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Generation of Tolerogenic Dendritic Cells for Cell Therapy in Multiple Sclerosis

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That the thesis entitled "Generation of Tolerogenic Dendritic Cells for Cell Therapy in Multiple Sclerosis" has been performed by Dàlia Raïch Regué under their direction and guidance, and is able to be presented and defended in order to apply for the PhD degree in Immunology of the Universitat Autònoma de Barcelona.

Badalona, 30th April 2012

Dr. Francesc E. Borràs Serres   Dr. Eva M. Martínez Cáceres

"

When a single man dreams,

it is just a dream.

But when many people dream together,

this is the beginning of a new reality.

"

Friedensreich Hundertwasser

(1928 – 2000, Austrian architect and artist)

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Abbreviations

Ads: Autoimmune diseases

APCs: Antigen-presenting cells

APLs: Altered peptide ligands

BBB: Blood-brain barrier

BM: Bone marrow

CCR7: chemokine receptor 7

CNS: Central nervous system

CSF: Cerebrospinal fluid

CTL: Cytotoxic T lymphocytes

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

DCs: Dendritic cells

DNA: Deoxyribonucleic acid

DNA: Deoxyribonucleic acid

EAE: Experimental autoimmune encephalomyelitis

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorting

FITC: Fluorescein isothiocyanate

G-CSF: Granulocyte colony–stimulating factor

GA: Glatiramer acetat

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GMP: Good manufacturing practices

HGF: Hepatocyte growth factor

HSCs: Hematopoietic stem cells

ICAM: Intercellular adhesion molecule

ICOSL: Inducible T cell co-stimulator ligand

iDCs: Immature monocyte-derived dendritic cells

IDO: Indoleamine 2,3-dioxygenase

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

ILT: Immunoglobulin like transcript

IR: Immune response

IRF: Interferon responsive factor

IS: Immune system

LCs: Langerhans cells

LPS: Lipopolysaccharides

mAb: Monoclonal antibody

MBP: Myelin basic protein

mDCs: Mature monocyte-derived dendritic cells

MDDCs: Monocyte-derived dendritic cells

MHC-I: Class I major histocompatibility complex

MHC-II: Class II major histocompatibility complex

MHC: Major histocompatibility complex

MRI: Magnetic resonance imaging

MS: Multiple sclerosis

mTECs: Medullar thymic epithelial cells

NFκ-B: Nuclear factor kappa B

NK: natural killer

PAMPS: Pathogen associated molecular patterns

PBMCs: Peripheral-blood mononuclear cells

PBS: Phosphate buffer saline

PD-L1: Programmed Death Ligand 1

PHA: Phytohaemagglutinin

PLP: Proteolipid protein

PRRs: Pattern-recognition receptors

RNA: Ribonucleic acid

RR-MS: Relapsing-remitting multiple sclerosis

TCR: T cell receptor

TCR: T cell receptor

TGF: Transforming growth factor

Th: T helper

TLRs: Toll-like receptors

TNF: Tumor necrosis factor

TRA: Tissue-restricted antigens

TSLP: Thymic stromal lymphopoietin

VDR: Vitamin D receptor

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Summary

Current treatments for relapsing-remitting multiple sclerosis (RR-MS) patients decrease the frequency of relapses and reduce inflammatory activity in a nonspecific manner, but their effect on disease progression is still unclear. Therefore, in order to modify the course of MS, new and more specific therapeutic approaches are necessary. Specific inhibition or deletion of autoreactive T cells represents an interesting goal for restoring peripheral tolerance in autoimmune diseases such as MS.

The main goal of this work has been to generate and characterize tolerogenic dendritic cells (tolDCs) from RR-MS patients, loaded with myelin peptides as specific antigen, as a therapeutic tool to re-establish tolerance to myelin-antigens in these patients.

Our results show that using different immunosuppressive drugs and different maturation stimulus permitted the generation of clinical-grade tolDCs products with differences that are relevant to therapeutic applicability. We evaluated the viability, phenotype, cytokine profile, stability and functionality of these tolDCs.

The comparison of different pharmacological grade tolerogenic agents (dexamethasone, rapamycin and vitamin-D3) led to the observation that dexamethasone-treated DCs showed a semi-mature phenotype and high IL-10 secretion; that rapamycin-treated DCs impaired IFN-γ in co-cultured T cells and expanded T regulatory cells; and finally that vitamin-D3-treated DCs presented a semi-mature phenotype, produced IL-10, and reduced IFN-γ in co-cultured T cells. These features, along with their reproducibility among different samples, made vitamin-D3 considered as the most convenient of the three compared agents to generate tolDCs for MS therapy. Regarding the maturation stimulus, the *cytokine cocktail* (composed by TNF-α, IL-1β and PGE-2) was determined as the optimal maturation stimulus to generate tolDCs (induced by

vitamin-D3 treatment) in our setting, since these tolDCs were the unique exhibiting functional stability and capability to suppress an immune response *in vitro*.

The generation and characterization of tolDCs from RR-MS patients (generated with vitD3 and matured with the pro-inflammatory cytokine cocktail), showed that there are no significant differences between tolDCs generated from healthy controls and from MS patients' cells, both presenting a tolerogenic profile. Importantly, myelin peptide-loaded tolDCs from MS patients induced antigen-specific and stable hyporesponsiveness in autologous myelin-reactive T cells *in vitro*.

Altogether this work has conducted to the development of a protocol to generate clinical-grade tolDCs and set up the bases for their use as a therapeutic tool to re-establish tolerance in RR-MS patients.

Introduction

1. Immune system and immune response

The immune system is composed by a complex group of tissues, cells and molecules that protect the organism from infectious agents like fungi, bacteria and viruses. To do so, the immune system has the ability to recognize danger signals from the infectious agents and to activate multiple mechanisms to eliminate them while preserving homeostasis. The immune system has developed different mechanisms to deal with invading pathogens from the milieu. These mechanisms are collectively named immune response (IR), and can be divided in two types: the innate or antigen-unspecific response and the adaptive or antigen-specific response (1,2). Each type of response has its own characteristics and includes cellular and soluble components.

The innate response takes place at the primary stage of an infection and it is constituted by quick and unspecific defenses beyond the physical barriers (epidermis, mucosa, etc.). The innate immune cells (such as mast cells, natural killer cells, granulocytes, monocytes, macrophages, dendritic cells and epithelial cells), together with the complement system, respond rapidly to invading microorganisms in the exposed tissues, releasing inflammatory cytokines and initiating antimicrobial activity. These innate immune cells detect pathogens through the pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), a set of evolutionary conserved hallmarks on the pathogens, or damage associated molecular patterns (DAMPs), released within tissues as a consequence of cellular distress. Some examples of PRRs are: toll-like receptors (TLRs), which bind to RNA or DNA from several pathogens and bacterial cell wall components such as lipopolysaccharides (LPS) (3,4), NOD-like receptors and RIG-I-like receptors, among others (5). Surveillance receptors also include scavenger receptors and C-type lectin receptors that induce endocytosis. Engagement of surveillance receptors

to pathogen domains leads to the activation of some of the immune cells, triggering the secretion of chemokines and inflammatory cytokines (i.e. tumor necrosis factor (TNF)-α and interleukin (IL)-1β), as well as the maturation and migration of antigen-presenting cells (APCs). This creates an inflammatory environment that allows the development of specific adaptive immune response.

The adaptive immune response is specifically designed for each pathogen, and provides long-lasting immunological memory that allows for a faster and more efficient response in later encounters. To start an adaptive immune response, APCs have to present a foreign antigen to T lymphocytes. Depending on the nature of the antigen, antigen presentation may occur through the classic pathway, involving the major histocompatibility complex (MHC), or the non-classic pathway through the CD1 family. Activation of APCs renders the clonal expansion of antigen-specific T and B lymphocytes, which are the main effector immune cells (together with APCs) in adaptive immunity. Thus, APCs orchestrate and act as a link between innate and adaptive immunity to achieve an effective immune response.

APCs could be classified into two categories: professional or non-professional. Dendritic cells, macrophages, monocytes, and B lymphocytes are classified as professional APCs, since they express MHC class II molecules constitutively and co-stimulatory molecules required for priming naive T cells. Oppositely, fibroblasts, glial cells, and endothelial cells are classified as non-professional APCs since they do not present such features. Remarkably, dendritic cells are the most potent activators of T cells (6).

1.1   Dendritic cells (DCs)

DCs were first described by P. Langerhans in 1868, and re-discovered by R. Steinman and Z. Cohn on 1973 (7). Nowadays, we know that DCs are a heterogeneous group of APCs widely distributed through different

tissues (8), and they are specialized in the regulation of immune responses.

DCs are generated from bone marrow (BM) precursors and are present in all mucosal tissues, typically sites of pathogen entry, like the gut and the lungs. DCs are also present in the skin, internal organs, blood, lymph, and all lymphoid tissues, including bone marrow (BM). DCs display a high degree of plasticity within organs and lymphoid tissues, and effector functions of DCs are often regulated by the microenvironment of the tissue (9).

1.1.1 DCs subsets

The population of DCs in humans is composed by distinct subsets with a great complexity of anatomical distribution, immunological function and cell-surface marker expression (10). Although DCs are widely distributed, they are present at very low frequencies in blood representing a 0.5–2% of the total peripheral-blood mononuclear cells (PBMCs) (11).

The different routes of DC differentiation from hematopoietic stem cells (HSCs) are yet another layer of complexity to the heterogeneity of DC populations and, as a matter of fact, classification of DCs subpopulations is still a matter of discussion (12,13). DCs were initially classified into 'conventional DCs' (found in steady-state conditions) and 'non-conventional DCs' (that arose in response to inflammatory stimuli), and are differentiated respectively by the expression or not of CD11c (14,15). Monocyte-derived DCs (MDDCs) and plasmacytoid DCs (pDCs) however, are considered non-conventional DCs despite also being encountered under steady-state condition (10,12,15).

According to work by Liu et al. (12) and Kushwah et al. (13), HSCs differentiate into 'common lymphoid progenitors' and 'common myeloid progenitors'. The latter, further differentiate into monocytes and pre-DCs in the BM and, subsequently, enter the blood and migrate to lymphoid

organs and peripheral tissues, giving rise to lymphoid DCs and tissue-resident DCs. Nonetheless, some studies have shown that lymphoid progenitors could also give rise to pDCs and conventional DCs (16). In addition, distinct cytokine signals produced during steady state or inflammation can have a different outcome on DC lineage commitment and differentiation (17,18).

As human DCs could have multiple routes of development, those that arise from pre-DCs can be regarded as conventional DCs, whereas non-conventional DCs can include pDCs and MDDCs (13).

<u>Conventional DCs</u> (cDCs), also initially termed 'myeloid DCs', are characterized by a monocytic morphology, due to secretion of high levels of interleukin-12 (IL-12), expression of CD11c, CD13, low levels of CD123, and also due to preferential presentation of TLR-1, 2, 3 and 8 (11). cDCs can be further subdivided into lymphoid DCs (found in thymus, spleen and lymph nodes) and migratory DCs (that can be found in the skin, lung, intestinal tract, liver and kidneys). Skin DCs include Langerhans cells and dermal DCs, which are widely distributed throughout the mucosal surfaces. In the circulatory system, cDCs subtypes can be distinguished on the basis of their expression of blood DC antigens (BDCA, types: BDCA-2, -3, and -4) (19).

<u>Plasmacytoid DCs</u> (pDCs), mainly circulate in blood and lymphoid tissue, but can be recruited to sites of inflammation, acquiring typical DC morphology after activation (11). These cells are phenotypically characterized by the expression of CD4, CD62L, CD123 and the lack of expression of CD11c. In humans, pDCs selectively express the activating *fragment crystallizable receptor* (FcR), as well as TLR-7 and TLR-9 but no other TLRs (20). When pDCs are bound to immune complexes containing DNA or RNA, they signal high levels of type I *interferons* (IFNs), promoting antiviral responses (20).

<u>Monocyte-derived DCs</u> (MDDCs); Monocytes can give rise to DCs under inflammatory as well as steady state conditions (21). MDDCs are

found in peripheral tissues such as the intestine, lung, skin and kidneys. During inflammation and infection it has been shown that monocytes can be mobilized to the dermis and differentiate into dermal DCs, which subsequently migrate into the lymph nodes (22). In fact, some studies support that Langerhans cells are originated from monocytes (23,24). Moreover, a recent study demonstrated that a subset of CD14+ monocytes can migrate across the inflamed human blood-brain barrier (BBB) and differentiate into CD83+ CD209+ DCs, under the influence of BBB-secreted *transforming growth factor* (TGF)-β and GM-CSF (25).

Owing to the low abundance of DCs in vivo, modeling of their immunological function is often approached using in vitro generated MDDCs, which exhibit similar characteristics to cDCs (26).

1.1.2   DC functions

From BM, DCs precursors home to sites of potential antigen entry, where they can differentiate locally into immature DCs (henceforth called iDCs) (27). The different subtypes of DCs are distributed through the body acting as sentinels in peripheral tissues or in lymphoid organs, where they scatter possible antigens. When pathogen invasion takes place, iDCs can capture microorganisms via endocytic surveillance receptors, through two distinct mechanisms: phagocytosis (internalization of large size particles) or pinocytosis (ingestion of fluid or solutes) (28).

After pathogen uptake, DCs initiate a process of pathogen degradation rendering antigenic peptides that are finally expressed in the context of the MHC) molecules (28). "Danger" signaling through PRRs in the presence of pro-inflammatory cytokines (released by cells of the innate IS) prompts a complex maturation process of iDCs. This maturation process (**Figure 1**) consists of profound phenotypic and functional modifications driven by changes in gene expression, including the activation of the nuclear factor kappa B (NFκ-B) and interferon responsive factor (IRF) members (29). During the maturation process, antigen–MHC complexes are redistributed from intracellular

compartments to the cell surface along with a down-regulation of the endocytosis, thus permitting a maximal antigen presentation (28,30). In summary, upon maturation, DCs redistribute the complex MHC-peptide to the cell-surface and increase the expression of T cell co-stimulatory molecules such as CD40, CD80, CD86, OX40L, and *inducible T cell co-stimulator ligand* (ICOSL or CD275), together with the secretion of cytokines including IL-1ß, IL-2, IL-6, IL-8, IL-12, and IL-18 (8,28). Such changes endow DCs with stimulatory capacity on effector immune cells.
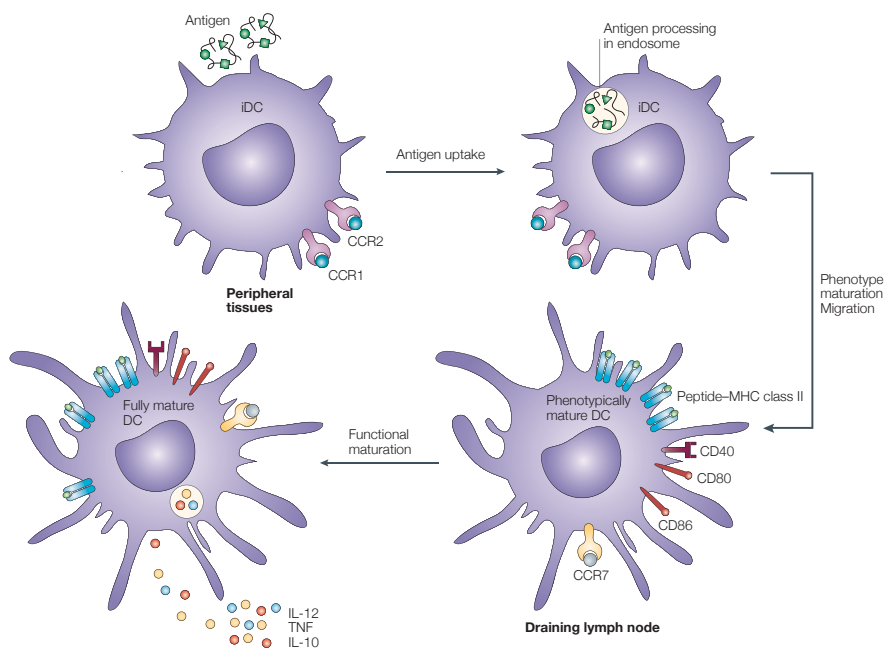
Figure 1. Maturation process of DCs (adapted from Nature Reviews Immunology (241)). iDCs in peripheral tissues continuously internalize antigens that can be processed by an endosomal MHC-II-restricted pathway. After antigen capture and depending on the nature of the antigen, DCs migrate to the draining lymphoid tissue and mature phenotypically, upregulating the expression of CD40, CD80, CD86, MHC class II molecules and CCR7. In the draining lymphoid tissue, they present peptide–MHC-II complexes on the cell surface, interacting with antigen-specific lymphocytes and mature functionally, producing pro-inflammatory cytokines, such as IL-12 and TNF.

In vivo, this maturation process is paralleled by a change in the repertoire of traffic molecules, such as the up-regulation of CCR7, a

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chemokine receptor that enables migration of DCs to the lymph nodes (31). There, mature DCs (mDCs) present antigen-derived peptides in association with MHC-II molecules to naive T helper (Th) lymphocytes, which recognize the MHC-II/peptide complex via the T cell receptor (TCR) (8,32). After recognition, and with the appropriate additional interactions mediated by co-stimulatory molecules, naive T lymphocytes become effector CD4+ T cells. The antigen specific crosstalk established between DCs and CD4+ T cells generates the "immunological synapse", which is supported by further cell contacts through adhesion molecules (33) (further explained in the section below).

Beyond pathogen capture and processing, DCs are also able to present antigens derived from endogenous proteins (degraded in the cytosol by the proteasome) to CD8+ T cells in MHC class I (MHC-I) molecules. This endogenous pathway allows DCs to trigger cytotoxic responses. Interestingly, mature DCs can also present exogenous antigens to CD8+ T cells through the MHC-I endogenous pathway (34). This process, known as cross-presentation, allows DCs to induce CD8+ T cell responses to immune complexes and dying cells, and also to non-replicating forms of microbes (35). DCs provide the link between the pathogen entry locations and the lymph nodes, presenting antigens to T cells in secondary lymphoid tissues that otherwise would not be able to respond to the distant invasion. Furthermore, a recent study showed that DCs also control the entry of naive lymphocytes to lymph nodes by modulating the phenotype of high endothelial venules, which are blood vessels specialized in lymphocyte recruitment (36). These results emphasize the role of DCs in the regulation of lymphocyte recirculation for immune surveillance.

In addition to the antigen-specific activation of T cells, DCs can influence the functions of other immune cells (37). DCs have been shown to play an important role in B-cell activation and differentiation into antibody forming cells (38), and also in priming and proliferation of natural killer (NK) cells and NKT cells (39).

1.2   T cell response

T cell response during antigen presentation depends on three different and integrative signals, involved in the identification and reciprocal activation of DCs and T cells:

- **Signal 1:** Antigen specific activation provided by MHC-II molecules (on APC surface) displaying a peptide to the cognate T cell receptor on the CD4+ T lymphocyte. This signal is of low affinity and needs the presence of adhesion molecules, such as LFA-1, CD11b, CD2, CD54/ICAM-1, CD58/LFA-3 or DC-SIGN, which stabilize the first step of the immunological synapse (40).

- **Signal 2:** Co-stimulatory signal by recognition and binding of co-stimulatory molecules on the surface of DCs such as CD40L and CD80/CD86 to their cognate receptors on the surface of T lymphocytes, CD40 and CD28 respectively. Interaction of CD40 with CD40L induces the total maturation of DCs, triggering an increase of CD80 and CD86 expression on DC surface and the secretion of cytokines IL-1, TNF and IL-12. Other molecules of the TNF family, such as 4-1BB-Ligand and OX40-Ligand, can also co-stimulate CD8+ and CD4+ T-cells respectively. The B7 family members ICOSL (Inducible Co-stimulator Ligand), PD-L1 (Programmed Death Ligand 1, B7-H1), PD-L2 (B7-DC), B7-H3 and B7-H4 can also play an important role during the T-cell priming (41).

- **Signal 3:** secretion of cytokines, such as IL-12/IFN-γ or IL-4/IL-10, by DCs or other micro-environmental sources that will bias the resultant immune response (42).

The '3 signals' theory is in line with the finding that the signal strength of the DC-T cell interaction is an important determinant of the fate of responding T cells (43). Low signal strength induces proliferation of naive T cells, a higher signal strength induces effector function and the capacity to migrate to inflamed tissues and an even higher signal strength induces T cell death (43).

Effector T lymphocytes could be differentiated in two types, CD8^{+} or T cytotoxic cells, and CD4^{+} or T helper cells, both of which are key mediators in multiple phases of the protective immune response. While CD8^{+} T cells recognize antigens presented in MHC class I molecules, CD4^{+} T cells recognize antigens in MHC class II molecules of an APC, mainly in secondary lymphoid organs. After antigen-recognition, the selected T cells undergo clonal expansion and differentiation to an array of cytotoxic and helper activities (**Figure 2**).

After priming, **CD8^{+} T cells** become effector cells that could be phenotypically and functionally heterogeneous, depending on the signals received from cytokines such as IL-2, IL-21, IL-12 and IL-27 (44,45). IL-2 signaling promotes the differentiation of *short-lived effector cells* (SLEC), representing the major population of effector cells that will mostly die off when infection is cleared. However, in the absence or decreased amounts of IL-2, CD8^{+} T cells preferentially become *memory precursor effector cells* (MPEC), contributing to the T cell memory pool (46). To achieve maximal expansion, CD8+ T cells need to integrate multiple signals including the TCR, co-stimulatory signals, and inflammatory cytokines, such as IL-12 and type I IFN (46). Effector CD8^{+} T cells (often termed cytotoxic T lymphocytes, CTL) can kill infected cells using granzymes and perforin. They can also release anti-viral cytokines such as IFNγ and TNF-α upon TCR ligation (47), and recent reports have shown that they may also play a regulatory role in preventing excessive tissue injury (IL-10^{+}CD8^{+} T cells) (48). Based on expression of the lymph-node homing receptors CD62L and CCR7, memory CD8+ T cells are often subdivided into non-lymphoid tissue resident effector memory cells (TEM, CD62L^{lo}CCR7^{lo}) and lymphoid tissue resident central memory cells (TCM, CD62L^{hi}CCR7^{hi}) (46). However, it is still unclear how different cell subsets are maintained, and which is the extent of their plasticity.

Like CD8^{+} T cells, **CD4^{+} T cells** need co-delivered antigenic and cytokine signals for optimal differentiation (49). Thus, CD4^{+} T cells can acquire the capacity to generate several responses depending on the

received signals, and they are defined according to their pattern of cytokines and functions, as detailed below:

T helper 1 (Th1) cells induction occurs via the ligation of TLRs (on APCs) and bacterial or viral products, which trigger the production of IL-12 (the essential Th1 cell differentiation factor) by DCs (50). Th1 cells produce IFN-γ, IL-2 and TNF-α among other cytokines, promoting a cellular immune response (type-1 immunity), such as the activation of macrophages to resist intracellular bacteria infections (51). Th1 cells express on their surface the CD40-ligand that activate the target cells, and the Fas-ligand that induce the cell death of Fas-expressing cells.

T helper 2 (Th2) cells secrete IL-4, IL-5, IL-9 and IL-13, and are required to fight parasitic infection but also promote allergic asthma responses in the lung (52). Numerous studies have shown that the type-2 effector cytokines are important for many aspects of type-2 immunity, including eosinophilic inflammation, mast cell proliferation and excessive mucus production at mucosal surfaces, IgE class-switching by B cells, and smooth muscle contraction (53). However, until recently, it was unclear how innate activation elicited this response. IL-25 and IL-33 were identified as type-2-inducing cytokines (54), and novel innate cell populations, such as 'nuocytes' or 'NHCs', have been recently discovered to be essential for type-2 responses against helminthic parasites (55). These novel populations respond to IL-25 and/or IL-33 (secreted by tissue resident cells), producing high amounts of IL-13 and IL-5, which in turn can trigger the differentiation of Th2 cells (56).

T helper 17 (Th17) cells develop through stimulation via IL-6 and TGF-ß. The former activates STAT3, enhancing expression of the transcription factors RORγt and RORα, which promote the expression of the cytokine products: IL-17A, IL-17F, IL-21, and IL-22. Th17 cells also secrete IL-23, which promote their own expansion and sustaining (57). Th17 cells are involved in promoting inflammation and host defense against certain infectious agents, and have been related with the immuno-pathogenesis of some autoimmune diseases, such as multiple sclerosis

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(58). Furthermore, non-CD4$^+$ T cell populations such as γδT cells and NK cells are also capable of producing Th17 cytokines.

T regulatory cells (Tregs) can be discriminated between two major types based on their origin (59): 'natural' and 'adaptive' Tregs. *Natural Tregs* (nTregs) originate during thymic development (60) and, upon recognition of self-antigens in the thymus, they up-regulate the transcription factor Foxp3, which controls the phenotype and suppressive program of CD4$^+$ nTregs (61). Innocuous self- and non-self-antigens that appear post-natally (like hormones, food, and commensal flora) may be transported into the thymus by migratory iDCs (62), which may induce new nTregs. In addition, effector T cells can be converted to *adaptive Tregs* (aTregs) in extra-thymic sites such as secondary lymphoid organs (SLOs) or peripheral tissues. aTregs are phenotypically heterogeneous, could be CD4$^+$ or CD8$^+$ and include *Tr1 cells* (that secrete IL-10), *Th3 cells* (that secrete TGF-β and IL-10), and Foxp3$^+$ Treg cells (63). Tregs may have different regulatory modes of action that include: secretion of anti-inflammatory cytokines, granzyme-perforin-induced apoptosis of effector lymphocytes, and inhibitory receptors such as cytotoxic T-lymphocyte antigen 4 (CTLA4), lymphocyte-activation gene-3 (LAG-3), glucocorticoid-induced tumor necrosis factor receptor (GITR), CD39, or CD73, among others, that can inhibit DCs function (64). Other lymphocyte subsets with regulatory function include: inducible CD8$^+$ Tregs, CD3$^+$CD4$^-$CD8$^-$ Tregs (double-negative), CD4$^+$Vα14$^+$ (NKTreg), and γδT-cells (63).

T helper 9 (Th9) cells are induced by TGF-β plus IL-4 and produce exceptionally large quantities of IL-9, high levels of IL-10 but only trace amounts of IL-17 or IFN-γ. Indeed Th9 cells act as key players in onset and progression of asthma, serving as strong inducers of mast cell responses (65).
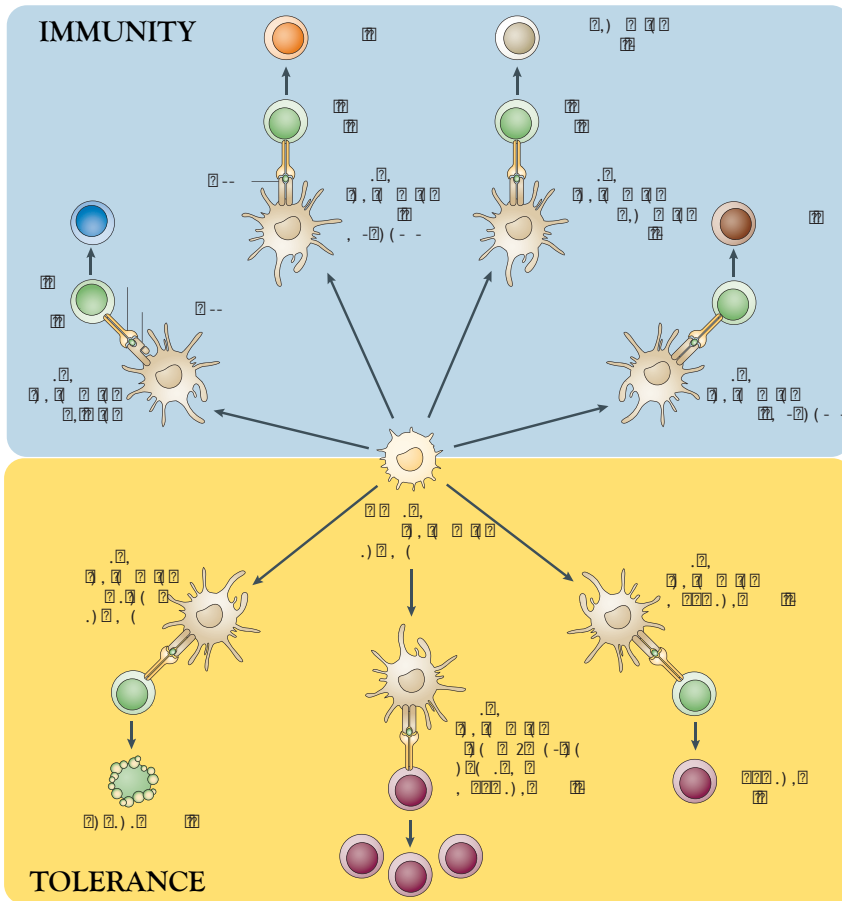
Figure 2. Effector function of DCs (adapted from Nature Reviews Immunology (96)). This illustration represents a view of DCs function, in which iDCs can give rise to multiple types of 'effector' DC that instruct distinct T-cell fates, including immunity, tolerance and immune deviation.

A remaining question is whether these T cell lineages are phenotypically and functionally stable. Recent studies have described certain plasticity of these T cell subsets. For example, it has been reported that the axis of PD-1 with its ligand PDL-1 converts human Th1 cells into regulatory T cells (66). Within the Treg subset there is also functional plasticity, since the loss of IL-10 and IL-35 by Tregs (double-deficient) did not deprive them to be fully functional *in vitro* and *in vivo*, but it was compensated for an increase in cathepsin E and TRAIL expression (67).

Therefore, cross-regulatory compensating pathways may exist to control the suppressive mechanisms of Tregs. In addition, the balance between Tregs and Th17 cells in culture is controlled by various factors (i.e. IL-6, IL-1, IL-23, and retinoic acid (68)), and Th17 cells are capable of converting into Tregs and vice versa. Therefore, effector Th17 cells are highly adaptable to their cytokine microenvironment, which may partially explain their association with pro- and anti-inflammatory functions (69). Interestingly, a recent study reported that plasticity of human Th17 cells and iTregs is orchestrated by different subsets of CD14$^+$ myeloid cells (70), shaping the outcome of immune reaction from inflammation to tolerance.

1.3   Immunological tolerance

The immune system is not only responsible to protect the organism from invading pathogens, but also to avoid self-responses that could destroy self-tissues. Thus, 'immunological tolerance' is also an active form of immune response aimed at avoiding autoimmune diseases. Based on their organic distribution, the immunological tolerance is divided in two scenarios, known as 'central' and 'peripheral' tolerance.

1.3.1   Central tolerance

Central tolerance is based on the positive and negative selection of T cell-precursors in the thymus. Only T-cells bearing receptors (TCRs) that recognize the own MHC molecules (on thymic epithelium) receive a survival signal leading to their positive selection. Those T lymphocytes that bear TCRs strongly reactive to self-peptides (autoreactive cells) are deleted and therefore eliminated from the repertoire by negative selection (71). Different cells contribute to this thymocite negative selection, such as *medullar thymic epithelial cells* (mTECs) that ectopically express *tissue-restricted antigens* (TRA) (i.e. insulin) under the control of a nuclear factor called *autoimmune regulator* (Aire). Medullary DCs also contribute to this process cross-presenting TRA (72), and peripheral DCs presenting

antigens from peripheral tissues when passing through the thymus (62), therefore expanding the antigen repertoire. Furthermore, the cytokine milieu in the thymus, like the influence of *thymic stromal lymphopoietin* (TSLP) produced by epithelial cells in Hassall's corpuscles, can promote the conversion of low-autoreactive CD4$^+$ CD25$^-$ thymocytes to CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs (73). Thus, within the thymus, T cells go through positive and negative selection processes to shape the entire peripheral T-cell repertoire.

1.3.2 Peripheral tolerance

Central tolerance is essential but not enough, since some autoreactive T cells escape from thymic deletion and, once in the periphery, may contribute to the development of autoimmune responses. Hence, peripheral tolerance mechanisms are designed to safe guard against these autoreactive T cells in peripheral tissues (basically lymph nodes and the spleen). Mechanisms of peripheral tolerance (74) can be divided into 'T cell-extrinsic' and 'T cell-intrinsic' mechanisms.

T cell-intrinsic mechanisms include:

a) Immunological ignorance to an antigen that could not be presented efficiently by APCs in secondary lymphoid organs, due to its low concentration. This phenomenon has been described to occur in the initial phases of some peripheral solid tumors (75).

b) Anergy is a state of long-term hyporesponsiveness in T cells that is characterized by an active repression of TCR signaling and IL-2 expression (76). Induction of anergy in T cells was initially described as the result of TCR (signal 1) without concomitant co-stimulatory signaling (signal 2). However, recent studies have demonstrated that the T cell actively sense its microenvironment, through mTOR dependent and independent mechanisms, for available nutrients and negative cues such as adenosine. This regulates the T cell commitment to switch its metabolic machinery and enter the S phase of the cell cycle, inducing

anergy and long-term tolerance in the T cell (77). Several forms of anergy have been described, and Schwartz *et al* proposed to classify them into two broad categories: *clonal anergy* (growth arrest state that can be reversed by adding IL-2) and *adaptive tolerance* or *in vivo anergy* (inhibition of proliferative and effector functions not reversible by IL-2) (76).

c) Deletion of T cell clones through induction of apoptosis is induced by the engagement of counter-regulatory receptors on T cells surface, such as CTLA4 with CD80/CD86, or PD1 with PD1L/ PDL2, during antigen presentation. Another mechanism to induce apoptosis is the Fas receptor engagement by FasL, and triggering of a Bcl-2 and Bcl-xL–regulated mitochondrial death pathway (78).

d) Phenotypic skewing or **immune deviation** is the shift of T cells towards a different effector subset expressing different cytokine patterns, i.e. from pro-inflammatory to anti-inflammatory phenotype, after activation. While Th1 and Th17 effector T cells are considered relevant for pathogenic immune response, it has been suggested that development of a Th2 effector T cell would counteract autoimmunity by promoting anti-inflammatory cytokines (79).

T cell-extrinsic mechanisms include the induction of Treg cells (explained earlier) and the induction of tolerogenic DCs. In fact, some authors suggested that tolerogenic DCs and Tregs regulate each other's homeostasis (80).

