriers.

For long-term treatments, the simplicity of our NVAs and the low dose (10 µL/cm^2) needed to enhance IMM skin penetration might be a remarkable competitive advantage in terms of patient compliance and safety. In order to understand the relevance *in vivo* of the increased transport of IMM across the skin performed by NVAs, further quantification of IMM serum levels need to be performed.

3.2.2. Interaction of NVAs with the skin by STEM and confocal imaging

The percutaneous penetration of small moderately lipophilic compounds is mainly driven by the intercellular pathway between the corneocytes. This route is limited to molecules smaller than 500 Da, which have around 1000 Van der Waals Surface Area (Aw). Consequently, it is unlikely that nanocarriers with size around 100 nm and 3 × 106 Aw would penetrate through this pathway. Apart from this, recent studies have shown the importance of the transappendageal route for the percutaneous penetration of nanocarriers [17,21,26,27]. Although skin appendages, such as hair follicles or sweat glands, represent just 0.1% of total skin surface, their contribution to the overall penetration might play an important role allowing the access of nanocarriers to deeper skin layers [28,29]. For this reason, we decided to evaluate the ability of our NVAs to preferentially penetrate through skin appendages using different imaging techniques.

First, we assessed the interaction of the NVAs with the skin surface using FESEM. For this, the suspension of NVAs was added to dehydrated skin and the samples were prepared for FESEM visualization. As shown in Fig. 5, NVAs (in green) are deposited in both skin surface and hair follicles (in brown).

Fig. 5 – **Colored SEM picture of hair follicle incubated with loaded NVAs during 24 hours**. Brown: skin surface and hair follicle, Green: NVAs. Scale bar = 10µm

Subsequently, the penetration of Cy5-labelled NVAs across the human skin upon skin exposure for 24 hours was investigated using CSLM. These fluorescent NVAs presented similar physicochemical properties (72 ± 0.7 nm, PDI 0.1 and -48 ± 2.4 mV) to the non-fluorescent NVAs. Confocal images taken from vertical sections confirmed the presence of Cy5-labelled NVAs with uniform distribution around the hair shaft down to the hair root (Fig. 6B). These results are in accordance with previously reported studies where small nanocarriers preferentially penetrate through skin appendages [16–18]. Nanocarriers' follicular accumulation has been shown to be time-dependent and enhanced when the application is done with massage [16,29]. This behavior is associated with hair movement, acting as a geared pump facilitating actively the accumulation of nanocarriers inside follicles [27]. In the case of our NVAs, it was observed that despite their retention in the skin superficial layers (Fig. 6C), their access to deeper skin layers was facilitated by their transfolicular transport (Fig. 6D). These images provide visual confirmation of the importance of hair follicles in IMM delivery

by NVAs, allowing the formulation to access deeper skin layers and as a result, enhancing IMM penetration.

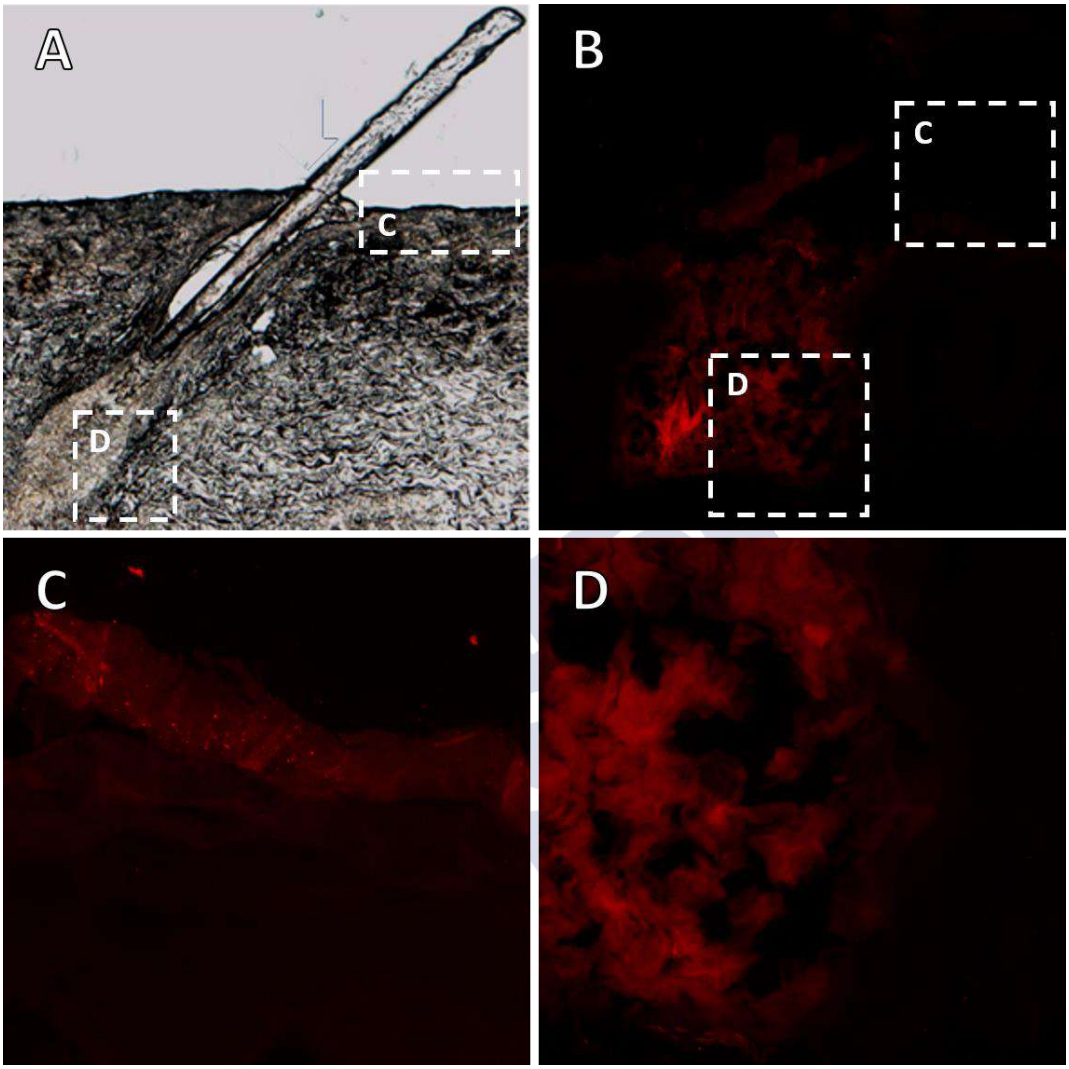

Fig. 6 – **Visualization of hair follicle incubated with Cy5-labelled NVAs for a period of 24 hours.** Electron microscopy image of a hair follicle adapted with permission from [30] **(A)**; CLSM cross section image (X-Z plane, 20x) of a hair follicle from human skin incubated with Cy5-labelled NVAs in red **(B)**; CLSM cross section image of the skin surface (63x) from the same skin sample **(C)**; CLSM cross section image of the skin deeper layers (63x) from the same skin sample **(D)**.

4. Conclusions

Herein we report the development of small polymeric nanocarriers (less than 100 nm) for the topical administration of IMM. *Ex vivo* human skin permeation studies showed the capacity of

NVAs to enhance the accumulation of IMM in the viable epidermis and, in a lower extent, in the dermis. The confocal images of the skin treated with fluorescent NVAs suggested that the IMM accumulation in the viable epidermis may be due to the transport of the NVAs across the hair follicles. Overall, these results suggest that IMM-loaded NVAs might enable safe and effective IMM supplementation by topical application. Further *in vivo* proof-of-concept studies would be necessary to assess the value of NVAs.

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General discussion

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Natural compounds have been used in medicine for preventing and treating some of the most prevalent diseases of our time, such as cancer or microbial infections. Among them, the immunomodulatory molecule selected in this thesis has been widely studied due to its wide influence in several physiological processes. The role of this molecule in apoptosis, immune response or proliferation have highlighted its potential for treating illnesses such as autoimmune diseases or cancer, among others.

However, this immunomodulatory molecule presents several problems for its clinic use. First, it requires high doses to achieve a therapeutic effect due to its non-targeted biodistribution, thereby leading to off-site toxicity. Furthermore, its oral supplementation plans have been associated with several drawbacks, such as the limited sources in the diet and the intestinal absorption fluctuations between individuals and in different disease scenarios. In addition, it has shown high instability both during storage and after its administration *in vivo*, illustrating the need of an effective formulation to assure their stability in both situations. In consequence, although good results have been obtained in some cases, they have not always been clearly confirmed in clinical trials.

In this sense, nanotechnology offers the possibility to improve biodistribution of compounds like this molecule by targeting different cell populations avoiding off-target effects. Moreover, nanocarriers can be adapted to different administration routes that could be convenient for its supplementation, such as the transdermal route. Furthermore, these nanocarriers can efficiently protect it by themselves or by co-encapsulating antioxidant agents, preserving their activity for longer time. Therefore, these characteristics make nanotechnology a great approach to enhance both therapeutic and preventive potential of the immunomodulatory molecule selected in this thesis.

Polymeric nanocarriers (NVs) present features that make them suitable for the delivery of highly lipophilic compounds like this immunomodulatory molecule. Their structure allows the encapsulation of lipophilic compounds. Moreover, their tunable composition facilitates the modulation of physicochemical properties critical for their biodistribution, such as size or surface charge [1,2]. In this context, our group has generated significant knowledge regarding the modulation of polymeric NVs properties and their influence on their biodistribution profile and interaction with different cells subsets [2–9].

