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Characterization of Biomarkers of the Generation and Functionality of Tolerogenic Dendritic Cells Differentiated with Vitamin D3

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Caracterització de biomarcadors de la generació i la funcionalitat de cèl·lules dendrítiques tolerogèniques diferenciades amb vitamina D3

Caracterización de biomarcadores de la generación y la funcionalidad de células dendríticas tolerogénicas diferenciadas con vitamina D3

Thesis presented by Juan Navarro Barriuso to qualify for the PhD degree in Advanced Immunology by the Universitat Autònoma de Barcelona.

The presented work has been performed in the Immunology Division, at the Germans Trias i Pujol Research Institute (IGTP), and has been directed by Dr. Eva M. Martínez Cáceres.

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Certifica:

Que el treball experimental i la redacció de la memòria de la Tesi Doctoral amb títol '*Characterization of Biomarkers of the Generation and Functionality of Tolerogenic Dendritic Cells Differentiated with Vitamin D3*' han estat realitzats per en Juan Navarro Barriuso sota la seva direcció, i considera que és apta per ser presentada per optar al grau de Doctor en Immunologia Avançada per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signa aquest document a Badalona,
el 19 de setembre del 2019.

Dra. Eva M. Martínez Cáceres

A mi madre
y a la memoria
de mi padre

Abbreviations

Abbreviations

AIRE: Autoimmune regulator

APC: Antigen presenting cell/s

cDC: Conventional dendritic cell/s

CNPase: 2′,3′-cyclic-nucleotide 3′-phosphodiesterase

CNS: Central nervous system

DAMP: Danger-associated molecular pattern/s

DC: Dendritic cell

dexa-tolDC: Dexamethasone-generated tolerogenic dendritic cell/s

DP: Double positive

EAE: Experimental autoimmune encephalomyelitis

EDSS: Expanded disability status scale

GM-CSF: Granulocyte-macrophage colony-stimulating factor

HHV6: Human herpesvirus 6

HLA: Human leukocyte antigen

iDC: Immature dendritic cell/s

IFN: Interferon

IL: Interleukin

IL10-tolDC: Interleukin 10-generated tolerogenic dendritic cell/s

ILC: Innate lymphoid cell/s

iNKT: Invariant natural killer T (cell/s)

iTreg: Induced regulatory T cell/s

LPS: Lipopolysaccharide

MAG: Myelin-associated antigen

MBP: Myelin basic protein

mDC: Mature dendritic cell

MDDC: Monocyte-derived dendritic cell/s

MHC: Major histocompatibility complex

MOBP: Myelin-associated oligodendrocyte basic protein

MOG: Myelin oligodendrocyte glycoprotein

MRI: Magnetic resonance imaging

MS: Multiple sclerosis

mTEC: Medullary thymic epithelial cell/s

NK: Natural killer (cell/s)

nTreg: Natural regulatory T cell/s

PAMP: Pathogen-associated molecular pattern/s

pDC: Plasmacytoid dendritic cell/s

PLP: Proteolipid protein

PPMS: Primary progressive multiple sclerosis

PRR: Pattern recognition receptor/s

qPCR: Quantitative polymerase chain reaction

RA: Rheumatoid arthritis

rapa-tolDC: Rapamycin-generated tolerogenic dendritic cell/s

RRMS: Relapsing-remitting multiple sclerosis

SPMS: Secondary progressive multiple sclerosis

T1D: Type 1 diabetes

T_C: Cytotoxic T (cell/s)

T_{CM}: Central memory T (cell/s)

TCR: T cell receptor

T_{EM}: Effector memory T (cell/s)

TGF: Transforming growth factor

T_H: Helper T (cell/s)

TNF: Tumor necrosis factor

tolDC: Tolerogenic dendritic cell/s

Treg: Regulatory T cell/s

TT: Tetanus toxin

VDR: Vitamin D receptor

vitD3-tolDC: Vitamin D3-generated tolerogenic dendritic cell/s

vtdx-tolDC: Vitamin D + dexamethasone-generated tolerogenic dendritic cell/s

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Summary

The last years have witnessed a breakthrough in the development of tolerance-inducing cell therapies for the treatment of autoimmune diseases, such as multiple sclerosis (MS), as well as other immune-mediated pathologies. In particular, tolerogenic dendritic cell (tolDC)-based therapies have become a promising approach in clinical trials worldwide due to their potential ability to restore immune tolerance without compromising the protective immunity of the patients, contrary to conventional and currently available treatments. However, the broad variety of protocols used to generate tolDC *in vitro* and their functional and phenotypical heterogeneity are evidencing the need to find robust biomarkers as a key point towards their translation into the clinic, as well as better understanding the mechanisms involved in the induction of immune tolerance.

In this regard, the main goal of this thesis was to identify and validate common transcriptomic biomarkers of the generation and functionality of three of the most common tolDC-inducing protocols, using either rapamycin (rapa-tolDC), dexamethasone (dexa-tolDC) or vitamin D3 (vitD3-tolDC) to generate them. However, after performing a microarray study using with cells differentiated from healthy donors, we could not identify any differentially expressed gene in common for these three different tolDC protocols compared to both mature (mDC) and immature dendritic cell (iDC) control conditions. Nevertheless, our results revealed that dexa-tolDC and vitD3-tolDC, but not rapa-tolDC, do actually share several immune regulatory and anti-inflammatory pathways towards tolerance induction.

Afterwards, and due to their potent regulatory properties, we focused on the study of vitD3-tolDC. We could validate that *CYP24A1*, *MAP7* and *MUCL1* genes were strongly up-regulated in these cells, in samples from healthy donors and MS patients. Therefore, these genes constitute robust biomarkers of the adequate generation of vitD3-tolDC, and thus can be tested as a quality control in clinical trials for MS before the administration of these cells into the patients. Furthermore,

we constructed a functional network of protein interactions which evidenced that *MAP7* and *MUCL1* —but not *CYP24A1*— were involved in the modulation of many relevant immune-related pathways, such as HLA class II presentation and anti-inflammatory responses, and could have a crucial role in the tolerogenic properties of vitD3-tolDC.

Finally, in order to better understand the mechanisms triggered by vitD3-tolDC for the induction of immune tolerance, we developed a protocol to generate and study the transcriptomic profile of T $CD4^+$ cells upon their antigen-specific interaction with vitD3-tolDC, using an RNA-seq analysis. In this regard, our results evidenced an important down-modulation of several genes involved in cell cycle and in cell responses to, mainly, pro-inflammatory immune-related stimuli. However, only *JUNB* gene could be identified as a potential biomarker of the functionality of vitD3-tolDC, since its expression was slightly induced in those T $CD4^+$ cells that interacted with them.

Altogether, the results presented in this thesis describe the whole process of screening, selection and validation of transcriptomic biomarkers of the generation —and functionality— of vitD3-tolDC towards their final translation into the clinic, serving as a bridge from the bench to the bedside.

Resumen

En los últimos años se ha producido un gran avance en el desarrollo de terapias celulares inductoras de tolerancia para el tratamiento de enfermedades autoinmunitarias tales como la esclerosis múltiple (EM) y otras patologías inmunomediadas. En concreto, el uso de células dendríticas tolerogénicas (tolDC) se ha convertido en una alternativa terapéutica prometedora en ensayos clínicos de todo el mundo, debido a su potencial capacidad de restablecer la tolerancia inmunitaria sin poner en riesgo la inmunidad protectora de los pacientes, a diferencia de los tratamientos convencionales disponibles actualmente. Sin embargo, la amplia variedad de protocolos existentes para la generación *in vitro* de estas tolDC, así como su gran heterogeneidad fenotípica y funcional, han puesto de manifiesto la necesidad de encontrar biomarcadores robustos como un paso fundamental para la aplicación de estas células en el ámbito clínico, así como para entender mejor los mecanismos involucrados en la inducción de tolerancia inmunitaria.

En este sentido, el principal objetivo de esta tesis consistió en identificar y validar biomarcadores comunes de la generación y la funcionalidad de tres de los protocolos más habituales para la generación de tolDC, utilizando ya sea rapamicina (rapa-tolDC), dexametasona (dexa-tolDC) o vitamina D3 (vitD3-tolDC). Sin embargo, después de realizar un estudio mediante *microarray* utilizando células diferenciadas de donantes sanos, no fuimos capaces de identificar ningún gen diferencialmente expresado, en comparación con células dendríticas maduras (mDC) e inmaduras (iDC), que fuera común para estos tres protocolos de diferenciación de tolDC. No obstante, nuestros resultados revelaron que las dexa-tolDC y las vitD3-tolDC, pero no las rapa-tolDC, compartían la modulación varias rutas inmunoreguladoras y antiinflamatorias para la inducción de tolerancia.

Posteriormente, debido a sus potentes propiedades reguladoras, nos centramos en el estudio de las vitD3-tolDC. Así, pudimos validar que la expresión de los

genes *CYP24A1*, *MAP7* y *MUCL1* estaba fuertemente inducida en estas células, tanto en muestras de donantes sanos como de pacientes con EM. Consecuentemente, dichos genes pueden ser utilizados como biomarcadores robustos de la correcta generación de vitD3-tolDC y, por lo tanto, pueden ser probados como controles de calidad en ensayos clínicos para la EM antes de la administración de estas células a los pacientes. Además, elaboramos una red funcional de interacciones proteicas que evidenció que los genes *MAP7* y *MUCL1* —pero no *CYP24A1*— estaban involucrados en la modulación de varias rutas relevantes relacionadas con el sistema inmunitario, tales como la presentación vía HLA de clase II o respuestas antiinflamatorias y, consiguientemente, podrían tener un papel crucial en las propiedades tolerogénicas de las vitD3-tolDC.

Finalmente, con el objetivo de entender mejor los mecanismos desencadenados por las vitD3-tolDC para la inducción de tolerancia inmunitaria, desarrollamos un protocolo para generar y a continuación estudiar el perfil transcriptómico de células T $CD4^+$ tras su interacción antígeno-específica con vitD3-tolDC mediante un análisis de *RNA-seq*. En este sentido, nuestros resultados pusieron de manifiesto una importante represión de la expresión de varios genes involucrados en el ciclo celular y en la respuesta celular ante, principalmente, estímulos proinflamatorios. Sin embargo, tan solo el gen *JUNB* pudo ser identificado como un potencial biomarcador de la funcionalidad de las vitD3-tolDC, dado que su expresión estaba ligeramente inducida en aquellas células T CD4+ que habían interactuado con las éstas.

En resumen, los resultados presentados en esta tesis describen el procedimiento completo de cribado, selección y validación de biomarcadores transcriptómicos de la generación —y de la funcionalidad— de vitD3-tolDC, enfocados a su traslación final al ámbito clínico y sirviendo, por tanto, como un puente entre el laboratorio y los pacientes.

Resum

En els darrers anys s'ha produït un gran avenç en el desenvolupament de teràpies cel·lulars inductores de tolerància per al tractament de malalties autoimmunitàries tals com l'esclerosi múltiple (EM) i altres patologies immunomediades. Concretament, l'ús de cèl·lules dendrítiques tolerogèniques (tolDC) s'ha convertit en una alternativa terapèutica prometedora en assaigs clínics arreu de tot el món, a causa de la seva potencial capacitat de restablir la tolerància immunitària sense posar en risc la immunitat protectora dels pacients, a diferència dels tractaments convencionals disponibles actualment. Però, l'àmplia varietat de protocols existents per a la generació *in vitro* d'aquestes tolDC, així com la seva gran heterogeneïtat fenotípica i funcional, han posat de manifest la necessitat de trobar biomarcadors robusts com a un pas fonamental per a l'aplicació d'aquestes cèl·lules dins de l'àmbit clínic, així com per a entendre millor els mecanismes involucrats en la inducció de tolerància immunitària.

En aquest sentit, el principal objectiu d'aquesta tesi ha consistit a identificar i validar biomarcadors comuns de la generació i la funcionalitat de tres dels protocols més freqüents per a la generació de tolDC, utilitzant ja sigui rapamicina (rapa-tolDC), dexametasona (dexa-tolDC) o vitamina D3 (vitD3-tolDC). Però, després de realitzar un estudi mitjançant *microarray* utilitzant cèl·lules diferenciades de donants sans, no vam ser capaços d'identificar cap gen diferencialment expressat en comparació amb cèl·lules dendrítiques madures (mDC) i immadures (iDC), que fos comú per a aquests tres protocols de diferenciació de tolDC. Tot i això, els nostres resultats van revelar que les dexa-tolDC i les vitD3-tolDC, però no les rapa-tolDC, compartien la modulació de vàries rutes immunoreguladores i antiinflamatòries per a la inducció de tolerància.

A continuació, a causa de les seves potents propietats reguladores, ens vam centrar en l'estudi de les vitD3-tolDC. Així doncs, vam poder validar que l'expressió dels gens *CYP24A1*, *MAP7* i *MUCL1* estava fortament induïda en aquestes cèl·lules, tant en mostres de donants sans com de pacients amb EM. En

conseqüència, aquests gens poden ser utilitzats com a biomarcadors robusts de la correcta generació de vitD3-tolDC i, per tant, poden ser provats com a controls de qualitat en assaigs clínics per l'EM abans de l'administració d'aquestes cèl·lules als pacients. A més, vam elaborar una xarxa funcional d'interaccions proteiques que va evidenciar que els gens *MAP7* i *MUCL1* —però no *CYP24A1*— estaven involucrats en la modulació de vàries rutes rellevants relacionades amb el sistema immunitari, tals com la presentació via HLA de classe II o respostes antiinflamatòries i, consegüentment, podrien tenir un paper crucial en les propietats tolerogèniques de les vitD3-tolDC.

Finalment, amb l'objectiu d'entendre millor els mecanismes desencadenats per les vitD3-tolDC per a la inducció de tolerància immunitària, vam desenvolupar un protocol per a generar i, a continuació, estudiar el perfil transcriptòmic de cèl·lules T $CD4^+$ després de la seva interacció antigen-específica amb vitD3-tolDC mitjançant una anàlisi de *RNA-seq*. En aquest sentit, els nostres resultats van posar de manifest una important repressió de l'expressió de diversos gens involucrats en el cicle cel·lular i la resposta cel·lular davant de, principalment, estímuls proinflamatoris. Però, només el gen *JUNB* va poder ser identificat com a un potencial biomarcador de la funcionalitat de les vitD3-tolDC, ja que la seva expressió estava lleugerament induïda a aquelles cèl·lules T CD4+ que van interactuar amb aquestes.

En resum, els resultats presentats en aquesta tesi descriuen el procediment complet de cribratge, selecció i validació de biomarcadors transcriptòmics de la generació —i de la funcionalitat— de vitD3-tolDC, enfocats a la seva translació final a l'àmbit clínic i, per tant, fent de pont entre el laboratori i els pacients.

Introduction

1. Immune system. Tolerance and autoimmunity

1.1. Danger model; own versus foreign

The immune system is constituted by a complex association of cells, tissues and organs that humans and other species possess to protect themselves against external agents, such as virus, bacteria or fungi. Very often, its definition is based on the danger model proposed by Matzinger *et al*., 2002 (1), which, in brief, considers the immune system to be responsible to discriminate between what is dangerous and what is not. Consequently, it remains tolerant in front of those components from the organism that naturally belong to the body —which are considered innocuous— while it specifically attacks and eliminates infecting pathogens and every other potentially dangerous element. In general, the immune system is constantly developing a sentinel function by which it performs an extensive and exhaustive screening of any substance —known as antigen— that penetrates into the organism.

When a determined antigen is recognized by the immune system and an immune reaction is mounted in order to clear it, this process is known as an **immunogenic response**. Consequently, the capability of a determined antigen to cause an immunogenic response is known as **immunogenicity**. However, if said antigen is either not recognized by the immune system or recognized but not attacked by it, this given situation is known as **immune tolerance**. However, in this case, the concept of **tolerogenicity** would be known as the capability of a determined element of the immune system or a determined treatment to reestablish immune tolerance towards a specific antigen that was previously causing an immunogenic response. Finally, there is another important situation that takes place if a determined self-antigen is recognized and attacked by the immune system. In this case, this phenomenon is known as **autoreactivity** or **autoimmunity**, and it will be discussed later on.

Considering its functionality, the immune system can be classified by its specificity and speed of response into the innate and the adaptive immune systems. Furthermore, based on the nature of its effector mechanisms, both can also be classified into **humoral response** (if the immune response is mainly mediated by soluble macromolecules) and **cellular response** (if the immune response mainly is mediated by cells).

1.2. Innate and adaptive immunity

On the one hand, the **innate immune system** consists on a group of molecules —complement system proteins— and cells —monocytes, macrophages, natural killer (NK) cells, mastocytes, platelets and others— with a limited repertoire of preexistent —innate— receptors, known as pattern recognition receptors (PRR). These PRR are in charge of the recognition of a series of pathogen-associate molecular patterns (PAMP) —typically molecules associated with different classes of virus, bacteria and/or fungi— and danger-associated molecular patterns (DAMP) —which are related to endogenous components that are released during cell damage or death— in order to orchestrate a quick response to protect the organism in front of them. These PAMP and DAMP are normally constituted by a pool of proteins, glycolipids, nucleic acids and other molecules that are highly conserved through evolution, and therefore allow the immune system to unequivocally recognize them. Once one of these PAMP and/or DAMP have been recognized, the innate immunity immediately recruits its different molecular and cellular components for the clearance and resolution of the threat. Thus, the innate immune response is fast —practically immediate—, but also limited to those pathogens that can be recognized by the PRR.

On the other hand, we have the adaptive immunity. While the innate immunity provides a first layer of defense, and it is present in almost every multicellular organism, the **adaptive immune system** has only developed in vertebrates and

constitutes an evolution in the immune system that allows it to, virtually, recognize any potential antigen. The main cellular components in this case are two types of hematopoietic lymphocytes known as T and B cells, differentiated in the thymus and in the bone marrow, respectively. While T cells are in charge of the cellular response, B cells orchestrate the humoral response of the adaptive immune system through the production of antibodies. However, for the purpose of this thesis, we will focus on the T cell-mediated immune response.

Nevertheless, apart from the subpopulations hereby described, the immune system is a much more complex and layered, and therefore, there exist several other immune cells that serve as a bridge between the innate and adaptive immunity, such as the invariant NK T (iNKT) cells or the innate lymphoid cells (ILC). Briefly, while iNKT cells constitute a population of lymphocytes with a semi-invariant T cell receptor (TCR) that recognizes a limited set of lipid and glycolipid antigens (2), ILC are capable to produce a range of effector cytokines in order to help T cell responses despite their lack of antigen receptor (3).

Finally, the autocrine, paracrine and endocrine signaling between the different components of both the innate and the adaptive immune system —among other biological functions— are modulated by a heterogeneous group of soluble mediators generally known as **cytokines**. This family of proteins includes chemokines, interleukins, interferons, tumor necrosis factors and lymphokines, and are produced by a broad range of immune cells, such as macrophages, T and B lymphocytes or mastocytes. Upon their recognition by their specific receptors, cytokines are responsible for the induction, maintenance or abrogation of immune responses —that can be either immunogenic or tolerogenic—, as well as of cell differentiation, proliferation and migration —chemotaxis—. Furthermore, each cytokine can be produced by several cell types and can be recognized by different receptors —which can also overlap with other cytokines— but, more importantly, each cytokine can develop very different roles depending on the cells producing

them, the cells or tissues being targeted and the specific immune context. Consequently, cytokines are crucial players in the modulation and coordination of both innate and adaptive immune responses (4).

1.3. The T cell-mediated immune response

The recognition of a determined antigen by the immune system is not mediated by the identification of the whole molecule, but rather by small and characteristic pieces of it, called **peptides** or **epitopes**. The phenomenon of processing an antigen into its immunogenic peptides is mainly developed by dendritic cells (DC), but also other antigen presenting cells (APC). Thus, once an antigen is recognized by DC on the periphery, these cells process it and migrate into the secondary lymphoid organs —either the lymph nodes or the spleen—. There, the immunogenic peptides of said antigen are presented to T cells via the major histocompatibility complex (MHC) molecules —also known as human leukocyte antigen (HLA)—, which are recognized by the TCR that is present on the surface of T cells. If a determined T cell recognizes and eventually interacts with the peptide-MHC complex present on the surface of an APC, an immunogenic response will be developed if an adequate co-stimulatory signal is provided by the interaction of CD28 —on the lymphocyte— with CD80 and/or CD86 molecules —on the APC—. If that is the case, those specific naïve T cell clones will activate, proliferate and differentiate into effector —and memory— cells, in a process known as **clonal expansion**. When this happens, the expression of a variety of molecules on the membrane of T cells is induced, such as CD2 and CD18 —which increase the interaction of T cells with APC— and several activation markers like CD25, CD38 or CD69. However, the characteristics and targets of the immune response will be different depending on the MHC-peptide and the immune context at the moment of T cell activation, as it will be discussed below. The whole process normally takes around a week and, on the meantime, the infection is managed by the innate immune response.

When a determined antigen is recognized for the first time, this phenomenon is known as primary immune response, and it is mainly mediated by naïve T cells differentiated into effector T cells —in cooperation, of course, with other elements of the immune system—. However, in subsequent encounters with said antigen, the reaction is known as secondary immune response and, in this case, it is mediated by memory T cells, that differentiate into effector cells within a couple of days. Memory T cells are a subset with very specific characteristics: these cells present a long lifespan and have the ability to quickly expand into effector cells. Consequently, they persist in the organism of the host during most of its life, if not all, and can quickly orchestrate a secondary immune response. Specifically, memory T cells can be further divided into two main subtypes, based on their function and on their surface markers. On the one hand, T central memory (T_{CM}) cells are characterized by their CD45RO surface expression and reside in the secondary lymphoid tissues. In addition, T_{CM} cells share several similarities with naïve T cells regarding their homing receptors —since they also express CCR7 and CD62L on their surface—. However, unlike them, T_{CM} cells present higher sensitivity to antigen stimulation and are less dependent on co-stimulation, thus proliferating and differentiating into effector cells much faster than naïve T cells. On the other hand, T effector memory (T_{EM}) cells also express CD45RO on their surface, but unlike T_{CM} cells, they lack CCR7 and the expression of CD62L is heterogeneous. Compared to T_{CM}, T_{EM} cells reside in peripheral tissues throughout the body and are characterized by their faster effector function after antigen stimulation —within hours— (5). Therefore, when a secondary immune response is developed, the infection is normally controlled much faster than in a primary immune response. In both cases, however, the adaptive immunity makes use of several mechanisms typical from the innate immunity —such as the complement system and other cell types such as mastocytes— for the development and maintenance of immune responses.

1.4. T cell development and specificity

As suggested above, each T lymphocyte clone recognizes one single specific antigen, determined by its TCR. This specificity is achieved after a process of somatic rearrangement and recombination of said TCR widely known as **clonal selection**, which is random and antigen-independent, being able to generate a repertoire of T cells that recognize up to 10^{11} different antigens. The development of T cells takes place in the thymus and consists on a sequential two-step process of positive selection —by which only those cells whose TCR can recognize self-MHC molecules are chosen— and negative selection —by which the repertoire of potentially autoreactive cells is depleted—.

In addition, during the abovementioned process of positive selection, T cells acquire a specific "signature" that determines their functionality during the adaptive immune response. In their early stages of T cell development, these cells express both CD4 and CD8 molecules on their surface and, therefore, they are known as double positive (DP) T cells. However, by the time they have finished their maturation, those T cells that recognized an MHC class I molecule during clonal selection will become T $CD8^+$ cells, while those that recognized an MHC class II molecule will become T $CD4^+$ cells (6). However, CD4 and CD8 molecules do not simply constitute lineage markers, since they develop a crucial role in TCR recognition of peptides presented via MHC, among other functions (7). Briefly, T $CD8^+$ cells will be mainly involved in the immune response against virus and intracellular pathogens, while T $CD4^+$ cells will be the main orchestrators of the adaptive immune response recognizing foreign antigens and activating other elements of the cellular immune response against them. Either way, whether they are differentiated into T $CD8^+$ or T $CD4^+$ cells, those cells that have been able to successfully undergo through clonal selection are known as naïve T cells, since they are antigen-specific but have not encountered said antigen yet. After a primary immune response, both T $CD4^+$ and T $CD8^+$ cells will be capable to generate memory T cell subpopulations (5).

1.4.1. The CD8$^+$ cell-mediated immune response

T CD8$^+$ cells constitute, in general, a short-lived effector subset of T cells, although there are some exceptions, such as T CD8$^+$ memory cells. As discussed above, if an antigen is presented via an MHC class I, it will be recognized by T CD8$^+$ cells and the immune response will be mediated by them. Briefly, the peptides presented this way come from the cytosolic compartment of the cells. Thus, the main function of T CD8$^+$ cells is to develop a cytolytic process to eliminate cells infected by intracellular pathogens, mainly virus, and most of these cells will die once the infection has been cleared. In addition, T CD8$^+$ cell responses require of a tighter regulation in order to avoid immunopathology and autoimmunity due to their potentially dangerous functionality (8,9). Thus, if there is a sufficient level of co-stimulation, T CD8$^+$ cells might become activated through their interaction with an APC alone. However, if the co-stimulation is weak, further co-operation with T CD4$^+$ cells might be required.

Once an antigen has been recognized and T CD8$^+$ lymphocytes become activated, a series of cytolytic molecules, such as perforins and granzymes, are recruited to the membrane of these cells and are secreted by exocytosis in order to eliminate the target cell. For this reason, T CD8$^+$ cells are also known as cytotoxic T (T_C) cells. In addition, T CD8$^+$ cells can also induce apoptosis by the interaction of their CD95L molecules with the CD95 receptor from the target cell.

1.4.2. The CD4+ cell-mediated immune response

In this case, the T CD4$^+$ responses are mediated by MHC class II-peptide complexes, and these cells are also known as helper T (T_H) cells. Contrary to class I, MHC class II molecules present extracellular antigens. Furthermore, T CD4$^+$ cells can be divided into different subpopulations that are differentiated at the moment of antigen recognition, based on different factors such as the nature of the antigen, the type of APC interacting with them —as well as the affinity with its

MHC and the engagement with its co-stimulatory molecules— and the cytokine milieu surrounding these cells. Each of these phenotypes is characterized by the up-modulation of determined transcriptomic factors and by the cytokine profile they produce, as well as the components of the immune system that these cells modulate and target (**Figure 1**).

T helper 1 (T_H1). These cells are induced by the presence of interferon (IFN)-γ and interleukin (IL)-12, and their differentiation is dependent on the up-modulation of the T-bet transcription factor. T_H1 cells mainly secrete IFN-γ, as well as IL-2 and tumor necrosis factor (TNF)-α, among other cytokines, and constitute the main activators of macrophages and T $CD8^+$ cells. For this reason, T_H1 cells are in charge of the immune response against bacterial infections (10,11).

T helper 2 (T_H2). Induced by the presence of IL-4, these cells are characterized by the up-modulation of the GATA3 transcription factor. T_H2 cells are the principal activators of B cell and eosinophilic responses due to their strong production of IL-4, IL-5, IL-9 and IL-13, and thus mediate immune responses against extracellular bacteria and macroscopic parasites, but also asthmatic and allergic responses (11,12).

T helper 17 (T_H17). This subset of T $CD4^+$ cells is induced by IL-6, IL-1β and transforming growth factor (TGF)-β, and are dependent on the transcription factor ROR-γt. T_H17 cells are strong producers of IL-17 and IL-22 cytokines, and consequently they are involved in the promotion of pro-inflammatory responses. Furthermore, this cell population has been related with the pathogenesis of determined autoimmune diseases (11,13).

Regulatory T cells (Treg). These cells constitute a very special subset of T cells due to their antigen-specific immune regulatory properties, and can be subdivided into natural Treg (nTreg) and induced Treg (iTreg). While the former

are differentiated in the thymus during T cell development, upon recognition of self-antigens, the latter are induced in the periphery at post-natal stages under the influence of TGF-β and IL-2. However, in both cases, these cells are characterized by the up-regulation of the transcription factor FoxP3 and the production of TGF-β (11,14–16).

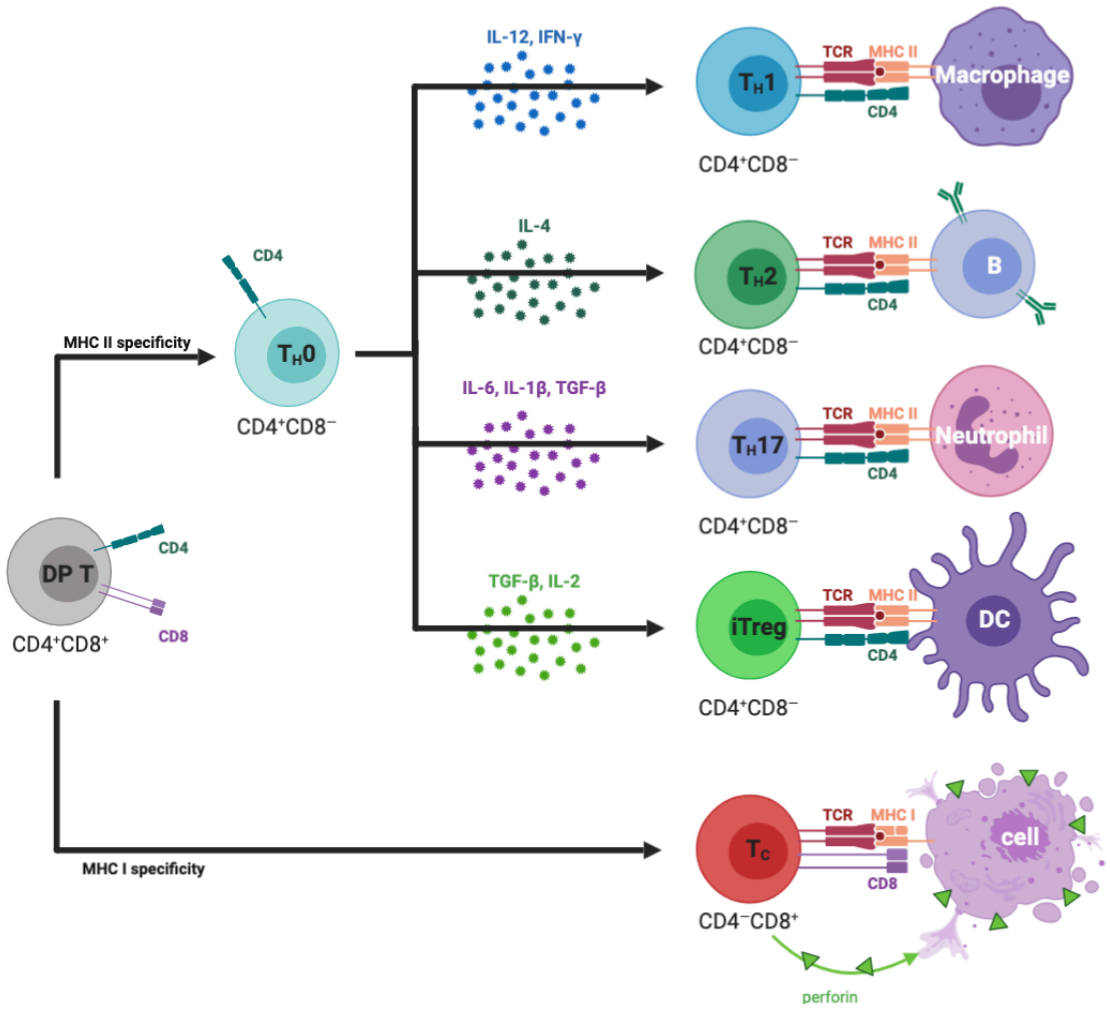

Figure 1. $CD4^+$ and $CD8^+$ T cell lineage. Depending on the conditions at the moment of antigen presentation, T $CD8^+$ and, especially, T $CD4^+$ cells, can differentiate into a variety of T cell subsets with very different functionalities and roles in the immune response. DC: dendritic cell; DP: double positive; IFN: interferon; IL: interleukin; MHC: major histocompatibility complex; T_C: cytotoxic T cell; TCR: T cell receptor; TGF: transforming growth factor; T_H: helper T cell;

Additionally, there also exist other T_H cell subpopulations that, although less studied, also mediate relevant responses over the immune system, such as T helper 3 (T_H3), T helper 9 (T_H9), T helper 22 (T_H22) and type 1 regulatory (Tr1) cells (11,17). Thus, the balance of the immune response between these subpopulations determines the type of reaction that is being mediated against a determined antigen, and therefore might become an indicator of whether it is more or less pro-inflammatory. In any case, the activation of macrophages via $CD4^+$ cell cytokines to kill facultative intracellular pathogens and the role of $CD8^+$ T cells in the killing of virally infected cells provides a control of intracellular infections that cannot be achieved by the innate system (18).

1.5. Generation and maintenance of immune tolerance

As stated above, the number of antigens that can be recognized by the adaptive immune system are practically unlimited, and furthermore, this repertoire is generated randomly. However, this particularity also implies that self-antigens might also be targeted by the immune system, but as a matter of fact, this is not the case. Therefore, there must exist some mechanisms to prevent this situation of autoreactivity and maintain the immune homeostasis between immunogenicity and tolerance. In this regard, immune tolerance is established at two different levels, and it consists on the elimination of developing T (and B) cells that are reactive to self-antigens. The first of them is called **central tolerance** and, briefly, it consists on the negative selection of developing autoreactive T cells during the second step of the abovementioned process of clonal selection. This phenomenon is mainly developed within the thymus by medullary thymic epithelial cells (mTEC). Specifically, mTEC drive a low-level expression of many tissue-specific self-antigens —regulated by a transcription factor known as autoimmune regulator (AIRE)— in order to present them to the TCR of developing thymocytes via MHC, causing its clonal deletion if the recognition of any auto-antigen happens (19–21). As a side note, for B cells, central tolerance is mediated within the bone marrow,

and those B cells that recognize any antigen present in this tissue are depleted by apoptosis or anergy.

This process of negative selection is rigorous and exhaustive, but yet some autoreactive cells manage to escape from the thymus and migrate to the periphery. If this happens, the second level of immune tolerance, the so-called **peripheral tolerance**, is able to control this escaped repertoire by several mechanisms such as the induction of anergy or deletion of autoreactive T cells, as well as the differentiation of Treg. However, in this case, peripheral tolerance is mainly controlled by a different subset of antigen presenting cells, the dendritic cells (DC). Nevertheless, DC are classically involved in T cell priming and activation in order to trigger the adaptive immune response against a specific antigen (21–23). Therefore, the role of DC in the balance between tolerance and autoimmunity is complex and bidirectional. In the case of B cells, however, the induction of peripheral tolerance mainly relies on the absence of autoreactive T_H cells, which is achieved by the abovementioned mechanisms, since B cells require the coordination with T_H cells for the development of an immune response.

1.6. Breach of tolerance and development of autoimmune diseases

When the immune homeostasis between immunogenicity and immune tolerance is disrupted, a situation of autoreactivity may be developed. Considering that, **autoimmunity** is defined as an induction of destructive responses against self-antigens mediated by the adaptive immune system, due to a loss of tolerance towards them, causing cell and tissue damage. This situation might result in a pathophysiological condition and, if maintained in time, in the development of **autoimmune diseases**, such as type 1 diabetes (T1D), rheumatoid arthritis (RA) or multiple sclerosis (MS). As a side note, it is important to remark that the innate immune system is unable to mediate an autoimmune response, since its repertoire of antigen recognition is limited and well established by their PRR to recognize

PAMP and DAMP only, and in any case a self-antigen. However, several of the innate mechanisms, such as the complement, might be involved in a situation of autoimmunity, but merely as effector tools mislead by an autoreactive response of the adaptive immune system. Consequently, autoimmune diseases are defined as complex and chronic disorders by which an inadequate attack against self-tissues and/or organs is mediated by the immune system.

The actual causes that lead to the development of autoimmune diseases are mostly unknown. However, several studies have shown that a combination of environmental factors and genetic susceptibility constitute the main risk factors for the loss of immune tolerance (24). Concerning genetic factors, even though autoimmune diseases can be monogenic —meaning that they are caused by a defect in one single gene—, in the majority of them multiple genetic and epigenetic factors are involved (**Figure 2**). For instance, a variety of preliminary and genome wide association studies have pointed towards MHC genes as one of the main susceptibility factors. However, all of them lack of a significant predictive value in the clinic, although they illustrate the predisposition to autoimmunity within families (25–27). As for environmental factors, the most commonly mentioned are the diet, infectious agents —due to epitope mimicry between certain structures from virus or bacteria and determined self-antigens—, the microbiota and xenobiotics, such as tobacco smoke, drugs, UV light or heavy metals (28–30). In any case, for an aberrant situation of autoimmunity to happen in an individual with genetic and/or environmental predisposition, a failure in one or more of the immune checkpoints —that regulate and maintain immune homeostasis— would be still necessary (31). Altogether, this combination of circumstances becomes significantly rare. Therefore, autoimmune diseases generally develop in susceptible individuals under specific environmental conditions, although not only on them and, especially, not in all of them.

However, even though autoimmune diseases are considered as conditions with a relatively low prevalence, its incidence increases every year and its aggregated prevalence is estimated by some authors in over the 9% of the population of Europe and North America (32). Separately, the year of onset, the prevalence and the incidence of each autoimmune disease varies. However, in general terms, these conditions tend to appear between 20 - 40 years of age, with an increased prevalence among women over men. Consequently, the socioeconomic impact of some of these pathologies has a high relevance, especially in developed countries. The general geoepidemiologic and organ-specific characteristics of the most prevalent autoimmune diseases are shown in **Table 1**.

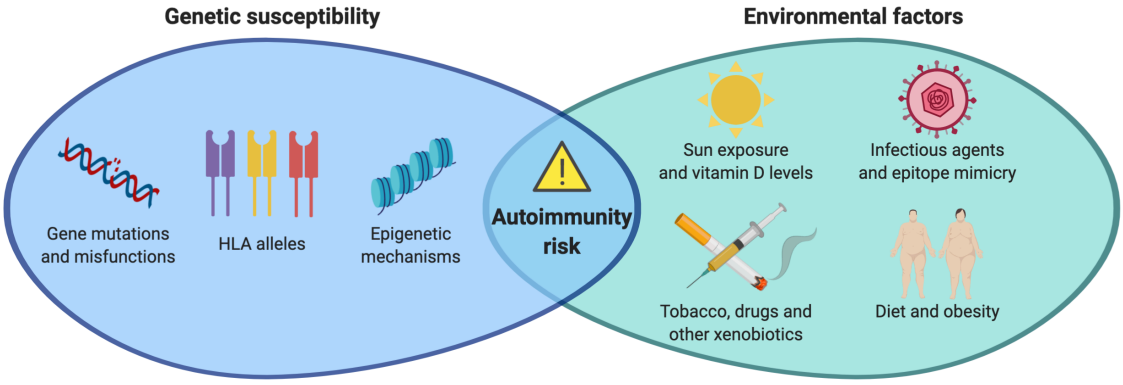

Figure 2. Genetic and environmental basis of autoimmunity. A variety of genetic mutations (i.e. *AIRE*, *TNFRSF6* or *FOXP3*) and misfunctions (i.e. *PRPN22* and *BACH2*), the presence of susceptible human leukocyte antigen (HLA) alleles and other epigenetic mechanisms (i.e. methylation, acetylation and microRNA), combined with determined environmental factors such as limited sun exposure (which leads to low vitamin D levels), several infectious agents (i.e. human herpesvirus and Epstein-Barr virus), diet and obesity, as well as tobacco, drugs and other xenobiotics, have been implicated with an increased risk for the development of specific autoimmune diseases.

Table 1. Organ-specific and geoepidemiologic features of the most prevalent autoimmune diseases in Europe.

Disease	**Target organ**	**Known antigen/s**	**Incidence in Europe** *(per 100,000 person-years)*	**Gender ratio** *(female/male)*
Coeliac disease	Small intestine	Tissue transglutaminase	1.5 - 8.7	1/1
Crohn's disease	Gastrointestinal tract	Desmin, Tubulin-β isoform 5	3.1 - 12.7	1/1.2
Graves' disease	Thyroid	TSHR, Na iodide transporter	21 - 50	5/1
Multiple sclerosis	Central nervous system	Myelin protein	0.8 - 8.7	2/1
Rheumatoid arthritis	Synovium of joints	Rheumatoid factor, Keratin, CCP, collagen, fibronectin	9 - 36	2/1
Systemic lupus erythematosus	Several organs (heart, joints, skin, lungs, blood vessels, liver, kidneys and nervous system)	Cardiolipin, carbonic anhydrase II, collagen, RNA polymerase I-III, fibronectin, golgin, C1q, histone H2A-H2B-DNA	1.0 - 5.0	9/1
Type 1 diabetes	Pancreas β-islets	Glutamate decarboxylase, insulin, insulin receptor	> 20	1/1
Ulcerative colitis	Colon	Desmin, Tubulin-β isoform 5	4.1 - 16.5	1/1

Table adapted from Wang *et al*., *Journal of International Medicine,* 2015 (27). CCP: cyclic citrullinated peptide; TSHR: thyroid stimulating hormone receptor.

2. Multiple sclerosis: a breach of tolerance against myelin peptides

2.1. General and demographic characteristics of multiple sclerosis

MS is a neurodegenerative, chronic disease of the central nervous system (CNS), characterized by inflammation, demyelination and axonal damage (33). MS normally affects people at the age of 20–50, having its mean age of onset at 30, and it is between 2 and 3 times more frequent among women than men (27,34). Currently, there is no cure for MS, and it is estimated that 50 % of patients will require help to walk within 15 years after the diagnosis. The combination of these factors makes of MS a disease with a highly important socioeconomic impact, being the main cause of neurological non-traumatic disability among young adults, and affecting about 60–80 per 100,000 persons in Europe, according to data proceeding from the World Health Organization.

There is a wide variety of neurological and physical symptoms related with MS as a consequence of the neurological impairment following the demyelization process; paresthesia, ataxia, fatigue, diplopia or incoordination are among the most recurrent manifestations. The progression of the disease is frequently followed based on clinical criteria, such as the determination of the Expanded Disability Status Scale (EDSS) score of the patients, and on the detection of white matter lesions by magnetic resonance imaging (MRI) techniques. However, associations between clinical and MRI measures are not always possible to be established. Currently, there is not an effective diagnostic test for MS, and thus the diagnosis primarily relies on the conjunction of clinical, radiological and laboratory data —mainly the detection of abnormalities in the cerebrospinal fluid of the patients, like the presence of oligoclonal bands—. In any case, none of these evidences alone are sufficient to perform a diagnosis, and thus there is always a strong dependence on the clinical expertise of the physician in order to demonstrate evidence of the dissemination of white matter lesions in space and time, as well as excluding other neurological disorders (34–38).

2.2. Clinical courses

The prognostic and clinical course of MS is highly variable between patients, but several forms of the disease can be differentiated. MS can be first classified into a relapsing (85 %) or a progressive (15 %) form, and both can present an active or not-active course according to disease activity by clinical and radiological criteria. On the one hand, the relapsing form, known as relapsing-remitting MS (RRMS), is marked by periods of relapses of symptoms (active disease) combined with phases of remission in which the symptoms whether improve or even fully disappear (not-active disease). On the other hand, in the progressive form, primary progressive MS (PPMS), the symptoms worsen gradually from the beginning, whether if it is with or without relapsing-remitting cycles. Furthermore, clinical courses are not static, and about 50 % of RRMS patients convert to a progressive form after 10–15 years, the so-named secondary progressive MS (SPMS), which can also be classified into active or not-active (39).

2.3. Etiology

Nowadays, MS is widely accepted as an autoimmune-mediated disease of the CNS that develops after a break of self-tolerance against myelin peptides, leading to inflammation, demyelization and finally axonal damage in the white matter, but the actual trigger of MS remains unknown (40). As discussed above, a complex combination of genetic and environmental factors is thought to be involved on the development of the autoreactive T cell repertoire. Specifically, several studies have pointed to determined factors conferring susceptibility to MS, such as several haplotypes from MHC class II molecules —such as HLA-DRB1*1501— (41,42) and miRNA signaling (miR-21) (43–45), a misfunction of *BACH2* gene (46) or the hypomethylation of the Src homology region 2 domain-containing phosphatase-1 (*SHP-1*) gene (47). For this reason, and as a demonstration of the genetic component of the disease, the risk to develop MS is of around 2-4 % in first-degree

relatives of patients —compared to around 0.1% in the general population—, and of around 30 to 50 % in monozygotic twins (48).

As for the environmental factors, low vitamin D levels and high geographic latitudes —which negatively correlate, since the latter directly affects sunlight exposure and thus modulates the former—, smoking, obesity and microbial infections by the human herpesvirus 6 (HHV6) —potentially due to epitope mimicry between the myelin basic protein and the HHV6-endoced U24 protein (49)— or the Epstein-Barr virus have also been related to a variable risk of developing MS (48,50,51,38). Consequently, a combination of both genetic and environmental factors is required.

The current hypothesis is that the attack against the myelin antigens starts with the disruption of the integrity of the blood-brain barrier in genetically predisposed people, for reasons that still remain unknown. This event allows the entrance of activated autoreactive T cells into the CNS, where they are able to recognize myelin antigens and other autoantigens presented by DC, triggering a complex immune response that leads to the formation of an acute inflammatory and demyelinating lesion (48,52). In order to elucidate the actual immune mechanisms that mediate demyelization, the animal model of MS, the experimental autoimmune encephalomyelitis (EAE), has been of a crucial importance, along with studies in MS patients (53).

2.4. Pathology and pathogenesis

The lesions caused by MS can appear throughout the whole CNS, including the optical nerve and the spinal cord —where they tend to induce more disability on patients—, and both on the gray and white matter (54–56). These lesions appear as focal areas of demyelination and inflammation, are heterogeneous and evolve unpredictably along the course of the disease —although recent studies suggest

that they may repair more effectively in young people (57)—. Furthermore, it remains unclear whether they can be eventually remyelinated (48,58).

The immune response against myelin antigens was classically thought to be mediated by autoreactive $CD4^+$ T_H1 cells, whose IL-12 production would initiate the inflammatory cascade leading to autoimmunity in the CNS. Nevertheless, its actual role in MS pathogenesis remains elusive, and it is remarkable that myelin-autoreactive T cells can also be retrieved from the blood and cerebrospinal fluid of healthy controls, as well as of patients (59). However, as shown in **Figure 3**, it has been evidenced that the pathogenesis of MS also involves many other mechanisms from the innate and adaptive immune system, such as $CD8^+$ cells, B cells, myelin autoantibodies and the complement, together with intrinsic processes of the CNS (34,52,60). Among these mechanisms, $CD4^+$ T_H17 cells have recently unveiled as one of the most relevant immune features in the pathogenesis of MS. The differentiation of this IL-17-producing subset mostly depends on IL-6, IL-23 and TGF-β, and they seem to be involved in the aggravation of the autoimmune processes. Several studies even point to IL-17 and IL-23 as the critical cytokines for the inflammatory process in the brain, instead of IL-12 (61). Consequently, a switch towards a T_H2 immune profile would potentially ameliorate the course of the disease as it would contribute to generate a less pro-inflammatory environment. On the other hand, a very recent study has evidenced the existence of a GM-CSF^+ $CXCR4^+$ T helper subset that appears selectively enriched in RRMS patients. Interestingly, these cells are directly affected by the effect of dimethyl fumarate, a commonly used disease-modifying therapy for MS, and thus constitute an interesting candidate for a therapeutic target (62).