1.3.3   Tolerogenic DCs (tolDCs)

DCs are important not only in the generation of T-cell immune responses, but also in immune tolerance. The ability of DCs to induce tolerance was initially demonstrated by experiments on iDCs residing in peripheral lymphoid tissues (81). Therefore, under steady-state conditions, iDCs may capture apoptotic bodies derived from natural cell turnover and, after migration to the draining lymph nodes, DCs present self antigens and silenciate autoreactive T cells (81). However iDCs are

not in a final differentiation state and can give rise to both immunogenic/pro-inflammatory mDCs as well as semi-mature DCs, which have the ability to establish and maintain tolerance. These natural tolDCs maintain tolerance in peripheral tissues against commensal microorganisms, antigens from food and airways, etc. within a steady-state environment. In addition, many pathogens and tumors can mimic or produce tolerogenic factors and instruct tolDCs as an immune escape mechanism (reviewed by Maldonado and von Adrian (82)). pDCs have also been described to play an important role in inducing immune tolerance (83).

TolDCs are characterized by reduced expression of co-stimulatory molecules (mainly CD40, CD80, CD86) and, usually, by reduced production of IL-12 and increased IL-10 secretion (84) together with a reduced ability to induce T cell proliferation. While these properties can explain their ability to induce Tregs rather than T effector cells, several other mechanisms may play a role in tolerance induction and regulation (85).

The molecular mechanisms involved in the tolerogenic function of DCs in the periphery include:

a) Antigen presentation with inappropriate co-stimulation (induction of anergy).

b) Presentation of very low levels of antigen in the absence of other stimuli, which promotes Treg differentiation (86)).

c) Production of cytokines such as IL-10, TGF-β, TNF-α, or *granulocyte colony–stimulating factor* (G-CSF) can induce functional properties to DCs (87). For instance, presence of IL-10 during differentiation of CD4^{+} T cells results in the development of Tr1 cells (82). In addition, some tissue-DCs can synthesize retinoic acid (RA), a metabolite of vitamin A that, besides imprinting T cells to express gut homing receptors, promote the differentiation to Foxp3^{+} Tregs (88).

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d) Expression of some molecules that induce T cell death (deletion), such as the *indoleamine 2,3-dioxygenase* (IDO), the rate-limiting enzyme of tryptophan catabolism that increases a cytotoxic metabolite for activated T cells (89). In this sense, some studies support that IDO-expressing DCs contribute to peripheral tolerance by depleting autoreactive T cells (90). Furthermore, DCs also express membrane receptors that may instruct T-cell deletion, such as the interaction through the Fas-L, or the PDL-1 and PDL-2 (mentioned earlier).

e) Expression of inhibitory receptors such as ILT3 (immunoglobulin like transcript 3), that contain a cytoplasmic tyrosine-based inhibitory motif (ITIM), have shown to negatively regulate activation of DCs (91). Furthermore, CD8$^+$CD28$^-$ suppressor T cells have demonstrated to up-regulate ILT3 and ILT4 expression on DCs rendering them tolerogenic (92).

Importantly, the role of DCs in maintaining tolerance is independent of their maturation state, since immature DCs, semi-mature DCs and also mature DCs have shown to expand antigen-specific Treg cells both *in vitro* and *in vivo* (93–95). For this reason, some authors recommend to define DCs based on their effector function on T cells rather than on their phenotype (87,96). Hence we can differentiate 'immunogenic DCs' from 'tolerogenic DCs' according to their functional properties.

DCs play key roles not only as an initiator of the immune response but also as a regulator of adaptive responses, ensuring the balance between immunity and maintenance of peripheral tolerance. This dual functionality of DCs is achieved by the integration of different signals: antigen dose, DC lineage and maturation status, DC stimulation by pathogen derived products, and cytokine milieu at sites of inflammation.

1.3.4   Lost of tolerance

Under homeostatic conditions, central and peripheral tolerance ensure the selective generation and regulation of functional, non-self-reactive T cells. However, despite these multiple mechanisms to maintain tolerance, some situations can elicit an eventual activation of autoreactive T cells, which can engage an IR against antigens produced from self-tissues. This breakdown of immune tolerance could trigger an adaptive autoimmune response, leading to the development of an autoimmune disease (97).

Autoimmune diseases (ADs) could be classified in 'organ-specific' when the IR is against a tissue-specific antigen (such as in multiple sclerosis or type-I diabetes), or 'systemic' when the IR is against an ubiquitous antigen (i.e. systemic lupus erythematosus). In addition, ADs could be also classified based on their mechanism of action: mediated by antibodies, by immunocomplexes or by autoreactive T cells (98).

An important issue in the field of autoimmunity is to identify the auto-antigens recognized by T and B lymphocytes, and use this information to design new strategies to induce antigen-specific therapeutic tolerance. One of the most studied ADs and their auto-antigens has been multiple sclerosis.

2.   Multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS). Epidemiologic studies show that MS is the most common cause of neurological disability among young adults (20-40 years), with a prevalence of 1/1000 in Caucasian populations (99).

The etiology of MS remains unclear, but according to current data, environmental and genetic factors are involved in the development of MS (100). Research of susceptibility genes has involved the MHC molecules, specifically the HLA-DR15 haplotype in Caucasians, which is thought to

account for 10%–60% of the genetic risk of MS (101,102). In addition, non-genetic factors also contribute to MS etiology, such as infectious agents and behavioral influences (103).

The autoimmune response is thought to be responsible for the pathological features of the disease, which include demyelination, oligodendrocyte loss and axonal injury (104).

The disease is characterized by episodes of inflammatory activity in the CNS associated with neurological impairment such as progressive paralysis in most patients (105). These episodes are caused by localized CNS demyelination plaques, and the symptoms of MS vary depending on the location of plaques within the CNS. Common symptoms include sensory disturbances in the limbs, optic nerve dysfunction, pyramidal tract dysfunction, bladder or bowel dysfunction, sexual dysfunction, ataxia, and diplopia (106).

The diagnosis is made primarily on the basis of the medical history and physical exam (formalized as the McDonald criteria and later reviewed) (107,108), and may take into account laboratory data, such as the characteristic oligoclonal bands in the cerebrospinal fluid (CSF). Over the past two decades, the diagnosis has included the identification of white matter lesions via evaluation of T2-hyperintense lesions and gadolinium-enhancing T1 lesions on *magnetic resonance imaging* (MRI) (109), the later serving as a marker of focal inflammation due to the local permeability and breakdown of the BBB.

According to Lublin *et al* (110) there are four different courses of MS (schematized in **Figure 3**):

a)   Relapsing-remitting (RR-MS) is characterized by acute attacks of neurologic dysfunction (relapses). Over the following weeks to months after the attacks, most patients experience a partial or complete recovery of function. Between the attacks the patient is neurologically and

symptomatically stable. This is the initial clinical form of 85% of MS patients.

b) <u>Secondary-progressive (SP-MS)</u>, begins as RR, but at some point the attack rate is reduced and the course becomes characterized by a steady deterioration in function without frequent relapses. 85% of RR-MS patients develop this form after 25 years of disease evolution (111).

c) <u>Primary-progressive (PP-MS)</u> is characterized by a progressive neurological worsening from the beginning without clear relapses. It is the initial clinical form of 15% patients.

d) <u>Progressive-relapsing (PR-MS)</u> also begins with a progressive course although these patients also experience occasional attacks.
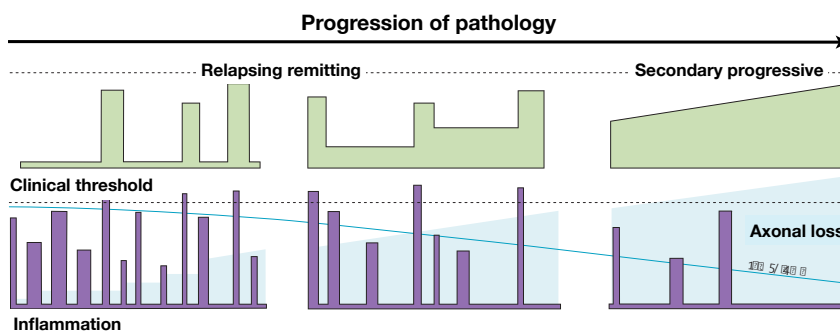
Figure 2. Frequent courses of MS (adapted from Lancet (106)). This scheme illustrates how the pathological processes of inflammation, demyelination, and axon degeneration explain the clinical course of multiple sclerosis. The 'clinical threshold' illustrates the fact that inflammatory lesions in CNS not always are manifested as a clinical episode; below this threshold the effects of individual inflammatory lesions can be compensated and above it they cause symptoms.

2.1 Immunopathology

The disease process in MS (reviewed in (112,113), and schematized in **Figure 4**) may start with the activation of autoreactive CD4$^+$ T cells in the periphery, for example by epitope mimicry, in an inflammatory context. Then, activated autoreactive T cells adhere to the BBB

endothelium via adhesion molecules such as LFA-1 and VLA-4, and transmigrate into the brain parenchyma. There, these autoreactive T cells are presumably reactivated by myelin antigen-presenting DCs that reside in the brain or have infiltrated into the CNS from the periphery. Activated encephalitogenic CD4+ T cells exert effector function by releasing pro-inflammatory cytokines (i.e. IFN-γ, IL-23, TNF-α) and chemokines (RANTES, IP-10, IL-8, and others), which activate microglia and macrophages in the CNS, and recruit other immune cells, such as monocytes, CD8$^+$ T cells, B cells, and mast cells from the peripheral blood. These pathological events result in formation of the inflammatory lesion, which is characterized by the release of proteases, pro-inflammatory molecules, and oxygen and nitrogen radicals from mast cells, monocytes, and T cells. Altogether lead to demyelination, neuronal damage, and axonal loss that is closely related to neurological disability (112).
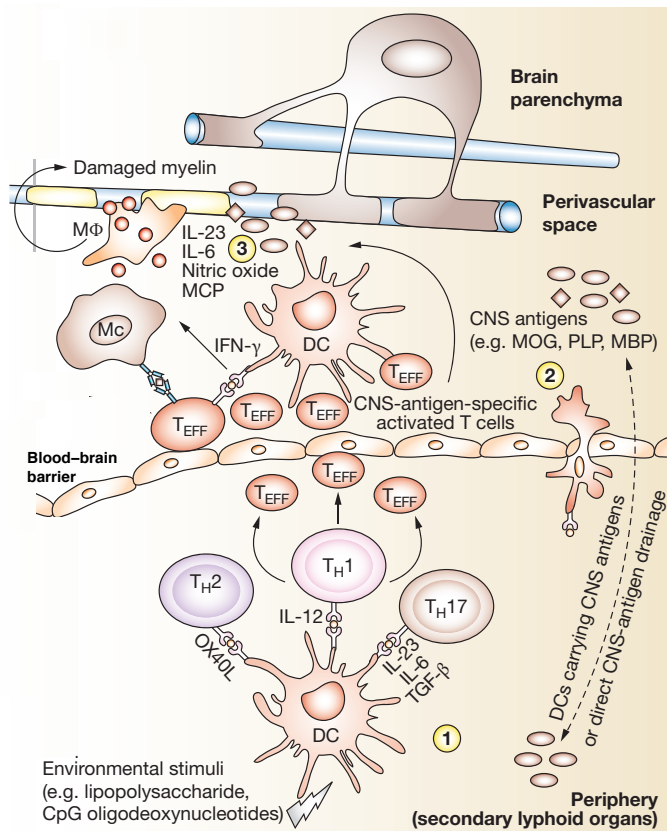
Figure 4. Pathogenic steps and factors that lead to tissue damage in MS (illustration adapted from Nature Reviews; Zozulya and Wiendl, 2008). (**1**) In response to environmental stimuli, DCs can prime and activate naive CD4+ T cells to produce IFN-□ (Th1 cells), IL-4 (Th2 cells), or IL-17 (Th17 cells). (**2**) Activated myelin-specific T cells travel through the peripheral circulation and across the blood–brain barrier into the perivascular space of the brain, where they are presumably reactivated by myelin antigen-presenting DCs that reside in the brain or have infiltrated into the CNS from the periphery. The T cells subsequently become pathogenic. (**3**) Activated encephalitogenic CD4+ T cells exert effector function by releasing pro-inflammatory cytokines, which activate microglia and macrophages, thereby contributing to a cascade of pathological events that result in demyelination and neuronal damage.

2.2   Autoreactive T cells

Although the pathogenic mechanisms of MS still remain unclear, some lines of evidence suggest a crucial role of CD4+ autoreactive T cells (114,115). Among these evidences are:

- The HLA-DR15 haplotype in Caucasians (DRB1-1501, DRB5-0101, DQA1-0102, DQB1-0602) carries the strongest genetic risk for MS, presumably via their role as antigen-presenting molecules to pathogenic CD4+ T cells (101).

- In experimental autoimmune encephalomyelitis (EAE) in rodents it is known that myelin reactive T cells are encephalitogenic, since the transfer of these cells from EAE mice to a healthy receptor developed the disease (116).

- A clinical trial demonstrated that MS patients treated with an altered peptide ligand based on the MBP(83-99) epitope developed clinical worsening, increased brain inflammation and increased anti-MBP responses (117).

- T cell responses to myelin antigens have been extensively studied in MS and control subjects. Interestingly, a higher level of activation of T cells reactive to myelin basic protein (MBP), an important constituent of CNS myelin, was found in MS patients as compared to healthy controls (118). In fact, many groups have confirmed the CD4+ T cell reactivity to myelin antigens in MS patients (119–121).

Some studies have reported evidences that these autoreactive CD4$^+$ T cells in MS patients have been previously activated *in vivo*. (114,122). The *in vivo* activation of this pathogenic autoreactive T cells could be explained by several microbial infection-mediated mechanisms, including molecular mimicry (i.e. the activation of autoreactive cells by cross-reactivity between self-antigens and foreign agents), bacterial superantigens and/or bystander activation (autoreactive cells are activated due to nonspecific inflammatory events during infections) (123,124). Recent studies have reported Th1 and Th17 as effector T cell subsets that play a key role in the regulation of IR during CNS infection and disease (125,126).

2.3 Myelin antigens

A lot of effort has been put in identifying myelin antigens that could be recognized by autoreactive T cells in MS patients (reviewed in (120)). *Myelin basic protein* (MBP) and *proteolipid protein* (PLP) rapidly gained interest due to their abundance and, most probably, MBP is the best-studied myelin protein in MS. Some reports have demonstrated that the frequencies and Th1-bias of MBP and PLP-specific T cells are increased in MS (115,127,128). *Myelin oligodendrocyte glycoprotein* (MOG), a less abundant myelin compound, has been reported to induce strong responses in MS patients as well (129). Similarly, elevated *myelin-associated glycoprotein* (MAG) specific T and B cell responses have been observed in the cerebrospinal fluid of MS patients by ELISPOT assays (130). Bielekova *et al.* demonstrated the presence of a higher proportion of high avidity myelin-specific CD4$^+$ T cells in MS patients compared to controls, which were mostly memory cells with a Th1 phenotype (115). Reactivity of these myelin-specific T cells was predominantly directed towards a group of immunodominant epitopes of myelin (MBP13–32, MBP11–129, MBP146–170, PLP139–154, MOG1–20 and MOG35–55), and also less dominant epitopes such as the *C-terminal area of 2',3'-Cyclic Nucleotide 3'-phosphodiesterase* (CNP-ase) (115).

Our group has recently confirmed a distinct reactivity to a selection of these immunodominant peptides (**Table 1**) in peripheral blood T-cells from a cohort of RR-MS patients compared to healthy controls (HC) (131) (**Figure 5**). A significant correlation was found between positive T-cell proliferation and the clinical score (a higher disability or EDSS score, a shorter relapse-free time and a higher frequency of relapses), and this reactivity was sustained over time in the majority of patients (131). These results supported the pathogenic significance of these set of myelin peptides in MS. Furthermore, these myelin peptides are expressed in the thymus (132) but are predicted to bind with low affinity to main MS-associated HLA-DR alleles (115,133), supporting a defective negative

selection in the thymus and consequently permitting a higher frequency in the periphery of these autoreactive CD4+ T cells.

Description	Sequence	aa
MBP(13-32)	KYLATASTMDHARHGFLPRH	20
MBP(83-99)	ENPVVHFFKNIVTPRTP	17
MBP(111-129)	LSRFSWGAEGQRPGFGYGG	19
MBP(146-170)	AQGTLSKIFKLGGRDSRSGSPMARR	25
PLP(139-154)	HCLGKWLGHPDKFVGI	16
MOG(1-20)	GQFRVIGPRHPIRALVGDEV	20
MOG(35-55)	MEVGWYRPPFSRVVHLYRNGK	21

Table 1. Selected myelin peptides
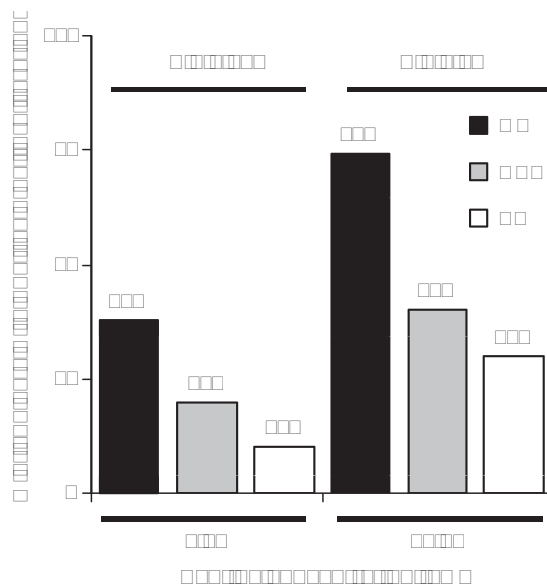
Figure 5. Proliferative response to myelin peptides. The figure summarizes the proportion of patients with RR-MS (black bars, n=42), other neurological diseases (OND) patients (grey bars, n=15) and healthy donors (HD) (white bars, n=40) showing positive proliferation to the mix of peptides used at 2μM (38% MS, 20% OND and 10% HD, P = 0.008) and at 10μM (74% MS, 40% OND and 30% HD, P=0.01). (Figure from Grau-López *et al, J Neurol* 2011).

Other myelin peptides involved in the T cell response and/or recognized by autoantibodies in cerebrospinal fluid and in serum of MS

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patients include: alpha-B cristallin, oligodendrocyte-specific glycoprotein (OSP), myelin-associated oligodendrocytic basic protein (MOBP), non-mielinic proteins as S-100 beta and transaldolase-H (Tal-H), heat shock proteins (HSP), or lipid components such as gangliosides (reviewed by (112)).

Furthermore, there is solid evidence that autoimmune response in MS changes with time and involves different antigens arising via "epitope spreading", both in humans (134) and in EAE (135). Therefore the ideal tolerization protocol in MS should be applied early in the disease course, and the strategy should allow tolerization of autoreactive T cells specific for multiple antigens.

2.4   Treatments for RR-MS

Current approved treatments for RR-MS are based in immunomodulatory and immunosuppressive drugs, which inhibit unspecifically the autoimmune response and show a modest effect on the natural progression of the disease. These MS treatments are summarized below.

Treatment of RR-MS typically consists of direct symptom management, brief corticosteroid administration for acute exacerbations, and regular use of disease-modifying drugs (136). Although corticosteroids are useful in reducing the severity and duration of relapses, they are not used as long treatment due to their fault of efficacy in disease progression and to have secondary effects (137).

2.4.1   Disease-modifying drugs in MS

The currently approved treatments that modify MS natural history can be classified as 'first-line' and 'second-line' treatments, depending on the severity of the disease (reviewed by (138).

First-line treatments are:

a) Interferon beta (IFN-ß): IFN-ß has been approved by the Food and Drug Administration (FDA) as treatment for RR-MS and has been used as a first-line therapy for many years. Although its mechanism of action has not been fully elucidated, this drug has been demonstrated efficacious by different clinical assays in reducing clinical relapses and number of active lesions (with MRI) in different clinical assays of 2-3 years of follow-up (139–141). However, besides the adverse effects that IFN-β can produce (being the most common flu-like symptoms, injection-site reactions, and lymphopenia (142)), interferon is not able to completely abolish disease activity neither administrated alone nor combined with azathioprine or prednisone (143). Although clearly a step forward in MS therapy, frequency of subcutaneous injections, adverse effects and treatment failures still motivate the search for alternative agents.

b) Glatiramer acetate (GA): GA is a synthetic copolymer made up of a random mixture of four aminoacids (glutamic acid, lysine, alanine, and tyrosine) in a specific molar ratio. Although the mechanism is still not well known, it is hypothesized that GA competes with the MBP for the binding to HLA molecules and it is able to induce immune deviation from a Th1/Th17 to a Th2 cell-type response (144). Some clinical trials demonstrated a reduction in relapses and in neural lesions (gadolinium capture) (145), RR-MS patients taking GA continuously for up to 22 years experience minimal disability progression. Although GA appears to be well tolerated, this treatment requires daily subcutaneous injections and is beneficial to only a minority of MS patients (146).

IFN-β and glatiramer acetate are generally selected based on clinician impression and patient preference as first-line therapies in RR-MS. When these agents are unable to stabilize disease progression with ongoing relapses, alternatives have to be considered (136).

Second line treatments are:

a) **Immunosupressive drugs:**

    i. *Azathioprine:* It is a purine analog derived from the mercapto-purine, and it mainly targets activation, proliferation, and differentiation of both T and B lymphocytes (147). There has been off-label use of azathioprine to treat MS for more than 30 years, and a systematic review of their efficacy indicated that it reduced relapses and disease progression (147). A major safety concern with chronic azathioprine treatment is the increased risk of non-Hodgkin lymphoma or other malignancies during long-term use (148).

    ii. *Cyclophosphamide:* Reduce the lymphocytes traffic through the blood and the synthesis of IgG in the CNS. Although it presents moderate adverse effects (149), it is an approved treatment for primary-progressive MS.

    iii. *Mitoxantrone:* Synthetic derived from the Anthracycline, with anti-tumoral activity. Suppresses the T lymphocytes proliferation and migration, antigen presentation and reduces the production of pro-inflammatory cytokines. It has been shown to increase the time between relapses, and has yielded promising results in induction regimens of mitoxantrone followed by IFN or GA preparations to stabilize disease and delay progression (150). It has however, serious adverse effects as cardiotoxicity and risk of leukaemia (142).

b) **Natalizumab:** It is a recombinant and humanized monoclonal antibody that binds integrin VLA-4 (on lymphocytes) blocking its complexing with receptor VCAM-1 (on endothelium). This blocking inhibits crossing of autoreactive T cells through the BBB, avoiding the CNS damage. Natalizumab was approved by the European Commission as RR-MS treatment after showing reduction in relapses and in neural lesions (151). Its use, however, has been associated with some cases of *progressive multifocal leukoencephalopathy* (PML), a CNS disease caused by Natalizumab-reactivated JC virus (152), which could be diagnosed on the

patient sera (JC virus antibodies) prior to the Natalizumab administration (153). Other adverse reactions documented include allergic and hypersensitivity reactions at the time of infusion and possibly more severe respiratory tract illnesses.

c) Fingolimod: It is a sphingosine 1-phosphate (S1P) receptor agonist, which binds with high affinity to S1P receptors, thereby sequestering lymphocytes in the lymph nodes and preventing their egress into the peripheral circulation (154). As a consequence, there is a reduction in the infiltration of autoreactive lymphocytes into the CNS. In large multinational trials in patients with RR-MS, oral fingolimod was more effective than oral placebo and intramuscular IFN-β in reducing the annualized relapse rate. Fingolimod was generally well tolerated in these trials of up to 2 years' duration. However, further clinical experience is required to fully determine its long-term safety profile (155). Common adverse events described in trials of fingolimod are bradycardia and atrioventricular conduction blockade during initial introduction of the drug, macular edema, liver function test abnormalities, lymphocytopenia, hypertension and also herpes virus infections have been documented (156).

d) Alemtuzumab: anti-CD52 monoclonal antibody present in lymphocytes and monocytes. Pulsed administration 12 months apart has been shown to significantly deplete T cells and modulate the lymphocyte repertoire (157). A phase II trial versus IFN-β 1a in patients with early RR-MS showed significant reductions in annualized relapse rate and sustained increase in disability. However it was to be ceased early due to reports of patients with immune thrombocytopenic purpura (ITP) (157). There was also a significant increase in infections, probably due to the profound lymphocyte depletion that occurs in the first 6 months after infusion.

Other monoclonal antibodies, as Rituximab (against CD20+ cells) and Daclizumab (against the IL-2 receptor alfa chain) have demonstrated efficacy in the treatment of RR-MS (158) but present some adverse effects

that may restrict their use (159). Currently, several new treatment strategies for MS are being evaluated in clinical assays. Some of them are oral drugs as Cladribine (approved in Australia but not in Europe or USA), Laquinimod, Teriflunomide or Dimethyl Fumarate (136). These treatments are effective in modifying MS natural course but reduction of long-term disability of MS patients has not been demonstrated yet. Moreover, they require long-term regular injection or parenteral infusions. All these inconveniences indicate that new therapeutic approaches need to be investigated for the treatment of MS.

2.4.2   Antigen-specific therapies in MS

The induction of antigen-specific tolerance has been considered the "holy grail" of immune therapy, because aims to specifically delete/inhibit pathogenic autoreactive cells while avoiding generalized long-term immunosuppression and, therefore, trying to correct the causes of autoimmune diseases at their roots. Thus, the ideal treatment would be an early intervention using an antigen-specific tolerance protocol that selectively targets both activated and naive autoreactive T cells, ideally blocking epitope spreading at an early stage and preventing progression of the disease.

Since CD4+ T cells are key contributors to the underlying pathogenic mechanisms responsible for the onset and progression of MS, they are also logical target for therapeutic intervention. Most strategies of antigen-specific tolerance interfere at the level of antigen presentation and activation of effector T cells by antigen presenting cells (APC). In this context they can modulate T cell activation either through direct interaction with the trimolecular complex (TCR/Ag/HLA) or via regulatory mechanisms through the induction of cytokines and regulatory cells. The basic tolerizing mechanisms include anergy, clonal deletion, immune deviation and induction of regulatory cells.

Despite many successes of antigen-specific therapies in animals, so far the attempts in humans resulted in several difficulties including lack of

efficacy, disease exacerbation and hypersensitivity reactions (160). The applied strategies in MS are summarized below:

a) **Tolerance induced by the mucosal (oral/nasal) route:** T cells found within the gastrointestinal surfaces are constantly exposed to exogenous foreign Ags and allow for protective tolerance against some (primarily food) Ags while at the same time serving as an immunological defense against other harmful (pathogenic) Ags. For this reason, the induction of tolerance using the mucosal route for the administration of soluble Ags is appealing, as it is antigen-specific, relatively easy to administrate, and it carries low risk of toxicity. In fact, orally administered antigens showed to induce deletion or Treg conversion depending on the dose treatment (161). Although the encouraging results obtained in EAE models (162) and in clinical trials phase I/II, a large phase III (placebo controlled) trial with oral MBP and GA failed to demonstrate significant effects on the clinical and immunological outcomes (163).

b) **Administration of myelin peptides:** Intravenous injection of high doses of soluble MBP leaded to the deletion of autoreactive T cells upon re-stimulation with the cognate peptide in EAE mice (164). Therefore, tolerance induced by soluble peptides may be useful for Ag-specific immunotherapy for human autoimmune diseases. The induction of tolerance to soluble MBP peptides was examined in two phase I clinical trials in primary-progressive MS patients, being well tolerated and showing favorable effect on disease progression (165,166). Based on these results, a phase III trial to assess the effect of the synthetic peptide MBP8298 i.v. injected was started, but results showed no efficacy in SP-MS patients (167).

c) **Intravenous injection of a solubilized MHC-peptide complex:** This strategy has the aim to engage the TCR of autoreactive T cells without delivering co-stimulatory signals, thereby inducing clonal anergy. Administration of the solubilized MHC-MBP84-102 complex ameliorated EAE in mice (168), but did not show significant effect in patients with SP-MS (169).

d) Altered peptide ligands (APLs): are analogues of immunogenic peptides that have been modified by introducing one or more amino-acid substitutions in positions essential for the contact with TCR, but retain the MHC binding motifs. APLs typically bind with lower affinity to the TCR than the native peptide, and function as either antagonists or partial agonists. APLs can block T cell responses or induce immune deviation from Th1/Th17 to Th2-/Th3-cell dependent responses, or bystander suppression through the induction of Tregs (170). In vivo administration of these myelin APLs were reported to prevent or reverse clinical disease progression in EAE (171). Based on this results, a phase II trial with an APL derived from the immunodominant MBP peptide 83-99 was started in MS, but the trial had to be halted because some patients developed relapses (117). Immunologic studies demonstrated that these relapses were driven by encephalitogenic T cells reactive to MBP83-99 that were stimulated by the APL. A second trial using another APL of MBP83-99 was suspended because of hypersensitivity reactions in 9% of the patients (172). In contrast to the aforementioned trials, Glatiramer acetate, which is thought to act as an APL, is the only approved semi-Ag-specific drug for the treatment of MS.

e) T cell or TCR vaccination: This treatment is based on the injection of inactivated antigen-specific T cells or TCR peptides with the aim of inducing an IR against autoreactive T cells of MS patients. The potential use of T cell vaccination has been tested in several clinical trials to treat MS patients (173–175), being well tolerated and accompanied by a reduction in the frequency of autoreactive T cells. Similarly, a pilot trial of a TCR peptide vaccine also boosted peptide-reactive T cells in patients with progressive MS, achieving a reduction of MBP response and remaining clinically stable without side effects during one year of therapy (176). These results hold promise for treatment of MS.

f) DNA vaccination: is based on the intramuscular injection of a plasmid encoding an auto-antigen, leading to a low-level expression of it in muscle cells. A DNA vaccine of whole MBP was well tolerated and

provided favorable trends on MRI in MS patients with active disease. This effect was paralleled by a marked decrease in peripheral blood Th1 CD4+ T cells reacting against myelin and reduced titers of myelin-specific auto-antibodies in CSF (177). A larger phase II trial is currently under way to further assess its clinical efficacy. As an advantage compared to other tolerization strategies, DNA vaccine offers the opportunity to combine expression of the auto-antigen with expression of anti-inflammatory cytokines in a single plasmid, or to act on different myelin epitopes at the same time, which might enhance the efficacy. In fact, a DNA vaccine encoding full-length human MBP has already been translated into the clinic, with the name of BHT-3009 (178). A phase II clinical trial showed that this DNA vaccine was safe, induced immune tolerance to MBP and to other myelin antigens, and reduced the number of active lesions, which was accompanied by a decrease in clinical relapse rates (178). BHT-3009 appears to be a promising new approach for the treatment of MS, although further clinical trials are warranted to confirm the early findings.

g) **Administration of ECDI-peptide-coupled cells:** Administration of peripheral blood lymphocytes (PBL) coupled with seven immunodominant peptides (MBP13-32, MBP111-129, MBP146-170, MBP83-99, MOG1-20, MOG35-55, PLP139-154) with the coupling agent *1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide* (ECDI) has been tested in the EAE model. The results shown that a single i.v. injection of murine myelin peptides-coupled splenocytes is highly efficient in inducing long-lived peptide-specific tolerance in vivo (179). This protocol effectively reduced the onset and severity of all subsequent relapses when given after EAE induction, indicating that specific tolerance can down-regulate an ongoing autoimmune response (180). Moreover, these studies in EAE have shown that tolerance can be simultaneously induced to multiple epitopes using a cocktail of encephalitogenic myelin peptides, thus providing the capacity to target autoreactive T cells with multiple specificities. In humans, tolerization of T cells by autologous antigen-coupled APCs treated with ECDI has shown to be effective in vitro (181).

Based on the current success in treating EAE, this therapy is near to be tested in a Phase I/IIa clinical trial for treating new-onset RR-MS patients (182).

There are other approaches under development that also aim to achieve antigen-specific immunoregulation in MS, for example:

- <u>Transfer of regulatory T cells</u>, which is typically achieved by initially extraction from patients, in vitro modulation and/or selection of autologous T cells, and subsequent administration of T cells back to the patients, has demonstrated some promising data both in animals and in humans (183,184). However, the main hurdles of this approach are the selective targeting of relevant autoreactive T cells, and that Treg-mediated suppressive activity can also contribute to the immune escape of pathogens or tumors.

- <u>Transfer of tolDCs</u>, with the aim of inducing tolerance to the specific antigens that elicit pathologic immune responses, without compromising the immune defense against pathogens or tumors. This approach has been demonstrated to be safe and effective in different animal models of autoimmunity (reviewed in (185)) and will be tested soon in clinical trials, as further explained in the next section.

- Targeting of DCs *in vivo* with monoclonal antibodies, such as anti-DEC205-mediated delivery of the $PLP_{139-151}$, which attempts to induce differentiation of tolDCs *in vivo* (186). However, accurate targeting of DCs in vivo and ensuring the function of modulated DCs remain difficult to accomplish.

3. DC-based immunotherapy (from bench to bedside)

As DCs are involved in the regulation of both immunity and tolerance, they could have many clinical applications for treatment of immune-based diseases. In fact, following the establishment of protocols for the generation of DCs from murine bone marrow (187) or human peripheral blood (188), the potential of DCs for clinical applications has been under extensive investigation (189–191). Up to now, DCs have been tested for therapy of infectious diseases such as HIV-1 (192,193), various cancers such as lymphoma, melanoma and renal carcinoma (190,194), post-transplant graft versus host disease (GVHD) (195,196) and various autoimmune diseases (reviewed in (185,197)). Many clinical trials of DC-based immunotherapies show that this method is reliable, safe and therapeutically efficient (198,199). However, there are still some challenges to the therapeutic development of DCs, such as the standardization of protocols for DCs generation. This standardization includes the definition of tissue sources, of growth factors and cytokines, the maturation stimulus or not, and the identification of antigens to be loaded, which are further developed below.

