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Review

Tolerogenic dendritic cells for reprogramming of lymphocyte responses in autoimmune diseases

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ABSTRACT

Dendritic cells (DCs) control immune responses by driving potent inflammatory actions against external and internal threats while generating tolerance to self and harmless components. This duality and their potential to reprogram immune responses in an antigen-specific fashion have made them an interesting target for immunotherapeutic strategies to control autoimmune diseases. Several protocols have been described for *in vitro* generation of tolerogenic DCs (tolDCs) capable of modulating adaptive immune responses and restoring tolerance through different mechanisms that involve anergy, generation of regulatory lymphocyte populations, or deletion of potentially harmful inflammatory T cell subsets. Recently, the capacity of tolDCs to induce interleukin (IL-10)-secreting regulatory B cells has been demonstrated. *In vitro* assays and rodent models of autoimmune diseases provide insights to the molecular regulators and pathways enabling tolDCs to control immune responses. Here we review mechanisms through which tolDCs modulate adaptive immune responses, particularly focusing on their suitability for reprogramming autoreactive $CD4^+$ effector T cells. Furthermore, we discuss recent findings establishing that tolDCs also modulate B cell populations and discuss clinical trials applying tolDCs to patients with autoimmune diseases.

Contents

Abbreviations: DCs, Dendritic cells; tolDcs, Tolerogenic dendritic cells; IL, Interleukin; APCs, Antigen presenting cells; TCR, T cell antigen receptor; MHC, Major histocompatibility complex; TLR, Toll-like receptor; RA, Rheumatoid arthritis; TNF-α, Tumor necrosis factor-α; iNOS, Inducible nitric oxide synthase; IFN-α, Interferon-α; HLA, Human leukocyte antigen; TGF-β, Transforming growth factor β; IDO, Indoleamine 2,3-dioxygenase; Treg, Regulatory T cells; T1D, Type 1 diabetes mellitus; SLE, Systemic lupus erythematosus; MS, Multiple sclerosis; LPS, Lipopolysaccharide; dex, Dexamethasone; vitD3, Vitamin D3; MPLA, Monophosphoryl lipid A; PGE2, Prostaglandin E2; Tr1, IL-10 secreting type 1 regulatory T cells; nTreg, Natural Treg; iTreg, Inducible Treg; ILTs, Immunoglobulin-like transcripts; SOCS3, Suppressor of cytokine signaling-3; TT, Tetanus toxin; Bregs, Regulatory B cells; EAMG, Experimental autoimmune myasthenia gravis; EAE, Experimental autoimmune encephalomyelitis; NOD, Non-obese diabetic; CIA, Collagen-induced arthritis; CII, Type II collagen.

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1. Introduction

Dendritic cells (DCs) are professional antigen presenting cells (APCs) capable of initiating and modulating antigen-specific immune responses. They link innate and adaptive immune systems and orchestrate the response of distinct cell types [1,2]. The "patrol and reply" skills of DCs consist in antigen sampling and processing, maturation upon encountering potentially harmful agents, and migration to secondary lymphoid organs, particularly to T cell zones [3], where DCs control the fate of T cells through three main signals: first, antigen presentation through HLA-peptide complexes, which are recognized by specific T cell antigen receptors (TCRs); second, costimulatory and co-inhibitory signals provided by cell surface ligands; and third, soluble mediators such as cytokines and chemokines that establish a pro-inflammatory or anti-inflammatory environment promoting the required T effector cell type [4–6]. Due to their critical role in regulating adaptive immune responses and their involvement in various autoimmune disorders [7–10], DCs have become an attractive target of immunotherapy. Several methods have been described to generate monocyte-derived DCs with tolerogenic properties (tolDCs) *in vitro*, able to silence or reprogram autoreactive T cells and to induce regulatory lymphocyte populations [11–13].

This review discusses strategies for the *in vitro* generation of tolDCs and mechanisms by which tolDCs modulate T and B cell subsets, regarding their suitability for therapeutic approaches to control autoimmune responses.

2. Dendritic cells in health and autoimmune disease

The ability of DCs to initiate and modulate immune responses is related to key features such as uptake, processing, and presentation of antigens via major histocompatibility complex (MHC) molecules, recognition of "danger" signals through pattern recognition receptors, chemotaxis to sites of interaction with lymphocytes, and provision of costimulatory or inhibitory ligands and soluble mediators that drive the differentiation of effector or regulatory lymphocyte populations, respectively [2,14,15].

In humans, DCs originate from bone marrow precursors, which migrate via the blood to lymph nodes and non-lymphoid tissues, where they undergo final differentiation [16]. Three DC subsets can be distinguished in human blood, including $CD123^+$ $BDCA\text{-}2^+$ plasmacytoid DCs, and two types of $CD11c^+$ myeloid DC: $CD1c^+$ and $CD141^+$. Plasmacytoid DCs are primarily found in the blood circulation and lymphoid tissues and are characterized by the secretion of large amounts of type I interferon (IFN) upon activation [17,18]. $CD1c^+$ and $CD141^+$ myeloid DCs are present in blood, secondary lymphoid organs, and non-lymphoid tissues, exert phagocytic activity and can be distinguished from one another by their respective toll-like receptor (TLR) repertoire and cytokine/chemokine pattern [19,20]. It has been described that, during inflammation, blood monocytes are recruited to affected tissues where they differentiate into highly inflammatory $CD14^+$ $CD1c^+$ DCs [21,22]. These inflammatory DCs were found in inflamed tissues of patients with autoimmune diseases such as psoriasis or rheumatoid arthritis (RA) and are thought to perpetuate disease pathogenesis by producing pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and inducible nitric oxide synthase (iNOS) [23,24].