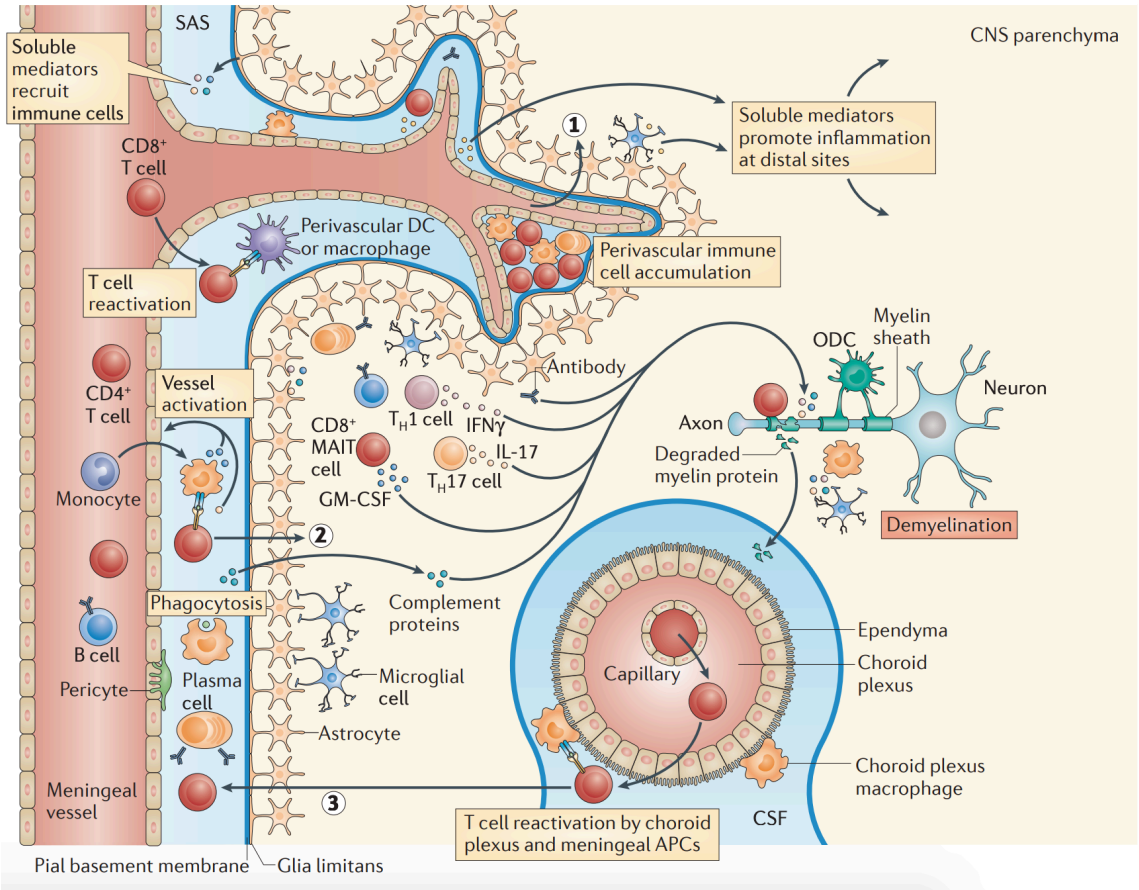

Figure 3. Immunopathogenesis of multiple sclerosis. Adapted from Dendrou *et al.*, Nature Reviews Immunology 2015 (63). Infiltration of both innate and adaptive peripheral immune cells into the central nervous system (CNS) can occur by direct crossing of the blood-brain barrier from the meningeal blood vessel (1), from the subarachnoid space (SAS; 2) or across the blood-cerebrospinal fluid (CSF) barrier from the choroid plexus (3). Once inside the CNS parenchyma T_H1 and T_H17 cells, along with T $CD8^+$ lymphocytes, complement proteins and antibodies mediate the demyelinating process and the subsequent oligodendrocyte (ODC) and neuroaxonal injury. APC: antigen-presenting cell; MAIT cell: mucosa-associated invariant T cell; DC: dendritic cell; FDC: follicular dendritic cell; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; T_H: Helper T cell.

The specific autoantigens in MS are still a matter of debate, and a lot of effort is being put in order to clarify which are the actual epitopes that are targeted by the autoreactive immune cells —although in many cases these cells can also be found in the healthy population—. In this regard, several peptides from myelin-

related proteins, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP), have been identified. Furthermore, different less prevalent epitopes from other myelin proteins like myelin-associated antigen (MAG), myelin-associated oligodendrocyte basic protein (MOBP) and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase), as well as peptides from S100β and transaldolase H proteins, have also been related with the pathophysiology of MS (64,65). Specifically, a previous study by our group evidenced that over 70 % of RRMS patients were reactive against a mix of $MBP_{13—32}$, $MBP_{111—129}$, $MBP_{146—170}$, $MBP_{83—99}$, $MOG_{1—20}$, $MOG_{35—55}$ and $PLP_{139—154}$ peptides (66). What seems already clear, according to observations in MS patients, is that one single antigen cannot be responsible for the whole pathogenic process, since as the disease progresses new autoreactive T cells are activated against new self-antigens, leading to a pathologic immune feedback that makes MS irreversible. This phenomenon is known as epitope spreading (67).

2.5. Current treatments

Nowadays, there is not a cure for MS. Current therapies are mostly palliative and unspecific, focused on achieving a general suppression of the immune system in order to slow down the course of the disease and diminish the clinical symptoms and the relapses. Furthermore, most of them are only indicated for the relapsing forms of the disease. Traditionally, these therapies have been classified into first-line treatments, prescribed on the first stages of the disease after its diagnose —such as IFN-β, dimethyl fumarate or glatiramer acetate—, and second-line treatments, which are prescribed after first line treatments have not succeeded to control the course of the disease —like Natalizumab or Fingolimod—. However, this classification strongly varies depending on the indications of medical organisms such as the Food and Drug Administration (FDA), in the USA, or the European Medicines Agency (EMA). Currently approved drugs for the treatment of MS, as well as their mechanism of action, are shown in **Table 2**.

Table 2. Disease modifying treatments approved by the European Medicines Agency for multiple sclerosis.

Drug	Administration route	Dosage	Mechanism of action	Approval	Refs.
Alemtuzumab	i.v	Cycles of 3 or 5 days per year	Anti-CD52 antibody. Depletes the existent T cell repertoire, thus forcing the immune system to generate a new one	2013	(68)
Cladribine	Oral	2 courses spread over 2 years	Purine analog with selective antiproliferative properties against B cells	2017	(69)
Dimethyl fumarate	Oral	Every 12 h	Not fully known. Inhibition of NF-κB pathway and activation of Nrf2 transcription factor.	2014	(70)
Fingolimod	Oral	Every 24 h	Sphingosine 1-phosphate receptor agonist that arrests lymphocytes within the lymph nodes	2011	(71)
Glatiramer acetate 20 mg	s.c.	Every 24 h	Deviation towards a T_H2 response by competing with the myelin basic protein for the binding to HLA molecules	2004	(72)
Glatiramer acetate 40 mg	s.c.	3 times a week	Deviation towards a T_H2 response by competing with the myelin basic protein for the binding to HLA molecules	2015	(73)
Interferon beta-1a	i.m.	Every week	General promotion of an anti-inflammatory response	1997	(74)
Interferon beta-1a	s.c.	3 times a week	General promotion of an anti-inflammatory response	1998	(75)
Interferon beta-1b	s.c.	Every 2 days	General promotion of an anti-inflammatory response	1995	(76)
Mitoxantrone	i.v.	Variable	Suppression of T cell proliferation and migration, antigen presentation and production of pro-inflammatory cytokines.	1998	(77)
Natalizumab	i.v.	Every 4 weeks	Binding to VLA-4 integrin, blocking lymphocyte migration through the blood bran barrier	2006	(78)
Ocrelizumab	i.v.	Every 6 months	Anti-CD20 antibody. Depletion of the B cell repertoire	2018	(79)
Teriflunomide	Oral	Every 24 h	Inhibition of the pyrimidine *de novo* synthesis with a selective antiproliferative effect over T cells	2013	(80)

HLA: human leukocyte antigen; i.m.: intramuscular; i.v.: intravenous; s.c.: subcutaneous; T_H: helper T cell

Due to their unspecific immunosuppressive nature, these currently available drugs present a lot of side effects —since they also weaken the protective immunity, thus exposing the organism to the infection of opportunistic pathogenic microorganisms— and, furthermore, they do not target the cause of the disease. Hence, the search of new specific and more effective therapies is mandatory, and

some new promising strategies are gaining importance in this field. In this regard, the most ambitious objective is to achieve a long-lasting or even permanent modulation of the immune system, specifically restoring peripheral tolerance against auto-antigens with several different strategies —while preserving the protective immunity—. The most relevant approaches are shown in **Table 3**. Among them, in the recent years, the tolerance-inducing antigen-specific DC-based therapies have postulated as one of the most outstanding approaches, as it will be discussed below.

Table 3. Experimental antigen-specific approaches for the treatment of multiple sclerosis

Approach	Route of administ.	MS form	Proposed mechanism of action	Outcome	Refs.
Glatiramer acetate administration	Oral/nasal mucosa	RRMS	T cells within the mucosa are constantly exposed to exogenous antigens and remain tolerant towards them, while remaining protective against harmful pathogens. This approach intends to use this mechanism to induce antigen-specific tolerance towards myelin peptides using glatiramer acetate	No significant effects	(81)
Peptide-coupled PBMC	i.v.	RRMS, SPMS	The administration of PBMC coupled with a mix of seven MBP, MOG and PLP peptides using ethylene carbodiimide (ECDI) induces long-lasted tolerance towards them by suboptimal T cell activation due to the absence of co-stimulation	Well tolerated, inducing a decrease in antigen-specific T cell responses	(64,82)
DNA vaccines	i.m.	RRMS, SPMS	Injection of a plasmid containing the gene encoding MBP leads to a low-production of this peptide, that induces immune tolerance towards it	Well tolerated, with favorable effects and induction of tolerance against MBP	(83,84)
Myelin peptides administration	i.v.	PPMS	The presence of high doses of soluble peptide might lead to the deletion of autoreactive T cells	Well tolerated; favorable effect on disease progression	(85,86)
HLA-MBP complexes	i.v.	SPMS	MBP-bounded HLA complexes would bind TCR without providing co-stimulation, thus leading to the induction of clonal anergy of autoreactive T cells	No significant effects	(87)
ATX-MS-1467	i.d.	RRMS	This molecule mimics myelin antigens and induces antigen-specific tolerance towards them	Well tolerated, with a decrease of MRI lesions	(88)
Altered myelin peptides	s.c.	RRMS	These peptides bind to TCR with lower affinity than natural peptides and can lead the immune response towards an anti-inflammatory or Treg profile	Clinical trial suspended due to AE (relapses)	(89)
T cell vaccination	s.c.	SPMS	Administration of inactivated antigen-specific T cells might lead to the induction of an immune response against autoreactive T cells	Well tolerated; Reduction of autoreactive T cells	(90–92)
TCR vaccination	i.d.	PPMS, SPMS	Administration of a TCR peptide might lead to the induction of an immune response against autoreactive T cells	Well tolerated, (no AE after 1 year); Reduction of myelin response	(93)
TolDC	Variable	Several	TolDC are dendritic cells with the ability to induce long-lasting antigen-specific immune tolerance	Discussed in Section 3	

AE: adverse effects; DNA: deoxyribonucleid acid; HLA: human leukocyte antigen; i.d.: intradermal; i.m.: intramuscular; i.v.: intravenous; MBP: myelin basic protein; MOG: myelin oligodendrocyte glycoprotein; MRI: magnetic resonance imaging; MS: multiple sclerosis; PBMC: peripheral blood mononuclear cells; PLP: proteolipid protein; PPMS: primary progressive multiple sclerosis; RRMS: relapsing-remmiting multiple sclerosis; s.c.: subcutaneous; SPMS: secondary progressive multiple sclerosis; TCR: T cell receptor; Treg: regulatory T cells; TolDC: tolerogenic dendritic cells.

3. Tolerogenic dendritic cells as a treatment for autoimmune diseases [1]

3.1. Biology of dendritic cells

DC are a heterogeneous subset of professional APC that includes classical (cDC), plasmacytoid (pDC), and monocyte-derived DC (MDDC). While pDC constitute a subset of $BDCA2^+$ cells with the ability to produce elevated levels of IFN-γ upon viral encounters, both cDC and MDDC are, in general terms, very similar between them morphologically, functionally and phenotypically. Briefly, cDC and MDDC can be identified by the surface expression of CD11c, CD209 and MHC class II (95). Both belong to the hematopoietic lineage, but the main difference between them is that, as suggested by their nomenclature, MDDC can be derived from human blood monocytes in the presence of GM-CSF and IL-4 (96). Within the immune system, DC have the potential to either stimulate or inhibit immune responses and, in consequence, these cells play a key role in the subtle balance between immunogenicity and immune tolerance (97,98). Briefly, DC are in charge of both the initiation of the adaptive immune response and the control or abrogation of the inflammatory processes once the immunogenic antigen has been cleared. For this regulatory role, DC can deploy several mechanisms such as the induction of anergy or deletion of the activated immune cells, as well as the activation of Treg in an antigen-specific manner. Therefore, since DC have the potential to both stimulate or inhibit immune responses, the role of these cells in the immune system is complex and bidirectional (21–23,97).

In their immature state (iDC), DC are mainly antigen-capturing cells with tolerance-inducing functionality. However, once in the presence of a pro-inflammatory stimulus such as TNF-α, lipopolysaccharide (LPS) or IL-1β, they

[1] Part of this section has been adapted from a review study (Navarro-Barriuso *et al*., Frontiers in Immunology, 2018). See Annex (94).

can differentiate into immunogenic mature DC (mDC). During the maturation process, an upregulation of the expression of HLA class II molecules, as well as of other costimulatory molecules such as CD40, CD80, CD83 or CD86, takes place, along with an increase in the production of IL-12 and other proinflammatory cytokines (96,97,99), as shown in **Figure 4**. Consequently, mDC are capable of priming and activating T cells to initiate an immune response after providing the three required activation signals of the immune synapsis once a specific and immunogenic antigen has been recognized:

First signal. It comes given by a low affinity interaction of the TCR of T cells with an antigen-loaded HLA molecule of DC (100).

Second signal. It is provided by the interaction of the co-stimulatory molecules from DC, such as CD80, CD86 and CD40L, with the CD28 and CD40 receptors —respectively— that are present on the surface of T cells. Analogously, CD80 and CD86 can also interact with CTLA-4 molecules on the surface of T cells, although in this case, an inhibition signal will be provided (101).

Third signal. The specific cytokine milieu secreted by DC —such as either IL-12/IFN-γ or IL-10/TGF-β— will strongly modulate the direction in which the immune response will be directed, as previously discussed (102).

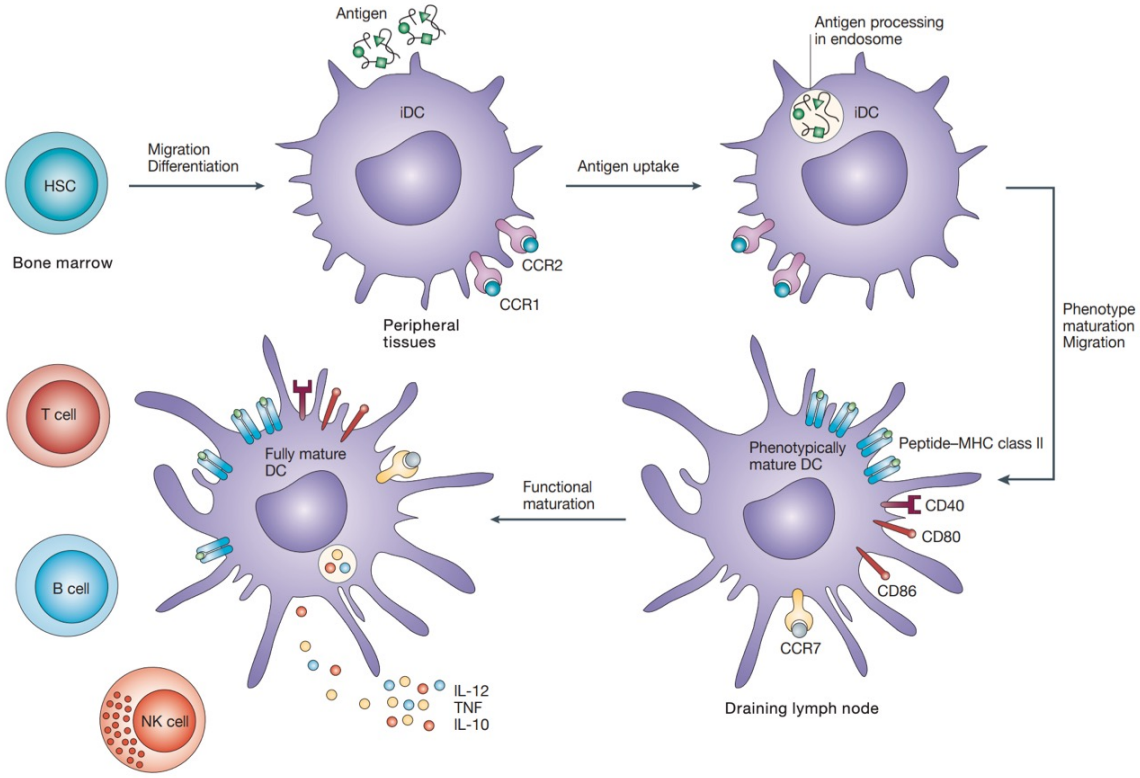

Figure 4. Maturation process of dendritic cells. Adapted from Hackstein and Thomson, Nature Reviews Immunology 2004 (103). Immature dendritic cells (iDC), differentiated from hematopoietic stem cell (HSC) precursors, are natural antigen-capturing cells. Once iDC recognize, internalize and process an antigen, these cells maturate and migrate into draining lymph nodes, where they up-regulate the expression of CD40, CD80, CD86 and other co-stimulatory molecules, as well as of peptide-bound major histocompatibility complex (MHC) class II complexes. Finally, when said peptide is presented to T cells, B cells or natural killer (NK) cells, dendritic cells (DC) undergo their functional maturation and start producing interleukin (IL)-12, tumor necrosis factor (TNF) and other soluble pro-inflammatory mediators.

3.2. Tolerogenic dendritic cells

Tolerogenic dendritic cells (tolDC) are currently defined as semimature DC that present an intermediate phenotype between iDC and mDC, and in this regard, tolDC combine the immune tolerance-inducing properties of iDC with the stability against maturation stimuli of mDC. However, it is not clear whether tolDC constitute a different DC subset by themselves or if they are mere maturation-

impaired iDC, although there seems to be a consensus about which features they have to possess in order to develop their regulatory function. In this regard, tolDC usually present one or more of these characteristics: a semi-mature phenotype, with low expression of co-stimulatory (CD80, CD86, CD83) and HLA class II molecules, a maintained CCR7-dependant migratory ability towards the secondary lymphoid organs, expression of T cell death-inducing ligands (CD95L) and inhibitory molecules (PDL1) and an increased IL-10 and/or TGF-β production accompanied by low or null IL-12 and IFN-γ secretion, among others (**Figure 5**). These features confer to tolDC a reduced capability to induce T cell proliferation and the possibility to prime Treg responses, thus potentially directing the immune response towards a regulatory context. Furthermore, the phenotype and functionality of tolDC are stable against maturation in front of a proinflammatory environment. This specific feature has been described in the majority of studies, and probably constitutes the most important characteristic among all of them (98,104,105).

Importantly, tolDC can be differentiated *in vitro* from peripheral blood monocytes in the presence of a determined tolerogenic-inducing agent. Indeed, a wide variety of protocols have emerged in the last 20 years describing the induction of tolDC with several stimuli, such as anti-inflammatory cytokines —IL-10 (106,107), TGF-β (107,108)—, pharmacological agents and immunosuppressant compounds —rapamycin (107,109,110), different corticosteroids (111), dexamethasone (107,110,112,113), vitamin D3 (107,110,114) or a combination of the last two (115)—, several drugs and blocking molecules —aspirin (116), mitomycin C (117), the NF-κB inhibitor BAY11-7082 (118)— and other strategies, such as genetic engineering for the selective repression or induction of key molecules and pathways (119,120), among many others. Generally, most of these protocols share several features in common, such as the differentiation of monocytes in the presence of GM-CSF and IL-4, as well as the addition of a maturation stimulus (which usually includes different

combinations of LPS, monophosphoryl lipid A, TNF-α, IL-1β, prostaglandin E2 and/or IL-6), with few exceptions.

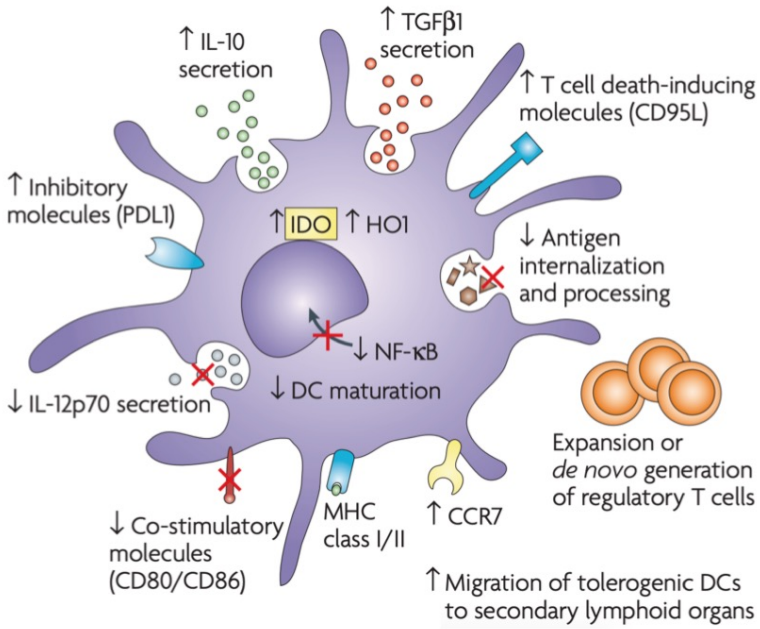

Figure 5. Main features of tolerogenic dendritic cells. Adapted from Morelli and Thompson, Nature Reviews Immunology 2007 (104). DC: dendritic cell; HO1: haem oxygenase-1; IDO: indoleamine 2,3-dioxygenase; IL: interleukin; MHC: major histocompatibility complex; PDL1: programmed cell death ligand 1; TGFβ1: transforming growth factor-β1.

3.3. Therapeutic potential of tolerogenic dendritic cells for autoimmune diseases

By definition, tolDC are capable of reestablishing immune tolerance in an antigen-specific manner against the peptide they are presenting on their HLA molecules, without compromising the protective immunity —or, in other words, without the side effects derived from the unspecific immunosuppression and/or other modulations that current treatments induce—. Consequently, it is not difficult to understand the crucial role that tolDC-based therapies could develop nowadays in the treatment of autoimmune diseases, since evidence from clinical studies and

experimental models involve DC in the pathogenesis of most of them (21). For instance, the number of peripheral blood DC has been reported elevated in MS patients (121), and recent studies even demonstrate the recruitment and accumulation of DC into the CNS white matter lesions in these patients (122). Therefore, the administration of myelin-loaded tolDC into MS patients could constitute a therapeutic approach that, for the first time, would directly address the cause of the disease instead of the symptoms. Furthermore, if immune tolerance could be permanently restored by tolDC-based therapies, they could constitute an actual cure for MS and other autoimmune diseases. However, for this approach to work, it would be necessary to identify all the peptide/s involved in the pathology of each autoimmune disease, which is not always the case.

So far, several tolDC-based approaches have been successfully translated from the bench to the bedside in the last few years, being tested in Phase I clinical trials in patients with autoimmune diseases —such as T1D, RA or Crohn's disease— as well as, kidney transplantation, demonstrating in all cases that tolerogenic cell therapies are safe and well tolerated, without relevant side effects (118,120,123–125). In addition, many other studies, shown in **Table 4**, are currently ongoing (126,127). These results, therefore, support the use of tolDC as novel and safe approaches aiming to restore immune tolerance. However, given the wide variety of protocols available for the generation of these cell products, finding objective and measurable biomarkers to characterize tolDC and compare their characteristics between different approaches and laboratories remains one of the main obstacles to overcome.

3.4. Generation of tolerogenic dendritic cells and characterization of biomarkers

A biological marker, commonly known as **biomarker**, is defined as a characteristic that is objectively measured and evaluated as an indication of normal

biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (128). Since its first dated use in 1980, describing an indirect indicator for breast carcinoma (129), its importance has increased quickly as they are usually cheaper, easier, quicker and earlier indicators than their respective true biological endpoints. Biomarkers do not necessarily have to be related with the biological process itself —it can happen just in parallel, which can be another advantage (130)—, and they can be of a very diverse nature — biochemical, immunological or genetic, to mention some examples—. Among them, genetic biomarkers have become one of the most relevant type in the last 20 years with the implementation of high throughput technologies, which allow the screening of thousands of genes in a given tissue or cell simultaneously (131). High throughput techniques such as microarrays, however, require further validation of each gene of interest by a quantitative polymerase chain reaction (qPCR) in order to corroborate the results, since microarray techniques tend to distort them with artifacts that can lead to mistaken conclusions due to the processes of array production, RNA extraction, probe labeling, hybridization conditions, and image analysis (132,133).

Applied to the objective of this thesis, either if we assume that tolDC are a specific DC subset *per se* or just a modified state of iDC, there must be some footprint left by this condition, which could be used a biomarker of their adequate generation and, probably, also of their functionality. In this context, the identification of differentially expressed genes (either up- or down-modulated) in tolDC constitutes one of the best tools for the definition of biomarkers of tolerogenicity, since they can provide more robust and reliable information compared to conventional methods, such as phenotypical characterization by flow cytometry (with high variability) or functional studies (which require several days).

In this regard, as already hinted above, some obvious downregulated candidate biomarkers of tolDC would be those genes encoding co-stimulatory molecules or pro-inflammatory cytokines. However, those features would be shared with steady state iDC, thus making them useless in terms of differentially characterizing tolDC. In fact, ideally, a comparison against both immature and immunogenic control conditions should be taken into account in the search of specific genetic biomarkers, something that has not been considered in the majority of the studies currently available in the literature. An ideal candidate should be, furthermore, clearly differentiated by a matter of full induction or repression, as a slight increase/decrease of its expression could be ambiguous and would always require the use of robust controls, which is not always possible. In any case an adequate biomarker should be able to guarantee the proper generation of the therapeutic cell product, ensuring that the cells are both safe and tolerogenic.

3.4.1. TolDC differentiated with glucocorticoids and immunomodulatory molecules

Since mDC are immunogenic cells or, in other words, promoters of inflammatory responses, the use of corticosteroids and other immunosuppressant drugs has been widely reported for the generation of tolDC. Rapamycin (107,109,110,134,135) and a combination of hydrocortisone and clobetasol-17-propionate (111), but especially dexamethasone (107,110,112,113,134–147), have all been used for the generation of tolDC. As a glucocorticoid-induced molecule, the expression of the gene encoding the anti-inflammatory mediator known as glucocorticoid-induced leucine zipper (*GILZ*) (148) has been reported strongly up-modulated in many of these studies, thus making it a good albeit predictable marker for tolDC generated with this kind of immunomodulatory agents. Furthermore, other molecules related with the complement and the immune system have been found commonly up- or down-modulated in several of these tolDC protocols, such as the anti-inflammatory cytokine IL-10 (up-regulated), the pro-inflammatory cytokine IL-12

Table 4. Disease modifying treatments approved by the European Medicines Agency for multiple sclerosis

Study ID	Phase	TolDC Protocol	Indication	Outcome	Center	Refs.
NCT00445913	I	Antisense ODN targeting CD40/CD80/CD86 tolDC	T1D	No adverse effects (AE); Increase of B220+CD11c+ B cells; Evidence for C-peptide reactivation	Pittsburgh (USA)	(120)
NCT02354911	II	Antisense ODN targeting CD40/CD80/CD86 tolDC	Recent onset T1D	Not recruiting	DiaVacs Inc. Pittsburgh (USA)	
NTR5542	I	VitD3 and Dexa tolDC pulsed with proinsulin peptide	T1D	Finished	Leiden (NL)	(125)
Rheumavax	I	NF-kB inhibitor Bay11-7082 tolDC pulsed with 4 citrullinated peptides	HLA-risk positive RA	Grade I AE; Decrease DAS28; Decrease Teff/Treg ratio; Decrease pro-inflammatory cytokines and chemokines	Brisbane (AUS)	(118)
NCT01352858 (AutoDECRA)	I	Dexa and VitD3 tolDC pulsed with autologous synovial fluid	nflammatory Arthritis	Safe, feasible, acceptable; Knee symptoms stabilized in 2 patients receiving the highest doses	Newcastle (UK)	
CreaVax-RA CRiS KCT0000035	I	tolDC pulsed with recombinant PAD4, RA33, citrullinated, fillagrin and vimentin	RA	Treatment well tolerated; Antigen-specific autoantibodies decreased in 5/9 positive patients	Seoul (KOR)	(149)
NCT03337165 (TolDCfoRA)	I	Dexa and IFN-α tolDC	RA	Recruiting	Novosibirk (RUS)	
NCT02283671	I	Dexa-tolDC pulsed with relevant disease peptides	MS and Neuro-myelitis optica	Recruiting	Barcelona (ES)	
NCT02618902	I/IIa	VitD3-tolDC pulsed with myelin-derived peptides	MS	Recruiting	Antwerp (BE)	
NCT02903537 (TolerVit-MS)	I/IIa	VitD3-tolDC pulsed with myelin-derived peptides	MS	Recruiting	Badalona (ES) Pamplona (ES)	
	I	Dexa and VitA tolDC	Crohn's Disease	No AE (3 patients withdrew due to worsening of symptoms); Clinical improvement in 3 patients; Increase of Treg and decrease of IFN-γ levels	Barcelona (ES)	(124)
NCT02622763	I	Dexa-tolDC	Crohn's Disease	Recruiting	Barcelona (ES)	
NCT02252055 (ONEatDC)	I/II	Low-dose GM-CSF-recipient tolDC	Kidney Tx from living donor		Nantes (FR)	(150)
NCT03726307	I	VitD3 and IL-10 donor tolDC (Dcreg)	Kidney Tx from living donor	Not yet recruiting	Pittsburgh (USA)	
NCT03164265	I	VitD3 and IL-10 donor tolDC (Dcreg)	Liver Tx	Enrolling by invitation	Pittsburgh (USA)	

Adapted from ten Brinke *et al*., Frontiers in Immunology, 2019 (127). i.m.: intramuscular; i.v.: intravenous; s.c.: subcutaneous. This table is based on information deposited on www.clinicaltrials.gov, www.clinicaltrialsregister.eu and/or indicated references. AE: adverse event; Dexa: dexamethasone; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL: interleukin; MS: multiple sclerosis; RA: rheumatoid arthritis; T1D: type 1 diabetes; Teff; effector T cells; Treg: regulatory T cells; Tx: transplantation; vitD3, vitamin D3.

or the fascin 1-encoding gene *FSCN1* (both down-modulated), which are common features that define these cells (134). The full list of differentially expressed molecules reported for each of the abovementioned protocols and their respective references can be found on **Table 5**.

Dexamethasone-induced tolDC (dexa-tolDC) are one of the most widely implemented approaches worldwide for the generation of human tolDC, and are being or have been tested on clinical trials for the treatment of numerous autoimmune diseases, such as Crohn's disease (http://www.clinicaltrials.gov, NCT02622763) (124), rheumatoid arthritis (http://www.clinicaltrials.gov, NCT03337165; NCT03337165) and both MS or neuromyelitis optica (http://www.clinicaltrials.gov, NCT02283671). Several studies have reported the differential up-modulation of genes *C1QA* (encoding the C1q complement protein, chain A) (142,145), *CD163* (142,145), *GILZ* (134,136,145), *MERTK* (encoding the MER Proto-Oncogene Tyrosine Kinase, also used as a marker in the abovementioned clinical trial for Crohn's disease) (113,124,145) and *ZBTB16* (encoding zinc finger and BTB domain containing protein 16) (142,145) in dexa-tolDC, thus making them the most relevant candidate biomarkers for this specific protocol. Additionally, the differential expression of *IDO1*, the gene encoding the indoleamine 2,3-dioxygenase —a molecule widely related to the induction of immune tolerance (151)—, has also been reported in dexa-tolDC. However, there is some controversy in this regard, as it has been found both up- (145) and down modulated (134) in different studies. Besides, other induced genes described in studies using dexamethasone, relevant by their role in the modulation and mediation of different mechanisms of the immune system —with their respective encoded proteins in brackets—, are *CD300LF* (CD300 molecule-like, family member F), *F13A* (coagulation factor XIII A), *FCGR2B* (Fc fragment of IgG receptor IIb), *FCGR3A* (Fc fragment of IgG receptor IIIa), *MRC1* (mannose receptor C-type 1) and *STAB1* (stabilin 1), as well as, other non-immune related genes like *FTL* (ferritin light chain), *IMDH2* (inosine monophosphate

dehydrogenase 2) and *SOD2* (superoxide dismutase 2). Furthermore, the combination of dexamethasone with rosiglitazone has also been reported for the generation of tolDC, highlighting the induction of *FABP4* (fatty acid-binding protein 4) with this protocol, but specially also of *GILZ* gene (152).

The generation of human rapamycin-modulated tolDC (rapa-tolDC) is the second most reported protocol of this group of pharmacological and immunomodulatory agents. However, transcriptomic studies in tolDC generated with this strong immunosuppressant drug are not as predominant as those induced with dexamethasone. Yet, several genes have been postulated as candidate biomarkers for rapa tolDC, both immune-related —*ANXA1* (annexin 1), *C1QC*, *CTSC* (cathepsin C) and *GILZ*— and non-immune-related —*GPX1* (Glutathione Peroxidase 1), *IMDH2*, *OSF1* (pleiotrophin) and *TPP1* (tripeptidyl peptidase 1)— . Interestingly, all these genes have also been described in common with dexa-tolDC (134).

Additionally, the immunostimulant TLR3 ligand polyinosinic:polycytidylic acid (poly I:C) has also been reported to induce human tolDC, although in an inconsistent and poorly efficient manner. Nevertheless, the differential up-modulation of both *IDO1* and *PD-L1*, two genes involved in the induction and maintenance of immune tolerance, has been confirmed by quantitative PCR for these cells (153,154). As for tolDC induced with hydrocortisone and clobetasol-17-propionate, no transcriptomic biomarkers have been reported to date.

3.4.2. TolDC differentiated with vitamin D3

As reviewed by Mora and colleagues, vitamin D3 exerts important immunomodulatory properties such as the inhibition of T cell proliferation and the reduction of IL-2 and IFN-γ secretion. Furthermore, vitamin D3 is naturally produced in the skin by the transformation of cholesterols upon sun exposure —

although it can also be obtained from the diet—, and its absence or low levels in the organism has been widely linked to an increase in the incidence of autoimmune diseases (155).

The tolerogenic-inducing properties of 1,25-dihydroxycholecalciferol, the active form of vitamin D3, in the generation of tolDC (vitD3-tolDC) have been widely reported *in vitro* in many studies performed with murine (156–160) and even cattle cells (161). However, for this thesis, we will only focus on human vitD3-tolDC (107,110,114,134–137,147,141,162–168). This molecule is the ligand of the vitamin D receptor (VDR), and its recognition has been reported to have an immunomodulatory impact on the differentiation, function and maturation of DC, resulting in T cell hyporesponsiveness. Specifically, vitD3-tolDC present a semi-mature profile, accompanied by an ability to inhibit allogenic T cell proliferation and to polarize the immune response towards an anti-inflammatory T_H2 profile (106–116,124). Furthermore, several studies using animal models of autoimmune diseases have demonstrated their functionality *in vivo* (116,117,119). In general, these cells are characterized by the suppression of the NF-κB pathway (108,134), accompanied by an increased activity of the oxidative metabolism of glucose, and indeed the glucose availability and the glycolytic activity mediated through mTOR signaling are crucial for the induction and maintenance of their tolerogenic function (114). However, despite the identification of several pathways involved in the anti-inflammatory role of vitD3-tolDC, the specific mechanisms for the induction of immune tolerance by these cells have not been clearly identified yet.

Such is the importance of vitD3-tolDC in the field of tolerogenic cell products that even two clinical trials using this cell product, generated and developed in the Germans Trias i Pujol Research Institute, are already ongoing for the treatment of MS patients in Badalona, Spain (http://www.clinicaltrials.gov, NCT02903537), and in Antwerp, Belgium (http://www.clinicaltrials.gov, NCT02618902). Several

transcriptomic and proteomic pre-clinical studies in human vitD3-tolDC have evidenced several genes and proteins strongly induced with this approach, including immune-related molecules —*CCL22* (164,167), *ILT3* (immunoglobulin-like transcript 3) (136), *CD300LF* (164) or *GILZ* (134), these last two in common with dexa-tolDC— and oxidative metabolism enzymes and regulators —*GLUT3* (glucose transporter 3), *LDHA* (lactate dehydrogenase A), *mTOR* (mammalian target of rapamycin), *PDHA1* (pyruvate dehydrogenase E1, subunit alpha 1) or *PFKFB4* (fructose-2,6-bisphosphatase) (167)—, as well as direct targets of the response to vitamin D3 through the interaction with its receptor, like *CYP24A1* (cytochrome P450, family 24, subfamily A, member 1) (147,162,164,167) and of course *VDR* (vitamin D receptor) (147). On the other hand, the repression of several co-stimulatory, pro inflammatory, and antigen presenting genes and molecules like *CD1A*, *CD1C*, *CD80*, *FSCN1* or the transcription factor *IRF4* have been reported at the transcriptomic and proteomic levels (137,164). Additionally, a synthetic structural analogue of vitamin D3, TX527, has also been used for the induction of human tolDC (169). However, and although the up-modulation of the ATP synthase F1 subunit alpha-encoding gene (*ATP5A1*) was reported in common with vitD3-tolDC, the transcriptomic resemblance was more relevant with tolDC induced with a combination of dexamethasone and vitamin D3, a strategy that will be further discussed below. Nevertheless, some of these induced molecules consist of mostly metabolic-related genes —*ACADVL* (Acyl-CoA dehydrogenase very long chain), ACO2 (aconitase 2), *FBP1* (fructose bisphosphatase 1), *IDH3A* (isocitrate dehydrogenase 3, subunit alpha), *PCK2* (phosphoenolpyruvate carboxykinase 2) and *PKM2* (pyruvate kinase M2)— and *CTSD*, encoding the protease cathepsin D (137,170,171). Other differentially expressed genes induced by vitamin D3 are shown in **Table 5**.

Table 5. Differentially up- and down-modulated genes and proteins in the most reported human tolDC-inducing protocols.

Protocol	Type	Up-modulated molecules	Down-modulated molecules	Refs.
Dexamethasone	Gene	***ANXA1, C1QA, C1QC, C1QTNF1, C3AR1, CCL17, CD163, CD300LF, CD32, CFH, CLIC2, CSGALNACT1, CTSC, DCR3, EP2, EP3, F13A, FCGR2A, FCGR2B, FKBP5, FOXO3, FPR1, GILZ, GPX1, IDO1, IL10,*** *IL12A, IL27B,* ***IMDH2, JAG1, MERTK, MRC1, MT1, NCF1, OSF1, P2RY14, SLC39A8, SOD2, STAB1, TPP1, ZBTB16***	***CCL22, CD1C, FCER1A, IDO1,*** *IL12B,* ***LAMP3, MMP12, ZNF366***	(113,134, 136,140, 142,144, 145,147)
	Protein	**CYP1B1, DAB2, DPYD, FCER1G, FCGR3A, FTL, GCLC, IVNS1ABP, LRRC25, MCTP1,** MERTK**, NUDT16, PDCD4, PECAM1, RNASE6, RNASET2, SIGLEC5, SLCO2B1**	**FSCN1**	(124,137, 142)
	miRNA	*miR-328-5P, miR-638, miR-663, miR-762, miR-1275, miR-1228, miR-1909*	*miR-142-5p*	(146)
Dexamethasone + rosiglitazone	Gene	***FABP4, GILZ***		(152)
Dexamethasone + vitamin D2	Protein	***ERK1/2, IDO, JNK/SAPK, mTOR, p38 MAPK, STAT3***		(172)
Dexamethasone + vitamin D3	Gene	*ACADM, ACADVL, ACO1, ACO2, ACOX2, ACSS1, ALDH2, ATP5G3, ATP5J, ATP5O, BLVRB, C1orf162, C1QA, CCR5,* ***CD14****, CD209, CD274,* ***CD52****, CLIC1, COX11, COX6A1, COX7A2, CTSB, CTSD, CTSH, CYC1, DHRS9, EIF3B, EIF3C, EIF3CL, EIF4A3, FBP1, FCGR2B, FCGR3A, FN1, FTH1, FTL, G6PD, GAPDH, IDH3A, IDH3B,* ***ILT3****, LDHB, LILRB4, MATK, MCEMP1, MDH2, ME1, ME3, NDUFB9, NDUFS1, NDUFS8, NOS3, PCK2, PDHA1, PDXK, PIK3R1, PKM2, PNP, PRDX3, PTPN6, RAC2, RGCC, RPS12, RPS19, RPS21, RPS6KA1, RPS6KA2, SDHA, SLC11A1, SLC27A5, SLC2A1, SLC2A5, SNCA, SUCLG1, SUCLG2, TCEB1,* ***TGFB1****, TP53, TPI1, UQCR10, UQCR11, UQCRB, UQCRC1*	*ACTB, ADAM12, ADAM19, ANKRD33B, AOC1,* ***CD25****,* ***CD40****,* ***CD80****,* ***CD83****,* ***CD86****, DPYSL2, EHF,* ***FSCN1****, GPR157, ICOSLG, IKZF1, IKZF4, IL12B, IL2RA, ORMDL3, PIK3CG, PLEKHA5, PPP1R16B, PTPN2, SH2B3, TYK2, WDR1*	(170,171, 173)
	Protein	ADK, AKR1A1, ALDH2, ALDOA, ATP5H, ECHS1, FBP1, FTL, G6PD, GPD2, GALK, MPDH2, PGAM, PGM1, PKM2, PNP, PRDX6, TALDO1, TKT, TPI1	DPYSL2, ENO1, FSCN1, HSPD1, PDIA3	(137)
Hepatocyte growth factor	Gene	***IL10***		(174)
IFN-γ	Gene		***IRF4, RELB, IL12p40***	(175,176)
IL-10	Gene	***ANXA1, C1QC, CTSB, CTSC, CTSL, F13A, FTH1, GILZ, HLA-DOB, IL8, LILRB3, MRC1, STAB1, THBS1, TPP1***	***CD74, LAMP3***	(134,147, 177)
IL-10 + IL-6	Gene	***CTSB, CTSL, FTH1, HLA-DOB, IL-8, THBS1***	***CD74***	(177)
Poly I:C	Gene	***IDO1, PDL1***		(153,154)
Rapamycin	Gene	***ANXA1, C1QC, CTSC, GILZ, GPX1, IMDH2, OSF1, TPP1***	***RALDH1***	(134)
Retinoic acid	Gene	***ALDH1A1, ALDH1A2, CD141, GARP***		(178,179)
TGF-β	Gene	***ANXA1, CTSL, CXCL1, CXCR3, FTH1, HLA-DOB, IL8, LILRB3, THBS1***	***CD74, STAB1***	(134,177)
TX527 (vitamin D3 analog)	Protein	ACADVL, ACO2, ACOX1, ATP5A1, CTSD, CTSS, COPG, FBP1, G6PD, HADHA, IDH3A, MnSOD, OGDH, PCK2, PKM2, PRX3, PTM, UQCRFS1	ACAT1, ARCN1, DLD, PA28beta, PTM, RabGDI	(169)
Vitamin D3	Gene	***ALOX5, ATP5A1, CAMP, CCL22, CD14, CD300LF, CMYC, CYP24, CYP24A1, CYP27B1, GILZ, GLUT3, HK3, ILT3, IRF8, LDHA, LGALS9, PDHA1, PFKFB4, PIK3CG, PRKAA1, THBD, VDR***	***CD1A, CD1C, CD1E, CD36, CD80, F13A, IER3, IRF4, LAMP3***	(134,136, 147,162, 164,167)
	Protein	**AKT, FTL, GSK-3b, mTOR**	**FSCN1, SOD2**	(137)
	miRNA	miR-378		(163)

Genes validated by qPCR or proteins validated by western blot are shown in bold. Table adapted from Navarro-Barriuso *et al*., Frontiers in Immunology, 2018 (94).

3.4.3. The synergic effect of dexamethasone and vitamin D in the differentiation of tolDC

Since dexamethasone and vitamin D treatments alone are able to generate tolDC, the combination of both of them is expected to induce synergic effects that would strengthen the tolerogenic functionality of these cells. Consequently, the simultaneous use of dexamethasone and vitamin D3, or vitamin D2 in a few cases (172,180), has become one of the most widely reported human tolDC generating protocols *in vitro*. Indeed, these cells have even reached the clinical phase for the treatment of rheumatoid arthritis, with successful results regarding the safety and tolerability of the product (http://www.clinicaltrials.gov, NCT01352858) (125).

As expected, the genetic signature of vitamin D + dexamethasone-induced tolDC (vtdx-tolDC) reported in pre-clinical studies partially overlaps with that reported for each or both of these treatments alone to generate human dexa- and vitD3-tolDC. In fact, the analysis of the reported data for these protocols showed that *C1QA*, *FCGR2B*, *FCGR3A*, *IDO1* genes were found induced in common with dexa-tolDC (140,142,145,171,180) and *CD14*, *ILT3*, *mTOR* and *PDHA1* were shared with vitD3-tolDC (136,164,167,170,171,180). Nevertheless, our analysis evidenced that the up-regulation of FTL and the suppression of FCSN1 genes were the only genetic modulations in common between these three protocols (137,142,167,171). Interestingly, the function of the proteins encoded by all these genes is strongly related to the modulation of the immune system. Surprisingly, however, there was a pool of genes that were only described for vtdx-tolDC but not for either dexa-tolDC nor vitD3-tolDC, such as *CTSB*, *DHRS9* (dehydrogenase/reductase 9), *FTH1* (ferritin heavy chain 1), *RGCC* (regulator of cell cycle), *SLC11A1* (solute carrier family 11 member 1), *TBET* or *TGFB1* (170,171,173). Indeed, after our study it is worth noting that out of 64 up-modulated genes and/or proteins reported for dexa-tolDC, 29 genes for vitD3-tolDC and 102 genes for vtdx-tolDC, only 4 genes could be found in common

between vtdx-tolDC and each treatment separately. The chances are, however, that many of these genes could simply not be detected or were overlooked in the validation process of the separated protocols due to intrinsic limitations of the methodologies used, as it is known that biases frequently appear in high throughput transcriptomic and proteomic techniques. For this same reason, for instance, some already mentioned immune-related and metabolic genes were detected simultaneously induced in vtdx-tolDC and tolDC generated in the presence of the vitamin D3 analogue TX527 —*ACADVL*, *ACO2*, *CTSD*, *FBP1*, *G6PD* (glucose-6-phosphate dehydrogenase), *IDH3A*, *PCK2*, *PKM2*)— (137,169–171). Although the down-modulation of genes is not as relevant towards the identification of transcriptomic biomarkers, it is nonetheless worth noting that the *FSCN1* gene has been found repressed in vtdx-tolDC, dexa-tolDC and vitD3-tolDC at the same time (137,171). **Table 5** shows a complete list of the differentially expressed genes reported in protocols using a combination of dexamethasone and vitamin D derivates.