Considering this background, the aim of this thesis was to develop polymeric NVs to improve the efficacy of the selected immunomodulatory molecule. First, the potential of polymeric NVs encapsulating this immunomodulatory molecule to prevent autoimmune diseases such as type 1 diabetes (T1D) was assessed. Second, we tested the ability of polymeric NVs for enhancing dermal supplementation of this molecule.

1. Tolerogenic NVs for prevention of autoimmune diseases

Dendritic cells (DCs) have a central role in the maintenance of peripheral self-tolerance and hence in the response against autoantigens developed during autoimmune diseases such as T1D [10]. In this context, the modulation of DCs towards a tolerogenic phenotype has been considered a good alternative to control the immune response against autoantigens and avoid disease onset [11]. Our approach for the development of a preventive treatment in the context of T1D has been to use an immunomodulatory molecule (IMM) encapsulated in polymeric NVs to enhance its immunomodulatory properties while avoiding off-target toxicity due to the targeting capacity of nanotechnology-based formulations. Furthermore, a peptidic autoantigen candidate in T1D was included in the formulation to achieve an antigen-specific response.

The interaction between nanocarriers and immune cells is dependent on their physicochemical characteristics and composition [12]. It has been described that parameters such as size and surface charge are key factors on the way this interaction is produced. In this sense, cationic nanocarriers have shown to enhance the interaction with DCs [13–16]. For that reason, we decided to provide our polymeric NVs with a positive surface charge using a cationic polymer (Fig. 1). In addition, control nanocarriers (NV) were formulated following the same protocol without adding the cationic polymer for its use as a control for *in vitro* and *in vivo* studies. Both blank cationic nanocarriers (NVCs) and control NV were found to have a narrow size distribution and stable under culture conditions up to 24 hours. IMM and an antigenic peptide in T1D selected by our collaborators (a fragment of preproinsulin, PPI $B_{10\text{-}18}$) were encapsulated in NVCs without huge changes in their physicochemical properties (Fig. 1).

Nanosystem	Particle Size (nm)	PDI	ζ-potential (mV)	PPI B_{10-18} EE (%)
Blank NV	158 ± 11	0.1	-50 ± 4	n/a
IMM-loaded NV	146 ± 25	0.2	-54 ± 5	n/a
Blank NVCs	224 ± 4	0.1	+55 ± 2	n/a
IMM-loaded NVCs	221 ± 4	0.2	+49 ± 1	n/a
(PPI B_{10-18} + IMM)-loaded NVCs	136 ± 12	0.1	+61 ± 7	34

Fig. 1 – Physicochemical properties of developed nanocarriers. Table with physicochemical properties of all nanocarriers**.** Values represent the mean ± SD of at least 3 replicates. PDI, polydispersity index; EE, encapsulation efficiency; NV, nanocarrier; n/a, not applicable; IMM, immunomodulatory molecule; PPI B_{10-18}, antigenic peptide; NVCs, cationic nanocarriers.

Due to the key role DCs have in autoimmunity, *in vitro* studies were performed using human DCs (hDCs). We evaluated the potential of our IMM-loaded NVCs to induce a tolerogenic phenotype in hDCs that could be translated in a preventive effect *in vivo* for T1D. First, cytotoxicity of blank NVCs was tested, showing a dose-dependent toxicity (Fig. 2A). Then, the interaction between NVCs with hDCs was analyzed by flow cytometry and confocal microscopy after their incubation with DiD-loaded NVCs at a non-toxic dose. As it is depicted in Fig. 2B, high interaction between NVCs and hDCs was shown with around 92% of positive cells. Furthermore, confocal images showed the internalization of DiD-loaded NVCs by hDCs. These results were consistent with previously described studies which indicated the preferential internalization of cationic nanocarriers of around 200 nm by hDCs [7,9].

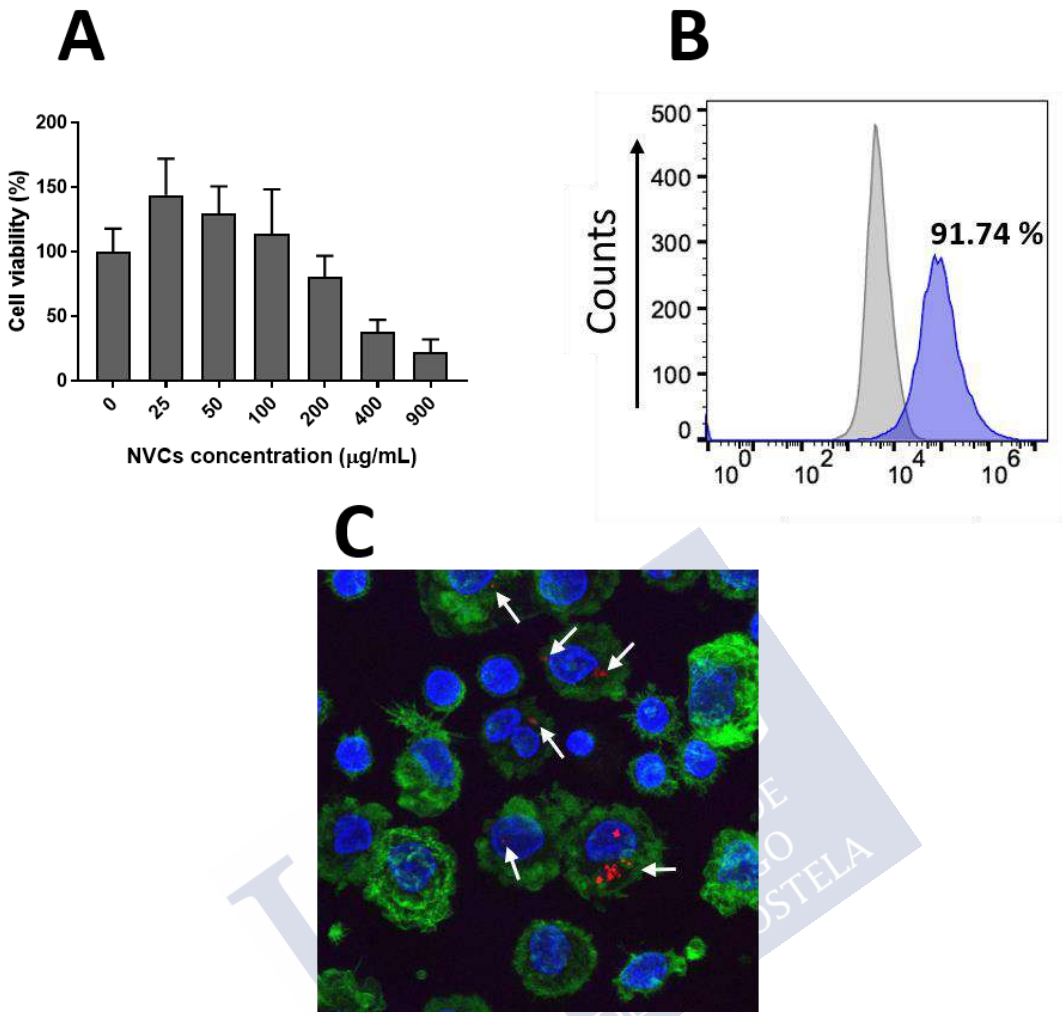

Fig. 2 – **Cell viability (A) and interaction (B-C) of NVCs upon incubation with hDCs**. Cells were incubated with of NVCs **(A)** at different concentration during 24h. Results are shown as the percentage of cell viability. Values represent mean ± SD of at least 2 replicates. Representative flow cytometry histogram showing the interaction of DiD-loaded NVCs at 100 µg/mL after 1-hour incubation with iDCs is shown in **(B)**. In grey non-treated cells are represented and percentage of positive cells is represented. Representative CSLM image (XY plane, 63x) of iDCs incubated with DiD-loaded NVCs under the same conditions **(C)**. White arrows point out red dots corresponding to DiD-loaded NVCs. Blue channel: nuclei, green channel: actin, red channel: DiD-loaded NVCs