In general, the maturation state of DCs depends on the nature of encountered stimuli as well as on the prevailing cytokine milieu and determines whether immunity or tolerance is induced. Maturation is also linked to specific functions. While immature DCs in peripheral non-lymphoid tissues act as phagocytes and sentinels for incoming antigens, mature DCs possess the ability to migrate to lymphoid organs and to efficiently present antigen for driving the differentiation of T lymphocytes into effector cells [25–27]. Maturation is induced upon encounter of "danger"-associated signals, recognized by pattern recognition receptors such as TLRs, or in the presence of IL-1β, TNF-α, and IFN-α pro-inflammatory cytokines [28]. DCs respond to these signals by increasing motility [29,30], transition from phagocytosis to antigen processing and presentation [31], chemokine secretion [32], synthesis of human leukocyte antigen (HLA) molecules and translocation of HLA-antigen peptide complexes to the cell surface [33], upregulation of costimulatory receptors like CD80/CD86 [34,35], and secretion of T cell polarizing cytokines [36,37]. Thus, mature DCs are highly immunogenic, capable of priming naive $CD4^+$ T cells and promoting T cell polarization toward pro-inflammatory type 1 T helper (Th1), Th2, or Th17 cells [36,37].

In contrast, immature and semi-mature DCs are rather tolerogenic. They express low levels of HLA and costimulatory molecules produce anti-inflammatory mediators, e.g., IL-10, transforming growth factor β (TGF-β) and indoleamine 2,3-dioxygenase (IDO), and favor the differentiation of T and B cell subsets with regulatory properties [38,39].

In the thymus, DCs are involved in the establishment of central tolerance, mediating negative selection and clonal deletion of autoreactive T cells that express high-affinity TCRs [40]. Thymic stromal lymphopoietin, produced by thymic epithelial cells of the Hassall's corpuscles, instructs DCs to convert remaining self-reactive thymocytes into natural occurring $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (Treg) [41,42]. But despite efficient selection in the thymus, autoreactive T cells with low affinity TCRs escape into the periphery and have to be kept at bay to prevent autoimmunity. Under homeostatic conditions, immature tissue-resident and semi-mature migratory DCs continuously present self-antigens and, due to insufficient levels of costimulatory signals, promote peripheral tolerance through deletion or anergy of potentially harmful autoreactive T lymphocytes, and/or conversion to Treg [43–45].

Alterations in DC subsets and function lead to aberrant immune responses [46–48]. In particular, presentation of autoantigen along with inappropriate activation signals and/or the loss of regulatory mechanisms might drive the priming of self-reactive effector cells promoting autoimmunity [46–48]. Experimental animal models and *in vitro* studies demonstrated the important role of DCs in the pathogenesis of different autoimmune diseases such as RA, type 1 diabetes mellitus (T1D) and systemic lupus erythematosus (SLE) [49–52]. Accordingly, DCs with inflammatory properties have been found in inflamed tissues of patients with autoimmune diseases such as synovial tissue of RA, cerebrospinal fluid of multiple sclerosis (MS), and inflamed lesions of inflammatory bowel disease [53,54]. These inflammatory DCs were characterized by upregulation of HLA-peptide complexes and secretion of high levels of pro-inflammatory cytokines, pointing to their involvement in the initiation and/or perpetuation of autoimmunity, mainly through presentation of self-antigens and activation of autoreactive T cells [46–48]. Based on these observations and the key role that DCs play in the immune system, researchers have focused on the modulation of DC functions to treat autoimmune diseases.

3. Generation of tolerogenic dendritic cells for the treatment of autoimmune diseases

The main approach to achieve long-term remission in autoimmune diseases is to differentiate patient-derived precursors *ex vivo* into DCs with tolerogenic properties (tolDCs), capable of restoring

self-tolerance, when loaded with autoantigens and re-infused in the patient [55].

DCs can be easily generated *in vitro* from monocytes [56], and numerous procedures have been described to obtain tolDCs with stable regulatory features [57] (Table 1). There are six main strategies for modulation: (i) cytokines, such as IL-10 [58], TGF-β [59,60], IL-21 [61], IL-6 [62], TNF-α [63], or the combination of IL-10 plus TGF-β [64]; (ii) short stimulation with microbial products like lipopolysaccharide (LPS) [65,66] or *Aspergillus oryzae* protease [67]; (iii) pharmaceutical agents, such as dexamethasone (dex) [68], rapamycin [69,70], aspirin [71,72], cyclosporine [73], rosiglitazone [74] or mycophenolic acid [75], the Janus kinase inhibitor tofacitinib [76], and the calcineurin inhibitor tacrolimus [76]; (iv) natural compounds such as resveratrol [77], curcumine [78], sulforaphane [79], andrographolide [74], 1α,25-dihydroxyvitamin D3, the active form of vitamin D3 (vitD3) [80,81], or the hormone vasoactive intestinal peptide [82]; (v) inhibitors of cell signaling, such as protein kinase C inhibitors [83], or the CTLA4-Ig fusion protein abatacept [84]; and (vi) genetic modifications such as transduction with the IL-10 or IL-4 genes [85–87] or knockdown of costimulatory molecules and inflammatory cytokines by RNA interference [88–90].