3.4.4. TolDC differentiated with vitamin A

Vitamin A, and specifically its main metabolite, retinoic acid, have been reported to have an influence in T cell differentiation and proliferation, as well as Treg induction (157). However, their use has not been so widely reported for the generation of human tolDC compared to vitamin D, and only the selective up regulation of *ALDH1A1* and *ALDH1A2* genes, encoding the aldehyde dehydrogenase 1 family members A1 and A2 —involved the metabolism of retinoic acid— has been reported, as well as the induction of *CD141* and *GARP* genes (178,179).

3.4.5. TolDC differentiated with cytokines and growth factors

Many different kinds of cytokines have been used for the induction of human tolDC, ranging from anti-inflammatory —IL-10 (106,107,134,135,147,177,181–183), TGF-β (108,134,135,177,184) or both (185)— to even immunostimulatory molecules —IFN-γ (175,176) or a combination of IL-6 with IL-10 (177)—, but also several growth factors —hepatocyte growth factor (174) and low-doses of GM-CSF alone (186)—.

As previously mentioned, the secretion of IL-10 is one of the most sought features of tolDC due to its anti-inflammatory and regulatory properties. Consequently, the generation of tolDC in the presence of exogenous IL-10 (IL10-tolDC) constitutes one of the most implemented protocols for the generation of this type of regulatory cell products. In fact, many of the genes and molecules already cited for other protocols, with immune or metabolic involvement, have also been found induced in IL10-tolDC, such as, *ANXA1*, *C1QC*, *CTSB*, *CTSC*, *CTSL* (cathepsin L), *F13A*, *FTH1*, *HLA-DOB*, *IL-8*, *LILRB3* (leukocyte immunoglobulin-like receptor B3), *MRC1*, *STAB1*, *THBS1*, *TPP1* and, especially for its repeated prevalence, *GILZ* (134,177). Also, and in line with the traditional concept of tolDC, the down-modulation of the antigen presenting molecule CD74 (also known as HLA-DR) (177) and LAMP3 (lysosomal-associated membrane protein 3), typically found on iDC (147), has been reported. Interestingly, the combined exposure in front of both of IL-10 and IL-6 for the generation of tolDC performed in one of the previously cited articles did not seem to change the transcriptomic profile of these cells, as many of the above mentioned genes were also found accordingly induced or repressed like they were in IL10 tolDC (177).

The use of TGF-β for the *in vitro* differentiation of tolDC is not as widely established as IL-10, but still some potential biomarkers have been described, both exclusively for this product (the immune related-genes *CXCL1* and *CXCR3*) and in common with other regulatory cells (*ANXA1*, *CTSL*, *FTH1*, *HLA-DOB*, *IL-8*,

LILRB3, *THBS1*) (134,177). Just like in IL10-tolDC, *CD74* appears differentially repressed in TGF-β-induced tolDC but, controversially, also does *STAB1*, reportedly up-modulated in the former protocol (177). As far as we are concerned, no potential transcriptomic or proteomic markers have been reported in cells induced with the combination of IL-10 and TGF-β for the generation of human tolDC.

Surprisingly, IFN-γ has also been described in a couple of publications for the generation of tolDC, even though it does not constitute the most obvious strategies due to its proinflammatory properties. Nevertheless, these studies have reported the selective reduction in the expression of the pro-inflammatory genes *IRF4*, *RELB* and *IL12p40* in this cell product (175,176). Consequently, the down-modulation of these genes is in line with the expected anti-inflammatory profile for tolDC, and even *IRF4* has also been reported as differentially repressed in vitD3-tolDC, as mentioned above (164). All the biomarkers described within the protocols mentioned in this section are shown in **Table 5**.

Finally, the differentiation of stable tolDC from monocytes in the presence of low doses of GM-CSF, in the absence of IL-4 in the culture, has also been reported in humans (186), but also in animal models (187–189). In fact, their clinical use is being tested under the context of a multicenter trial named The ONE Study ATDC in living-donor renal transplantation (http://www.clinicaltrials.gov, NCT02252055) (150). However, any potential biomarker in human low dose GM-CSF-induced tolDC has been reported yet.

3.4.6. TolDC generated by genetic engineering and other strategies

The pharmacological agents and factors mentioned so far comprise the most predominant strategies in the literature for the induction of tolDC, but there is still a wide variety of drugs, proteins and several treatments with the potential of

generating this type of regulatory DC products. However, provided that the aim of this review is to look for universal biomarkers of immune tolerance, we have also considered these approaches. In fact, a significant amount of studies have reported the differential expression of several genes and molecules that could become potential biomarkers for their respective and specific protocols, generating tolDC in the presence of different organic compounds —such as the Aspergillus cell wall (134), curcumin (190), mitomycin C (191), paeoniflorin (192), phosphatidylserine liposomes imitating apoptotic bodies (193)—, other cell types —mast cells (194) and trophoblasts (195)— and a variety of agents, conditions and/or molecules —for instance a combination of the complement protein C5a and LPS (196), seminal plasma (197), the Wnt-5a protein (198) or even the deprivation of tryptophan in the culture (199)—. However, there are still many other different strategies without transcriptomic or proteomic studies reported in the literature that are therefore outside of the objective of this review. The full list of differentially expressed genes and molecules in the protocols mentioned in this section is presented in **Table 6**.

A totally different approach to generate tolDC consists in using targeted genetic engineering in order to achieve cells with specific functional features either silenced or induced. There are several strategies reported in this regard, ranging from the impairment of immunogenic properties —such as silencing the expression of CD40, CD80 and CD86, already tested in type 1 diabetes patients, which was the first clinical trial using a tolerogenic cell therapy (http://www.clinicaltrials.gov, NCT00445913) (120,200)— to selectively inducing the production of several anti-inflammatory cytokines like IL-10 and TGF-β (119,201), overexpressing the IL-12 and IL-23-suppressor factor SOCS-3 (202) or transfecting the cells with a modified CTLA4 construct that inhibits the expression of the co-stimulatory molecules CD80 and CD86 (203). Surprisingly, some approaches using genetic manipulation achieved to generate human IL-10-producing DC through the induction of, a priori, immunogenic

functions such as the CD40-CD40L signaling pathway (204). However, the definition of transcriptomic biomarkers for tolDC induced by genetic engineering would not be of much utility, provided that the differentially expressed genes or proteins to check would be precisely those that have been specifically induced or repressed by the procedure itself.

Table 6. Differentially up- and down-modulated genes and proteins in other human tolDC-inducing protocols.

Protocol	Type	Up-modulated molecules	Down-modulated molecules	Refs.
Aspergillus cell wall	Gene	***ANXA1, STAB1, GILZ, IDO, RALDH1, RALDH2***	***F13A, MRC1***	(134)
C5a and LPS	Gene	***RGCC, FERMT2, SLC39A14, TNFSF14, TGFB1***	***IL12B, FOXO1***	(196)
Curcumin	Gene		***RELB***	(190)
Mast cells	Gene	***IDO1, NFKB1, NFKB2, RELB, SOCS5***	***SOCS3***	(194)
Mitomycin C	Gene	***ADM, CSF2RA, DDIT3, FDXR, GAB2, LILRB4, LRDD, MAFB, MAP4K4, PERP, TNFRSF10B, TRAF4, TSC22D3***	***CFLAR (FLAME-1, I-FLICE, Usurpin), NRG2***	(191)
Paeoniflorin	Gene	***IDO1***		(192)
Phosphatidyl-serine lyposomes	Gene	*CLCN6,* ***CYTH4****, IFNLR1, LAIR1, LDLR, MFSD2A, NFKBIA, PLAUR, PPME1, SHB, SLC43A3, TNFAIP3,* ***TNFSF14****, VEGFA*	*ALKBH1, ATP10D, AURKA, BCL2L1, BLCAP, BST1, BTBD3, BTK, BUB1, C9orf64, CASP3, CBX4, CD1D, CDC23, CDC42SE1, CDK13, CDYL2, CKAP2, CLCN3, CSRP2BP, CUL3, DAPP1, DCAF12, DCAF7, DCLRE1A, DCTD, DDO, DYRK2, EHBP1, ERLIN1, FBXO25, FBXO36, FRAT2, FZD5, GIMAP4, GLRX, GOLPH3L, GTF2B, HHEX, HPGD, ICK, KBTBD6, KIF11, KIF20B, LMNB1, LNX2, MAPRE2, MCM4, MCPH1, MDM1, MEF2C, MEGF9, MIER3, MLH1, MNDA, MSH2, MYB, N4BP1, NCAPG2, NET1, NFIA, NSMCE4A, NUP160, PAQR8, PARG, PAXIP1, PCNA, PMP22, PROS1, RAB32, RAD51C, RCSD1, RMDN1, RMND5A, SCYL3, SEC22C, SKI, SLAMF6, SLC10A7, SLC40A1, SMC2, SNN, SNX18, SOCS2, STIM2, STX3, TIMMDC1, TNFRSF11A, TPK1, TRIM5, UBE2E3, UBFD1, UNC50, VWA5A, WRNIP1, ZBED3, ZBTB39, ZBTB5, ZFP36L2, ZNF436*	(193)
Seminal plasma	Gene	***COX2, TGFB1***	***CD1A***	(197)
Trophoblasts	Gene	***IDO1***		(195)
Tryptophan-deprived	Gene	***CHOP, ILT3***		(199)
Wnt5a	Gene	***ID3, IRF1, IRF2, SOCS3, TLR1***	***ID2, IRF8, TLR3, TLR4, TLR5***	(198)

Genes validated by qPCR or proteins validated by western blot are shown in bold. Table adapted from Navarro-Barriuso *et al*., Frontiers in Immunology, 2018 (94).

3.5. The role of biomarkers in the translationality of tolDC-based therapies

The search for new biomarkers is nowadays a constant in medicine, in order to provide an early diagnose of a disease or to guarantee the biosafety and effectiveness of a new cell-based therapy. And due to its high cost and their potentially dangerous side effects on patients if the product is not appropriately generated, tolDC-based therapies are not an exception. In this regard, the identification of robust biomarkers for the characterization of tolerogenic and immunoregulatory cell products constitutes one of the final steps needed to take the final leap towards the broad application of these novel autologous antigen-specific therapies in the clinic. Specifically, their key importance resides in their capability to provide a reliable quality control of the proper generation, functionality and safety of tolDC, while optimizing their production cost by providing a cheaper, faster and more reliable way of validating the effectiveness of the generated cellular product prior to the administration back into the patient, avoiding other time-consuming or not-so-reliable methods.

However, as previously discussed, albeit many genes and molecules have been found separately induced using different strategies to generate tolDC, so far, there is not a biomarker or a pool of biomarkers that can functionally characterize or at least identify the entirety of the studied protocols. Still, despite this, a significant amount of differentially expressed genes encoding several anti-inflammatory and immunomodulatory molecules have been reported in very different protocols, like for instance *IDO1* (in 7 approaches) *GILZ* (in 6 approaches) or *ANXA1* (in 5 approaches), which can be combined with other protocol-specific biomarkers for the robust characterization of each cell product. Consequently, this fact evidences that, although a universal transcriptomic profile of immune tolerance induction might not be achievable, the elaboration of useful panels of biomarkers can still be feasible for determined pools of tolerogenic products.

Thus, the combination of both stimulus-specific and some other partially common differentially expressed genes could potentially lead to the development of transcriptomic panels of tolerogenic functionality. After all, provided that the relevance of tolerance-inducing cell therapies in the treatment of autoimmune diseases and solid organ transplantation rejection is becoming hugely relevant in the last years, the need for adequate and objective biomarkers is increasing accordingly. And in this context, the definition of panels of tolerogenic functionality for at least a limited pool of protocols would, consequently, provide a robust tool for the establishment of reliable quality and safety controls for trials using tolDC-based therapies in the near future. In addition, these panels would also allow to properly compare tolDC products and, therefore, to dramatically accelerate their translation into the clinic. Furthermore, the definition of potential biomarkers of response to the treatment with tolDC would also be of a huge relevance, since they would allow to follow up the evolution of patients during clinical trials. However, in this regard, and due to the early stages in which tolDC-based therapies still are, not a single biomarker of treatment response has been identified yet.

Hypothesis and objectives

Current treatments for MS and other autoimmune diseases are chronic and unspecific, and since they do not target the cause of the disease, they are also uncapable to cure it. Furthermore, due to their unspecific nature, they are normally accompanied by side effects, which can be very severe. For that reason, there is an increasing need for new, more effective and more specific treatments. In that context, tolDC-based therapies have postulated as one of the most promising alternatives, since they can potentially restore the lost tolerance in autoimmune diseases in an antigen-specific, without compromising the protective immunity of the patients. However, for their full development and translation into the clinic, adequate and robust biomarkers of their generation, safety and functionality still need to be defined.

In this regard, our hypothesis is that, as biological entities, there exists a transcriptomic footprint that allows us to define biomarkers to differentially characterize tolDC in general, and vitD3-tolDC in particular, which could be used in clinical trials as a quality control and monitoring of the treatment of patients. Furthermore, those biomarkers could potentially provide crucial information about the mechanisms that are being developed within and by tolDC in order to induce immune tolerance.

Consequently, the specific objectives targeted in this thesis are:

1. Characterization and validation of biomarkers of tolDC.

- **a.** Comparative study of the transcriptomic profile of vitD3-tolDC, dexa-tolDC and rapa-tolDC differentiated from healthy donors.
- **b.** Identification of common biomarkers of the generation of these tolDC.
- **c.** Validation of the candidate biomarkers in tolDC differentiated from healthy donor samples.
- **d.** Validation of the candidate biomarkers in tolDC differentiated from MS patient samples.

2. Identification of biomarkers of response to vitD3-tolDC treatment.

- **a.** Standardization of a protocol for the generation, analysis and co-culture of autologous antigen-specific vitD3-tolDC and T $CD4^+$ cells.
- **b.** Comparative transcriptomic study of isolated T $CD4^+$ cells after their co-culture with mDC and vitD3-tolDC.
- **c.** Identification of transcriptomic biomarkers of the modulation mediated by vitD3-tolDC over T $CD4^+$ cells.

Results

The results of this thesis are presented as a compendium of two publications on the identification and validation of biomarkers of the generation of vitD3-tolDC and an annexed manuscript studying the antigen-specific transcriptomic changes induced by these cells over autologous T $CD4^+$ cells. However, even though our group has focused on the study of MS, the aim of these manuscripts is the applicability of said biomarkers to other autoimmune diseases and tolDC-generating protocols. A brief summary of these studies is shown below.

Results I

Comparative transcriptomic profile of tolerogenic dendritic cells differentiated with vitamin D3, dexamethasone and rapamycin

Juan Navarro-Barriuso, María José Mansilla, Mar Naranjo-Gómez, Alex Sánchez-Pla, Bibiana Quirant-Sánchez, Aina Teniente-Serra, Cristina Ramo-Tello, Eva M. Martínez-Cáceres. *Scientific Reports*. 8(1):14985 (2018).

In this study, we compared the transcriptomic profile of three of the most common tolDC-inducing protocols, vitD3-tolDC, dexa-tolDC and rapa-tolDC, using a microarray experiment with healthy donor samples. Our aim was to find one or more differentially expressed genes in common between these protocols that could serve as universal biomarkers of tolDC. However, we observed that, even though vitD3-tolDC and dexa-tolDC shared several transcriptomic similarities —while rapa-tolDC exhibited a mostly down-regulated profile, being the opposite to the other two protocols in many cases— we could not find a single differentially expressed gene in common.

Consequently, we focused on the identification of candidate biomarkers of each protocol separately, identifying that *CYP24A1*, *MUCL1* and *MAP7* genes for vitD3-tolDC; *CD163*, *CCL18*, *C1QB* and *C1QC* genes for dexa-tolDC; and

CNGA1 and *CYP7B1* genes for rapa-tolDC could constitute good candidate biomarkers for each respective cellular product. Consequently, we concluded that, provided the huge transcriptomic differences that we observed, which evidenced the possibility to induce immune tolerance through different mechanisms, the identification of a common biomarker of tolDC generation was not possible, and that the validation of protocol-specific biomarkers —or in the best case of biomarkers that could serve to a limited number of protocols—, should be considered.

Results II

MAP7 and *MUCL1* are biomarkers of vitamin D3-induced tolerogenic dendritic cells in multiple sclerosis patients

Juan Navarro-Barriuso, María José Mansilla, Bibiana Quirant-Sánchez, Alicia Ardiaca-Martínez, Aina Teniente-Serra, Silvia Presas-Rodríguez, Anja ten Brinke, Cristina Ramo-Tello, Eva M. Martínez-Cáceres. *Frontiers in Immunology*. 10:1251 (2019).

Due to their potent immunoregulatory properties —and provided that this cell product was selected by our group to be tested in a clinical trial—, in this work we focused on the validation of transcriptomic biomarkers of vitD3-tolDC, using the same microarray data from the previous study as the source of information. Furthermore, we aimed to further test the translationality of our results by validating the expression of our candidate genes in vitD3-tolDC differentiated from not only healthy donors but also MS patients, in order to evaluate their potential and direct application in clinical trials for MS. For that reason, we performed a screening process throughout the transcriptome of these cells, looking for genes that were differentially expressed in vitD3-tolDC compared to both iDC and mDC control conditions.

Even though we could validate the differential expression of several genes as biomarkers of vitD3-tolDC generation in healthy donor samples, the analysis on MS patient samples only allowed us to validate *CYP24A1*, *MAP7* and *MUCL1* genes as transcriptomic biomarkers of the generation of vitD3-tolDC. However, by constructing a network of protein interactions based on our microarray data and previously reported information on the literature, we could observe that *MAP7* and *MUCL1* —but not *CYP24A1*— were closely involved in the modulation of many relevant immune-related pathways such as HLA class II presentation and anti-inflammatory responses. In order to confirm these results, we also analyzed and observed that the expression of the proteins encoded by *MAP7* and *MUCL1* genes was also differentially expressed in vitD3-tolDC, both in healthy donors and in MS patient samples.

Furthermore, the differential expression of *MUCL1* was also confirmed in IL10-tolDC, indicating the potential of this gene as a broad-use biomarker for other tolDC-inducing protocols other than vitD3-tolDC.

Results III

Vitamin D3-induced tolerogenic dendritic cells modulate the transcriptomic profile of T CD4$^+$ cells towards a functional hyporesponsiveness

Juan Navarro-Barriuso, María José Mansilla, Bibiana Quirant-Sánchez, Aina Teniente-Serra, Cristina Ramo-Tello, Eva M. Martínez-Cáceres. Under review.

Once we had characterized and validated several biomarkers of the generation of vitD3-tolDC, in this work we wanted to perform a preliminary study to identify potential transcriptomic biomarkers of the modulations induced by vitD3-tolDC over T cells, after an antigen specific interaction, with the aim to identify the potential mechanisms of immune tolerance induction that might be being

triggered. For that, we needed to standardize the whole protocol of cell culture, in parallel, of autologous vitD3-tolDC and PBMC from the same original sample, and in order to make it as versatile as possible, our whole procedure was performed using healthy donor samples and tetanus toxin (TT) as an immunogenic peptide. Thus, the protocol could be easily adapted to become an experimental model for the study of antigen-specific vitD3-tolDC and PBMC interactions in any autoimmune disease or immune-mediated disorder, simply by substituting the donor samples with patient samples and the TT with specific peptide/s of the determined disease or condition of interest.

In our case, we decided to focus on the study of vitD3-tolDC-modulated T $CD4^+$ cells, since this population directly interacts with DC via their HLA class II molecules. Once we co-cultured TT-loaded DC cells and PBMC, we isolated T $CD4^+$ cells by flow cytometry and performed a transcriptomic analysis by RNA-seq, comparing those T $CD4^+$ cells that interacted with vitD3-tolDC with those who did with immunogenic mDC. Our results evidenced that vitD3-tolDC were inducing a generalized and antigen-specific repression of the transcriptome of T $CD4^+$ cells, which corresponded with the induction of a functional hyporesponsiveness that could explain the tolerogenic properties of vitD3-tolDC, while no Treg induction could be observed. Furthermore, we could identify the up-regulation of *JUNB* gene as a potential candidate biomarker of the antigen-specific vitD3-tolDC-mediated modulation of T $CD4^+$ cells. This study is presented as an annexed manuscript to the compendium of publications.

Results I

Comparative transcriptomic profile of tolerogenic dendritic cells differentiated with vitamin D3, dexamethasone and rapamycin

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Comparative transcriptomic profile of tolerogenic dendritic cells differentiated with vitamin D3, dexamethasone and rapamycin

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Tolerogenic dendritic cell (tolDC)-based therapies have become a promising approach for the treatment of autoimmune diseases by their potential ability to restore immune tolerance in an antigen-specific manner. However, the broad variety of protocols used to generate tolDC *in vitro* and their functional and phenotypical heterogeneity are evidencing the need to find robust biomarkers as a key point towards their translation into the clinic, as well as better understanding the mechanisms involved in the induction of immune tolerance. With that aim, in this study we have compared the transcriptomic profile of tolDC induced with either vitamin D3 (vitD3-tolDC), dexamethasone (dexa-tolDC) or rapamycin (rapa-tolDC) through a microarray analysis in 5 healthy donors. The results evidenced that common differentially expressed genes could not be found for the three different tolDC protocols. However, individually, *CYP24A1*, *MUCL1* and *MAP7* for vitD3-tolDC; *CD163*, *CCL18*, *C1QB* and *C1QC* for dexa-tolDC; and *CNGA1* and *CYP7B1* for rapa-tolDC, constituted good candidate biomarkers for each respective cellular product. In addition, a further gene set enrichment analysis of the data revealed that dexa-tolDC and vitD3-tolDC share several immune regulatory and anti-inflammatory pathways, while rapa-tolDC seem to be playing a totally different role towards tolerance induction through a strong immunosuppression of their cellular processes.

In the last decade, tolerogenic dendritic cells (tolDC) have become one of the most promising approaches for the treatment of immune-mediated disorders such as autoimmune diseases (i.e. type 1 diabetes, multiple sclerosis or rheumatoid arthritis), but also for allergies or transplant rejection. In a healthy organism, immature dendritic cells (iDC) are specialized antigen-capturing cells that, when exposed to a pro-inflammatory millieu, differentiate into mature dendritic cells (mDC) in order to orchestrate an immunogenic response against the potentially pathogen-related peptide they previously recognized, captured and presented. Autoimmune disorders are characterized by the loss of immune tolerance against determined self-peptides, thus causing a pathological response of the immune system that leads to different diseases depending on which antigen/s are equivocally attacked. In this context, the main advantage of potential tolDC-based therapies resides in their presumed role to restore the immune tolerance against self-peptides in an antigen-specific manner, acting only over the cause of the pathologic process without compromising the protective immunity from the patient.

A wide variety of protocols has been developed to generate tolDC *in vitro*, for instance by the action of several immunomodulatory agents (such as 1,25-dihydroxycholecalciferol, the active form of vitamin D3[1–4], dexamethasone[3–6] or rapamycin[3,4,7]), cytokines (IL-10[4,8], IFN-β[4,9]) or by genetic engineering[10,11] and, in all cases, they remain stable against maturation. Furthermore, the leap from the bench to the bedside has already been taken, there existing several clinical trials, either completed or ongoing, that have demonstrated the safety of autologous

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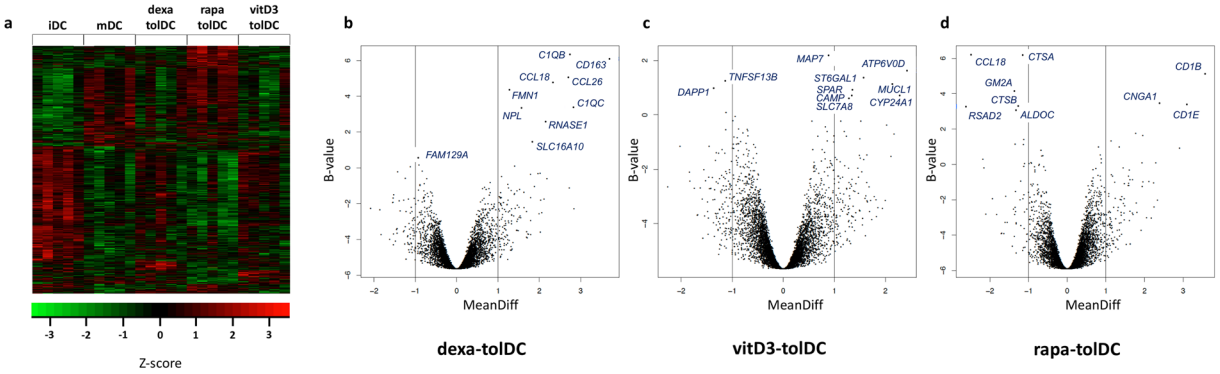

Figure 1. Comparative transcriptomic analysis of vitD3-tolDC, dexa-tolDC, rapa-tolDC, iDC and mDC. (**a**) Heat map representation of the transcriptomic expression profile of the different DC. Volcano plots of the top differentially expressed genes based on mean differences of expression (MeanDiff) and B scores of (**b**) dexa-tolDC, (**c**) vitD3-tolDC and (**d**) rapa-tolDC versus mDC.

tolDC-based therapies in rheumatoid arthritis, type 1 diabetes and Crohn's disease, while further studies to evaluate their actual efficacy are currently being developed[11–16]. However, the characteristics of these tolerogenic cells are heterogeneous depending on which protocol was used to differentiate them, presenting, for instance, variable phenotypical characteristics or producing different cytokines. For this reason, a wide range of analyses has to be carried out to characterize them. Currently, the most reliable evidence of the regulatory properties of tolDC comes given by functional assays. However, these tests normally take days and require the generation of control immunogenic conditions in parallel, which also translates into an increase in the cost of an already expensive production process due to the strict good manufacturing practice (GMP) conditions that are required to generate clinical grade tolDC. Therefore, the need for common pathways or strong biomarkers that could define the concept of tolerogenicity and unequivocally characterize tolDC is one of the pending questions to be answered, as they would help to better understand the molecular mechanisms of tolerance as well as saving time and money during the manufacturing of the cell products.

Vitamin D3, dexamethasone and rapamycin are three of the most widely used drugs to induce the differentiation of tolDC *in vitro*. Our previous studies have shown that vitamin D3-induced tolDC (vitD3-tolDC) and dexamethasone-induced tolDC (dexa-tolDC) generate cells with rather similar characteristics in terms of presenting a semi-mature phenotype, increased IL-10 secretion and reduced allogeneic T cell proliferation priming. In contrast, rapamycin-induced tolDC (rapa-tolDC) seemed to develop their tolerogenic role through regulatory T cell (Treg) induction, despite their mature phenotype and not secreting IL-10. In all cases, however, an allogeneic T cell proliferation suppression was observed, and the three tolDC types remained stable upon LPS re-stimulation[3].

Consequently, provided the heterogenous characteristics of these cells, we performed a microarray analysis of vitD3-, dexa- and rapa-tolDC, differentiated from 5 healthy donors, in order to obtain their transcriptomic profile and look for common pathways and/or mechanisms of tolerance induction. Indeed, our hypothesis is that the identification and definition of these effector routes could provide useful biomarkers for the characterization of these cells, specially thinking of their application in future clinical trials, since they may be helpful to compare results in studies worldwide and thus accelerate the translation of tolDC-based therapies from the bench to the bedside.

Results

Gene expression analysis revealed two different transcriptomic profiles in tolDC. The preprocessing steps described in the methods section left 7864 probesets to be included in the analysis. In order to look for potential common biomarkers for the three tolDC conditions, the expression between each tolDC vs mDC, as well as between mDC vs iDC, was compared in cells differentiated from 5 healthy donors, using the linear models approach described in the methods section. Among them, an additional comparison was carried out between dexa-tolDC, rapa-tolDC and vitD3-tolDC versus both mDC and iDC, yielding a total of 1216 genes showing a statistically significant differential expression in at least one comparison (p-value < 0.01).

The representation of the transcriptomic profile of the 5 types of DC (iDC, mDC, dexa-tolDC, rapa-tolDC and vitD3-tolDC) in a heat map evidenced a segregation into two clusters of 492 and 724 genes with opposed expression (Fig. 1a). As expected, iDC and mDC exhibited an opposed genetic signature. However, rapa-tolDC showed a similar profile to mDC, while vitD3- and dexa-tolDC presented more resemblance to iDC.

TolDC showed several differentially expressed genes involved in the immune response modulation, signaling and trafficking compared to mDC. To find the genes involved in the tolerogenic function of vitD3-, dexa- and rapa-tolDC, the expression of each tolDC condition was compared with the expression of mDC. The results are presented as mean differences of the signals (MeanDiff) for each gene, and the B-statistic values were also considered. As a result, only those genes presenting $B > 0$ and $p < 0.01$ values were selected for each of the different tolDC conditions, as they would constitute the most relevant and likely candidates for being involved in tolerance.

When looking at the differentially expressed genes (DEG) between dexa-tolDC and mDC (Table 1), we found that there were several overexpressed genes (MeanDiff > 1.2) directly involved in immune-related functions such as the complement activation (*C1QB* and *C1QC*) and the immune-related chemotaxis (*CCL18* and *CCL26*), while others are mainly involved in metabolism and cell interaction. In contrast, only 3 down-regulated genes (MeanDiff < −0.6) presented a positive value for the B-statistic, *LSM14B* (which may play a role in mRNA translation), *FAM129A* (a regulator of p53-mediated apoptosis) and *PIWIL4* (involved in the development and maintenance of germline stem cells). The volcano-plot representation of the results can be observed in Fig. 1b.

In the case of vitD3-tolDC, the up- and down-regulated genes compared to mDC were not so directly related to immune functions (Table 1). Metabolism, as well as cell differentiation, structure and signaling, were the most predominant related functions, with genes such as *MAP7*, *MUCL1* or *SPARC* strongly up-regulated (MeanDiff > 0.7). Nevertheless, genes encoding antimicrobial proteins (*GZMB* and *CAMP*) and proteins related with the direct metabolism of vitamin D3 (*CYP24A1*) could also be found, making a total of 9 up-regulated genes with B > 0. Among the down-regulated genes, only 3 fulfilled our criteria in the microarray, once again *PIWIL4* (demonstrating certain similarity between vitD3-tolDC and dexa-tolDC), *TNFSF13B* and *DAPP1* (both outstanding for being involved in immune regulation). All three of them showed strong reductions on their expression (MeanDiff < −0.6). The volcano-plot representation of the data is shown in Fig. 1c.

As for rapa-tolDC, as shown in Table 1, a total of 27 genes were selected. We found 3 genes with a strong up-regulation (MeanDiff > 2.2), encoding proteins developing innate immunity-related functions (*CD1B*, *CD1C* and *CD1E*), as well as, surprisingly, 2 genes related with the metabolism of fat soluble vitamins such as vitamin D3 (*CNGA1* and *CYP7B1*). Among the down-regulated genes, most of them were related with the metabolism of different molecules and proteins, especially outstanding *CTSB*, *ALDOC* and *GM2A* for their high B values (>3) and their strong down-modulation (MeanDiff < −1.2). The down-modulation of *FAS* gene, mediating the induction of cell death, was also relevant. Analogously, a volcano-plot representation of the results in rapa-tolDC is shown in Fig. 1d.

A common genetic biomarker could not be found for the three tolDC conditions. Provided that a biomarker should unequivocally characterize a determined biological condition, we restricted even more our filtering parameters, selecting only those genes that were differentially expressed in the tolDC conditions versus both iDC and mDC at the same time. Once again, we made use of the P and B-statistic values as filtering criteria, selecting only those genes presenting B > 0 and p < 0.01 values for both comparisons. Consequently, we obtained those DEG that not only appeared to be differentially expressed, either over- or down-regulated, but that also their differential expression had high enough odds of being reliable.

As a result, 26 different genes, many of them already mentioned in the previous section, were compliant with the filtering parameters in at least one tolDC condition; 3 of them were overexpressed in vitD3-tolDC, 7 genes in dexa-tolDC and, in the case of rapa-tolDC, 4 genes were up-regulated and 13 were down-regulated (Fig. 2). Among all those genes, only *CCL18* appeared in 2 out of the 3 tolDC conditions, showing a MeanDiff > 2.30 in dexa-tolDC but a MeanDiff < −1.69 in rapa-tolDC (p-value < 0.01). As for the other reported genes, many of them were related with immune functions or cell differentiation, interaction or signaling mechanisms, such as *MUCL1*, *MAP7*, *CD163*, *C1QB* or *C1QC*, indicating important changes in the status of the different tolDC conditions respect of iDC and mDC that might be relevant for the tolerogenic function of the cells, or simply induced by the different tolerogenic agents used. These genes presented at least a MeanDiff > 0.79 for the up-regulated ones and a MeanDiff < −0.60 for those down-regulated. In all cases, statistical significance was reached (p < 0.01). These and further details can be found in Table 2.

VitD3 and dexa-tolDC share several common regulatory pathways, although none of them with rapa-tolDC. After determining which DEG could be found on each condition, we decided to perform a more comprehensive study of the transcriptome by analyzing which pathways and protein sets were up- or down-modulated on each DC condition. To do this, a Gene Set Enrichment Analysis (GSEA) was performed, and only those pathways and protein sets that showed a statistically significant enrichment (p-value < 0.05) on each tolDC condition compared to mDC were considered. Additionally, all those pathways that were up-modulated on iDC versus mDC were excluded as they would not constitute differential pathways of tolerance for our tolDC products, with the exception of the induction of Treg lymphocytes, immune response and hemophilic cell adhesion via plasma membrane adhesion molecules protein sets, due to their functional relevance in tolerance. Finally, a total of 49 pathways and protein sets, differentially expressed versus mDC, were selected, either due to their relevance or for being shared between at least 2 tolDC conditions (Table 3). A graphical representation of them is presented in Fig. 3. The analysis could not reveal any pathway up- or down-modulated in common between the three tolDC conditions versus mDC.

When taking the comparisons two by two, a total of 18 pathways were simultaneously up-regulated in both dexa and vitD3-tolDC versus mDC, and 3 protein sets, mainly related with the plasma membrane, appeared up-regulated in dexa and rapa-tolDC versus mDC, with different behaviors regarding the comparisons between the remaining conditions. Any common enriched protein sets could be found between vitD3- and rapa-tolDC. Further 13 pathways were enriched at the same time in rapa-tolDC and either dexa or vitD3-tolDC, but with opposite modulation. Among these 34 mentioned protein sets, only 8 were differentially induced versus mDC in at least two tolDC conditions, being them dexa- and vitD3-tolDC in all cases, and with no differences between iDC and mDC. In addition, in 5 of those cases, the protein sets were also simultaneously down-modulated in rapa-tolDC. Of them, 3 were related with extracellular components (extracellular region, extracellular space and extracellular exosome) and the other 2 with a response to inflammation stimuli (inflammatory response and cellular response to IL-1).

Gene	EntrezID	MeanDiff vs mDC	B-statistic	p-value	GO annotations
dexa-tolDC					
CD163	9332	3.70	6.08	<0.0001	Protein binding, scavenger receptor acivity
C1QC	714	2.83	3.39	<0.0001	Innate immune response, immune complement
C1QB	713	2.74	6.33	<0.0001	Innate immune response, immune complement
CCL26	10344	2.71	5.05	<0.0001	Chemotaxis, signal transduction, inflammatory response
CCL18	6362	2.34	4.79	<0.0001	Chemotaxis, signal transduction, inflammatory response
RNASE1	6035	2.16	2.59	<0.0001	Nucleic acid binding
SLC16A10	117247	1.83	1.45	0.0001	Amino acid transport
NPL	80896	1.57	3.37	<0.0001	Protein binding
RGL1	842953	1.40	0.11	0.0003	Protein binding
FMN1	342184	1.27	4.36	<0.0001	Microtubule cytoskeleton
LSM14B	149986	−0.91	0.15	0.0003	RNA binding
FAM129A	116496	−0.92	0.55	0.0002	Protein binding
PIWIL4	143689	−1.12	0.07	0.0004	RNA binding
vitD3-tolDC					
ATP6V0D2	245972	2.42	1.64	0.0001	Protein binding
CYP24A1	1591	2.27	0.72	0.0002	Metabolism
MUCL1	118430	2.13	1.13	0.0001	Metabolism
ST6GAL1	6480	1.56	1.38	0.0001	Metabolism
CAMP	820	1.35	0.71	0.0002	Innate immune response
SPARC	6678	1.35	0.93	0.0001	Protein binding
SLC7A8	23428	1.27	0.62	0.0002	Amino acid transport
MAP7	9053	0.88	2.23	<0.0001	Microtubule cytoskeleton
GZMB	3002	0.74	0.34	0.0003	Protein binding, immunological synapse
PIWIL4	143689	−1.12	0.09	0.0004	RNA binding
TNFSF13B	10673	−1.15	1.25	0.0001	Protein binding
DAPP1	27071	−1.37	0.99	0.0001	Protein binding
rapa-tolDC					
CD1B	910	3.56	5.12	<0.0001	Adaptive immune response
CD1E	913	3.09	3.38	<0.0001	Adaptive immune response
CNGA1	1259	2.40	3.46	<0.0001	Protein binding, plasma membrane
CD1C	911	2.29	0.09	0.0004	Adaptive immune response
CYP7B1	9420	1.95	1.63	0.0001	Oxidation-reduction process
LOC100128175	100128175	1.77	1.76	0.0001	N/A
KIAA1586	57691	1.13	1.34	0.0001	Ligase activity
SFMBT1	51460	0.88	0.08	0.0004	Protein binding, negative regulation of transctiption
FAM129A	116496	0.88	0.31	0.0003	Protein binding
PSIP1	11168	0.81	0.99	0.0001	RNA binding
PSAP	5660	−0.63	0.49	0.0003	Lipid binding
P4HB	5034	−0.66	0.48	0.0003	Metabolism
FTL	2512	−0.68	0.80	0.0002	Protein binding
FAS	355	−0.72	0.01	0.0005	Cell death induction
RRAGD	58528	−0.93	0.07	0.0004	Protein binding
SOAT1	6646	−0.99	0.05	0.0004	Protein binding
SERINC2	347735	−1.10	0.05	0.0004	Metabolism
SCD	6319	−1.12	1.40	0.0001	Oxidation-reduction process
TPP1	1200	−1.13	1.13	0.0001	Protein binding
CTSA	5476	−1.14	6.20	<0.0001	Protein metabolism
CTSB	1508	−1.26	3.32	<0.0001	Protein metabolism
ALDOC	230	−1.32	3.07	<0.0001	Protein binding
CTSD	1509	−1.35	1.14	0.0001	Protein metabolism
GM2A	2760	−1.37	4.19	<0.0001	Metabolism
GPNMB	10457	−2.18	0.30	0.0003	Protein binding
CCL18	6362	−2.47	6.23	<0.0001	Chemotaxis, signal transduction, inflammatory response
RSAD2	91543	−2.62	3.27	<0.0001	Protein binding

Table 1. Differentially expressed genes in dexa-tolDC, vitD3-tolDC and rapa-tolDC versus mDC. Results shown as mean difference of expression (MeanDiff). In all cases, $B > 0$ and $p < 0.01$. GO: Gene Ontology.

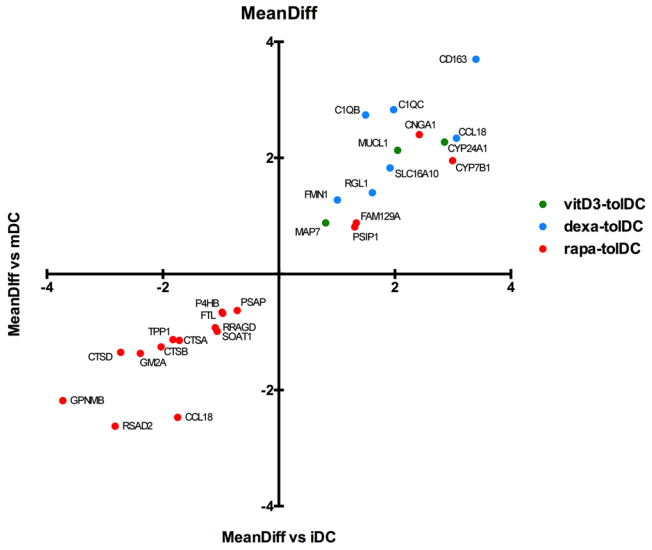

Figure 2. Differentially expressed genes in vitD3-, dexa- or rapa-tolDC versus both iDC and mDC with a B-statistic value > 0. Results shown as mean difference of expression (MeanDiff).

Additionally, a GSEA was also performed comparing tolDC and mDC versus iDC, and in this case both nucleosome assembly and autoreactivity and multifocal inflammation protein sets were found differentially over-expressed only in dexa, rapa and vitD3-tolDC at the same time, as there were no differences in their expression between iDC and mDC. However, again, the comparison of any pathway or biomarker in tolDC versus iDC could potentially provide results caused by the maturation process that these cells were exposed to, just like mDC, and not exclusively by the tolerogenic features of the cells. In fact, these same two protein sets did not show the same pattern in the previous GSEA versus mDC. Consequently, those sets which were upregulated in mDC versus iDC were excluded from the analysis. The results can be seen in Supplementary Table S1.

VitD3-tolDC presented an increased metabolic activity combined with a reduction in the apoptotic processes. When considering the pathways simultaneously regulated in vitD3-tolDC versus both iDC and mDC, we encountered that, as expected, those related with oxidative phosphorylation and the metabolism of vitamin D3 were overexpressed. In addition, the protein O-linked glycosylation pathway was also found over-expressed in vitD3-tolDC in comparison to mDC, as already reported in previous studies[17,18]. Furthermore, the ERK1/2 signaling cascade and the SP1 signaling factor, both involved in important tolerogenic functions, were induced in vitD3-tolDC respect of mDC. Consequently, the tolerance-inducing functionality of vitD3-tolDC is suggested to be driven by the up-regulation of the Treg lymphocyte induction genes and an increased expression of the extracellular region protein set compared to both iDC and mDC, together with the results shown in the previous section. These protein sets contain, in fact, important immune-related genes such as *CCL4* and *CCL7*, which determine T cell and monocyte chemotaxis respectively, as well as *MUCL1*, previously mentioned as a potential biomarker. Other up-regulated protein sets included viral and inflammatory response activities. In contrast, only the apoptosis pathway appeared to be differentially down-regulated in vitD3-tolDC. All the results are presented in Table 3 and Table 4.

A strongly down-regulated transcriptome is observed in rapa-tolDC. Contrary to vitD3-tolDC, the transcriptomic profile of rapa-tolDC was mostly consisting on down-modulated protein sets, evidencing 64 pathways that were repressed in comparison with both iDC and mDC. Of note, many of these down-modulated pathways were related with inflammation, chemotaxis and lipid metabolism (Table 4). Among them, 11 of these protein sets were those that appeared simultaneously up-regulated in dexa- and vitD3-tolDC, as mentioned above. As for the rest, many signaling, metabolic and transportation processes were inhibited in rapa-tolDC, such as the ERK1 and ERK2 cascade or the glycosphingolipid metabolism and cholesterol transport. Moreover, several protein sets related with the inflammatory and innate immune responses were also found inhibited, evidencing the potent immunosuppressant effect of rapamycin. Confirming previous reports, and as therefore expected, the mTOR pathway also appeared down-modulated in rapa-tolDC referred to mDC but not to iDC[19–21] (Table 3). On the other hand, only 3 protein sets were upregulated, being especially relevant the methylated histone binding and the DNA-templated transcription, as they indicate that deep changes might be happening regarding the DNA processing and epigenetics of rapa-tolDC.

Immune complement and macrophage features are expressed in dexa-tolDC. Similarly to vitD3-tolDC, dexa-tolDC presented a mostly up-regulated differential transcriptomic profile (Table 4). However, the induction of immune-related protein sets was much more relevant in this condition, with the positive regulation of immune complement activation and macrophage chemotaxis pathways. In addition, the up-modulation of immunosuppressant and DC tolerogenicity protein sets, along with the induction of the ERK1/2 signaling

Cell type	Gene	EntrezID	Coding protein	*MeanDiff*		*B-statistic*		*p-value*	
				vs iDC	vs mDC	vs iDC	vs mDC	vs iDC	vs mDC
vitD3-tolDC	*CYP24A1*	1591	Vitamin D3 24-Hydroxylase	2.86	2.27	3.54	0.72	<0.0001	0.0002
	MUCL1	118430	Mucin-Like Protein 1	2.05	2.13	0.97	1.13	0.0002	0.0001
	MAP7	9053	Microtubule Associated Protein 7	0.80	0.88	1.60	2.23	0.0001	<0.0001
dexa-tolDC	*CD163*	9332	Cluster of Differentiation 163	3.40	3.70	6.06	6.08	<0.0001	<0.0001
	C1QC	714	Complement C1q C Chain	1.98	2.83	0.01	3.39	0.0005	<0.0001
	C1QB	713	Complement C1q B Chain	1.50	2.74	0.04	6.33	0.0004	<0.0001
	CCL18	6362	C-C Motif Chemokine Ligand 18	3.06	2.34	9.83	4.79	<0.0001	<0.0001
	SLC16A10	117247	Solute Carrier Family 16 Member 10	1.92	1.83	2.37	1.45	<0.0001	0.0001
	RGL1	842953	RalGDS-Like 1	1.61	1.40	1.76	0.11	0.0001	0.0003
	FMN1	342184	Formin 1	1.01	1.27	2.21	4.36	<0.0001	<0.0001
rapa-tolDC	*CNGA1*	1259	Cyclic Nucleotide Gated Channel Alpha 1	2.42	2.40	3.77	3.46	<0.0001	<0.0001
	CYP7B1	9420	Oxysterol 7-Alpha-Hydroxylase	3.00	1.95	7.67	1.63	<0.0001	0.0001
	FAM129A	116496	Cell Growth-Inhibiting Gene 39 Protein	1.34	0.88	5.46	0.31	<0.0001	0.0003
	PSIP1	11168	PC4 And SFRS1 Interacting Protein 1	1.31	0.81	7.49	0.99	<0.0001	0.0001
	PSAP	5660	Prosaposin	−0.72	−0.63	1.88	0.49	0.0001	0.0003
	P4HB	5034	Prolyl 4-Hydroxylase Subunit Beta	−0.98	−0.66	5.45	0.48	<0.0001	0.0003
	FTL	2512	Ferritin Light Chain	−0.97	−0.68	5.35	0.80	<0.0001	0.0002
	RRAGD	58528	Ras Related GTP Binding D	−1.10	−0.93	1.79	0.07	0.0001	0.0004
	SOAT1	6646	Sterol O-Acyltransferase 1	−1.07	−0.99	0.72	0.05	0.0002	0.0004
	TPP1	1200	Tripeptidyl Peptidase 1	−1.82	−1.13	7.71	1.13	<0.0001	0.0001
	CTSA	5476	Cathepsin A	−1.72	−1.14	13.71	6.20	<0.0001	<0.0001
	CTSB	1508	Cathepsin B	−2.03	−1.26	11.08	3.32	<0.0001	<0.0001
	CTSD	1509	Cathepsin D	−2.73	−1.35	11.59	1.14	<0.0001	0.0001
	GM2A	2760	GM2 Ganglioside Activator	−2.39	−1.37	13.72	4.19	<0.0001	<0.0001
	GPNMB	10457	Glycoprotein NMB	−3.73	−2.18	7.27	0.30	<0.0001	0.0003
	CCL18	6362	C-C Motif Chemokine Ligand 18	−1.75	−2.47	1.94	6.23	0.0001	<0.0001
	RSAD2	91543	Viperin	−2.82	−2.62	4.48	3.27	<0.0001	<0.0001

Table 2. Differentially expressed genes in vitD3-tolDC, dexa-tolDC and rapa-tolDC versus both mDC and iDC. Results shown as mean difference of expression (MeanDiff). In all cases, $B > 0$ and $p < 0.01$.

cascade and the SP1 transcription factor, supports the tolerogenic functionality of dexa-tolDC. Moreover, the increased expression of the extracellular region protein set was also directed towards the immune function, with *CCL2*, *CCL4, CD163* and several other immune-related protein-encoding genes up-regulated. However, STAT1 appeared to be up-modulated in dexa-tolDC, which constituted an unexpected result due to its generally pro-inflammatory-related functionality. Another similarity with previously reported results for vitD3-tolDC was the up-modulation of the response to hypoxia also in dexa-tolDC[17].