3.1   Cell sources used for DC generation

The first approach to obtain DCs for a DC-based therapy was the use of peripheral blood DCs, but they are present at very low numbers in human blood (around 1% of the PBMCs) (11), which add a difficulty working with them. Interestingly, it is well established that DCs can be differentiated in vitro from various cellular sources, including bone marrow (BM), cord blood (CB) or PBMCs, from which it can be obtained both CD34+ (stem cells) and CD14+ (monocytes) cells. The most widely used cell source for DC generation are monocytes (MDDCs) since the first protocol reported by Sallusto and colleagues (200), probably due to they can be easily obtained from peripheral blood without previous mobilization.

Monocytes are usually isolated from PBMCs, which are usually obtained performing a leukapheresis (method based on differential centrifugation that permits to separate millions of PBMCs) (201). The techniques used for enrichment and purification of monocytes from PBMCs (and subsequent DCs generation) are plastic adherence (202)], density gradient centrifugation (203), and the most used for DC-based therapy protocols: positive and negative immunomagnetic selection (204) and elutriation (technique based on a counter-flow centrifugation to physically separate cells depending on their size and density) (201), both showing high monocyte recovery from the leukapheresis product (around 80% and 109 of monocytes) (201,205).

A different way to obtain DCs *in vitro* has been recently proposed by Silk et al., using human induced pluripotent stem cells (iPSCs) or human embryonic stem cells (hESCs) as progenitors (206), but it will require further work to be fully characterized.

3.2 Role of cytokines in DCs generation

Although a wide variety of conditions have been reported to be able to support DC generation, the majority of research and clinical protocols to date differentiate DCs from precursors using granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF is involved in monocyte survival and differentiation both in humans and in mice (207). Lower concentrations of GM-CSF promote only cell survival, while higher concentrations of GM-CSF lead to cell survival and cell proliferation, differentiation or functional activation (208). This factor has been usually used in combination with either TNF-α or IL-4 (200,209).

Several cytokines have been shown to be able to induce DC differentiation under a variety of conditions. According to recent reports, cytokines such as IL-2, IL-6, IL-7, IL-13, IL-15 and hepatocyte growth factor (HGF), can contribute to the generation of DCs from monocytes (210). For instance, IL-3 is an important cytokine for generating DCs from monocytes (211), by promoting their survival and exhibiting

phenotypic and functional properties highly similar to those DCs generated with GM-CSF and IL-4 (212). As well, Flt3 ligand (Flt3-L) combined with stem cell factor has been reported to enhance *in vivo* DC recovery (213), according with recent findings (17,18). However, Flt3-L seems to act on a distinct population of DCs compared to GM-CSF (214,215).

Monocytes cultured with GM-CSF and IL-4 or TNF-α render immature DCs (200) that are inefficient in antigen presentation and migration. Upon treatment with maturation factors, DCs acquire the characteristics of mature DC including morphology, loss of monocyte markers (such as CD14+), up-regulation of co-stimulatory molecules and MHC-II to the cell surface, and finally the capacity to efficiently prime naive T cells. Also, maturation induces de novo expression of CD83 that is therefore used as a surrogate marker for mature DCs (216). Maturation increases the antigen presentation and migratory capacity of DCs. Certainly the ability of DCs to generate an effective immune response has been correlated with their maturation state (217,218). For this reason, the generation of DCs for immunotherapy requires the addition of a potent maturating stimulus.

3.3 Maturation stimuli for DC generation

A wide variety of stimuli are able to induce DC maturation and these include signals from T cells such as CD40L (219), inflammatory cytokines such as TNF-α, IFN-γ and IL-1, bacterial stimuli such as LPS (200), live Gram positive bacteria (220), bacterial DNA and double-stranded RNA (221). In addition, infection by pathogens such as the influenza virus can also directly induce the maturation of DCs (217).

Some molecules such as CD40 ligand (CD40L) can play a dual function in DC activity: to generate DCs from monocytes, and to be a potent maturation factor for DCs, enhancing their stimulatory ability (222). However, some studies suggest that CD40 ligation might not be sufficient for optimal maturation (223). Hence, a combination of

signaling through CD40 and IFN-γ was required to optimally mature DCs that produced high levels of IL-12p70, which the authors suggest as a more accurate marker for mature DCs than CD83 expression (223). In fact, TNF-α-matured DCs have also shown a lack of IL-12 secretion and consequently, a reduced ability to activate T cell responses *in vitro* (224).

LPS, due to its bacterial origin and its predominant role as a PAMP, represents a prototypical model for DC maturation. Hence it has been described that iDCs generated with TNF-α and GM-CSF can be converted into mature DCs in the presence of LPS, which are then able to stimulate both Th1 and Th17 immune responses (225). Additionally, new TLR agonists have been brought to the clinical setting as a new generation of vaccine adjuvants and immunomodulators (226,227). These clinical-grade TLR agonists may also be used under the restrictive good manufacturing practice (GMP) conditions to generate MDDC for clinical treatments (228). In this sense, the low toxicity LPS-derivative monophosphoryl lipid A (LA) in combination with IFN-γ showed to induce potently immunogenic MDDCs (229).

The first maturation medium in which iDCs were cultured was monocyte-conditioned medium (MCM) (230). In fact, MCM is the supernatant of the cultured monocytes and contains a variety of cytokines that one cannot have control over its composition. Various cytokine cocktails have therefore been developed to replace MCM, with the goal of defining a standard procedure for clinical and experimental purposes. The first and most frequently used cocktail for DC maturation contains TNF-α, IL-1ß, IL-6, and prostaglandin E2 (PGE2). The rationale for the use of this cocktail is to enhance the pro-inflammatory effects of TNF-α in an attempt to mimic the physiologically inflammatory environment. IL-6 is a potent, pleiotropic, inflammatory cytokine that mediates many physiological functions (231). PGE2 is believed to play an important role in DC migration and lymph node homing (31), but it is also considered to have some inhibitory properties on DCs, such as the induction of IDO and the increased secretion of IL-10 (232). These properties may permit

the expansion of Tregs or the induction of a Th2 response by such matured DCs (233), which could be a reason for the limited efficacy of MDDC-based tumor vaccines (194).

An alternative cytokine cocktail contains IL-1β, TNF-α, Poly (I:C), IFN-α and IFN-γ (234). This maturation regime seems to generate DCs with the capacity to induce a Th1 response, but its in vitro T-cell stimulation failed to show a clear improvement in relation to DCs matured with the PGE2 cocktail (235). Another cytokine cocktail without PGE2 is the composed by LPS and IFN-γ (236) that has shown to generate DCs much more stimulators of antigen-specific T cells in vitro than PGE2-matured DCs (237).

Other cytokines such as IL-16 or IL-17 have been involved in promoting DC maturation in vitro. IL-16 is a natural ligand for the CD4 receptor and has the ability to up-regulate CD25 and CD83 expression in MDDC (238). IL-17 is a T cell-derived cytokine that stimulates stromal cells and macrophages to secret pro-inflammatory cytokines (239). In fact, it has been hypothesized that IL-17 may contribute to allogeneic immune responses during organ and stem cell transplantation (240).

Remarkably, molecular gene expression analyses (using DNA array and SAGE: Serial Analysis of Gene Expression) suggested that exist fundamental differences between DC populations differently matured, which may have functional implications (241). In this sense, the comparison and study of the effect of different maturation protocols will permit to improve the effectiveness of DC-based immunotherapy (242).

3.4   TolDCs generation

In the last decades, researchers attempted to emulate the conditions leading to tolDCs differentiation and function, in order to understand the underlying biology and to use tolDCs for immunotherapy (84,243,244). But, what constitutes a tolDC? Generally, the low constitutive expression of surface MHC and co-stimulatory molecules,

resistance to maturation in response to 'danger signals', the ability to acquire and present antigen to T cells, and finally the ability to expand Tregs and/or to delete/anergize T cells. All these features can be induced in vitro using anti-inflammatory biologicals, pharmacologic agents, or genetic modification of DCs (82,244) (**Figure 6**).
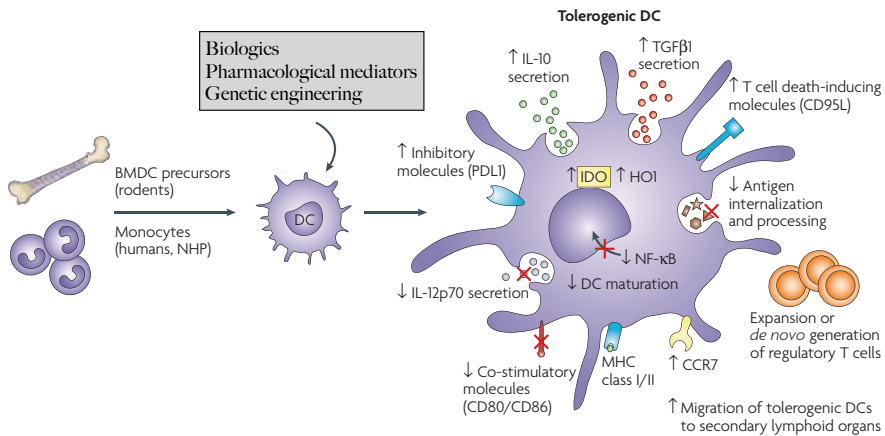

Figure 6. Generation of tolDCs *in vitro* (illustration adapted from Nature Reviews Immunology; 244). DCs that are generated *in vitro* from bone marrow (BMDC) precursors in rodents or blood monocytes in humans and non-human primates (NHP) have been rendered tolerogenic by controlling their culture conditions through exposure to biologics, pharmacological mediators, or by genetic engineering.

3.4.1   Generation of tolDCs using biologics

Diverse biomolecules that are physiologically encountered in tolerogenic situations in vivo, can also induce tolDC differentiation in vitro, and are commented below.

Incubation of DCs with IL-10, alone or in combination with other cytokines, confers a certain capacity to induce Tregs, including CD4+CD25+, CD8+, and invariant NK cells (245). The suppressive capacity of these Tregs has been extensively tested in models of allograft rejection, allergies, and graft-versus-host disease (recently reviewed in (82)). Signaling through the IL-10 receptor maintains iDCs in their immature state even in the presence of maturation signals (246).

TGF-β, a cytokine produced by Tregs and other cells, has also profound effects on DCs in vitro. The use of animal models has revelead that TGF-β allows DCs to attenuate the neuropathology associated with EAE (247).

Other bioderivatives instructing tolDCs are HGF and the active metabolite of vitamin D: 1α,25-dihydroxyvitamin D3 (vitD3). When treated in vitro with these compounds, DCs initiate the expression of gene products that have been implicated in immune tolerance, including IDO, CCL2, IL-10, TGF-β, TRAIL, and the inhibitory receptors CD300LF and CYP24A1 (87,248).

Several other factors, such as estrogen, vasoactive intestinal peptide (VIP), binding immunoglobulin protein (BiP), thymic stromal lymphopoietin (TSLP), GM-CSF, PGE2, and TNFα, may also promote Treg-inducing ability on tolDCs. For instance, DCs generated in the presence of GM-CSF and TSLP may induce proliferation of naive CD4+ T cells and also CCL22 and CCL17 chemokine production, both attracting CCR4-expressing Th2 cells (249). Culture of murine BM precursors in low doses of GM-CSF has also shown to produce DCs that induce alloantigen-specific T cell hyporesponsiveness in vitro (250).

TolDCs could be also generated using antibodies and synthetic soluble ligands of surface receptors. As an example, HLA-G (a non-classical histocompatibility molecule and the natural ligand for ILT4) impaired the maturation of human MDDCs in vitro and induced suppressive autologous CD25+CTLA4+ T cells (251). Co-stimulatory blockers such as anti-CD40L have also shown to induce tolDCs (252).

3.4.2   Pharmacologically induced tolDCs

The use of immunosuppressive drugs and anti-inflammatory agents has been crucial for the treatment of many diseases. Not surprisingly, both frequently affect DC immunogenicity often by intervening with their maturation. Possibly, this effect is via inhibition of the NF-kB

transcription factor family, which down-regulates many cytokines, such as IL-1, IL-6, and TNF-α, but increase IL-10 production (243,253,254). Thus, pharmacological agents have been successfully employed to manipulate DCs function both in vitro and in vivo in many disease models (243,255), and include: anti-inflammatory agents (such as acetilsalicylic acid), histamine, adenosin receptor agonists, and immunosuppressive drugs including corticosteroids, cyclosporin A, rapamycin, deoxyspergualin, tacrolimus (FK506), mycophenolate mofetil (MMF), and BAY-117085 (256). Apart from these classical immunosuppressive drugs, there are other agents less addressed, such as resveratrol, imiquimod, curcumin, or triptolide, that have also shown to induce tolDCs (recently reviewed by Svajger et al (257)).

Glucocorticoids (GCs) were the first immunosuppressants to be used in a clinical setting (258). Treatment of human MDDCs or mouse BMDCs with prednisolone or dexamethasone leads to tolDCs differentiation with the ability to instruct aTregs (259,260). The binding of GCs to their receptor regulates DC activation, negatively modulating the canonical NFκB pathway, inflammatory cytokines, chemokines, and antigen presentation molecules (258). In addition to repress DC maturation, dexamethasone also induces a discrete set of anti-inflammatory gene products and chemo-attractants, including IL-10, GITRL, IDO, CCL2 (MCP-1), CCL8 (MCP-2), CCR2, CCL9 (MIP-1c), and CCLl2 (MIP-2) (261). This impairs the DCs' ability to migrate and provokes them to assume a tolerogenic phenotype capable of instructing T cells to express CD25, Foxp3, and IL-10.

Recent observations suggest that cellular metabolism also plays a role in DC immunogenicity, for example the pathway of serine/threonine kinase mammalian target of rapamycin (mTOR). Inhibition of mTOR by **rapamycin**, a macrolide from Streptomyces hygroscopicus, exerts immunosuppressive effects in humans and animals (262) and has shown efficacy in both clinical and preclinical settings of autoimmunity and

inflammatory disease (263,264). Treatment of DCs with rapamycin has shown to stimulate Treg expansion *in vivo* and *in vitro* (263,265,266).

One of the most efficient pharmacological agents is the **vitD3** which, besides its function as an important secosteroid hormone for the regulation of body calcium homeostasis, exerts a large number of biological effects as an essential organic compound with important immunoregulatory properties (267). The biological effects of vitD3 are mediated by the vitamin D receptor (VDR), which function as a transcription factor binding to specific genes (268). DCs are key targets of VDR agonists both *in vivo* and *in vitro*, and modulate their phenotype and function towards semi-mature DCs (269–271). The phenotype of vitD3-conditioned MDDCs showed a down-regulated expression of MHC class II molecules and the co-stimulatory molecules CD40, CD80 and CD86; the up-regulation of inhibitory molecules ILT3; impaired production of IL-12 and enhanced IL-10 production (recently reviewed in (271)). This tolDC phenotype resist further differentiation into a mature one, even when exposed to strong maturation stimuli including pro-inflammatory cytokines, LPS or CD40L (272,273). In addition, Széles et al. (248) suggested that vitD3 regulates a large set of its targets autonomously and not via the inhibition of differentiation and maturation of MDDCs, leading to the tolDC state. Different mechanisms have been attributed to vitD3-treated DCs, as the induction of T cell anergy (due to the reduction in antigen presentation and T cell co-stimulation (274)), the dampening of Th1 responses, and recruitment and differentiation of regulatory T cells (270).

3.4.3   Genetically induced tolDCs

Several genetic manipulations have been used to modulate the maturation of DCs and inducing tolDCs (244). With this end, the selected genes can be transferred to DCs through viral or nonviral delivery systems (including liposomes and electroporation) (275), or could be knock-down by selective gene silencing such as anti-sense

oligodeoxynucleotides (ODNs) and small interfering RNAs (siRNA) (276). Using these techniques, tolDCs have been generated by either inducing the expression of different immunomodulatory molecules (such as IL-4, IL-10, TGF-β, CTLA-4, or PDL-1 among others) or, in contrast, by inhibiting specific molecules involved in DC activation (i.e. IL-12p35, CD40, or CD86) (reviewed in (185,244)). These genetically induced tolDCs have shown in some cases to induce hyporesponsiveness and prolong allograft survival in mice (277), to induce Treg differentiation (278), and to suppress diabetes or delayed-type hypersensitivity (DTH) in mice (279). However such technology will require further developments and studies before establishing clinical-grade tolDCs.

3.5   Clinical-grade tolDCs

Clinical-grade DCs were initially designed and tested in clinical trials for cancer treatment. Since then, the knowledge and expertise around DC-based vaccines have increased considerably, and tolDCs have been explored for their potential use in transplantation and in treating autoimmunity. Indeed, the prophylactic and therapeutic potential of tolDCs has been proven predominantly in experimental animal models of human autoimmune diseases, such as type-I diabetes, MS, and rheumatoid arthritis (RA) (summarized in (185)) and also in transplantation (195,196,244). Importantly, the results obtained by these studies aimed to consider the application of tolDC therapy to human autoimmune pathologies.

As clinical studies progress, the regulatory agencies require to accomplish the restrictive good manufacturing practice (GMP) guidelines when preparing DCs (as well as other cell types) for immunotherapy (212). When manufacturing clinical-grade DCs it is important to monitor the quality of DCs before their release to the clinical setting. The predefined criteria for release of clinical-grade DCs are sterility testing and the evaluation of viability, purity, stability, and potency to ensure consistency and quality of the cell product (280).

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Currently, two clinical trials are ongoing to test tolDC-based immunotherapy in patients with autoimmune diseases. Specifically, tolDCs designed to express low levels of CD40, CD80, and CD86 (using ODN) are in a Phase I clinical trial to evaluate safety in an adult cohort with insulin-requiring type-1 diabetes (M.Trucco, University of Pittsburgh (PE, USA), ClinicalTrials.gov identifier: NCT00445913) (281). Soon will start a clinical trial to treat patients with rheumatoid arthritis using autologous tolDCs treated with dexamethasone and vitD3 (C.M.U. Hilkens and J.D. Isaacs, University of Newcastle (U.K.), ClinicalTrials.gov identifier: NCT01352858 (282)). Another clinical trial is being prepared in rheumatoid arthritis with BAY117082 treated-DCs (256) (Thomas et al., University of Queensland (Australia)).

Considering this scenario, we believe that these imminent clinical trials will reveal the security and efficacy of treating autoimmune diseases with tolDC-based vaccines, and will permit to do a step torwards their cure.

Hypothesis and Objectives

Current treatments for RR-MS patients decrease the frequency of relapses and reduce inflammatory activity in a nonspecific manner, but their effect on disease progression is still unclear. Therefore, in order to modify the course of MS, new and more specific therapeutic approaches are necessary. Specific inhibition or deletion of autoreactive T cells is an interesting goal for restoring peripheral tolerance in autoimmune diseases such as MS.

We hypothesize that infusion of antigen-specific autologous tolDCs (previously generated *in vitro*) to MS patients may inhibit autoreactive T cells, hence re-establishing tolerance to self-antigens. The main goal of this work is to generate and characterize tolDCs from RR-MS patients, loaded with myelin peptides as specific antigen, as a prof-of-concept study for evaluating the feasibility of re-establishing tolerance using such cells in a future clinical trial.

The specific objectives pursued in this thesis are outlined below:

1) Standardization of a protocol to generate tolDCs in GMP conditions.

   a) Determination of the most convenient tolerogenic agent to generate tolDCs for MS therapy.

   b) Determination of the optimal maturation stimulus to generate tolDCs for MS therapy.

2) Generation and characterization of tolDCs from MS patients following the pre-established protocol, specifically:

   a) Comparison of phenotype and function of HC and MS patient-derived tolDCs;

   b) Determination of the best conditions for loading tolDCs with myelin peptides;

c) Investigation antigen-specificity and tolerogenic function of tolDCs.

Results

Results are presented as a compendium of three published manuscripts on the generation of tolDCs for cell therapy in MS. Altogether these manuscripts have contributed to the development of a protocol to generate clinical-grade tolDCs and have set up the bases for the use of tolDCs as a therapeutic tool to re-establish tolerance in RR-MS patients. A summary of these publications is detailed below.

Results I

Comparative study of clinical grade human tolerogenic dendritic cells.

Naranjo-Gómez M, Raïch-Regué D, Oñate C, Grau-López L, Ramo-Tello C, Pujol-Borrell R, Martínez-Cáceres E, Borràs FE. *Journal of Translational Medicine*. 9, 89 (2011)

In this study we compared the capabilities of three different GMP-grade immunosuppressive drugs (dexamethasone, rapamycin and vitamin D3) in obtaining tolDCs. Our results show relevant differences exhibited by the three types of clinical-grade tolDCs. Regarding the maturation phenotype, only Dexa-and VitD3-DCs showed a reduced expression of surface maturation markers, oppositely to Rapa-DCs that express co-stimulatory molecules at similar level than fully mature DCs. Cytokine profile experiments revealed that Dexa-DCs and moderately VitD3-DCs produced IL-10, in contrast to Rapa-DCs, whereas the secretion of IL-12p70 was not detected in any case. Functionally, all three tolDCs sustained a poor alloantigen T cell proliferation compared to mature DCs, and reduced the secretion of IFN-γ. In addition, only Rapa-DCs promoted $CD4^+CD127^{lo/neg}CD25^{hi}$ Foxp3$^+$ T cells.

These contrasting influences of the three clinical-grade pharmacological agents on tolDCs generation should be considered when applying these tolDCs for a particular disease. In our case, we chose vitD3 as a tolerance-inducing agent for generating tolDCs for MS therapy.

Differential effects of monophosphoryl lipid A and cytokine cocktail as maturation stimuli of immunogenic and tolerogenic dendritic cells for immunotherapy.

In this work we performed a comparative study to find the best maturation stimulus to generate tolDCs. We compared three clinical-grade maturation stimuli: the classic cytokine cocktail (CC)—composed of IL-1b, TNF-a and PGE-2–, the monophosphoril lipid A (LA) and their combination (CC+LA). We evaluated the *in vitro* effects of these maturation stimuli on both immunogenic and tolerogenic (1α,25-dihydroxyvitamin D3-treated) MDDCs.

The results point to the combination of CC+LA as the best stimuli for immunogenic DCs, since they induced the production of IFN-g and IL-17 in allogeneic co-cultures, besides a fully mature phenotype. In contrast, we identified the CC as the best stimuli for tolerogenic DCs, since CC permitted to obtain functionally stable tolDCs along with a strikingly capability of suppressing an immune response.

Altogether these results demonstrate the importance of choosing an appropriate stimulus to modulate the therapeutic potential of DCs suitable for immunotherapy.

Stable antigen-specific T cell hyporesponsiveness induced by tolerogenic dendritic cells from multiple sclerosis patients.

In this study we investigate the generation and characterization (phenotypic and functionally) of tolDCs from relapsing-remitting (RR)-MS patients with the aim of developing a cellular immunotherapy for MS. Based on our previous studies, we generated tolDCs from monocytes in the presence of vitD3 and matured with the pro-inflammatory cytokine cocktail.

We demonstrate that there are no significant differences between tolDCs generated from HC and from MS patients' cells, both presenting a tolerogenic profile. We also investigate the optimal conditions for myelin-peptide loading to tolDCs. Importantly, myelin peptide-loaded tolDCs from MS patients induced stable antigen-specific tolerance, or hyporesponsiveness state, in autologous myelin-reactive T cells in vitro.

These results suggest that myelin peptide-loaded tolDCs may be a powerful tool for inducing myelin-specific tolerance in RR-MS patients.

As a global summary of the results, we have standardized a protocol to generate clinical-grade tolDCs. First we defined the best tolerogenic inducing agent (vitD3). Secondly, we have set the optimal maturation stimulus (proinflammatory cytokine cocktail) in our setting. We have used this protocol to generate tolDCs from RR-MS patients. These cells will be ultimately loaded with the pool of immunodominant myelin peptides for tolerogenic antigen presentation. Importantly, these tolDCs demonstrated to induce stable antigen-specific hyporesponsiveness in myelin-reactive T cells of MS patients. Altogether, these findings provide the basis for the use of autologous tolDCs as a therapeutic tool to re-establish myelin-tolerance in RR-MS patients.

Results I

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RESEARCH **Open Access**

Comparative study of clinical grade human tolerogenic dendritic cells

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Abstract

Background: The use of tolerogenic DCs is a promising therapeutic strategy for transplantation and autoimmune disorders. Immunomodulatory DCs are primarily generated from monocytes (MDDCs) for *in vitro* experiments following protocols that fail to fulfil the strict regulatory rules of clinically applicable products. Here, we compared the efficacy of three different tolerance-inducing agents, dexamethasone, rapamycin and vitamin D3, on DC biology using GMP (*Good Manufacturing Practice*) or clinical grade reagents with the aim of defining their use for human cell therapy.

Methods: Tolerogenic MDDCs were generated by adding tolerogenic agents prior to the induction of maturation using TNF-α, IL-β and PGE2. We evaluated the effects of each agent on viability, efficiency of differentiation, phenotype, cytokine secretion and stability, the stimulatory capacity of tol-DCs and the T-cell profiles induced.

Results: Differences relevant to therapeutic applicability were observed with the cellular products that were obtained. VitD3-induced tol-DCs exhibited a slightly reduced viability and yield compared to Dexa-and Rapa-tol-DCs. Phenotypically, while Dexa-and VitD3-tol-DCs were similar to immature DCs, Rapa-tol-DCs were not distinguishable from mature DCs. In addition, only Dexa-and moderately VitD3-tol-DCs exhibited IL-10 production. Interestingly, in all cases, the cytokine secretion profiles of tol-DCs were not modified by a subsequent TLR stimulation with LPS, indicating that all products had stable phenotypes. Functionally, clearly reduced alloantigen T cell proliferation was induced by tol-DCs obtained using any of these agent. Also, total interferon-gamma (IFN-γ) secretion by T cells stimulated with allogeneic tol-DCs was reduced in all three cases, but only T cells co-cultured with Rapa-tol-DCs showed impaired intracellular IFN-γ production. In addition, Rapa-DCs promoted CD4+ CD127 low/negative CD25high and Foxp3+ T cells.

Conclusions: Our results demonstrate contrasting influences of different clinical-grade pharmacological agents on human tol-DC generation. This should be taken into account for decisions on the use of a specific agent for the appropriate cellular therapy in the context of a particular disease.

Background

Autoimmune diseases are characterized by the loss of tolerance toward self-antigens and the induction of destructive immune responses leading to tissue damage. Most patients with autoimmune diseases are treated with immunosuppressive drugs that induce a generalized immune suppression, which increases the risk of infectious diseases and cancer [1]. Thus, induction of tolerance is an important goal for treating autoimmune disorders or to prevent undesirable immune responses against allogeneic transplants [2-8].

Research in recent years has primarily focused on developing more selective immunosuppressive or immunomodulatory therapies with fewer side effects and with the potential for long-term disease remission. In this context, the use of antigen-specific tolerogenic dendritic cells (tol-DCs) that target autoreactive T cells is an attractive strategy, with the aim of reprogramming the

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immune system for the treatment of autoimmune disorders [9-11].

Dendritic cells (DCs) are professional antigen-presenting cells that have the potential to either stimulate or inhibit immune responses [12-15]. Their broad range of powerful immune stimulatory and regulatory functions has placed DCs at centre stage of active immunotherapy [16-23]. Dendritic cells maintain immune tolerance to self-antigens by deleting or controlling the pathogenicity of autoreactive T-cells. Modifications of DCs in the laboratory can enhance and stabilise their tolerogenic properties, and several pharmacological agents, such as dexamethasone (Dexa), rapamycin (Rapa) and vitamin D3 (VitD3), may promote the tolerogenic activities of DCs [24,25]. It has been widely reported that such maturation-resistant DCs can regulate autoreactive or alloreactive T-cell responses and promote or restore antigen-specific tolerance in experimental animal models [26-36].

Yet, the current challenge is to move tol-DCs from the bench to the bedside [37-41], and one of the major tasks is to translate laboratory protocols into clinically-applicable procedures. Currently, information on different tolerogenic cellular products can be found at the research level. Therefore, a systematic comparison of the required functional characteristics of the various clinical tolerogenic DCs is necessary.

In this study, we compared the effects of three immunomodulatory agents: Dexa, Rapa and VitD3, on tol-DCs generation using clinical grade reagents. We describe both the convenient and inconvenient aspects of each different "tolerogenic cellular products" to induce tolerance and discuss the eligibility of each cellular product for particular therapeutic scenarios.

Methods
Culture Media and reagents
Culture medium used was X-VIVO 15 (BioWhittaker®, Lonza, Belgium) supplemented with 2% (vol/vol) heat-inactivated AB human serum (BioWhittaker®, Lonza, Belgium), 2 mM L-glutamine (Sigma-Aldrich Company LTD, Saint Louis, MO, USA), 100 U/mL penicillin (Cepa S.L, Madrid, Spain), and 100 μg/mL streptomycin (Laboratorios Normon S.A, Madrid, Spain).

Monoclonal Antibodies
The following murine mAbs were used. FITC-labelled mAbs: CD86 and Foxp3 (BD Biosciences, CA, USA); PE-labelled mAbs: CD14 (ImmunoTools GmbH, Germany), CD40 and CD127 (BD Biosciences); PerCP-labelled mAb: CD3 (BD Biosciences); PE-Cyanine dye 5-labelled mAb: CD25 (BD Biosciences); PE-Cyanine dye 7-labelled mAb: CD14 (BD Biosciences); Allophycocyanin (APC)-labelled mAbs: CD83, CD4 and anti-IFN-γ

(BD Biosciences); APC-H7-labelled mAb: HLA-DR (BD Biosciences).

Immunostaining and flow cytometry
Cells were washed, resuspended in 50 μl of PBS and incubated with mAbs for 15-18 minutes at room temperature (RT). After washing, acquisition used a Facs-Canto II flow cytometer with Standard FacsDiva software (BD Biosciences). Subsequent analyses used FlowJo software (Tree Star, Inc, OR, USA). Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris.

Cell Isolation
Buffy coats, provided by our Blood Bank department, were obtained from healthy blood donors following the institutional Standard Operating Procedures for blood donation and processing. Peripheral Blood Mononuclear Cells (PBMCs) were isolated by Ficoll-Paque (Lymphoprep, Axis Shield, Oslo, Norway) density gradient centrifugation at 400 × g for 25 min. Recovered cells were washed twice in PBS and counted using Perfect Count microspheres (Cytognos SL, Salamanca, Spain) following the manufacturer's instructions. 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).

Establishing Monocyte-derived DCs
PBMCs were depleted of CD3+ T cells using a Rosette-Sep™ Human CD3 Depletion Cocktail (StemCell Technologies, Seattle, WA, USA). Monocytes were obtained by positive selection using an EasySep® Human CD14 Positive Selection Kit (StemCell Technologies, Seattle, WA, USA). For all samples, the purity and viability of the monocyte populations were greater than 95% and 90% respectively, as assessed by the expression of specific markers and Annexin V + and 7-Amino-actinomycin D (7AAD) labelling (BD Biosciences).

Monocytes were cultured at 1-1.1 ×10^6/ml for 6 days in cGMP-grade XVIVO15 containing penicillin (100 U/ml) and streptomycin (100 μg/ml) in the presence of clinical-grade granulocyte-macrophage colony-stimulating factor (GM-CSF: 1000 U/ml; CellGenix, Freiburg, Germany) and interleukin 4 (IL-4: 1000 U/ml; CellGenix, Freiburg, Germany). Cells were replenished on day 2 with a half volume of fresh medium and cytokines, and complete fresh medium and cytokines on day 4. To induce mature DCs (Mat-DCs), DCs were treated with a cGMP-grade cytokines cocktail: TNF-α (1000 U/mL) and IL-β (10 ng/mL) (both from CellGenix); and PGE2 (1 μM) (Pfizer, New York, USA) on day 4. Tol-DCs were established by treatment with either Dexa (1 μM, Fortecortín, Merck Farma y Química, S.L, Spain),

Rapa (10 nM, Rapamune, Wyeth Farma S.A, Spain) on days 2 and 4, or VitD3 (1 nM, Calcijex, Abbott) on days 0 and 4. Tol-DCs were stimulated as mature DCs at day 4 with the cytokine cocktail. On day 6, DCs were harvested and washed extensively twice before functional assays were performed.

Allostimulatory assays

PBMCs were labelled with CFSE and plated (10^5 cells/well) in 96-well round-bottom plates. Mononuclear cells were co-cultured for 6 days with MDDCs at a 1:20 ratio (DC: PBMC). Cell proliferation was determined by the sequential loss of CFSE fluorescence of CD3 positive cells, as detected by flow cytometry.

Intracellular cytokine staining

Mononuclear cells isolated from healthy donors were seeded in 96-well round bottom plates (Nunc) at a density of 1×10^5 cells/well and stimulated for 6 days with allogeneic DCs (5×10^3 DC/well). Then, total cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) plus 500 ng/mL ionomycin (Sigma) for 5 h in the presence of 10 µg/ml brefeldin A (Sigma). After stimulation, cells were washed with PBS and stained for 18 min at RT with PerCP-conjugated anti-human CD3 mAb (BD Biosciences). Cells were then washed, fixed and permeabilised using an IntraStain kit (Dako) and incubated for 28 min at RT with anti-human IFNγ APC mAb (eBioscience). Cells were washed and analysed with a BD-FACScanto II flow cytometer equipped with FACSDiva software (Becton-Dickinson).

Measurements of cytokine production

Interleukin 10 (IL-10), IL-12p70 and IL-23 were determined in supernatants of activated DCs using MILLI-PLEX Multi-Analyte Profiling (MAP; Millipore Corporate Headquarters, MA, USA) following the manufacturer's instructions. These supernatants were collected after 48 h upon maturation and also after strong TLR (LPS: 100 ng/mL from E. Coli 0111:B4, Sigma. Reference: L4391) re-stimulation for 24 h and analysed for the presence of the indicated cytokines.

Supernatants from allogeneic co-cultures were collected after 6 days, stored at -20°C, and analyzed by MILLIPLEX Multi-Analyte Profiling (IL-10) and ELISA (TGFβ, eBioscience).

Determination of CD4+ CD127 low/negative CD25high and Foxp3+ T cells

CD3+ T lymphocytes were purified from mononuclear cells by negative selection using an EasySep® Human T Cell Enrichment Kit (StemCell Technologies) following the manufacturer's instructions. Purity was > 95% in all experiments. Enriched T cells were plated (10^5 cells/well) in 96-well round-bottom plates. After 6 days of co-culture (1DC:20T), we used flow cytometry to determine the percentages of Tregs defined as CD4+, CD127$^{low/negative}$, CD25^{high} and intracellular Foxp3+, as previously reported [42] (Human Regulatory T Cell Staining Kit; eBioscience, San Diego, CA, USA).