Besides some variations of tolDC properties between protocols, there is consensus about fundamental features that tolDCs must display. These include low levels of costimulatory molecules, high production of anti-inflammatory cytokines, particularly IL-10, minimal secretion of pro-inflammatory cytokines, and the ability to induce T cell hyporesponsiveness and/or Treg [12,91,92] (Table 1).

Alternative activation of DCs, i.e., partial maturation after or during DC modulation, was suggested to be beneficial for immunotherapeutic purposes, as it endows tolDCs with some essential features of mature DCs, namely, robust antigen presentation and lymph node homing

Table 1
Mechanisms of tolerance induction by different human tolDC preparations.

Modulatory agent	DC phenotype	Targeted lymphocyte subset	Mechanism of tolerance	Ref.
IL-10	↓Costimulatory molecules and MHCII expression ↓IL-6, IL-23, and IL-12 secretion ↓CCR7 expression and invariant migratory capacity ↑IL-10, PGE2, IL-6, and TGF-β secretion ↓Antigen presenting capacity	Alloreactive $CD4^+$ T cells Naive $CD4^+$ T cells Antigen-specific $CD4^+$ T cells [a] Peptide-specific syngeneic $CD4^+$ T cell clone Allogeneic $CD4^+$ and $CD8^+$ T cells [a] Autologous naive $CD4^+$ T cells [a]	Anergy T cell suppressor capacity Induction of IL-10-secreting Tr1 cells Induction of $CD127^{-/low}$ $CD25^{high}$ $FoxP3^+$ Treg cells [a] Induction of $IL10^+$ $TGF\text{-}\beta^+$ $FoxP3^+$ Treg cells [a]	[11,12,106,107, 114,131–134]
IL-10 + TGF-β	↓Costimulatory molecules and MHCII expression, ↓IL-6, IL-12, IL-18, and IL-23 secretion ↑CCR7 expression and invariant migratory capacity ↑IL-4, IL-5, PGE2, and TGF-β secretion ↓Antigen presenting capacity	Autologous effector/memory $CD4^+$ T cells	Anergy Promotion of an $IL2^{low}$ $IFN\text{-}\gamma^{low}$ $IL\text{-}10^{high}$ cytokine profile on stimulated T cells	[64]
IL-10 + IL-6	Invariant MHCII and costimulatory molecules expression ↓IL-12, IL-23 and TGF-β secretion ↑IL-10, IL-6, PGE2 secretion ↑CCR7 expression	Allogeneic $CD4^+$ T cells	Anergy Proliferation of IL-10-secreting $CD4^+$ $CD45RA^-$ T cells	[12]
TGF-β	↓Costimulatory and maturation markers expression ↑inhibitory molecules ILT4, PD-Ll/2 expression ↑IL-10 production ↓IL-1 β, IL-6, IL-12, IL-23	Allogeneic $CD4^+$ T cells Naive $CD4^+$ T cells	Anergy Induction of regulatory $CD4^+$ T cells Inhibition of INFγ secretion by $CD4^+$ T cells	[11,59,172]
Dex	↓Costimulatory molecules and MHCII expression ↓IL-12, IL-23, TNF-α secretion ↑IL-10 secretion [a] ↑CCR7 and CXCR4 expression and migratory capacity [a]	Resting $CD4^+$ T cells Alloreactive $CD4^+$ T cells [a] Th1-polarized $CD4^+$ T cells [a]	Anergy [a] Induction of IL-10-secreting Tr1 cells [a]	[13,92,94,97,130]
Dex + VitD3	↓Costimulatory molecules and MHCII expression ↑Inhibitory molecules PD-L2 [a] ↓IL-5, IL-6, IL-12 and TNF-α secretion ↑IL-10, IL-8 secretion [a] ↑CCR7 and CXCR4 expression and migratory capacity [a]	Naive $CD4^+$ T cells (a) $CD4^+$ memory T cells (a) $CD8^+$ memory T cells Monocyte-depleted PBL	Anergy Apoptosis T cell suppressor capacity [a] Induction of IL-10-secreting Tr1 cells [a] Induction of $CD19^+$ $IL\text{-}10^+$ Breg cells	[15,91,93,95, 105,136]
VitD3	↓Costimulatory molecules expression Invariant MHCII molecule expression ↑IL-10 secretion ↓ IL-6, IL-12 and TNF-α secretion [a] ↓CCR7 expression and migratory capacity [a]	Alloreactive $CD4^+$ T cells Resting $CD4^+$ T cells Autoantigen-specific $CD4^+$ T cell clones Autoreactive $CD4^+$ T cells	Apoptosis Induction of IL-10-secreting Tr1 cells Induction of Tr1-like cells	[94,103,104]
Rapamycin	↑Costimulatory molecules ↑Inhibitory molecules PD-L1/2 [a] ↑CCR7 expression and migratory capacity [a] ↓ IL-6, IL-12 and IL-23 secretion [a]	Allogeneic $CD4^+$ memory T cells $CD4^+$ T cells	Anergy Induction of $CD127^{-/low}$ $CD25^{high}$ $FoxP3^+$ Treg cells	[69,70,91]
Acetylsalicylic acid	↓Costimulatory and MHCII molecules expression ↑Inhibitory molecules ILT3 and PD-L1 ↓IL-10 and TNF-α secretion ↑IL-12 secretion	Allogeneic $CD4^+$ T cells Naive $CD4^+$ T cells Memory $CD4^+$ T cells	Anergy Induction of $CD25^{high}Foxp3^+$ Treg cells Inhibition of INFγ secretion by $CD4^+$ T cells	[71,173]
Complement Factor H	↓ Costimulatory molecules expression [a] ↓IL-12, TNF-α, IFN-γ, IL-6, and IL-8 secretion [a] ↑IL-10 secretion and TGF-β gene expression [a] ↓Endocytic capacity [a] ↓CCR7 expression and migratory capacity [a]	Allogeneic $CD4^+$ T cells [a]	Induction of $CD127^{-/low}$ $CD25^{high}$ $FoxP3^+$ Treg cells [a] Inhibition of INFγ secretion by $CD4^+$ T cells [a]	[174]
IFN-γ (early exposure)	↓ Costimulatory and maturation molecules expression [a] ↓IL-12 secretion [a] ↓Endocytic capacity [a]	Autologous naive $CD4^+$ T cells Alloreactive $CD4^+$ T cells [a]	Induction of $CD127^{-/low}$ $CD25^{high}$ $FoxP3^+$ Treg cells Induction of $IL10^+$ $TGF\text{-}\beta^+$ $FoxP^+$ Treg cells [a]	[106,107]