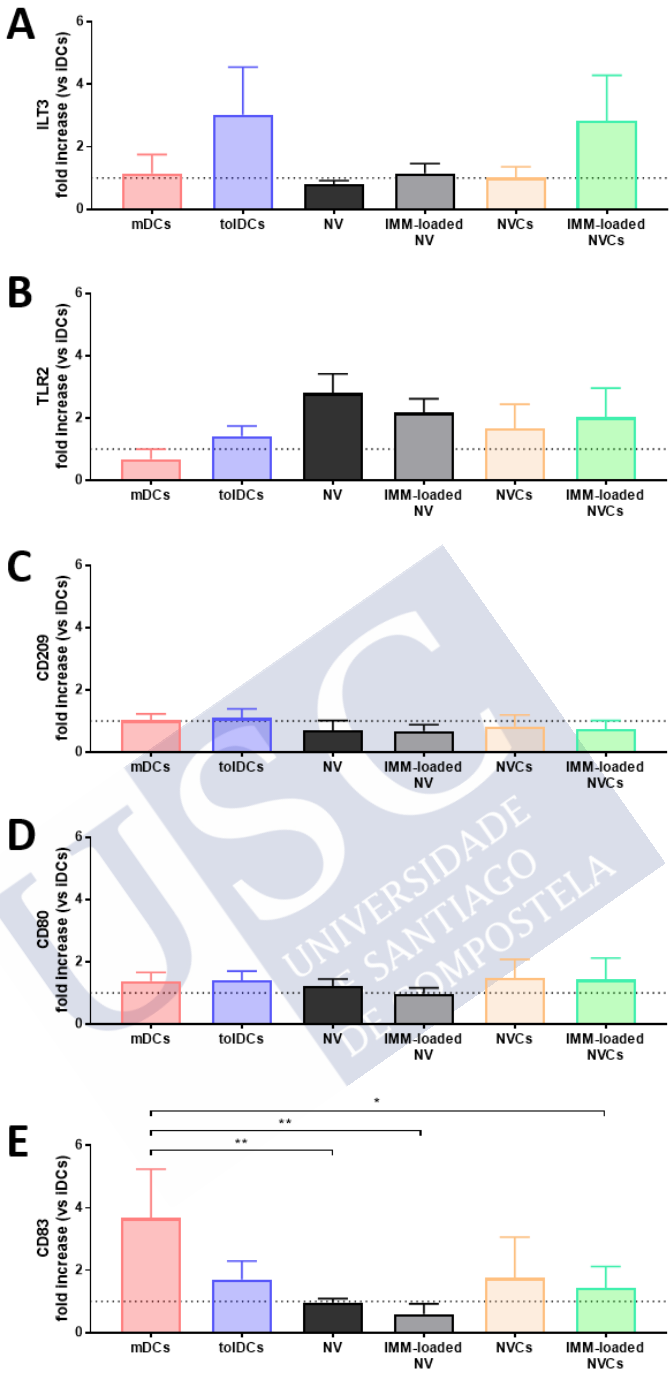

Fig. 3 – **Expression of surface markers ILT3 (A), TLR2 (B), CD209 (C), CD80 (D) and CD83 (E) in iDCs after incubation with different nanosystems.** Surface markers expression was determined by flow cytometry after incubation of iDCs with nanosystems during 2h. Data is shown as expression fold increase compared to untreated iDCs. Dotted line represents iDCs expression levels. Values represent mean ± SD of at least 4 replicates. Statistical analyses were done using a one-way ANOVA followed by a Tukey test. Significance levels * $p < 0.05$ and ** $p < 0.01$. Statistical comparison was done between IMM-loaded NVCs against the other groups. ILT, immunoglobulin-like transcript; TLR, toll-like receptor; iDCs, immature dendritic cells; mDCs, mature dendritic cells; tolDCs, tolerogenic dendritic cells; NV, nanocarrier; IMM, immunomodulatory molecule; NVCs, cationic nanocarrier.

For the evaluation of the influence of IMM-loaded NVCs on hDCs phenotype we determined different parameters, such as surface markers expression, cytokine secretion and indoleamine 2,3-dioxygenase (IDO) activity. First, we evaluated the expression of several surface markers known to be upregulated in tolerogenic DCs. It has been described that DCs with high expression of surface markers such as ILT3, TLR2 or CD209 among others is associated with a tolerogenic phenotype and T cell anergy promotion [17–21]. Our results showed an increase in ILT3 and TLR2 expression in DCs incubated with IMM-loaded NVCs similar to tolerogenic DCs (tolDCs), even though statistical significance was not reached (Fig 3A-B). In the case of CD209, similar expression levels were found for all the conditions tested (Fig. 3C).

In addition, the expression of surface markers related with a pro-inflammatory phenotype of DCs was evaluated. In this context, it has been described that the upregulation of co-stimulation receptors, such as CD80 or CD83, is necessary to activate T lymphocytes [22,23]. Our results showed a significant decrease in CD83 expression of both IMM-loaded nanosystems compared to mDCs, similar to the levels achieved by tolDCs (Fig. 3E). However, in the case of CD80, similar expression levels were found for all the conditions tested (Fig. 3D). Considering surface markers expression, these results highlight a tendency in promoting a tolerogenic phenotype in DCs after the incubation with IMM-loaded NVCs. Furthermore, the activity of IMM *in vitro* is maintained once incorporated in the nanosystems, showing similar effect as free IMM used for tolDCs differentiation.

Next, IDO activity was determined. The activity of this enzyme, implicated in the tryptophan catabolism, has been linked to tolerance promotion by DCs [24]. In the specific case of non obese diabetic (NOD) mice, the development of autoreactivity has been linked to a defect in tryptophan catabolism related to impaired IDO activity [25]. As depicted in Fig. 4, incubation of iDCs with IMM-loaded NVCs significantly increases IDO activity compared to untreated iDCs, mDCs and the incubation with blank NVCs.

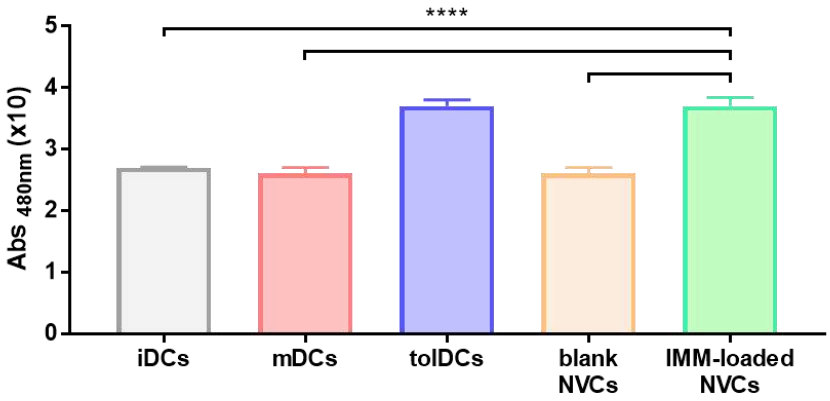

Fig. 4 – **IDO activity of iDCs incubated with NVCs.** Determination of kineurin in the culture medium was done after incubation of iDCs with blank and IMM-loaded NVCs for 24h. Data is shown as the absorbance at 490 nm. Values represent mean ± SD (n=4). Statistical analyses were done using a one-way ANOVA followed by a Tukey test. Significance levels **** $p < 0.0001$. Statistical comparison was done between IMM-loaded NVCs against the other groups. iDCs, immature dendritic cells; mDCs, mature dendritic cells; tolDCs, tolerogenic dendritic cells; IMM, immunomodulatory molecule; NVCs, cationic nanocarriers.

Finally, cytokine secretion of hDCs after incubation with both blank and IMM-loaded NVCs was determined. As we can observe in Fig. 5, maturation of DCs promotes a general increase of cytokine secretion. In this sense, pro-inflammatory cytokines such as IL-12p70, IL-8, TNF-α and IFN-γ increased greatly its expression (Fig. 5A-C-D-E). Surprisingly, the expression of IL-10, a known anti-inflammatory cytokine, was also increased (Fig. 5B). In the case of tolDCs, results showed a generalized decrease in cytokine secretion. This was also shown by DCs incubated with either blank or IMM-loaded NVCs. Regarding cytokine expression, these results showed that our NVCs do not promote a pro-inflammatory state in DCs, as blank NVCs showed similar expression levels as iDCs. Furthermore, cytokine expression levels of iDCs incubated with blank or IMM-loaded NVCs are similar to tolDCs, indicating that incubation with our NVCs maintained an immature tolerogenic state in DCs.

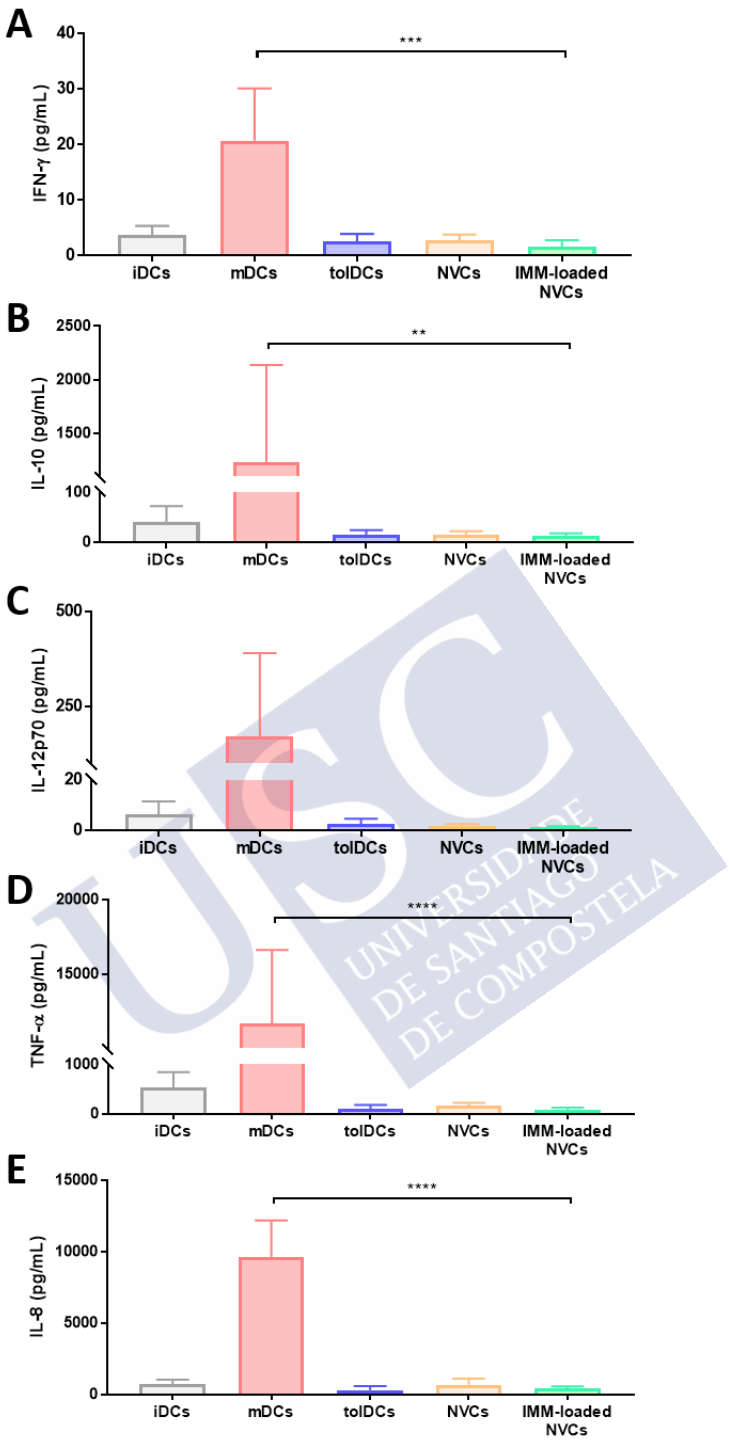

Fig. 5 – **Cytokine expression by iDCs incubated with NVCs.** Determination of cytokines in the culture medium was done after incubation of iDCs with blank and IMM-loaded NVCs for 24h. Values represent mean ± SD (n=4). Statistical analyses were done using a one-way ANOVA followed by a Tukey test. Significance levels ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Statistical comparison was done between IMM-loaded NVCs against the other groups. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; iDCs, immature dendritic cells; mDCs, mature dendritic cells; tolDCs, tolerogenic dendritic cells; IMM, immunomodulatory molecule; NVCs, cationic nanocarriers.