Discussion

The number of clinical trials using autologous tolDC to treat autoimmune diseases is increasing each year, and the first results from several phase I studies have demonstrated that this tolerogenic therapy is safe for the patients[11–16]. Therefore, the role of these cells is gaining a huge relevance in the field of personalized medicine. Due to the wide variety of protocols that exist nowadays to generate tolDC *in vitro*, a deep study of the cells generated by them has become of key importance to elucidate which mechanisms of tolerance induction are being triggered. Establishing adequate quality controls and biomarkers that can ensure not only the functionality but also the safety of tolDC has become one of the main concerns towards its translation into the clinic[22]. Thus, determining if common pathways of tolerance are being promoted or whether each treatment is activating different mechanisms in the cellular product is important, as it would set up the first steps towards the finding of potential biomarkers of tolDC. Ideally, however, they should be able to generically identify these cells despite the protocol used to generate them.

To our knowledge, our microarray analysis constitutes the first study directly comparing three of the most widely used tolDC-inducing protocols. Unfortunately, it was not possible to find a common DEG in the transcriptomic profile of vitD3-, dexa- and rapa-tolDC. In fact, just a brief analysis of the whole transcriptomic profile looking at the heat map already evidenced that different protocols came with different prints, as rapa-tolDC showed not only a different but a completely opposite genetic signature compared to dexa- and vitD3-tolDC. These results are in accordance with a previous study by our group that evidenced different phenotypical and functional characteristics of dexa, rapa and vitD3-tolDC[3]. Our current study allowed us to go deeper in that direction and, in fact, we could identify some potential biomarkers for both rapa- and dexa-tolDC, *CCL18* and *FAM129A* genes. However, they showed an opposed behavior pattern –while *CCL18* appeared to be differentially induced in dexa-tolDC, it was down-modulated in rapa-tolDC, and vice versa for *FAM129A*–, evidencing that the

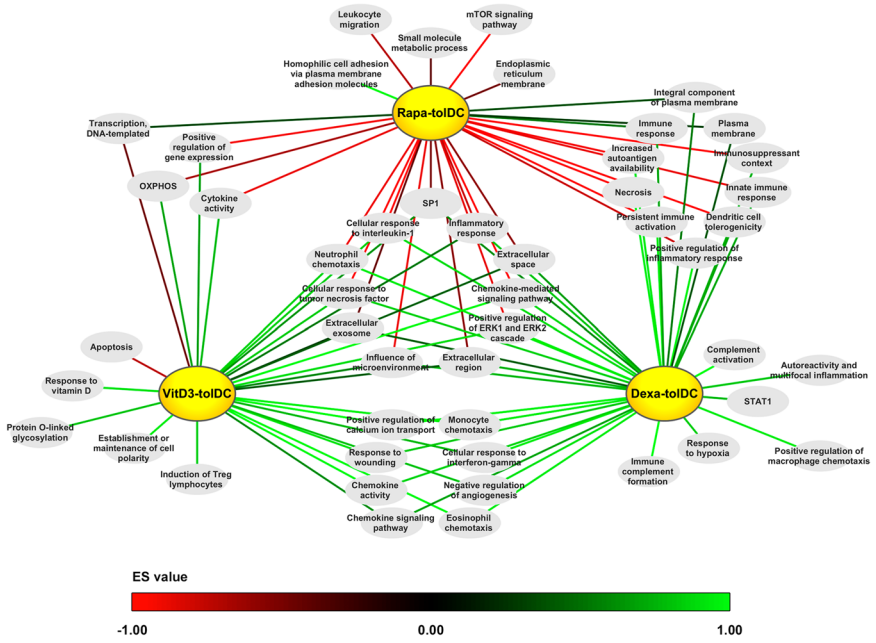

Figure 3. Graphical representation of the enriched pathways and protein sets in vitD3-, dexa- and/or rapa-tolDC. The color code indicates the degree of enrichment of each protein set based on their ES, from red (ES = −1) to green (ES = 1) as indicated by the color scale bar.

molecular tolerance-inducing mechanisms triggered by these two immunomodulatory agents must be different. Specifically, *CCL18* is of a notorious relevance, since the cytokine encoded by this gene has been reported to have a fundamental role in the cell differentiation process towards the development of semi-mature DC with the ability to produce IL-10 and prime Treg[23].

The fact that common candidate biomarkers could not be found for our three tolDC-inducing protocols, however, does not decrease the value of those genes that appeared as differentially expressed on each one of the studied conditions versus both iDC and mDC. For instance, as indicators of the up-regulation of the protein O-linked glycosylation, of the response to vitamin D treatment, and of the establishment of cell polarity, detected by the GSEA in vitD3-tolDC, *MUCL1*, *CYP24A1*, and *MAP7* genes, respectively, were found strongly induced versus iDC and mDC at the same time. Provided that the O-linked glycosylation has been directly related with the regulation of microtubule-associated proteins of the cytoskeleton[24], combined with previous studies reporting *CYP24A1* and *CAMP* genes as directly related to the vitamin D3 metabolism[25–28], all these genes apparently make good candidates to become strong biomarkers of vitD3-tolDC. Additionally, these candidates have also been related to many other key cell processes such as the glucose metabolism, stress response and cell cycle, as reviewed by Hart *et al.*[29]. Moreover, their discovery is also supported by the positive B-statistic values showed by these genes in the microarray. Further supporting our results, an induction of the oxidative metabolism could also be detected, which constitutes a key feature of vitD3-tolDC, as previously reported[17].

In the case of dexa-tolDC, apart from *CCL18*, already discussed above, the overexpression of the genes encoding CD163 and two different chains of the complement C1q protein (*C1QB* and *C1QC*) versus both iDC and mDC are the most relevant results as potential biomarkers, due to their immune-related implications. It is also worth mentioning that *MERTK* has been previously reported as a biomarker for dexa-tolDC in several studies[6,30]. Our results, however, do not evidence the differential expression of this gene, but it might be explained due to the intrinsic limitations of microarrays regarding false negatives results. As for *CD163*, its role in tolerance induction has already been reported in M2 macrophages but, so far, not for tolDC. We have also detected an enrichment on the macrophage chemotaxis protein set. Therefore, our results suggest that both regulatory macrophages and dexa-tolDC might be triggering similar tolerogenic mechanisms, probably through the STAT3 and Wnt5a signaling pathway, as its interaction with CD163 has already been reported in cancer studies[31,32]. Regarding *C1QB* and *C1QC* genes, their overexpression is aligned with the up-modulation of the complement activation protein set. The role of the immune complement system, as a promoter of immune tolerance in dendritic cells, has been overlooked until the last few years. Nevertheless, as reviewed by Luque *et al.*[33], recent studies demonstrate that C1q is involved in key tolerogenic processes such as an increased surface PD-L2 and decreased CD86 expression, linked to a reduced induction of Th1 and Th17 proliferation[34], the inhibition of the production of pro-inflammatory mediators[35] and an increased production of anti-inflammatory cytokines, such as IL-10[36,37]. As a matter of fact, *C1QB* has previously been proposed as a potential biomarker for tolerogenicity[38], and its differential expression along with other genes encoding the C1q protein has been reported in previous studies[30]. Therefore, our results seem to indicate that dexa-tolDC might be developing their tolerogenic properties through the mentioned mechanisms, among others that will be discussed below. Conversely, our microarray also detected an induction of the STAT1 signaling pathway, which has been reported as pro-inflammatory and opposed to STAT3, switching

GSEA versus mDC

DATABASE	PROTEIN SET NAME	Dexa-tolDC	Rapa-tolDC	VitD3-tolDC	iDC	DATABASE	PROTEIN SET NAME	Dexa-tolDC	Rapa-tolDC	VitD3-tolDC	iDC
KEGG	Chemokine signaling pathway	▲	=	▲	=	GO	Leukocyte migration	=	▼	=	=
GO	Chemokine activity	▲	=	▲	=	GO	Small molecule metabolic process	=	▼	=	=
GO	Response to wounding	▲	=	▲	=	GO	Transcription, DNA-templated	=	▲	▼	=
GO	Cellular response to interleukin-1	▲	▼	▲	=	BED	Smooth muscle apoptosis	=	=	▼	=
GO	Extracellular exosome	▲	▼	▲	=	GO	Cellular response to interferon-gamma	▲	=	▲	▼
GO	Extracellular region	▲	▼	▲	=	GO	Eosinophil chemotaxis	▲	=	▲	▼
GO	Extracellular space	▲	▼	▲	=	GO	Monocyte chemotaxis	▲	=	▲	▼
GO	Inflammatory response	▲	▼	▲	=	GO	Positive regulation of calcium ion transport	▲	=	▲	▼
GO	Establishment or maintenance of cell polarity	=	=	▲	=	GO	Cellular response to tumor necrosis factor	▲	▼	▲	▼
GO	Protein O-linked glycosylation	=	=	▲	=	GO	Chemokine-mediated signaling pathway	▲	▼	▲	▼
GO	Response to vitamin D	=	=	▲	=	BED	Influence of microenvironment	▲	▼	▲	▼
GO	Cytokine activity	=	▼	▲	=	GO	Neutrophil chemotaxis	▲	▼	▲	▼
BED	OXPHOS	=	▼	▲	=	GO	Positive regulation of ERK1 and ERK2 cascade	▲	▼	▲	▼
GO	Positive regulation of gene expression	=	▼	▲	=	TRRUST	SP1	▲	▼	▲	▼
BED	Autoreactivity and multifocal inflammation	▲	=	=	=	BED	Induction of Treg lymphocytes	=	=	▲	▲
GO	Complement activation	▲	=	=	=	GO	Immune response	▲	▲	=	▲
BED	Immune complement formation	▲	=	=	=	GO	Integral component of plasma membrane	▲	▲	=	▼
GO	Positive regulation of macrophage chemotaxis	▲	=	=	=	GO	Plasma membrane	▲	▲	=	▼
GO	Response to hypoxia	▲	=	=	=	BED	Dendritic cell tolerogenicity	▲	▼	=	▼
TRRUST	STAT1	▲	=	=	=	BED	Immunosuppressant context	▲	▼	=	▼
BED	Persistent immune activation	▲	▼	=	=	BED	Increased autoantigen availability	▲	▼	=	▼
GO	Positive regulation of inflammatory response	▲	▼	=	=	GO	Innate immune response	▲	▼	=	▼
KEGG	mTOR signaling pathway	=	▼	=	=	BED	Necrosis	▲	▼	=	▼
GO	Endoplasmic reticulum membrane	=	▼	=	=	GO	Homophilic cell adhesion via plasma membrane adhesion molecules	=	▲	=	▲

Table 3. Enriched protein sets in dexa-tolDC, rapa-tolDC, vitD3-tolDC and/or iDC versus mDC. Green arrow: upregulation of said set; Yellow bar: unchanged regulation of said set; Red arrow: downregulation of said set. BED: Biological Effectors Database; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; TRRUST: Transcriptional Regulatory Relationships Unraveled by Sentence-based Text-mining.

between both responses under the control of NOD1 after an IL-10-dependant activation[39]. However, other studies also reported that, in regulatory dendritic cells such as dexa-tolDC, STAT1 can be activated in response to TLR stimuli in order to attract Th1 cells through an increased CXCL10 production and subsequently inhibit them[40].

Comparative studies between vitD3- and dexa-tolDC have been reported before, demonstrating many similarities between the two conditions regarding their semi-mature status and the inhibition of allogeneic proliferation[3], the NF-κB pathway suppression[41–43] and the polarization of the immune response towards a Th2 profile[44]. Some differences, however, have been described regarding the antigen-specific induction of Treg[45], and a proteomic comparative study also evidenced differences in the protein expression profile, despite confirming that vitD3- and dexa-tolDC were very similar on the phenotypical and functional aspects[46]. Furthermore, most of the mentioned studies also evidenced that the effect of both drugs was syngeneic, enhancing the tolDC-inducing effect of vitamin D3 and dexamethasone when used in combination, instead of each one independently. In fact, this approach has even been tested on a clinical trial, with successful results regarding the tolerability and safety of the cell product[16,47,48]. Our study confirmed this resemblance between vitD3- and dexa-tolDC, as well as their tolerogenic potential, since a strong up-modulation of the ERK1/2 and SP1 pathways was observed in

vitD3-tolDC

DATABASE	PROTEIN SET NAME	vs iDC	vs mDC	DATABASE	PROTEIN SET NAME	vs iDC	vs mDC
BED	Immune response to skin-stage plasmodium	▲	▲	GO	Response to calcium ion	▲	▲
BED	OXPHOS	▲	▲	GO	Response to drug	▲	▲
BED	Treg immunosuppression	▲	▲	GO	Viral entry into host cell	▲	▲
GO	Virus receptor activity	▲	▲	GO	Response to vitamin D	▲	▲
GO	Extracellular region	▲	▲	TRRUST	VDR	▲	▲
GO	Inflammatory response	▲	▲	KEGG	Apoptosis	▼	▼

dexa-tolDC

DATABASE	PROTEIN SET NAME	vs iDC	vs mDC	DATABASE	PROTEIN SET NAME	vs iDC	vs mDC
BED	Autoreactivity and multifocal inflammation	▲	▲	GO	Immune response	▲	▲
BED	Immune complement formation	▲	▲	GO	Positive regulation of macrophage chemotaxis	▲	▲
BED	Dendritic cell tolerogenicity	▲	▲	GO	Complement activation	▲	▲
BED	Immunosuppressant context	▲	▲	GO	Response to hypoxia	▲	▲
GO	Extracellular region	▲	▲	TRRUST	STAT1	▲	▲
GO	Collagen trimer	▲	▲	GO	Endosome membrane	▼	▼
GO	Inflammatory response	▲	▲				

rapa-tolDC

DATABASE	PROTEIN SET NAME	vs iDC	vs mDC	DATABASE	PROTEIN SET NAME	vs iDC	vs mDC
GO	Methylated histone binding	▲	▲	GO	Sphingolipid metabolic process	▼	▼
GO	Transcription, DNA-templated	▲	▲	GO	Positive regulation of ERK1 and ERK2 cascade	▼	▼
GO	Homophilic cell adhesion via plasma membrane adhesion molecules	▲	▲	GO	Positive regulation of vascular endothelial growth factor receptor signaling pathway	▼	▼
BED	Increased autoantigen availability	▼	▼	GO	Cellular response to tumor necrosis factor	▼	▼
BED	Degradation of extracellular matrix	▼	▼	GO	Negative regulation of tumor necrosis factor production	▼	▼
BED	Influence of microenvironment	▼	▼	GO	Small molecule metabolic process	▼	▼
BED	Innate immune response	▼	▼	GO	Extracellular matrix disassembly	▼	▼
BED	Skin inflammation	▼	▼	GO	Keratan sulfate catabolic process	▼	▼
GO	Innate immune response	▼	▼	GO	Inflammatory response	▼	▼
BED	Imbalanced lipid metabolism	▼	▼	GO	Cell-substrate junction assembly	▼	▼
BED	OXPHOS	▼	▼	GO	Response to virus	▼	▼
GO	Cholesterol transport	▼	▼	BED	Joint inflammation - sacroiliitis	▼	▼
BED	Necrosis	▼	▼	GO	Response to ethanol	▼	▼
GO	Collagen binding	▼	▼	GO	Protein catabolic process	▼	▼
GO	Cellular response to interleukin-1	▼	▼	GO	Drug transmembrane transport	▼	▼
GO	Lysosomal lumen	▼	▼	GO	Ion transmembrane transport	▼	▼
GO	Positive regulation of vascular endothelial growth factor production	▼	▼	GO	Vacuolar proton-transporting V-type ATPase complex	▼	▼
GO	Extracellular exosome	▼	▼	GO	Ganglioside catabolic process	▼	▼
GO	Extracellular region	▼	▼	GO	Heparin binding	▼	▼
GO	Lipid particle	▼	▼	GO	Collagen catabolic process	▼	▼
GO	Melanosome	▼	▼	GO	Extracellular space	▼	▼
GO	Lysosome	▼	▼	GO	Chemotaxis	▼	▼
GO	Chemokine-mediated signaling pathway	▼	▼	GO	Proton-transporting V-type ATPase, V0 domain	▼	▼
GO	Endoplasmic reticulum membrane	▼	▼	GO	Glycosaminoglycan metabolic process	▼	▼
GO	Low-density lipoprotein particle	▼	▼	GO	Extracellular matrix organization	▼	▼
GO	Autophagy	▼	▼	GO	Regulation of autophagy	▼	▼
GO	Proteolysis	▼	▼	TRRUST	SPI1	▼	▼
GO	Glycosphingolipid metabolic process	▼	▼	TRRUST	SP1	▼	▼
GO	Neutrophil chemotaxis	▼	▼	TRRUST	USF1	▼	▼
GO	Cellular iron ion homeostasis	▼	▼	TRRUST	JUN	▼	▼
GO	Leukocyte migration	▼	▼	TRRUST	SP3	▼	▼
BED	T3/T4 production	▼	▼				

Table 4. Enriched pathways and protein sets, versus both iDC and mDC, in vitD3-tolDC, rapa-tolDC and dexa-tolDC. Green arrow: upregulation of said set; Yellow bar: unchanged regulation of said set; Red arrow: downregulation of said set. BED: Biological Effectors Database; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; TRRUST: Transcriptional Regulatory Relationships Unraveled by Sentence-based Text-mining.

both conditions, among other protein sets. These specific pathways have been reported to be involved in key mechanisms of tolerance induction, such as, TGF-ß secretion[49–51], dendritic cell survival[52], TLR-dependent and independent IL-10 production[51,53,54], and functional stability[55]. Surprisingly, however, we could not find any DEG in common for both dexa- and vitD3-tolDC respect of mDC, despite sharing the induction of such key pathways.

In addition, our results also showed that not only rapa-tolDC do not share the up-regulation of any of the discussed pathways in common with the other studied tolDC conditions, but that they are even down-modulated

after rapamycin treatment. Furthermore, mTOR signaling has been reported as a crucial and even indispensable mechanism to maintain the tolerogenic functionality of vitD3- and dexa-tolDC in some of the same reports cited above[17,55]. Therefore, and provided that rapamycin is, indeed, the natural inhibitor of the mTOR signaling pathway, the transcriptomic and functional incompatibility of both dexa- and vitD3-tolDC with rapa-tolDC becomes evident. Consequently, our results suggest that different mechanisms might be triggered in rapa-tolDC to induce immune tolerance.

The down-modulation of the mTOR signaling by the response to rapamycin constitutes the main signature of these cells, and through the inhibition of its dependent pathways, several immune-related mechanisms have been reported to play a role in the induction of tolerance, as reviewed by Stallone *et al.*[56]. For instance, rapamycin has been described to both induce the up-regulation of CCR7 and dampen the production of IL-10 in monocyte-derived DC, but also that the surface expression of the former is inhibited by the latter[57]. Furthermore, the rapamycin-mediated inhibition of mTOR also reportedly induces the expression of ILT3 and ILT4 in DC, through the down-modulation of CD40, in order to prime $Foxp3^+$ Treg and switch the immune response towards a Th2 profile[58]. Consequently, and in accordance to our results, the low IL-10 secretion by rapa-tolDC is functionally logical and demonstrates that tolerance can be achieved by different mechanisms that look apparently contradictory at first sight. Apart from the inhibition of mTOR, the effect of rapamycin comes along with the repression of many other immune-related genes, pathways and proteins. Many of them are involved in pro-inflammatory and chemotactic processes, thus demonstrating the strong immunosuppressant effect of this drug. In fact, while only overexpressed genes could be detected as potential biomarkers in the case of vitD3- and dexa-tolDC, for rapa-tolDC, from a total of 17 selected DEG, 13 of them were repressed and only 4 appeared up-modulated respect both iDC and mDC. A similar situation was evidenced for the selected protein sets after the GSEA analysis, both exclusively and in comparison to the other tolDC conditions, as discussed above.

In any case, the incapability to find common biomarkers arises the idea that, although a normalized transcriptomic profile of immune tolerance induction might not be achieved, at least a small pool of the most representative genes of each condition, constituting a "generic" tolDC signature, could be established. Nevertheless, it is worth stating that single results obtained from microarrays are highly prone to be biased, as the generally low B-statistic values found in our results suggest. Therefore, we cannot fully discard the possibility of having overlooked a determined universal genetic biomarker of tolerance, just like we did, for instance, with MERTK in dexa-tolDC. Nevertheless, this scenario seems unlikely given the strong differences that we have observed among the transcriptomic profiles of our tolDC conditions, and that were confirmed by the GSEA. Indeed, enrichment analyses provide an increased reliability to microarray studies, as they are based in the grouped expression of genes instead of single results and, as a matter of fact, many of the genes and pathways found in our array for each individual tolDC protocol have been previously reported and even evidenced in similar transcriptomic and proteomic studies[7,17,30,46,59], thus strengthening our results.

In conclusion, and despite further validation is required, *CYP24A1*, *MUCL1*, *MAP7*, *CD163*, *CCL18*, *C1QB*, *C1QC*, *CYP7B1* and *CNGA1* genes, among several others, have been identified as potential biomarkers for the different individual tolDC-generating protocols. Furthermore, we have also been able to identify several pathways that are being differentially modulated by the pharmacological tolDC-inducing treatments, suggesting that immune tolerance is a complex status that can be achieved through different mechanisms. After all, several publications have demonstrated the capability of these protocols to generate functional immune regulatory cells, despite their differences. This functional heterogenicity, however, also suggest that determined tolDC-inducing protocols might be more suitable than others for the treatment of specific autoimmune diseases. For instance, a defect on the functionality and activation of Treg has been described in patients with type 1 diabetes and myasthenia gravis[60–62]. Consequently, based on both the literature and our current and previous results[3,45,56,58], vitD3-tolDC and rapa-tolDC might constitute better therapeutic alternatives than dexa-tolDC in these two specific examples, since the induction of Treg plays an important role in their tolerogenic functionality. On the other hand, in diseases in which the presence of autoreactive T cells plays a main role, such as multiple sclerosis[63,64], the vitD3-tolDC-mediated induction of hyporesponsiveness over these pathologic cells might have a more beneficial effect. However, this is far from demonstrated yet and, provided the complexity of the mechanisms of tolerance induction within the immune system, several *in vitro* experiments and clinical trials should be conducted in order to compare the efficacy of different protocols. In any case, and although our results seem to indicate that finding a common biomarker of tolerogenicity might be utopic, they also reinforce the role of tolDC as a promising therapeutic approach for the immediate future.

Methods

Sample collection and *in vitro* tolDC generation. Five samples from healthy donors of iDC, mature mDC and the three conditions of tolDC differentiated in the presence of either vitamin D3 (vitD3-tolDC), dexamethasone (dexa-tolDC) or rapamycin (rapa-tolDC) were selected from previous experiments by our group[3]. The Ethical Committee of Germans Trias i Pujol Hospital approved the study, and all subjects gave their informed consent according to the Declaration of Helsinki (BMJ 1991; 302: 1994). Briefly, for the DC differentiations, buffy coats provided by the *Banc de Sang i Teixits* (Barcelona, Spain) were processed, first depleting T $CD3^+$ cells using a RosetteSep Human CD3 Depletion Cocktail (StemCell Technologies, Vancouver, Canada) during a ficoll-hypaque (Rafer, Zaragoza, Spain) gradient separation and later isolating monocytes by positive selection using the EasySep Human CD14 Positive Selection Kit (StemCell Technologies). In all cases, purity was greater than 95% and viability greater than 90%. Monocytes were cultured for 6 days in cGMP-grade X-VIVO 15 medium, supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, in the presence of 1000 U/mL clinical-grade granulocyte-macrophage colony- stimulating factor (GM-CSF; CellGenix, Freiburg, Germany) and 1000 U/mL clinical-grade interleukin 4 (IL-4; CellGenix). Respectively, half and total volume of fresh medium and cytokines were replenished on days 2 and 4. All the conditions except for iDC were treated on day 4 with a maturation

cocktail of clinical-grade cytokines containing 1000 U/mL tumor necrosis factor alpha (TNFα; CellGenix), 10 ng/mL IL-1β (CellGenix) and 1 μM prostaglandin E2 (PGE2; Pfizer, New York, NY, USA). While mDC did not receive any additional stimulus, the different tolDC conditions were obtained adding either 1 nM vitamin D3 (Calcijex, Abbott, Chicago, IL, USA) on days 0 and 4, 1 μM dexamethasone (Fortecortín, Merck, Spain) on days 2 and 4 or 10 nM rapamycin (Rapamune, Wyeth, Spain) on days 2 and 4. In order to determine optimal and comparable concentrations of each of these immunomodulatory agents, dose-dependent experiments were set up using mDC as reference. Cells were harvested on day 6 for further characterization and functional assays, and later centrifuged and stored as dry pellets at −80 °C. The complete characterization of vitD3-, dexa- and rapa-tolDC regarding phenotype, cytokine secretion and functionality can be found in our previous study by Naranjo-Gómez *et al.*[3].

Preparation of RNA samples and microarray analysis. Total RNA was isolated from the dry pellet samples using RNeasy Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and RNA Integrity Number (RIN) was assessed. Only samples with good quality were considered (RIN $\geq$ 6). Total RNA was later retrotranscribed, and the resulting cDNA was further preamplified using the Ovation® PicoSL WTA System V2 kit (NuGEN Technologies, San Carlos, CA, USA) at the *Unitat Cientificotècnica de Suport* of the Vall d'Hebron Research Institute (Barcelona, Spain), due to the low amount of RNA initially obtained in some of the samples (1–300 ng). Subsequently, the cDNA was fragmented, labeled and hybridized to the 33297 probes of a GeneChip 1.0 microarray chip (Affymetrix, Santa Clara, CA, USA). The statistical analysis was performed using R software and the libraries developed for microarray data analysis by the Bioconductor Project (www.bioconductor.org). All the samples demonstrated high quality cDNA characteristics, with a 3′/5′ ratio of probe sets for glyceraldehyde-3-phosphate dehydrogenase and beta-actin of $<$1.5.

Differentially expressed genes selection. All the images generated by the microarray were processed at the Department of Statistics from the University of Barcelona. The raw data obtained from the image ("CEL") files were pre-processed using the robust multi-array average method[65], which performs a three-step process consisting of background correction, normalization and summarization at gene level. The resulting expression values were then submitted to a two-step non-specific filtering process; First, those genes whose mean signal per group was below the 50th percentile of all signals were removed. From the remaining genes, those whose standard deviation was below the 50th percentile of all standard deviations were further filtered out. These normalized filtered values were used for all the analysis. The selection of DEG was based on a linear model analysis with empirical Bayes moderation of the variance estimates, following the methodology developed by Smyth[66]. The Benjamini and Hochberg method[67] was used to adjust the p-values in order to obtain a strong control over the false discovery rate. For each gene, B-statistic values were calculated. Briefly, this parameter roughly indicates the logarithm of the odds of a gene to be effectively differentially expressed, and the higher the B value, the more likely that one determined result is reliable.

Identification of enriched pathways and protein sets. A GSEA was performed by Anaxomics (Barcelona, Spain) over our microarray data in order to determine the presence of enriched pathways and protein sets between our different tolDC conditions, following previously described methodology[68]. The analysis was performed over protein sets from several databases, including Gene Ontology (GO) terms (biological process, cellular component and molecular function) according to the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI)/UniProt-GO[69], Biological Effectors Database (BED, property of Anaxomics), Kyoto Encyclopedia of Genes and Genomes (KEGG)[70], Pharmacogenomics Knowledgebase (PharmGKB)[71], Small Molecule Pathway Database (SMPDB)[72] and the regulatory molecular mechanisms included in the Transcriptional Regulatory Relationships Unraveled by Sentence-based Text-mining (TRRUST) database[73]. The degree of enrichment of a determined protein set was evaluated based on their respective enrichment score (ES). Cytoscape 3.5.1. software was used to create the representation of the common and individual enriched protein sets between each tolDC condition, based on their ES score.

Accession code. Microarray data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6937 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6937).

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Author Contributions

C.R.-T. and E.M.M.-C. conceived the experiment. M.N.-G. performed the cell cultures and sample preparations. A.S.-P. performed the bioinformatic analysis. J.N.-B. and M.J.M. analyzed the results. E.M.M.-C., J.N.-B. and M.J.M. interpreted the results. J.N.-B. wrote the manuscript. A.S.-P., A.T.-S., B.Q.-S., C.R.-T., E.M.M.-C., J.N.-B., M.J.M. and M.N.-G. reviewed the manuscript.

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Results II

MAP7 ***and*** **MUCL1** ***are biomarkers of vitamin D3-induced tolerogenic dendritic cells in multiple sclerosis patients***

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MAP7 and *MUCL1* Are Biomarkers of Vitamin D3-Induced Tolerogenic Dendritic Cells in Multiple Sclerosis Patients

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The administration of autologous tolerogenic dendritic cells (tolDC) has become a promising alternative for the treatment of autoimmune diseases, such as multiple sclerosis (MS). Specifically, the use of vitamin D3 for the generation of tolDC (vitD3-tolDC) constitutes one of the most widely studied approaches, as it has evidenced significant immune regulatory properties, both *in vitro* and *in vivo*. In this article, we generated human vitD3-tolDC from monocytes from healthy donors and MS patients, characterized in both cases by a semi-mature phenotype, secretion of IL-10 and inhibition of allogeneic lymphocyte proliferation. Additionally, we studied their transcriptomic profile and selected a number of differentially expressed genes compared to control mature and immature dendritic cells for their analysis. Among them, qPCR results validated *CYP24A1*, *MAP7* and *MUCL1* genes as biomarkers of vitD3-tolDC in both healthy donors and MS patients. Furthermore, we constructed a network of protein interactions based on the literature, which manifested that *MAP7* and *MUCL1* genes are both closely connected between them and involved in immune-related functions. In conclusion, this study evidences that *MAP7* and *MUCL1* constitute robust and potentially functional biomarkers of the generation of vitD3-tolDC, opening the window for their use as quality controls in clinical trials for MS.

Keywords: tolerogenic dendritic cells, multiple sclerosis, biomarkers, vitamin D3, immune tolerance

INTRODUCTION

The role of dendritic cells (DC) within the immune system is crucial, since they are in charge of orchestrating immune responses and maintaining the homeostasis between immunogenicity and tolerance. Under normal conditions, DC remain in an immature status (iDC), characterized by their ability to capture and present antigens and other signals in their environment. However, these cells are not stable, and when exposed to a danger signal, iDC become activated and differentiate into professional, antigen-presenting mature DC (mDC). This pro-inflammatory status is

characterized by an increase in the expression of MHC and co-stimulatory molecules, thus enabling mDC to induce an efficient and potent immunogenic response (1–4).

If the immune homeostasis is lost, and a breach of tolerance causes mDC to recognize and present specific self-antigens to T cells, different autoimmune pathologies may develop depending on which protein or tissue is targeted, such as multiple sclerosis (MS), rheumatoid arthritis or type 1 diabetes. These complex disorders involve many innate and adaptive mechanisms of the immune system, and their etiology still remains unknown. For this reason, a cure has not been found yet, and the currently available treatments consist in strong immunomodulatory or immunosuppressive drugs. In general, these are focused on addressing the symptoms in a poorly effective and unspecific manner, with potentially severe side effects. Hence, there is an increasing need for new, more specific and effective therapies. Over the last years, tolerogenic DC (tolDC) have been postulated as a novel and promising alternative to treat these disorders (5). In fact, several approaches have already been tested in Phase I clinical trials for autoimmune diseases, as reviewed by ten Brinke et al. (6), and other clinical studies are still ongoing. In all cases, these treatments have demonstrated to be safe, with no relevant side effects on the patients. Consequently, many initiatives are now focused on assessing the actual efficacy of tolerogenic cell therapies.

In general, tolDC are defined as a stable and semi-mature subset of DC with the potential to restore immune tolerance in an antigen-specific manner if loaded with one or more determined peptides, thus not compromising the protective immunity of the patients. Compared to mDC, these cells are typically characterized by their low —or lower— expression of MHC and co-stimulatory molecules (such as CD40, CD80, CD83 or CD86), and by their reduced or null secretion of IL-12, IFN-γ and other pro-inflammatory cytokines, combined with an increment in the production of IL-10 or TGF-β. These features confer to tolDC a reduced capability to induce T cell proliferation and the possibility to prime regulatory T cell (Treg) responses, thus potentially directing the immune response toward a regulatory context (7–9). However, these characteristics can sometimes be very variable, since there is a wide variety of protocols to generate tolDC *in vitro* from human peripheral blood monocytes. These approaches include the use of several compounds, cytokines and immunomodulatory drugs such as IL-10 (10, 11), dexamethasone (11–15) or rapamycin (11, 12, 16), as well as different genetic engineering techniques (17, 18).

Among all of them, the use of 1,25-dihydroxyvitamin D3 —the active form of vitamin D3— to generate tolDC is one of the most widely established protocols. Specifically, vitamin D3-induced tolDC (vitD3-tolDC) present a semi-mature profile, accompanied by an ability to inhibit allogenic T cell proliferation and to polarize the immune response toward an anti-inflammatory T_H2 profile (12, 19–28). Furthermore, several studies using animal models of autoimmune diseases have demonstrated their functionality *in vivo* (29–31). In general, these cells are characterized by the suppression of the NF-κB pathway (21, 32), accompanied by an increased activity of the oxidative metabolism of glucose, and indeed the glucose availability and the glycolytic activity mediated through mTOR signaling are crucial for the induction and maintenance of their tolerogenic function (27). However, despite the identification of several pathways involved in the anti-inflammatory role of vitD3-tolDC, the specific mechanisms for the induction of immune tolerance by these cells have not been clearly identified yet.

Previously, our group has successfully generated human vitD3-tolDC —demonstrating their tolerogenic properties *in vitro* using cells generated from both healthy donors and MS patient samples—, and has studied their transcriptomic profile compared to other tolDC protocols (12, 25, 26, 33). Additionally, in further *in vivo* studies, we also reported a positive and beneficial effect of antigen-specific vitD3-tolDC treatment over the course of the murine model of MS, the experimental autoimmune encephalomyelitis (30, 31). Altogether, these results have led to the development of an ongoing Phase I clinical trial in MS patients with peptide-loaded vitD3-tolDC (http://www.clinicaltrials.gov, NCT02903537).

However, for the full translation of an autologous, antigen-specific, tolerogenic cell therapy into the common clinical practice, several additional steps yet need to be taken. Among them, the definition of adequate, robust and objective biomarkers constitutes one of the priorities. These markers would, on the one hand, guarantee the proper generation and functionality of tolDC, without compromising the safety for the patients. On the other hand, these biomarkers would enable the comparison of results with other research groups, hereby accelerating the translation of tolDC therapies into the clinic in a collaborative endeavor (6). However, although many efforts have been made in this regard, and despite several genes and molecules have been identified for a variety of tolDC protocols separately, such as *IDO1*, *GILZ*, or *ANXA1*, the definition of universal biomarkers of tolerance-inducing cell products has not been possible so far, and it seems unlikely provided the wide heterogenicity of approaches being used (34). For this reason, in this study we have analyzed the transcriptomic profile of vitD3-tolDC in order to select and validate several differentially expressed genes (DEG) that may be used as transcriptomic biomarkers of these cells in a clinical trial for MS patients.

MATERIALS AND METHODS

Sample Collection

Buffy coat samples from 24 randomized healthy donors were obtained from the *Banc de Sang i Teixits* (Barcelona, Spain), following the institutional Standard Operating Procedures for blood donation, which included a signed informed consent. Whole blood samples from 10 MS patients were collected by standard venipuncture in lithium heparin tubes. Patients did not receive any corticoid or disease-modifying therapy during at least the previous 2 months, and both relapsing and progressive forms of the disease were considered. The same procedure was followed for whole blood obtention from 34 healthy donors for the functional assays (see below). This study was approved by the Germans Trias i Pujol Hospital ethical committee, and all patients and healthy controls signed an informed consent.

Complementary DNA (cDNA) of paired IL-10-induced tolDC (IL10-tolDC) and mDC samples from 5 healthy donors were obtained from Sanquin Bloodbank (Amsterdam, The Netherlands) after informed consent. These samples were generated as described in Boks et al. (11).

Monocyte Isolation

Samples from healthy donors were processed first depleting $CD3^+$ cells using the RosetteSep® Human Monocyte Enrichment Cocktail kit (StemCell Technologies, Vancouver, Canada) prior to a ficoll-hypaque (Rafer, Zaragoza, Spain) density gradient separation. Subsequently, $CD14^+$ cells were isolated by positive selection using the EasySep® Human CD14 Positive Selection Kit (StemCell) following manufacturer's instructions. For the isolation of monocytes from MS patients, peripheral blood monocytes were isolated from 50 mL of whole blood by ficoll-hypaque density gradient separation, followed by the abovementioned CD14 positive selection step. The initial $CD3^+$ cells depletion step was not performed due to the limited amount of blood. Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and cell viability was determined using 7-amino-actinomycin D (7-AAD) (BD Biosciences) and phycoerythrin (PE)-conjugated annexin V (Immunotools, Friesoythe, Germany) staining for 20 min at 4°C, protected from light. Cell counts were quantified using PerfectCount microspheres (Cytognos, Salamanca, Spain) and monocyte purity was determined using forward and side scatter gating strategies on FACSDiva software (BD Biosciences).

VitD3-tolDC Generation

Monocytes from both healthy donors and MS patients were cultured at a density of 1×10^6 cells/mL in 24-well plates at 37°C and a 5% CO_2 atmosphere for 6 days in 1 mL X-VIVO 15 medium (Lonza, Basel, Switzerland), supplemented with 2% heat-inactivated human AB serum, 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin (Reig Jofre, Sant Joan Despí, Spain) and 100 μg/mL streptomycin (Normon, Tres Cantos, Spain). For the generation of iDC, monocytes were differentiated in the presence of 200 U/mL granulocyte macrophage colony-stimulating factor (GM-CSF) and 250 U/mL IL-4 (both from Peprotech, London, UK). Whole volume of fresh medium and cytokines was replenished on day 4. In addition, a maturation cocktail containing 1,000 U/mL IL-1β, 1,000 U/mL TNF-α (both from Peprotech) and 1 μM prostaglandin E2 (PGE2) (Pfizer, New York, NY, USA) was added on day 4 to both mDC and vitD3-tolDC conditions. Finally, for the induction of vitD3-tolDC, these cells were besides treated with 1 nM vitamin D3 (Calcijex, Abbott, Chicago, IL, USA) on days 0 and 4. On day 6, all three conditions were harvested following an incubation with accutase (Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C to detach the cells from the plate, and washed twice. Cell counts and viability were determined by flow cytometry, as shown above, and after the phenotypical and functional characterization, dry pellets of each condition were generated by centrifugation and stored at −80°C.

Phenotype Analysis

Surface protein expression of CD11c, CD14, CD25, CD83, CD86 and HLA-DR of iDC, mDC and vitD3-tolDC was determined by flow cytometry. For each measurement, DC suspensions were incubated for 20 min, protected from light, with the adequate amounts of monoclonal antibodies anti-: CD11c PE-Cyanine dye 7 (PE-Cy7), CD14 Violet 450 (V450), CD25 allophycocyanin (APC), CD83 APC, CD86 fluorescein isothiocyanate (FITC) and HLA-DR Violet 500 (V500) (all of them from BD Biosciences). Afterwards, at least 10,000 $CD11c^+$ events of each sample were acquired in a FACSCanto II flow cytometer and analyzed using FACSDiva software.

Functionality Test

Allogeneic peripheral blood mononuclear cells (PBMC) from whole blood of healthy donors were isolated by ficoll-hypaque density gradient separation. Cells were washed twice afterwards, and their absolute number and viability was determined as shown above.

Subsequently, a proliferation assay was performed in 96-well round bottom plates with co-cultures of 10^5 allogeneic PBMC and 5,000 iDC, mDC, or vitD3-tolDC (1:20 ratio) in a total volume of 200 μL of supplemented X-VIVO 15 medium. The same number of PBMC cultured in the presence of either supplemented X-VIVO 15 medium or 50 ng/mL phorbol 12-myristate-13-acetate (PMA) and 500 ng/mL ionomycin were used as negative and positive controls, respectively. Six replicates of each condition were performed. Cells were incubated for 4 days at 37°C in a 5% CO_2 atmosphere.

Finally, 1 μCi [^{3}H]-thymidine (PerkinElmer, Waltham, MA, USA) was added to each well and the plate was incubated for further 18 h under the same conditions. Cells were then collected using a HARVESTER96 2M cell harvester (Tomtec Inc, Hamden, CT, USA) and read on a 1450 MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland).

Cytokine and Soluble Protein Production

The production of granzyme B (GZMB) and vascular endothelial growth factor (VEGF), as well as IL-10, IFN-γ and IL-12p70 cytokines, was quantified in the culture supernatants of tolDC using the Human Soluble Protein CBA Flex Set (BD biosciences) according to manufacturer's instructions. Samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FACSDiva software.

The production of TGF-β was determined using the Human/Mouse TGF beta 1 Uncoated ELISA kit (Invitrogen) in 100 μL of supernatant samples, again following manufacturer's instructions. The optical density of each well was measured at 450 nm, and the optical density at 570 nm was then substracted as background signal, using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA).

Differential Expressed Genes Selection and Generation of a Network of Protein Interactions

The data of a comparative transcriptomic analysis of vitD3-tolDC, iDC and mDC from 5 healthy donors, previously

performed by our group in a microarray study (33), was used to select several DEG. For that, the mean difference of expression (MeanDiff) of each gene was evaluated, and only those genes that were specifically induced or repressed in vitD3-tolDC vs. both iDC and mDC conditions (either MeanDiff $_{\text{vitD3-tolDC vs. mDC}} > 0.5$ —first criterium—, while MeanDiff $_{\text{iDC vs. mDC}} < 0.5$, —second criterium—; or MeanDiff $_{\text{vitD3-tolDC vs. mDC}} < -0.5$, while MeanDiff $_{\text{iDC vs. mDC}} > -0.5$), with a statistically significant differential expression in the first criterium ($p < 0.01$), were selected in order to validate them. Unlike in our previous study, B-statistic —an indicator of the likelihood of the results— was not considered for the selection of candidate genes this time. Microarray raw and processed data were deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6937.

Additionally, a bioinformatic analysis was performed in order to characterize the molecular mechanisms involved in the tolerogenicity of vitD3-tolDC. Briefly, the Therapeutic Performance Mapping System technology (35, 36) was used to generate a mathematical model of protein interactions from our transcriptomic microarray data, based on an effectors database of tolDC biology and functionality (Anaxomics, Barcelona, Spain). This database was generated using information available in the literature as well as public and private repositories. From this model, a network of protein interactions between all the effectors found in our microarray data was built using an Artificial Neural Networks analysis and represented using Cytoscape 3.6.1 software.

RNA Extraction and qPCR Validation

Total RNA was isolated from dry pellets using the RNeasy Mini Kit (Qiagen, Hilden, Germany) along with a complementary DNAse treatment with the RNAse-free DNAse Set (Qiagen), following manufacturer's instructions. Quantity and purity of the samples was then determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and the RNA was subsequently retrotranscribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Finally, 250 ng cDNA were preamplified using the TaqMan™ PreAmp Master Mix Kit (Applied Biosystems).

The expression of genes *CA2*, *CAMP*, *CLEC5A*, *CYP24A1*, *DHRS9*, *GAPDH*, *GZMB*, *IL1R1*, *MAP7*, *MUCL1*, *OS9*, *PPIA*, *SNORD30*, *SPARC*, *ST6GAL1*, *TBP* and *THBS1* was determined by quantitative PCR (qPCR) using the respective TaqMan Gene Expression Assays (Applied Biosystems) shown in **Supplementary Table 1**, following the instructions provided by the manufacturer, in a LightCycler 480 System thermocycler (Roche, Basel, Switzerland). Housekeeping genes *CYPA*, *TBP* and *GAPDH* were used as controls. The quantitative expression of each gene was calculated based on the $2^{-\Delta Cp}$ method (37), using the mean Cp values of the 3 housekeeping genes. The decimal logarithm of fold change (logFC) expression values of each gene were considered for the definition of validation criteria. Similar to the MeanDiff parameter from the microarray, in this case genes were considered as differentially expressed in vitD3-tolDC vs. both iDC and mDC when either logFC $_{\text{vitD3-tolDC vs. mDC}} > 0.5$ —first criterium—, while logFC $_{\text{iDC vs. mDC}} < 0.5$, —second criterium—; or logFC $_{\text{vitD3-tolDC vs. mDC}} < -0.5$, while logFC $_{\text{iDC vs. mDC}} > -0.5$, if statistical significance was reached for the first criterium ($p < 0.05$).