Statistical analyses

Results are given as means ± standard deviations (SD) for n samples per group. Results are the means of at least 5 replicates for each experiment. Comparisons used either parametric paired t-tests or non-parametric Wilcoxon tests, as appropriate. A p-value ≤ 0.05 was considered statistically significant. Prism software (GraphPad v4.00 software. CA, USA) was used for statistical analysis.
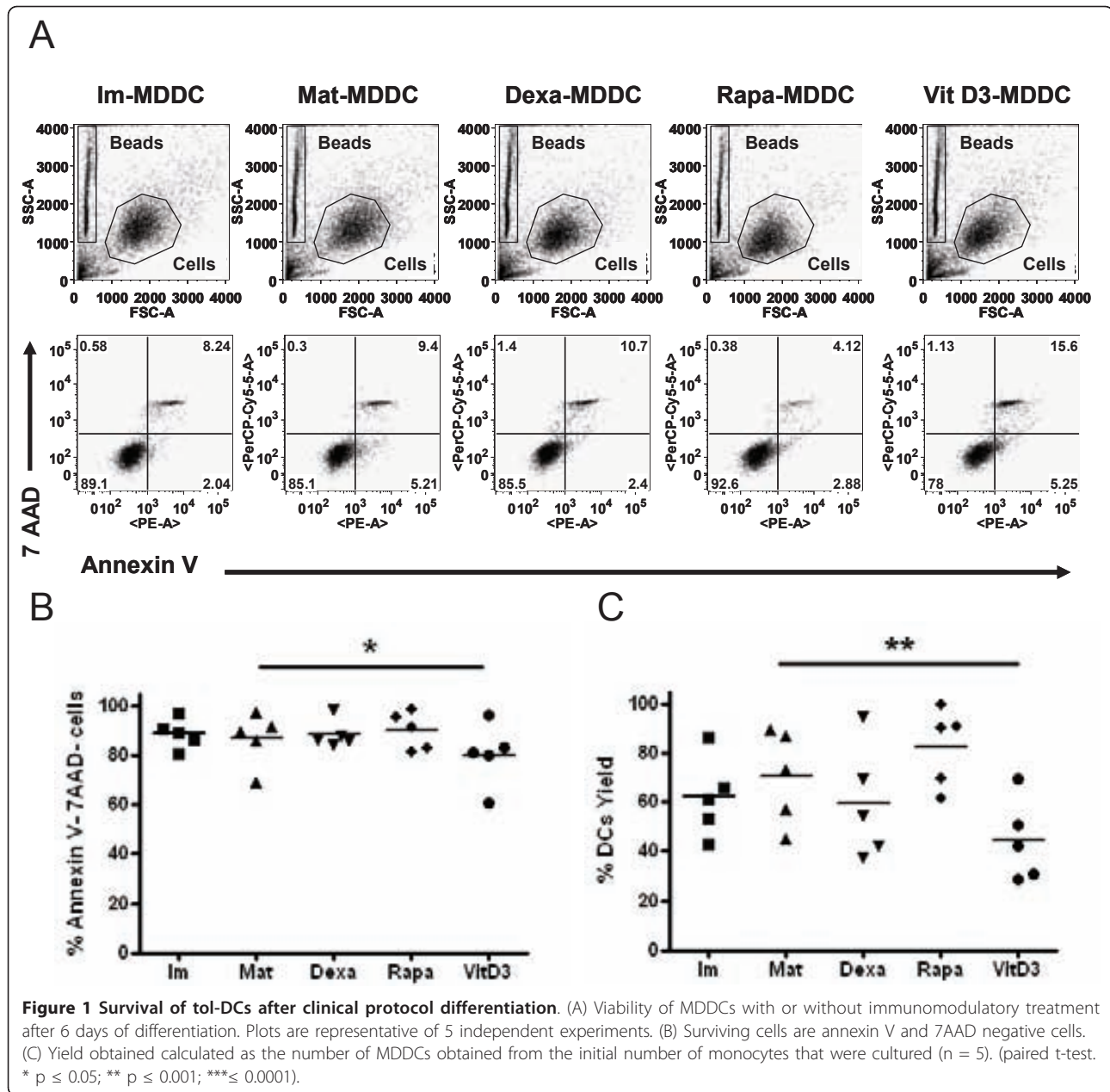
Results

Dexa, Rapa and VitD3 generate tol-DCs under GMP conditions

Most clinical studies use MDDCs to obtain adequate numbers of cells to warrant clinical doses for patients. We first evaluated the viabilities and yields of the differentiation processes using parallel conditions for the same individual for each of 5 different donors. In order to establish a common, objective baseline for comparative purposes, dose-dependent experiments were set up to obtain the optimal concentration of each immunomodulatory agent that induced an arbitrary 50% reduction of allostimulatory capacity compared to mature DCs (similar to immature DCs) with high viability (≥ 85% viable cells) (additional file 1:, Figure S1). Rapa-and VitD3-tol-DCs exhibited 50-70% reductions of T proliferation at 10 nM and 1 nM, respectively, while Dexa required a concentration 100-1000 times higher (1 µM) to achieve similar results. These criteria allowed us to evaluate equivalent tolerogenic products using the following final concentrations: 1 µM Dexa, 10 nM Rapa and 1 nM VitD3.

Simultaneous staining of cells with PE-annexin V and with the non-vital dye 7AAD was used to discriminate viable cells (Figure 1A). These results showed that, compared to mature DCs, only VitD3 treatment slightly reduced the cell viability (80 ± 13% vs. 87 ± 11% of mature DCs, p = 0.01, paired t-test; Figure 1B) and yield of DCs (45 ± 17% vs. 70 ± 19%, p = 0.0071, paired t-test; Figure 1C) (n = 5). Treatment with Dexa and Rapa did not affect these outcomes (viability: 89 ± 6% and 90 ± 8% and yield: 60 ± 23% and 83 ± 16%; respectively, n = 5).

Dexa-and Vit D3-tol-DC phenotypes change and produce IL-10

The tolerogenic functions of DCs may depend on their maturation stage and their anti-inflammatory profile.

Figure 1 Survival of tol-DCs after clinical protocol differentiation. (A) Viability of MDDCs with or without immunomodulatory treatment after 6 days of differentiation. Plots are representative of 5 independent experiments. (B) Surviving cells are annexin V and 7AAD negative cells. (C) Yield obtained calculated as the number of MDDCs obtained from the initial number of monocytes that were cultured (n = 5). (paired t-test. * p ≤ 0.05; ** p ≤ 0.001; ***≤ 0.0001).

Thus, in our initial studies, we investigated the surface phenotypes and cytokine milieus of tol-DCs obtained using the 3 different immunomodulatory agents.

After 6 days of differentiation, immature DCs (Im-DCs) expressed low surface levels of MHC II and co-stimulatory molecules (CD86 and CD83; n = 15) as compared with mature DCs (Mat-DCs) (Table 1 and Figures 2A and 2B). Tol-DC generation in the presence of Dexa and VitD3 was associated with an immature phenotype as compared to Mat-DCs. This phenotypic impairment may affect the whole population or may be observed as a partial maturation induced in a relatively low

Table 1 Surface markers on tolerogenic DCs

	CD86	CD83	HLA-DR	n
Im-DC	15737 ± 7681 ***	1316 ± 673 ***	39405 ± 33712 **	15
Mat-DC	22704 ± 13632	4371 ± 3189	70692 ± 66038	15
Dexa-DC	12291 ± 11364 ***	2811 ± 2343 *	50928 ± 62830	11
Rapa-DC	23782 ± 10961	4785 ± 2786	75297 ± 56014	15
VitD3-DC	6398 ± 6243 **	1941 ± 3096 **	20851 ± 38803 **	11

Surface markers expression was measured by flow cytometry on MDDC. Results are the averages ± SDs of Mean Fluorescence Intensity (MFI) from different donors; n (number of samples). Mature DCs were used as a reference group for all comparisons. * p ≤ 0,05; ** p ≤ 0,001; *** p ≤ 0,0001 (paired t-test) indicating significant differences compared to MDDCs.
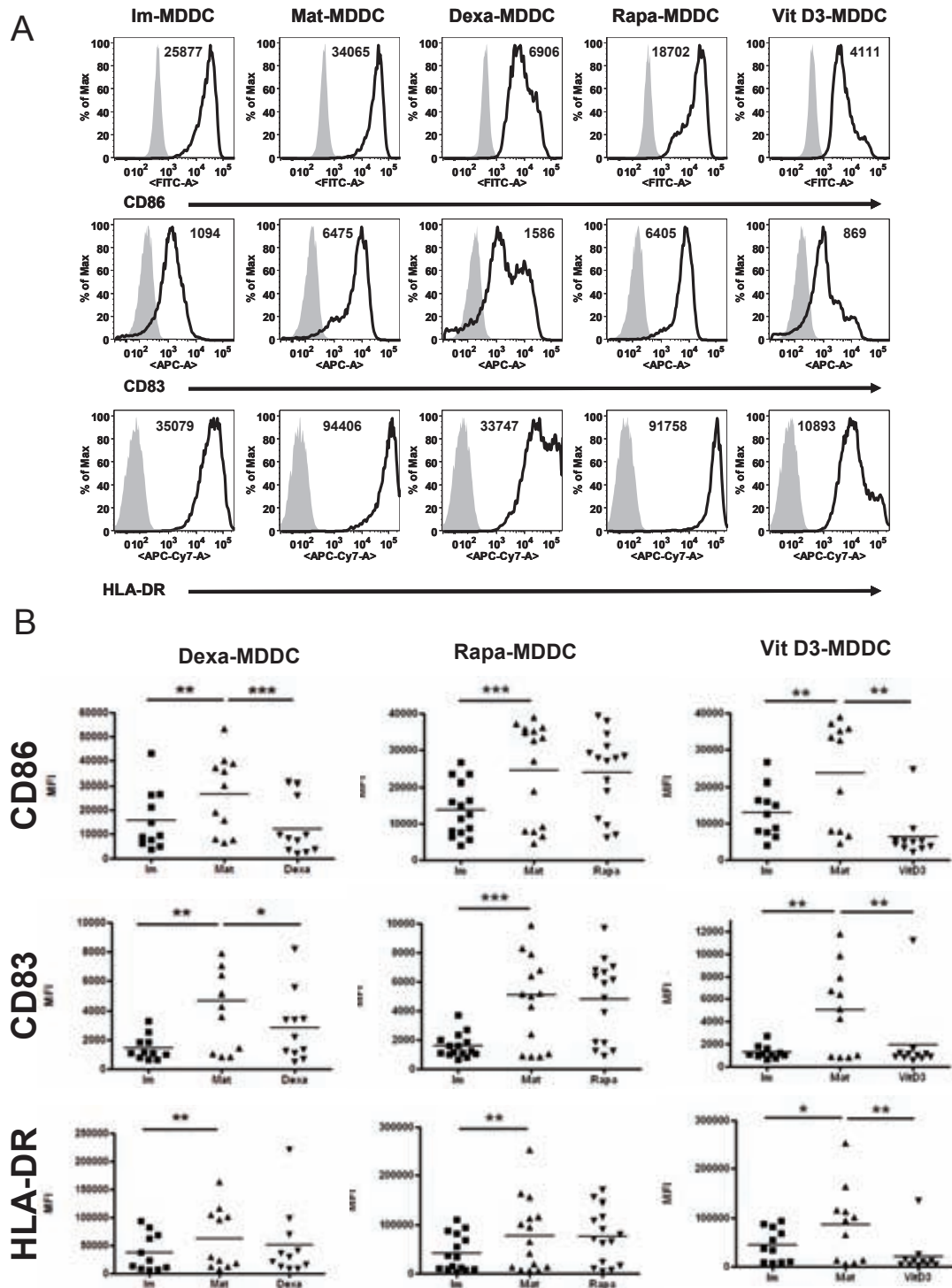
Figure 2 Dexa-and VitD3-DCs exhibit a semi-mature phenotype as compared with Mat-DCs. (A) DC expression of maturation-associated markers of immature DCs (Im-DCs), mature DCs (Mat-DCs) and tol-DCs. Surface expression of CD86-FITC, CD83-APC and HLA-DR-APCH7 staining on MDDCs. Each histogram is representative of 15 independent experiments. Isotype controls are shown in grey. (B) Results are mean fluorescence intensities from n = 11 cultures in the presence of Dexa, n = 15 cultures with Rapa-DCs and n = 11 cultures with VitD3-DCs. (paired t-test. * p ≤ 0.05; ** p ≤ 0.001; ***≤ 0.0001).

proportion of cells compared to the mature situation. The latter was often observed in most cases of our results. Indeed, in several experiments the percentage of cells with low CD83 and HLA DR levels ("semi-mature") was over 75%. As our study aimed for the comparison of the populations obtained under different tolerogenic regimes, we considered that the analyses of the whole population would better reflect these comparisons. VitD3-DCs showed a significantly reduced expression of CD86, CD83 and HLA-DR (n = 11). Dexa-tol-DCs exhibited a similar pattern, although only CD86 and CD83 showed significantly reduced expression levels (n = 11). In contrast, Rapa-tol-DCs were not phenotypically different from Mat-DCs (n = 15) (Table 1 and Figures 2A and 2B).

In addition, we measured the secretion of IL-10 and IL-12p70 after 48 h upon maturation. We found IL-10 production in cultures with either Dexa or VitD3, but not with Rapa (Figure 3A). Of note, the production of IL-10 in the presence of dexamethasone was 6 times higher compared to mature DCs (1305 ± 846 pg/mL vs. 204.5 ± 160.5 pg/mL; p = 0.0135, n = 6, paired t-test). Also, VitD3 tol-DCs produced slightly more IL-10 than mature cells (243 ± 272.9 pg/mL vs. 204.5 ± 160.5 pg/mL, n = 11). In contrast, IL-12 was notably undetectable in all culture conditions (data not shown).

Stability of Tol-DCs after restimulation with LPS
To evaluate whether DCs were resistant to an exogenous maturation stimulus, tol-DC stability was investigated by culturing tol-DCs for 24 h in XVIVO medium containing LPS (without immunomodulatory agent). As shown in Figure 3B, tol-DCs were phenotypically refractory to secondary stimulation, and retained their typical cytokine profile of IL-10 production. Dexa tol-DCs restimulated with LPS produced 19 times more IL-10 than Dexa-DCs (165.1 ± 203.7 pg/mL vs. 3244 ± 828.6 pg/mL, p = 0.0046, n = 4, paired t-test). Regarding VitD3-DCs, LPS-restimulation did not greatly modified the IL-10 production. Again, Rapa tol-DCs did not exhibit any IL-10 production.

Importantly, while primary stimulation of the DCs with this strong TLR4 ligand induced greater IL-23 production by immature DCs (10.86 ± 6.5 fold increase), no increased IL-23 production was detected by tol-DCs in any culture condition (Dexa-DC: 1.11 ± 0.46; Rapa: 1.22 ± 0.84; VitD3: 1.08 ± 0.51 fold changes), which supported a stable non-proinflamatory profile for tol-DCs. Mat-DC also showed some refractoriness to the ulterior stimulation with LPS, meaning there was a faint production of cytokines "de novo" as opposite to Im-DCs.

DC-tols do not promote a Th1 profile
To analyze the effect of the different tol-DCs, allostimulated T cells were further studied. An example of the proliferation of T cells allostimulated by tol-DCs is shown in Figure 4A. We have also summarized the relative results achieved using mature-DCs for different donors in Figure 4B. Of mention, we found that Dexa-DCs inhibited T cell proliferation only partially in some donors (4/12 subjects, data not shown).

To further investigate the effect of tol-DCs on T cells, we also determined whether inhibition of T cell proliferation was due to increased T cell apoptosis. We found that the reduced stimulation of T cell proliferation was not due to a reduction in cell viability induced by a particular type of tol-DC (% of both Annexin V and 7AAD negative cells) of allostimulated T cells (Im: 61.76 ± 9.28%; Mat: 65.92 ± 10.13%; Dexa: 62.08 ± 9.21%; Rapa: 61.02 ± 11.12% and VitD3: 60.43 ± 11.72%; n = 4) (Figure 4C).

To gain some insight into the cytokines secreted by these responding T cells, CFSE^{low} alloproliferative T lymphocytes were re-stimulated with PMA + ionomycin and IFN-γ production was measured by intracellular staining. These results confirmed a reduction of about 50-60% in IFN-γ production relative to mature DCs for all conditions tested (Figures 5A and 5B: 50.18 ± 16.65% IFN-γ producing cells among T cells allostimulated by Dexa-DC, p = 0,0093, n = 4, paired t-test; 39.83 ± 16.76% Rapa-DC, p < 0,0001, n = 7, paired t-test; and 37.97 ± 44.08 VitD3-DC, p = 0,0098, n = 7, paired t-test). When only CFSE^{low} proliferating T cells were analysed, Rapa-DCs stimulated T cells showed a significant decrease in IFN-γ production relative to Mat-DCs (Figure 5C: 40.99 ± 9.2% vs. 52.47 ± 10.85% IFN-γ among CFSE^{low} CD3+ cells, n = 7, p = 0,0057, paired t-test). VitD3-DCs also suppressed IFN-γ production in co-cultures with allogeneic mononuclear cells, but only in some donors and Dexa-DCs did not reduce the capability of responding T cells to produce IFN-γ in any of the experiments.

In addition, we determined the production of IL-10 and TGFβ in the supernatants from T cells co-cultured with tol-DC. We could measure IL-10 production in allostimulated T cells by Dexa-DC in 3 of 4 donors. Interleukin 10 values obtained were 57.47 ± 29.47 pg/mL (T cells + Dexa-DCs) compared to 33.37 ± 2.66 pg/mL (T cells allostimulated with Mat-DCs). Conversely, we did not find major differences in T cells stimulated with Rapa-DC (15.7 ± 13.61 pg/mL) or VitD3-DC (38.7 ± 7.28 pg/mL) compared to mature DCs (n = 3). Regarding TGFβ, all the measures were below the limit of detection of the assay (60 pg/mL) in the different stimulatory conditions analyzed.

Finally, the presence of Tregs cells defined as CD4+ CD127 low/negative CD25high and Foxp3+ as reported before (72) was estimated in these culture conditions. After one round of stimulation for 6 days, we analysed the induction of CD4+ Foxp3+ and CD25^{high}, CD127$^{low/negative}$
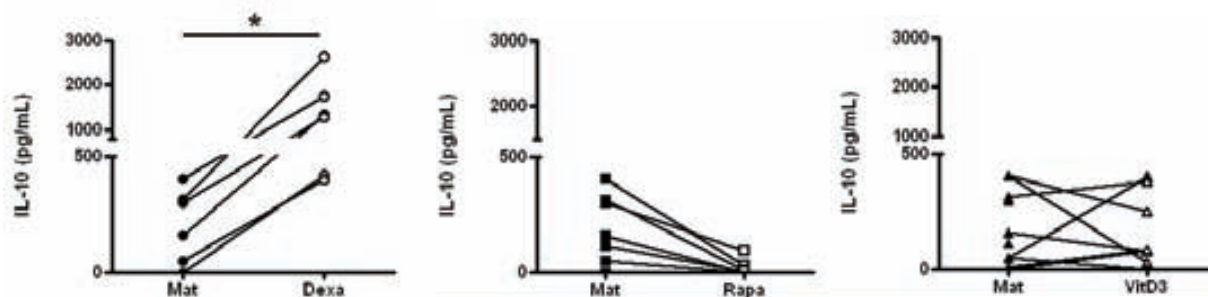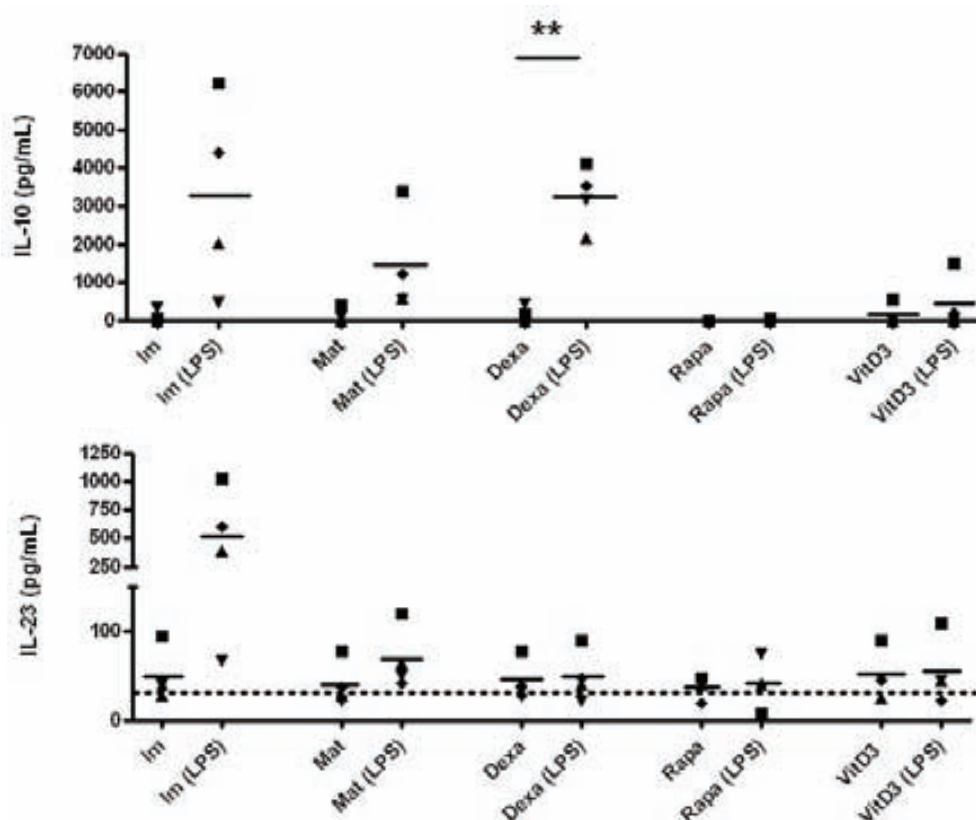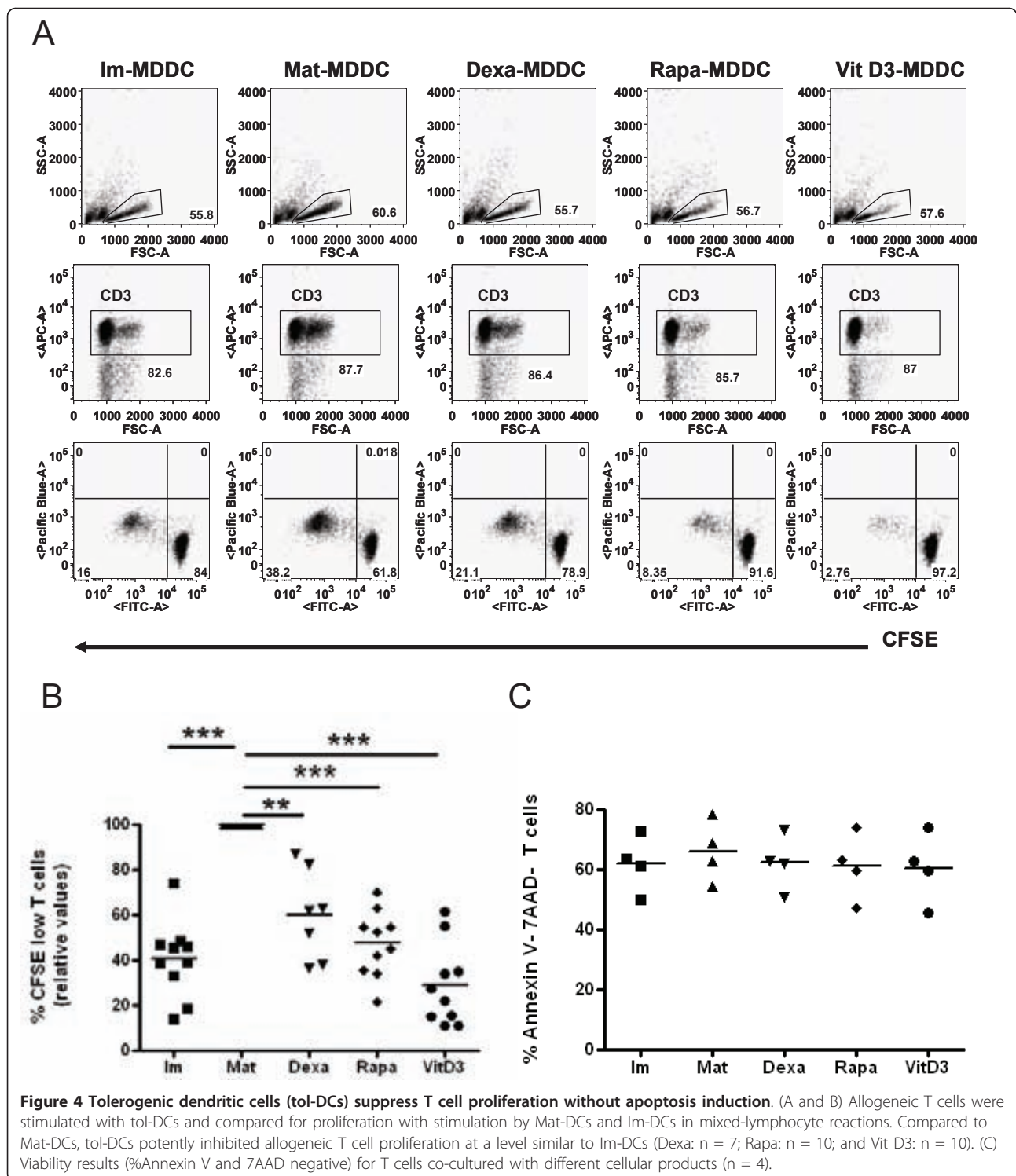
Figure 3 Tolerogenic dendritic cells (tol-DCs) exhibit an anti-inflammatory cytokine profile and stable phenotype. (A) IL-10 release by DCs in the presence or absence of immunomodulatory agents (Dexa, Rapa or VitD3) was measured after 48 h stimulation with a maturation cocktail. Supernatants were harvested and analysed for IL-10 production by MILLIPLEX (Dexa: n = 6; Rapa: n = 7 and VitD3: n = 11). (B) Stability of tol-DCs was evaluated after culture for 24 h in XVIVO medium containing LPS (without immunomodulatory agent). IL-10 and IL-23 production was determined for all DC conditions (with or without LPS). (n = 4. Statistical significance derived from a paired t-test. * p ≤ 0.05).

cells as shown in Figure 6A. Then, as depicted, only those T cells stimulated by Rapa-DCs showed a significantly increase of the percentages of CD4+ Foxp3+ and CD25^{high}, CD127$^{low/negative}$ cells (5.4 ± 1.9% vs. 3.5 ± 1.7% with Mat-DCs, p = 0.0211, n = 6, paired t test) (Figure 6B).

Discussion

Induction of therapeutic tolerance is of increasing interest in autoimmunity, allograft rejection, allergy, asthma, and various forms of hypersensitivity. Because of their capacity to orchestrate immune responses, DCs can be used as therapeutic agents. The classical concept that

Figure 4 Tolerogenic dendritic cells (tol-DCs) suppress T cell proliferation without apoptosis induction. (A and B) Allogeneic T cells were stimulated with tol-DCs and compared for proliferation with stimulation by Mat-DCs and Im-DCs in mixed-lymphocyte reactions. Compared to Mat-DCs, tol-DCs potently inhibited allogeneic T cell proliferation at a level similar to Im-DCs (Dexa: n = 7; Rapa: n = 10; and Vit D3: n = 10). (C) Viability results (%Annexin V and 7AAD negative) for T cells co-cultured with different cellular products (n = 4).

immature DCs induce tolerance and that mature DCs induce immune responses has changed completely, and several lines of evidence demonstrate that the maturation state of DCs does not always correlate with their tolerising or activating functions [43]. In this sense, the definition of tol-DCs must include a maturation-resistant cell that acts as "an immature DC" with a stable phenotype that is preserved, even in the presence of pro-inflammatory signals. This tolerogenic state of DCs can be induced using several pharmacological agents [44-46].
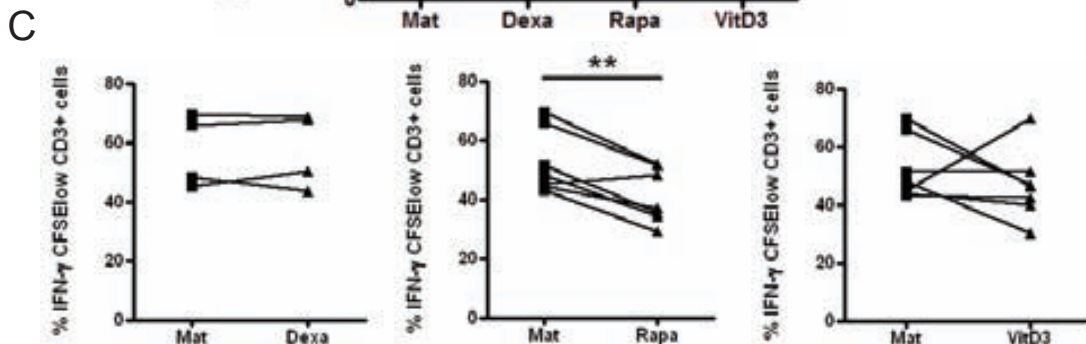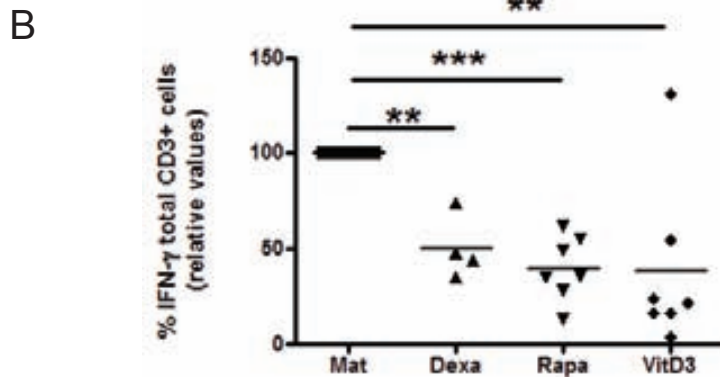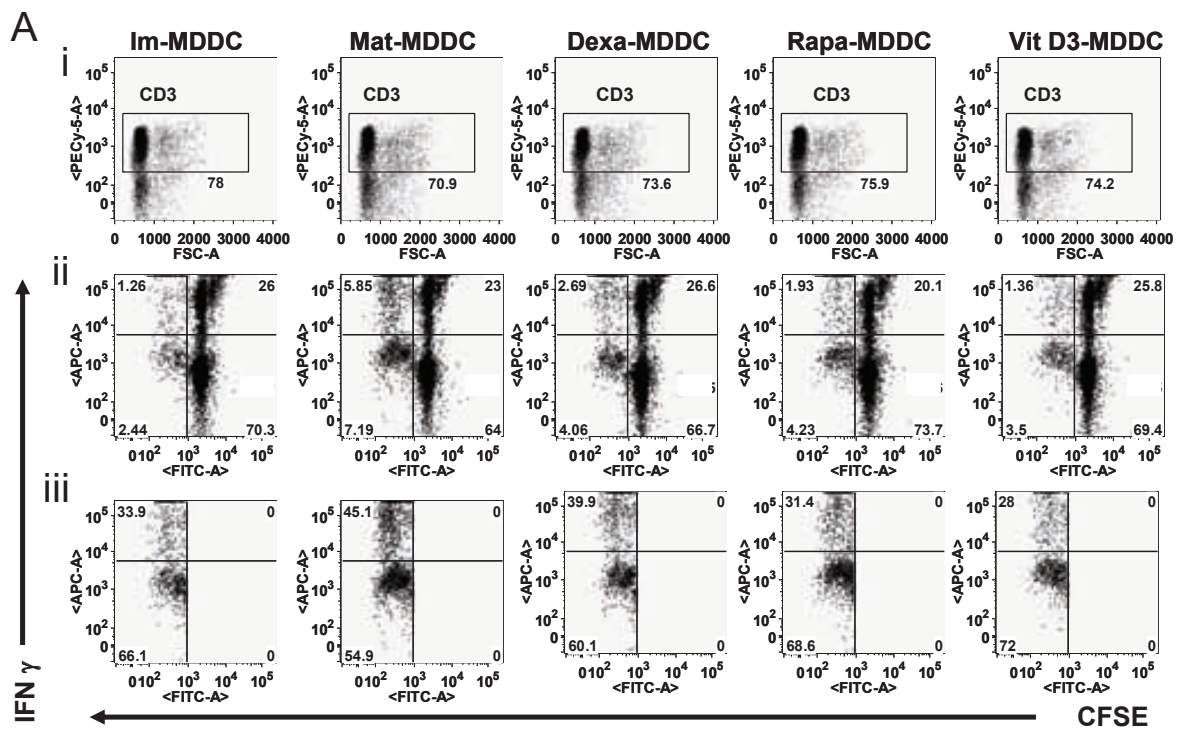
Figure 5 Decreased production and secretion of IFN-γ by T lymphocytes stimulated with tol-DCs. Proliferating T lymphocytes were obtained from allostimulatory cultures. The production of interferon (IFN)-γ was measured by intracellular staining after restimulating the cells with PMA+Io in the presence of brefeldin for 5 h. (A) First row (i) shows gating CD3+ cells. The second row plots (ii) indicate the proportion of total IFN-γ producing cells. Third row (iii) shows the percentages of cells that responded to allostimulation (CFSElow) and produced IFN-γ. The numbers inside the plots indicate the percentage of cells in each quadrant or boxes (a representative experiment). (B) Summary of the results of the total intracellular IFN-γ (Upper Left, UL) production with Dexa-(n = 4), Rapa-(n = 7) and Vit D3 (n = 7) activated cultures relative to Mat-DCs (taken as 100% production). (C) Percentage of IFN-γ producing T cells that responded to allostimulation (CFSE^low CD3+ cells). Each symbol represents an individual sample. Significant differences are indicated (** p < 0,001; paired t-test).
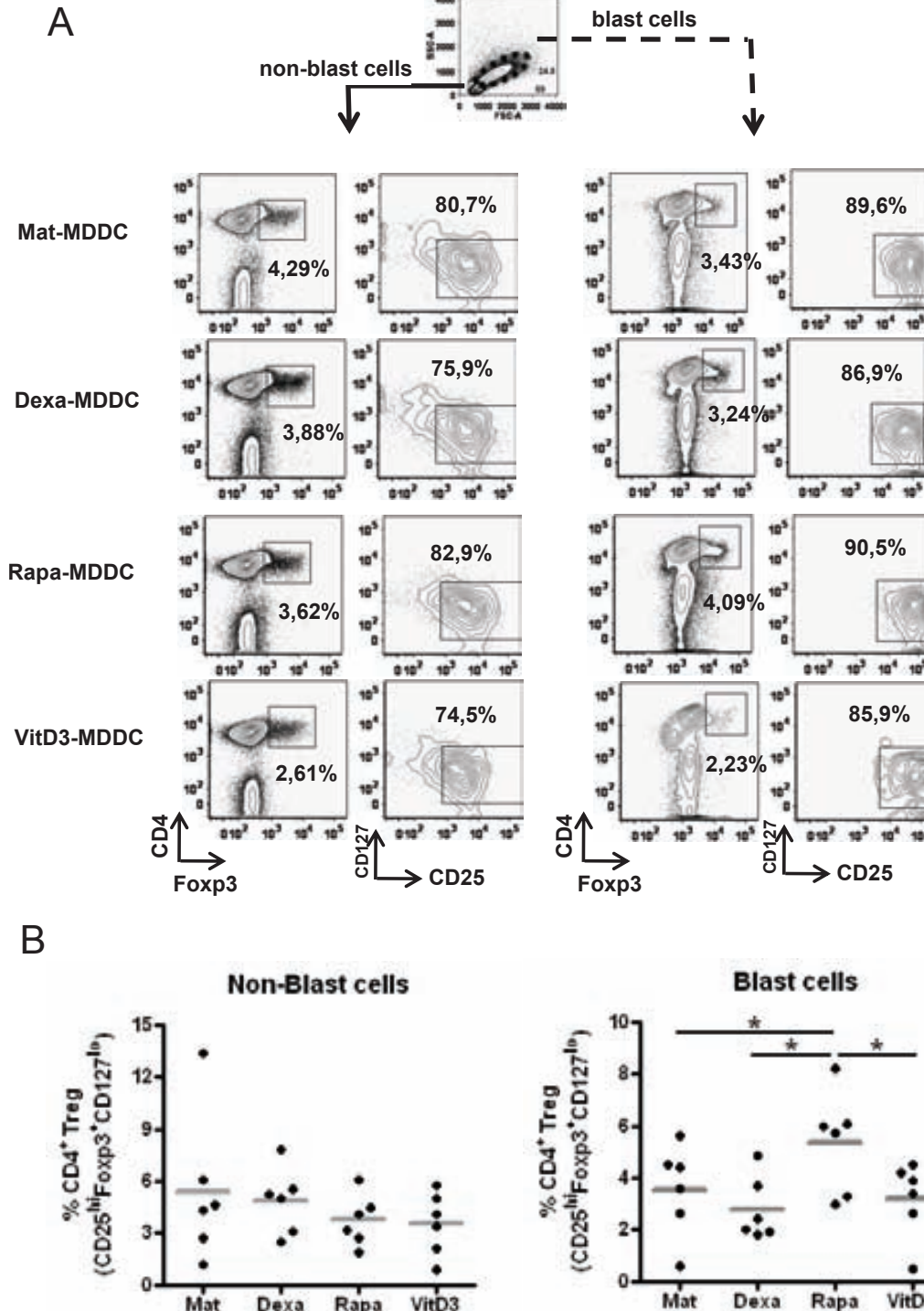
Figure 6 Rapa-DCs promote CD4+CD25^hiCD127^lowFoxP3+ induction from blast T cells. After 6 days of culture without re-stimulation and any supplemental cytokines, cell sizes were evaluated by FACS by plotting forward scatter (FSC) versus side scatter (SSC) parameters. Small (solid line) non-blast cells and large (dotted line) blast cells are circled. (A) Phenotype of T cells as CD4+, Foxp3+ and CD25+ with low or null CD127 expression. One of 6 representative experiments is shown. (B) Summary of percentages of T cells in non-blast (left) and blast (right) cells. (* p ≤ 0.05, n = 6, paired t-test).