Overall, from these *in vitro* studies we could conclude that IMM-loaded NVCs were able to promote a tolerogenic phenotype on iDCs. This effect is similar to soluble IMM, used to differentiate iDCs to tolDCs, suggesting that the loading of IMM in NVCs does not affect its immunological properties.

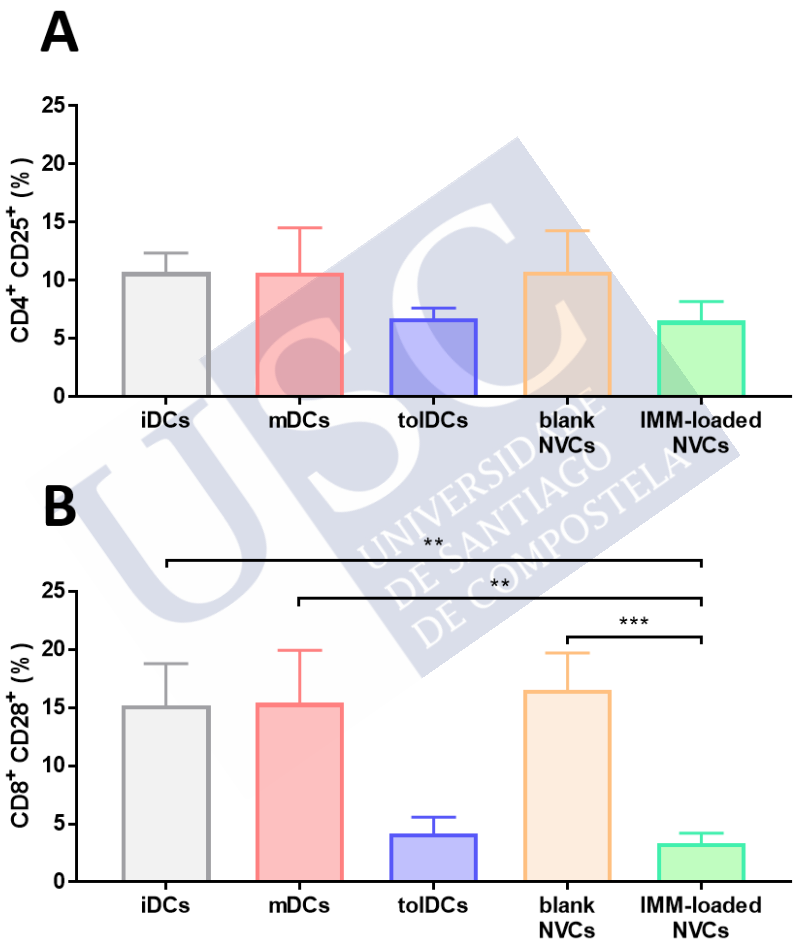

Fig. 6 – T lymphocyte activation capacity of pre-incubated iDCs in allogeneic culture. The percentage of activated $CD4^+$ $CD25^+$ T lymphocytes **(A)** and $CD8^+$ $CD28^+$ T lymphocytes **(B)** was determined by flow cytometry after co-culture of allogeneic PBLS with iDCs pre-treated with blank or IMM-loaded NVCs for 7 days. Values represent mean ± SD of at least 3 replicates. Statistical analyses were done using a one-way ANOVA followed by a Tukey test. Significance levels ** $p < 0.01$ and *** $p < 0.001$. Statistical comparison was done between IMM-loaded NVCs against the other groups. iDCs, immature dendritic cells; mDCs, mature dendritic cells; tolDCs, tolerogenic dendritic cells; IMM, immunomodulatory molecule; NVCs, cationic nanocarriers.

Once we confirmed the promotion of a tolerogenic state in hDCs promoted by IMM-loaded NVCs, the following step was to determine if this effect was translated into decreased activation of T lymphocytes. For that, pre-incubated hDCs with IMM-loaded NVCs were incubated with allogeneic T lymphocytes and their activation was evaluated by flow cytometry. As depicted in Fig. 6A, preincubation of iDCs with IMM-loaded NVCs slightly decreased the percentage of activated $CD4^+$ T lymphocytes compared to untreated iDCs, mDCs and iDCs pre-treated with blank NVCs, although without statistical significance. This reduction in lymphocyte activation by iDCs pre-treated with IMM-loaded NVCs was greatly accentuated in $CD8^+$ T lymphocytes, showing a significant decrease compared to iDCs, mDCs and blank NVCs (Fig. 6B). In both cases, this behavior was similar to the one promoted by soluble IMM, epitomized by tolDCs.

Finally, we performed animal studies to test if the immunomodulatory potential shown *in vitro* by our IMM-loaded NVCs could be translated into a delayed onset of T1D *in vivo*. It has been described in the literature that the use of soluble IMM has shown potential for T1D treatment. However, its systemic distribution resulted toxic effects limiting its use. We hypothesized that its inclusion in a nanocarrier would allow its targeted delivery to immune cells avoiding *in vivo* off-target toxicity. On the other hand, our additional hypothesis has been that the co-encapsulation of IMM and PPI $B_{10\text{-}18}$ autoantigen would enhance the prevention of T1D. This was based in reported findings showing that the use of high doses of soluble antigens promotes tolerance induction by inhibiting T lymphocytes proliferation or promoting their selective depletion [26,27]. Furthermore, the use of autoantigens could enhance the generation of selective tolerance for one specific antigen, maintaining the capacity to elicit immune responses against other antigens.

For this *in vivo* experiment, we selected NOD.B6-Tg(HLA-A2.1)Enge/DvSJ (NOD-HHD) mice. This animal model mimics several features observed in human T1D patients. In general, NOD mice have early infiltration of immune cells in pancreatic islets, such as DCs, macrophages or neutrophils [28–31]. Furthermore, antigenic specificity of T lymphocytes in NOD mice is similar to humans, with common antigens being recognized [32–36]. In addition, NOD-HHD mice are transgenic for HLA class I A*02:02 allele coding for the HLA-A human protein of the major histocompatibility complex class I. This molecule is capable of selecting autoreactive T $CD8^+$

lymphocytes accelerating significantly disease development compared to other NOD strains [37,38]. Here in, female NOD-HHD mice were treated intraperitoneally starting during the prediabetic period (4 weeks old) and following the administration schedule described in Fig. 7A. The doses of IMM and PPI B_{10-18} per administration were 5 µg/kg and 4 mg/kg, respectively. Mice with glycosuria were confirmed diabetic when urine glucose was ≥ 500 mg/dL after two consecutive weeks.

As expected, similar incidence was shown by groups treated with saline, soluble IMM or PPI B_{10-18} (Fig. 7B-C-D). In contrast, IMM-loaded NVCs showed a T1D incidence significantly lower than all control groups (Fig. 7B-C-D). Therefore, a straight-forward conclusion was that under the experimental protocol described, only encapsulated IMM was efficient in terms of preventing T1D. On the other hand, the results presented in Fig. 7F, indicate that a control IMM-loaded NV was not efficient at preventing T1D. Therefore, these results indicate not only that the preventive effect of IMM-loaded NVCs comes from IMM immunomodulatory properties but also that the cationic surface charge of the NVCs is necessary to promote this effect. This could be related to the preferential uptake by DCs showed by cationic nanocarriers [13–16]. However, specific *in vivo* biodistribution studies would be necessary to understand the mechanistic behavior of NVCs.