Immunocytochemistry Validation

In order to confirm the qPCR results and analyze the protein expression and distribution of MAP7 and MUCL1 molecules, an indirect immunocytochemistry (ICC) staining was carried out in vitD3-tolDC, mDC and iDC from healthy donors and MS patient samples. Cell culture differentiations of vitD3-tolDC, mDC and iDC were performed in 24-well plates, following the same protocol described above, over 12 mm round coverslips. After 6 days of culture, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% TWEEN20 (Sigma-Aldrich) and subsequently blocked with 10% goat serum for 15 min. Afterwards, samples were incubated for 1 h at room temperature or overnight at 4°C with the primary antibodies mouse anti-human α-tubulin (Invitrogen) and either rabbit anti-human MAP7 (Invitrogen) or rabbit anti-human MUCL1 (Sigma-Aldrich). Next, cells were washed and later incubated with AlexaFluor (AF) 488 goat anti-mouse IgG and AF 594 goat anti-rabbit IgG secondary antibodies (both from Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) for 30 min at room temperature, protected from light. Cells were washed again, and the coverslips were finally mounted using ProLong® Gold Antifade Mountant medium with DAPI (Invitrogen) for nucleus staining. Samples were analyzed on an Axio Observer Z1 fluorescence microscope (Zeiss, Oberkochen, Germany) with a 63x objective, using ZEN software (Zeiss), and the expression of MAP7 and MUCL1 was calculated as the corrected total cell fluorescence (CTCF) of each protein (38, 39) using the FIJI distribution of ImageJ software (40, 41).

Statistical Analysis

All the statistical analyses were performed with either parametric or non-parametric tests depending on the normality of each compared data set, as determined by the D'Agostino & Pearson test, using Prism 6.0 software (GraphPad, La Jolla, CA, USA). For multiple comparisons, either the one-way ANOVA test with Geisser-Greenhouse correction or the non-parametric Friedman test with Dunn's correction were used depending on the normality of the groups. Analogously, for comparisons between two groups, either the t-Student or the Wilcoxon test were used if the samples were normally distributed or not, respectively. When $N \leq 6$, parametric tests were used anyway due to the small sample size (42). Results were expressed as mean ± standard deviation (SD), unless noted otherwise, and they were considered statistically significant when $p < 0.05$.

RESULTS

VitD3-tolDC Show Phenotypical Characteristics of tolDC

TolDC were generated from samples of 24 healthy donors and 10 MS patients with 83.1 ± 0.01% purity of monocytes and 98.4 ±

0.03% viability after $CD14^+$ cells positive selection. After *in vitro* differentiation, DC were harvested, and their phenotype, purity and viability were characterized by flow cytometry following the gating strategy depicted in **Supplementary Figure 1a**. As determined by the percentage of $CD11c^+$ cells, purity of DC was always >90%, with 91.1 ± 0.04% viability in both healthy donor and MS patient cells (**Supplementary Figure 1b**). When we analyzed the phenotype of the cells, the median fluorescence intensity (MFI) values for CD83, CD86 and HLA-DR on each condition were considered. As displayed in **Supplementary Figures 1c–e**, vitD3-tolDC from healthy donors showed reductions of 83 ± 34%, 59 ± 15% and 71 ± 15% in the expression of CD83, CD86 and HLA-DR, respectively, compared to mDC, and similar to the expression levels showed by iDC. In the case of MS patient-derived vitD3-tolDC, a similar behavior was observed, with reductions of 77 ± 30%, 60 ± 13% and 61 ± 14% in CD83, CD86 and HLA-DR expression, respectively, compared to mDC, and similar to iDC. All the results reached statistical significance for both healthy donors and MS patients ($p < 0.05$).

VitD3-tolDC Induce Allogeneic Hyporresponsiveness and Produce Anti-inflammatory Cytokines

The functional assay results evidenced that vitD3-tolDC significantly inhibited the proliferation of allogeneic PBMC. As shown in **Supplementary Figure 2a**, a 73.6 ± 16.6% reduction compared to mDC was observed in healthy donors, similar to the 84.1 ± 10.7% reduction exhibited by iDC. Analogously, although in a less strong degree, for MS patient-derived DC, a 47.9 ± 25.6% and a 46.3 ± 28.9% reduction in the proliferation induction was observed for vitD3-tolDC and iDC, respectively, in comparison to mDC. In all four comparisons, statistical significance was reached ($p < 0.05$).

When the cytokine secretion profile of vitD3-tolDC was compared to mDC and iDC, an increase in the production of the anti-inflammatory cytokines IL-10 (**Supplementary Figure 2b**) and TGF-β (**Supplementary Figure 2c**) was detected in vitD3-tolDC differentiated from healthy donors (IL-10 $_{\text{vitD3-tolDC}}$: 166.0 ± 287.7 pg/mL vs. IL-10 $_{\text{mDC}}$: 44.0 ± 51.4 pg/mL; $p = 0.003$; and TGF-β $_{\text{vitD3-tolDC}}$: 306.5 ± 159.5 pg/mL vs. TGF-β $_{\text{mDC}}$: 188.1 ± 165.3 pg/mL; $p = 0.046$), but only of IL-10 in the case of vitD3-tolDC generated from MS patients (IL-10 $_{\text{vitD3-tolDC}}$: 148.6 ± 141.7 pg/mL vs. IL-10 $_{\text{mDC}}$: 62.1 ± 54.1 pg/mL; $p = 0.043$). Furthermore, IL-12 production could not be detected in any condition (data not shown). Finally, no statistically significant changes were found in the production of GZMB (**Supplementary Figure 2d**), nor IFN-γ (**Supplementary Figure 2e**), between the different conditions.

CYP24A1, *MAP7* and *MUCL1* Genes Are Induced in vitD3-tolDC From Healthy Donors and MS Patients

Following the study previously performed by our group in a comparative microarray study of vitD3-tolDC, iDC and mDC from 5 healthy donor samples (33), we applied the filtering criteria described in the methods section to the data results (either MeanDiff $_{\text{vitD3-tolDC vs. mDC}} > 0.5$ —first criterium—, while MeanDiff $_{\text{iDC vs. mDC}} < 0.5$, —second criterium—; or MeanDiff $_{\text{vitD3-tolDC vs. mDC}} < -0.5$, while MeanDiff $_{\text{iDC vs. mDC}} > -0.5$, with a statistically significant differential expression in the first criterium). Briefly, these parameters spotted those genes that were specifically induced by the effect of vitamin D3 over DC according to our microarray analysis, since they were differentially expressed in vitD3-tolDC compared to both mDC and iDC, and therefore could be considered as potential transcriptomic biomarkers of vitD3-tolDC (**Table 1**). As a result, we selected *CA2*, *CAMP*, *CLEC5A*, *CYP24A1*, *GZMB*, *IL1R1*, *MAP7*, *MUCL1* and *SNORD30* genes for validation.

The subsequent qPCR analysis of the actual expression of these genes in healthy donors evidenced that all of them showed an expression pattern compliant with the expression thresholds that were established for the validation —analogously to those from the microarray but depicted in logFC, as defined in the methods section—, except for *CA2* (uncompliant with the second criterium), *GZMB* (uncompliant with the second criterium) and *SNORD30* (uncompliant with the first criterium), as shown in **Table 1** and **Figure 1A**. Consequently, these 3 genes were discarded from further analysis and the expression of the remaining genes was tested on MS patient samples. In this case, **Table 1** and **Figure 1B** show that, however, neither *CAMP*, nor *CLEC5A*, nor *IL1R1* fulfilled our expression criteria. As a result, only *CYP24A1*, *MAP7* and *MUCL1* could be validated as DEG for our vitD3-tolDC product —reaching statistical significance for the first criterium, as required, in all cases ($p < 0.05$)—, and therefore were selected as transcriptomic biomarkers of our tolDC-inducing protocol.

MUCL1 Is Also Induced in tolDC Generated With IL-10

Given the positive results, we therefore intended to test the expression of some of these genes in 5 samples of IL10-tolDC differentiated from healthy donors, in order to assess their potential value as biomarkers of a different tolDC-inducing protocol. While the expression of *CA2*, *CAMP*, *CLEC5A*, *IL1R1* (data not shown) and *MAP7* (**Figure 1C**) was not altered in these cells, we observed the up-modulation of *MUCL1* gene expression in IL10-tolDC compared to mDC (logFC $_{\text{IL10-tolDC vs. mDC}} = 0.960$ ± 0.395; $p = 0.034$), similar to that observed for vitD3-tolDC (**Figure 1C**). The expression of *CYP24A1* was not analyzed, since this gene is directly related to the response of the cells to vitamin D3, which was not present in these specific cultures.

MAP7 and *MUCL1* Are Functionally Related in vitD3-tolDC Through a Network of Protein Interactions

In order to find potentially common genes related to *CYP24A1*, *MAP7* and *MUCL1* that could provide a mechanistic insight

TABLE 1 | Expression by microarray and qPCR of the selected genes in dendritic cells differentiated from healthy donors and multiple sclerosis patients.

MICROARRAY DATA FROM HEALTHY DONORS [FROM NAVARRO-BARRIUSO ET AL. (33)]				
	iDC vs. mDC		vitD3-tolDC vs. mDC	
Gene	***MeanDiff***	***p-value***	***MeanDiff***	***p-value***
CA2	1.044	0.097	1.680	**0.010**
CAMP	0.337	0.279	1.351	**<0.001**
CLEC5A	0.015	0.975	1.573	**0.003**
CYP24A1	−0.585	0.264	2.271	**<0.001**
GZMB	0.097	0.582	0.734	**<0.001**
MAP7	0.075	0.663	0.880	**<0.001**
MUCL1	0.084	0.857	2.132	**<0.001**
SNORD30	−0.040	0.949	1.616	**0.016**

HEALTHY DONORS						
	iDC vs. mDC		vitD3-tolDC vs. mDC		vitD3-tolDC vs. iDC	
Gene	***logFC ± SD***	***p-value***	***logFC ± SD***	***p-value***	***logFC ± SD***	***p-value***
CA2	0.566 ± 0.391	**0.001**	0.858 ± 0.445	**<0.001**	0.292 ± 0.276	**0.042**
CAMP	0.290 ± 0.397	**0.042**	0.957 ± 0.447	**<0.001**	0.668 ± 0.433	**0.003**
CLEC5A	−0.254 ± 0.371	0.063	0.602 ± 0.324	**0.001**	0.856 ± 0.346	**<0.001**
CYP24A1	−1.680 ± 0.422	**0.008**	1.532 ± 0.637	**0.004**	3.212 ± 0.914	**0.004**
GZMB	0.588 ± 0.426	**0.001**	0.607 ± 0.554	**0.001**	0.019 ± 0.515	>0.999
IL1R1	−0.133 ± 0.259	0.250	−0.662 ± 0.353	**<0.001**	−0.528 ± 0.293	**<0.001**
MAP7	0.473 ± 0.353	**0.002**	1.015 ± 0.220	**<0.001**	0.542 ± 0.308	**0.007**
MUCL1	−0.318 ± 0.453	0.130	1.511 ± 0.419	**<0.001**	1.829 ± 0.405	**<0.001**
SNORD30	−0.025 ± 0.204	0.056	−0.001 ± 0.207	0.233	0.024 ± 0.278	>0.999

MULTIPLE SCLEROSIS PATIENTS						
	iDC vs. mDC		vitD3-tolDC vs. mDC		vitD3-tolDC vs. iDC	
Gene	***logFC ± SD***	***p-value***	***logFC ± SD***	***p-value***	***logFC ± SD***	***p-value***
CAMP	0.431 ± 0.342	0.042	0.441 ± 0.331	0.076	0.010 ± 0.459	>0.999
CLEC5A	−0.837 ± 0.539	**0.002**	0.111 ± 0.249	0.668	0.947 ± 0.490	**0.002**
CYP24A1	−0.762 ± 0.739	0.160	1.465 ± 0.908	**<0.001**	2.227 ± 1.448	**<0.001**
IL1R1	0.143 ± 0.158	0.103	−0.307 ± 0.179	**0.002**	−0.450 ± 0.214	**0.008**
MAP7	0.320 ± 0.296	0.081	0.506 ± 0.297	**0.003**	0.187 ± 0.292	0.174
MUCL1	−0.283 ± 1.209	0.221	0.790 ± 0.325	**0.042**	1.073 ± 1.362	**<0.001**

Gene expression values from a microarray study with healthy donors (n = 5) or from qPCR analysis from healthy donors (n = 24, except for CYP24A1, in which n = 10, and GZMB and SNORD30, in which n = 20) and multiple sclerosis patients (n = 10). Data presented as either the mean difference of expression (MeanDiff) for the microarray data or as the mean decimal logarithm of fold change (logFC) ± SD for the qPCR results. Housekeeping genes GAPDH, TBP and CYPA were used as controls. One qPCR experiment was performed for each donor or patient, with triplicated measurements for each sample. Microarray p-values < 0.01 and qPCR p-values < 0.05 are highlighted in bold. iDC, immature dendritic cells; mDC, mature dendritic cells; vitD3-tolDC, vitamin D3-induced tolerogenic dendritic cells. Friedman test with Dunn's correction or one-way ANOVA test with Geisser-Greenhouse correction.

in their functionality and the metabolic pathways triggered in vitD3-tolDC, we constructed a network of protein interactions. Briefly, previously reported data from the literature and several public and private databases were crossed with our microarray results in order to link the function of each gene of our transcriptomic study between them. This approach would allow us to reveal potential interactions between our biomarkers that might explain their involvement in immune regulation. Our study revealed that, while *CYP24A1* seemed to be functionally separated from the rest of the genes, *MAP7* and *MUCL1* were closely related through common immune-related mechanisms —such as HLA class II antigen presentation and different anti-inflammatory mediators— and other cellular processes and pathways —highlighting the metabolism of retinoic acid and the oxidative metabolism—, as shown in **Figure 2**. Furthermore, *CAMP*, *CLEC5A*, *GZMB* and *IL1R1* genes appeared closely related to *MAP7* and *MUCL1* in our network.

To validate these results and confirm the close functional relation between *MUCL1* and *MAP7*, we studied the expression of *DHRS9*, *OS9*, *SPARC*, *ST6GAL1* and *THBS1* genes. As shown in **Table 2**, their expression in our microarray indicated an overexpression of *DHRS9*, *SPARC* and *ST6GAL* in vitD3-tolDC

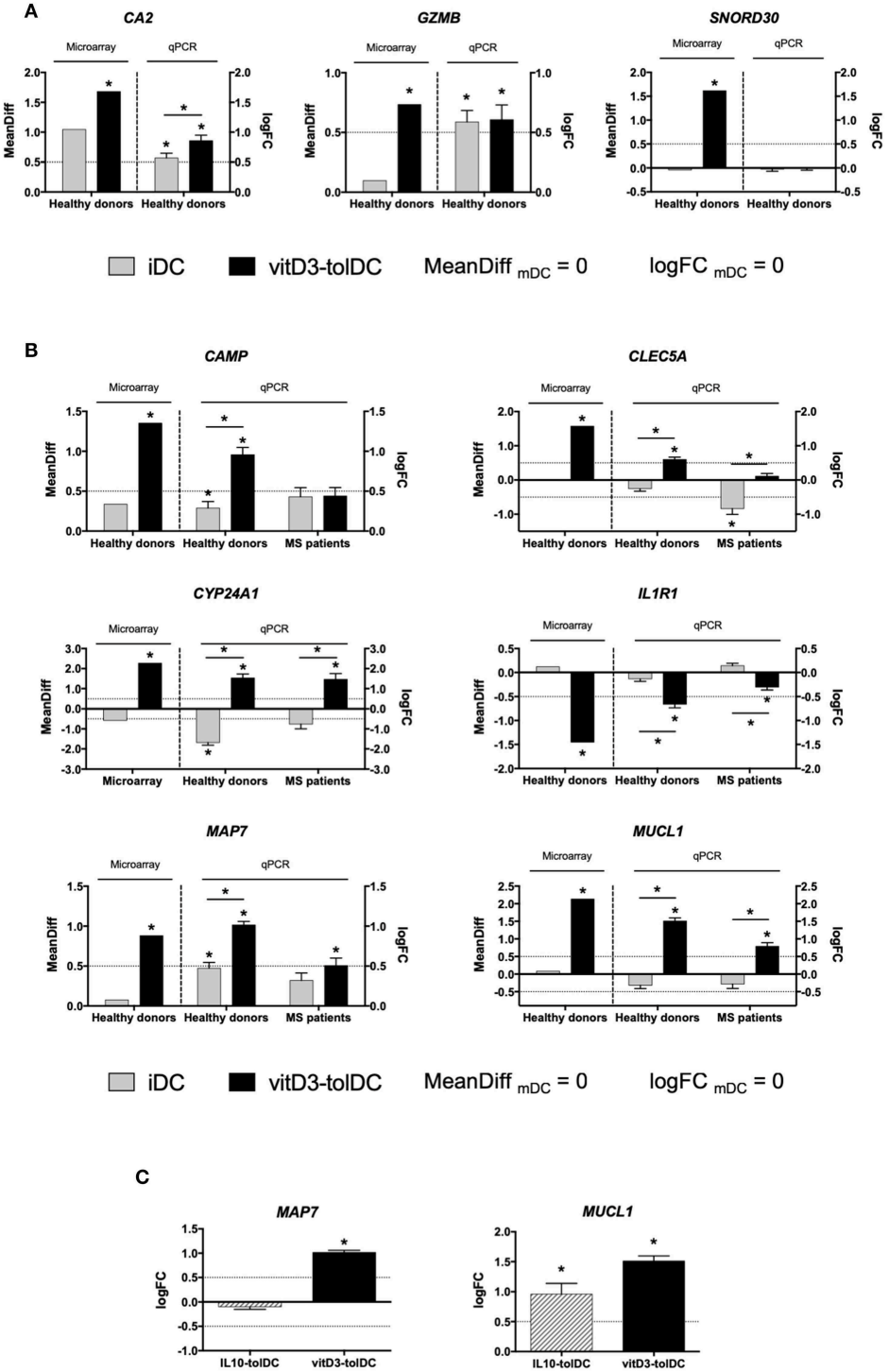

FIGURE 1 | Expression of the selected genes as candidate biomarkers of vitD3-tolDC. **(A)** Expression of *CA2*, *GZMB* and *SNORD30* in healthy donors by microarray ($n = 5$) and quantitative PCR (qPCR; $n = 20$, except *CA2*, in which $n = 24$) in immature dendritic cells (iDC), mature DC (mDC) and vitD3-tolDC. **(B)** Expression of *CAMP*, *CLEC5A*, *CYP24A1*, *MAP7* and *MUCL1* in healthy donors both by microarray ($n = 5$) and qPCR analysis ($n = 24$, except *CYP24A1*, in which $n = 10$) in iDC, mDC and vitD3-tolDC, and in multiple sclerosis (MS) patients by qPCR only ($n = 10$). **(C)** Expression of *MAP7* and *MUCL1* in vitD3-tolDC ($n = 24$) and in IL10-tolDC ($n = 5$) by qPCR. Data presented as the mean difference of expression (MeanDiff) or the decimal logarithm of fold change (logFC) expression for the microarray and qPCR results, respectively, in both cases normalized to mDC expression. Housekeeping genes *GAPDH*, *TBP* and *CYPA* were used as controls. One qPCR experiment was performed for each donor or patient, with triplicated measurements for each sample. Error bars corresponding to SEM. Dotted lines represent the logFC = 0.5 or −0.5 expression threshold. $^*p < 0.05$. Friedman test with Dunn's correction, one-way ANOVA test with Geisser-Greenhouse correction or paired *t*-test.

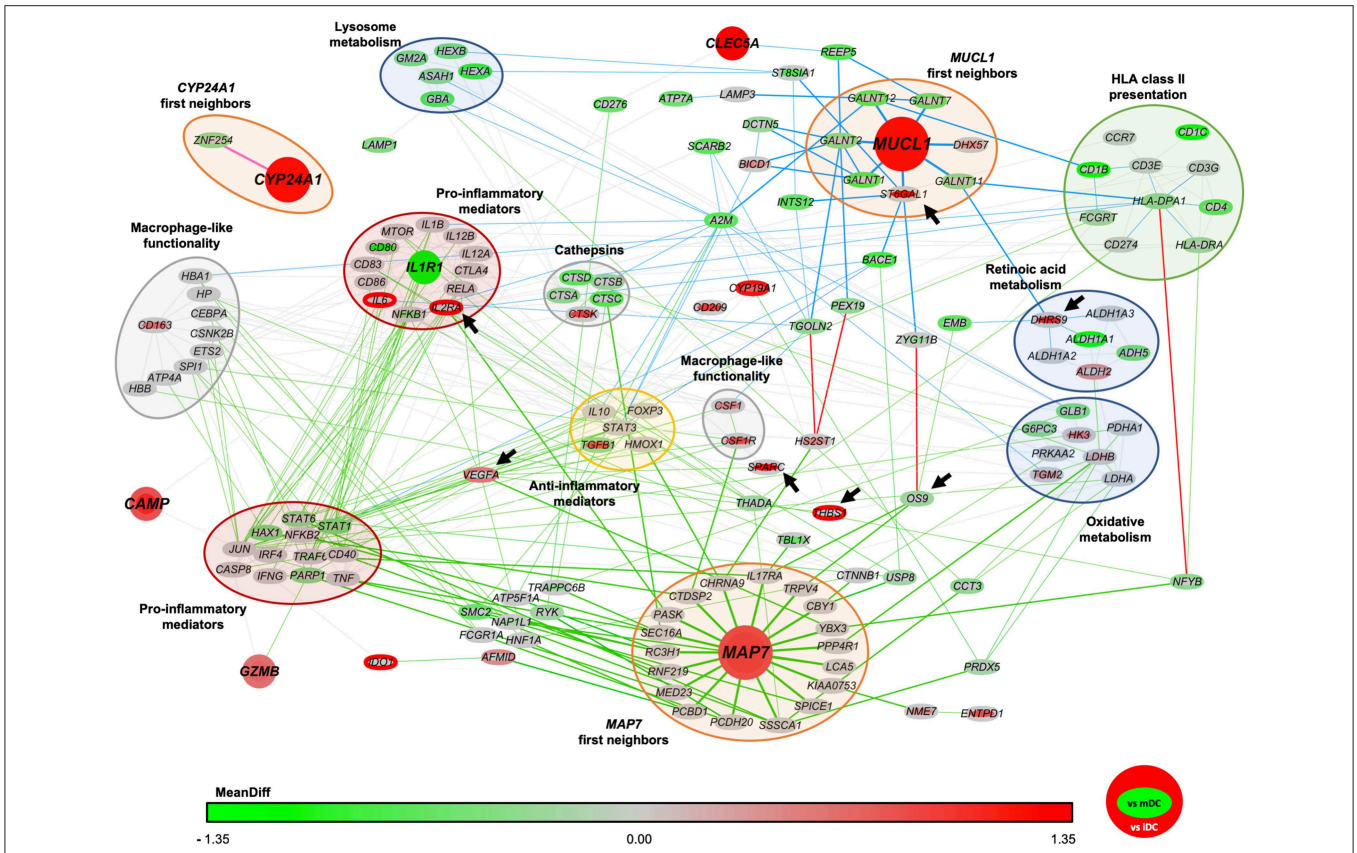

FIGURE 2 | Network of protein interactions between *CYP24A1*, *MAP7* and *MUCL1*. The protein interactions network was built based in both our microarray results and previously reported data. Each node represents a different protein encoding gene. The color of the border of each node indicates the level of expression of each gene in vitD3-tolDC compared to immature dendritic cells (iDC) and the color of the body of each node indicates the expression of each gene compared to mature dendritic cells (mDC). The color scale indicates the level of mean difference expression (MeanDiff) of each gene according to our microarray study, from green (MeanDiff ≤ -1.35) to red (MeanDiff ≥ 1.35). Green and blue lines indicate protein interactions that are related to *MAP7* and *MUCL1* genes (respectively) in 3 or less parenthood levels, and the red lines indicate interactions that are shared by both genes in <6 parenthood levels. Genes have been grouped in functional clusters, as indicated by the colored bubbles. Arrows indicate those genes that were selected for further validation studies.

vs. mDC, but not iDC, while *THBS1* and *OS9* appeared induced and slightly down-modulated, respectively, in vitD3-tolDC compared to iDC. We, therefore, validated their expression in 10 samples from healthy donors and 10 samples from MS patients, and observed that the expression pattern of these 5 genes in healthy donor samples was in accordance to that shown in our microarray data (**Table 2**). Furthermore, and with the only exception of *DHRS9* and *OS9*, these expression patterns were also confirmed in DC samples obtained from MS patients, as shown in **Table 2**.

Additionally, *VEGFA* and *IL2RA* were also studied due to their relevance in our network, but their expression was validated at the protein level. Their gene expression in our microarray study is shown in **Table 2**. VEGF production (encoded by the *VEGFA* gene) was analyzed by cytometric bead array (CBA) in supernatant samples from 10 healthy donors and 8 MS patients, and the surface expression of CD25 (encoded by the *IL2RA* gene) was assessed by flow cytometry in 6 healthy donor samples. First, in accordance with the results from the microarray (**Table 2**), an increase in the production of VEGF was evidenced in vitD3-tolDC from healthy donors compared to iDC —since it could not be detected in this condition—, but no statistical significance could be reached in the reduction found compared to mDC (VEGF $_{\text{vitD3-tolDC}}$: 205.5 $\pm$ 276.9 pg/mL; VEGF $_{\text{mDC}}$: 422.2 $\pm$ 497.6 pg/mL). Furthermore, similar results were evidenced in samples from MS patients, and VEGF production could not be detected on iDC either (VEGF $_{\text{vitD3-tolDC}}$: 331.8 $\pm$ 321.5 pg/mL; VEGF $_{\text{mDC}}$: 369.2 $\pm$ 243.1 pg/mL). On the other hand, the study of the expression of CD25 in healthy donor samples from the microarray evidenced a strong up-modulation of the *IL2RA* gene in vitD3-tolDC compared to iDC only. The expression of this gene in vitD3-tolDC compared to mDC, however, did not reach statistical significance (**Table 2**). Accordingly, our results in samples from 6 healthy donor DC cultures showed a statistically significant increase ($p = 0.040$) in the MFI values of this marker in vitD3-tolDC compared to iDC, but not mDC, probably due to the high variability observed in this specific condition (CD25 $_{\text{vitD3-tolDC}}$: 433.8 $\pm$ 194.9; CD25 $_{\text{mDC}}$: 796.3 $\pm$ 621.2; CD25 $_{\text{iDC}}$: 175.0 $\pm$ 16.8).

TABLE 2 | Expression by microarray and qPCR of the selected genes from the protein interaction network in dendritic cells differentiated from healthy donors and multiple sclerosis patients.

MICROARRAY DATA FROM HEALTHY DONORS [FROM NAVARRO-BARRIUSO ET AL.(33)]				
	vitD3-tolDC vs. mDC		**vitD3-tolDC vs. iDC**	
Gene	***MeanDiff***	***p-value***	***MeanDiff***	***p-value***
DHRS9	1.160	**0.007**	−0.161	0.685
IL2RA	−1.539	0.040	2.297	**0.003**
OS9	−0.014	0.926	−0.478	**0.004**
SPARC	1.347	**<0.001**	0.506	0.101
ST6GAL1	1.564	**<0.001**	0.189	0.570
THBS1	−1.052	0.048	1.944	**<0.001**
VEGFA	0.200	0.509	0.696	0.028
qPCR DATA FROM HEALTHY DONORS				
	vitD3-tolDC vs. mDC		**vitD3-tolDC vs. iDC**	
Gene	***logFC ± SD***	***p-value***	***logFC ± SD***	***p-value***
DHRS9	0.683 ± 0.387	**0.002**	n/a	
OS9	−0.011 ± 0.172	0.917	−0.265 ± 0.182	**0.015**
SPARC	1.210 ± 0.346	**0.003**	n/a	
ST6GAL1	0.571 ± 0.223	**0.002**	n/a	
THBS1	−0.023 ± 0.285	0.694	2.508 ± 0.303	**0.004**
qPCR DATA FROM MULTIPLE SCLEROSIS PATIENTS				
	vitD3-tolDC vs. mDC		**vitD3-tolDC vs. iDC**	
Gene	***logFC ± SD***	***p-value***	***logFC ± SD***	***p-value***
DHRS9	0.264 ± 0.358	0.557	n/a	
OS9	−0.036 ± 0.152	>0.999	−0.190 ± 0.222	0.076
SPARC	0.552 ± 0.194	**0.011**	n/a	
ST6GAL1	0.403 ± 0.331	**0.001**	n/a	
THBS1	−0.027 ± 0.216	0.420	2.391 ± 0.381	**0.002**

Gene expression values from a microarray study with healthy donors (n = 5) or from qPCR analysis from healthy donors (n = 10) and multiple sclerosis patients (n = 10). Data presented as either the mean difference of expression (MeanDiff) for the microarray data or as the mean decimal logarithm of fold change (logFC) ± SD for the qPCR results. Housekeeping genes GAPDH, TBP and CYPA were used as controls for the qPCR experiments. One qPCR experiment was performed for each donor or patient, with triplicated measurements for each sample. Microarray p-values < 0.01 and qPCR p-values < 0.05 are highlighted in bold. iDC, immature dendritic cells; mDC, mature dendritic cells; vitD3-tolDC, vitamin D3-induced tolerogenic dendritic cells. Friedman test with Dunn's correction, one-way ANOVA test with Geisser-Greenhouse correction, Wilcoxon matched-pairs signed rank test or paired t-test.

Protein Expression of Both *MAP7* and *MUCL1* Is Strongly Induced in vitD3-tolDC

Since *MAP7* and *MUCL1* proved themselves as transcriptomic biomarkers of vitD3-tolDC in both healthy donors and MS patient samples and, additionally, they were closely related between them in our functional network, we further analyzed them and determined the actual expression of their respective encoded proteins in order to provide more reliability to our qPCR results. The ICC analysis in 4 healthy donor samples evidenced that, in fact, a strong up-modulation of these proteins was observed in vitD3-tolDC compared to both iDC and mDC conditions. As shown in **Figure 3A**, the microtubule-associated protein 7, encoded by *MAP7*, showed a 2.81-fold and a 4.00-fold higher CTCF expression in vitD3-tolDC compared to iDC and mDC, respectively (MAP7 $_{\text{vitD3-tolDC}} = 198.35 \pm 40.45 \times 10^3$; MAP7 $_{\text{mDC}} = 49.58 \pm 17.16 \times 10^3$; MAP7 $_{\text{iDC}} = 70.54 \pm 36.39 \times 10^3$), reaching statistical significance in both cases ($p = 0.016$; $p = 0.007$, respectively). The induction of the *MUCL1*-encoded protein was even stronger, as evidenced by the 6.72-fold and 13.02-fold CTCF expression in vitD3-tolDC vs. iDC and mDC, respectively (MUCL1 $_{\text{vitD3-tolDC}} = 1204.85 \pm 509.91 \times 10^3$; MUCL1 $_{\text{mDC}} = 92.54 \pm 94.19 \times 10^3$; MUCL1 $_{\text{iDC}} = 179.19 \pm 32.93 \times 10^3$). In this case, however, statistical significance was only reached in the comparison vs. mDC ($p = 0.024$), probably due to the small sample size (**Figure 3B**). Representative microscopy pictures of the expression of both proteins in healthy donor samples are shown in **Figures 3C,D**. Furthermore, the ICC analysis in 3 MS patient samples presented a similar tendency. On the one hand, as shown in **Figure 3A**, the study of MAP7 expression presented a 1.57-fold and a

2.14-fold higher CTCF expression in vitD3-tolDC compared to iDC and mDC, respectively (MAP7 $_{\text{vitD3-tolDC}} = 132.68 \pm 91.61 \times 10^3$; MAP7 $_{\text{mDC}} = 61.74 \pm 31.52 \times 10^3$; MAP7 $_{\text{iDC}} = 84.32 \pm 66.48 \times 10^3$). On the other hand, and as shown in **Figure 3B**, the expression of MUCL1 was, again, even stronger, with a 2.76-fold and 4.49-fold CTCF expression in vitD3-tolDC compared to iDC and mDC, respectively (MUCL1 $_{\text{vitD3-tolDC}} = 132.34 \pm 46.44 \times 10^3$; MUCL1 $_{\text{mDC}} = 29.47 \pm 11.66 \times 10^3$; MUCL1 $_{\text{iDC}} = 47.97 \pm 24.65 \times 10^3$). However, statistical significance for MS patient samples could only be reached for the expression of MUCL1 in vitD3-tolDC compared to mDC ($p = 0.049$), again, probably due to the small sample size. Representative microscopy pictures of the expression of both proteins in MS patient samples are shown in **Figures 3C,D**.

DISCUSSION

The identification of biomarkers is a key point for the translation of tolDC into the clinic. In this article, we have evidenced that *CYP24A1*, *MAP7* and *MUCL1* genes appear strongly induced in vitD3-tolDC, both in healthy donors and MS patients. Therefore, the differential expression of these genes in our tolerogenic cell product gives them the potential to unequivocally identify vitD3-tolDC with a simple qPCR analysis, without ambiguity, thus ensuring that they are not immunogenic (not mDC), nor susceptible of maturation (not iDC), and consequently characterizing their tolerogenic potential. Additionally, the study of the protein expression of *MAP7* and *MUCL1* further supported these results. The study of these genes in the context of the whole transcriptome of the vitD3-tolDC has also elucidated that the role of *MAP7* and *MUCL1* —but not of *CYP24A1*— seems to be closely related with important and widely described immune- and non-immune-related pathways, which correlates with many of the results that we have obtained at the phenotypical (reduction of co-stimulatory molecules and HLA-DR expression), functional (increased secretion of IL-10 and TGF-β; non detectable production of IL-12) and transcriptomic levels (reduction of *IL1R1* gene expression). Furthermore, many of the interactions suggested by our network have also been confirmed in both healthy donor and MS patient cells. Therefore, even though the specific role of *MAP7* and *MUCL1* in the tolerogenic functionalities of vitD3-tolDC is not fully clear, our results manifested that they are at least having an effect on several relevant immune regulatory mechanisms and different metabolic pathways. On the other hand, *CYP24A1* gene, encoding the vitamin D3 24-hydroxylase, might well serve as a strong and robust biomarker of vitD3-tolDC, although it seems to have little to no influence in the actual regulatory properties of these cells. After all, this gene is directly involved in the metabolism of vitamin D3 (43, 44), and thus could constitute a robust indicator of the response of the cell product to the treatment with this compound. Even though we previously suggested the potential of *CYP24A1*, *MAP7* and *MUCL1* as candidate biomarkers of vitD3-tolDC in a previous microarray study (33), the current report constitutes their first validation as such.

Several years ago, mucin-like 1, the protein encoded by *MUCL1* gene, was initially identified as a breast-specific gene expressed in more than 90% of breast cancer cell lines, developing an important role in the proliferation of these tumor cells (45–48). However, no other specific role has been reported for it. In fact, its name comes given by the structural analogy of MUCL1 protein with mucin proteins, characterized by regions of high tandem repeated serine and threonine content with extensive O-glycosylation of these residues (46). On the other hand, we have *MAP7*. This gene encodes the microtubule-associated protein 7, a cytoskeleton component that has been mainly related with the development of collateral axon morphogenesis and development in neurons (49, 50). For both MUCL1 and MAP7, a potential role in DC function has not been reported yet. However, interestingly, microtubule associated proteins have been described to be O-glycosylated —just like MUCL1—, although the functional significance of this modification remains to be determined (51). In the case of vitD3-tolDC, our results have demonstrated a strong induction of gene and protein expression of both *MUCL1* and *MAP7*, as already hinted in our previous microarray study, thus suggesting that they might be developing an important role in the functionality of these cells. However, further functional studies should be performed first in order to elucidate to what extent these genes might be involved in the mechanisms of immune tolerance. In any case, the fact that our results also evidence a strong induction of *MUCL1* in IL10-tolDC indicates that this gene might constitute a potential biomarker of the regulatory function of tolDC, and it would be interesting to test its expression in other protocols generating tolerance-inducing cell products. Nevertheless, on the other hand, it is also true that our above-mentioned preliminary microarray results did not show the overexpression of *MUCL1* in dexamethasone nor rapamycin-induced tolDC, although proper PCR validations should be performed in order to confirm this.

In concordance with previous reported studies by our own group (26), and with the objective to finally develop an autologous tolerogenic cell-therapy for MS, our results demonstrated that the generation of vitD3-tolDC in both healthy donors and MS patients did not significantly differ regarding their phenotypic and functional characteristics —with the exception of an increase on TGF-β production in healthy donor samples only—. In all cases, a consistent decrease in the surface expression of CD86 and HLA-DR, as well as in the induction of allogeneic proliferation, was observed, accompanied by a significant increase in the production of IL-10 and no *IL-12* detection. However, the tolerogenic potential of vitD3-tolDC generated from monocytes of MS patients, although sufficient, did not seem as robust as in healthy donors, provided that the changes in their functional and phenotypic markers were not as pronounced. Interestingly, though, the study of *CYP24A1* expression raised another concern in this regard. Even though this gene constitutes a robust biomarker of the generation of vitD3-tolDC, as discussed above, its expression also manifested important differences in iDC from healthy donors and MS patients; compared to mDC, a strong repression of *CYP24A1* was

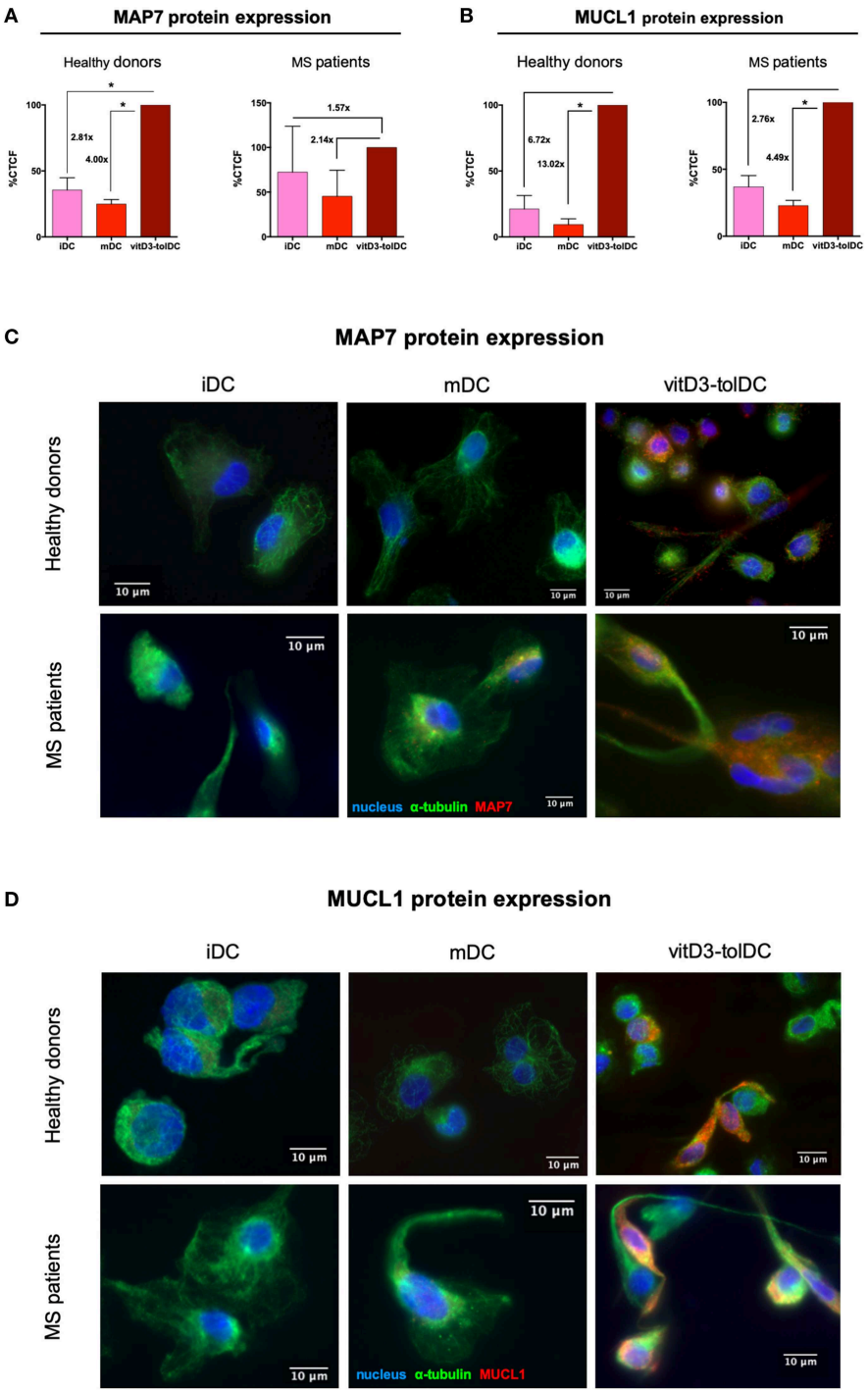

FIGURE 3 | Immunocytochemistry study of MAP7 and MUCL1 protein expression in dendritic cells. **(A)** Relative levels of expression of microtubule-associated protein 7 (MAP7) in dendritic cells differentiated from healthy donor samples ($n = 4$) and MS patient samples ($n = 3$). **(B)** Relative levels of expression of mucin-like 1 (MUCL1) in dendritic cells differentiated from healthy donor samples ($n = 4$) and MS patient samples ($n = 3$). Results are calculated as percentage (%) of corrected total cell fluorescence (CTCF) in immature dendritic cells (iDC) and mature DC (mDC) compared to vitD3-tolDC. One single immunocytochemistry experiment was performed for each sample. Error bars corresponding to SEM. *$p < 0.05$. One-way ANOVA test with Geisser-Greenhouse correction or paired *t*-test. Representative pictures of the expression of **(C)** MAP7 and **(D)** MUCL1 in iDC, mDC and vitD3-tolDC from healthy donor and MS patient samples. α-tubulin staining is shown in green; either MAP7 or MUCL1 staining is shown in red; nuclei staining is shown in blue. The immunocytochemistry analysis was performed on a fluorescence microscope using a 63x objective. Immunocytochemistry primary staining was performed using mouse anti-human α-tubulin and either rabbit anti-human MAP7 or rabbit anti-human MUCL1 antibodies. Secondary stainings were performed using AlexaFluor (AF) 488 goat anti-mouse IgG and AF 594 goat anti-rabbit IgG antibodies. Nuclei staining was performed using DAPI.

observed in healthy donor samples, but the expression of this gene in iDC from MS patients was similar to mDC. Therefore, this result implicates that the transcriptomic profile of DC in MS patients is already different before receiving the same treatment with vitamin D3, thus suggesting a more pro-inflammatory baseline status in these cells. However, the strong induction of *CYP24A1* observed in vitD3-tolDC still indicates that these cells are adequately responding to the effect of vitamin D3, suggesting that the problem has to be downstream.

These differences between healthy donors and MS patient samples also forced us to discard another interesting candidate transcriptomic biomarker, *DHRS9*, the gene encoding the dehydrogenase/reductase 9, involved in the metabolism of retinoic acid (52). The relevance of this gene comes given by its potential as a broad-use biomarker of immune tolerance, since it has been already described as differentially induced in vitamin D3 + dexamethasone-induced tolDC (53) and, especially, in regulatory macrophages (54). It is also worth noting that, even though *DHRS9* could not be confirmed as a biomarker of vitD3-tolDC from MS patients, this does not mean that it could not still be useful in cells generated from patients with a different autoimmune disease, such as rheumatoid arthritis or type 1 diabetes. In this regard, our results already allowed us to point out some transcriptomic differences between healthy donors and MS patients. We observed that *CLEC5A* could not be validated in MS patient samples either, and when we studied our network, we noticed that both *CLEC5A* and *DHRS9* were closely related to *MUCL1* through *GALNT2*, *GALNT7* and *GALNT11* genes. Specifically, these 3 genes have been described to be in charge of the processes of O-linked glycosylation. Consequently, this might be an indicator of a potential misfunction in the glycosylation mechanisms of MS patients, that might be indirectly affecting the expression of *CLEC5A, DHRS9* and probably many other genes. In fact, several glycosylation defects have already been related with the pathogenesis of MS, as reviewed by Grigorian et al. (55). However, *MUCL1* gene expression would also be expected to be affected by a defect in these genes, but our results showed that this was not the case. As a possible explanation, the glycosylation of *MUCL1* might be sufficiently mediated by *ST6GAL1* gene alone —whose expression still remains slightly induced in vitD3-tolDC from MS patient samples—. In any case, our results have raised the need to explore the mechanisms that might be different between healthy donors and MS patients. On the other hand, most of the genes closely related to *MAP7* showed a similar behavior in both healthy donor and MS patient vitD3-tolDC, such as *SPARC*, *THBS1* or, specially, *VEGFA*, which has been reported to develop a role in the recruitment, inhibition of maturation and IDO1 induction in DC (56–58).

In conclusion, our results evidenced that, despite not having an obvious involvement in the tolerogenic functionality of the cells in all cases, several genes have shown a strong differential expression in vitD3-tolDC from healthy donors, compared to both mature and immature control conditions. Among them, *CYP24A1*, *MAP7* and *MUCL1* have also been validated as robust transcriptomic biomarkers of vitD3-tolDC generated from MS patient samples. Thus, this finding opens a promising window for the use of these genes as a reliable quality control in clinical trials with a simple qPCR analysis, before administering the cell product into the patients. Furthermore, the role of *MAP7* and *MUCL1*, but not of *CYP24A1*, seems to be strongly related to important immune-related functions. Specifically, the case of *MUCL1* is of significant relevance, since this gene has also demonstrated an interesting potential as a broad-use biomarker of tolerance, based on its validation both in vitD3-tolDC and in IL10-tolDC. Consequently, *MUCL1* sets an interesting path for future experiments with the objective to validate the role of this gene as a potential biomarker of other tolDC protocols, and thus, hopefully as a wide biomarker of tolerogenic cell products and their mechanisms of immune tolerance induction.

ETHICS STATEMENT

This study was approved by the Germans Trias i Pujol Hospital ethical committee, and all patients and healthy controls signed an informed consent.

AUTHOR CONTRIBUTIONS

EM-C, JN-B, and MM conceived the experiments. CR-T and SP-R obtained the patient samples. AtB provided cDNA samples from IL10-tolDC. AA-M, BQ-S, JN-B, and MM performed the cell cultures. AA-M, BQ-S, JN-B, and MM performed the cell characterization analyses. JN-B and MM analyzed the results. EM-C, JN-B, and MM interpreted the results. JN-B wrote the manuscript. AA-M, AtB, AT-S, BQ-S, CR-T, EM-C, JN-B, MM, and SP-R reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01251/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Results III

Vitamin D3-induced tolerogenic dendritic cells modulate the transcriptomic profile of T CD4$^+$ cells towards a functional hyporesponsiveness

Vitamin D3-induced tolerogenic dendritic cells modulate the transcriptomic profile of T CD4$^+$ cells towards a functional hyporesponsiveness

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MANUSCRIPT UNDER REVIEW

Background: The use of autologous tolerogenic dendritic cells (tolDC) has become a promising alternative for the treatment of autoimmune diseases. Among the different strategies available, the use of vitamin D3 for the generation of tolDC (vitD3-tolDC) constitutes one of the most robust approaches due to their immune regulatory properties, which are currently being tested in clinical trials. However, the mechanisms that vitD3-tolDC trigger for the induction of tolerance remain elusive.

Methods: We generated fresh antigen-loaded vitD3-tolDC and autologous peripheral blood mononuclear cells from healthy donors, and after 6 days we co-cultured both cell populations for 5 days. Afterwards, we isolated T CD4$^+$ cells using flow cytometry cell sorting and performed a full phenotypical, functional and transcriptomic characterization of these T CD4$^+$ cells upon their antigen-specific interaction with vitD3-tolDC.