At present, scattered knowledge from different tolerogenic cellular products can be found. A better understanding of clinical grade cellular therapies may offer new opportunities for treating different disorders. However, several gaps in our knowledge remain to be filled in before a perfect tolerogenic DC (one best suited for targeting a particular process) may be envisaged. Thus, our work aimed to determine the capabilities of those GMP-grade immunosuppressive drugs (dexamethasone, rapamycin and vitamin D3) that are used to obtain tol-DCs in comparative scenarios and identify the "array" of their individual characteristics, such as phenotypes, cytokine profiles, resistance to maturation, and T-cell profiles, in order to define the best DCs for a particular situation.

Hence, we report for the first time a comparative study of clinical-grade tolerogenic cellular products for therapeutic applications that fulfil the regulatory medical rules for human therapy. Our results show that all clinical-grade tol-DCs that were analysed function as "negative cellular vaccines," which are comparable to previously characterised research-grade tol-DCs [47]. In terms of viability, we observed that VitD3 had a slight tendency to promote DC apoptosis, in accordance with previous reports [48]. However, this minor reduction in cell viability does not compromise either DC functionality or the eventual use of these cells in therapy. Although apoptosis induction in DCs by pharmacological agents has been controversial, several reports demonstrated that Dexa did not induce cell death in MDDCs at any of the tested concentrations [49,50]. Also, use of Rapa for DC maturation did not increase apoptosis [51], in agreement with our results.

When analysing the phenotypes of the generated tol-DCs, we observed that only Dexa-and VitD3-DCs had reduced classical markers of mature cells on their surfaces. However, Rapa-DCs did not show an immature phenotype, thus being characterized as "mature DCs" with respect to their exhibited phenotype. In this context, it is obvious that the definition of DC maturation using phenotype markers is not a distinguishing feature of immunogenicity nor tolerogenicity [40]. Thus, a set of "biomarkers" for tolerance induction in our cellular products have to be defined to better monitor the putative tolerogenic cells [17,37], as phenotypic identification of tol-DCs may not be as accurate as expected. Ideally, quality controls for tol-DCs should be based on markers that are quickly and readily detectable and that are reliable.

From the cytokine profile results, Dexa-and moderately VitD3-derived DCs showed increased IL-10 production, whereas the secretion of IL-12p70 was not detected in all cases. It is well known that IL-10 blocks IL-12 synthesis by DCs, downregulates the expression of co-stimulatory molecules and potentiates their tolerogenicity [43,52]. This tolerogenic feature was not observed with Rapa-DCs, as was previously reported [53]. Most likely, DCs modified by Rapa use some other mechanism to induce tolerance, as discussed below.

Resistance to maturation is considered a prerequisite of tolerogenic potential for "negative cellular vaccines". Under the influence of inflammation, the administered immature DCs should potentially undergo maturation and lose their tolerogenic function. Thus, for good clinical applications, tol-DCs should show a stable immunosuppressive phenotype that will not be transformed to immunostimulatory DCs after injection into patients. In this context, several methods have been described for designing maturation-resistant DCs [54-57]. Our results show that Dexa-DCs, and to a lesser extent VitD3-DCs, exhibit a durable "immaturity," as high IL-10 production and no IL-12/IL-23 production was maintained upon subsequent TLR stimulation. In agreement with this, Xia et al. previously demonstrated that this tolerogenic product preserves this feature up to 5 days after removing Dexa [58]. As described in the literature, immature DCs undergo maturation and lose their tolerogenic functions. Interestingly, the cytokine profiles of the generated tol-DCs were not modified by a strong TLR stimulation, indicating that they maintained a stable profile.

Another functional property of tol-DCs is their decreased T cell-stimulatory capability. We further investigated the immunoregulatory capability of clinical-grade tol-DCs using direct T cell activation in mixed-lymphocyte reactions. Our results showed differential potentials for reducing proliferation: Rapa and VitD3 worked in the nM range, while Dexa required higher concentrations in the μM range. In fact, tolerogenic MDDCs conditioned with Dexa from 1/3 of the individuals (4/12) did not acquire regulatory properties at the concentration used, and even showed a "semi-mature" phenotype. In this regard, the possibility of combining Dexa with VitD3 to prevent de-sensitization of the DCs to the actions of Dexa has been reported [11]. Furthermore, both immunomodulatory agents used in combination inhibit DC maturation and function in an additive manner [7,59,60].

In addition, total IFN-γ production was significantly reduced when these T cells were stimulated by tol-DCs. To extend our analyses, we evaluated IFN-γ in T cells that had responded to allostimulation and observed that IFN-γ production was only reduced when Rapa-DCs were used as stimulators. This property in the deviation of Th differentiation was also observed previously by Monti P. et al [61].

It has been described that tolerogenic DCs induce immune tolerance through several pathways, including

clonal T cell depletion or exhaustion, anergy, deviation of Th differentiation or generation of Tregs [15,62-68]. To deduce which mechanisms that tol-DCs might have exerted, the possibility of apoptosis induction was evaluated. However, we did not find any differences in cell death by allostimulated T cells, indicating that this mechanism was not acting in our cellular products. In contrast, it has been reported that Dexa-and VitD3-DCs induced a hyporesponsiveness as a strategy to dampen autoreactive responses [50], and our own observations (Raïch-Regué D. et al) support these results.

Finally, we tested for the induction of CD4+CD25^{hi}CD127^{low}FoxP3+ T cells. Regulatory T cells suppress the responses of alloreactive or self-reactive CD4+ T cells and are supposed to maintain immunologic self-tolerance or control autoimmunity [69-71]. Rapa-DC-primed T cells exhibited reduced alloproliferation along with a concomitant expansion of CD4+CD25^{hi}CD127^{low}FoxP3+ cells [72-74]. This effect may have been in response to the expression of high levels of CD86 and is consistent with previous reports that described that co-stimulation is required for induction and expansion of FoxP3+ Tregs [53,75,76]. In contrast, Dexa and VitD3 did not induce this phenotype on T cells. This discrepancy with the literature could be due to the particular experimental approaches. It is important to note that we analyzed these T cells in co-cultures of MDDCs with allogenic T cells for one round of stimulation. However, it has been demonstrated that VitD3-DCs convert naive T cells into Tregs after several rounds of priming and boosting [77]. Another possibility to explore was the presence of other CD4+ Treg subsets, including CD4+CD25-FoxP3-IL-10 producing Tr1 cells [78,79] and transforming growth factor-β (TGF-β+) Th3 cells [80]. In this sense, our results show IL-10 production on T cells stimulated by Dexa-DCs but not TGF-β in any of cultured conditions.

Conclusions

In summary, in these comparative analyses of clinical grade tol-DCs, Dexa-and VitD3-DCs exhibited a "semi-immature" phenotype and IL-10 secretion. In contrast, Rapa-DCs induced CD4+CD25^{hi}CD127^{low}FoxP3+ and inhibited IFN-γ secretion by allostimulated T cells. This comparative study emphasises the fact that a simple phenotypic determination of maturation markers does not guarantee a tolerogenic function and that a complete set of functional determinations is mandatory in order to clearly define a tolerogenic "functional" phenotype. This also stresses the necessity to define reliable biomarkers for applications in GMP labs. Finally, this may also help with decisions on which tolerogenic product will be the best for a particular situation. Phase I-II studies with quality control measures and appropriate

clinical and immunological outcomes must be performed to evaluate potential tol-DC functions.

Additional material

Additional file 1: Figure S1-Dose-dependent experiments to establish equivalent tol-DCs. Summary of the dose-dependent experiments set up to obtain the optimal concentration of each immunomodulatory agent. The results reflected the relative values of the alloproliferation of T cells co-cultured with different tol-DCs (A: Dexa-DCs, n ≥ 2; B: Rapa-DCs, n = 3; C: VitD3-DCs, n = 4).

List of abbreviations
DC: dendritic cell; Dexa: dexamethasone; GMP: Good Manufacturing Practice; IFN-γ: Interferon-gamma; Io: ionomycin; MDDC: Monocyte Derived DC; PBMCs: Peripheral Blood Mononuclear Cells; PMA: phorbol 12-myristate 13-acetate; Rapa: rapamycin; tol-DC: tolerogenic DCs; Tregs: regulatory T cells; VitD3: vitamin D3.

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Authors' contributions
MNG conceived and designed the study, performed most of the experiments and drafted the manuscript. DRR carried out the immunophenotyping and the determination of Tregs, participated in the design of the study and helped in writing the manuscript. CO contributed in cell culture techniques and analysed data. LGL participated in the statistical analysis and interpretation of data. CR participated in the analysis and revised the manuscript. RPB, head of the lab, critically revised the manuscript. EMC participated in the coordination of the study and helped to draft manuscript. FEB, author for correspondence, participated in the design of the study, supervised the research, and revised the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Results II

Differential effects of monophosphoryl lipid A and cytokine cocktail as maturation stimuli of immunogenic and tolerogenic dendritic cells for immunotherapy

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ABSTRACT

Immunotherapy using monocyte-derived dendritic cells (MDDC) is increasingly being considered as alternative therapeutic approach in cancer, infectious diseases and also in autoimmunity when patients are not responsive to conventional treatments. In general, generation of MDDC from monocytes is induced in the presence of GM-CSF and IL-4, and a maturation stimulus is added to the culture to obtain mature DCs suitable for therapy. For DC maturation, different combinations of pro-inflammatory mediators and Toll-like receptor ligands have been tested, obtaining DCs that differ in their properties and the type of immune response they promote. Therefore, it is necessary to find an optimal cytokine environment for DC maturation to obtain a cellular product suitable for DC-based immunotherapeutic protocols.

In this study, we have evaluated in vitro the effects of different maturation stimuli on the viability, phenotype, cytokine profile, stability and functionality of immunogenic and tolerogenic (1α,25-dihydroxyvitamin D_3-treated) MDDC. Maturation was induced using the clinical grade TLR4-agonist: monophosphoryl lipid A (LA), compared to the traditional cytokine cocktail (CC; clinical grade TNF-α, IL-1β, PGE2) and a combination of both.

Our results showed the combination of CC+LA rendered a potent immunogenic DC population that induced the production of IFN-γ and IL-17 in allogeneic co-cultures, suggesting a Th17 polarization. Moreover, these immunogenic DCs showed a high surface expression of CD83, CD86, HLA-DR and secretion of IL-12p70. When aiming to induce tolerance, using LA to generate mature TolDC did not represent a clear advantage, and the stability and the suppressive capability exhibited by CC-matured TolDC may represent the best option. Altogether, these findings demonstrate the relevance of an appropriate maturation stimulus to rationally modulate the therapeutic potential of DCs in immunotherapy.

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1. Introduction

Since dendritic cells (DCs) were identified as master cells in the modulation of the immune response, a lot of effort has been made to bring their potential to the clinical setting [1–3]. The definition of culture methods to derive DCs from monocytes (MDDC) [4,5] established the starting point to the development of new potential therapeutic approaches for several diseases that are refractory to conventional treatments. Among those, cancer and infectious diseases were first treated with MDDC, reporting partially satisfactory results [1,6–8]. The extensive knowledge generated in the past has even increased the interest in considering immunotherapy using MDDC as an alternative therapeutic approach in patients non-responding to current treatments in cancer, infectious diseases and lately, in autoimmune disorders.

Initially, MDDC are obtained using similar procedures, but each target disease must be particularly envisaged, as the final desired effect would be different. To date, most clinical studies have used a combination of pro-inflammatory cytokines to obtain the so-called "mature dendritic cells" in vitro from monocyte precursors [9]. The standard cytokine cocktail (CC) containing TNF-α, IL-1β, IL-6 and PGE-2, has largely proved to effectively mature DCs in vitro [10].

However, it has been argued that the effectiveness of these DCs regarding their capacity to produce the pro-inflammatory cytokine IL-12p70, which is involved in the generation of Th1 immune responses (as requested in the cancer setting), may require additional activation via toll-like receptor (TLR) signalling [11].

In the last 10 years, TLR agonists have been brought to the clinical setting as a new generation of vaccine adjuvants and immunomodulators, due to their ability to stimulate innate and adaptive immune responses [12,13]. In fact, TLR3- (poly I:C) and TLR4- (LPS) ligand clinical-derivatives have been recently introduced in clinical trials. Apart from their direct use as adjuvants in vaccination [14,15], clinical-grade TLR agonists may also be used under the restrictive good manufacturing practices (GMP) conditions to generate MDDC for clinical treatments [16]. In this sense, it has been shown that the low toxicity LPS-derivative monophosphoryl lipid A (LA) in combination with IFN-γ generates MDDC that produce IL-12p70 and induce Th1 polarization, which may favour anti-tumour responses [17,18].

In this work, we report the use of LA in the generation of both tolerogenic and immunogenic MDDC, compared to the traditional CC and the combination of both (CC + LA). Throughout this article, we named 'MatDC' the immunogenic DCs that promote the immune response, and 'TolDC' the tolerogenic DCs with the capacity to modulate the immune response promoting the induction of tolerance [19]. Comparison among the different DCs has been conducted taking in account their state of maturation and their functional role. Hence, MatDC have been compared to immature DC along the study, while TolDC have been compared to MatDC and/or immature DC to better define their characteristics and function. Our results point to some fine-tuning features of DCs that may be important for the specific therapeutic activity to which the cells are being designed for.

2. Material and methods

2.1. Isolation of cells from peripheral blood

Buffy coats, provided by our Blood Bank department, were obtained from healthy blood donors following the Institutional Standard Operating Procedures for blood donation and processing. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Paque PLUS^{TM} (GE-Healthcare®, Sweden). Monocytes were isolated by positive magnetic selection using anti-CD14 (Easysep®, Stemcell Technologies, France). T cells were isolated by CD3+ negative magnetic selection (Easysep®, Stemcell Technologies). The Ethical Committee of HUGTIP approved the study and all subjects gave their consent according to the declaration of Helsinki (BMJ 1991; 302:1994).

2.2. Generation of monocyte-derived DCs

Monocytes were cultured at 1–1.5×10^6 cells/mL in presence of GM-CSF and IL-4 (1000 U/mL for both, CellGenix^{TM} GmbH, Germany) for 6 days to obtain monocyte-derived DCs (MDDC). The culture medium was X-VIVO^{TM} 15 medium (BioWhittaker®, Lonza, Belgium) supplemented with pooled AB human serum (2%, supplied by Blood and Tissue Bank), L-glutamine (2 mM, Sigma Aldrich, USA), penicillin (100 U/mL, Cepa, Spain) and streptomycin (100 μg/mL, Normon Lab., Spain). Medium supplemented with cytokines was refreshed at day 2 (half volume) and 4 (total volume). Maturation was induced at day 4 using LA (1 μg/mL, Avanti Polar Lipids Inc., AL, USA), or a proinflammatory cytokine cocktail (CC) consisting of TNF-α (100 ng/mL), IL-1β (10 ng/mL, both from CellGenix), and prostaglandin-E2 (1 μM, Pfizer, NY, USA), or the

combination of both (CC + LA) for 48 h. TolDC were generated by adding 1α,25-dihydroxyvitamin D$_3$ (1 nM, Abbot) to the culture at days 0 and 4.

2.3. Flow cytometry analysis

The following monoclonal antibodies were used for cell surface marker analysis: CD83-APC, CD86-FITC, CD40-PE, HLA-DR-APCH7, CD4-APC, CD25-PECy5, CD127-PE (all from BD Pharmingen, San Diego, CA, USA). Staining followed standard protocols (incubation for 30 min at 4 °C, and extensively wash in PBS). Regulatory T cells (CD4+ CD25^{high} Foxp3+ CD127$^{low/-}$) were stained as previously reported [20] (Foxp3 Ab from eBioscience, San Diego, CA, USA). Events were acquired on a FacsCanto II cytometer (BD) using the standard FacsDiva software (BD) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). MDDC were gated according to their Forward scatter (FSC) and Side scatter (SSC) properties. Cell viability was assessed by Annexin V-PE apoptosis detection kit (BD Pharmingen).

2.4. Assessment of DCs stability

All generated DCs were extensively washed with PBS, and then re-stimulated for 24 h in complete medium with or without LPS (100 ng/mL, Sigma–Aldrich), with no other reagent added. Subsequently, DCs were recollected, washed and further evaluated for their viability, phenotype, cytokine secretion profile and allostimulatory inducing ability.

2.5. Allostimulatory assays

DC were washed twice with an excess of PBS to exclude dead cells and debris and to avoid a possible direct effect of residual vitD3 on PBMCs. Allogeneic PBMC cells (10^5) were stimulated in vitro with the indicated DCs at a 20:1 ratio in 96 round-well plate. After 4 days, the cells were pulsed with 1 μCi of (^{3}H)-thymidine (Amersham, Germany) for additional 16 h. Cells were harvested (Harvester 96®, Tomtec, USA) and analyzed using a scintillation counter (1450 Microbeta reader, Trilux Wallac, Finland).

2.6. TolDC-suppression assay

PBMCs from blood donors were tested for CMV-specific positive proliferation (CMV, dilution 1:300, Peptivator-CMV pp65, Miltenyi Biotec). Some of the donors were used for the suppression assay. Peripheral blood T cells (10^5) from CMV-responders were cocultured with autologous CMV-loaded MatDC (CC-matured, 5×10^3) in presence or not of CMV-loaded TolDC (CC, LA or CCLA-matured) at different ratios (2:1, 1:1, 1:5 relative to MatDC) for 5 days. Proliferation was then determined as above by (^{3}H)-thymidine incorporation for 16 h.

2.7. Cytokine production

Supernatants of DCs were collected 48 h after activation with the maturation stimuli and stored at −20 °C. Cytokine production was determined by multiplex assay (Milliplex^{TM} MAP, Millipore Corporation, MA, USA) and analyzed by Luminex 100^{TM} IS (Millipore Corp.).

Supernatants from allogeneic co-cultures were collected after 96 h (time prior to addition of thymidine to the culture), stored at −20 °C, and analyzed by cytometric bead array (CBA, BD).

To determine the intracellular production of cytokines, day 6 alloproliferative T cells were washed and re-stimulated for 5 h with phorbol-12-myristate 13-acetate (PMA, 50 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) in the presence of brefeldin A (10 μg/mL,

Sigma). Cells were washed with PBS and stained with PerCP-conjugated anti-human CD3 mAb (BD Biosciences). Then, cells were washed, fixed and permeabilized using an IntraStain kit following the manufacturer instructions (Dako Cytomation, Glostrup, Denmark). Finally, cells were labelled with anti-human IFN-γ APC and anti-IL17 FITC mAb (eBioscience) or the corresponding fluorescence minus one (FMO) control. Cells were washed and analyzed with a FACScanto II flow cytometer equipped with FACSDiva software (BD).

2.8. Statistical analysis

Data are given as mean ± standard deviation (SD) of n samples. Statistics were performed using the Prism 4.0 software (GraphPad software Inc., San Diego, CA). Analysis of variance (ANOVA) was used for comparisons between multiple groups, and Mann–Whitney U or Student's t test for comparisons between two groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. MDDC morphology, viability and yield

Comparison of the three different maturation strategies (CC, LA and CC + LA) was firstly evaluated by the cellular morphology, viability and yield. MDDC matured using the CC predominantly showed round-shape core morphology, exhibiting prominent dendrites (Fig. 1A). Conversely, in both LA and CC + LA conditions, a higher number of cells remained attached to the culture plate showing elongated-shape morphology (Fig. 1A). Nevertheless, these cells were otherwise easily detached by moderate pipetting. A similar pattern was obtained when cells were cultured in the presence of 1α,25-dihydroxyvitamin D₃ (vitD3) to generate tolerogenic DCs (TolDC) in each condition (Fig. 1A).

Best differentiation yield (72% ±10) for immunogenic DCs was observed when using CC + LA, while lower cell recovery (49% ±15) occurred when using only LA as maturation stimulus (Fig. 1B). Under tolerogenic conditions, yield was not massively modified by any of the three maturation combinations studied (50–60%, Fig. 1B). In addition, no major differences were found either when cell viability was examined (Fig. 1C). In all conditions, either immunogenic and tolerogenic viable cells were about 80–90% of total counts (Fig. 1C), as determined by Annexin-V and 7AAD negative staining (Fig. 1D).

3.2. MDDC phenotype and cytokine profile

Phenotypic changes, such as up-regulation of CD83, costimulatory molecules and HLA-DR among others, are considered gold-standard markers of DCs activation. In this study (CC + LA)-immunogenic DCs expressed higher levels of CD83, CD86 and HLA-DR compared to CC or LA alone, and this increase was statistically significant when compared to immature DC expression (Fig. 2A). Regarding TolDC, no significant differences were observed among the three maturation stimuli used (Fig. 2A). In all TolDC, the expression of CD83, CD86 and HLA-DR was reduced when compared to their immunogenic counterparts (MatDC), as expected for the tolerogenic phenotype (Fig. 2A and B).

As for cytokine secretion, using CC + LA on immunogenic DCs resulted in a higher production of IL-12p70 compared to CC or LA stimuli (Fig. 2C). Moreover, IL-10 was detected in some samples when matured in the presence of LA and CC + LA, but less with CC alone (Fig. 2C). For tolerogenic regime, all TolDC produced very low levels of IL-12p70 under the different maturation conditions (less than 1.5 pg/mL per 10⁶ cells). Interestingly, IL-10 was preferentially produced by (LA)- or (CC + LA)-TolDC, when compared with

(CC)-TolDC secretion (Fig. 2C). However, when comparing the IL-10 secretion by TolDC with their immunogenic DCs counterparts (CC)-TolDC showed 1.4 times more of IL-10 production (144 ± 120 vs. 101 ± 79 pg/mL per 10⁶ cells, respectively), in contrast with (LA)-TolDC and (CCLA)-TolDC that produced about 50% less IL-10 than their MatDC counterparts.

3.3. MDDC allostimulatory capability and T cell cytokine polarization

T lymphocyte induced alloresponse are often considered a surrogate marker of the DC's immunomodulatory potential. Thus, the alloresponse induced in allogeneic T cells was evaluated using MDDC matured with each combination. The maturation regimes CC + LA and CC, but not LA alone, endowed a significant higher allostimulatory capacity to immunogenic DCs, compared to immature DCs (Fig. 3A, left panel). As expected, TolDC were modest inducers of T cell alloproliferation, similar to immature DCs, but no major differences were found among the three groups (Fig. 3A, right panel). However, only (CC)-TolDC induced a statistically significant lower alloproliferation than their immunogenic counterparts (CC)-MatDC (19,290 ± 10,330 vs. 40,570 ± 9925 cpm, respectively; $n \geq 7$; $p < 0.05$, Tukey's multiple comparison test).

Analyses of the supernatants from allostimulatory cultures revealed that IL-6 production was slightly (but not significantly) increased when using LA alone or CC + LA, compared to CC alone and to immature DCs values, by both MatDC (60 ± 60, 70 ± 52, 61 ± 68 pg/mL respectively, $n = 6$) and TolDC (47 ± 40, 56 ± 42, 40 ± 36 pg/mL respectively, $n = 6$) (Fig. 3B). However, IL-6 secretion was lower in presence of TolDC than MatDC ($p < 0.05$, paired T test, for CC + LA condition). The secretion capacity of IL-10 was increased in T cells primed by (CC + LA)-MatDC (27 ± 18 pg/mL, $n = 5$) in comparison with all TolDC ($p < 0.05$ paired T test) (Fig. 3B). Strikingly, the secretion of IFN-γ and IL-17 showed a particular behaviour. IFN-γ was mostly detected in T cells stimulated with immunogenic DCs, being CC or CC + LA matured DCs the most efficient (1204 ± 1306 and 1359 ± 1169 pg/mL respectively, $n = 5$) (Fig. 3B). Interestingly, IL-17 was mainly secreted by T cells stimulated by (LA)-immunogenic DCs (9 ± 10 pg/mL) and more efficiently by (CC + LA)-immunogenic DCs (11 ± 4 pg/mL), in contrast to TolDC (CC: 2 ± 2 pg/mL, LA: 4 ± 6 pg/mL, CC + LA: 3 ± 5 pg/mL) and immature DC (2 ± 3 pg/mL) (Fig. 3B). The results obtained for IFN-γ and IL-17 secreted in supernatants were further confirmed by intracellular staining upon re-stimulation (Fig. 3C).

3.4. TolDC suppressive ability on MatDC-induced T cell proliferation

To test whether the differently matured TolDC were also able to suppress in vitro proliferation, T cell proliferation was induced on CMV-responders using autologous CMV-loaded MatDC. Differently matured TolDC (CC, LA or CC + LA) were added to these cultures at different ratios. In two independent experiments, CC-matured TolDCs were the unique cells able to suppress the CMV-loaded MatDC induced proliferation at all ratios tested (Fig. 4). In contrast, (LA)-matured TolDC only suppressed proliferation at ratio 2:1 (TolDC:MatDC), whereas (CC + LA)-matured TolDC could not suppress the proliferation induced by CMV-loaded MatDC (Fig. 4). Therefore, the different maturation regimes confer different suppressive ability to matured TolDC.

3.5. Phenotypic and functional stability of MDDC

Due to their potential use in the clinical setting, we aimed to study whether an ulterior activation would modify the phenotype
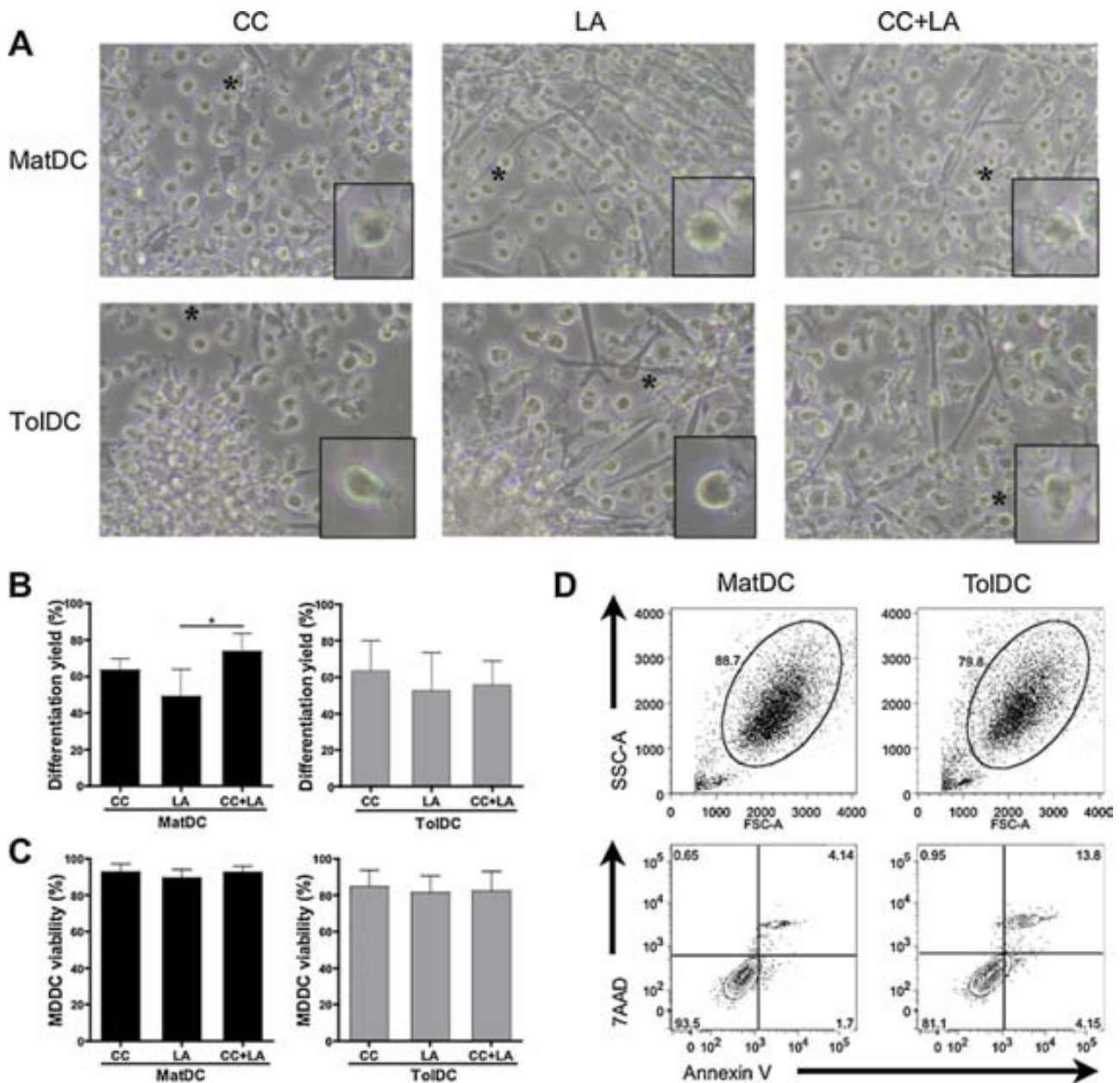
Fig. 1. Morphology, yield and viability of different MDDC. (A) MDDC morphology was observed the last day of the differentiation protocol (1000×). (B) MDDC differentiation yield, calculated as the percentage of MDDC obtained from the initial monocytes (n = 6; $*p$ < 0.05, Tukey's multiple comparison test). (C) MDDC viability, determined as the percentage of both Annexin-V and 7AAD negative cells were also measured. (D) One representative plot of (CC + LA)-maturated DCs is shown. Graphs represent the results as mean ± SD of six independent experiments.

and function of MDDC. To do so, MatDC and TolDC were matured as mentioned above, and then re-stimulated using LPS for 24 h.

Re-stimulation using LPS did not induce significant changes in MDDC viability in any of the situations studied (Fig. 5A). Phenotypically, cells did not show any remarkable change, although both immunogenic and tolerogenic (LA)-matured DCs showed a modest increase of CD83 and CD86 markers upon LPS re-stimulation (Fig. 5B).