Abbreviations used: Dex, dexamethasone; VitD3, 1α,25-dihydroxyvitamin D3; ILT3/4, Immunoglobulin-like transcript 3/4; PD-L1/2, Programmed death ligand 1/2; PGE2, Prostaglandin E2; [a], features were also determined after alternative activation.

capacity [93]. In particular, migration of tolDCs to T cell priming sites might help to tackle autoimmunity at its roots and to restore self-tolerance. Alternative activation can be achieved by the addition of LPS [94–96], its non-toxic analog monophosphoryl lipid A (MPLA) [97], or a cytokine cocktail, containing TNF-α, TGF-β, IL-1β, IL-6, and prostaglandin E2 (PGE2) [91]. Activated tolDCs were shown to maintain their tolerogenic phenotype and the capacity to induce regulatory T cell even under pro-inflammatory conditions [13,98], which is a prerequisite for therapeutic application, concerning the highly inflammatory environment in patients with autoimmune disease.

4. Tolerogenic dendritic cells and the modulation of adaptive immune responses

4.1. T cell regulation

Autoreactive T cells that escaped from negative selection in the thymus are controlled by DCs in the periphery. Under steady-state conditions, DCs induce antigen-specific tolerance by presenting self-peptides to $CD4^+$ T cells in the context of low costimulation and high levels of inhibitory ligands and/or anti-inflammatory cytokines [55]. Antigen presentation under such sub-optimal conditions may generate Treg which suppress antigen-specific T effector cell responses, skew T effector cells toward a hyporesponsive state, or lead to deletion of autoreactive T cells (Table 1).

4.1.1. Induction of regulatory T cell subsets

Regulatory T cells possess potent immunosuppressive abilities and comprise IL-10 secreting type 1 regulatory T cells (Tr1), TGF-β secreting Th3, as well as $FoxP3^+$ natural and inducible Treg (nTreg and iTreg) [99,100]. TolDCs are crucial for Treg induction, and tolDC subtypes favor the differentiation of particular subsets through distinct mechanisms [99,100] (Fig. 1, Table 1).

Priming with dex-modulated tolDCs was shown to decrease the portion of IFN-γ-producing T cells, while increasing the number of IL-10-producing T cells, both *in vitro* and *in vivo* [101]. Similarly, dex-modulated tolDCs, alternatively activated with LPS, were poor stimulators of proliferation and IFN-γ production of allogenic T cells, but promoted Tr1-like cells that suppressed allo-proliferation and IFN-γ production of previously primed effector T cells in an IL-10 dependent manner [13,98]. TolDCs modulated with vitD3 exhibited a reduced capacity to activate alloreactive T cells [102], but induced $CD4^+$ $FoxP3^+$ Treg with suppressive activity [102]. Priming of autoreactive T cells by tolDCs that were treated with the physiological precursor of active vitD3, calcidiol, resulted in IL-10-producing Treg with suppressive capacity but sustained IFN-γ production [80,102–104]. Unger and colleagues showed that both dex-treated tolDCs and vitD3-treated tolDCs were able to convert resting $CD4^+$ T cells into IL-10-producing Tr1, capable of suppressing the proliferation of responder T cells by a cell-contact-dependent mechanism [94]. However, only Tr1 cells induced by vitD3-modulated tolDCs were antigen-specific [94]. Naive T cells primed by TolDCs that were generated by a combined treatment with vitD3 and dex, proliferated upon restimulation but were skewed toward a Tr1-like phenotype with high IL-10 and low IFN-γ secretion [93,95,105].

IL-10-modulated tolDCs have been shown to produce high levels of IL-10 and to induce Tr1 capable of suppressing primed $CD4^+$ T cells via IL-10 and TGF-β [11]. Likewise, $CD4^+$ naive T cells that were primed with IFN-γ-treated and TNF-α-activated DCs expressed high levels of FoxP3, IL-10, and TGF-β mRNA and were able to suppress alloreactive T cells [106]. TolDCs, obtained by early exposure of monocytes to IFN-γ were shown to induce differentiation into $CD4^+$ $CD127^{-/low}$ $CD25^{high}$ $FoxP3^{high}$ cells, able to suppress the proliferation of autologous naive T cells in an antigen-specific way [107]. Nevertheless, comparative studies revealed opposing results concerning the efficacy of each tolDC subtype in inducing Treg [11]. While Boks and colleagues found IL-10-modulated tolDCs to be most efficient in inducing Tr1