Results: We observed a strong antigen-specific reduction of T cell proliferation, combined with a decrease in the relative prevalence of T_H1 subpopulations and IFN-γ production. The analysis of the transcriptomic profile of T CD4+ cells evidenced an important down-modulation of genes involved in cell cycle and cell response to mainly pro-inflammatory immune-related stimuli, highlighting the role of *JUNB* gene as a potential biomarker of these processes.

Conclusions: Our results show the induction of a strong antigen-specific hyporesponsiveness combined with a reduction on the T_H1 immune profile of T cells upon their interaction with vitD3-tolDC, which manifests the regulatory properties of these cells and therefore their therapeutic potential in the clinic.

Keywords: Tolerogenic dendritic cells, immune tolerance, T cells, antigen-specific response, transcriptomic study

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INTRODUCTION

In the last years, tolerogenic dendritic cells (tolDC) have become one of the most promising alternatives for the treatment of autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis or type 1 diabetes. In fact, several Phase I clinical trials have already finished or are currently ongoing, with positive results regarding the safety and the tolerability of this therapeutic cell-based approach (1). In general, tolDC are commonly defined as a stable and semi-mature subset of dendritic cells (DC), between antigen-capturing immature DC (iDC) and immunogenic mature DC (mDC) —characterized by their increased expression of MHC and co-stimulatory molecules—. But most importantly, tolDC are presumably capable to induce immune tolerance towards the peptides these cells are presenting, in an antigen-specific manner (2–5).

TolDC can be generated *in vitro* from peripheral blood monocytes. In the last years, a wide variety of protocols for their production have been reported, ranging from the use of different drugs and chemical agents to genetic engineering techniques (6,7). In this regard, the use of 1,25-dyhydroxyvitamin D3, the active form of vitamin D3, constitutes one of the most widely studied approaches for the differentiation of tolDC. Briefly, vitamin D3-induced tolDC (vitD3-tolDC) are thought to develop their regulatory properties through a semi-mature profile, their ability to inhibit T cell responses and a switch of the immune response towards a T_H2 profile (8–18). Furthermore, vitD3-tolDC are characterized by a reduced NF-κB-mediated activity and an increase of mTOR-mediated glucose metabolism (10,19).

Even though tolDC —and vitD3-tolDC in particular— have been characterized with a developing knowledge over their metabolism, molecular mechanisms and functional pathways, the specific effect of these cells over the rest of the immune-related components still remains elusive. It is known that tolDC can usually induce either anergy, hyporesponsiveness or depletion over activated T cells, as well as regulatory T cell (Treg) differentiation (20). However, to our knowledge, so far only one study has focused its attention on the actual processes that autologous T cells might be undergoing upon tolDC interaction —reporting an induction of hyporesponsiveness of CD4+ memory and naïve T cells towards antigen-specific stimulation mediated by dexamethasone-induced tolDC (21)—, but neither at the transcriptomic level nor with vitD3-tolDC in particular.

In previous studies, our group has already extensively characterized vitD3-tolDC phenotypically, functionally and transcriptomically, evidencing the regulatory potential of these cells both *in vitro* and *in vivo* in the animal model of MS, experimental autoimmune encephalomyelitis (13,16,22–24). Consequently, we wanted to take one step further for the elucidation of the mechanisms of immune tolerance induction of vitD3-tolDC. With that aim, here we present a full phenotypical, functional and transcriptomic characterization of T CD4+ cells after their interaction in co-culture with autologous vitD3-tolDC, loaded with tetanus toxin (TT), in order to study the antigen-specific effect mediated by these cells, compared with TT-loaded mDC. Hopefully, this study could allow us to identify one or several potential biomarkers of the immune modulation developed by vitD3-tolDC over T cells, which could constitute an interesting tool for the monitoring of patients treated with these cells in clinical trials and the understanding of the mechanisms of tolerance induction.

MATERIALS AND METHODS

Sample collection

Buffy coat samples from 16 randomized healthy controls were obtained from the *Banc de Sang i Teixits* (Barcelona, Spain), according to the institutional Standard Operating Procedures for blood donation, including a signed informed consent. In parallel, whole blood samples from 12 different healthy donors were collected by standard venipuncture in lithium heparin tubes for the allogeneic functional assays (see below).

Monocyte isolation

Healthy donor buffy coat samples were processed first depleting CD3+ cells using the RoseetteSep® Human Monocyte Enrichment Kit (StemCell Technologies, Vancouver, Canada) prior to a density gradient separation using ficoll-hypaque (Rafer, Zaragoza, Spain). Afterwards, CD14+ cells were isolated using the EasySep® Human CD14 Positive Selection Kit (StemCell), according to manufacturer's instructions. Cell viability was determined using 7-amino-actinomycin D (7-AAD) (BD Biosciences, Franklin Lakes, NK, USA) and phycoerythrin (PE)-conjugated annexin V (Immunotools, Friesoythe, Germany) staining for 20 min at 4 ºC, protected from light, and cell counts were quantified simultaneously using PerfectCount beads (Cytognos, Salamanca, Spain). Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences), and monocyte purity was determined using forward and side scater gating strategies on FACSDiva software (BD Biosciences).

TT-loaded DC cultures

The protocol for the generation of antigen-loaded tolDC was adapted from a previous study (25). Briefly, the isolated monocytes were cultured for 6 days in 24-well plates at 37ºC at a density of $1x10^6$ cells/mL in X-VIVO 15 medium, in the presence of 400 U/mL granulocyte macrophage colony-stimulating factor (GM-CSF) and 500 U/mL IL-4 (both from Peprotech, London, UK). Whole volume of medium and cytokines was replenished on day 4. If no further treatment was performed, monocytes were differentiated into iDC. For the generation of mDC, we further added a maturation cocktail, containing 1000 U/mL IL-1β, 1000 U/mL TNF-α (both from Peprotech) and 1 μM prostaglandin E2 (PGE2) (Pfizer, New York, NY, USA) on day 4. Finally, in addition to the maturation cocktail, we added 1 nM vitamin D3 (Calcijex, Abbott, Chicago, IL, USA) on days 0 and 4 for the differentiation of vitD3-tolDC. For the generation of TT-loaded mDC (mDC-TT) and TT-loaded vitD3-tolDC (vitD3-tolDC-TT) as antigen-specific experimental conditions, 0.1 μg/mL of whole TT (Sigma-Aldrich, St. Louis, MO, USA) were added to the mDC and

vitD3-tolDC cultures on day 3, 18 h before the addition of the maturation stimulus, while still in an immature status. On day 6, cells were harvested after an accutase (Invitrogen, Carlsbad, CA, USA) detaching treatment for 30 min, and washed twice. As shown above, cell counts and viability were determined by flow cytometry.

Autologous PBMC isolation, co-culture and sorting

For the isolation of autologous PBMC, 3 mL of the buffy coat samples from each healthy donor were processed using a ficoll-hypaque density gradient separation and washed twice. Afterwards, cells were counted by flow cytometry, as described above, and plated in round-bottom 96 well-plates at a density of $1x10^6$ cells/mL in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine (Lonza, Basel, Switzerland), 100 U/mL penicillin (Reig Jofre, Sant Joan Despí, Spain) and 100 µg/mL streptomycin (Normon, Tres Cantos, Spain). The plates were then incubated for 6 days at 37 ºC in a 5% CO_2 atmosphere. Afterwards, the cells were harvested, and cell counts and viability were determined by flow cytometry.

Subsequently, an antigen-specific proliferation experimental set-up was performed in 96-well round bottom plates with co-cultures of 10^5 autologous PBMC and 5000 either mDC-TT or vitD3-tolDC-TT (1:20 ratio) in a final volume of 200 µL of supplemented RPMI medium. For each condition, 48 replicates were performed. Cells were then incubated for 5 days at 37 ºC in a 5% CO_2 atmosphere. Afterwards, cells were harvested and the whole volume of each cell suspension was incubated for 20 minutes, protected from light, with the adequate amounts of monoclonal antibodies anti-CD3 Violet 450 (V450) and anti-CD4 PerCP-Cyanine dye (Cy)5.5. Finally, the cells were washed and the whole CD3+CD4+ cell subpopulation was isolated and counted using a FACSAria II cell sorter (BD Biosciences). Finally, dry pellets of mDC-TT-conditioned CD3+CD4+ cells (mDC-Tcell) and vitD3-tolDC-TT-conditioned CD3+CD4+ cells (vitD3-Tcell) were obtained by centrifugation and stored at -80 ºC.

Phenotype characterization of DC and autologous PBMC

Surface protein expression of CD11c, CD14, CD83, CD86 and HLA-DR of iDC, mDC, mDC-TT, vitD3-tolDC and vitD3-tolDC-TT was determined by flow cytometry. On each case, DC suspensions were incubated for 20 minutes, protected from light, with the appropriate amounts of monoclonal antibodies anti-: CD11c PE-Cy7, CD14 V450, CD83 allophycocyanin (APC), CD86 fluorescein isothiocyanate (FITC) and HLA-DR Violet 500 (V500) (all of them from BD Biosciences). Subsequently, at least 10,000 CD11c+ events of each condition were acquired using a FACSCanto II flow cytometer and analysed using FACSDiva software.

For the phenotypical characterization of mDC-Tcell and vitD3-Tcell, cell suspensions of these conditions were incubated for 20 minutes with the adequate amounts of monoclonal antibodies indicated below. Afterwards, samples were washed twice and acquired on a LSRFortessa flow cytometer, setting the stopping gate at 300,000 peripheral blood mononuclear cells. The definition of each peripheral blood mononuclear cell subpopulation was determined as specified in **Supplemental Table 1**, using several combinations of the following monoclonal antibodies anti-: CXCR3 AlexaFluor (AF)488, CD4 PerCP-Cy5.5, CCR7 PE, CD45RA PE-Cy7, CD38 APC, CD45 AF700, CD8 APC-H7, CD3 V450, HLA-DR V500, CCR6 Brilliant Violet (BV) 605, CD25 PE, CCR4 PE-Cy7, CD127 AF647, CD45RO APC-H7, CD49b FITC and LAG-3 PE (BD Biosciences). Results were analyzed with FACSDiva software (BD Biosciences). A forward and side scatter gating strategy was used in order to select the desired lymphocyte subpopulations, and their relative percentages were analyzed for each cell subset.

Allogeneic and autologous cell proliferation assays

For the determination of the reactivity of PBMC from each donor against TT, $2x10^5$ PBMC were plated in 96-well round bottom plates at day 0 of each culture in supplemented RPMI medium containing 0.1 µg/mL TT. As control conditions, the same number of cells was cultured with either supplemented RPMI medium only (negative control) or 50 ng/mL phorbol 12-myristate-13-acetate (PMA) and 500 ng/mL ionomycin (positive control). Ten replicates were performed for the negative control and the condition of analysis, and six replicates for the positive control. The cells were then cultured for 5 days at 37 ºC in a 5% CO_2 atmosphere. Afterwards, 1 µCi [^{3}H]-thymidine (PerkinElmer, Waltham, MA, USA) was added to each well and the plate was incubated for further 18 h under the same conditions. Cells were then collected using a HARVESTER96 2M cell harvester (Tomtec Inc, Hamdem, CT, USA) and read on a 1450 MicroBeta TriLux liquid scintillation counter (Wallac, Turku, Finland). Donors were considered positive for TT reactivity when the counts per minute (cpm) of at least 5 replicates from the condition of analysis were over the mean plus 2 times the standard deviation (SD) of the negative control.

For the isolation of allogeneic PBMC, whole blood samples of different healthy donors were processed by ficoll-hypaque density gradient separation. Cells were washed twice, and afterwards, their absolute number and viability was determined as shown above. Subsequently, 10^5 either allogeneic or autologous PBMC were co-cultured with 5000 either iDC, mDC, mDC-TT, vitD3-tolDC or vitD3-tolDC-TT (1:20 ratio) in 96-well round bottom plates, in a total volume of 200 µL of supplemented RPMI medium. Again, as negative and positive controls, either supplemented RPMI medium or either a mix of 50 ng/mL PMA and 500 ng/mL ionomycin were used, respectively. Six replicates of each condition were performed. Cells were then plated for 4 days at 37 ºC in a 5% CO_2 atmosphere, and afterwards, 1 µCi [^{3}H]-thymidine was added to each well and the plates were incubated, harvested and read as described above.

Cytokine and soluble protein production

The production of granzyme B (GZMB), as well as IL-1β, IL-6, IL-10, IFN-γ, IL-12p70 and TNF-α cytokines, was quantified in the supernatants of mDC-TT and vitD3-tolDC-TT with autologous PBMC co-cultures, using the Human Soluble Protein CBA Flex Set (BD biosciences) according to manufacturer's instructions. The results were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FACSDiva software. The production of TGF-β was determined using the Human/Mouse TGF beta 1 Uncoated ELISA kit (Invitrogen) in 100 µL of the co-culture

supernatants after sample activation with HCl 1N, following manufacturer's instructions. The optical density of each well was measured at $\lambda = 450$ nm, and the optical density at $\lambda = 570$ nm was then substracted as background signal, using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Waltham, MA, USA).

RNA extraction and RNA-seq analysis

Total RNA of autologous mDC-Tcell and vitD3-Tcell samples was isolated using the automated Maxwell 16 LEV simplyRNA Purification Kit (Promega Biotech, Madison, WI, USA), including a DNAse I digestion step, according to manufacturer's instructions. The samples were quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific), and subsequently stored at -80ºC in RNAse-free tubes. RNA integrity number (RIN) was determined in an Agilent BioAnalyzer with the RNA6000 Pico assay (Agilent Technologies, Santa Clara, CA, USA). Afterwards, the sequencing libraries were prepared using the TruSeq Stranded Total RNA Sample Preparation kit (Illumina, San Diego, CA, USA) with 200 ng of total RNA per sample as input. Paired-end sequencing (2x75 bp) was then performed on a HiSeq-2500 instrument (Illumina). Reads were quality trimmed and adapters removed using Trimmomatic V0.30. TopHat software v2.1.0 was used to map RNA-seq reads to the human reference genome (Ensembl release 78) (26). FeatureCounts function was used to assign reads to genomic features focusing on RNA biotypes. A matrix with summarized raw counts of reads assigned through mapping to high confidence protein coding genes only was generated ("golden" annotation label, corresponding to stable and unlikely to change transcripts from the Consensus CDS (CCDS) Project. Data exploration results from hierarchical clustering and principal component analysis (PCA) in R software were used to exclude any outliers and assess sample similarities based on global gene expression patterns, and to guide the modeling design to be used for subsequent analyses.

Differentially gene expression analysis

Transcriptional changes at the gen level between mDC-Tcell and vitD3-Tcell were assessed using the Bioconductor DeSeq2 package in R (27). A paired samples comparison design, factoring in inter-individual differences, was applied. The results were considered statistically significant with an adjusted p-value (padj) < 0.05. We set a 20% fold change (FC) cutoff as the threshold for relevant biological effects ($|FC| > 1.2$).

Gene Ontology enrichment analysis

Unranked lists of the significant differentially upregulated ($FC > 1.2$ and $padj < 0.05$) and downregulated genes ($FC < -1.2$ and $padj < 0.05$) were tested for enrichment in Gene Ontology (GO) functional categories using the GOrilla web tool, applying the default settings for comparison to the background list of genes found in the dataset (28). We tested for enrichment in three types of GO categories: "biological process" (GOPROCESS), "molecular function" (GOFUNCTION) and "cellular component" (GOCOMPONENT). Enrichment score (ES) was defined as $ES = (b/n) / (B/N)$, where "N" is the total number of genes in the background list, "B" is the total number of genes in N associated with a specific GO term, "n" is the number of differentially expressed genes being tested for enrichment and "b" is the number of n intersecting with B. Enrichment p-value is computed according to the hypergeometric (HG) model. FDR q-value is the Benjamini and Hochberg multiple testing correction adjusted p-value. For the i^{th} term (ranked according to p-value), the FDR q-value is the p-value multiplied by the number of GO terms assessed and divided by i.

Statistical analysis

All the statistical analyses were performed with either parametric or non-parametric tests depending on the normality of each compared data set, as determined by the D'Agostino & Pearson test using Prism 6.0 software (GraphPad, La Jolla, CA, USA). For multiple comparisons, either the non-parametric Friedman test with Dunn's correction or the one-way ANOVA test with Geisser-Greenhouse correction were used, and analogously, either t-Student or Wilcoxon tests for the comparisons between two groups if they were normally distributed or not, respectively. Results were expressed as mean ± SD, unless noted otherwise, and they were considered statistically significant when $p < 0.05$.

RESULTS

Functional and phenotypical characteristics of vitD3-tolDC-TT

Monocytes from 16 healthy donor samples were isolated (94.4 ± 2.8% purity) with viabilities of CD14+ cells above 95%. After their differentiation into DC, with or without exposition to TT, the cells were harvested and their purity, viability and phenotype were determined by flow cytometry, as shown in a previous report (25). In all cases, purity was >90%, as determined by the percentage of $CD11c^+$ cells, with a mean viability of 94.2 ± 3.3%. The study of the phenotype of vitD3-tolDC-TT showed important reductions in the surface expression of CD86 (77.2 ± 8.7%) and HLA-DR (79.5 ± 7.7%) compared to mDC, but more importantly, evidenced that the exposure of DC to TT on day 3 of the culture did not have an effect *per se* over the expression of these molecules, neither in vitD3-tolDC nor in mDC, since there were no differences on the percentages of reduction (**Supplemental Figures 1A, 1B**). The same could be observed regarding the functionality of these cells. As shown in **Supplemental Figures 1C**, both vitD3-tolDC and vitD3-tolDC-TT exhibited a similar and strong reduction of allogeneic proliferation compared to mDC (vitD3-tolDC: 50.6 ± 30.7, $p < 0.001$; vitD3-tolDC-TT: 49.2 ± 36.7, $p = 0.001$). On the other hand, there were no statistically significant differences in the mean induction of allogeneic proliferation induced by mDC-TT compared to mDC ($p = 0.916$). Altogether, our results evidence that vitD3-tolDC-TT show the same tolerogenic properties as vitD3-tolDC, thus demonstrating that loading these cells with TT does not have an effect over their phenotype nor their functionality.

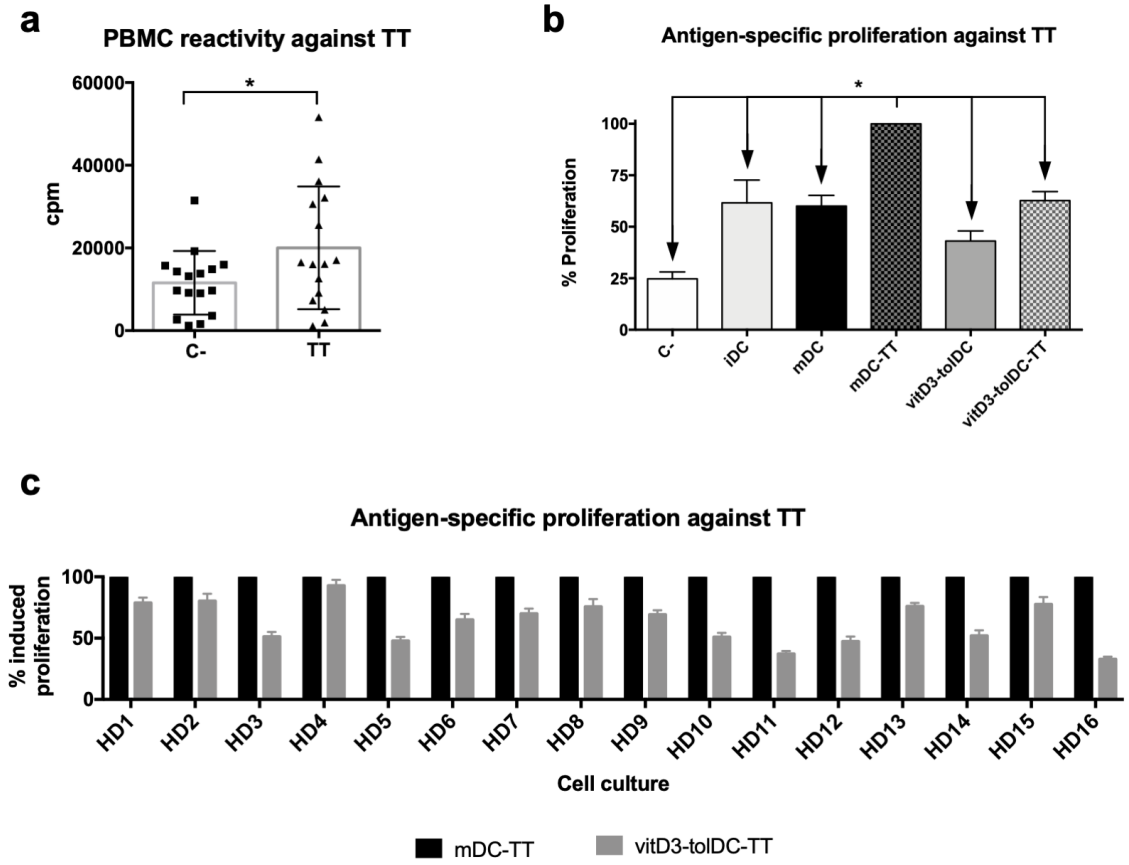

FIGURE 1 | PBMC reactivity and antigen-specific induction of autologous proliferation mediated by dendritic cells against tetanus toxin. (a) Induction of proliferation of PBMC without stimuli (C-) and against tetanus toxin (TT) after 5 days of culture (n = 16). Data presented as counts per minute (cpm), measured as tritiated thymidine incorporation after 18 h. Ten replicated measurements of each condition were performed. (b) Induction of antigen-specific autologous proliferation against TT mediated by immature DC (iDC), mature DC (mDC), TT-loaded mDC (mDC-TT), vitamin D3-induced tolerogenic DC (vitD3-tolDC) and TT-loaded vitD3-tolDC (vitD3-tolDC-TT), as well as a negative control (C-), without any stimuli (n = 16) and (c) comparison of autologous antigen-specific proliferation against TT mediated by mDC-TT and vitD3-tolDC-TT on each donor. Data presented as relative percentage of induced proliferation compared to mDC-TT, measured as tritiated thymidine incorporation after 18 h. Six replicated measurements of each condition were performed. Error bars corresponding to SEM. ns = not significant; * $p < 0.05$. One-way ANOVA test with Geisser-Greenhouse correction or paired t test.

VitD3-tolDC-TT induce an antigen-specific response over autologous PBMC

In order to test the antigen-specific functionality of vitD3-tolDC-TT in an autologous set up, first we assessed the baseline reactivity of each donor against the TT itself to measure the potential of each donor to respond under these conditions. As shown in **Figure 1A**, we were able to assess the TT reactivity in all of our healthy donors, but only 9 of them resulted positive, according to the criteria described in the methods section —when the mean proliferation of at least 5 out of the 10 replicates was over the mean plus 2 times the SD of the control condition—, and reaching statistical significance ($p < 0.05$).

Subsequently, we analyzed the capability of our cells to induce proliferation over autologous PBMC. As shown in **Figure 1B**, a significant proliferation was only induced by mDC-TT, as evidenced by the statistically significant differences observed with the remaining conditions. Specifically, reductions of a $38.4 \pm 44.3\%$ ($p = 0.020$), a $40.0 \pm 21.0\%$ ($p < 0.001$), a $56.9 \pm 19.2\%$ ($p < 0.001$) and a $37.3 \pm 17.4\%$ ($p < 0.001$) were observed in iDC, mDC, vitD3-tolDC and vitD3-tolDC-TT, respectively, compared to mDC-TT. Our results therefore evidence that the autologous proliferation is only primed if an antigenic peptide is presented by an immunogenic DC condition, such as mDC-TT, confirming the antigen-specific modulation developed by our cells. Furthermore, this reduction of autologous proliferation mediated by vitD3-tolDC-TT was observed in all of our donors (**Figure 1C**).

VitD3-tolDC-TT drive a reduction of T_H1 CD4+ cell subpopulations

Once determined that an antigen-specific modulation was established by TT-loaded DC, we studied which changes were being induced over the autologous T lymphocytes. Therefore, we characterized the phenotype of CD3+CD4+ and CD3+CD8+ cells using an exhaustive multiparametric flow cytometry panel, described in previous studies (29). First, our results evidenced a reduction in the prevalence of activated T CD4+ cells, determined by HLA-DR and/or CD38 staining, in vitD3-Tcell compared to

TABLE 1 | Up-modulated genes in vitD3-Tcell compared to mDC-Tcell.

GeneSymbol	GO annotation	FC vs mDC-Tcell	Adj. p-value
TMIE	NA	1.62	0.01199
PRH2	NA	1.61	0.04136
ARHGEF26	NA	1.53	0.01404
GRTP1	G-Protein Modulator; Cysteine Protease	1.50	0.03547
AKAP6	NA	1.45	0.01310
CKMT2	Amino Acid Kinase	1.43	0.03068
SLC10A1	Cation Transporter	1.40	0.00264
C17orf107	NA	1.40	0.01419
SULT1B1	NA	1.38	0.00117
TTC16	NA	1.37	0.03271
TEC	NA	1.35	0.02676
KRT72	NA	1.31	0.00543
ABCC2	ATP-Binding Cassette (ABC) Transporter	1.30	0.00598
KRT73	NA	1.28	0.02747
SORBS3	NA	1.26	0.00279
AK5	Nucleotide Kinase	1.24	0.02996
EDAR	NA	1.24	0.00041
ADAM23	Metalloprotease	1.24	0.01495
RALGPS2	Guanyl-Nucleotide Exchange Factor	1.23	0.02922
ALS2CL	NA	1.22	0.04815
KBTBD11	NA	1.22	0.00543
JUNB	Basic Leucine Zipper Transcription Factor; Nucleic Acid Binding	1.22	0.00360
RASA4	G-Protein Modulator	1.22	0.01328
C9orf72	NA	1.22	0.00818
ZC4H2	NA	1.21	0.04572
ADPRM	NA	1.21	0.01438
SCML1	Chromatin/Chromatin-Binding Protein; Transcription Factor	1.21	0.03955
MEGF6	Extracellular Matrix Protein	1.21	0.00102
LMTK3	NA	1.21	0.03156

Results shown as mean fold change (FC) of expression. GO: Gene Ontology; NA: not available; padj: adjusted p-value.

mDC-Tcell (Activated CD4+ $_{\text{mDC-Tcell}}$: 23.57 ± 15.81 vs Activated CD4+ $_{\text{vitD3-Tcell}}$: 18.52 ± 14.16; p = 0.002). The same effect was observed over T CD8+ cells (Activated CD8+ $_{\text{mDC-Tcell}}$: 15.94 ± 12.48 vs Activated CD8+ $_{\text{vitD3-Tcell}}$: 11.33 ± 9.81; p = 0.002). Furthermore, we found a reduction in the relative percentages of CD4+ T_H1 Central Memory (CM) and Effector Memory (EM) subpopulations in vitD3-Tcell (T_H1 CM $_{\text{mDC-Tcell}}$: 33.98 ± 6.44 vs T_H1 CM $_{\text{vitD3-Tcell}}$: 30.23 ± 7.48; p = 0.013; T_H1 EM $_{\text{mDC-Tcell}}$: 44.46 ± 8.72 vs T_H1 EM $_{\text{vitD3-Tcell}}$: 40.95 ± 8.08; p = 0.001). All these results are shown in **Figures 2A, 2B**. Thus, our data suggest that vitD3-tolDC-TT are driving an antigen-specific switch towards a more anti-inflammatory —or less T_H1-like— profile over T CD4+ lymphocytes. On the other hand, we could not detect any significant changes over any other T cell subpopulation, nor Treg nor Tr1 subpopulations (data not shown).

Next, we analyzed the cytokine secretion profile present in the autologous co-cultures of mDC-TT and vitD3-tolDC-TT. Our results, as shown in **Figure 2C**, evidenced a statistically significant increased secretion of the cytokine IL-6 and lower levels of IFN-γ in the co-culture of autologous PBMC with vitD3-tolDC-TT compared to mDC-TT (IL-6 $_{\text{mDC-TT}}$: 61.4 ± 84.3 pg/mL vs IL-6 $_{\text{vitD3-tolDC-TT}}$: 77.7 ± 94.5 pg/mL; p = 0.039; and IFN-γ $_{\text{mDC-TT}}$: 3.3 ± 2.9 pg/mL vs IFN-γ $_{\text{vitD3-tolDC-TT}}$: 2.2 ± 2.8 pg/mL; p = 0.002). Therefore, the reduction in the production of IFN-γ combined with the increase of IL-6, again suggest a reduction of the T_H1-like cytokine profile, in line with the phenotype results. No statistically significant changes could be found in the production of GZMB, IL-1β, IL-10, TGF-β nor TNF-α.

VitD3-tolDC induce a general transcriptomic repression over T CD4+ cells

For all the 16 donors, at least 700,000 CD3+CD4+ cells in both conditions (mDC-Tcell and vitD3-Tcell) were successfully isolated by flow cytometry cell sorting. The gating strategy is shown in Supplemental **Figure 1D**. Afterwards, we extracted their RNA and selected 10 donors that showed a good nucleic acid integrity for the RNA-seq analysis (RIN > 7) in both conditions and a reduction in the induced autologous proliferation of PBMC in vitD3-tolDC-TT compared to mDC-TT. Consequently, donors HD4, HD5, HD9, HD10, HD11 and HD12 were discarded from downstream studies.

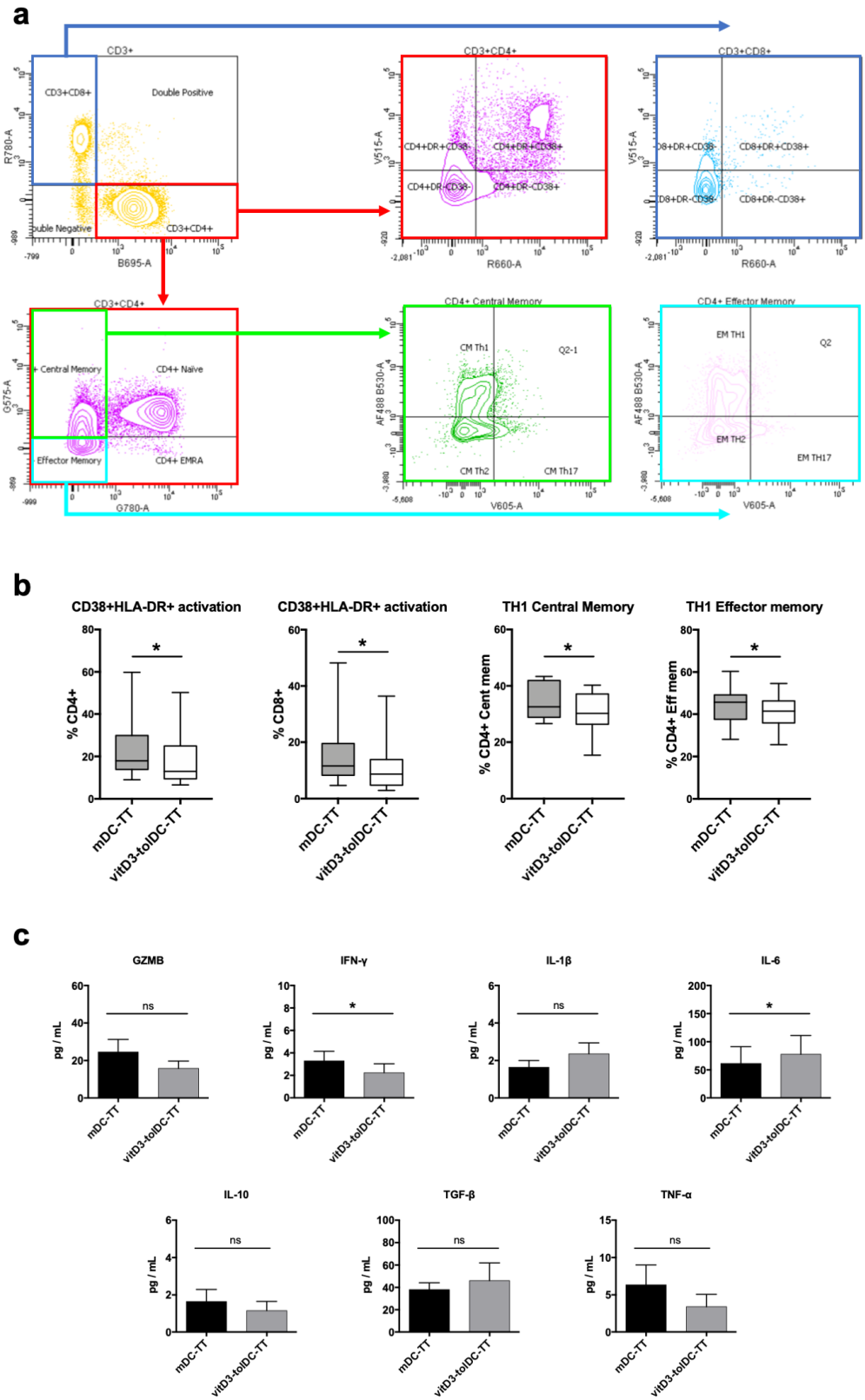

FIGURE 2 | Phenotype and cytokine production of dendritic cell-co-cultured autologous PBMC. (a) Gating strategy. (b) Relative percentages of $CD4^+CD38^+HLA\text{-}DR^+$, $CD8^+CD38^+HLA\text{-}DR^+$, T_H1 Central Memory and T_H1 Effector Memory subpopulations on PBMC co-cultured with either tetanus toxin (TT)-loaded mature DC (mDC-TT) or TT-loaded vitamin D3-induced tolerogenic DC (vitD3-tolDC-TT) after 5 days of culture (n = 10). Data presented as the relative percentage of each lymphocyte subpopulation within its respective parent subpopulation, measured by multiparametric flow cytometry, in a box and whiskers representation, Error bars corresponding to the maximum and minimum values of each condition. (b) Analysis of the secretion of granzyme B (GZMB) (n = 12), IFN-γ (n = 12), IL-1β (n = 8), IL-6 (n = 8), IL-10 (n = 10), TGF-β (n = 7), and TNF-α (n = 8) in the supernatants of PBMC co-cultured with either tetanus toxin mDC-TT or vitD3-tolDC-TT after 5 days of culture using either ELISA (TGF-β) or cytometric bead array (GZMB, IFN-γ, IL-1β, IL-6, IL-10, and TNF-α) techniques. One ELISA experiment was performed for all samples, with duplicated measurements for each sample. One single cytometric bead array experiment was performed for the analysis of all the samples, with one single measurement for each sample. Error bars corresponding to SEM. ns: not significant; * $p < 0.05$. Paired t test or Wilcoxon test.

TABLE 2 | Top 50 down-modulated genes in vitD3-Tcell compared to mDC-Tcell.

GeneSymbol	GO annotation	FC vs mDC-Tcell	padj
IL13	Cytokine	-2.59	0.00082
C1orf106	NA	-2.43	0.00486
RYR2	Ligand-Gated Ion Channel	-2.35	0.00802
HLA-DQA2	Major Histocompatibility Complex Antigen	-2.25	0.03271
UBE2C	NA	-2.23	0.00000
DEPDC1	Nucleic Acid Binding	-2.23	0.00367
NEK2	Protein Kinase	-2.20	0.00206
EBI3	Cytokine; Defense/Immunity Protein	-2.18	0.00009
CCL17	Chemokine	-2.18	0.01852
AURKB	Non-Receptor Serine/Threonine Protein Kinase	-2.14	0.00006
CYP1B1	Oxygenase	-2.12	0.02334
SPC25	Enzyme Modulator	-2.11	0.01556
KIF4A	Microtubule Binding Motor Protein	-2.09	0.00001
KIF18B	Microtubule Binding Motor Protein	-2.07	0.00001
CCNB2	Kinase Activator	-2.06	0.00000
HLA-DRB5	Major Histocompatibility Complex Antigen	-2.02	0.00970
MCM10	NA	-2.01	0.00003
HIST1H3C	Histone	-2.01	0.00000
KIF15	Microtubule Binding Motor Protein	-1.97	0.00000
BIRC5	Protease Inhibitor	-1.96	0.00002
CHST3	NA	-1.95	0.01782
FAM111B	NA	-1.95	0.00000
MYBL2	NA	-1.92	0.00000
ATP8B4	Cation Transporter. Hydrolase	-1.92	0.00000
SKA1	NA	-1.91	0.00898
KIAA0101	NA	-1.91	0.00292
TK1	Nucleotide Kinase	-1.90	0.00001
HIST1H3J	Nucleic Acid Binding; Transcription Factor	-1.89	0.03504
E2F8	Nucleic Acid Binding; Transcription Factor	-1.89	0.00209
HLA-DQA1	Major Histocompatibility Complex Antigen	-1.88	0.00011
HIST1H3F	Reductase	-1.88	0.00005
RRM2	Reductase	-1.88	0.00337
GNG4	Heterotrimeric G-Protein	-1.87	0.01479
PRR11	NA	-1.86	0.00041
CEP55	NA	-1.85	0.00008
CKAP2L	NA	-1.84	0.00151
CDCA8	NA	-1.84	0.00015
HIST1H3G	Histone	-1.83	0.00000
CDK1	Non-Receptor Serine/Threonine Protein Kinase; Non-Receptor Tyrosine Protein Kinase	-1.83	0.00283
HMMR	NA	-1.83	0.00012
PKMYT1	Non-Receptor Serine/Threonine Protein Kinase	-1.83	0.00825
CCL22	Chemokine	-1.82	0.02508
CREB3L3	NA	-1.81	0.02749
CDC25A	Protein Phosphatase	-1.81	0.02454
DTL	NA	-1.81	0.00008
RAD51AP1	NA	-1.80	0.00912
ESCO2	NA	-1.79	0.01020
LIF	Cytokine	-1.78	0.00000
KIFC1	Microtubule Binding Motor Protein	-1.78	0.00000
ASB2	NA	-1.78	0.00000

Results shown as mean fold change (FC) of expression. GO: Gene Ontology; NA: not available; padj: adjusted p-value.

After processing the samples through the RNA-seq analysis, 39% of total reads could be assigned to different known RNA classes (**Figure 3A**), and out of them, around 47% of these assigned reads could be related to protein coding genes (**Figure 3B**). Interestingly, the hierarchical clustering analysis revealed that our samples tended to cluster by individual rather than by treatment (**Figure 3C**), but also that there is a consistent pattern by which vitD3-Tcell samples ranked higher on both axes from the PCA (**Figure 3D**). These results led to choose a paired comparative analysis approach for the differential expression analysis.

After the subsequent filtering process described in the methods section, a total of 16333 protein coding genes with detectable reads were tested for differential expression. Among all of them, 546

genes showed a statistically significant change in their expression (adjusted $p < 0.05$) in vitD3-Tcell compared to mDC-Tcell, and only 373 also presented an absolute value of FC superior to 1.20 ($|FC_{\text{vitD3-Tcell vs mDC-Tcell}}| > 1.20$). While only 29 of these genes were up-modulated in vitD3-Tcell compared to mDC-Tcell, the majority of them, 344 genes, were down-modulated, indicating a strong transcriptomic repression induced by vitD3-tolDC-TT over these cells (**Figure 3E**).

T CD4+ cells selectively undergo a strong functional and immune-related transcriptomic down-modulation upon interaction with vitD3-tolDC

When we studied those differentially expressed genes that appeared up-modulated ($FC_{\text{vitD3-Tcell vs mDC-Tcell}} > 1.20$) in our analysis (**Table 1**), we did not find many relevant nor immune-related genes. Specifically, 18 of these 29 genes did not have any GO annotation, and among the rest, we could only find the genes encoding the JUNB and SCML1 transcription factors and several other genes encoding different molecule transporters (*ABCC2* and *SLC10A1*), G-protein modulators (*GRTP1* and *RASA4*) and kinases (*AK5* and *CKMT2*).

However, as mentioned above, the study of the down-modulated genes ($FC_{\text{vitD3-Tcell vs mDC-Tcell}} < 1.20$) yielded many more results. Within the 50 most down-regulated results ($FC_{\text{vitD3-Tcell vs mDC-Tcell}} < 1.78$) we found several genes encoding proteins involved in the immune response (*CCL17*, *CCL22*, *EBI3*, *IL13* and *LIF*), antigen presentation (*HLA-DQA1*, *HLA-DQA2* and *HLA-DRB5*) and microtubule binding (*KIF4A*, *KIF15*, *KIF18B* and *KIFC1*), among others (**Table 2**). Furthermore, when we analyzed the whole list, we could find not only several more genes included in these categories, but also many other genes encoding proteins related to cytoskeleton and cell adhesion (*ARPC1B*, *CAPG*, *CTNNA1*, *LGALS1*, *MYL6B*, *LGALS9* or *SDC4*), actin related functionalities (*ACTB*, *ACTG1*, *PARVB* or *TPM4*), G-proteins and modulators (*GBP2*, *GBP4*, *GNA15*, *GNG4*, *IQGAP3*, *MYO1G*, *MYO1E* or *SRGAP3*), nucleic acid binding (*ASF1B*, *DEPDC1*, *EXO1* or *FEN1*), histones (*HIST1H2BL*, *HIST2H2BF* or, *HIST1H4H*), the pro-inflammatory transcription factor *STAT1* and other pro-inflammatory mediators (*TNFSF4*), different kinase activators and modulators, proteases and protease inhibitors, oxydases, oxygenases, transferases and many other metabolic mediators. The whole list is shown in **Supplemental Table 2**. Altogether, these results indicate that vitD3-tolDC-TT mediate a strong down-modulation of metabolic and immune-related functions over vitD3-Tcell.

VitD3-Tcell present decreased cell cycle and mitotic activity

In this regard, the GO enrichment analysis further supported the results observed in the differential gene expression (DGE) study. Thus, first, the enrichment analysis produced a total of 482 protein sets and pathways with $p < 0.001$, 4 of them up-modulated — although none of them showed a false discovery rate (FDR) value below 0.25— and the remaining 478 down-modulated (**Supplemental Table 3**). We further filtered the results to analyze only those GO terms that presented a much more significant enrichment ($p < 10^{-9}$). This process left us with a total of 66 down-modulated GO terms, but none up-modulated. These 66 elements, ordered by decreasing GO enrichment score (ES), are shown in **Table 3**. Interestingly, among the most significantly enriched down-modulated pathways, we found several GO annotations referring to immune-related functionality (for instance *Interferon-Gamma-Mediated Signaling Pathway*, ES: 10.65; *Cytokine-Mediated Signaling Pathway*, ES: 5.09; or *Immune Response*, ES: 3.03), class II-related antigen presentation (like *MHC Class II Protein Complex*, ES: 30.23; *Antigen Processing And Presentation Of Exogenous Peptide Antigen Via MHC Class II*, ES: 8.08; or *Antigen Processing And Presentation Of Exogenous Peptide Antigen*, ES: 6.74), cell response to different stimuli (*Cell Surface Receptor Signaling Pathway*, ES: 2.21; *Response To Stress*, ES: 1.93; or *Cellular Response To Stimulus*, ES: 1.80) and, specially, to cell cycle and mitotic division (for instance *Condensed Chromosome Outer Kinetochore*, ES: 21.98; *Mitotic Spindle Organization*, ES: 8.79; *Microtubule Cytoskeleton Organization Involved In Mitosis*, ES: 7.48; or *Cell Cycle Checkpoint*, ES: 6.05). Our results, in line with the DGE analysis, would suggest that vitD3-Tcell are undergoing a process of transcriptomic down-modulation leading to reduced immune-related, metabolic and proliferative functionalities.

DISCUSSION

In this study we analyzed the specific effect of vitD3-tolDC over $CD4^+$ T cells. Thus, we switched the attention from the study of tolDC themselves —widely studied so far— to focus on the study of the functional effect that these cells develop over T cells upon their interaction. In homeostatic conditions, either depletion, inactivation and/or induction of anergy is often induced on T cells due to a lack of one or more of the three immunogenic activation signals. This causes T cells to become hyporesponsive or to die (20,30). However, in the case of autoimmunity, where T cells are already activated and developing an immunogenic response, an antigen-specific process of tolerance induction is required. In this regard, previous *in vivo* studies with vitD3-tolDC in the EAE model showed that an antigen-specific set-up —and therefore an active process— is required, provided that a beneficial effect of this therapy was only observed when vitD3-tolDC were pulsed with the adequate immunogenic peptide (23,24). Consequently, we developed an experimental model for the generation of autologous antigen-specific vitD3-tolDC and T cells from healthy donors, using an immunogenic peptide presented via class II MHC with the aim to reproduce antigen presentation in the context of $CD4^+$-mediated autoimmune diseases. In this regard, we selected TT for its compliance with this feature —since the vaccination against TT is included in European health systems—, but with the idea that the immunogenic peptide/s might be replaced depending on the disease of interest that needs to be addressed.