With regard to the cytokine secretion, immunogenic DCs showed refractoriness to the ulterior stimulation with LPS, meaning there was no detectable IL-10, IL-12p70 and IL-23 production "de novo". In contrast, an interesting fact was that in some experiments, LPS-restimulated TolDC still produced some IL-10 (around 500 pg/mL × 10^6 cells, Fig. 5C), but no IL-12p70 nor IL-23 (data not

shown), thus confirming their stable non-proinflammatory profile. As a control of the LPS functionality on DCs, we evaluated the secretion of IL-10 (2149 pg/mL × 10^6 cells), IL-12 (28 pg/mL × 10^6 cells) and IL-23 (621 pg/mL × 10^6 cells) on immature DCs upon first LPS stimulation (Fig. 5C).

Alloproliferation experiments with the re-stimulated MDDC produced an interesting observation: in most experiments, immature DCs alloproliferative induction increased after LPS treatment (1.6 times higher, p < 0.05, paired T test), however LPS-restimulation of immunogenic DCs did not result in an increase of T cell alloproliferation (1.1 times for CC; 1.3 for LA; 0.8 for CCLA-matured DCs) (Fig. 6A). Similarly to the immunogenic DC behaviour, TolDC did not increase their alloproliferative induction ability after LPS challenge, but (LA) and (CCLA)-TolDC failed to maintain their capacity to induce lower levels of alloproliferation after 24 h,
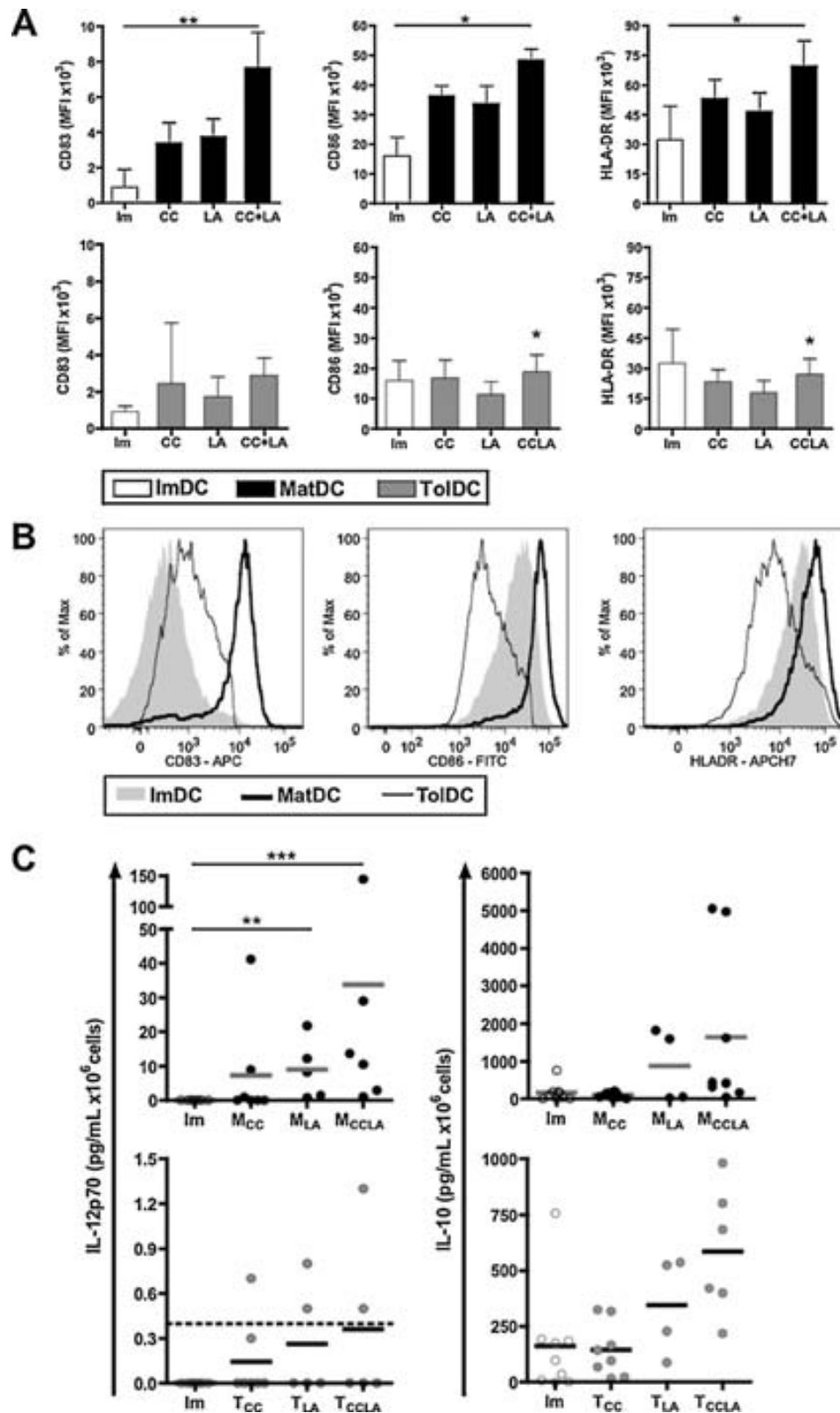
Fig. 2. MDDC phenotype and cytokine secretion. Surface markers of MDDC analyzed by flow cytometry, from different healthy donors ($n \geq 5$). (A) Plots represented the mean ± SD of the mean fluorescence intensity (MFI) for each marker, which shows the surface expression of immature DCs (white bars), immunogenic DCs (black bars) and TolDC (grey bars). Significant differences for TolDC were relative to their immunogenic DCs counterparts ($n = 5–9$; *$p < 0.05$, Tukey's multiple comparison test). (B) One representative histogram, which shows the surface expression of immature DCs (grey), immunogenic DCs (thick line) and TolDC (thin line) of CC + LA matured DCs. (C) Cytokines from MDDC culture supernatants from healthy donors ($n \geq 5$) were analyzed by multiplex assay. Plots showed the values obtained by immature DCs (Im, white circles), immunogenic DCs (M, black circles) and TolDC (T, grey circles), represented as pg/mL produced by 10^6 cells. Dotted line indicates the minimum detectable level of each cytokine. Significant differences are shown (**$p < 0.01$, ***$p < 0.001$, Dunn's multiple comparison test).

Fig. 3. Allostimulatory capacity of MDDC. (A) Allogeneic T cell proliferation induced by differentially matured DC, at 1:20 ratio. Proliferation was measured by (^{3}H)-thymidine incorporation after 4 days of co-culture. Plots show the mean (line) of $n \geq 7$ independent experiments (circles, which in torn are the mean of 5 replicates each). Results are dep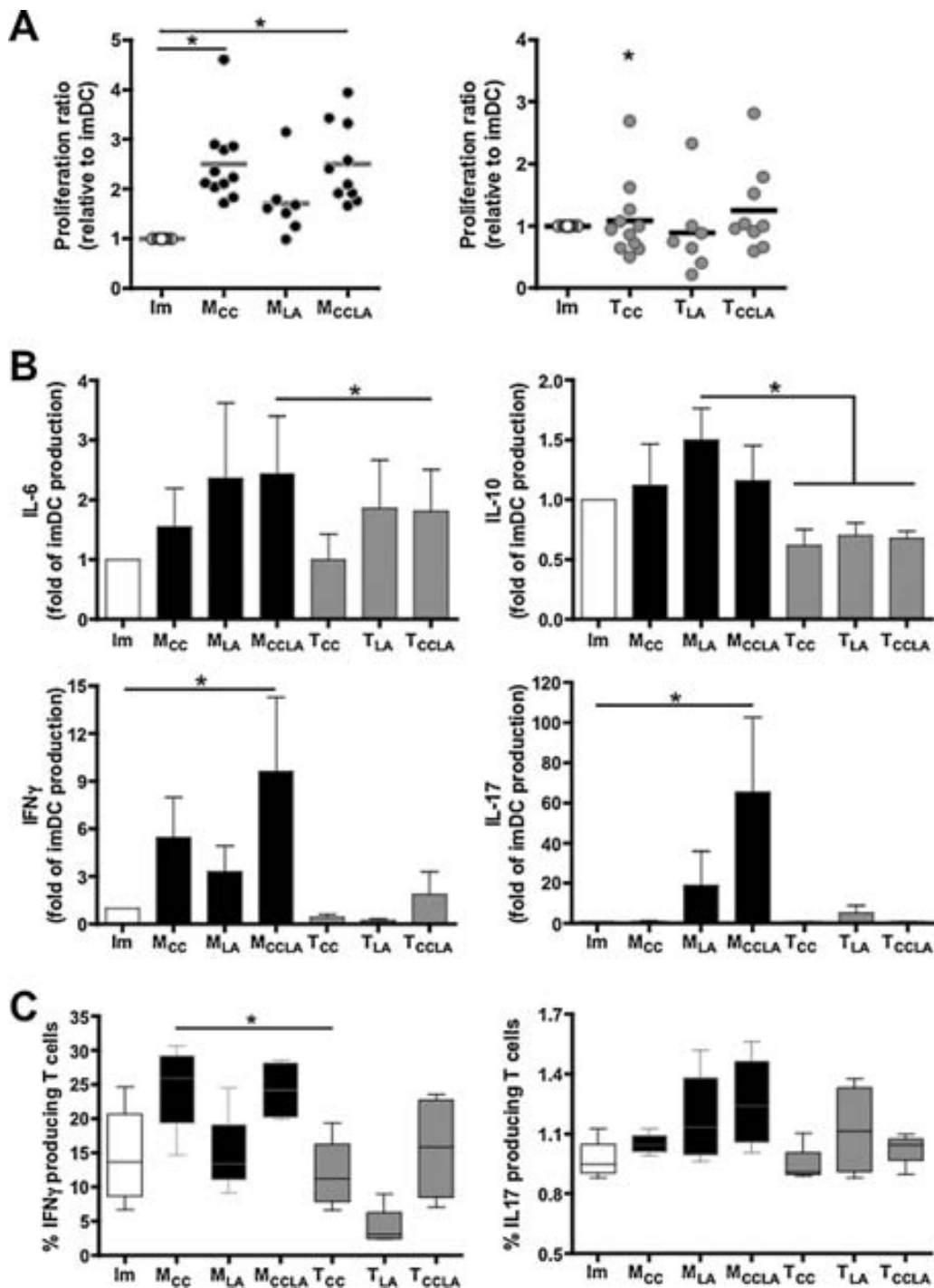icted as the proliferation value relative to immature DCs' one, to minimize alloresponse mismatch variability. Significant differences for TolDC (grey circles) were relative to their immunogenic DCs (black circles) counterparts ($^*p < 0.05$, Tukey's multiple comparison test). (B) The cytokine profile was determined in supernatants from alloproliferative assays induced by DC, collected after 96 h of culture and quantified by multiplex assay (Millipore). To minimize donor variability, the results were represented as fold of immature DCs (Im) cytokine production for each cytokine ($n \geq 5$; $^*p < 0.05$ paired T test). The detection limit for IL-6, IL-10, IFN-γ and IL-17 is 3.2 pg/mL. (C) IFN-γ and IL17 production by the different DC-primed T cells was detected intracellularly, at day 6 of coculture, using a cytokine secretion assay upon re-stimulation (as described in Section 2). Box plots represent the mean, percentiles and SD of four experiments with different donors' cells. We considered the IFN-γ and IL-17 secretion by immature DC-stimulated T cells as the basal levels.

independently of the LPS-restimulation (Fig. 6B). Interestingly, only (CC)-TolDC exhibited similar levels of induced alloproliferation (comparable with the initial levels), hence showing stability of their tolerogenic functionality (Fig. 6B).

4. Discussion

Despite the fact that DCs loaded with tumour-associated antigens induce anti-tumoral cytotoxic T cells *in vitro* and *in vivo* [21],
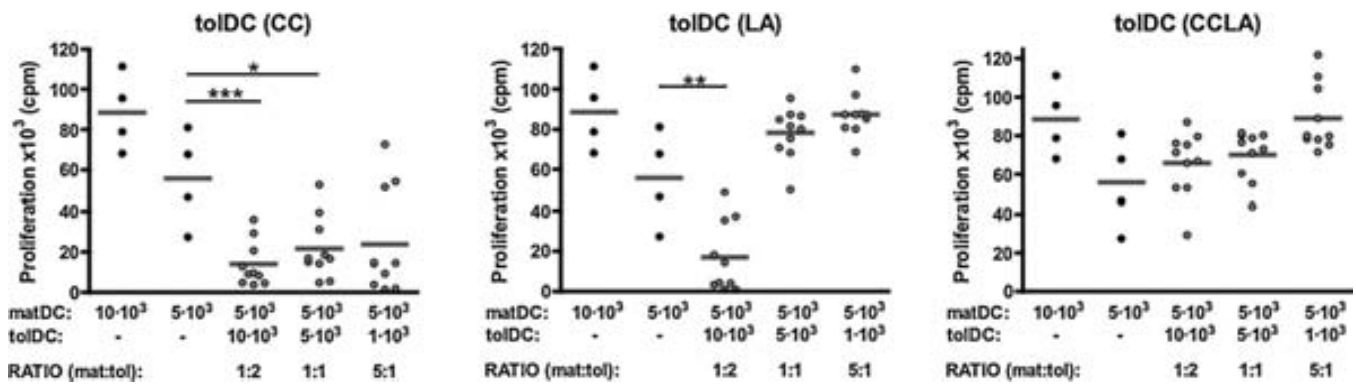
Fig. 4. TolDC suppressive ability of T cell proliferation. T cells (10^5) were cocultured with autologous CMV-loaded MatDC (CC-matured, 5×10^3) in presence of CMV-loaded (CC)-TolDC (A), or (LA)-TolDC (B) or (CCLA)-TolDC (C) at different ratios (1:2, 1:1, 5:1 relative to MatDC) for 5 days. Proliferation was determined by (^{3}H)-thymidine incorporation for 16 h. The results of a representative experiment out of two are shown (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$ Mann–Whitney test).

and DCs-immunotherapy has provided a proof of principle for therapeutic immunity in cancer [1], clinical trials using *ex vivo*-generated DCs have failed to demonstrate wide clinical efficacy. Several reasons may account for this disappointing result. Among those, authors consider that the full potential of DCs has not been effectively exploited [22]. Thus, although some cancer treatments are envisaged to target DCs with tumour antigens *in vivo*, improved *ex vivo* generated immunogenic DCs may have an important role to play in cellular therapy. An important contribution to the immunogenicity of *ex vivo* DCs was the use of TLR-activating agents. In fact, different TLR-agonists have been shown to instruct DCs to induce different types of T-cell responses [23–25]. Thus, the definition of GMP-compliant TLR-agonists has permitted new combinations of maturating regimens allowing the definition of more immunogenic DCs [17,26]. One of these GMP-compliant TLR agonists is the low toxicity LPS-derivative monophosphoryl lipid A (LA), which activates TLR4 on both DCs and T cells [27]. It has been well established that MDDC matured with LA plus IFN-γ are endowed with a higher capacity of Th1 polarization compared to the traditional CC and the IFN-α cocktail [17].

In our experiments, the combination of CC + LA rendered a potent MDDC population with higher surface expression of CD83, CD86, HLA-DR and secretion of IL-12p70. A relevant novel finding was the identification of IL-17 secretion, additional to IFN-γ, mainly in supernatants from allostimulated T cells responding to (CC + LA)-immunogenic DCs. Intracellular staining confirmed the results, thus suggesting the generation of Th17 cells (as a major source of IL-17) induced by these immunogenic DCs. In fact, the induction of human IL-17 producing cells mediated by DCs has been reported in the context of myeloma patients [28] and also in the inflamed joints of rheumatoid arthritis patients [29]. Hence, the maturation stimulus CC + LA it is likely to "simulate" an environment of pathogen-induced inflammation, activating the MDDC and entitling them to promote a Th17 response. Though the role played by Th17 cells in cancer is still controversial [30], it is postulated that IL-17-producing cells may have a decisive role in anticancer immune responses [31]. Furthermore, some studies showed the important role that Th17 cell play in immunity and host responses to extracellular [32,33] and also to intracellular pathogens [34,35]. Thus, the induction of IL-17 producing T cells could contribute to immunotherapy. Taking these results together, (CC + LA)-immunogenic DCs may yield superior anti-tumour or anti-pathogen immune responses than the immunity induced by DCs matured with CC or LA alone.

Beyond the induction of potent immunoresponses, DCs may also induce tolerance. In this sense, the use of *ex vivo* tolerogenic DCs as cellular treatment in autoimmune disorders and in transplantation is just at the initial steps, and will undoubtedly

benefit from the vast knowledge generated in DC-based tumour vaccination. Some studies reported the use of immature DCs for tolerance immunotherapy [36], but under inflammatory conditions these cells could undergo maturation and acquire immunogenic DCs features. To overcome this undesired effect, it is necessary to generate mature DCs with a stable tolerogenic profile. Hence, one important feature to consider is the maturation stimuli used, which would condition the TolDC behaviour in terms of the stability of tolerogenicity. In this sense, not all maturation stimuli may confer such stability. For example, it has been reported that TNF-α matured-DCs could still be further activated *in vitro* and *in vivo* [37]. Therefore, we evaluated on TolDC (generated in the presence of vitD3) the effect of the different maturation regimens tested in immunogenic DCs (in matched donors). As expected, all TolDC were phenotypically "semi-mature", failed to produce the proinflammatory cytokine IL-12p70, and induced a moderate alloresponse in T cells compared to donor-matched immunogenic DCs. However, fine-tuning of TolDC features using differential maturation regimens may be observed. It is remarkable that (LA) or (CC + LA)-matured TolDC produced higher levels of IL-10 (Fig. 2C) than (CC)-matured TolDC, which could be an important feature in DC tolerance immunotherapy. In sharp contrast, (CC)-TolDC exhibited the most suppressive potential of T cell proliferation *in vitro*, when compared to (CC + LA)-matured TolDC, whereas (LA)-TolDC also showed certain suppressive capability, which is partially in line with a previous report [38]. Furthermore, only (CC)-TolDC showed functional stability since did not induce increased T cell proliferation after LPS challenge or after additional 24 h of culture. Such stability was not observed in (LA) and in (CC + LA)-TolDC (Fig. 6B). These results suggest that CC-matured TolDC may be best suited as negative cellular vaccine for immunotherapy. A previous report has also studied the use of LA as maturation signal for TolDC generation [38]. Harry et al. used Dexamethasone and a low dose of VitD3 to generate LA-matured TolDC that exhibited stable phenotype and cytokine secretion when exposed to different proinflammatory stimuli [38]. In a recent study, our group compared different tolerogenic pharmacological agents and found that dexamethasone and vitD3 TolDCs were similar in terms of phenotype and alloproliferative responses [39] and therefore similar functional properties could be expected. However, dexamethasone induced-TolDC secreted high levels of IL10 compared to VitD3-TolDCs [39]. Therefore, the different reagents used to induce TolDC may undoubtedly account for minor differences found between Harry et al. and our study. As different tolerogenic agents may be best suited for specific treatments, our results extent the knowledge on maturation signals to generate functionally stable TolDC to use them as negative cellular vaccine for immunotherapy.
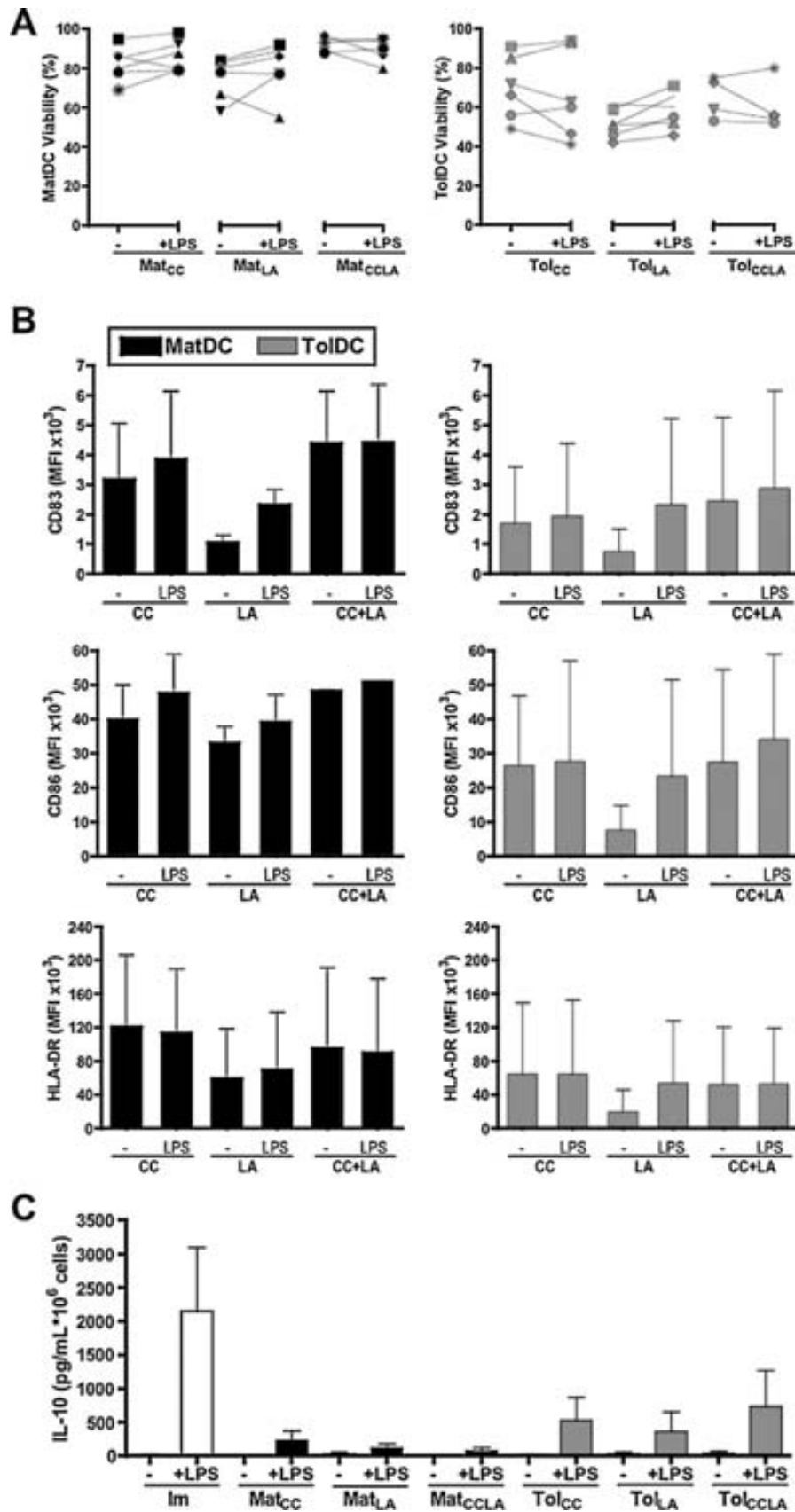
Fig. 5. Phenotypic stability of MDDC. Stability of immunogenic DCs (Mat) and tolerogenic DCs (Tol) was determined in response to LPS re-stimulation. At day 6 of differentiation, DCs were washed and recultured in the absence of vitD3 for 24 h, with (+LPS) or without (−) lipopolysaccharide (100 ng/mL). (A) DCs viability, expressed as the percentage of surviving DCs (determined as both Annexin-V and 7AAD negative) was analyzed in six independent experiments. (B) DCs were analyzed for cell surface phenotype (CD83, CD86 and HLA-DR) by flow cytometry. Data were expressed as the mean MFI ±SD of three independent donors. (C) IL-10 production by immature (Im), mature (Mat) and tolerogenic (Tol) DCs was measured in supernatants 24 h after the LPS stimulation (+LPS) or not (−). Results of five independent donors are represented (mean ± SD).

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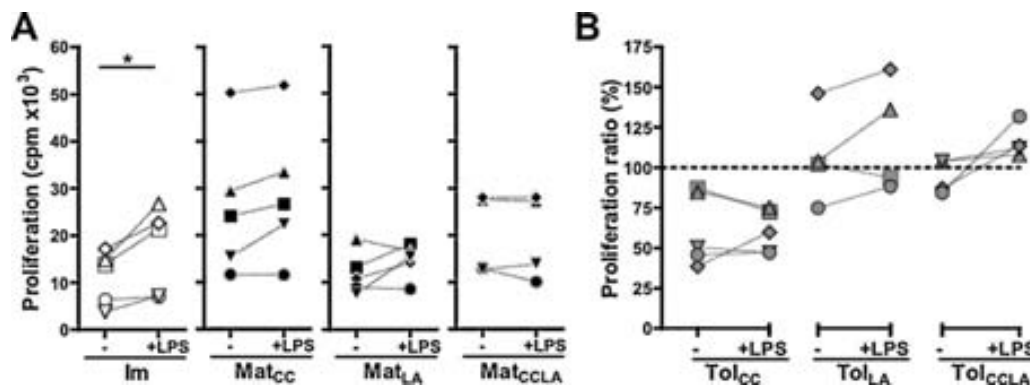
Fig. 6. Functional stability of MDDC. Following 24 h in culture with or without LPS, DCs were washed extensively with PBS, and then cocultured with allogeneic peripheral blood mononuclear cells (PBMC) for 5 days. (^{3}H)-thymidine was added to the culture for the last 16 h. Data were expressed as counts per minute (cpm) (A), showing the mean of five replicates for 6 different donors. Each donor is represented by a different symbol (*$p < 0.05$, paired T test). (B) The results for TolDC proliferation are expressed as the percentage of the donor-matched MatDC proliferation, considered as 100%.

In summary, this study compares the features of clinical-grade generated immunogenic and tolerogenic DCs differently matured using the traditional CC in combination or not with the TLR-activating LA. Fine-tuning features observed may be relevant in the translation of these DCs to human immunotherapy. Specifically, in our experimental conditions, the maturation stimulus CC + LA rendered fully immunogenic DCs that induced IFN-γ and IL-17 production by allostimulated T cells, suggesting a Th17 polarization. Clinical immunotherapy strategies that are aimed to induce immune activation against tumours and infectious agents may take advantage of this feature. Conversely, in the tolerance field it is important to notice that DCs maturation using LA promoted the secretion of high levels of IL-10, while CC-matured TolDC produced IL10 at lower levels but were unique to show a potent suppressive ability and stable tolerogenic functionality. Altogether, these findings point to the selection of appropriate maturation stimuli for optimizing the therapeutic potential of DCs, when aiming to design a DC-based vaccine.

Contributions

DRR performed the experiments, analyzed data, made the figures and wrote the manuscript; MNG performed some experiments and revised the manuscript; LGL performed flow cytometry; CRT directed statistical analyses; RPB and EMC revised the manuscript; FEB designed and conducted the study, organized the data and wrote the paper.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

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Results III

Stable antigen-specific T-cell hyporesponsiveness induced by tolerogenic dendritic cells from multiple sclerosis patients

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Multiple sclerosis (MS) is a chronic demyelinating autoimmune disease of the central nervous system. Current therapies decrease the frequency of relapses and limit, to some extent, but do not prevent disease progression. Hence, new therapeutic approaches that modify the natural course of MS need to be identified. Tolerance induction to self-antigens using monocyte-derived dendritic cells (MDDCs) is a promising therapeutic strategy in autoimmunity. In this work, we sought to generate and characterize tolerogenic MDDCs (tolDCs) from relapsing-remitting (RR) MS patients, loaded with myelin peptides as specific antigen, with the aim of developing immunotherapeutics for MS. MDDCs were generated from both healthy-blood donors and RR-MS patients, and MDDC maturation was induced with a proinflammatory cytokine cocktail in the absence or presence of $1\alpha,25$-dihydroxyvitamin-D_3, a tolerogenicity-inducing agent. tolDCs were generated from monocytes of RR-MS patients as efficiently as from monocytes of healthy subjects. The RR-MS tolDCs expressed a stable semimature phenotype and an antiinflammatory profile as compared with untreated MDDCs. Importantly, myelin peptide-loaded tolDCs induced stable antigen-specific hyporesponsiveness in myelin-reactive T cells from RR-MS patients. These results suggest that myelin peptide-loaded tolDCs may be a powerful tool for inducing myelin-specific tolerance in RR-MS patients.

Keywords: $1\alpha,25$-dihydroxyvitamin D3 · Autoimmune disease · Cell immunotherapy · DCs · Tolerance induction

See accompanying Commentary by Wiendl et al.

Supporting Information available online

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Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system, characterized by leucocyte infiltration, demyelination, and axonal loss. Although the pathogenic mechanisms of MS remain unclear, some lines of evidence suggest a crucial role of autoreactive CD4$^+$ T cells [1]. In fact, some groups have confirmed CD4$^+$ T-cell reactivity to myelin antigens [2,3]. In addition, a higher proportion of high-avidity myelin-specific CD4$^+$ T cells was demonstrated in MS patients in comparison with controls, and their specificities were predominantly directed toward a group of immunodominant epitopes of myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG) [4]. Furthermore, our group has recently confirmed the reactivity to the aforementioned set of immunodominant peptides in peripheral blood T cells from MS patients compared with healthy controls (HCs) [5]. Hence, specific inhibition or depletion of autoreactive T cells represents an interesting goal for future therapies aiming to restore peripheral tolerance in autoimmune diseases such as MS.

Current treatments for relapsing-remitting MS (RR-MS) patients decrease the frequency of relapses and reduce inflammatory activity in a nonspecific manner, but their effect on disease progression is still unclear [6,7]. Therefore, in order to modify the course of MS, new and more specific therapeutic approaches, such as the induction of antigen-specific tolerance are necessary. So far, current strategies for induction of antigen-specific tolerance in MS include T-cell vaccination [8], T-cell receptor blockade using antibodies [9], and immunization with DNA-encoding MBP [10]. These have been effective in the experimental autoimmune encephalomyelitis (EAE) model, but not in MS patients [11]. The administration of an altered peptide ligand also prevented EAE induction in animal models [12], but clinical trials in MS patients were discontinued due to worsening of the disease [13]. As has recently been reviewed by Turley and Miller [14], other antigen-specific strategies used to induce tolerance in the EAE model include the mucosal administration of antigens, and the intravenous injection of soluble peptide or ECDI-peptide coupled cells.

A novel approach for targeting autoreactive T cells is the use of antigen-loaded dendritic cells (DCs) [15,16]. DCs are a heterogeneous group of antigen presenting cells (APCs) involved in immunity and tolerance. Tolerogenic DCs induce tolerance through antigen presentation along with inadequate costimulation and biased cytokine production, resulting in T-cell silencing, deletion, immune deviation, and/or induction of regulatory T (Treg) cells [17]. Use of tolDCs to induce antigen-specific anergy and/or Treg cells has been tested in several autoimmune animal models [18], including the EAE model [19]. Hence, several groups have designed immunotherapeutic approaches involving administration of tolDCs to humans to induce T-cell tolerance in transplantation and autoimmune diseases [20,21]. Different strategies have been evaluated to generate tolDCs in vitro, as recently reviewed [18,22]. We have focused on the use of 1α,25-dihydroxyvitamin D3 (vitD3) to generate tolDCs. In vitro treatment of DCs with vitD3 or other vitamin D-receptor agonists decreased expression of costimulatory molecules such as CD40, CD80, CD86, reduced interleukin (IL)-12 production, enhanced IL-10 secretion, and down-modulated T-cell activation [23], indicating a tolerogenic functional phenotype. Moreover, these effects have recently been suggested to occur in vivo as well [24]. Also, high serum levels of vitD3 in MS patients have been correlated with a slower progression of the disease [25]. Finally, our own results have confirmed the possibility of obtaining clinical grade vitD3-tolDCs [26].

Here we sought to generate and characterize tolDCs from RR-MS patients' monocytes, as a pilot study to investigate the feasibility of reestablishing tolerance using such cells in a future clinical trial.

Our results show that tolDCs generated from RR-MS patients behave similarly to those of HCs, and are able to induce a stable state of hyporesponsiveness in myelin-specific autologous T cells.

Results

Differentiation to tolDCs is equally efficient from monocytes of both HCs and MS patients

Generation of tolDCs was evaluated in a group of eleven RR-MS patients and eleven HCs to assess the possible differences in differentiation efficiencies (percentage of monocyte-derived dendritic cells (MDDCs) obtained from the initial cultured monocytes). No statistical difference was observed in differentiation yield ((number tolDC / number initial monocytes) × 100), or in the percentage of surviving tolDCs at day 6 of culture, between MS patients and HCs (Table 1). Therefore, differentiation of monocytes to tolDCs was as efficient in MS patients as it was in HCs.

TolDCs from RR-MS patients induce hyporesponsiveness in allogeneic T cells

During the study, tolDCs (matured in presence of vitD3) were compared with immunogenic dendritic cells (matDCs; matured without vitD3) and/or immature dendritic cells (imDCs; without vitD3 and not matured) to better define their characteristics and function. In relation to cell surface phenotype, the expression of CD83, CD86, CD40, and human histocompatibility leukocyte Ag (HLA)-DR was significantly upregulated in matDCs compared with imDCs, except for CD40 in MS patients (Fig. 1A).