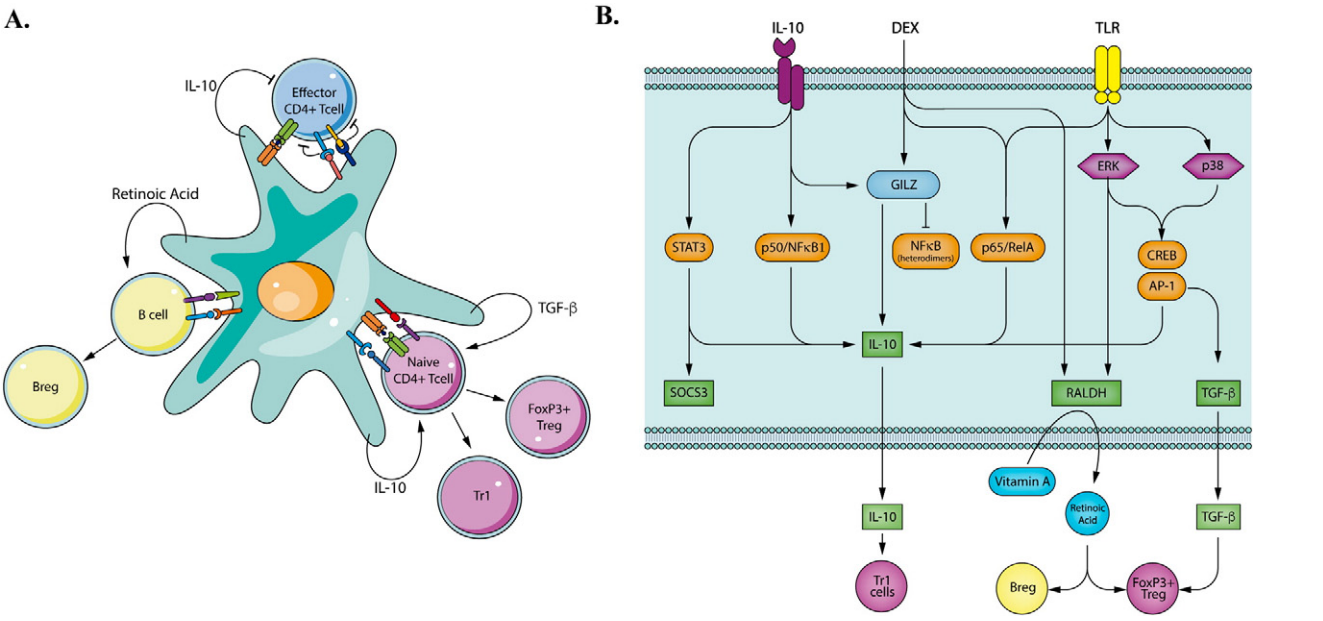

Fig. 1. *Tolerogenic dendritic cells (tolDCs) control adaptive immune responses A*: TolDCs modulate antigen-specific adaptive immune responses by presenting self-peptides to $CD4^+$ T cells in the presence of inhibitory signals, anti-inflammatory cytokines, or other molecules that promote regulatory B and T cell populations, and induce anergy or deletion of autoreactive T cells. *B*: Different modulating agents, used to generate tolDCs, induce regulatory properties through distinct signaling pathways. TLR engagement promotes expression of IL-10, TGF-β, and retinoic acid metabolizing enzymes RALDH1/2 via selective activation of Erk and p38 MAPK. Stimulation with glucocorticoids and TLR triggering induce p65/RelA homodimers that bind to the IL-10 promoter and enhance its transcription. Glucocorticoids such as dexamethasone induce upregulation of the transcription factor GILZ, which promotes IL-10 expression and restrains pro-inflammatory responses through inhibition of NF-κB heterodimers (p50/RelA, p52/RelB). Furthermore, IL-10 can drive its own expression in an autocrine manner, through STAT3 activation and nuclear translocation of p50 homodimers, which promotes IL-10 expression and represses the transcription of inflammatory molecules. Retinoic acid and TGF-β produced by tolDCs induce the differentiation and expansion of $FoxP3^+$ Treg cells, while IL-10 secretion promotes IL-10-producing Tr1 cells. Retinoic acid is furthermore involved in tolDC-mediated induction of regulatory B cells (Breg).

[11], Naranjo-Gomez and co-workers demonstrated that rapamycin-modulated DCs were superior in expanding $CD4^+$ $CD127^{-/low}$ $CD25^{high}$ $FoxP3^+$ T cells [11].

The mechanisms tolDCs use to program Treg are not fully understood but may involve different pathways that can either function alone or in combination with others (Fig. 1). Besides insufficient costimulation during antigen presentation, tolDCs produce soluble or membrane-bound inhibitory molecules that act either directly on T cells or create an anti-inflammatory environment [108]. The PD-L1-PD1 ligand-receptor pathway, immunoglobulin-like transcripts (ILTs), aryl hydrocarbon receptor agonists, and the vitamin A metabolite all-trans retinoic acid were shown to enhance Treg conversion [109,110]. Anti-inflammatory cytokines such as IL-10 and TGF-β have been described to be crucial to Treg differentiation and expansion in several models. Human and mouse $CD4^+$ T cells, repeatedly stimulated in the presence of IL-10, differentiate into Tr1 that secrete high levels of IL-10 and regulate Th1 and Th2 responses *in vitro* and *in vivo* [111,112]. Furthermore, IL-10 signaling is required to stabilize the Treg phenotype toward strong inflammatory signals [113]. Gregori and colleagues proposed a "tolerogenic loop" induced by IL-10: IL-10-modulated tolDCs secrete high levels of IL-10, which enhances the expression of ILTs and HLA-G on DCs and T cells. ILT4/HLA-G interaction confers negative signals to T cells, inducing anergy and Tr1, while amplifying IL-10 secretion in DCs and promoting de novo differentiation of tolDCs [114]. Other studies showed that IL-27 produced by tolDCs, either alone or together with TGF-β, favors the differentiation of Tr1 cells [115,116]. Antigen presentation in the presence of TGF-β has been shown to convert $CD4^+$ $CD25^-$ precursors to $FoxP3^+$ Treg, producing either IL-10 or TGF-β and exerting suppressive activity [117,118].