After validating our experimental setup —meaning that vitD3-tolDC-TT were able to induce an antigen-specific response—, we focused on the study of the actual phenotypic, functional and transcriptomic modulations induced by vitD3-tolDC. On the one hand, the analysis of the phenotype of T CD4+ cells evidenced that their interaction with vitD3-tolDC-TT caused a relative reduction in the activation of these cells. More importantly, a switch in the immune response of these cells towards a more immunoregulatory profile was induced, with a reduction in the prevalence of T_H1 memory subpopulations. These results were

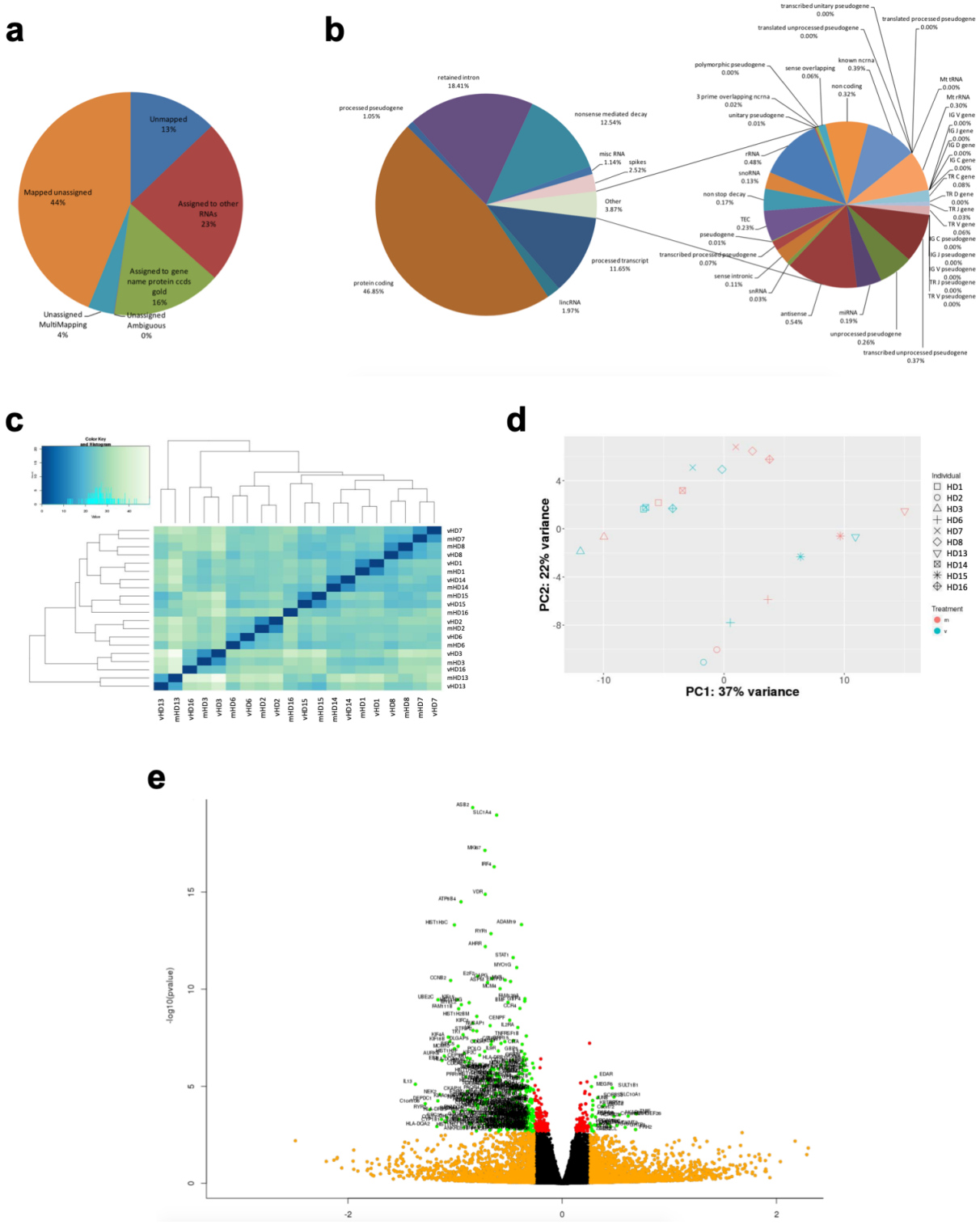

FIGURE 3 | Exploratory analysis of the RNA-seq study of T CD4+ cells co-cultured with autologous antigen-specific dendritic cells. (a) Assignment of the total reads from the RNA-seq analysis to known RNA classes. (b) Classification of the assigned reads into known RNA functionalities. (c) Hierarchical clustering analysis by gene expression of the 20 samples of $CD4^+$ T cells of the RNA-seq study. (d) Representation of the first two principal component analysis (PCA) components at the gene level. Each symbol corresponds to a different sample of CD4+ T cells, and the red and green colors stand for the co-culture condition of each sample, either tetanus toxin (TT)-loaded mature DC (m) or TT-loaded vitamin D3-induced tolerogenic DC (v), respectively, as depicted on the legend. (e) Volcano plot showing the significant differentially expressed genes. Axis is the log2 fold change. Color code: green, significantly regulated genes (padj < 0.05; |FC| > 1.2) considered in the Gene Ontology enrichment analysis; orange, genes with |FC| > 1.2 below the significance threshold; red, genes with padj < 0.05 below the relevant fold change cutoff.

TABLE 3 | Most significantly down-regulated Gene Ontology terms in vitD3-Tcell compared to mDC-Tcell.

GO category	GO term	ES	p-value	FDR
GOCOMPONENT	MHC Class II Protein Complex	30.23	5.24E-16	2.39E-13
GOCOMPONENT	Condensed Chromosome Outer Kinetochore	21.98	3.96E-10	4.81E-08
GOCOMPONENT	MHC Protein Complex	18.71	1.94E-12	5.88E-10
GOCOMPONENT	Clathrin-Coated Endocytic Vesicle Membrane	16.92	6.86E-10	7.81E-08
GOFUNCTION	Peptide Antigen Binding	16.92	6.86E-10	7.06E-07
GOPROCESS	Interferon-Gamma-Mediated Signaling Pathway	10.65	1.35E-13	1.44E-10
GOCOMPONENT	DNA Packaging Complex	10.26	5.61E-19	5.11E-16
GOPROCESS	Nuclear Chromosome Segregation	10.21	9.93E-10	2.98E-07
GOCOMPONENT	Nucleosome	9.82	2.16E-16	1.31E-13
GOPROCESS	Mitotic Spindle Organization	8.79	1.87E-11	8.07E-09
GOPROCESS	Antigen Processing And Presentation Of Exogenous Peptide Antigen Via MHC Class II	8.08	1.20E-12	8.70E-10
GOPROCESS	Regulation Of Chromosome Segregation	7.98	9.85E-14	1.24E-10
GOPROCESS	Antigen Processing And Presentation Of Peptide Or Polysaccharide Antigen Via MHC Class II	7.98	1.50E-12	9.88E-10
GOPROCESS	Antigen Processing And Presentation Of Peptide Antigen Via MHC Class II	7.98	1.50E-12	9.43E-10
GOPROCESS	Microtubule Cytoskeleton Organization Involved In Mitosis	7.48	1.93E-11	8.07E-09
GOPROCESS	Chromosome Segregation	7.21	8.19E-13	7.07E-10
GOPROCESS	Mitotic Cell Cycle	7.20	1.49E-15	2.29E-12
GOPROCESS	Nucleosome Assembly	7.08	9.78E-14	1.35E-10
GOPROCESS	Antigen Processing And Presentation Of Exogenous Peptide Antigen	6.74	1.09E-11	5.59E-09
GOPROCESS	Antigen Processing And Presentation Of Exogenous Antigen	6.62	1.57E-11	6.98E-09
GOCOMPONENT	Protein-DNA Complex	6.57	5.01E-14	1.82E-11
GOPROCESS	Antigen Processing And Presentation Of Peptide Antigen	6.32	3.73E-11	1.47E-08
GOPROCESS	Spindle Organization	6.11	2.12E-10	7.32E-08
GOPROCESS	Cell Cycle Checkpoint	6.05	2.87E-11	1.16E-08
GOPROCESS	Antigen Processing And Presentation	5.91	1.55E-11	7.15E-09
GOCOMPONENT	Midbody	5.75	9.66E-12	1.95E-09
GOPROCESS	Nucleosome Organization	5.71	1.12E-11	5.52E-09
GOPROCESS	Regulation Of Mitotic Nuclear Division	5.46	7.83E-11	2.92E-08
GOPROCESS	Regulation Of Nuclear Division	5.39	1.37E-11	6.52E-09
GOPROCESS	Cytokine-Mediated Signaling Pathway	5.09	3.61E-27	2.49E-23
GOCOMPONENT	Spindle	5.07	1.32E-10	1.85E-08
GOCOMPONENT	Chromosome	4.67	9.10E-12	2.07E-09
GOPROCESS	Mitotic Cell Cycle Process	4.64	3.48E-28	4.81E-24
GOPROCESS	Positive Regulation Of Cell Cycle Process	4.55	4.10E-11	1.57E-08
GOPROCESS	Protein-DNA Complex Assembly	4.47	7.59E-10	2.33E-07
GOPROCESS	Cell Division	4.25	5.31E-16	9.17E-13
GOPROCESS	Negative Regulation Of Cell Cycle Process	4.04	6.43E-10	2.02E-07
GOPROCESS	Regulation Of Mitotic Cell Cycle Phase Transition	3.99	1.04E-12	8.42E-10
GOPROCESS	Chromosome Organization	3.94	1.50E-12	1.04E-09
GOPROCESS	Regulation Of Cell Cycle Phase Transition	3.82	1.92E-12	1.15E-09
GOPROCESS	Cell Cycle Process	3.63	2.27E-25	1.05E-21
GOPROCESS	Cell Cycle	3.56	1.11E-12	8.53E-10
GOPROCESS	Regulation Of Cell Cycle Process	3.47	7.37E-17	1.69E-13
GOFUNCTION	Protein Heterodimerization Activity	3.44	5.36E-10	7.36E-07
GOCOMPONENT	Chromosomal Part	3.27	8.53E-20	1.55E-16
GOCOMPONENT	Nuclear Chromosome Part	3.14	3.70E-11	6.75E-09
GOPROCESS	Regulation Of Mitotic Cell Cycle	3.12	2.72E-12	1.44E-09
GOPROCESS	Negative Regulation Of Cell Cycle	3.12	2.28E-10	7.66E-08
GOPROCESS	Immune Response	3.03	2.51E-12	1.45E-09
GOPROCESS	Regulation Of Cell Cycle	2.82	6.87E-17	1.90E-13
GOPROCESS	Positive Regulation Of Immune System Process	2.68	5.07E-10	1.67E-07
GOFUNCTION	Protein Dimerization Activity	2.58	1.83E-12	3.76E-09
GOPROCESS	Immune System Process	2.36	3.18E-16	6.27E-13
GOPROCESS	Regulation Of Immune System Process	2.31	1.35E-10	4.91E-08
GOCOMPONENT	Cytoskeletal Part	2.26	7.15E-12	1.86E-09
GOPROCESS	Cell Surface Receptor Signaling Pathway	2.21	2.80E-13	2.76E-10
GOPROCESS	Response To Stress	1.93	6.04E-13	5.56E-10
GOCOMPONENT	Extracellular Region Part	1.83	1.79E-10	2.33E-08
GOCOMPONENT	Non-Membrane-Bounded Organelle	1.82	1.01E-10	1.67E-08
GOCOMPONENT	Intracellular Non-Membrane-Bounded Organelle	1.82	1.01E-10	1.53E-08
GOPROCESS	Cellular Response To Stimulus	1.80	6.19E-10	1.99E-07
GOPROCESS	Response To Stimulus	1.78	6.82E-17	2.35E-13
GOPROCESS	Signal Transduction	1.76	9.95E-14	1.14E-10
GOPROCESS	Regulation Of Cellular Process	1.28	1.91E-10	6.76E-08
GOFUNCTION	Protein Binding	1.26	4.87E-15	2.01E-11
GOPROCESS	Cellular Process	1.15	2.64E-12	1.46E-09

ES: Enrichment Score; FDR: False Discovery Rate; GO: Gene Ontology; GOCOMPONENT: GO cellular component; GOFUNCTION: GO molecular function; GOPROCESS: GO biological process.

further supported by the decrease of IFN-γ production in the autologous co-culture supernatants, consequently supporting that vitD3-tolDC were inducing a switch towards a more anti-inflammatory immune profile.

When we deepened into the analysis of the vitD3-tolDC-mediated transcriptomic profile of T cells, we observed several genes and GO terms regulated in line with the abovementioned phenotypical and functional switch towards a less activated an more immunoregulatory profile; for instance, a down-modulation of *STAT1* gene and the interferon-gamma-mediated signaling pathway was observed, which on the other hand supported the robustness of our RNA-seq study. In general, the results pointed towards a generalized down-modulation of the transcriptomic profile of vitD3-Tcell, compared to the immunogenic control provided by mDC-Tcell. Thus, the down-modulation of genes and pathways involved in crucial cellular processes, in particular of those related to cell proliferation —cell cycle and mitosis-related GO terms— and with the response to immune stimuli, suggests that the antigen-specific interaction of vitD3-tolDC with autologous T CD4+ cells is mediating an induction of hyporesponsiveness over these cells. This situation, potentially, might lead to the abrogation of an autoimmune immunogenic response in patients. Previous studies from our group already pointed in this direction (16). Moreover, our current results evidence that these modulations are taking place at the transcriptomic level in T $CD4^+$ cells, indicating that the antigen-specific modulation induced by vitD3-tolDC is deeper than expected and, in consequence, probably also long-lasting. Unfortunately, the lack of strongly up-regulated genes among the protein coding RNA transcripts did not allow us to point towards many clear candidate biomarkers that might be indicators of the response of T cells upon their interaction with vitD3-tolDC. The only relevant exception came given by *JUNB* gene, encoding a member of the AP-1 family of transcription factors. In experimental models, this gene has been reported to be crucial in maintaining Treg suppressive function (31), although it is also apparently involved in the induction and maintenance of IL-23-related pathogenicity of T_H17 cells (32,33). However, neither of these functionalities seem to fit in our model based on our results, since neither T_H17, nor Treg induction was evidenced. Consequently, it would be interesting to elucidate the specific role of JunB in our experimental setting. Furthermore, if this hypothesis proves to be valid, either *JUNB* and/or other related genes might also constitute potential biomarkers of response to vitD3-tolDC treatment in a clinical trial. In addition, it is also worth noting that we may have overlooked other potential biomarkers that might be found among the non-protein coding and alternate splicing RNA transcripts. Although this possibility, if true, would have a limited functional value in our experimental model, it could be addressed in future studies.

On the other hand, our results do not allow us to reach any conclusion regarding a potential induction of anergy, and, as discussed above, they also rule out any kind of Treg or Tr1 induction. Even though our previous *in vivo* experiments with the murine EAE model pointed towards an induction of Treg mediated by vitD3-tolDC (23,24), we have observed that, at least in this experimental setting, this is not the case with human cells. These results are in line with what previous studies from both our group and other authors have already reported (13,16,34), although there seems to be some controversy (35,36). However, it is also worth mentioning that in these reports, Treg induction was only observed after two rounds of stimulation of T cells, which might explain why we have not detected it. This is definitely something to be taken into account, since Treg induction is, undoubtedly, one of the main mechanisms for the induction of immune tolerance of tolDC and other antigen presenting cell approaches (37,38). Indeed, Treg themselves, when expanded *in vitro*, present a huge therapeutic potential as a cell therapy for autoimmune diseases in humans (39). Consequently, the transcriptomic study of vitD3-tolDC-induced Treg should probably be addressed separately in future studies, since two rounds of T cell stimulation might have masked some of the results that we have reported here.

Our current study presents some limitations. First, since we focused on the study of $CD4^+$ T cells alone, we were naturally omitting the potential modulation that vitD3-tolDC might be mediating through other subpopulations, such as regulatory B cells or regulatory NK cells. Furthermore, the election of the timepoint for the RNA-seq analysis intrinsically establishes another limitation, which is the status of the transcriptomic profile at different timepoints of the co-culture. However, our selection came based on the phenotypical and functional results shown on the study, which evidence that by day 5 of the co-culture there is an evident differential modulation mediated by vitD3-tolDC over T $CD4^+$ cells. Consequently, even though it is true that other timepoints might provide valuable additional information, we think that our election provides the best compromise, and the full time course characterization of the antigen-specific transcriptomic changes induced by vitD3-tolDC could be addressed in future studies. On the other hand, we cannot fully discard the presence of non-antigen-specific $CD4^+$ cells by the time the cell sorting was performed. However, even with a residual amount of non-antigen-specific T cells, the obtained results were robust and consistent, not only within the different techniques used in this study but also in line with the above referenced literature.

CONCLUSSIONS

Our results clearly evidence that vitD3-tolDC are inducing a strong antigen-specific transcriptomic down-modulation over autologous T CD4+ cells, with a reduced ability to respond to immune- and non-immune-related stimuli. Consequently, our study constitutes one of the first attempts to fully understand the changes that T cells are undergoing at the transcriptomic level upon an antigen-specific interaction with a tolerogenic cell product, such as vitD3-tolDC. In that regard, we have identified several specific genes and pathways selectively down-modulated, as well as the induction of *JUNB*, which might constitute a putative biomarker of the modulation mediated by vitD3-tolDC over $CD4^+$ T cells and, consequently, a potential biomarker to monitor the effect of vitD3-tolDC in a clinical trial. Therefore, the results presented in this article allowed us to better understand the process of T cell hyporesponsiveness at the molecular level and, more importantly, to set the path for future studies to fully elucidate the specific processes that are taking place in one of the most important mechanisms that the promising tolDC-based therapies can trigger in order to restore tolerance in autoimmune diseases.

LIST OF ABBREVIATIONS

DC: Dendritic cells; **iDC:** Immature dendritic cells; **mDC:** Mature dendritic cells; **mDC-Tcell:** Mature dendritic cell-conditioned T cells; **mDC-TT:** Tetanus toxin-loaded mature dendritic cells; **MS:** Multiple sclerosis; **tolDC:** Tolerogenic dendritic cells; **TT:** Tetanus toxin; **vitD3-Tcell:** Vitamin D3-induced tolerogenic dendritic cell-conditioned T cells; **vitD3-tolDC:** Vitamin D3-induced tolerogenic dendritic cells; **vitD3-tolDC-TT:** Tetanus toxin-loaded vitamin D3-induced tolerogenic dendritic cells

DECLARATIONS

Ethics approval and consent to participate

This study was approved by the Germans Trias i Pujol Hospital ethical committee, and an informed consent was signed by all the donors.

Availability of data and materials

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (40) and are accessible through GEO Series accession number GSE128816 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128816).

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

EM-C, JN-B and MM conceived the experiments. JN-B and MM performed the cell cultures, the cell sortings, and prepared the samples for the RNA-seq analysis. JN-B and MM analyzed the results. EM-C, JN-B and MM interpreted the results. JN-B wrote the manuscript. AT-S, BQ-S, CR-T, EM-C, JN-B, and MM reviewed the manuscript.

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Supplemental Table 1. Definition of T cell subpopulations in the flow cytometry panel.

Cell population	Marker signature
T lymphocytes	CD3+
T CD4 lymphocytes	CD3+CD4+
CD4 Naïve	CD3+CD4+CCR7+CD45RA+
CD4 Central Memory	CD3+CD4+CCR7+CD45RA-
T_H1	CD3+CD4+CCR7+CD45RA-CCR6-CXCR3+
T_H2	CD3+CD4+CCR7+CD45RA-CCR6-CXCR3-
T_H17	CD3+CD4+CCR7+CD45RA-CCR6+CXCR3-
T_H1T_H17	CD3+CD4+CCR7+CD45RA-CCR6+CXCR3+
CD4 Effector Memory	CD3+CD4+CCR7-CD45RA-
T_H1	CD3+CD4+CCR7-CD45RA-CCR6-CXCR3+
T_H2	CD3+CD4+CCR7-CD45RA-CCR6-CXCR3-
T_H17	CD3+CD4+CCR7-CD45RA-CCR6+CXCR3-
$T_H1/17$	CD3+CD4+CCR7-CD45RA-CCR6+CXCR3+
CD4 T_{EMRA}	CD3+CD4+CCR7-CD45RA+
Treg	CD3+CD4+CCR4+CD25+CD127low
Tr1	CD3+CD4+CD49b+LAG-3+
T CD8 lymphocytes	CD3+CD8+
CD8 Naïve	CD3+CD8+CCR7+CD45RA+
CD8 Central Memory	CD3+CD8+CCR7+CD45RA-
CD8 Effector Memory	CD3+CD8+CCR7-CD45RA-
CD8 T_{EMRA}	CD3+CD8+CCR7-CD45RA+

EMRA: terminally differentiated effector memory cells re-expressing CD45RA

Supplemental Table 2. Down-modulated genes in vitD3-Tcell compared to mDC-Tcell. This file is provided in the attached USB drive (file name *Supplemental Table 2.xlsx*).

Supplemental Table 3. Differentially enriched Gene Ontology terms in vitD3-Tcell compared to mDC-Tcell. This file is provided in the attached USB drive (file name *Supplemental Table 3.xlsx*).

Discussion

The characterization of robust biomarkers of cell-based tolerance-inducing therapies has become one of the key pillars for the translation of tolDC into the clinics. On the one hand, they would enable to easily compare the results obtained in different laboratories all around the world, subsequently allowing the standardization of certain procedures and accelerating the development of these treatments. But more importantly, on the other hand, the definition of adequate biomarkers would also guarantee that tolDC have been properly differentiated. Or in other words, they would serve as a quality control that certifies that the cell product is completely safe before administering it into the patients. In this regard, the search of transcriptomic biomarkers of tolDC constitutes one of the most convenient approaches, since gene expression can be analyzed easily, reliably and faster than other conventional methods. In addition, transcriptomic biomarkers can also potentially provide functional information about the metabolic processes that are being modulated within the cells themselves.

In this regard, the study of the actual mechanisms of tolerance induction deployed by tolDC can also be developed not only on these cells, but also on those cells with which they are interacting. For this reason, in this thesis we wanted to cover the full transcriptomic study of both vitD3-tolDC —the cell product in which we focused our research, for reasons that will be discussed below— and autologous T $CD4^+$ lymphocytes that have interacted with them in an antigen-specific manner. With this approach, our objective was to identify several differentially expressed genes that could serve as transcriptomic biomarkers of both the generation of vitD3-tolDC and the response to the treatment with these cells.

1. Characterization and validation of biomarkers of vitD3-tolDC

1.1. The transcriptomic profile of vitD3-tolDC, dexa-tolDC and rapa-tolDC

Given the importance of the definition of new and robust biomarkers, our first objective was focused on the characterization of, if possible, general transcriptomic biomarkers of tolDC. For this reason, our first main objective was to study the transcriptomic profile of three of the most relevant tolDC-inducing protocols previously reported on the literature, vitD3-tolDC, dexa-tolDC and rapa-tolDC. The selection of these protocols was made especially considering that the use of vitamin D3 and dexamethasone for the induction of tolDC, both separately and in combination, is currently being tested in several finished and ongoing Phase I clinical trials around the world (126,127).

Our comparative transcriptomic study (*Chapter 1 of Results*), unfortunately, evidenced that there is not a single differentially expressed gene in common between vitD3-tolDC, dexa-tolDC an rapa-tolDC that could be considered as a potential biomarker of the generation of these cell products. Instead, several differentially expressed genes and functional pathways could be identified for each protocol separately, which in any case could still constitute, upon further validation, robust biomarkers for their use as quality controls in a clinical trial for their respective tolDC approaches. Even though this was something that we were expecting, based on the literature reviewed in a previous study (94), we could extract some interesting ideas.

First, despite all 3 protocols exhibited their own transcriptomic signature, we could also observe that, while vitD3-tolDC and dexa-tolDC shared several metabolic and immune-related pathways, on the other hand, rapa-tolDC presented a profile that was not only different but in many cases the opposite of the other two tolDC approaches. Second, and taking into account that all of the protocols considered in this study have previously shown strong immunoregulatory

properties *in vitro* (110), our results therefore support that the induction of immune tolerance can be achieved by several and different mechanisms. Thus, for instance, while on the one hand vitD3-tolDC seem to tackle autoreactivity by the induction of functional hyporesponsiveness (166), rapa-tolDC on the other hand seem to be more capable to induce Treg-mediated immunoregulatory responses. Consequently, this leads to our third point, which is that determined tolDC could be more suitable for the treatment of determined immune-mediated conditions and pathologies, and that this suitability could be determined based on the immune modulation that is preferred for each situation.

1.2. Selection of vitD3-tolDC as the tolerance-inducing cell product of study

After having analyzed the transcriptomic profile of three of the most reported tolDC-inducing products, and considering that we could not continue our research focused on the study of broad-use biomarkers of tolerance for different tolDC approaches since we could not find any, we decided to focus on vitD3-tolDC alone. Previous studies by our group had already compared the phenotype and functionality of human vitD3-tolDC, dexa-tolDC and rapa-tolDC (110), and evidenced that vitD3-tolDC exhibited a marked semi-mature phenotype, robust stability against maturation and, specially, the strongest ability to inhibit allogeneic responses of the three protocols. In addition, the solid immunoregulatory properties shown *in vivo* in the EAE murine model, being able to abrogate the course of the disease in an antigen-specific manner (158–160), rounded up our choice of vitD3-tolDC as our candidate cell product for the full study and validation of transcriptomic biomarkers.

1.3. Transcriptomic differences between healthy donors and MS patients

Our experiments have allowed us to validate the gene expression of *CYP24A1*, *MAP7* and *MUCL1* as transcriptomic biomarkers of the generation of vitD3-tolDC

differentiated from MS patients —and, potentially, also functionally-related in the case of the last two— (*Chapter 2 of Results*). However, several other promising candidates that were strongly differentially expressed in healthy donors were lost along the way when we analyzed them on MS patient samples. This fact made us notice that we were observing significant differences in the transcriptomic profile of healthy donors and MS patients.

Since the first characterizations —in which we studied the phenotype and the functionality of our vitD3-tolDC— we started noticing that, although sufficient, vitD3-tolDC differentiated from MS patient samples were not exhibiting a semi-mature phenotype nor a reduction of allogeneic proliferation as marked as those showed by healthy donors. Even though the monocytes of the two conditions come from slightly different samples —healthy donor monocytes come from buffy coat samples, while MS patient monocytes come from peripheral blood—, the differentiation process was essentially the same for both and should not be making such a difference. Conversely, after performing the first validations of our candidate biomarkers in healthy donor samples, we started experiencing that some of our most robust genes, such as *CAMP* or *CLEC5A* were being lost in the process, as we did not observe a sufficient differential expression in MS patient samples. Furthermore, the differential expression of *MAP7* and *MUCL1* themselves was not as marked either. However, the most striking difference came when we performed a second round of gene validations in order to enrich our initial analysis. Then, we noticed that *DHRS9*, the gene encoding the dehydrogenase/reductase 9, involved in the metabolism of retinoic acid (205), was strongly induced in healthy donors but not in MS patients. Interestingly, *DHRS9* has been reported as a robust biomarker of vtdx-tolDC and regulatory macrophages (170,206), and therefore, after its initial validation in our healthy donor samples, this result in MS patients was not expected.

Altogether, these findings made us realize that there might be some significant transcriptomic differences between vitD3-tolDC from healthy donors and MS patients. Subsequently, we re-studied our initial results with a new perspective, and we were able to observe several more relevant discrepancies. First, for *CYP24A1*, which was robustly validated in both cases, there was a significant difference in the expression of this gene in iDC between healthy donors and MS patients, compared to their respective mDC —*CYP24A1* was much more down-modulated in iDC from healthy donors—, which suggested that there were baseline differences in the transcriptomic profile of our control conditions already. On the other hand, we could also observe that, mostly, those genes that could not be validated in MS patients —but perfectly did on healthy donors— were functionally closer to *MUCL1* in our protein interaction network, while those that were closer to *MAP7* generally maintained an expression pattern similar to healthy donors. Since *MUCL1* encodes an extensively O-glycosylated protein at its serine and threonine repeated tandems (207), and the genes that are closer to it in our protein interaction analysis, such as *GALNT2*, *GALNT7* and *GALNT11*, precisely mediate glycosylation, we hypothesized that there might be a misfunction in the mechanisms of glycosylation of MS patients —something that has already been reported (208)—. In any case, this might only constitute one of the many alterations that probably exist among the transcriptomic profile of MS patients, since it does not seem likely that a defect in the glycosylation explains all the differences that we have observed. Consequently, further studies comparing the transcriptomic profile of vitD3-tolDC from healthy donors and MS patients should be conducted in order to improve the efficiency of the generation of these cells under pathologic conditions and, potentially, to boost their therapeutic potential.

1.4. Potential validation of our vitD3-tolDC biomarkers in other autoimmune diseases

In line with the previous section, the fact that only *MAP7*, *MUCL1* and *CYP24A1* could be validated as biomarkers of vitD3-tolDC in MS patients allow us to postulate them as candidates in MS samples, and MS only. In other words, based in our results, we do not have enough evidence to get to a conclusion regarding if:

1. *CYP24A1*, *MAP7* and/or *MUCL1* genes could also serve as biomarkers of vitD3-tolDC differentiated from patients with other autoimmune diseases, such as T1D or RA.

2. Those genes that could be validated in healthy donors only, such as *CAMP*, *CLEC5A* and/or *DHRS9*, could also serve —together with *CYP24A1*, *MAP7* and *MUCL1*— as biomarkers of vitD3-tolDC differentiated from patients with other autoimmune diseases.

Consequently, before extrapolating our results to other autoimmune diseases —or even allergies and transplantation rejection—, it would be necessary to repeat the whole validation process in vitD3-tolDC differentiated from cells of patients with the respective condition of interest.

1.5. *MUCL1* as a broad-use biomarker of tolDC generation

Since the differential expression of *MUCL1* gene could be validated not only in vitD3-tolDC but also in IL10-tolDC as a biomarker of the generation of these cell products, this finding opens the window to the possibility of *MUCL1* constituting a robust biomarker of the generation of, potentially, several other tolDC-inducing protocols. Naturally, this should be validated in as many protocols as possible in the near future. However, we can already preliminary discard the possibility of *MUCL1* being a biomarker of dexa-tolDC and/or rapa-tolDC, since its differential

expression in these two cell products was not observed in our microarray study. In any case, it is also worth stating that, due to the tendency to bias of microarray studies and other high throughput techniques, these results should also be confirmed first.

2. Transcriptomic study of treatment response to vitD3-tolDC

2.1. Standardization of a protocol to generate and co-culture autologous vitD3-tolDC and T CD4$^+$ cells

In the last part of this thesis (*Chapter 3 of Results*), we have developed a protocol for the generation, in parallel, of autologous antigen-loaded vitD3-tolDC and PBMC from healthy donors with one single sample of origin. Consequently, this procedure has allowed us to co-culture them and perform an exhaustive study of the antigen-specific interactions that are being developed between our tolDC and, specifically, T CD4$^+$ cells. Nevertheless, the versatility of our approach also allows us —and other authors— to make use of this experimental setup in order to focus on different cell subsets or specific interactions, if needed, in future studies. Furthermore, the cell sorting process by flow cytometry could be easily adjusted, by just changing the antibodies used, to isolate and study other or more specific T or B cell subsets from the co-cultures.

It is true, however, that due to the significant amount of sample of origin, we needed to perform our experiment with healthy donors. Specifically, the volume of whole blood required would have been so elevated in order to obtain enough monocytes and PBMC for the cell cultures that we were forced to use buffy coat samples from routine blood donations. Consequently, we were furthermore losing the disease-related pro-inflammatory context that patients might present, but this proved to be inevitable since trying to reproduce it introducing a pro-inflammatory

stimuli on the culture would be completely blind —since the specific cytokine environment is not known neither for any autoimmune disease in general, nor in particular—, would force us to focus on a determined pathology —thus losing the versatility of our approach, and still being forced to use TT as a peptide provided that our donors should not react no any specific auto-antigen— and probably too strong and unreliable —since it would potentially bias the downstream RNA-seq study—. For this reason, the conclusions reached in this part of the thesis should be considered as a solid starting point for future research, and any result must be validated first in the specific disease-related setup. In this regard, it is worth mentioning that using leukapheresis samples from autoimmunity patients might constitute a viable option in terms of culture yield —although it needs to be confirmed— but for us, at least for a first approach, it presented sufficient inconveniencies compared to buffy coat samples regarding sample obtention and manipulation to focus on the latter. In any case, the leukapheresis approach could be considered for future studies in order to validate —or characterize— treatment response biomarkers or different diseases-specific particularities. But even in that case, the protocol described in this thesis would still be applicable, despite some slight modifications in the first steps of sample manipulation.

2.2. Selection of the tetanus toxin peptide

Naturally, the selection of TT as our immunogenic stimulus was not trivial. First, for the reasons discussed above, since we decided to use healthy donor samples, we could not make use of any disease-related peptide —such as myelin or insulin— since we would not expect any reactivity against them. Consequently, we needed to find an alternative that presented enough functional similarities to make our experimental approach as close as possible to the immunopathological situation that takes place in patients. On the one hand, since many autoimmune diseases such as MS, T1D or RA are mediated by T $CD4^+$ cells, we needed to find a candidate that was presented via HLA class II. However, on the other hand, we

also needed that the peptide of election was predictably immunogenic in the majority of our healthy donors, if not in all of them. These two circumstances led us to choose TT for our study, provided that the peptides of this protein are typically presented via HLA class II —since it comes from an extracellular bacteria— and the majority of the European and North American population has been vaccinated against tetanus during their infancy and/or later on —thus ensuring that a good immune response against TT is to be expected—.

Furthermore, another consideration that we made was to introduce the whole TT protein in the culture on day 3, instead of directly providing specific immunogenic peptides after the full differentiation of the DC. The reason was that, in a preliminary study (data not shown), we observed that the immunogenicity of a determined TT peptide —previously described on the literature (209,210)—, loaded to the cells during 2 hours by the end of the 6th day of culture, was significantly lower to that induced by the whole protein. Consequently, we discarded the use of specific peptides and decided to use the whole TT protein. Our hypothesis was that, by adding the whole TT protein directly to the cell culture on day 3, 24 hours before the addition of the maturation stimuli and thus while DC are still in an immature status, cells capture the antigen, process it and present it on their HLA class II molecules in a more physiological manner, while also reducing the manipulation of the cells during their differentiation, thus optimizing the whole process. And as a matter of fact, our results evidence that our TT-loaded DC present the same phenotypic and functional properties of our "non-manipulated" DC, but with the ability to induce TT-specific responses over autologous T CD4^{+} cells, thus validating our experimental design.

2.3. Selection of the time point for the transcriptomic analysis

Due to logistic and economic limitations, for this study we were forced to choose one specific timepoint of co-culture of our autologous TT-loaded vitD3-tolDC and

T $CD4^+$ cells, and we did it after 5 days. Performing the experiment at 2 or more different timepoints would have significantly increased not only the cost of the process (since it would duplicate the number of conditions for the subsequent RNA-seq study), but more importantly the amount of blood samples needed, which was very limiting even for the setup presented in this thesis. Consequently, we needed to make a compromise, and we chose a timeframe that would yield us the best results possible under the considered conditions. For that reason, we based our decision on our previous experience with both allogeneic and, especially, autologous co-cultures. By day 5, we were already obtaining significant differences between T $CD4^+$ cells co-cultured with either mDC or vitD3-tolDC regarding phenotypic and functional evidences, as presented in *Chapter 3 of the Results*. Before that timepoint, we were not observing significant differences in the antigen-specific proliferation induced by our vitD3-tolDC (data not shown), and afterwards we were afraid that our T $CD4^+$ cells might be starting to become exhausted and experience cell death.

Furthermore, transcriptomic differences are normally induced earlier than they can be observed macroscopically, and therefore, we discarded the possibility to consider any timepoint past the 5^{th} day since these changes would probably be lost by that time. However, it is also true that they are likely induced before our final time of analysis. Consequently, we have to be aware that, by performing the RNA-seq at different timepoints, the results might have varied significantly, something that can be addressed in future and more detailed studies in order to complement the information provided in this thesis. In any case, we are also convinced that we have enough evidence, especially based on our results, to conclude that the timepoint selected has proved itself correct, since we have been able to extract a handful of useful information that has allowed us to specifically depict some of the transcriptomic changes that have been induced by our antigen-specific tolDC.

2.4. Adaptability of the protocol to other immune-mediated disorders

As stated above, and even though our research group is focused on MS, our idea from the beginning was to develop a versatile, antigen-specific protocol for the co-culture of vitD3-tolDC and T cells, that could be easily adapted for the study of different autoimmune diseases and other immune-mediated disorders. The only way to study the viability of this approach, and to keep it as flexible as possible, was to design our protocol using samples from healthy donors and TT as the immunogenic peptide. This way, our protocol can be easily adapted to study, potentially, any autoimmune disease or any other immune-mediated disorder —such as allergies— mediated by a known antigen —or pool of antigens— by just replacing the peptide and using sample cells proceeding from patients with the particular disease of interest. Consequently, the potential of our experimental setup is almost unlimited, and could constitute a very interesting and solid starting point for future research.

2.5. Induction of an antigen-specific transcriptomic hyporesponsiveness mediated by vitD3-tolDC and identification of treatment response biomarkers

Our results indicate that TT-loaded vitD3-tolDC are inducing an antigen-specific hyporesponsiveness over autologous T $CD4^+$ cells upon their interaction, thus inducing immune tolerance without the induction of Treg or Tr1 subpopulations. In this regard, and as discussed in *Chapter 3 of the Results*, we are aware that, for the induction of a Treg-mediated response, longer co-culture times or repeated challenges with TT-loaded vitD3-tolDC might be required, although that should be addressed properly in a different and specific study.

However, since the transcriptomic profile of T cells is so remarkedly repressed due to this hyporesponsiveness induced by vitD3-tolDC —down-modulating important immune-related pathways and cell cycle processes—, the identification

of potential candidate biomarkers became harder, since there were very few genes that were differentially up-modulated. And yet, the induction of the expression of *JUNB* might constitute a putative biomarker of the modulation mediated by these cells over $CD4^+$ T cells, since this gene has been reported to be decisive in sustaining Treg suppressive function in experimental models, although there is some controversy since it is also apparently involved in the induction and maintenance of IL-23-related pathogenicity of T_H17 cells. In any case, an adequate validation of these findings should be conducted before reaching any conclusion, preferably under autoimmunity circumstances for its full translationality to clinical trials, in a similar fashion to that described in *Chapter 2 of the Results*. However, if proven true, the analysis of the expression of *JUNB* gene in peripheral blood T cells of patients treated with vitD3-tolDC in a clinical trial for a determined autoimmune disease could constitute a direct indicator of the adequate effect of these cells.

3. General considerations and concluding remarks

The results presented in this thesis describe the whole process of screening, selection and validation of transcriptomic biomarkers of the generation —and functionality— of vitD3-tolDC, which constitute a crucial process for the acceleration and final translation of tolDC-based therapies for autoimmune diseases into the clinic. Furthermore, in the last part, we also present the first steps of the development and standardization of protocols, screening and selection of candidate biomarkers of response to the treatment with, precisely, those same vitD3-tolDC, which in this case could allow to monitor the efficacy of these cells after their administration into patients in a clinical trial. Consequently, this thesis serves as a bridge of tolDC-based therapies from the bench to the bedside.

Nowadays, tolDC-based therapies have come a long way, and are currently being tested in clinical trials, in which their safety has been already widely demonstrated, even though their efficacy will not be able to be assessed until the development of phase II clinical trials. Definitely, tolDC generation still presents some issues to overcome, such as an elevated price and a complex cell culture process, especially compared to other approaches such as peptide-encapsulating liposomes and nanoparticles (211). However, tolDC have demonstrated very promising results in terms of efficacy in experimental models, and with the definition of adequate biomarkers —such as those validated in this thesis, that allow to reduce the cost of the production by making it more efficient and easier to characterize—, tolDC-based therapies present one of the most bright perspectives to effectively treat —and maybe even cure— immune-mediated diseases.

Conclusions

In this thesis, we performed an exhaustive study of the transcriptomic profile of vitD3-tolDC, which has allowed us to identify and validate the differential expression of *CYP24A1*, *MAP7* and *MUCL1* genes as biomarkers of the generation of these cells —both in healthy donors and MS patients—. In addition, we found that *MAP7* and *MUCL1* have a potential implication in the tolerogenic functionality of vitD3-tolDC. Subsequently, we evidenced that these cells are modulating the transcriptomic profile of autologous T $CD4^+$ cells towards a functional hyporesponsiveness upon their antigen-specific interaction. This work has allowed us to reach to the following conclusions:

1. Comparing the transcriptomic profile of dexa-tolDC, rapa-tolDC and vitD3-tolDC, we could not identify any gene as a common candidate biomarker of the generation of these tolDC. However, several genes could be identified as candidate biomarkers for each of these protocols separately.

2. The differential up-modulation of the expression of *CYP24A1*, *MAP7* and *MUCL1* genes in vitD3-tolDC, compared to mature and immature control conditions, makes them robust transcriptomic biomarkers of the generation of these cells, both in healthy donors and MS patients. Furthermore, these biomarkers possess a remarkable potential of translationality, since their use can be immediately tested in ongoing clinical trials with tolDC.

- The functionality of the proteins encoded by *MAP7* and *MUCL1* genes — but not by *CYP24A1*— seems to be closely related to important immune-related pathways, which could explain, in part, the tolerogenic properties of vitD3-tolDC and their role as biomarkers of these cells.

- The up-regulation of *MUCL1* gene expression can also be used as a biomarker of the generation of IL10-tolDC, which opens the possibility for

this gene to become a broad-use biomarker of the generation of other tolDC-inducing protocols.

- The transcriptomic differences found in vitD3-tolDC between healthy donors and MS patients suggest that significant metabolic and regulatory pathways might be affected in the latter. These alterations could potentially be involved in the loss of tolerance that characterize MS pathogenesis, but require further research. These differences could be considered in future studies to improve the efficiency of the generation of vitD3-tolDC in MS patients and, potentially, to boost their therapeutic potential.

3. Our experimental setting has evidenced that vitD3-tolDC modulate the transcriptomic profile of autologous T $CD4^+$ cells towards a functional hyporesponsiveness upon their antigen-specific interaction, causing the down-modulation of important immune-related pathways and cell cycle processes. Furthermore, this modulation could potentially be mediated through the induction of JunB transcription factor. Consequently, this gene could constitute a biomarker of the response to the treatment with vitD3-tolDC.

- The protocol standardized for this study, developed using cells from healthy donors, is versatile and can be easily adapted to study multiple timepoints and cell subpopulations in, virtually, any immune-mediated disorder with known immunodominant antigenic peptides.

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Annex

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REVIEW
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Searching for the Transcriptomic Signature of Immune Tolerance Induction—Biomarkers of Safety and Functionality for Tolerogenic Dendritic Cells and Regulatory Macrophages

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The last years have witnessed a breakthrough in the development of cell-based tolerance-inducing cell therapies for the treatment of autoimmune diseases and solid-organ transplantation. Indeed, the use of tolerogenic dendritic cells (tolDC) and regulatory macrophages (Mreg) is currently being tested in Phase I and Phase II clinical trials worldwide, with the aim of finding an effective therapy able to abrogate the inflammatory processes causing these pathologies without compromising the protective immunity of the patients. However, there exists a wide variety of different protocols to generate human tolDC and Mreg and, consequently, the characteristics of each product are heterogeneous. For this reason, the identification of biomarkers able to define their functionality (tolerogenicity) is of great relevance, on the one hand, to guarantee the safety of tolDC and Mreg before administration and, on the other hand, to compare the results between different cell products and laboratories. In this article, we perform an exhaustive review of protocols generating human tolDC and Mreg in the literature, aiming to elucidate if there are any common transcriptomic signature or potential biomarkers of tolerogenicity among the different approaches. However, and although several effectors seem to be induced in common in some of the most reported protocols to generate both tolDC or Mreg, the transcriptomic profile of these cellular products strongly varies depending on the approach used to generate them.

Keywords: biomarkers, tolerogenic dendritic cells, regulatory macrophages, tolerance mechanisms, genetic markers, immune tolerance, regulatory dendritic cells

INTRODUCTION

The immune system develops complex and sophisticated reactions, which are able to differentiate between what is dangerous and what is innocuous for the host (1), thus specifically attacking pathogens and other potentially dangerous antigens while remaining unresponsive against whether non-dangerous or self-molecules. This balance between immunogenicity and tolerance is orchestrated in the periphery by professional antigen-presenting cells (APC), such as dendritic

cells (DC) and, in a lesser extent, macrophages, which direct the immune response depending on the characteristics of the antigen and the cytokine milieu they encounter (2). Briefly, DC are in charge of both the initiation of the adaptive immune response and the control or abrogation of the inflammatory processes once the immunogenic antigen has been cleared. For this regulatory role, DC can deploy several mechanisms such as the induction of anergy or deletion of the activated immune cells, as well as, the activation of regulatory T cells (Treg) in an antigen-specific manner. Therefore, since DC have the potential to both stimulate or inhibit immune responses, the role of these cells in the immune system is complex and bidirectional (3–6). By their part, macrophages also play a minor role as APC, developing some of the regulatory processes mentioned above, although their main function consists in the clearance of cell debris, pathogens and other molecules after the immune response has concluded (7).

Eventually, the immune homeostasis can be disturbed due to a malfunction of the immune system, thus setting up immunogenic responses toward self-antigens from specific tissues and organs, which may lead to the development of autoimmune diseases. In the last years, there has been a significant progress in the knowledge of the mechanisms of immune regulation mediated by APC. Consequently, the development of novel autologous cell therapies capable of re-educating the immune system toward a tolerogenic profile has been postulated as a promising therapeutic alternative to conventional, unspecific immunomodulatory and immunosuppresive drugs, which often present severe side effects and a relatively poor efficacy (8).

So far, a wide variety of *in vitro* protocols has been established for the generation of immune tolerance-inducing DC—or tolerogenic DC (tolDC)—and regulatory macrophages (Mreg). Moreover, some of these cell products have been successfully translated from the bench to the bedside in the last few years, being tested in Phase I clinical trials in patients with autoimmune diseases—such as type 1 diabetes, rheumatoid arthritis or Crohn's disease—as well as, kidney transplantation, demonstrating in all cases that tolerogenic cell therapies are safe and well tolerated, without relevant side effects (9–13). In addition, many other studies are currently ongoing (14). These results, therefore, support the use of tolDC and Mreg as novel and safe approaches aiming to restore the immune tolerance. However, given the wide variety of protocols available for the generation of these cell products, finding objective and measurable biomarkers to characterize tolDC and Mreg and compare their characteristics between different approaches and laboratories remains one of the main obstacles to overcome.

In this context, the identification of differentially expressed (up- or down-modulated) genes in tolDC and/or Mreg constitutes one of the best tools for the definition of biomarkers of tolerogenicity, since they can provide more robust and reliable information compared to conventional methods such as phenotypical characterization by flow cytometry (with high variability) or functional studies (which require several days), as it will be further discussed below. In the case of tolDC and Mreg, these biomarkers would be able to guarantee the proper generation of the therapeutic cell product, ensuring that the cells are both safe and tolerogenic. Therefore, the ideal biomarker would be one that is selectively overexpressed or repressed in the tolerance-inducing cell product compared to its respective mature immunogenic steady-state control condition.

With that purpose, here we review the main human tolDC- and Mreg-inducing protocols reported on the literature. We specifically focus on the different agents and drugs used to generate these cell products, in order to define a catalog of genes and/or proteins induced by these stimuli and thus try to find potential and universal biomarkers of tolDC and Mreg.

TOLEROGENIC DENDRITIC CELLS AS KEY TOLERANCE-INDUCING PLAYERS AND THEIR TRANSCRIPTOMIC SIGNATURE

DC constitute an heterogeneous subset that includes classical, plasmacytoid, and monocyte-derived myeloid DC (15). In their immature state (iDC), DC are mainly antigen-capturing cells with tolerance-inducing functionality. However, once in the presence of a pro-inflammatory stimulus such as TNF-α, lipopolysaccharide (LPS) or IL-1β, they can differentiate into immunogenic mature DC (mDC). By their part, mDC are capable of priming and activating T cells to initiate an immune response after providing the three required activation signals of the immune synapsis once a specific and immunogenic antigen has been recognized. During this maturation process, an upregulation of the expression of human leukocyte antigen (HLA) molecules, as well as, of other costimulatory molecules such as CD40, CD80, CD83, or CD86 takes place, along with an increase in the production of IL-12 and other proinflammatory cytokines (2, 3, 8).

However, a third type of DC has been defined in the last years, combining immune tolerance-inducing properties with a stability against maturation stimuli, called tolerogenic DC (tolDC). It is not clear whether tolDC constitute a different DC subset by themselves or if they are mere maturation-impaired iDC, although there seems to be a consensus about which features they have to possess in order to develop their regulatory function. Thus, tolDC usually present one or more of these characteristics: a semi-mature phenotype, with low expression of co-stimulatory (CD80, CD86, CD83) and HLA molecules, a maintained CCR7-dependant migratory ability toward the secondary lymphoid organs, an increased IL-10 production accompanied by low or null IL-12 and IFN-γ secretion, a lowered T cell-proliferation priming capability, potential to induce Treg and stability against maturation in front of a proinflammatory milieu. Specifically the latter, which has been described in the majority of these studies, probably constitutes the most important feature among all of them (16–18).