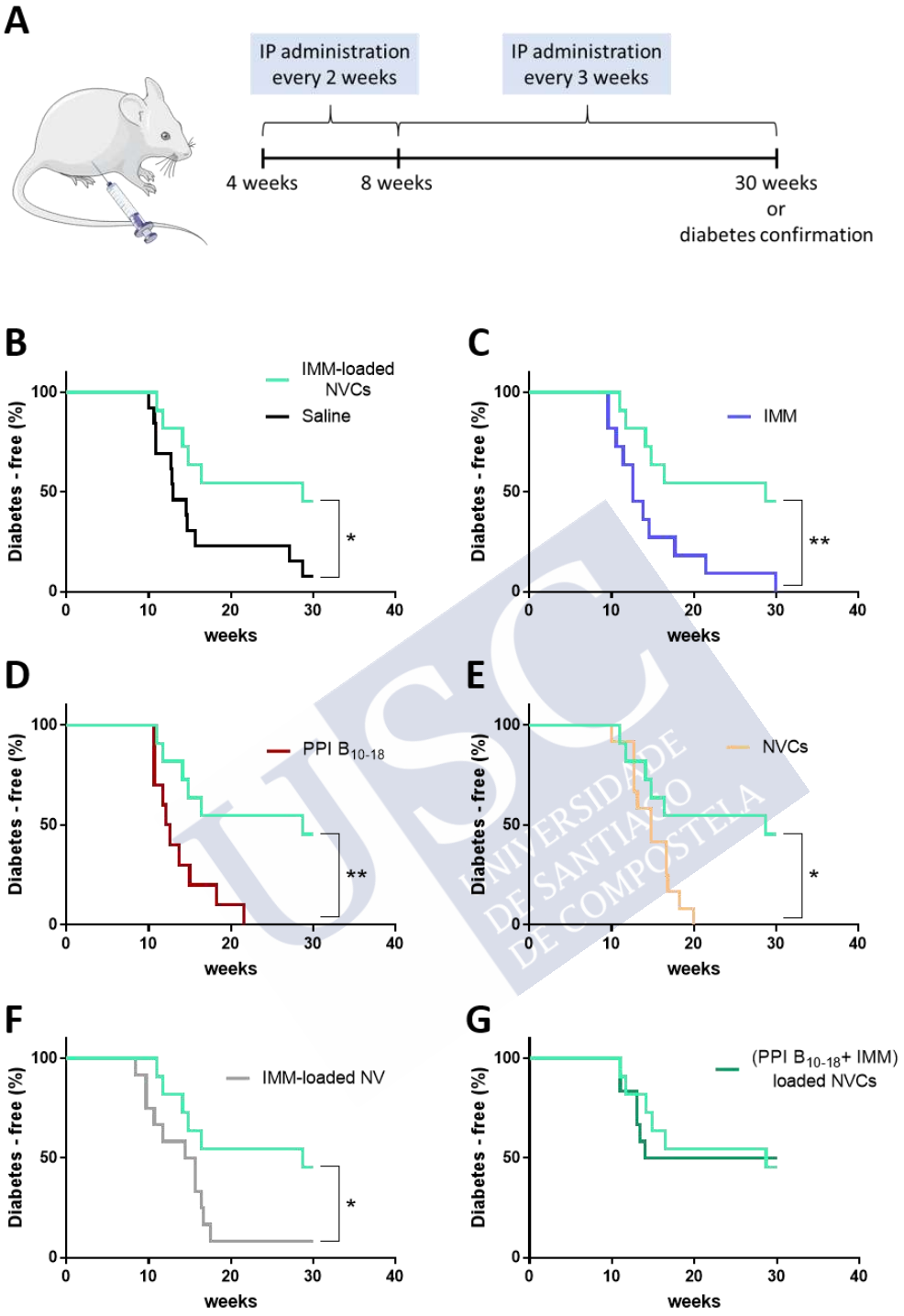

Fig. 7 – **Preventive effect of IMM-loaded NVCs in diabetes onset.** Administration schedule **(A)** and diabetes incidence in female NOD-HHD mice treated intraperitoneally with IMM-loaded NVCs compared with several groups: saline **(B)**, soluble IMM **(C)**, soluble PPI B_{10-18} **(D)**, blank NVCs **(E)**, IMM-loaded NE **(F)** and (PPI B_{10-18} + IMM)-loaded NVCs **(G)** (n of at least 10 animals per group). Statistical analyses of survival curves were done using a Kaplan-Meier log-rank analysis. Significance levels * $p < 0.05$ and ** $p < 0.01$. IP, intraperitoneal; NV, nanocarrier; IMM, immunomodulatory molecule; PPI B_{10-18}, antigenic peptide; NVCs, cationic nanocarriers.

Contrary to what was expected, the co-encapsulation of the antigenic peptide PPI B_{10-18} and IMM in NVCs did not show a synergistic effect to delay diabetes onset (Fig. 7G). This indicates that the main effect in diabetes prevention observed for NVCs comes from the IMM immunomodulatory activity and not from its synergy with this specific autoantigen. It has been described that the selection of the autoantigen, the administration route, dose and vaccination time are key aspects to induce tolerance and prevent diabetes onset [39–42]. Therefore, adjusting the administration schedule and dose of the selected autoantigen or including different ones could improve the performance of (PPI B_{10-18} + IMM) loaded NVCs. Nevertheless, the inclusion of antigens in nanocarriers designed for diabetes prevention have shown controversial results. It seems that the effect of the autoantigen is dependent on the route selected for the generation of tolerance. In the case of the use of immunomodulatory molecules, results showed no tolerogenic activity of the antigen selected [43]. However, when apoptosis signaling molecules are used, such as phosphatidylserine, the combination of the antigen with these molecules enhances tolerance generation in comparison with the signaling molecule alone [44]. On the other hand, nanocarriers loaded with immunomodulatory molecules in combination with an antigen have shown to promote a tolerogenic response specific for the selected antigen while maintaining the response against different antigens [45]. Considering this, further studies evaluating the specificity of the tolerogenic response developed by NVCs would be necessary in order to understand the effect of the antigen.

These preliminary studies indicate that the use IMM-loaded NVCs could be a good strategy to delay T1D onset. The simplicity of our IMM-loaded NVCs would be a remarkable competitive advantage for its translation to the clinics.

2. Dermal supplementation of natural compounds using nanocarriers

Taking advantage of the NVCs developed in Chapter 2 for enhancing the immunomodulatory properties of the selected molecule, we hypothesized that our NVCs could be optimized to improve the supplementation of this molecule by the dermal route. Its accumulation in the viable epidermis may result in an improved bioavailability. However, its transport through the stratum corneum (StC) is limited due to its high lipophilicity. In this sense, the inclusion of IMM

in a nanocarrier offers the possibility to enhance its dermal penetration without disturbing the natural barrier properties of the skin. Furthermore, the versatile composition of polymeric NVs allows the modulation of their physicochemical properties, such as size or surface charge, which may influence their performance in terms of interaction with the skin. Indeed, it has been reported that the penetration of nanocarriers across the skin is dependent on nanocarrier's particle size due to the favored transfolicular permeation of smaller nanocarriers [46–48]. Namely, small nanoparticles of around 70 nm and composed of PLGA have shown an enhanced skin permeation in both, healthy and inflamed skin [49]. Based on this knowledge, the first step in this work was the reduction of the size of previously developed cationic nanocarriers (NVCs, Chapter 2). In addition, anionic nanocarriers (NVAs) were also developed to determine the influence of surface charge in IMM skin penetration.

The formulation parameters affecting the particle size of NVs have been previously investigated by our group [50,51]. In this sense, increasing the volume of both, water and solvent, and injecting the solvent diminished the size of NVCs from 224 nm to 88 nm. In the case of NVAs, adjusting those formulation parameters resulted in particle size of about 90 nm. These formulations encapsulated IMM with high efficiency (Fig. 8).

Nanosystem	Particle Size (nm)	PDI	ζ-potential (mV)	EE (%)
Blank NVCs	85 ± 4	0.2	+58 ± 5	n/a
Loaded NVCs	80 ± 1	0.1	+61 ± 9	98
Blank NVAs	77 ± 1	0.1	-44 ± 4	n/a
Loaded NVAs	86 ± 6	0.2	-20 ± 5	103

Fig. 8 – **Physicochemical properties of small polymeric nanocarriers containing IMM.** Table with physicochemical properties of all developed nanocarriers. Values represent the mean ± SD of at least 3 replicates. PDI, polydispersity index; EE, encapsulation efficiency; n/a not applicable; NVCs, cationic nanocarriers; NVAs, anionic nanocarriers.

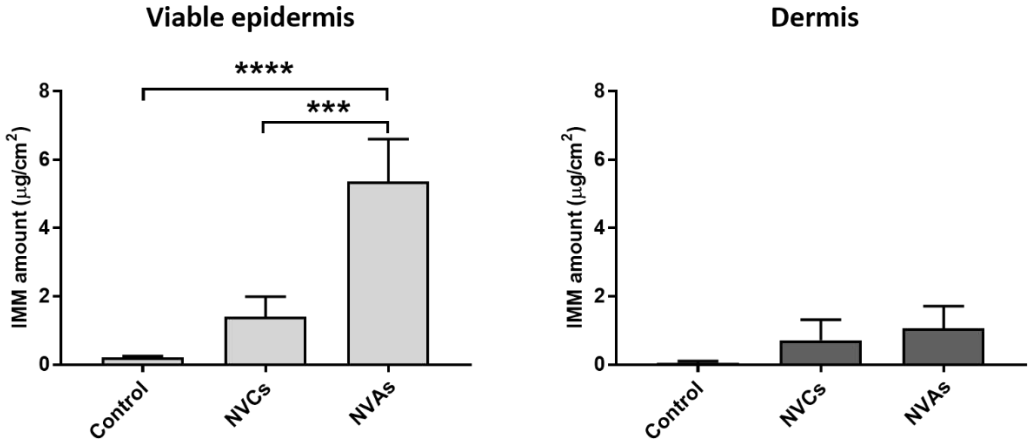

Fig. 9 – **Penetration of IMM into different skin layers after 24 hours of incubation with a finite dose (≈ 10 µL/cm²) of loaded nanocarriers**. Control was done with a solution of IMM in 0.25% oil at the same concentration as in the nanocarriers. Values represent mean ± SD (n=4). Statistical analyses were done using one-way ANOVA followed by a Tukey test. Significance levels *** $p < 0.001$ and **** $p < 0.0001$. IMM, immunomodulatory molecule; NVCs, cationic nanocarriers; NVAs, anionic nanocarriers.