Signaling via TLR2 triggers in DCs the phosphorylation of extracellular signal-regulated kinase p44/p42 (Erk1/2) and stabilizes the transcription factor c-Fos [119], promoting IL-10 production and expression of retinoic acid metabolizing enzyme RALDH2 [120]. The latter converts retinal to retinoic acid, acting on both DCs and Treg. In DCs, retinoic acid induces the expression of suppressor of cytokine signaling-3 (SOCS3), which suppresses activation of p38 mitogen-activated protein kinase (MAPK) and thereby inhibits the production of pro-inflammatory cytokines [120]. In T cells, retinoic acid promotes the differentiation of regulatory $FoxP3^+$ $CD4^+$ T cells from naive and activated $CD4^+$ T cells while suppressing differentiation of inflammatory Th1 and Th17 cell subsets [121–123]. Likewise, β-catenin signaling was shown to support $FoxP3^+$ Treg conversion through induction of RALDH expression and secretion of IL-10 and TGF-β in DCs, whereas it inhibits TLR-induced production of pro-inflammatory cytokines and differentiation of inflammatory effector T cells [124,125]. Additionally, IDO activity in DCs has been demonstrated to regulate T cell fate at metabolic level, by causing tryptophan starvation, which promotes Treg conversion and anergy of effector T cells [126,127].

4.1.2. Induction of anergy

Anergic T lymphocytes are functionally inactivated following antigen encounter but remain alive in a hyporresponsive state for an extended period and may even exert suppressive functions [128]. Anergy induction is an active process that occurs when T cell receptors recognize antigen in the absence of costimulation or when inhibitory signals overcome activating signals provided by APCs during priming (Fig. 1). As a consequence, IL-2 production is blocked and T cells become incapable of proliferating upon recognition of antigen presented by fully competent APCs [129].

Several reports describe that tolDCs are capable of inducing antigen-specific anergy and hyporesponsiveness of T cells [128] (Table 1). Dex-treated and LPS-activated tolDCs were demonstrated to induce anergy of allogeneic $CD4^+$ T cells toward secondary stimulation with LPS-matured DCs [92]. Likewise, tolDCs modulated with dex and activated by CD40 ligation were shown to render Th1 cells hyporesponsive to antigen-specific restimulation [130]. Another study reported that IL-10-treated tolDCs induced alloantigen-specific anergy in $CD4^+$ T cells and peptide-specific anergy in the influenza hemagglutinin-specific $CD4^+$ T cell clone HA1.7, characterized by impaired proliferation, reduced IL-2 and IFN-γ production, and a lack of Th2-polarizing cytokines [58]. Further analysis revealed that these anergic T cells dose-dependently suppressed the proliferation of naive syngeneic T cells or activated $CD4^+$ T cell clones with the same specificity and that suppression required cell-to-cell contact between anergic and responder T cells, mainly via CTLA-4, as well as activation by antigen-loaded DCs [131,132]. Similarly, naive $CD4^+$ T cells primed by allogeneic IL-10-treated tolDCs presented antigen-specific hyporesponsiveness to restimulation with LPS-matured DCs, upregulated expression of CTLA-4 and granzyme B, and were capable of suppressing primary $CD4^+$ T cell responses induced by mature DCs [114]. Other study indicated that IL-10-modulated tolDCs, activated by LPS or TNF-α and pulsed with tetanus toxin (TT) or cellular fragments from necrotic allogeneic fibroblasts, induced anergy of antigen-specific $CD4^+$ and $CD8^+$ T cells via the presentation of the internalized protein and cross-presentation of phagocytosed cellular fragments, respectively [133]. Likewise, IL-10-treated and LPS-activated tolDCs were shown to render an autologous TT-specific $CD4^+$ T cell clone and allogeneic $CD4^+$ and $CD8^+$ T cells hyporesponsive toward restimulation with TT-pulsed DCs [134]. Transwell experiments demonstrated that inhibition of T cell proliferation was not due to the lack of pro-inflammatory cytokines or the release of soluble inhibitory factors by IL-10/LPS-modulated tolDCs and could not be overcome by the addition of soluble factors derived from LPS-treated mature DCs [134]. In contrast, allogeneic T cells cultured with plasma membranes from IL-10/LPS-treated tolDCs showed a reduced proliferation compared to T cells that were cultured with plasma membranes of LPS-treated mature DCs, pointing to a membrane-associated contact-mediated mechanism of T cell anergization [134]. Correspondingly, Tuettenberg and colleagues proposed that induction of both anergy and suppressive capacity of $CD4^+$ T cells by IL-10-treated tolDCs is dependent on cell-to-cell contact via inducible costimulator (ICOS)-ICOS ligand interaction [135].