Importantly, tolDC can be differentiated *in vitro* from peripheral blood monocytes in the presence of a determined tolerogenic-inducing agent. Indeed, a wide variety of protocols have emerged in the last 20 years describing the induction of tolDC with several stimuli, such as anti-inflammatory cytokines—IL-10 (19, 20), TGF-β (20, 21)—, pharmacological agents and immunosuppressant compounds—rapamycin (20, 22, 23), different corticosteroids (24), dexamethasone (20, 23, 25, 26),

vitamin D3 (20, 23, 27) or a combination of both dexamethasone and vitamin D3 (28)—, several drugs and blocking molecules—aspirin (29), mitomycin C (30), the NF-κB inhibitor BAY11-7082 (11)—and other strategies, such as genetic engineering for the selective repression or induction of key molecules and pathways (10, 31), among many others further discussed below. Generally, most of these protocols share several features in common, such as the differentiation of monocytes in the presence of GM-CSF and IL-4, as well as, the addition of a maturation stimulus (which usually includes different combinations of LPS, monophosphoryl lipid A, TNF-α, IL-1β, prostaglandin E2, and/or IL-6), with few exceptions.

Either if we assume tolDC are a specific DC subset *per se* or just a modified state of iDC, there must be some footprint left by this condition. At the transcriptomic level, as already hinted, some obvious downregulated candidates would be the genes encoding co-stimulatory molecules or pro-inflammatory cytokines. However, those features would be shared with steady state iDC, thus making them useless in terms of differentially characterizing tolDC. In fact, ideally, a comparison against both immature and immunogenic control conditions should be taken into account in the search of specific genetic biomarkers, something that has not been considered in the majority of the reviewed studies. An ideal candidate should be, furthermore, clearly differentiated by a matter of full induction or repression, as a slight increase/decrease of its expression could be ambiguous and would always require the use of robust controls, which is not always possible.

Consequently, many research groups have been working on the identification of genetic markers for human tolDC, and deep transcriptomic studies are becoming more frequent each year. However, and although several studies have described a pool of markers for some specific tolerogenic cell products, common genetic biomarkers have not been found yet.

Glucocorticoids and Immunomodulatory Molecules in the Generation of Tolerogenic Dendritic Cells

Since mDC are immunogenic cells, or, in other words, promoters of inflammatory responses, the use of corticosteroids and other immunosuppressant drugs has been widely reported for the generation of tolDC. Rapamycin (20, 22, 23, 32, 33) and a combination of hydrocortisone and clobetasol-17-propionate (24), but especially dexamethasone (20, 23, 25, 26, 32–45), have all been used for the generation of tolDC. As a glucocorticoid-induced molecule, the expression of the gene encoding the anti-inflammatory mediator known as glucocorticoid-induced leucine zipper (GILZ) (46) has been reported strongly up-modulated in many of these studies, thus making it a good albeit predictable marker for tolDC generated with this kind of immunomodulatory agents. Furthermore, other molecules related with the complement and the immune system have been found commonly up- or down-modulated in several of these tolDC protocols, such as the anti-inflammatory cytokine IL-10 (up-regulated), the pro-inflammatory cytokine IL-12 or the fascin 1-encoding gene *FSCN1* (both down-modulated), which are common features that define these cells (32). The full list of differentially expressed molecules reported for each of the abovementioned protocols and their respective references can be found on **Table 1**.

Dexamethasone-induced tolDC (dexa-tolDC) are one of the most widely implemented approaches worldwide for the generation of human tolDC, and are being or have been tested on clinical trials for the treatment of numerous autoimmune diseases, such as Crohn's disease (http://www.clinicaltrials.gov, NCT02622763) (12), rheumatoid arthritis (http://www.clinicaltrials.gov, NCT03337165; NCT03337165) and both multiple sclerosis or neuromyelitis optica (http://www.clinicaltrials.gov, NCT02283671). Several studies have reported the differential up-modulation of genes *C1QA* (encoding the C1q complement protein, chain A) (34, 35), *CD163* (34, 35), *GILZ* (32, 35, 36), *MERTK* (encoding the MER Proto-Oncogene Tyrosine Kinase, also used as a marker in the abovementioned clinical trial for Crohn's disease) (12, 26, 35) and *ZBTB16* (encoding zinc finger and BTB domain containing protein 16) (34, 35) in dexa-tolDC, thus making them the most relevant candidate biomarkers for this specific protocol. Additionally, the differential expression of *IDO1*, the gene encoding the indoleamine 2,3-dioxygenase —a molecule widely related to the induction of immune tolerance (65)—, has also been reported in dexa-tolDC. However, there is some controversy in this regard, as it has been found both up- (35) and down-modulated (32) in different studies. Besides, other induced genes described in studies using dexamethasone, relevant by their role in the modulation and mediation of different mechanisms of the immune system—with their respective encoded proteins in brackets—, are *CD300LF* (CD300 molecule-like, family member F), *F13A* (coagulation factor XIII A), *FCGR2B* (Fc fragment of IgG receptor IIb), *FCGR3A* (Fc fragment of IgG receptor IIIa), *MRC1* (mannose receptor C-type 1), and *STAB1* (stabilin 1), as well as, other non-immune related genes like *FTL* (ferritin light chain), *IMDH2* (inosine monophosphate dehydrogenase 2), and *SOD2* (superoxide dismutase 2). Furthermore, the combination of dexamethasone with rosiglitazone has also been reported for the generation of tolDC, highlighting the induction of *FABP4* (fatty acid-binding protein 4) with this protocol, but specially also of *GILZ* gene (47).

The generation of human rapamycin-modulated tolDC (rapa-tolDC) is the second most reported protocol of this group of pharmacological and immunomodulatory agents. However, transcriptomic studies in tolDC generated with this strong immunosuppressant drug are not as predominant as those induced with dexamethasone. Yet, several genes have been postulated as candidate biomarkers for rapa-tolDC, both immune-related—*ANXA1* (annexin 1), *C1QC*, *CTSC* (cathepsin C) and *GILZ*—and non-immune-related —*GPX1* (Glutathione Peroxidase 1), *IMDH2*, *OSF1* (pleiotrophin) and *TPP1* (tripeptidyl peptidase 1)—. Interestingly, all these genes have also been described in common with dexa-tolDC (32).

Additionally, the immunostimulant TLR3 ligand polyinosinic:polycytidylic acid (poly I:C) has also been reported to induce human tolDC, although in an inconsistent and poorly efficient manner. Nevertheless, the differential up-modulation

TABLE 1 | Differentially up- and down-modulated genes and proteins in the most reported human tolDC-inducing protocols.

	Protocol	Type	Up-modulated molecules	Down-modulated molecules	References
tolDC	Dexamethasone	Gene	***ANXA1, C1QA, C1QC, C1QTNF1, C3AR1, CCL17, CD163, CD300LF, CD32, CFH, CLIC2, CSGALNACT1, CTSC, DCR3, EP2, EP3, F13A, FCGR2A, FCGR2B, FKBP5, FOXO3, FPR1, GILZ, GPX1, IDO1, IL10,*** *IL12A, IL27B,* ***IMDH2, JAG1, MERTK, MRC1, MT1, NCF1, OSF1, P2RY14, SLC39A8, SOD2, STAB1, TPP1, ZBTB16***	***CCL22, CD1C, FCER1A, IDO1,*** *IL12B,* ***LAMP3, MMP12, ZNF366***	(26, 32, 34–36, 38, 39, 41)
		Protein	**CYP1B1, DAB2, DPYD, FCER1G, FCGR3A, FTL, GCLC, IVNS1ABP, LRRC25, MCTP1,** MERTK**, NUDT16, PDCD4, PECAM1, RNASE6, RNASET2, SIGLEC5, SLCO2B1**	**FSCN1**	(12, 34, 37)
		miRNA	*miR-328-5P, miR-638, miR-663, miR-762, miR-1275, miR-1228, miR-1909*	*miR-142-5p*	(40)
	Dexamethasone + rosiglitazone	Gene	***FABP4, GILZ***		(47)
	Dexamethasone + vitamin D2	Protein	**ERK1/2, IDO, JNK/SAPK, mTOR, p38 MAPK, STAT3**		(48)
	Dexamethasone + vitamin D3	Gene	*ACADM, ACADVL, ACO1, ACO2, ACOX2, ACSS1, ALDH2, ATP5G3, ATP5J, ATP5O, BLVRB, C1orf162, C1QA, CCR5,* ***CD14****, CD209, CD274,* ***CD52****, CLIC1, COX11, COX6A1, COX7A2, CTSB, CTSD, CTSH, CYC1, DHRS9, EIF3B, EIF3C, EIF3CL, EIF4A3, FBP1, FCGR2B, FCGR3A, FN1, FTH1, FTL, G6PD, GAPDH, IDH3A, IDH3B,* ***ILT3****, LDHB, LILRB4, MATK, MCEMP1, MDH2, ME1, ME3, NDUFB9, NDUFS1, NDUFS8, NOS3, PCK2, PDHA1, PDXK, PIK3R1, PKM2, PNP, PRDX3, PTPN6, RAC2, RGCC, RPS12, RPS19, RPS21, RPS6KA1, RPS6KA2, SDHA, SLC11A1, SLC27A5, SLC2A1, SLC2A5, SNCA, SUCLG1, SUCLG2, TCEB1,* ***TGFB1****, TP53, TPI1, UQCR10, UQCR11, UQCRB, UQCRC1*	*ACTB, ADAM12, ADAM19, ANKRD33B, AOC1,* ***CD25****,* ***CD40****,* ***CD80****,* ***CD83****,* ***CD86****, DPYSL2, EHF,* ***FSCN1****, GPR157, ICOSLG, IKZF1, IKZF4, IL12B, IL2RA, ORMDL3, PIK3CG, PLEKHA5, PPP1R16B, PTPN2, SH2B3, TYK2, WDR1*	(49–51)
		Protein	ADK, AKR1A1, ALDH2, ALDOA, ATP5H, ECHS1, FBP1, FTL, G6PD, GPD2, GALK, MPDH2, PGAM, PGM1, PKM2, PNP, PRDX6, TALDO1, TKT, TPI1	DPYSL2, ENO1, FSCN1, HSPD1, PDIA3	(37)
	Hepatocyte growth factor	Gene	***IL10***		(52)
	IFN-γ	Gene		***IRF4, RELB, IL12p40***	(53, 54)
	IL-10	Gene	***ANXA1, C1QC, CTSB, CTSC, CTSL, F13A, FTH1, GILZ, HLA-DOB, IL8, LILRB3, MRC1, STAB1, THBS1, TPP1***	***CD74, LAMP3***	(32, 41, 55)
	IL-10 + IL-6	Gene	***CTSB, CTSL, FTH1, HLA-DOB, IL-8, THBS1***	***CD74***	(55)
	Poly I:C	Gene	***IDO1, PDL1***		(56, 57)
	Rapamycin	Gene	***ANXA1, C1QC, CTSC, GILZ, GPX1, IMDH2, OSF1, TPP1***	***RALDH1***	(32)
	Retinoic acid	Gene	***ALDH1A1, ALDH1A2, CD141, GARP***		(58, 59)
	TGF-β	Gene	***ANXA1, CTSL, CXCL1, CXCR3, FTH1, HLA-DOB, IL8, LILRB3, THBS1***	***CD74, STAB1***	(32, 55)
	TX527 (vitamin D3 analog)	Protein	ACADVL, ACO2, ACOX1, ATP5A1, CTSD, CTSS, COPG, FBP1, G6PD, HADHA, IDH3A, MnSOD, OGDH, PCK2, PKM2, PRX3, PTM, UQCRFS1	ACAT1, ARCN1, DLD, PA28beta, PTM, RabGDI	(60)
	Vitamin D3	Gene	***ALOX5, ATP5A1, CAMP, CCL22, CD14, CD300LF, CMYC, CYP24, CYP24A1, CYP27B1, GILZ, GLUT3, HK3, ILT3, IRF8, LDHA, LGALS9, PDHA1, PFKFB4, PIK3CG, PRKAA1, THBD, VDR***	***CD1A, CD1C, CD1E, CD36, CD80, F13A, IER3, IRF4, LAMP3***	(32, 36, 41, 61–63)
		Protein	**AKT, FTL, GSK-3b, mTOR**	**FSCN1, SOD2**	(37)
		miRNA	*miR-378*		(64)

Genes validated by qPCR or proteins validated by western blot are shown in bold.

of both *IDO1* and *PD-L1*, two genes involved in the induction and maintenance of immune tolerance, has been confirmed by quantitative PCR for these cells (56, 57). As for tolDC induced with hydrocortisone and clobetasol-17-propionate, no transcriptomic biomarkers have been reported.

Vitamins A and D Modulate the Transcriptomic Footprint of Tolerogenic Dendritic Cells

As reviewed by Mora et al. (66), vitamins A and D exert important immunomodulatory properties. While vitamin A and specifically its metabolite, retinoic acid, have been reported to have an influence in T cell differentiation and proliferation, as well as, Treg induction, vitamin D plays an important role as an immunoregulatory agent in the inhibition of T cell proliferation and the reduction of IL-2 and IFN-γ secretion. Furthermore, the absence or low levels of vitamin D in the organism has been widely linked to an increase in the incidence of autoimmune diseases.

The tolerogenic-inducing properties of 1,25-dihydroxycholecalciferol, the active form of vitamin D3, over DC (vitD3-tolDC) have been widely reported *in vitro* in many studies performed with murine (67–70) and even cattle cells (71), although we will only focus on biomarkers of human vitD3-tolDC (20, 23, 27, 32, 33, 36, 37, 41, 44, 61–64, 72–74). As a measurement of its relevance, such is the importance of vitD3-tolDC in the field of tolerogenic cell products that even two clinical trials are already ongoing for the treatment of multiple sclerosis using this cell product in Badalona, Spain (http://www.clinicaltrials.gov, NCT02903537) and in Antwerp, Belgium (http://www.clinicaltrials.gov, NCT02618902). Several transcriptomic and proteomic pre-clinical studies in human vitD3-tolDC have evidenced several genes and proteins strongly induced with this approach, including immune-related molecules—*CCL22* (62, 63), *ILT3* (immunoglobulin-like transcript 3) (36), *CD300LF* (62) or *GILZ* (32), these last two in common with dexa-tolDC—and oxidative metabolism enzymes and regulators—*GLUT3* (glucose transporter 3), *LDHA* (lactate dehydrogenase A), *mTOR* (mammalian target of rapamycin), *PDHA1* (pyruvate dehydrogenase E1, subunit alpha 1) or *PFKFB4* (fructose-2,6-bisphosphatase) (63)—, as well as direct targets of the response to vitamin D3 through the interaction with its receptor, like *CYP24A1* (cytochrome P450, family 24, subfamily A, member 1) (41, 61–63) and of course *VDR* (vitamin D receptor) (41). By their part, the repression of several co-stimulatory, pro-inflammatory, and antigen presenting genes and molecules like *CD1A*, *CD1C*, *CD80*, *FSCN1* or the transcription factor *IRF4* has been reported at the transcriptomic and proteomic levels (37, 62). Additionally, a synthetic structural analog of vitamin D3, TX527, has also been used for the induction of human tolDC (60). However, and although the up-modulation of the ATP synthase F1 subunit alpha-encoding gene (*ATP5A1*) was reported in common with vitD3-tolDC, the transcriptomic resemblance was more relevant with tolDC induced with a combination of dexamethasone and vitamin D3, a strategy that will be further discussed in the next section. Nevertheless, some of these induced molecules consist of mostly metabolic-related genes—*ACADVL* (Acyl-CoA dehydrogenase very long chain), *ACO2* (aconitase 2), *FBP1* (fructose bisphosphatase 1), *IDH3A* (isocitrate dehydrogenase 3, subunit alpha), *PCK2* (phosphoenolpyruvate carboxykinase 2) and *PKM2* (pyruvate kinase M2)—and *CTSD*, encoding the protease cathepsin D (37, 49, 50).

The use of vitamin A-derived molecules like retinoic acid, however, has not been so widely reported for the generation of human tolDC and only the selective up-regulation of *ALDH1A1* and *ALDH1A2* genes, encoding the aldehyde dehydrogenase 1 family members A1 and A2—involved the metabolism of retinoic acid—has been reported, as well as, the induction of *CD141* and *GARP* genes (58, 59). Other differentially expressed genes induced by the protocols mentioned in this section are shown in **Table 1**.

The Synergic Effect of Dexamethasone and Vitamin D

Since dexamethasone and vitamin D treatments alone are able to generate tolDC, the combination of both of them is expected to induce synergic effects that would strengthen the tolerogenic functionality of these cells. Consequently, the simultaneous use of dexamethasone and vitamin D3, or vitamin D2 in a few cases (48, 75), has become one of the most widely reported human tolDC-generating protocols *in vitro*. Indeed, these cells have even reached the clinical phase for the treatment of rheumatoid arthritis, with successful results regarding the safety and tolerability of the product (http://www.clinicaltrials.gov, NCT01352858) (13).

As expected, the genetic signature of dexamethasone + vitamin D-induced tolDC (vtdx-tolDC) reported in pre-clinical studies partially overlaps with that reported for each or both of these treatments alone to generate human dexa- and vitD3-tolDC. In fact, the analysis of the reported data for these protocols showed that *C1QA*, *FCGR2B*, *FCGR3A* and *IDO1* genes were found induced in common with dexa-tolDC (34, 35, 38, 48, 50) and *CD14*, *ILT3*, *mTOR* and *PDHA1* were shared with vitD3-tolDC (36, 48–50, 62, 63). Nevertheless, our analysis evidenced that the up-regulation of *FTL* and the suppression of *FCSN1* genes were the only genetic modulations in common between these three protocols (34, 37, 50, 63). Interestingly, the function of the proteins encoded by all these genes is strongly related to the modulation of the immune system. Surprisingly, however, there was a pool of genes that were only described for vtdx-tolDC but not for either dexa-tolDC nor vitD3-tolDC, such as *CTSB*, *DHRS9* (dehydrogenase/reductase 9), *FTH1* (ferritin heavy chain 1), *RGCC* (regulator of cell cycle), *SLC11A1* (solute carrier family 11 member 1), *TBET* or *TGFB1* (49–51). Indeed, after our study, it is worth noting that out of 64 up-modulated genes and/or proteins reported for dexa-tolDC, 29 genes for vitD3-tolDC and 102 genes for vtdx-tolDC, only 4 genes could be found in common between vtdx-tolDC and each treatment separately, as shown in the Venn diagram in **Figure 1**. The chances are, however, that many of these genes could simply not be detected or were overlooked in the validation process of the separated

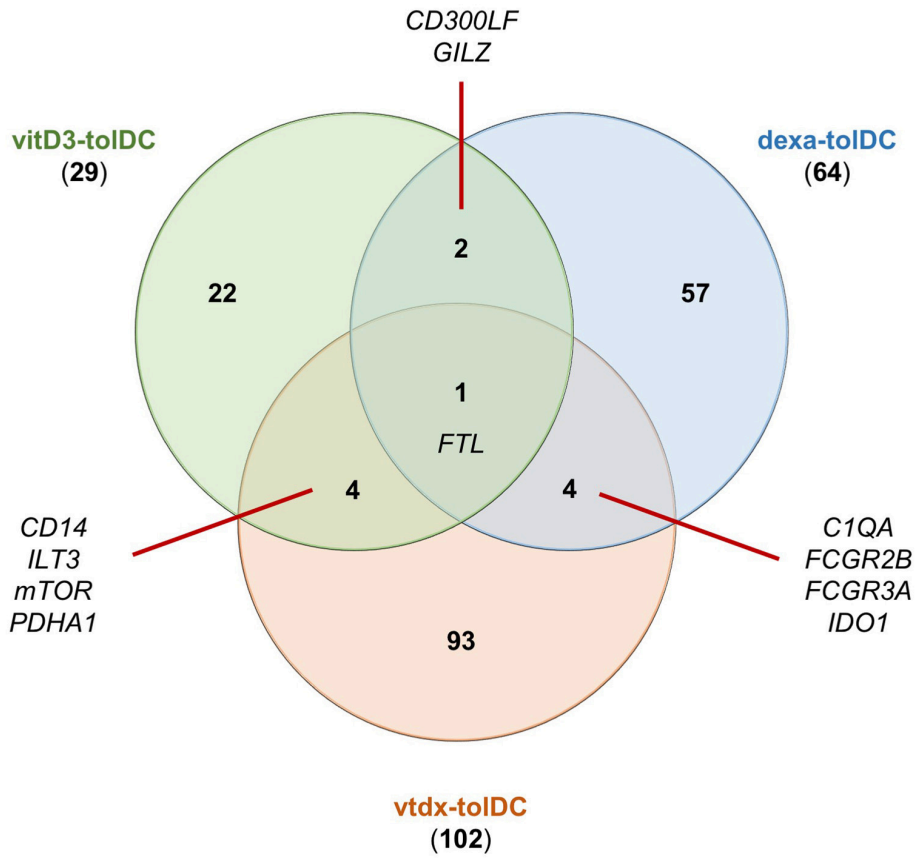

FIGURE 1 | Common up-modulated genes in tolDC induced with either vitamin D3, dexamethasone or the combination of both. The numbers in the Venn diagram indicate the number of reportedly induced genes for each condition alone or in combination with one or both of the others. Dexa-tolDC: dexamethasone-induced tolDC; vitD3-tolDC: vitamin D3-induced tolDC; vtdx-tolDC: dexamethasone + vitamin D3-induced tolDC.

protocols due to intrinsic limitations of the methodologies used, as it is known that biases frequently appear in high throughput transcriptomic and proteomic techniques. For this same reason, for instance, some already mentioned immune-related and metabolic genes were detected simultaneously induced in vtdx-tolDC and tolDC generated in the presence of the vitamin D3 analog TX527—*ACADVL*, *ACO2*, *CTSD*, *FBP1*, *G6PD* (glucose-6-phosphate dehydrogenase), *IDH3A*, *PCK2*, *PKM2*)—(37, 49, 50, 60). Although the down-modulation of genes is not as relevant toward the identification of transcriptomic biomarkers, it is nonetheless worth noting that the *FSCN1* gene has been found repressed in vtdx-tolDC, dexa-tolDC, and vitD3-tolDC at the same time (37, 50). **Table 1** shows a complete list of the differentially expressed genes and proteins reported in protocols using a combination of dexamethasone and vitamin D derivates.

The Effect of Cytokines and Growth Factors in the Induction of Tolerogenic Dendritic Cells

Many different kinds of cytokines have been used for the induction of human tolDC, ranging from anti-inflammatory—IL-10 (19, 20, 32, 33, 41, 55, 76–78), TGF-β (21, 32, 33, 55, 79) or both (80)—to even immunostimulatory molecules—IFN-γ (53, 54) or a combination of IL-6 with IL-10 (55)—, but also several growth factors—hepatocyte growth factor (52) and low-doses of GM-CSF alone (81).

As previously mentioned, the secretion of IL-10 is one of the most sought features of tolDC due to its anti-inflammatory and regulatory properties. Consequently, the generation of tolDC in the presence of exogenous IL-10 (IL10-tolDC) constitutes one of the most implemented protocols for the generation of this type of regulatory cell products. In fact, many of the genes and molecules already cited for other protocols, with immune or metabolic involvement, have also been found induced in IL10-tolDC, such as, *ANXA1*, *C1QC*, *CTSB*, *CTSC*, *CTSL* (cathepsin L), *F13A*, *FTH1*, *HLA-DOB*, *IL-8*, *LILRB3* (leukocyte immunoglobulin-like receptor B3), *MRC1*, *STAB1*, *THBS1*, *TPP1* and, especially for its repeated prevalence, *GILZ* (32, 55). Also, and in line with the traditional concept of tolDC, the down-modulation of the antigen presenting molecule *CD74* (also known as HLA-DR) (55) and *LAMP3* (lysosomal-associated membrane protein 3), typically found on iDC (41), has been reported. Interestingly, the combined exposure in front of both of IL-10 and IL-6 for the generation of tolDC performed in one of the previously cited articles did not seem to change the transcriptomic profile

of these cells, as many of the above mentioned genes were also found accordingly induced or repressed like they were in IL10-tolDC (55).

The use of TGF-β for the *in vitro* differentiation of tolDC is not as widely established as IL-10, but still some potential biomarkers have been described, both exclusively for this product (the immune related-genes *CXCL1* and *CXCR3*) and in common with other regulatory cells (*ANXA1*, *CTSL*, *FTH1*, *HLA-DOB*, *IL-8*, *LILRB3*, *THBS1*) (32, 55). Just like in IL10-tolDC, *CD74* appears differentially repressed in TGF-β-induced tolDC but, controversially, also does *STAB1*, reportedly up-modulated in the former protocol (55). As far as we are concerned, no potential transcriptomic or proteomic markers have been reported in cells induced with the combination of IL-10 and TGF-β for the generation of human tolDC.

Surprisingly, IFN-γ has also been described in a couple of publications for the generation of tolDC, even though it does not constitute the most obvious strategy due to its proinflammatory properties. Nevertheless, these studies have reported the selective reduction in the expression of the pro-inflammatory genes *IRF4*, *RELB,* and *IL12p40* in this cell product (53, 54). Consequently, the down-modulation of these genes is in line with the expected anti-inflammatory profile for tolDC, and even *IRF4* has also been reported as differentially repressed in vitD3-tolDC, as mentioned above (62). All the biomarkers described within the protocols mentioned in this section are shown in **Table 1**.

Finally, the differentiation of stable tolDC from monocytes in the presence of low doses of GM-CSF, and in the absence of IL-4 in the culture, has also been reported in humans (81), but also in animal models (82–84). In fact, their clinical use is being tested under the context of a multicentre trial named *The ONE Study ATDC* in living-donor renal transplantation (http://www.clinicaltrials.gov, NCT02252055) (85). However, any potential biomarker in human low-dose GM-CSF-induced tolDC has been reported yet.

Genetic Changes Induced in Tolerogenic Dendritic Cells Generated With Other Strategies

The pharmacological agents and factors mentioned so far comprise the most predominant strategies in the literature for the induction of tolDC, but there is still a wide variety of drugs, proteins and several treatments with the potential of generating this type of regulatory DC products. However, provided that the aim of this review is to look for universal biomarkers of immune tolerance, we have also considered these approaches. In fact, a significant amount of studies have reported the differential expression of several genes and molecules that could become potential biomarkers for their respective and specific protocols, generating tolDC in the presence of different organic compounds—such as the *Aspergillus* cell wall (32), curcumin (86), mitomycin C (87), paeoniflorin (88), phosphatidylserine liposomes imitating apoptotic bodies (89)—, other cell types—mast cells (90) and trophoblasts (91)—and a variety of agents, conditions and/or molecules—for instance a combination of the complement protein C5a and LPS (92), seminal plasma (93), the Wnt-5a protein (94) or even the deprivation of tryptophan in the culture (95)—. However, there are still many other different strategies without transcriptomic or proteomic studies reported in the literature that are, therefore, outside of the objective of this review. The full list of differentially expressed genes and molecules in the protocols mentioned in this section is presented in **Table 2**.

A totally different approach to generate tolDC consists in using targeted genetic engineering in order to achieve cells with specific functional features either silenced or induced. There are several strategies reported in this regard, ranging from the impairment of immunogenic properties—such as silencing the expression of *CD40*, *CD80,* and *CD86*, already tested in type 1 diabetes patients, which was the first clinical trial using a tolerogenic cell therapy (10, 98) (http://www.clinicaltrials.gov, NCT00445913)—to selectively inducing the production of several anti-inflammatory cytokines like IL-10 and TGF-β (31, 99), overexpressing the IL-12 and IL-23-suppressor factor *SOCS-3* (100) or transfecting the cells with a modified *CTLA4* construct that inhibits the expression of the co-stimulatory molecules CD80 and CD86 (101). Surprisingly, some approaches using genetic manipulation achieved to generate human IL-10-producing DC through the induction of, *a priori*, immunogenic functions such as the CD40-CD40L signaling pathway (102). However, the definition of transcriptomic biomarkers for tolDC induced by genetic engineering would not be of much utility, provided that the differentially expressed genes or proteins to check would be precisely those that have been specifically induced or repressed by the procedure itself.

GENERATION OF REGULATORY MACROPHAGES: DIFFERENCES AND SIMILARITIES WITH TOLEROGENIC DENDRITIC CELLS

Mreg constitute one of the three main macrophage subtypes, being the other two the classical macrophages and the so-called alternatively activated macrophages, or M2 macrophages. However, and as reviewed by Fleming and Mosser (103), Mreg present unique features: they are characterized by their ability to modulate the immune system toward a regulatory T_H2 response through the production of IL-10 and a limited or absent secretion of IL-12 mediated by the activation of the ERK cascade. In addition, these cells present an increased antigen-presenting functionality with an elevated expression of HLA class II and B7 co-stimulatory molecules. And this is, probably, the crucial feature in which Mreg and M2 macrophages differ the most, because although both subsets exhibit immunoregulatory properties, the ability to induce antigen-specific responses is limited in M2 macrophages due to their low HLA expression. However, Mreg are considered to deploy their potent T cell suppressor functions mainly through three non-antigen-specific mechanisms: via IFN-γ-induced IDO activity, by a contact-dependent deletion of activated T lymphocytes or mediating the induction of $TIGIT^+$ $FoxP3^+$ Treg (9, 96, 104, 105).

Just like tolDC, human macrophages can be generated *in vitro* by differentiating them from monocytes. Briefly, classical

TABLE 2 | Differentially up- and down-modulated genes and proteins in other human tolDC- and Mreg-inducing protocols.

	Protocol	Type	Up-modulated molecules	Down-modulated molecules	References
tolDC	*Aspergillus* cell wall	Gene	***ANXA1, STAB1, GILZ, IDO, RALDH1, RALDH2***	***F13A, MRC1***	(32)
	C5a and LPS	Gene	***RGCC, FERMT2, SLC39A14, TNFSF14, TGFB1***	***IL12B, FOXO1***	(92)
	Curcumin	Gene		***RELB***	(86)
	Mast cells	Gene	***IDO1, NFKB1, NFKB2, RELB, SOCS5***	***SOCS3***	(90)
	Mitomycin C	Gene	***ADM, CSF2RA, DDIT3, FDXR, GAB2, LILRB4, LRDD, MAFB, MAP4K4, PERP, TNFRSF10B, TRAF4, TSC22D3***	***CFLAR* (FLAME-1, I-FLICE, Usurpin)**, ***NRG2***	(87)
	Paeoniflorin	Gene	**IDO1**		(88)
	Phosphatidyl-serine lyposomes	Gene	*CLCN6,* ***CYTH4****, IFNLR1, LAIR1, LDLR, MFSD2A, NFKBIA, PLAUR, PPME1, SHB, SLC43A3, TNFAIP3,* ***TNFSF14****, VEGFA*	*ALKBH1, ATP10D, AURKA, BCL2L1, BLCAP, BST1, BTBD3, BTK, BUB1, C9orf64, CASP3, CBX4, CD1D, CDC23, CDC42SE1, CDK13, CDYL2, CKAP2, CLCN3, CSRP2BP, CUL3, DAPP1, DCAF12, DCAF7, DCLRE1A, DCTD, DDO, DYRK2, EHBP1, ERLIN1, FBXO25, FBXO36, FRAT2, FZD5, GIMAP4, GLRX, GOLPH3L, GTF2B, HHEX, HPGD, ICK, KBTBD6, KIF11, KIF20B, LMNB1, LNX2, MAPRE2, MCM4, MCPH1, MDM1, MEF2C, MEGF9, MIER3, MLH1, MNDA, MSH2, MYB, N4BP1, NCAPG2, NET1, NFIA, NSMCE4A, NUP160, PAQR8, PARG, PAXIP1, PCNA, PMP22, PROS1, RAB32, RAD51C, RCSD1, RMDN1, RMND5A, SCYL3, SEC22C, SKI, SLAMF6, SLC10A7, SLC40A1, SMC2, SNN, SNX18, SOCS2, STIM2, STX3, TIMMDC1, TNFRSF11A, TPK1, TRIM5, UBE2E3, UBFD1, UNC50, VWA5A, WRNIP1, ZBED3, ZBTB39, ZBTB5, ZFP36L2, ZNF436*	(89)
	Seminal plasma	Gene	***COX2, TGFB1***	***CD1A***	(93)
	Trophoblasts	Gene	***IDO1***		(91)
	Tryptophan-deprived	Gene	***CHOP, ILT3***		(95)
	Wnt5a	Gene	***ID3, IRF1, IRF2, SOCS3, TLR1***	***ID2, IRF8, TLR3, TLR4, TLR5***	(94)
Mreg	M-CSF + IFN-γ	Gene	***ALDH1A1, ALDH1A2, CD1D, DHRS9***		(96)
	M-CSF + LPS	Protein		**IL-12p35, IL-12p40, IL23-p19**	(97)

Genes validated by qPCR or proteins validated by western blot are shown in bold.

macrophages are obtained in the presence of GM-CSF, and M2 and Mreg macrophages are generated in the presence of M-CSF, but with different supplementary treatments. While M2 macrophages are normally achieved using M-CSF + IL-4 and/or other T_H2 cytokines, Mreg are treated with M-CSF + LPS or IFN-γ for a brief period of time (106, 107). This combination of M-CSF and a short and complementary pro-inflammatory treatment is precisely the responsible for the strong induction of IL-10 production, something that both stimuli alone fail to achieve in macrophages (108, 109).

The generation of human Mreg is not as widely extended as tolDC, and consequently the number of protocols describing the differentiation of these cells is much more reduced. However, several molecules have already been postulated as potential biomarkers for these cell products. So far, transcriptomic studies have only been performed over LPS (LPS-Mreg) and IFN-γ-activated Mreg (IFN-Mreg). The former, LPS-Mreg, were initially described as IL-10-producing M2 macrophages, and their impaired IL-12 and IL-23 production was confirmed by qPCR (97, 110). However, IFN-Mreg are more widely reported and studied, especially considering their translation into the clinic, where they have already been used for the treatment of living-donor renal transplant-recipient patients (http://www.clinicaltrials.gov, NCT00223067 and NCT02085629) (9, 105). This product is obtained by the stimulation of M-CSF-differentiated macrophages with IFN-γ, after 7 days of culture (9, 105, 111), and a strong up-modulation of *ALDH1A1, ALDH1A2* and *CD1D* genes has been reported, as well as the induction of *DHRS9* as an specific IFN-Mreg biomarker (96). The detailed list of markers reported in pre-clinical human Mreg protocols is shown in **Table 2**.

Nevertheless, although the list of genetic biomarkers described in Mreg is short, the identification of *DHRS9* in IFN-Mreg achieves a high relevance in the context of immune tolerance biomarkers, provided that the enzyme encoded by these gene seems to be involved in the biosynthesis of retinoic acid (112). As commented above, this compound is a vitamin A-derived molecule that can be used to differentiate human monocytes into tolDC. Interestingly, both *ALDH1A1* and *ALDH1A2* genes have been identified as differentially induced

TABLE 3 | Differentially expressed genes reported in at least two different protocols for the generation of human tolDC and/or Mreg.

Gene	Name	Modulation	Repeats	Protocols	References
ACADVL	Acyl-CoA Dehydrogenase Very Long Chain	Up	2	Dexa+vitD3, TX527	(49, 60)
ACO2	Aconitase 2	Up	2	Dexa+vitD3, TX527	(49, 60)
ALDH1A1	Retinaldehyde Dehydrogenase 1	Up	3	Asp, IFNg Mreg, RA	(58, 96)
ALDH1A2	Retinaldehyde Dehydrogenase 2	Up	2	IFNg Mreg, RA	(58, 96)
ANXA1	Annexin A1	Up	5	Asp, dexa, IL10, rapa, TGFb	(32)
ATP5A1	ATP Synthase 5 Alpha Subunit 1	Up	2	TX527, vitD3	(60, 63)
C1QA	Complement C1q A Chain	Up	2	Dexa, dexa+vitD3	(34, 35, 50)
C1QC	Complement C1q C Chain	Up	3	Dexa, IL10, rapa	(32)
CD14	Cluster of Differentiation 14	Up	2	dexa+vitD3, vitD3	(50, 62)
CD1A	CD1a Receptor	Down	2	Sem, vitD3	(50, 93)
CD1C	CD1c Receptor	Down	2	Dexa, vitD3	(35, 62)
CD300LF	CD300 Molecule Like Family Member F	Up	2	Dexa, vitD3	(34, 62)
CD80	Cluster of Differentiation 80	Down	2	Dexa+vitD3, vitD3	(50, 62)
CTSB	Cathepsin B	Up	3	Dexa+vitD3, IL10, IL10+6	(50, 55)
CTSC	Cathepsin C	Up	3	Dexa, IL10, rapa	(32)
CTSD	Cathepsin D	Up	2	Dexa+vitD3, TX527	(49, 60)
CTSL	Cathepsin L	Up	3	IL10, IL10+6, TGFb	(55)
DHRS9	Dehydrogenase/Reductase 9	Up	2	Dexa+vitD3, IFNg Mreg	(49, 96)
F13A	Coagulation Factor XIII A	Up	2	Dexa, IL10	(32)
		Down	2	Asp, vitD3	(32)
FBP1	Fructose-Bisphosphatase 1	Up	2	Dexa+vitD3, TX527	(37, 49, 60)
FCGR2B	Fc Fragment Of IgG Receptor IIb	Up	2	Dexa, dexa+vitD3	(34, 49)
FCGR3A	Fc Fragment Of IgG Receptor IIIa	Up	2	Dexa, dexa+vitD3	(34, 49)
FSCN1	Fascin Actin-Bundling Protein 1	Down	3	Dexa, dexa+vitD3, vitD3	(37, 50)
FTH1	Ferritin Heavy Chain	Up	4	Dexa+vitD3, IL10, IL10+6, TGFb	(50, 55)
FTL	Ferritin Light Chain	Up	3	Dexa, dexa+vitD3, vitD3	(34, 37, 50)
G6PD	Glucose-6-Phosphate Dehydrogenase	Up	2	Dexa+vitD3, TX527	(37, 50, 60)
GILZ	Glucocorticoid-Induced Leucine Zipper	Up	6	Asp, dexa, RGZ, IL10, rapa, vitD3	(32, 35, 36, 47)
GPX1	Glutathione Peroxidase 1	Up	2	Dexa, rapa	(32)
HLA-DOB	Human Leukocyte Antigen Class II, DO Beta Chain	Up	3	IL10, IL10+6, TGFb	(55)
IDH3A	Isocitrate Dehydrogenase 3 Alpha	Up	2	Dexa+vitD3, TX527	(49, 60)
IDO1	Indoleamine 2,3-Dioxygenase	Up	7	Asp, dexa, dexa+vitD2, mast, pae, pIC, tropho	(32, 35, 48, 57, 88, 90, 91)
IL-10	Interleukin 10	Up	2	Dexa, hepa	(35, 52)
IL-12	Interleukin 12	Down	5	C5a, dexa, dexa+vitD3, IFNg, LPS Mreg	(35, 38, 50, 53, 54, 92, 97)
IL-8	Interleukin 8	Up	2	IL10, IL10+6	(55)
ILT3	Immunoglobulin-Like Transcript 3	Up	4	Dexa+vitD3, mitC, tryp, vitD3	(36, 50, 87, 95)
IMDH2	Inosine Monophosphate Dehydrogenase 2	Up	2	Dexa, rapa	(32)
IRF4	Interferon Regulatory Factor 4	Down	2	IFNg, vitD3	(54, 62)
LAMP3	Lysosome-Associated Membrane Protein 3	Down	3	Dexa, IL10, vitD3	(41)
LILRB3	Leukocyte Immunoglobulin Like Receptor B3	Up	2	IL10, TGFb	(55)
MRC1	Mannose Receptor C-Type 1	Up	2	Dexa, IL10	(32)
mTOR	Mammalian Target Of Rapamycin	Up	2	Dexa+vitD2, vitD3	(48, 63)
OSF1	Pleiotrophin	Up	2	Dexa, rapa	(32)
PCK2	Phosphoenolpyruvate Carboxykinase 2	Up	2	Dexa+vitD3, TX527	(49, 60)
PDHA1	Pyruvate Dehydrogenase E1 Alpha 1 Subunit	Up	2	Dexa+vitD3, vitD3	(49, 60)

(Continued)

TABLE 3 | Continued

Gene	Name	Modulation	Repeats	Protocols	References
PIK3CG	Phosphatidylinositol-3-Kinase Subunit Gamma	Up	2	Dexa+vitD3, vitD3	(49, 50, 63)
PKM2	Pyruvate Kinase Muscle Isozyme M2	Up	2	Dexa+vitD3, TX527	(37, 49, 60)
RELB	RelB Transcription Factor, NF-κB Subunit	Down	2	Cur, IFNg	(53, 54, 86)
RGCC	Regulator Of Cell Cycle	Up	2	C5a, dexa+vitD3	(50, 92)
STAB1	Stabilin 1	Up	3	Asp, dexa, IL10	(32)
TGFB	Transforming Growth Factor Beta	Up	3	C5a, dexa+vitD3, sem	(51, 92, 93)
THBS1	Thrombospondin 1	Up	3	IL10, IL10+6, TGFb	(55)
TNFSF14	TNF Superfamily Member 14	Up	2	C5a, lipo	(88, 92)
TPP1	Tripeptidyl Peptidase 1	Up	3	Dexa, IL10, rapa	(32)

The column "Modulation" indicates if a determined gene has been found up- or down-modulated, and the field "Repeats" indicates the amount of different protocols in which each gene or protein has been described. The abbreviations stand for either tolDC induced with asp, Aspergillus cell wall; C5a, C5a, and LPS; cur, curcumin; dexa, dexamethasone; dexa+vitD2, dexamethasone + vitamin D2; dexa+vitD3, dexamethasone + vitamin D3; hepa, hepatocyte growth factor; IFNg, IFN-γ; IL10, IL-10; IL10-6, IL-6 + IL-10; mast, mast cells; mitC, mitomycin C; pae, paeoniflorin; pIC, Polyinosinic:polycytidylic acid; RA, retinoic acid; rapa, rapamycin; RGZ, rosiglitazone; sem, seminal plasma; TGFb, TGF-β; tropho, trophoblasts; tryp, tryptophan deprivation; vitD3, vitamin D3; or regulatory macrophages induced with IFNg Mreg, IFN-γ; LPS Mreg, lipopolysaccharide.

in retinoic acid-generated tolDC (58) as well as IFN-Mreg, making them two interesting candidates for the characterization of at least this couple of different tolerance-inducing cell products. Furthermore, the differential up-modulation of *DHRS9* has also been reported in vtdx-tolDC, also discussed above (49). Consequently, since these cells are generated with both dexamethasone and vitamin D3, a clear relation between the transcriptomic profile of both IFN-Mreg and tolDC induced with either vitamin A or D is likely to exist. For this reason, further studies and validations in this direction could be of great interest, as potential common biomarkers of two different immune-regulatory myeloid cell-derived products could be identified.

SUMMARY AND CONCLUDING REMARKS

The identification of robust biomarkers for the characterization of tolerogenic and immunoregulatory cell products constitutes one of the last steps needed to take the final leap toward the broad application of these novel autologous antigen-specific therapies in the clinic. Specifically, their key importance resides in their capability to provide a fast and reliable quality control of the proper generation, functionality and safety of tolDC and Mreg.

In this article we have performed an exhaustive review of the currently published human tolDC- and Mreg-generating protocols that have reported potential biomarkers for these cells, with the aim of elucidating if a common transcriptomic or proteomic pattern relating all of them could be drawn. However, as it has been discussed, albeit many genes and molecules have been found separately induced using different strategies to generate these immunoregulatory cell products, so far, there is not a biomarker or a pool of biomarkers that can functionally characterize or at least identify the entirety of the studied protocols. Nonetheless, this is not necessarily bad news, as the chances of identifying a common biomarker were slim given the overwhelming variety of approaches and cell types reported in this review. As already mentioned above, the immune system can deploy several strategies for the induction of tolerance that modulate many different immune and non-immune related pathways and transcriptomic cascades, thus making this goal even more unlikely. However, it is also worth noting that finding biomarkers provided only by the tolerance-inducing mechanisms could also be misleading; for instance, DC subtypes like iDC are capable of developing some tolerogenic functions, but still they could not be applied as a therapeutic approach in autoimmune diseases provided their lack of stability against pro-inflammatory stimuli, as discussed above.

Still, despite the consideration of such a wide variety and heterogenicity of protocols for the induction of regulatory cells, a significant amount of differentially expressed genes encoding several anti-inflammatory and immunomodulatory molecules has been reported in very different protocols, for instance *IDO1* (in 7 approaches) *GILZ* (in 6 approaches) or *ANXA1* (in 5 approaches). Similarly, the down-modulation of the pro-inflammatory cytokine IL-12 has been reported in 5 different tolDC-inducing strategies. In other words, in this review we have gathered all the genes and proteins that have been described separately with each of the approaches for the generation of tolDC and Mreg in the literature, and we have subsequently compared and put them all together in order to evidence potential common biomarkers between them. The complete list of the genes that have been reported in studies with at least two different approaches for the generation of human tolDC and/or Mreg are shown in **Table 3**. Therefore, the general idea that lies behind these reported molecules is that all the considered tolerogenic-inducing agents are modulating the cells toward a regulatory profile that might be partially shared between some approaches, but that is often achieved through different mechanisms and biological pathways that are strongly dependent on the stimuli used to generate them.

Consequently, this review evidences that the definition of strong biomarkers for tolDC and Mreg is still needed, but also that, although a universal transcriptomic profile of immune

tolerance induction might not be achievable, the elaboration of useful panels of biomarkers can still be feasible for determined pools of tolerogenic products. Bearing that in mind, our work could therefore serve as a starting point for developing and guiding further research in this field. For instance, one of the next steps that could be taken in this regard could be to specifically try to validate some of the above discussed genes in different protocols in which they have not been explicitly reported, either because they have been already identified in several approaches—like *IDO1* or *GILZ*—or because the stimuli used to induce the tolerogenic status share some functional or structural resemblance that might translate into the induction of common pathways and metabolic processes. In other words, with this review we intend to provide a useful reference of currently described biomarkers from which direct the investigation of new genes and proteins, most likely protocol-specific.

Thus, the combination of both stimulus-specific and some other partially-common differentially expressed genes could potentially lead to the development of transcriptomic panels of tolerogenic functionality. After all, provided that the relevance of tolerance-inducing cell therapies in the treatment of autoimmune diseases and solid organ transplantation rejection is becoming hugely relevant in the last years, the need for adequate and objective biomarkers is increasing accordingly. And in this context, the definition of panels of tolerogenic functionality for at least a limited pool of protocols would consequently provide a robust tool for the establishment of reliable quality and safety controls for trials using tolDC- and/or Mreg-based therapies in the near future, which would also allow to properly compare them and therefore to dramatically accelerate their translation into the clinic.

AUTHOR CONTRIBUTIONS

EM-C, JN-B, and MM conceived the manuscript. JN-B wrote the manuscript. EM-C and MM reviewed the manuscript.

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