Once we developed the formulations, the following step was to determine IMM penetration in human skin. As depicted in Fig. 9, the transport of free IMM to the viable epidermis and dermis was negligible (0.26 ± 0.84 µg/cm2), in agreement with its high lipophilicity (logP ≈ 7.5). In fact, free IMM is expected to interact with the lipids in the StC and remain retained in this skin layer. On the contrary, the permeation of IMM administered in the encapsulated form was much higher and dependent on the NVs composition. IMM-loaded NVAs were found to be the most efficient formulation to deliver the drug to the viable epidermis (Fig. 9). However, this increased accumulation in the epidermis was not translated into a greater accumulation in the dermis layer. We hypothesized that in the *ex vivo* conditions, the transport mechanism of IMM to systemic circulation might be saturated, limiting the accumulation in the dermis and promoting epidermal accumulation of IMM. In addition, we believe that the amount deposited in the viable epidermis could act as a reservoir for the controlled release of IMM. On the other hand, NVCs enhanced drug penetration to the viable epidermis, but the amount permeated from NVAs was significantly higher. In accordance with our findings, other authors have shown enhanced skin penetration for anionic nanocarriers [52–55]. The hypothesis

behind this behavior is the closer interaction between cationic nanocarriers and skin surface due to its net negative charge, promoting their retention in superficial skin layers.

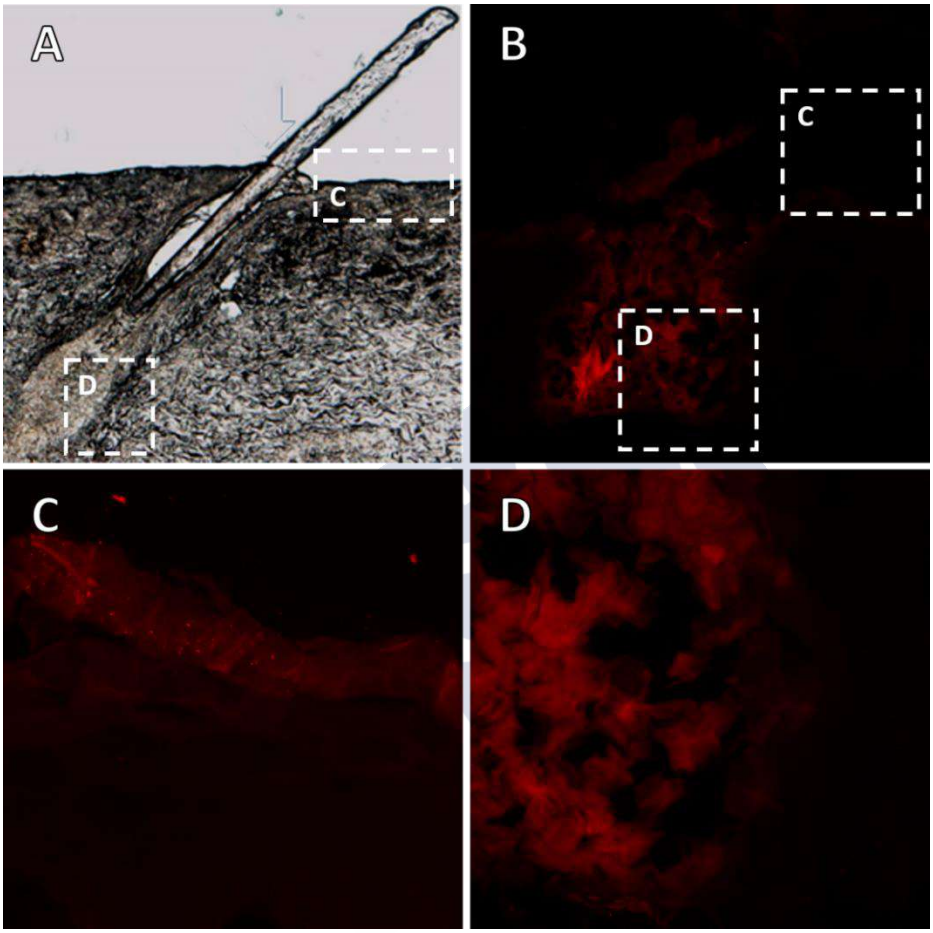

Fig. 10 – **Visualization of hair follicle incubated with Cy5-labelled NVAs for a period of 24 hours.** Electron microscopy image of a hair follicle adapted with permission from [59] **(A)**; CLSM cross section image (X-Z plane, 20x) of a hair follicle from human skin incubated with Cy5-labelled NVAs in red **(B)**; CLSM cross section image of the skin surface (63x) from the same skin sample **(C)**; CLSM cross section image of the skin deeper layers (63x) from the same skin sample **(D)**.

To understand the mechanism behind the preferential penetration of NVAs, we evaluated their interaction with the skin using confocal scanning laser microscopy. As depicted in Fig. 10B, Cy5-labelled NVAs presented uniform distribution around the hair shaft down to the hair

root. Although the nanocarriers were found to be retained in the skin superficial layers (Fig. 10C), their transport through hair follicles may allow them to access deeper skin layers (Fig. 10D). These results are in accordance with recent studies showing the importance of the transappendageal route for the percutaneous penetration of nanocarriers [48,56–58].

To sum up, these preliminary results suggest that IMM-loaded NVAs might enable safe and effective IMM supplementation by topical application. Further *in vivo* proof-of-concept studies would be necessary to assess the value of NVAs.

Overall, in this thesis we have shown the potential of polymeric nanocarriers for the delivery of immunomodulatory molecules in different contexts. The possibility of tuning their composition and properties allowed us to enhance their interaction with immune cells and dermal penetration. As a result, IMM immunomodulatory activity was maintained and translated to a delay in diabetes onset *in vivo*, and to an enhanced IMM dermal penetration.

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Conclusions

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The objective of this thesis was to evaluate the potential of nanotechnology for the delivery of an immunomodulatory molecule, according to two different therapeutic scenarios: prevention of type 1 diabetes (T1D) and its topical administration. Within the frame of T1D prevention, the objective was to design and develop polymeric nanocarriers for the targeted delivery of the selected immunomodulatory molecule to immune cells, thereby aiming to enhance its immunomodulatory potential while avoiding systemic toxicity. On the other hand, the objective was also to improve the penetration of this molecule across the skin.

The experimental work performed during this thesis led us to conclude:

1. Cationic nanocarriers were developed for the encapsulation of the immunomodulatory molecule (IMM). *In vitro*, IMM-loaded cationic nanocarriers exhibited the capacity to promote a tolerogenic phenotype in human dendritic cells (hDCs) in terms of cytokine expression, surface markers expression and IDO activity. Furthermore, the treatment of hDCs with IMM-loaded NVCs led to a significant reduction of lymphocyte activation in allogeneic culture. Therefore, the immunomodulatory activity of IMM was maintained after its encapsulation in cationic nanocarriers.
2. The administration of IMM-loaded cationic nanocarriers in NOD-HHD mice by intraperitoneal injections showed a delay in diabetes onset. Similar results were shown when an autoantigenic peptide was co-encapsulated, suggesting a leading role of IMM in this effect. Furthermore, this effect was not observed when mice were treated with free IMM or IMM-loaded control nanocarriers, thus, showing the importance of both the encapsulation and the surface charge of the nanocarriers.
3. Polymeric nanocarriers were optimized in terms of size in order to enhance their capacity to improve the penetration of IMM across the skin. Nanocarriers with cationic or anionic surface charge of around 100 nm were prepared by adjusting several parameters of the formulation technique. IMM was successfully loaded in both prototypes. *Ex vivo* human skin permeation studies showed the capacity of anionic nanocarriers to enhance the accumulation of IMM in the viable epidermis and, in a lower extent, in the dermis. The confocal images of the skin treated with fluorescent

nanocarriers suggested that the IMM accumulation maybe due to the transport of the nanocarriers across the hair follicles.

Overall, we have shown the potential of polymeric nanocarriers for the targeted delivery of the selected immunomodulatory molecule to the immune system and for its transport across the skin. The targeted delivery to the immune system opens new opportunities for the exploitation of the immunomodulatory properties of this molecule. On the other hand, its delivery across the skin offers a new avenue in its supplementation.