It is of note that once an autoimmune response is established, it is particularly important to silence autoreactive memory T cell populations since they expand rapidly, exert their effector functions immediately upon antigen encounter, and are more resistant to tolerance induction than antigen-specific naive T cells. To address this issue, Anderson and colleagues demonstrated that dex plus vitD3-modulated and LPS-activated tolDCs rendered $CD4^+$ memory T cells hyporesponsive in terms of proliferation and cytokine production, and that this anergic state could be partially reversed by IL-2 [95]. Although these hyporesponsive memory T cells did not exhibit a Treg phenotype, they were capable of inhibiting the proliferation of $CD4^+CD25^-$ and $CD8^+$ T cells in co-cultures with mature DCs [95]. In a similar way, Torres-Aguilar and colleagues described that tolDCs, generated with different combinations of the cytokines IL-10, TGF-β, and IL-6 induced anergy of TT-specific memory $CD4^+$ T cells, whereas the proliferative response to an unrelated antigen remained intact [12]. The authors proposed that thrombospondin-1 expression, adenosine production, as well as the secretion of IL-10 and prostaglandins by tolDCs might be involved in the induction of antigen-specific tolerance [12].

4.1.3. T cell killing

Another mechanism of tolerance induction by tolDCs is T cell deletion (Table 1). This mechanism has been particularly described for tolDCs generated with vitD3 or its nonhypercalcemic analogue TX527, which do not only alter the cytokine pattern of committed autoreactive T cells but also selectively induce apoptosis in a portion of T cells that recognize cognate antigen peptides [103]. Repeated stimulation of $CD8^+$ memory T cells with tolDCs that were modulated by dex and vitD3 led to depletion of this population, probably due to withdrawal of positive signals via cytokines and costimulatory molecules and

inhibition by regulatory soluble factors such as IL-10 and TGF-β, which create a less favorable environment for cytotoxic T cell survival and expansion [136]. Furthermore, it has been described that expression of galectins by DCs was responsible for apoptosis of effector T cells [137,138]. In particular, galectin 9 was shown to induce the differentiation of naive T cells into Treg and its ligation to T cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3) on Th1-cells-induced apoptosis [137–139].

4.2. Induction of regulatory B cells

B lymphocytes were originally known for their antibody-producing capacity, although, in recent years, evidence has accumulated that functions of these cells are far more diverse. Different B cell subsets with distinct tasks have been described, of which regulatory B cells (Bregs) gained special attention, as they are able to dampen immune responses. Bregs were shown to suppress the differentiation of pro-inflammatory T lymphocyte subsets, while inducing Treg, through production of high levels of IL-10 [140–142]. However, involvement of TGF-β and IL-35 secretion [140–142] and contact-mediated mechanisms have been also suggested [140–142].

Recent studies propose that tolDCs, in addition to modulating T cell responses, promote the generation of Breg cells (Fig. 1, Table 1). Volchenkov and colleagues observed an increase in the frequency of Bregs in co-cultures of non-adherent cells and tolDCs generated with dex and vitD3 [15]. Similarly, Li and colleagues demonstrated in a rat model of experimental autoimmune myasthenia gravis (EAMG) that atorvastatin-modified bone marrow-derived tolDCs increased the percentages of both $FoxP3^+$ and $IL\text{-}10^+$ Breg cells and ameliorated autoimmune-like disease [143]. Qian and colleagues described that tolDCs, generated by exposure to splenic stroma, induce B cells to differentiate into a $CD19^{high}Fc\gamma IIb^{high}$ Breg subset, which secretes IL-10 and exerts phagocytic capacity as well as regulatory functions [144]. The authors propose that tolDC-derived IFN-β and DC-B cell interaction via CD40L-CD40 is responsible for Breg differentiation [144].

In humans, plasmacytoid DCs were shown to govern the differentiation of immature B cells into Bregs that restrain inflammation via IFN-α release and CD40 engagement [145]. Giannoukakis and colleagues discovered the upregulation of Bregs in peripheral blood of T1D patients that were treated with autologous costimulation-impaired tolDCs during a phase I clinical trial [146,147]. Additional *in vitro* studies by the same group demonstrated that tolDCs induced an increase in the frequency of $CD19^+CD24^+CD38^+$ B cells with suppressive properties in co-cultures with PBMC [148]. Breg generation was proposed to be dependent on retinoic acid, since those tolDCs produced retinoic acid and Bregs expressed retinoic acid receptor RARα [148]. Furthermore, the increase in the Breg population was shown to be the consequence of both expansion of existing Bregs and conversion of $CD19^+$ B cells into IL-10 expressing Bregs [148,149].

These findings indicate that tolDCs not only modulate T cell responses but also induce a B cell population with suppressive capacities. Nonetheless, specific mechanisms by which tolDCs induce Breg have to be explored.

5. Modulation of T cell responses in autoimmune diseases

Tolerance to self is mainly maintained through induction of antigen-specific anergy and Treg conversion. Alterations in frequencies and functional defects of Treg favor the activation of autoreactive effector T cells which might trigger autoimmune diseases such as RA, SLE, T1D, and MS, among others [150–154]. Since tolDCs play an important role in the induction of T cell anergy/hyporesponsiveness and the generation of Treg subsets, *in vitro* generated tolDCs are a promising therapeutic tool to restore tolerance to specific tissue-derived autoantigens.