Table 1. TolDC differentiation yield ((number tolDC / number initial monocytes) ×100) and viability (annexin-V and 7AAD double negative) obtained from healthy controls (HCs) and RR-MS patients (MS) cells

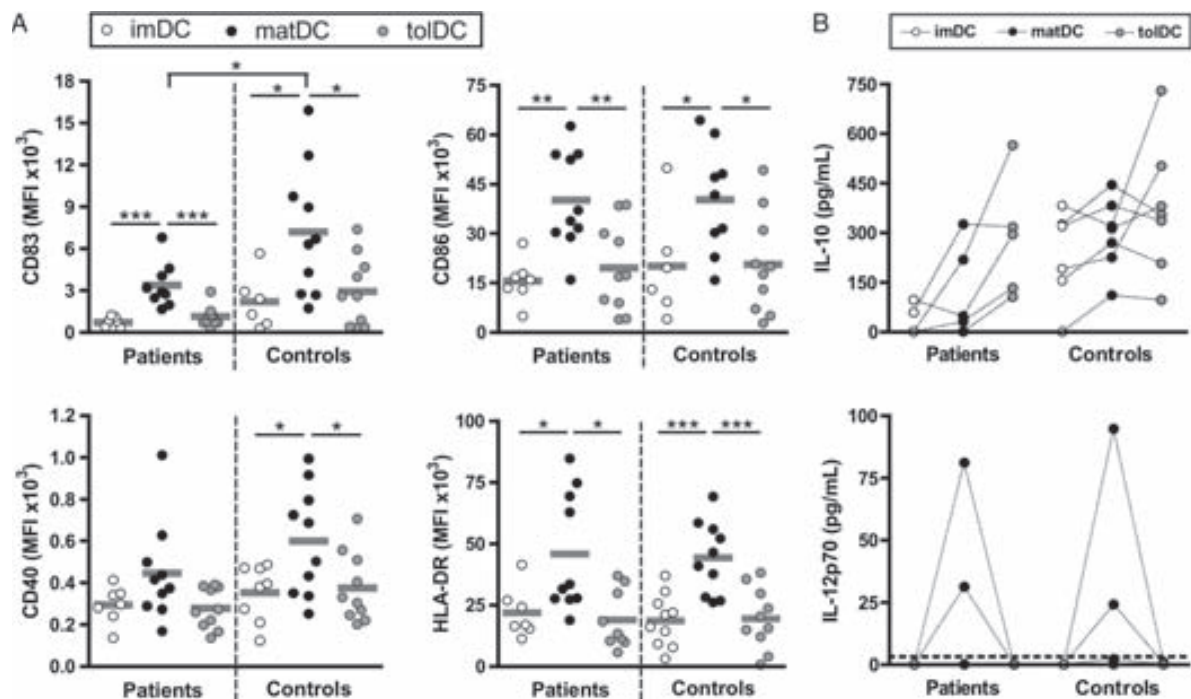|	n	tolDC differentiation yield (%)	tolDC viability (%)
HC	11	45 ± 15	83 ± 8
MS	11	32 ± 13	79 ± 7

Figure 1. Characterization of tolDCs from MS patients reveals a semimature phenotype and antiinflammatory cytokine profile. (A) Expression of DCs surface markers (CD83, CD86, CD40, and HLA-DR) in imDCs, matDCs, and tolDCs of MS patients ($n = 9$) and controls ($n = 10$) were analyzed by flow cytometry. Dots represent the MFI of the analyzed marker for each individual (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Unpaired t-test). The gray bar represents the mean. (B) IL-10 and IL-12p70 levels in culture supernatants of DCs isolated from MS patients ($n = 5$) and HCs ($n = 7$) as determined by multiplex assay. The minimum detectable level of IL-12p70 is indicated by a dotted line.

Conversely, the expression of all these markers remained unaltered in tolDCs in contrast to imDCs (Fig. 1A). When comparing HCs and RR-MS patients, an intriguing low expression level of the maturation marker CD83 was observed in matDCs generated from RR-MS patients, compared with HCs (2954 ± 989 vs. 7175 ± 4654 mean fluorescence intensity (MFI), $n = 9$, $p < 0.05$, unpaired t-test) (Fig. 1A) while no significant changes were observed in the other markers analyzed.

When analyzing cytokine production, although differences did not reach statistical significance, it was observed that tolDCs produced higher levels of IL-10 (3.1 times more for MS patients and 1.6 times more for HCs) and produced very low IL-12p70 levels compared with matDCs (Fig. 1B). Additionally, IL-23 production by matDCs and tolDCs was also evaluated, but most samples were below the detection level of the technique (23 pg/mL) (data not shown). These data suggest an antiinflammatory profile of tolDCs from both HCs and MS patients (Fig. 1B).

TolDCs from RR-MS patients induce hyporesponsiveness in allogeneic T cells

The immunostimulatory capability of tolDCs was first determined in allogeneic mixed leukocyte reaction experiments. Compared with matDCs, tolDCs showed a reduced ability to stimulate T-cell proliferation, observed equally in MS patients and HC subjects

($51.6 \pm 20.1\%$ and $53.5 \pm 19.9\%$, respectively, $n = 9$) (Fig. 2A). The supernatants of these allogeneic cocultures contained significantly reduced amounts of tumor necrosis factor (TNF)-α, IL-6, and interferon (IFN)-γ in the presence of tolDCs, compared with those of matDCs ($p < 0.05$ paired t-test, $n = 6$) (Fig. 2B). The IL-4 and IL-10 levels were below the detection limit (data not shown). To confirm that this reduction in proinflammatory cytokine secretion was not uniquely due to the lower number of proliferating T cells in the presence of tolDCs, intracellular cytokine experiments were performed. tolDCs were less potent in inducing IFN-γ secretion by T cells than matDCs, for total CD3^{+} cells ($p < 0.05$ Wilcoxon test, $n = 6$; Fig. 2C) and also for proliferating CD3^{+} cells (carboxyfluorescein diacetate succinimidyl ester (CFSE)[low]) ($p < 0.05$ paired test, $n = 6$; Fig. 2D).

tolDCs have a stable tolerogenic profile in vitro

Due to their potential use in the clinical setting, we evaluated whether the vitD3 induced-DCs were phenotypically and functionally stable after a potent proinflammatory stimulus. MatDCs and tolDCs were restimulated for 24 h with or without lipopolysaccharide (LPS). Under these conditions, matDC and tolDC surface expression of CD83, CD86, and HLA-DR remained stable following LPS restimulation in comparison to the nonstimulated phenotype (Fig. 3A). Furthermore, tolDCs
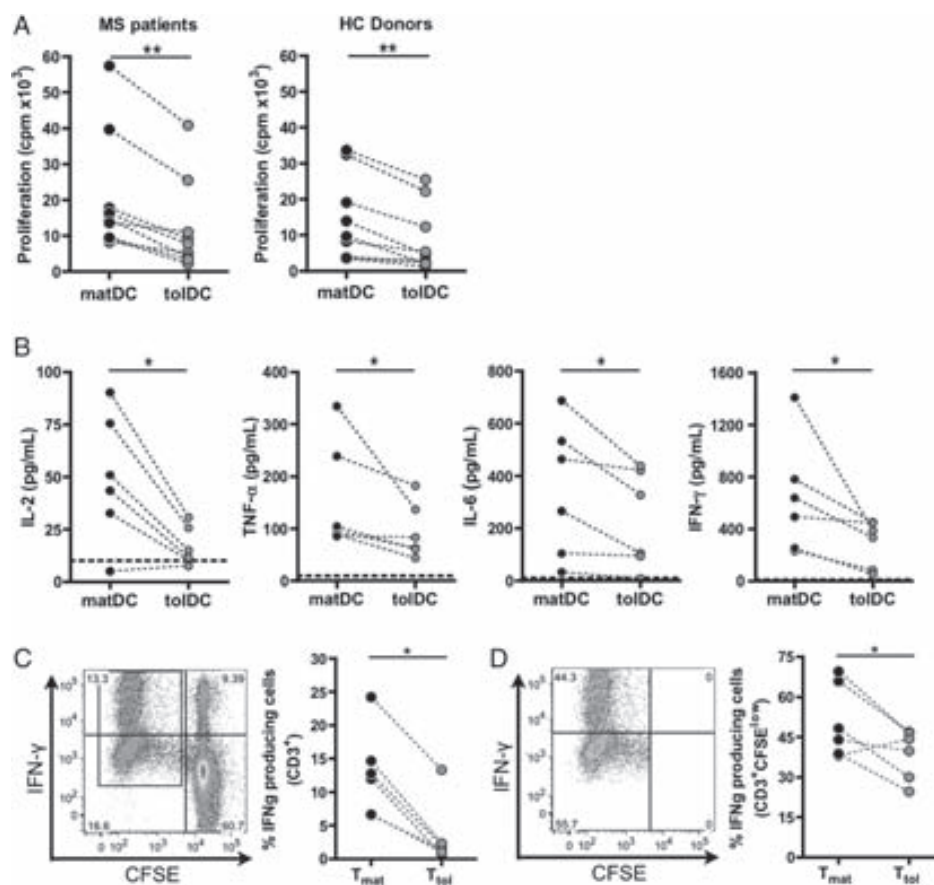
Figure 2. tolDCs from RR-MS patients induced hyporesponsiveness of and impaired proinflammatory cytokine secretion by allogeneic T cells. (A) Allogeneic T-cell proliferation induced by matDCs and tolDCs, at a 1:20 ratio, from MS patients ($n = 9$) and HCs ($n = 9$). Proliferation was measured by (^{3}H)-thymidine incorporation and data were expressed as cpm. The discontinuous line indicates the reduction of alloproliferation in the presence of tolDCs compared with that of matDCs derived from the same patient (**$p < 0.01$ Wilcoxon test). The background levels (unstimulated T cells) were 297 ±180 cpm for HCs and 459 ±207 cpm for MS, and the positive control (pokeweed mitogen, 1 μg/mL): 18.89 ± 25.22 × 10^3 cpm for HCs and 37.56 ± 38.47 × 10^3 cpm for MS. (B) IL-2, TNF-α, IL-6, and IFN-γ profiles were analyzed in supernatants of the alloproliferative assays induced by either matDCs or tolDCs (from MS patients), collected after 96 h and quantified by cytometric bead array. The minimum detection level of each cytokine is indicated by a dotted line (*$p < 0.05$ paired t-test, $n = 6$). (C and D) IFN-γ production by matDC- and tolDC-primed T cells was measured on day 6 of coculture by intracellular staining and flow cytometry, after restimulating the cells (phorbol myristate acetate (PMA) + ionomycin) in the presence of brefeldin A for 5h. A representative flow cytometry plot (left) and percentage of IFN-γ producing cells from $n = 6$ independent experiments are shown for (C) the total T cell population and (D) those T cells that responded to allostimulation (CFSE^{low}). The numbers inside the plots indicate the percentage of cells in each quadrant and dots represent the mean of five replicates per experiment (*$p < 0.05$ Wilcoxon and paired t-test for CD3$^+$ and CD3$^+$CFSE^{low} cells, respectively).

maintained a lower expression of CD83, CD86, and HLA-DR compared with matDCs (Fig. 3A).

Additionally, LPS did not induce the secretion of IL-23 from tolDCs but small amounts of IL-10 were still detectable in these supernatants (Fig. 3B). As positive control, LPS-stimulated imDCs secreted significant amounts of IL-10 and IL-23 whereas non-stimulated imDCs did not produce detectable levels of these cyto-kines ($p < 0.005$ Mann–Whitney Test, $n = 5$) (Fig. 3B). Secretion of IL-12p70 was only detected for LPS-stimulated imDCs (28 pg/mL per 10^6 cells in two assays out of five, data not shown).

Furthermore, alloproliferation experiments indicated that allo-proliferative responses induced by imDCs increased after LPS treatment (1.6-fold higher, $p < 0.05$ paired t-test, $n = 5$), whereas

no significant increase was observed in alloproliferative responses induced by matDCs after LPS restimulation (Fig. 3C). Similarly, the alloproliferative induction ability of tolDCs after LPS challenge was not increased, indicating stability of their tolerogenic functionality (Fig. 3C).

Binding of myelin peptides to DCs

To assess tolDC hyporesponsiveness induction to autologous myelin-reactive T cells, we first estimated the peptide-loading capability of tolDCs using a biotinylated form of a single myelin peptide (PLP$_{(139–154)}$) in time-course experiments on both matDCs and tolDCs. Results from RR-MS patients' cells ($n = 3$) showed
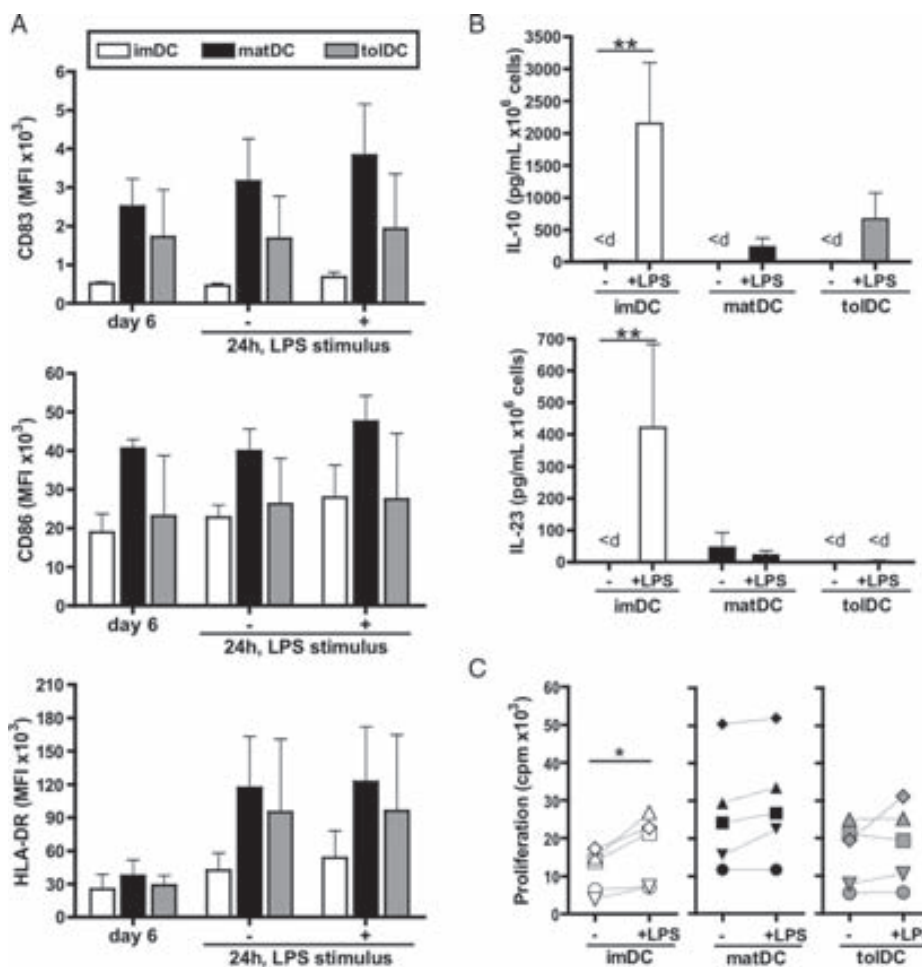
Figure 3. tolDCs have a highly stable tolerogenic profile. DCs were washed and recultured for 24 h with (+) or without (-) LPS (100 ng/mL) on day 6 of differentiation. (A) Cell surface phenotype (CD83, CD86, and HLA-DR) of imDCs, matDCs, and tolDCs were analyzed by flow cytometry. Data are expressed as the mean MFI +SD of three independent donors. (B) IL-10 and IL-23 production by DCs at 24h after LPS stimulation (+LPS) or unstimulated (–) was measured by multiplex assay. Results are represented as the mean + SD of five independent donors (**$p < 0.01$ Mann–Whitney Test). (C) Alloproliferation-inducing ability of DCs, following LPS stimulation (+LPS) or not (–), on cocultured PBMCs for 108 h was measured by (^{3}H)-thymidine uptake during the final 16 h. Data are expressed as cpm showing the mean of five replicates for five different donors analyzed (indicated with different symbols) (*$p < 0.05$ paired t-test).

that optimal binding was obtained between 12 and 18 h after peptide loading at 37°C (Fig. 4A). No significant difference was observed in the loading capacity exhibited by matDCs or tolDCs for any patient (Fig. 4B and C). To ascertain the specificity of peptide loading, a competition experiment was performed. When increasing amounts of unlabeled PLP$_{(139–154)}$ were added to tolDCs in the presence of a constant concentration of biotinylated PLP$_{(139–154)}$, a gradual decline of the biotinylated signal was observed
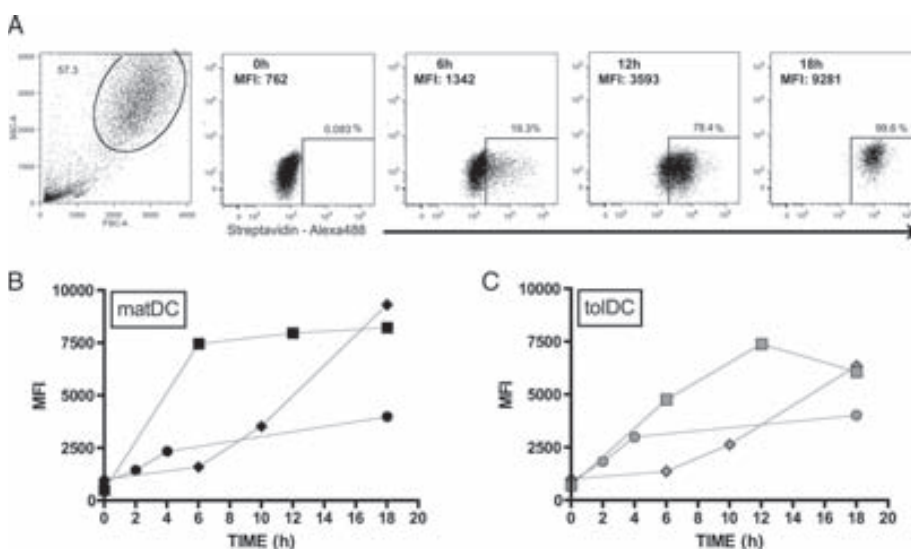
Figure 4. Time course of myelin peptide binding to DCs. (A) DCs (indicated by FSC/SSC) were incubated with bio-tinylated PLP$_{(139–154)}$ (50 μM) for the indicated times. After extensive washing, bound peptide was detected with Streptavidin-Alexa488 by flow cytometry (MFI). Time course of binding of biotinylated PLP$_{(139–154)}$ to (B) matDCs and (C) tolDCs from RR-MS patients ($n = 3$, indicated with different symbols) are shown.

(Supporting Information Fig. 1), showing specificity in peptide binding.

Myelin peptide-loaded tolDCs induce stable hyporesponsiveness in autologous RR-MS patients

We investigated whether the immunomodulatory ability of tolDCs observed in allogeneic cultures was also maintained in autologous conditions. For this purpose, enriched myelin-specific T cells from RR-MS patients ($n = 3$) were stimulated for 6 days with either autologous matDCs or tolDCs, which had been previously loaded with the same mix of myelin peptides. It was observed that T cells stimulated by myelin peptides-loaded tolDCs exhibited a lower proliferation (reduction of $45 \pm 17\%$, $n = 3$) compared with those induced by myelin peptides-loaded matDCs (Fig. 5A). Supernatants from coculture experiments were analyzed for secreted cytokines and a reduction of IL-17 (62%), IL-6 (76%), and IFN-γ (58%) production in the presence of tolDCs compared with that of matDCs was observed (Table 2). Therefore, tolDCs from RR-MS patients induced hyporesponsiveness in autologous myelin-reactive T cells accompanied by a reduction of proinflammatory cytokines.

To further evaluate the T-cell response promoted by tolDCs, T cells were recovered from the primary coculture with myelin-loaded matDCs ($T_{mat+ptd}$) or tolDCs ($T_{tol+ptd}$), and following 3 days of resting, were restimulated with autologous myelin-loaded peripheral blood mononuclear cells (PBMCs) as APCs. In the three patients tested, $T_{mat+ptd}$ readily responded after restimulation with myelin-loaded PBMCs, in contrast to $T_{tol+ptd}$, as shown by the reduced proliferation ($35 \pm 8\%$ reduction compared with $T_{mat+ptd}$, $p < 0.01$ paired t-test, $n = 3$) (Fig. 5B). Taken together, these results support the induction of stable hyporesponsiveness of myelin-reactive T cells by TolDCs from RR-MS patients.

tolDC-induced hyporesponsiveness is antigen specific

Finally, we evaluated whether the induction of hyporesponsiveness by tolDCs was antigen specific, meaning that tolDC-primed T cells would still respond to an unrelated antigen. This possibility was tested using T cells from four donors reactive to tetanus toxin (TT) and two MS patients reactive to myelin peptides (ptd). In both cases, the unrelated antigen was cytomegalovirus (CMV). T cells were cocultured with either TT- or ptd-loaded matDCs (TT/ptd-matDCs) or TT- or ptd-loaded tolDCs (TT/ptd-tolDCs). Following a resting period, the T cells were restimulated with unloaded, TT/ptd-loaded, or CMV-loaded matDCs separately, and the induced response was measured by IFN-γ enzyme-linked immunospot (ELISpot) assay. In both HCs and patients, the results showed that TT/ptd-matDC-primed T cells (T_{mat+TT} in Fig. 6A, and $T_{mat+ptd}$ in Fig. 6B) secreted large amounts of IFN-γ when restimulated with TT/ptd-matDCs or CMV-matDCs (Fig. 6A and B). In contrast, TT/ptd-tolDC-primed T cells (T_{tol+TT}
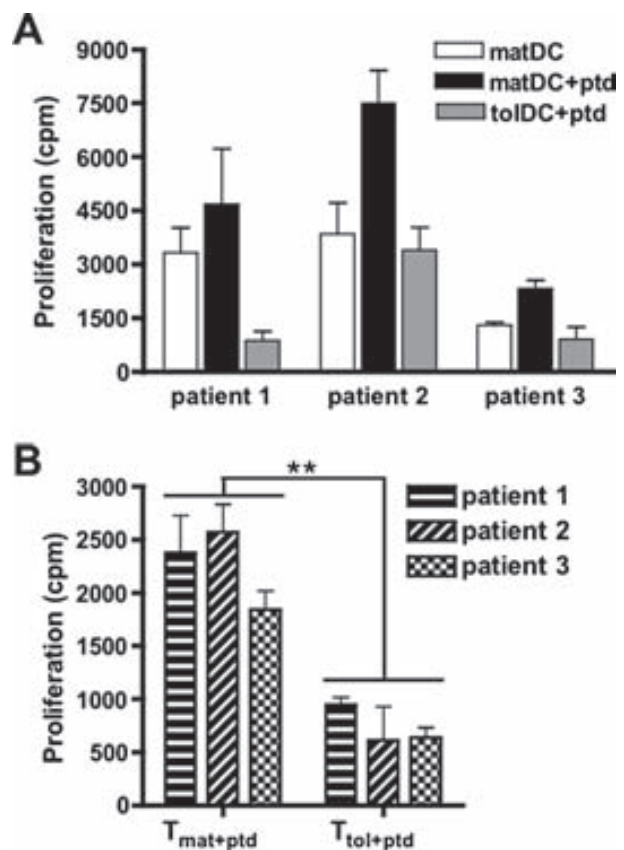
Figure 5. Myelin-peptide-loaded tolDCs induce hyporesponsiveness in autologous T cells from RR-MS patients. (A) The graph shows the mean + SD (of 21 replicates) of myelin-specific proliferation induced by DCs from three different RR-MS patients. Specific T cells generated by sequential myelin-peptide stimulation were cocultured with unloaded matDCs (matDC), with myelin-peptide-loaded matDCs (matDC+ptd) or with myelin-peptide-loaded tolDCs (tolDC+ptd), at a 1:20 ratio for 6 days. Proliferation was determined by (^{3}H)-thymidine uptake (cpm) during the final 16 h. The proliferation of unstimulated T cells was 705 $\pm$599 cpm and 23645 $\pm$4517 cpm for the positive control (pokeweed mitogen, 1 μg/mL). (B) T cells primed as described in (A), after 3 days of resting, were restimulated (ratio 1:10) with irradiated autologous PBMCs loaded with the mix of myelin peptides. Proliferation was determined by (^{3}H)-thymidine uptake after 72 h of culture. Bars represent the mean + SD of three replicates for each of the RR-MS patients (**$p < 0.005$ Wilcoxon test).

Table 2. Cytokine secretion analyzed in supernatants from autologous proliferation assays induced by either matDC or tolDC from RR-MS patients loaded with the mix of myelin peptides, collected after 96 h and quantified by multiplex assay (detection limit: 3.2 pg/mL) (paired t-test)

(pg/mL)	n	matDC mean (range)	tolDC mean (range)	p
IL-17	5	4.2 (0.3–12)	1.6 (0–6.98)	0.057
IL-6	3	124 (21–289)	29 (10–44)	0.236
IFN-γ	3	306 (224–407)	128 (34–256)	0.336
IL-10	5	646 (14–1975)	576 (25–2529)	0.724
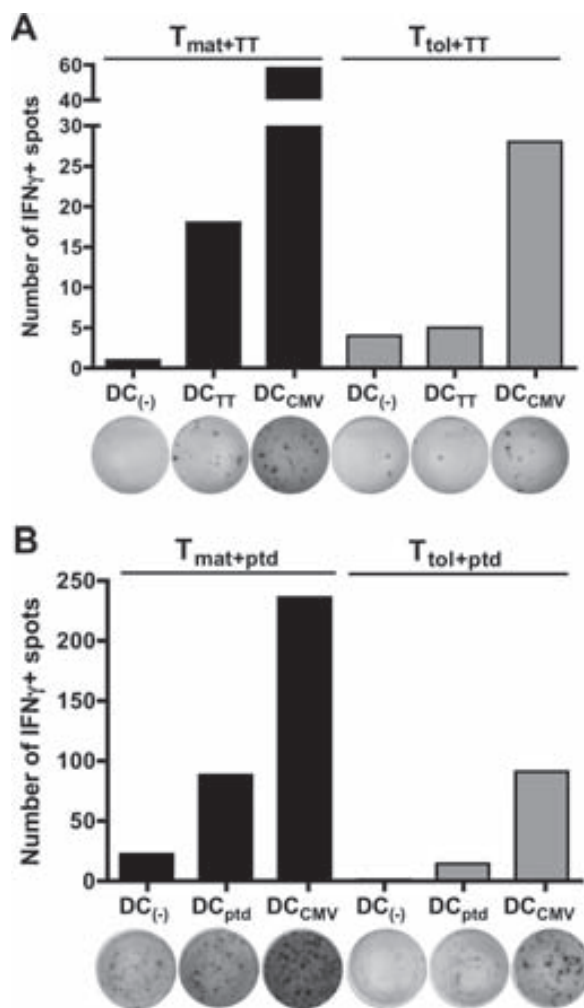
Figure 6. tolDCs induce antigen-specific hyporesponsiveness. Unlabeled CD3+ lymphocytes, from (A) donors or (B) MS patients with positive reaction to both CMV and TT or myelin peptides (ptd), respectively, were cocultured with (A) autologous TT-loaded or (B) ptd-loaded matDC or tolDC at a 20:1 T cell/DC ratio for 5 days. After this time, lymphocytes ((A) T_{mat+TT} and T_{tol+TT} or (B) $T_{mat+ptd}$ and $T_{tol+ptd}$) were harvested and left to rest for three additional days, and then were rechallenged in a second proliferation assay with autologous unloaded (DC$_{(-)}$), TT/ptd-loaded (DC$_{TT}$/DC$_{ptd}$), or CMV-loaded (DC$_{CMV}$) matDCs at a 20:1 T cell/DC ratio for 24 h. The secretion of IFN-γ in these cocultures was analyzed with ELISpot. Data show the number of counted IFN-γ spots (upper panel) and the corresponding IFN-γ ELISpot of one representative assay of (A) a healthy donor ($n = 4$) and (B) a MS patient ($n = 2$).

in Fig. 6A, and $T_{tol+ptd}$ in Fig. 6B) secreted large amounts of IFN-γ only when restimulated with CMV-matDCs but not when restimulated with TT/ptd-matDCs (Fig. 6A and B). Hence, these results indicate that TT/ptd-tolDCs selectively induced hyporesponsiveness to TT/ptd-specific T cells but did not alter the ability of other T cells to respond to an unrelated antigen such as CMV. Therefore, these results demonstrate that myelin peptides loaded-tolDCs from MS patients are able to induce an antigen-specific state of hyporesponsiveness to myelin-autoreactive T cells.

Discussion

Given the limited effectiveness of the approved treatments for MS, the design of new and more specific therapeutic approaches is necessary. To this end, induction of T-cell antigen-specific tolerance administering tolDCs has been tested in the EAE model [19, 27]. Different strategies have been evaluated to generate tolDCs in vitro using different tolerogenic agents as recently reviewed [18, 20]. Among those agents, some benefits of vitD3 in MS patients have been previously reported [28]. As a potential tolerogenic agent, we have focused on the use of vitD3 to generate tolDCs from RR-MS patients. The role of vitD3 in the generation of tolDCs has been previously reported in HCs [29, 30].

Our in vitro data demonstrate that vitD3 has a comparable effect on the differentiation and function of DCs derived from either HCs or MS patients, indicating that it is able to overcome any immune-activating factors associated with the autoimmune response in RR-MS patients. Comparative analyses of MDDCs between healthy donors and MS patients did not show any significant difference, except for the reduced expression of CD83 observed in matDCs and tolDCs from MS patients. Intriguingly, this phenomenon has also been reported in myeloid DCs from systemic lupus erythematosus patients [31]. Additionally, a proinflammatory phenotype of blood-derived DCs from MS patients has been described (higher levels of IFN-γ, IL-6, TNF-α, and IL-23 than their counterparts from HCs) [32, 33]. In contrast, the results obtained in this study and by another group [34] clearly indicate that monocyte-derived DCs from MS patients became tolDCs and exhibited an antiinflammatory profile following treatment with vitD3.

In addition to their tolerogenic potential, a key consideration for tolDC therapy should be the ability to maintain a tolerogenic function under inflammatory conditions in vivo, once tolDCs are reinfused into the patient. In vitro tolDCs maintained their tolerogenic phenotype and cytokine profile and secreted neither IL-12p70 nor IL-23, even when rechallenged with LPS in the absence of vitD3. Moreover, in agreement with other studies [26, 35], these tolDCs also retained their reduced T-cell allostimulatory ability after challenge with LPS, hence showing stability of their tolerogenic phenotype and functionality.

Our study reports for the first time that tolDCs from RR-MS patients are capable of inducing stable hyporesponsiveness in both allogeneic and myelin-autoreactive T cells. MS patients' autoreactive T cells are generally considered strong secretors of proinflammatory cytokines [36]. Interestingly, our results showed that T cells primed by tolDCs were refractory to further stimulation using myelin peptides-loaded APC, thus emphasizing the tolerogenic potential of tolDCs in MS patients. We evaluated some putative mechanisms involved in the hyporesponsive state induced by tolDCs in T cells (data not shown). The results ruled out specific deletion (apoptosis) of autoreactive T cells as shown with dexamethasone induced-DCs [37]. The phenotype of these primed T cells did not support the induction of Treg cells, in agreement with a previous report [38], but in contrast to the results of other authors [35]. Finally, the inability of exogenous

IL-2 to restore proliferation of tolDC-primed T cells suggests it is not T-cell clonal anergy [39] in accordance with previous findings [40]. However, our results clearly suggest that the reduction in secretion of proinflammatory cytokines is a key mechanism of tolerance induction by tolDCs in T cells. While MS patients' autoreactive T cells are strong secretors of proinflammatory cytokines including IFN-γ [41], tolDCs obtained from RR-MS patients are able to readily reduce the secretion of IFN-γ in antigen-experienced T cells. Thus, both hyporesponsiveness and reduction in the secretion of proinflamatory cytokines are key mechanisms of tolerance induction by vitD3-treated DCs in our setting.

In the design of an antigen-specific cellular therapy, antigen loading of APC is of important relevance. Current cancer treatments undergoing investigation include DCs loaded with tumor lysates [42] and genetically engineered DC vaccines expressing relevant tumour antigens [43]. Another approach for tolerance-inducing therapies is the use of apoptotic cells as a "pool of antigens," which has the potential to induce a tolerogenic profile in DCs [44, 45]. Induction of therapeutic tolerance in EAE has also been reported by targeting of DCs with monoclonal antibodies, such as anti-DEC205-mediated delivery of the PLP$_{139-151}$ [46], and also administration of splenocytes coupled with a myelin-peptide cocktail [47]. In our experiments, mature DCs loaded with a selected pool of seven immunodominant myelin peptides [4] induced specific proliferation of autoreactive T cells while tolDCs loaded with the same pool of myelin peptides induced hyporesponsiveness of autoreactive T cells from RR-MS patients. Importantly, this hyporesponsiveness shown by autoreactive antigen-experienced T cells obtained from MS patients was stable, since it was not overcome by myelin-loaded autologous APC. Moreover, using a combination of two different antigens, we could demonstrate the antigen specificity of this hyporesponsiveness in HCs (TT–CMV) and MS patients (myelin peptides–CMV). Therefore, tolDCs had the ability to induce specific tolerance to the antigen they present to T cells. These results confirm previous observations on the induction of antigen-specific hyporesponsiveness [16].

In summary, our data show that tolDCs generated from RR-MS patients using vitD3 exhibit a stable tolerogenic phenotype and antiinflammatory cytokine profile, with the capability to induce stable and antigen-specific hyporesponsiveness in autoreactive (myelin-primed) T cells from RR-MS patients. The tolerogenic potential of these autologous tolDCs may be an effective tool to reestablish myelin tolerance in RR-MS patients and provide the basis for future designs of clinical trials.

Material and methods

Patients

Eleven patients (ten females and one male) with RR-MS (age: 44 $\pm$ 9 years, median of the Expanded Disability Status Scale (EDSS): 4.0 (2.5–4.5) points, and illness duration: 11.8 $\pm$ 5.8 years) were included. All patients were in remission and had not received any immunosuppressive or immunomodulatory therapy for 3 months prior to their inclusion in the study. Sixteen control individuals (ten females and six males) aged 25–40 years were included. The Ethical Committee of Germans Trias i Pujol Hospital approved the study, and all subjects gave their consent according to the Declaration of Helsinki (59th WMA General Assembly, Korea, 2008).

Isolation of cells from peripheral blood

Samples were obtained from buffy coats from healthy blood donors (HCs) supplied by Blood and Tissue Bank (Spain), or by vein puncture of RR-MS patients (70 mL). PBMCs were isolated by density gradient centrifugation on Ficoll-Paque PLUS^{TM} (GE-Healthcare$^®$, Life Sciences, Uppsala, Sweden). Monocytes were isolated by positive selection using anti-CD14 (Miltenyi Biotec GmbH, Cologne, Germany). T cells were isolated by CD3$^+$ negative magnetic selection (Easysep$^®$, Stemcell Technologies, Seattle, WA, USA). Purity of both CD14$^+$ and CD3$^+$ was always >85%.