List of abbreviations

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°C: degree Celsius

5-ASA: 5-aminosalicylic acid

aa: amino acids

ADAs: antidrug antibodies

Ag: antigen

AIA: adjuvant-induced arthritis

ANOVA: analysis of variance

APCs: antigen presenting cells

Aw: Van der Waals surface area

BSA: bovine serum albumin

BTLA: B and T lymphocyte attenuator

CA: California

CACTUS: Centro de Apoyo Científico-Tecnológico de la Universidad de Santiago de Compostela

CAD: cyclophosphamide-accelerated model of diabetes

CD: Crohn disease

cDNA: complementary deoxyribonucleic acid

CII: type II collagen

CIA: collagen-induced arthritis

CiMUS: Center for Research in Molecular Medicine and Chronic Diseases

CLSM: confocal laser scanning microscopy

CMCS: carboxymethyl chitosan

COVID-2019: coronavirus disease 2019

Cy5: cyanine dye

DAMP: danger-associated molecular pattern

DCMCS: *N,N*-dimethylhexadecyl carboxymethyl chitosan

DCs: dendritic cells

DiD: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate

dL: deciliter

DLS: dynamic light scattering

DMARDs: disease-modifying anti-rheumatic drugs

DMT: disease-modifying therapies

EAE: experimental allergic encephalomyelitis

EDC: N-(3-dimetrylaminopropyl)-N'-ethylcarbodiimide hydrochloride

EE: encapsulation efficiency

FBS: fetal bovine serum

FESEM: field emission scanning electron microscopy

Fig: figure

GA: glatiramer acetate

GALT: gut-associated lymphoid tissue

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GI: gastrointestinal

GIT: gastrointestinal tract

GM-CSF: granulocyte-macrophage colony-stimulating factor

HA: hyaluronic acid

HACS: high amylose corn starch

hDCs: human dendritic cells

HLA: human leukocyte antigen

HPLC: high performance liquid chromatography

IBD: inflammatory bowel disease

ICH: International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use

ID: intradermal

iDCs: immature dendritic cells

IDO: indoleamine 2,3-dioxygenase

IFN-β: interferon β

IFN-γ: interferon γ

IL: interleukin

ILT: immunoglobulin-like transcript

IM: intramuscular

IMM: immunomodulatory molecule

InsA: insulin A chain

InsB: insulin B chain

IP: intraperitoneal

ITE: 2-(1'*H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester

IV: intravenous

LDA: laser-doppler anemometry

LDHA: lactate dehydrogenase A

LNPs: lipid nanoparticles

LOD: limit of detection

LOQ: limit of quantification

LPS: bacterial lipopolysaccharide

MA: Massachusetts

MAPK: mitogen-activated protein kinase

MBP: myelin basic protein (MBP)

MCR: mean count rate

MD: Maryland

mDCs: mature dendritic cells

mg: milligrams

MHC: major histocompatibility complex

MI: Michigan

min: minutes

mL: milliliters

MO: Missouri

MOG: myelin oligodendrocyte protein

MPs: microparticles

MS: multiple sclerosis

MTS: 3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

Mw: molecular weight

n/a: no applicable

NASH: non-alcoholic steatohepatitis

NCs: nanocapsules

NF-κβ: nuclear factor kappa-light-chain-enhancer of activated B cells

NHS: N-hydroxysuccinimide

NJ: New Jersey

NLCs: nanostructured lipid carriers

nm: nanometers

NPs: nanoparticles

NOD: non obese diabetic

NOD-HHD: NOD.B6-Tg(HLA-A2.1)Enge/DvSJ

NOD.SCID: immunodeficient NOD mice

NSAIDs: non-steroidal anti-inflammatory drugs

NTA: nanoparticle tracking analysis

NVs: nanocarriers

NVAs: anionic nanocarriers

NVCs: cationic nanocarriers

OAE: oleoyl alginate ester

OCT: optimal cutting temperature

PBLs: peripheral blood lymphocytes

PBMCs: peripheral blood mononuclear cells

PBS: phosphate buffer saline

PCS: photon correlation spectroscopy

PDI: polydispersity index

PD-L1: programmed death-ligand 1

pDNA: plasmid DNA

PEG: polyethylene glycol

PEG-DTR: polyethylene glycol-desaminotyrosyl-tyrosine ester triblock copolymers

PFA: paraformaldehyde

PFKFB4: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4

pg: picograms

PGA: poly(L-glutamic acid)

PGIA: proteoglycan-induced arthritis

PGE_2: prostaglandin E_2

PLA: poly(D, L) lactic acid

PLGA: poly(lactic-co-glycolic acid)

PLL: poly(L-lysine)

PLP: proteolipid protein

pMHC: antigen-MHC complexes

PPI: preproinsulin

PS: phosphatidylserine

PSG: penicillin-streptomycin-glutamine

qPCR: quantitative polymerase chain reaction

R2: RPMI medium containing 2% FBS and 1% PSG

R10: RPMI medium containing 10% FBS and 1% PSG

RA: rheumatoid arthritis

R-EAE: relapsing experimental allergic encephalomyelitis

Ref: reference

RNA: ribonucleic acid

rpm: revolutions per minute

RPMI: Roswell Park Memorial Institute

RT: room temperature

SC: subcutaneous

SD: standard deviation

SGF: simulated gastric fluid

SGI: simulated gastrointestinal

SIF: simulated intestinal fluid

siRNA: small interfering RNA

SLE: systemic lupus erythematous

SPI: soy protein isolate

StC: stratum corneum

STEM: scanning transmission electron microscopy

T1D: type 1 diabetes

TFA: trifluoroacetic acid

TGF-β1: transforming growth factor beta 1

tLNPs: targeted lipid nanoparticles

TLR: toll-like receptor

TNF-α: tumor necrosis factor α

tolDCs: tolerogenic dendritic cells

UC: ulcerative colitis

UK: United Kingdom

UPLC: ultra performance liquid chromatography

US: United States

USC: Universidade de Santiago de Compostela

UV: ultraviolet

VT: Vermont

v/v: volume / volumen

WI: Wisconsin

WPI: whey protein isolate

w/v: weight / volume

µg: micrograms

µL: microliters

µm: micrometer

Ethical considerations and Permissions

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Animal studies

The studies in mice described in Chapter 2 were done at the Centre for Research in Molecular Medicine and Chronic Diseases (CiMUS) of the Universidade de Santiago de Compostela (Spain). The protocol used was approved by the "Comité Ético para la Experimentación Animal" of the Universidade de Santiago de Compostela (project number: 15010/10/002). The experiments were done following National (RD 53/2013) regulations for transport, housing, and care of laboratory animals, in compliance with the Directive 2010/63/EU of the European Parliament and Council of 22nd September 2010 on the protection of animals used for scientific purposes.

Human-derived samples

Evaluation of nanocarriers in human dendritic cells described in Chapter 2 was conducted at the Faculty of Pharmacy of the Universidade de Santiago de Compostela (Spain). The experiments were approved by the "Comité Autonómico de Ética da Investigación de Galicia" (project number: 2014/543). Experiments were conducted following the Spanish and European laws. Primary human immune cells from healthy donors for the preparation of dendritic cells were recovered from the buffy coats of blood donations. Buffy coats were obtained from anonymous healthy blood donor at an authorized organization (Agencia de Donación de Órganos y Sangre).

Evaluation of nanocarriers penetration in human skin was done in the CiMUS of the Universidade de Santiago de Compostela. Human skin samples were collected immediately after surgery from the Department of Plastic, Aesthetic & Reconstructive Surgery (Complexo Hospitalario Universitario de Santiago, Universidade de Santiago de Compostela). The experiments were approved by the "Comité Autonómico de Ética da Investigación de Galicia" (project number: 2017/103) and carried out following the Spanish and European laws.

Authorization of Experiments using Human-Derived Samples (Chapter 2)

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DITAME DO COMITÉ DE ÉTICA DA INVESTIGACIÓN DE SANTIAGO-LUGO

Juan Manuel Vázquez Lago, Secretario do Comité de Ética da Investigación de Santiago-Lugo

CERTIFICA:

Que este Comité avaliou na súa reunión do día 20/01/2015 o estudo:

Título: Diseño de nanovacunas terapéuticas basadas en péptidos: aplicación al tratamiento de enfermedades autoinmunes
Promotor: África González Fernández, María Josefa Alonso Fernández, Rubén Varela Calviño
Tipo de estudo: Outros
Versión:
Código do Promotor:
Código de Rexistro: 2014/543

E, tomando en consideración as seguintes cuestións:

- A pertinencia do estudo, tendo en conta o coñecemento dispoñible, así coma os requisitos legais aplicables, e en particular a Lei 14/2007, de investigación biomédica, o Real Decreto 1716/2011, de 18 de novembro, polo que se establecen os requisitos básicos de autorización e funcionamento dos biobancos con fins de investigación biomédica e do tratamento das mostras biolóxicas de orixe humana, e se regula o funcionamento e organización do Rexistro Nacional de Biobancos para investigación biomédica, a ORDE SAS/3470/2009, de 16 de decembro, pola que se publican as Directrices sobre estudos Posautorización de Tipo Observacional para medicamentos de uso humano, e a Circular nº 07/2004, investigacións clínicas con produtos sanitarios.
- A idoneidade do protocolo en relación cos obxectivos do estudo, xustificación dos riscos e molestias previsibles para o suxeito, así coma os beneficios esperados.
- Os principios éticos da Declaración de Helsinki vixente.
- Os Procedementos Normalizados de Traballo do Comité.

Emite un **INFORME FAVORABLE** para a realización do estudo **polo/a investigador/a do centro:**

Centros	Investigadores Principais
Universidade de Vigo	África González Fernández
Univerisdade de Santiago de Compostela	María Josefa Alonso Fernández
	Rubén Varela Calviño

En Santiago de Compostela, a 20 de xaneiro de 2015
O secretario

juan.manuel.vazquez.lago@sergas.es

Juan M. Vázquez Lago

Authorization of Animal Experiments (Chapter 2)

REXISTRO XERAL DA XUNTA DE GALICIA
REXISTRO DO EDIFICIO ADMINISTRATIVO DA CORUÑA
A CORUÑA

Data 03/08/2016 13:11:59

XUNTA DE GALICIA
DELEGACIÓN TERRITORIAL DA CORUÑA
Xefatura Territorial da Consellería do Medio Rural

Edificio Administrativ
Vicente Ferrer, 2
15071 A Coruña

SAÍDA 98655 / RX 1124140

José Manuel Cifuentes
Departamento de Anatomía
Facultade de Veterinaria
Campus Universitario
27002 Lugo

NOTIFICACIÓN DE RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 15010/16/002
Interesado: Rubén Varela Calviño
Procedemento: resolución de autorización

Data de inicio: 1-07-2016
Forma de inicio: solicitude do interesado

Notifícolle que con data 1 de agosto de 2016, o xefe territorial da Consellería do Medio Rural emitiu unha resolución de autorización do proxecto de experimentación animal, cuxo texto íntegro é o seguinte:

ANTECEDENTES

O interesado, como representante do centro CIMUS (Universidade de Santiago de Compostela), presentou con data 30-06-2016 e rexistro de entrada 80870 RX 1767387, unha solicitude para a realización do proxecto de experimentación animal, cuxos datos se detallan a continuación:

Denominación do proxecto: Deseño de nanovacinas terapéuticas baseadas en péptidos: aplicación ao tratamento de enfermidades autoinmunes
Nome do centro usuario: CIMUS (USC)
Persoa responsable do proxecto: Rubén Varela Calviño
Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): CIMUS (USC)
Clasificación do proxecto : Tipo I ☐ Tipo II ☒ Tipo III ☐

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1. O Real decreto 53/2013, de 1 de febreiro (BOE núm. 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

2. O artigo 89 da Lei 30/1992, de 26 de novembro, do réxime xurídico das administracións públicas e do procedemento administrativo común (BOE núm. 285, 27 de novembro de 1992), modificada pola Lei 4/1999, de 14 de xaneiro, establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

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3. O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto, realizada polo órgano habilitado Sección de Experimentación Animal do Comité de Bioética da Universidade de Santiago de Compostela.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co artigo 11 do Decreto 245/2009 de 3 de abril, polo que se regulan as delegacións territoriais da Xunta de Galicia e o Decreto 46/2012, de 19 de xaneiro, polo que establece a estrutura orgánica da Consellería do Medio Rural e do Mar e do Fondo Galego de Garantía Agraria.

De acordo con todo o indicado, RESOLVO:

1. Autorizar o proxecto solicitado.
2. Notificarlle esta resolución ao interesado.

O mencionado proxecto non precisa someterse a unha avaliación retrospectiva.

A autorización deste proxecto terá unha duración de 5 anos, transcorridos os cales, deberá ser renovada esta autorización.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida, no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpor recurso de alzada ante a conselleira de Medio Rural da Xunta de Galicia no prazo dun mes contado a partir da recepción da notificación da presente resolución, conforme coa Lei 30/1992, do 26 de novembro, (BOE núm.: 285, 27 de novembro de 1992), de réxime xurídico das administracións públicas e do procedemento administrativo común na súa redacción dada pola Lei 4/1999, do 13 de xaneiro.

A Coruña, 2 de agosto de 2016
O xefe do Servizo de Gandaría
PA: o xefe de área

José Luis Puerta Villegas

Authorization of Experiments using Human-Derived Samples (Chapter 3)

XUNTA DE GALICIA
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Secretaría Xeral Técnica

Secretaría Técnica
Comité Autonómico de Ética da Investigación de Galicia
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DICTAMEN DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN DE SANTIAGO-LUGO

Guillermo José Prada Ramallal, Secretario del Comité de Ética de la Investigación de Santiago-Lugo,

CERTIFICA:

Que este Comité evaluó en su reunión del día 23 de marzo de 2017 el estudio:

Título: Evaluación del transporte de fármacos antiinflamatorios a través de la Piel
Promotor: M.ª José Alonso Fernández
Tipo de estudio: Outros
Versión:
Código del Promotor:
Código de Registro: 2017/103

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humana, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Postautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07/2004, de investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos da Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del Comité.

Emite un dictamen **FAVORABLE** para la realización del estudio **por el/la investigador/a del centro:**

Centros	Investigadores Principales
CIMUS. Universidade de Santiago de Compostela (USC)	M.ª José Alonso Fernández

En Santiago de Compostela, a 30 de marzo 2017.

El Secretario del Comité Territorial de Ética de la Investigación de Santiago Lugo,

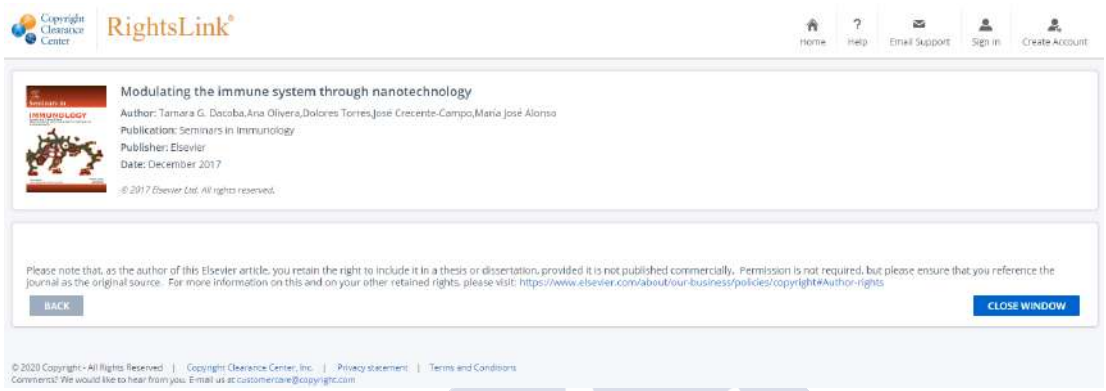
Firmado digitalmente por: guillermo.jose.prada.ramallal@sergas.es
Fecha: 2017.03.30 14:55:37 +02'00'

Guillermo José Prada Ramallal

Attributions to the figure employed in the Introduction

Permission to use and extract from a published review in Chapter 1

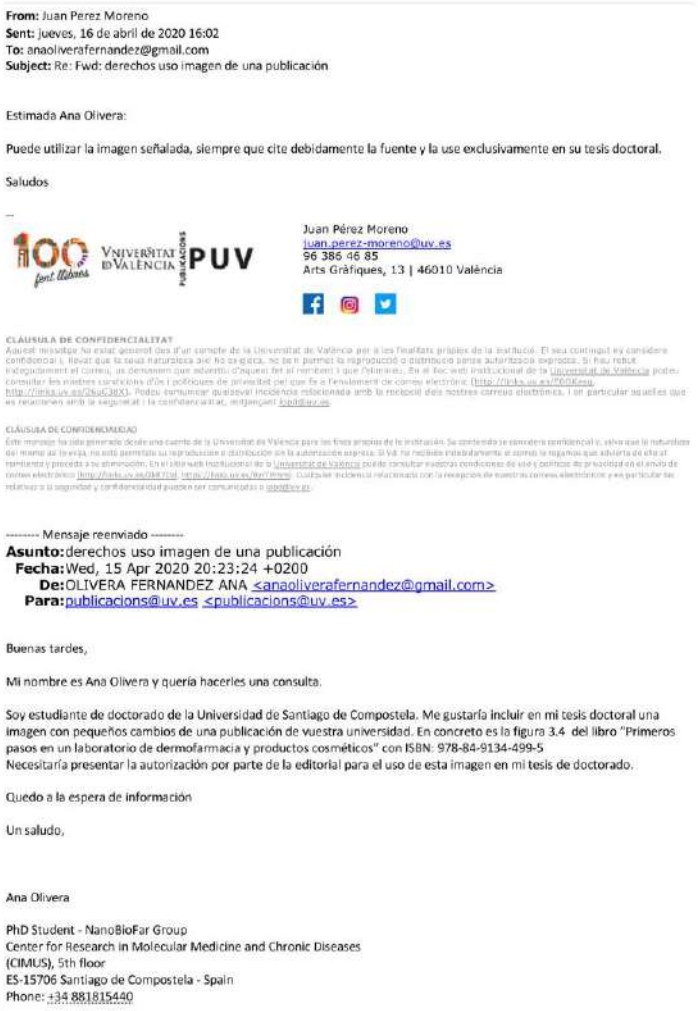
Origin review: T.G. Dacoba, A. Olivera, D. Torres, J. Crecente-Campo, M.J. Alonso, Modulating the immune system through nanotechnology, Semin. Immunol. 34 (2017) 78-102. Doi: 10.1016/j.smim.2017.09.007.



Permission to use an image from a published book in Chapter 3

Figure 6A was adapted with permissions from an already published book.

Original image: figure 3.4 in S. Del Río-Sancho, V. Santer, C.E. Serna Jiménez, A. López Castellano, V. Merino, Primeros pasos en un laboratorio de dermofarmacia y productos cosméticos, Universitat de València, Valencia, 2019.



From: Juan Perez Moreno
Sent: jueves, 16 de abril de 2020 16:02
To: anaoliverafernandez@gmail.com
Subject: Re: Fwd: derechos uso imagen de una publicación

Estimada Ana Olivera:

Puede utilizar la imagen señalada, siempre que cite debidamente la fuente y la use exclusivamente en su tesis doctoral.

Saludos

--

Juan Pérez Moreno
juan.perez-moreno@uv.es
96 386 46 85
Arts Gràfiques, 13 | 46010 València

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CLÁUSULA DE CONFIDENCIALIDAD

-------- Mensaje reenviado --------
Asunto: derechos uso imagen de una publicación
Fecha: Wed, 15 Apr 2020 20:23:24 +0200
De: OLIVERA FERNANDEZ ANA <anaoliverafernandez@gmail.com>
Para: publicacions@uv.es <publicacions@uv.es>

Buenas tardes,

Mi nombre es Ana Olivera y quería hacerles una consulta.

Soy estudiante de doctorado de la Universidad de Santiago de Compostela. Me gustaría incluir en mi tesis doctoral una imagen con pequeños cambios de una publicación de vuestra universidad. En concreto es la figura 3.4 del libro "Primeros pasos en un laboratorio de dermofarmacia y productos cosméticos" con ISBN: 978-84-9134-499-5
Necesitaría presentar la autorización por parte de la editorial para el uso de esta imagen en mi tesis de doctorado.

Quedo a la espera de información

Un saludo,

Ana Olivera

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