Generation of monocyte-derived DCs

Monocytes were cultured at 1–1.5 $\times$ 10^6 cells/mL in the presence of granulocyte-macrophage colony forming unit and IL-4 (1000 U/mL both, CellGenix^{TM} GmbH, Freiburg, Germany) for 6 days to obtain MDDCs. Complete medium contained X-VIVO^{TM} 15 medium (BioWhittaker$^®$, Lonza, Walkersville, MD, USA) supplemented with pooled AB human serum (2% v/v, supplied by Blood and Tissue Bank), L-glutamine (2 mM, Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/mL, Cepa S.L., Madrid, Spain), and streptomycin (100 μg/mL, Normon Lab. S.A., Madrid, Spain). Complete medium was refreshed at days 2 (half volume) and 4 (total volume). Maturation was induced at day 4 using a proinflammatory cytokine cocktail containing TNF-α (100ng/mL), IL-1β (10 ng/mL, both from CellGenix), and prostaglandin E2 (1 μM, Pfizer, New York, NY, USA) for 48 h to obtain matDCs. TolDCs were generated by adding vitD3 (1 nM, Calcijex$^®$, Abbott Lab., North Chicago, IL, USA) to the culture on days 0 and 4, and cytokine cocktail on day 4. imDCs were generated without either vitD3 or the proinflammatory stimulus. At day 6, DCs were loaded with the mix of myelin peptides for 18 h for the indicated assays.

Flow cytometry analysis

The following monoclonal antibodies were used for cell surface marker analysis: CD83-allophycocyanin, CD86-FITC, CD40-PE, HLA-DR-APC-H7, CD4-allophycocyanin, CD25-PECy5, CD127-PE (BD Pharmingen, San Diego, CA, USA). Staining followed standard protocols (incubation for 30 min at 4°C, and wash in phosphate-buffered saline (PBS)). CD4$^+$ CD25^{high} Foxp3$^+$ CD127$^{low/-}$ Treg cells were stained as previously reported [48] (Foxp3-Ab from eBioscience, San Diego, CA, USA). Events were

acquired on a FacsCanto II cytometer using the standard FacsDiva software and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). DCs were gated according to their forward scatter (FSC) and side scatter (SSC) properties. Cell viability was assessed using the Annexin V-PE apoptosis detection kit.

Assessment of DC stability

DCs were extensively washed with PBS, and then restimulated for 24 h in complete medium with or without LPS (100 ng/mL, from *Escherichiacoli* 0111:B4, Sigma-Aldrich) in the absence of vitD3. Subsequently, DCs were collected, washed, and further evaluated for their viability, phenotype, cytokine secretion profile, and allostimulatory inducing ability.

Allostimulatory assays

DCs were washed twice with an excess of PBS to exclude dead cells and debris, and to avoid a possible effect of residual vitD3. Allogeneic PBMCs were stimulated in vitro with the indicated DCs at a 20:1 ratio in 96 round-well plates. After 4 days, the cells were pulsed with 1 μCi of (^{3}H)-thymidine (Amersham Pharmacia Biotech, Munich, Germany) for an additional 16 h. Cells were harvested (Harvester 96 ®, Tomtec Inc., Hamden, CT, USA) and analyzed using a scintillation counter (1450 Microbeta®, Trilux Wallac, Turku, Finland).

Cytokine production

Supernatants were collected as indicated and stored at –20°C. Cytokine production was determined by multiplex assay (Milliplex™ MAP, Millipore Co., Bedford, MA, USA) and analyzed by Luminex 100™ IS . Supernatants from allogeneic cocultures were collected after 96 h (prior to addition of thymidine), stored at –20°C, and analyzed by cytometric bead array (CBA). To determine the intracellular production of cytokines, day 6 alloproliferative T cells were washed and restimulated for 5 h with phorbol-12-myristate 13-acetate (PMA, 50 ng/mL, Sigma) and ionomycin (500 ng/mL, Sigma) in the presence of brefeldin A (10 μg/mL). Cells were washed, fixed, and permeabilized using the IntraStain kit following the manufacturer instructions (Dako Cytomation, Glostrup, Denmark). Finally, cells were labeled with anti-IFN-γ allophycocyanin monoclonal antibodies, or the corresponding isotype antibody (fluorescence control), and analyzed by flow cytometry.

Peptide-binding assays

For peptide binding assays (based on previous literature [49]), matDCs and tolDCs were incubated in complete medium with the biotinylated myelin peptide PLP$_{(139–154)}$ (PolyPeptide group, Strasbourg, France) at different time points (from 2 to 18 h) at 37°C.

Peptide competition assay (based on previous literature [50]) was performed adding 50 μM of biotinylated PLP$_{(139–154)}$ with increasing concentrations of unlabeled PLP$_{(139–154)}$ (0, 50, 250, 500 μM) to either MatDCs or TolDCs (2 × 10^5 DCs in 100 μL of complete medium), and incubated the mixture for 18 h at 37°C. After peptide excess was washed off with PBS, the bound peptide was detected by staining with Alexa-488 labeled streptavidin (1/800 dilution, Molecular Probes, Inc., Eugene, OR, USA), and with Annexin V-PE to only analyze living cells by flow cytometry.

Enrichment of myelin-specific T-cell lymphocytes

For enrichment of myelin-specific T cells from MS patients, we followed the protocol described by Bielekova et al. [4] that expanded the high-avidity myelin-specific T cells, which were >90% CD45RA$^-$/RO$^+$. Briefly, PBMCs were isolated from peripheral blood of RR-MS patients by density gradient centrifugation. A total of 2 × 10^5 PBMCs/well were seeded into fresh 96-well plates in X-vivo 15 medium (BioWhittaker, Walkersville, MD, USA) enriched with IL-7 (10 ng/mL; PeproTech, Rocky Hill, NJ, USA) and a mix of seven myelin peptides: MBP$_{13–32}$, MBP$_{83–99}$, MBP$_{111–129}$, MBP$_{146–170}$, MOG$_{1–20}$, MOG$_{35–55}$, PLP$_{139–154}$ (5 μM each peptide, NeoMPS, San Diego, CA, USA). Sixty wells without antigen served as negative control. Following 7 days of incubation at 37°C at 5% CO$_2$, 50% of the individual cultures were transferred into new 96-well plates ("daughter plates"), which were pulsed for 16 h with 1 μCi/well (^{3}H)-thymidine to measure incorporated radioactivity (counts per minute (cpm)). Individual wells were considered positive if their cpm was at least three standard deviation (SD) above the average cpm of the 60 control wells. Hundered microliter of X-VIVO15 medium containing IL-2 (20 IU/mL; ImmunoTools GmbH, Friesoythe, Germany), 2-mM L-glutamine, and 100-U/mL penicillin/streptomycin and 5% pooled human AB sera were added to original plates. Positive cultures were restimulated on day 12 with 10^5/well autologous irradiated Ag-pulsed (5 μM of each peptide) PBMCs with IL-2 (20 IU/mL). Cell culture was refreshed every 3–4 days adding IL-2 (20 IU/mL). On days 24 and 36, T cells were restimulated again in 48 or 24 well plates with 1 × 10^6/well or 2 × 10^6/well (respectively) autologous irradiated Ag-pulsed (5 μM of each peptide) PBMCs with IL-2 (20 IU/mL). The phenotypic analysis of these cells, at the end of the third in vitro restimulation cycle (days 45–80 ex vivo), showed that 80–90% were CD3$^+$ and 75–80% of them were CD4$^+$.

Myelin-specific proliferation assay

Enriched myelin-specific T lymphocytes from three RR-MS patients were cocultured with matDCs or tolDCs (DC:T ratios 1:20 and 1:40 for both) loaded or not with the same mix of peptides. Proliferation was determined after 6 days by (^{3}H)-thymidine incorporation for the final 16 h.

T-cell restimulation

Following stimulatory cultures, myelin-loaded matDC- and/or tolDC-primed T cells were counted and equal numbers were plated. After 3 days of resting (without IL-2), T cells were restimulated with irradiated (60Gy) (IBL 437C, CIS Bio International S.A., Gif-sur-Yvette, France) autologous PBMCs as APC (ratio 1:10) loaded with the mix of myelin peptides. Proliferation was determined after 84 h of culture by (^{3}H)-thymidine incorporation during the final 16 h.

Analysis of antigen-specific responsiveness

imDCs from HCs with positive reaction (significant proliferation of PBMCs) to both TT (from *Clostridium tetani*, Sigma-Aldich) and CMV (PepTivator® CMV pp65, Miltenyi Biotec) were loaded with TT, CMV, or no antigen for 8 h prior to the addition of the maturation cocktail. To detect antigen-specific responsiveness, unlabeled CD3^{+} lymphocytes were cocultured with autologous TT-loaded matDCs or tolDCs at a 20:1 T cell/DC ratio for 5 days. Following this, lymphocytes were harvested and rested in fresh medium for 2 additional days. After that, T cells were rechallenged in a second stimulation assay with autologous TT-loaded, CMV-loaded, or unloaded matDCs at a 20:1 T cell/DC ratio for 24 h. Secretion of IFN-γ was analyzed with ELISpot[PRO] (Mabtech AB, Nacka Strand, Sweden) after 20 h of additional incubation, following the manufacturer's instructions. Spots were counted and analyzed with a CTL ImmunoSpot® Analyzer using the CTL ImmunoSpot® Academic software (CTL-Europe GmbH, Bonn, Germany). The same experiment was performed using the mix of myelin peptides instead of TT to detect antigen-specific responsiveness with MS patients' cells with positive reaction to both myelin peptides and CMV.

Statistical analysis

Data are given as mean $\pm$ SD of n samples. Comparisons between two groups were performed using the paired and unpaired t-test for parametric data, and the Wilcoxon test (paired data) or Mann–Whitney test (unpaired data) for nonparametric data. Comparisons among multiple groups were performed using analysis of variance. Statistical analysis was performed using the Prism 4.0 software (GraphPad software Inc., San Diego, CA, USA). The preset limit of statistical significance was $p < 0.05$.

Conflict of interest: The authors declare no financial or commercial conflicts of interest.

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Discussion

Given the limited efficacy of the approved treatments for MS, the design of new and more specific therapeutic approaches is necessary. To this end, several authors proposed tolDC administration for inducing T-cell antigen-specific tolerance, as a potential treatment for autoimmune pathologies (87,185,283,284). Altogether the findings of this thesis, set up the bases for using autologous tolDCs as a therapeutic tool to re-establish tolerance in RR-MS patients. During last decade the amount of bibliography concerning the DC-based therapy in tolerance has increased, and some key considerations relating to their clinical application have emerged (185,254), summarized below.

- Standardizing protocols for their generation:
  - Choice of type and source of DC
  - Choice of growth factors, tolerance-inducing agent and maturation stimulus
  - Choice and loading of auto-antigen/s
- Ensuring:
  - Stability of tolerogenicity and specificity
  - Safety: GMP compliance
- Identification of quality control/s of tolerogenicity
- Identifying the optimal route, timing, dose and frequency of administration
- Migration after administration
- Identification of biomarker of tolerance induction

Our studies have covered some of these issues focusing on MS therapy, with a special interest on the cell-product manufacturing process, discussed below.

1. Standardization of tolDC generation protocol

1.1. Source of DCs and growth factors

Our first decision to standardize the protocol for tolDC generation was the type and source of cells to be used. As mentioned in the introduction section, DCs can be differentiated *in vitro* from different cellular sources, including bone marrow, cord blood and PBMCs. In humans, blood monocytes are the most common source for tolDC generation, although tolerance induction with pDCs is being investigated too (285). MDDCs offer the advantages of being the most accessible, since they do not require previous precursor mobilization, and are very well characterized both from animal models and from humans. For these reasons we decided to use autologous dendritic cells derived from monocytes as a therapeutic tool in our setting. Regarding the growth factors to differentiate monocytes to MDDCs, we used the classical combination of IL-4 and GM-CSF, like most of the studies performed with MDDCs (286,287).

1.2. GMP compliance

The standardized protocol for generating tolDCs that has been developed fulfills the regulatory medical rules for human therapy. All reagents used are (or are available) in GMP-grade, including growth factors (IL-4 and GM-CSF), tolerance-inducing agents (vitD3, dexamethasone and rapamycin) used in the clinical practice, maturation agents (IL-1 β , TNF-α , PGE-2 and MPLA), cell-culture medium and human serum.

1.3. Tolerance-inducing agent

To decide which tolerance-inducing agent was the most convenient to generate tolDCs for MS therapy, we performed a comparative study of three different clinical-grade tolDCs products (Part I of Results). Several immunosuppressive drugs were investigated for tolDC generation:

dexamethasone (Dexa), rapamycin (Rapa) and vitamin D3 (vitD3). Our results show that all clinical-grade tolDCs were functionally tolerogenic in comparison with previously characterized research-grade tolDCs (260). Only Dexa-DCs and VitD3-DCs presented reduced expression of surface maturation markers and increased IL-10 production, in contrast with Rapa-DCs that showed a "mature phenotype" and no IL-10 secretion. In agreement with previous reports (288) Rapa-DCs also exhibited reduced ability to induce T-cell alloproliferation along with a concomitant decrease of T-cell IFN-γ secretion and $CD4^+ CD25^{hi} CD127^{lo} FoxP3^+$ Tregs expansion.

Despite its interesting features, we ruled out the use of Rapa since Rapa-tolDCs showed a phenotype and cytokine profile comparable to that of immunogenic DCs, not allowing the distinction between tolDCs and immunogenic DCs. Nevertheless, some groups reported reduced expression of co-stimulatory molecules (especially CD86) on Rapa-treated DCs (266,289), which could be explained for differences on the DC source or in the DC differentiation protocol. Other works suggest that Rapa selectively up-regulates CCR7 expression on MDDCs (290) and that it could be used as a distinguishable biomarker. In fact, some groups selected Rapa-conditioned DCs for tolerance promotion in animal models of transplantation (291,292).

We also ruled out the use of Dexa because in approximately 1/3 of the tested samples, Dexa-conditioned DCs did not acquire regulatory properties at the concentration used. These findings could be explained by a de-sensitization of DCs to the immunomodulatory action of Dexa in some individuals (293). Precisely to overcome this phenomena Hilkens and co-workers decided to use a combination of Dexa and vitD3 in rheumatoid arthritis (294). The combined use of both immunomodulatory agents has been shown to inhibit DC maturation and function in an additive manner (185,295), rendering effective tolDCs in a colitis murine model (273).

We selected vitD3 for tolDCs-generation in the MS setting, although the different features presented by tolDCs obtained with Dexa or Rapa may be suitable for other disease contexts. VitD3 as tolerogenic agent, besides having immunomodulatory potential for MS treatment (296), has been repeatedly shown to generate tolDCs with anti-inflammatory profile and tolerogenic function. In a similar study, Pedersen and co-workers also compared differently generated tolDCs, treated with IL-10, dexamethasone or vasoactive intestinal peptides, concluding that, compared to others, vitD3-treated DCs were superior in terms of tolerogenic functional stability (271), finding that reinforced our choice for vitD3.

1.4. Quality control of tolerogenicity

Our comparative study of differently generated tolDCs revealed that features such as phenotype markers and cytokine profiles do not always permit to distinguish immunogenic from tolerogenic DCs, as is the case of Rapa-DCs. For this reason, a set of "biomarkers" or quality control (QC) measures of tolerogenicity in cellular products have to be defined for a better monitoring of putative tolerogenic cells (297,298). The definition of QC measures will be especially important for clinical trials. Unlike recent efforts in developing QC for immunogenic DCs (297), QC measures on clinically applicable tolDCs are still in its infancy. Ideally, QC for DCs should be based on reliable markers that can be assessed rapidly prior to release of the generated tolDC product, validating their suitability for clinical use. Current *in vitro* functional assays for assessing functional tolerogenicity of DCs (such as generation of Tregs) are labor-intensive and take several days, being not suitable as QC of a tolDC-based vaccine (299). In this line, some studies proposed PD-L1 and ILT molecules as markers for vitD3-treated DCs (92,259,300), but they were not confirmed. Pedersen and co-workers (271) recently suggested the lack of IL-23 secretion and the up-regulated expression of microRNA-155 (miR-155) as markers to distinguish vitD3-modified regulatory DCs (273).

Importantly, these markers can be analyzed with standard laboratory techniques such as ELISA or RT-PCR, and may be combined with the classical phenotype markers for tolDC characterization (i.e. IL-10 production).

1.5. Functional stability and maturation stimulus

Another issue of critical importance for the clinical use of tolDCs is the functional stability of the tolDC product. We refer to functional stability as the incapacity of tolDC to revert into immunogenic DC in response to inflammatory signals (such as pro-inflammatory cytokines, TLR ligands or CD40 ligation) when injected back to the patient. This major concern comes up with the finding that some regulatory DCs showed a versatile function, such as immature DCs or TNF-α matured DCs, which could be reverted into immunogenic DCs under potent inflammatory conditions (301,302). For this reason, ensuring the functional stability of *in vitro*-generated tolDCs is extremely crucial prior to patient administration.

Maturation stimulus used for obtaining tolDCs is an important feature to be considered, since it conditions the stability of their tolerogenicity. In this regard, DCs with contrasting properties have been obtained using different combinations of pro-inflammatory mediators and TLR ligands, but not all maturation stimulus confer such stability to tolDCs (227,242,303). Therefore, to find an optimal cytokine environment for DC maturation to obtain a cellular product suitable for DC-based immunotherapeutic protocols, we performed a comparative study of three different maturation stimuli on vitD3-induced DCs and on matched immunogenic DCs (Part II of Results). Using differential maturation regimens: the traditional CC in combination or not with the TLR4-activating LA, we obtained tolDCs (and also MatDCs) with distinctive features. Specifically, we found that CC-matured tolDCs were unique in showing stable tolerogenic functionality, since they did not induce T cell proliferation after LPS challenge or after additional 24

hours of culture. Such functional tolerogenic stability was not observed in (LA) neither in (CC+LA)-TolDC, which could be explained by a study of Griffin and co-workers (304). They suggest that DCs cultured with vitD3 (as other VDR ligands) selectively suppress the expression of the promoter RelB (a NF-κB family member), inhibiting DCs maturation (305). Interestingly, RelB is up-regulated by the presence of LPS (304). This downstream regulation could explain our finding that vitD3-induced DCs matured with the TLR4 agonist LA did not result in functionally stable tolDCs. These results are supported by the fact that treatment of human monocytes with vitD3 suppressed the expression of TLR2 and TLR4 mRNAs and proteins (306,307). This mechanism may permit to prevent excessive TLR activation and inflammation at a later stage of an infection (306).

Thus, we selected to stimulate our tolDCs with the CC, since CC-matured tolDCs were unique to show stable tolerogenic functionality besides a potent suppressive ability on T-cell induced proliferation. CC-matured tolDCs were the best suited to be used as negative cellular vaccine for immunotherapy in our setting.

1.6. Choice and loading of auto-antigens

Since the main aim of tolDC-based vaccines is to induce antigen-specific tolerance, loading of tolDCs with the relevant autoantigen(s) will have to be taken into consideration. In the cancer setting, immunogenic DC-vaccines tested in clinical trials have been loaded with disease-specific antigens in the form of DNA, RNA, peptides, proteins, cell lysates, or exosomes (308–310). Best options for antigen loading of DCs need to be exhaustively studied and analyzed for each application (311). On the other hand, many of the animal studies conducted so far for tolDC vaccination did not employ antigen-loaded DCs (94). Administration of unloaded tolDCs can lead to either untargeted tolerance induction or to tolerance to non-related disease antigens taken up *in vivo*. Therefore it is desirable that tolDCs to be used in human clinical settings are loaded

with defined antigens. Although several methods of Ag-pulsing tolDC (cell-free lysate, MHC peptides, early apoptotic cells, or exosomes) have been described to be successful in the context of transplantation in rodents (244), an optimal approach for an *in vivo* use in humans has not yet been determined.

The choice of autoantigen is an important consideration that depends on the particular disease to be targeted. In Grau-López et al (131) we demonstrated distinct reactivity to a selection of seven immunodominant myelin peptides in T cells from a cohort of MS patients compared to HC. We decided to load tolDCs with this pool of seven immunodominant myelin peptides (Table 1), in order to develop an antigen-specific tolerogenic tool for MS. Due to the antigenic epitope spreading during disease development, using a pool of peptides as opposed to a single one, offers a clear advantage widening the repertoire of potentially relevant auto-antigens and epitopes in MS patients. Promising results supporting the use of multiple peptide-loaded tolDCs have been showed by Smith et al, which have demonstrated to ameliorate on-going relapsing EAE after the infusion of multiple peptide-coupled cells (312).

Investigation of loading the myelin peptides to tolDCs showed that the optimal timing is achieved after incubating the peptides between 12h and 18h at 37°C, which was confirmed functionally (antigen-specific proliferation, part III of Results). These conditions are in agreement with other publications (313), and in contrast with other groups that only incubate the peptides for 2h or 4h (273,314–316). This divergence could be due to differences in the length and hydrophobicity of peptides (273), to the fact that the peptides used were not HLA-type restricted (314), or to the source (mice or human) of DCs (316). Remarkably, there are not many studies focusing on the antigen loading on DCs for immunotherapy, and most of them incubate the cells with autologous tissue lysate/fluid (294) or antigenic peptide in the T-cell coculture (269).

Long determined times for peptide-loading could be explained by the fact that DCs may process the peptides before presenting them in the context of MHC molecules. It is generally assumed that after the maturation process, DCs reduce their ability to capture and present antigens compared with immature DCs (28,30). However it has been recently reported that mature DCs continue to accumulate antigens, especially by receptor-mediated endocytosis and phagocytosis (317). Thus, matured tolDCs are likely to internalize myelin peptides, transport them to late endosomes and lysosomes, and finally load them onto MHC-II molecules for T-cell presentation. Such complex peptide processing could explain to long DC presentation times to T cells.

Another question that remains to be solved is whether these peptides remain on the surface of tolDCs after their infusion or whether they are replaced by other antigens found *in vivo*. Several authors have avoided the uncertainty with alternative methods such as the use of ECDI (*ethylenecarbodiimide*) that fixes the peptides to the cell surface (318), or the targeting of DCs *in vivo* with peptide-bound monoclonal antibodies like anti-DEC205-mediated delivery of the PLP139-151 (186). Both approaches have shown to be effective in EAE (186,312). Interestingly, the use of an artificial multi-epitope protein (composed by selected MS-relevant epitopes of five major myelin antigens) was reported to be more efficient than the peptide cocktail in reversing chronic EAE (319). These different approaches offer an optimization of the effectiveness of immune-specific therapies.

1.7. Specificity and mechanisms of tolDCs

In part III of results we detected specific proliferation of autoreactive T cells from RR-MS patients against myelin peptides when presented by immunogenic DCs, whilst tolDCs loaded with the same myelin peptides induced T cell hyporesponsiveness, as also found by Pedersen et al using human vitD3-DCs (273). Induction of hyporesponsiveness to T cells was accompanied by a reduction of the pro-inflammatory cytokines IFN-γ ,

IL-6 and IL-17 in the co-culture with tolDC. These results are very relevant since MS patients' autoreactive T cells are strong secretors of pro-inflammatory cytokines including IFN-γ and IL-17 (128). We demonstrated that this hyporesponsiveness is antigen-specific, since T cells tolerized to myelin peptides are able to secrete IFN-γ after CMV re-stimulation but not when re-stimulated with the myelin peptides. These results confirm previous observations on the induction of antigen-specific hyporesponsiveness with tolDCs (284).

What remains to be fully investigated are the molecular mechanisms behind this T-cell induced hyporesponsiveness. Our results ruled out the specific deletion (apoptosis) of autoreactive T cells, in contrast with a previous study where vitD3-treated DCs induced apoptosis to autoreactive T cells (269). These controversial results can be explained by the fact that T cells were antigen-specific clones and the shorter timing in which apoptosis was measured. Similarly to our results, dexamethasone-induced DCs do not induce apoptosis in alloreactive T cells (320). We also considered the possibility of induction of regulatory T cells, which has been observed in tolDCs generated using rapamycin (265,321). However, our phenotypic data seem to not support this hypothesis, in agreement with a previous report (322) and in contrast with results obtained from mouse model of transplantation (323) and from non-obese diabetic mice (324). Additional experiments such as suppressive assays are necessary to fully characterize alternative subpopulations of T regulatory cells. In fact, it has been described that vitD3-treated DC could convert CD4+ into IL-10 secreting T cells potently suppressing the proliferation of responder T cells (259). Interestingly, our results confirmed the production of IL-10 by vitD3-DCs stimulated T-cells (Results, part III). Finally, stimulation with immunogenic DCs and exogenous IL-2 was ineffective to restore proliferation of tolDC-primed T cells. This suggests that the hyporesponsive state induced by tolDC is not due to T cell clonal anergy, as previously described (76,325) and in accordance with reported data by Penna et al (272).

Our results ruled out specific deletion (apoptosis), induction of Treg and clonal anergy as possible mechanisms of the tolDC-induced hyporesponsiveness. A similar behavior was reported in T cells stimulated using dexamethasone-induced tolDC (320). Thus, the functional characterization of the tolDC-induced hyporesponsiveness in T cells merits further efforts. In this sense, several molecules have been identified that may be involved in this function. For instance, secretion of IL-10 is considered a common feature of many tolDCs and has been related with their ability to induce IL-10–producing T cells both *in vitro* and *in vivo*, and also to prevent EAE (94). Although vitD3-DCs also secrete IL-10 ((272,273) and our results), the low levels of expression do not contribute to support a prominent role for IL-10 as a unique mechanism to induce tolerance.

Other molecules related with the regulatory ability of tolDCs, such as IDO, TGF-beta and Fas signaling were discarded to be involved in the T-cell tolerance induction by vitD3-DCs (259,273). Interestingly, inhibitory receptors ILT3 and PD-L1 are up-regulated in vitD3-treated DCs (92,255,259,326), and both have been described to play a key role in the suppression of T cell activation (92,327,328), suggesting an important role in the tolerogenic function of DCs. Although PD-1 signaling limits the extent of CD4^{+} T-cell accumulation in response to an immunogenic stimulus, it is not required for either the induction or maintenance of peptide-induced tolerance (329), in contrast with the reported requirement for PD-1 signals in CD8(+) T-cell tolerance (330). This finding has to be taken into account since most of the autoreactive T cells employed in our work were CD4+ (80%). Therefore, with our current knowledge, it is unlikely that PD-L1 played a key role in the tolDC induced hyporesponsiveness on T cells.

Expression of ILT3 has been postulated as a general feature of tolDCs that enables them to anergize T cells and convert them into Treg cells (331). Although it is also reported that ILT3 expression is not required for vitD3-DCs to induce FoxP3+ Treg cells (326), this molecule

may be involved in the induction of T-cell anergy. Indeed, PD-L1, ILT3, and also CTLA-4 (constitutively expressed by Treg (332)) are described to modulate the phenotype and function of DCs, emphasizing the importance of the cross talk between tolDCs and T cells in the suppression of the immune response. Thus it is likely that, instead of existing only one molecular mechanism, there is a complex interrelation during the encounter between tolDCs and T cells that results in the induction of T-cell hyporesponsiveness.

2. Considerations about clinical application of tolDCs

Further relevant considerations related to the clinical application of tolDCs include time, frequency and route of administration, as well as migration and effectiveness after infusion (185). Animal models have been instrumental in answering some of these concerns [ref] although *in vivo* tolDC treatment requires of further investigations using EAE mice.

2.1. Route of administration and migration of tolDCs

The most common routes for tolDC administration are intraperitoneal (i.p.), intravenous (i.v.) or subcutaneous (s.c.). Comparison of the three administration routes of DCs in EAE has showed different influence in tolerance versus immunity induction (315). For example, i.v. and i.p. were shown to be ineffective for EAE induction (315), but could be the most effective for tolerance induction, as suggested by some authors (333). In fact, in the eventual scenario of tolerogenic treatment, i.v. injection of tolDC has been proved as the preferential route to induce peripheral tolerance in mouse models of transplantation (250,274) and autoimmunity (313,316,334).

Depending on the route of injection, tolDCs may be distributed differently among the tissues. In fact, Morita *et al* reported that after i.v. and i.p. administration, most tolDCs accumulated in the spleen, whereas s.c. vaccination directed tolDC migration to the lymph nodes (335).

Moreover, upon i.v. injection into arthritic mice, tolDCs migrated to the spleen, liver, lung, feet, and draining lymph nodes (334). A similar distribution was observed in humans after DCs i.v. administration, where most of the DCs were found in the spleen few hours after injection and could remain there few days later (336).

However, it should be noted that the capacity of different types of tolDCs to migrate is likely to vary, especially if the expression of chemokine receptors such as CCR7 is altered by the tolerization treatment. Several authors agreed that TLR-activation and PGE-2 endows DCs with the ability to migrate in response to the lymph node chemokine CCL19 through the expression of CCR7 (31,337), which has been confirmed with immunogenic DCs matured with a combination of PGE-2 and TLR-stimulation (338). Remarkably, the CCR7 expression of vitD3-treated DCs is reduced compared with untreated DCs (300,326), suggesting that these tolDCs will not migrate to lymph nodes after being injected. Despite this fact and since the main mechanism of our tolDCs is the induction of hyporesponsiveness to autoreactive T cells, it becomes logical from the function point of view that migration to lymph nodes is unnecessary. We hypothesize that it would be in the spleen where our tolDCs may encounter the circulating auto-antigen experienced T cells (131,339,340), leading to the induction of 'tolerogenic signals'. Nevertheless, further studies are needed to address tolDCs migration in our setting. These studies will also allow defining the optimal dose of tolDCs and the number of injections for therapeutic treatment of established EAE.

The route of tolDC delivery also influences their efficacy of inducing tolerance *in vivo* (341). The efficacy and safety of TolDC therapy in EAE mice has been demonstrated by several studies (reviewed in ref.(185)). Although some of them showed to prevent the EAE, the most relevant for the tolDC human application are those that demonstrate to abrogate the ongoing disease progression (313,342,343).

2.2. Biomarker of tolerance induction

To better evaluate the *in vivo* effectiveness of the DC-based therapy proper biomarkers of tolerance induction need to be defined (185,344). TolDC therapies could take several weeks or even months to induce their full effects, since these could depend on the tolerization of T-cells or the generation of Tregs. For this reason, we need to develop readily quantifiable biomarkers that tell us that the therapy is prompting the recipient's immune system in the right direction (185). In our case, to read out the effectiveness of our tolDCs in MS patients, we could use PBMCs reactivity (proliferation) in front of the pool of myelin peptides, as a quantifiable biomarker of tolerance induction. This technique could be accompanied by the current diagnostic methodology in MS (such as MRI). These biomarkers, besides allowing designs of more robust trials, they will provide us with feedback information on whether enough number of tolDCs are being administered or whether it is done frequently enough.

2.3. Other considerations

TolDC administration may have the potential to reinstate immune tolerance, but the successful suppression of autoimmune response might require some time. Therefore, with the aim of improving the efficacy of the treatment, the combined administration of short-term immunomodulatory drugs with tolDCs may be considered, as suggested in the transplantation setting (195,196). Hopefully, this combined therapy will synergize and prevent the expansion of pathogenic T cells (inducing hyporesponsiveness), and favor the expansion of Treg cells. Mouse models of EAE will be useful for testing such combination therapies. Anyhow, no animal model can substitute well-designed and robust clinical trials to address the efficacy of tolDC-based therapies in human autoimmunity. Finally, we are confident that the results obtained during the course of this thesis will allow the translation of tolDC-based

therapy into clinical application for RR-MS patients, being able to fulfill the long-sought goal of a definitive cure.

Conclusions

This study defines a clinical-grade protocol to generate autologous myelin-peptide loaded tolerogenic DCs able to induce antigen-specific tolerance in RR-MS patients.

This general conclusion derives from specific studies which conclusions are summarized as follows:

1. The comparison of different pharmacological grade tolerogenic agents (Dexa, Rapa and vitD3) led to the generation of tolDCs exhibiting different properties. Specifically:

    1.1. A semi-mature phenotype and high IL-10 secretion shown by Dexa-treated DCs. Although these are ideal features of tolDC, some individuals may be refractory to Dexa treatment, making this agent not reliable.

    1.2. The impairment of IFN-γ in co-cultured T cells and expansion of T regulatory cells (CD4$^+$ CD25^{hi} FoxP3$^+$ CD127^{lo}) induced by Rapa-treated DCs. However, their "mature phenotype" (CD83^{hi} CD86^{hi} HLA-DR^{hi}) impairs the phenotypic distinction between tolDCs and immunogenic DCs in a particular sample.

    1.3. A semi-mature phenotype, production of IL-10, and reduction of IFN-γ in co-cultured T cells by vitD3-treated DCs. These features, along with their reproducibility among different samples, made vitD3 considered as the most convenient of the three compared agents to generate tolDCs for MS therapy.

2. The maturation stimulus is crucial to induce a "stable" phenotype in tolDCs. In this sense:

    2.1. The combination of *cytokine cocktail* and *monophosphoryl lipid A* (CC+LA) pointed as the best stimuli for obtaining immunogenic DCs, since they showed a high surface expression of CD83, CD86, HLA-DR and IL-12p70 secretion, and induced the production of IFN-γ and IL-17 in allogeneic co-cultures, suggesting a Th17 polarization.

    2.2. The *cytokine cocktail* (CC) was determined as the optimal maturation stimulus to generate tolDCs (induced by vitD3 treatment) in our setting, since CC-stimulated tolDCs were the unique functionally stable and capable of suppressing an immune response *in vitro*.

3. It is feasible to generate monocyte-derived vit-D3 tolDCs from relapsing-remitting MS patients. These cells show an equivalent yield, viability, phenotype and functionality compared to tolDCs from healthy donors.

4. Loading of myelin peptides to tolDCs for optimal antigen presentation is established between 12 and 18 hours at 37ºC.

5. Myelin-loaded tolDCs from MS-patients induce antigen-specific and stable hyporesponsiveness in autologous myelin-reactive T cells *in vitro*.

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Publications

1. <u>Raïch-Regué D</u>, Grau-López L, Naranjo-Gómez M, Ramo-Tello C, Pujol-Borrell R, Martínez-Cáceres E, Borràs FE. 2012. **Stable antigen-specific T cell hyporesponsiveness induced by tolerogenic dendritic cells from multiple sclerosis patients.** *European Journal of Immunology.* 42(3):771-82.

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