5.1. Multiple sclerosis

Although neurological deficits in MS are the result of combined cellular and humoral attack on myelin sheaths, this autoimmune disease is considered to be predominantly T cell mediated. This is supported by the presence of autoreactive T cells recognizing myelin-derived antigens in cerebrospinal fluid of MS patients [155].

In vitro generated vitD3-treated tolDCs, derived from monocytes of MS patients and loaded with myelin peptides, induced robust antigen-specific hyporesponsiveness of autologous T cells [156]. In a murine model of experimental autoimmune encephalomyelitis (EAE), adoptive transfer of IDO expressing bone marrow-derived and vitD3-treated tolDCs increased the percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg in lymph nodes and ameliorated the clinical course of the disease [157]. In the same model, administration of vitD3-treated tolDCs pulsed with 40–55 peptide of the myelin oligodendrocyte glycoprotein (MOG_{40-55}) reduced both disease incidence and severity through induction of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg and IL-10 production and impairment of antigen-specific lymphoproliferation [158].

5.2. Type 1 diabetes

In T1D, insulin-producing beta cells are selectively killed by autoreactive $CD4^+$ and $CD8^+$ T lymphocytes. Studies in non-obese diabetic (NOD) mice demonstrated that bone marrow-derived tolDCs, obtained by exposure to apoptotic bodies or IL-10, prevented the onset of T1D-like disease through induction T cell hyporesponsiveness and generation of Treg [159,160]. A similar effect was observed in a murine model of islet allotransplantation, where vitD3-treated tolDCs loaded with islet antigen, diminished proliferation and skewed the cytokine profile of T cells toward IL-10, TGF-β, TNF-α, and IL-4 secretion *in vitro* and prevented hyperacute islet allograft rejection *in vivo* [161]. Human monocyte-derived tolDCs, modulated by vitD3 or its analogue TX527, were shown to alter the cytokine secretion profile and decrease survival of committed autoreactive T cells, specific for the T1D-associated antigen glutamic acid decarboxylase (GAD) 65 [103,162]. Likewise, IL-10/TGF-β-treated and insulin-pulsed tolDCs were shown to render insulin-specific $CD4^+$ memory T cells from T1D patients hyporesponsive toward second challenge with fully competent insulin-pulsed DCs [64]. High levels of IL-10 detected in co-cultures of these cells suggest the induction of Tr1 [64]. Concerning $CD8^+$ T cells, Kleijwegt and colleagues reported that vitD3/dex-treated tolDCs interfere with $CD8^+$ T priming, preventing the expansion of antigen-specific naive $CD8^+$ T cells while inducing the deletion of memory $CD8^+$ T cells after second contact [136].

5.3. Rheumatoid arthritis

The hallmark of RA is inflammation and swelling of synovial tissue, primarily due to the infiltration of $CD4^+$ T cells that express an activated/memory phenotype and secrete inflammatory factors. TolDCs that were generated from monocytes of RA patients, by modulation with vitD3 plus dex and activation with MPLA, impaired antigen-specific T cell proliferation as well as IFN-γ and IL-17 secretion, and rendered T cells hyporesponsive to subsequent stimulation [81]. *In vivo*, the development of collagen-induced arthritis (CIA), a mouse model of RA, was prevented by the administration of tolDCs that had been modulated by TNF-α, dex, or IL-10 [163]. Likewise, treatment of CIA with vitD3/dex-treated tolDCs reduced the severity of disease and enhanced the number of immunoregulatory IL-10-producing Tr1 cells, while decreasing the percentage of pathogenic Th17 cells [164]. In the same model, our group has demonstrated that tolDCs, obtained by short-term stimulation with LPS and loaded with type II collagen (CII), modulated $CD4^+$ T cell responses *in vivo*, through diminution of the Th17 population and induction of Treg expressing IL-10 and TGF-β [65]. A recent study demonstrated that

CII-pulsed DCs, treated with the calcineurin inhibitor tacrolimus/FK506, act as a cellular drug delivery system that targets antigen-specific T cells and prevents development of CIA [165]. The authors showed that upon encounter with antigen-specific tacrolimus-treated DCs, T cells initiated a program of activation and subsequently underwent apoptosis *in vitro* [165]. Another report describes the potential of allogenic bone-marrow-derived tolDCs, modulated by IL-10 plus TGF-β and loaded with CII, to dose-dependently diminish the severity and progression of CIA [166]. The improvement of disease was attributed to the modulation of cytokine secretion, suppression of antigen-specific T cell proliferation and enhanced induction of $CD4^+$ T cell death, along with Th17 to $FoxP3^+$ Treg shift, and inhibition of anti-CII antibody secretion [166].

5.4. Tolerogenic dendritic cells in clinical trials of autoimmune diseases

Although tolDCs constitute a promising strategy for the treatment of autoimmune diseases, assessment of their efficacy in clinical trials is challenging [167]. The first attempt of applying tolDCs to humans was made by Ralph Steinman's group, who discovered that autologous immature DCs pulsed with influenza matrix peptides (MP) and subcutaneously delivered to healthy individuals were well tolerated and able to inhibit antigen-specific $CD8^+$ T cell responses while promoting the appearance of MP-specific IL-10-producing T cells [168,169].

But it was not until recently that the first clinical trial using tolDCs in autoimmune disease was published by Giannoukakis and colleagues. The investigators delivered autologous monocyte-derived tolDCs, treated with a mixture of antisense oligonucleotides targeting the costimulatory molecules CD40, CD80, and CD86, intradermally to T1D patients and confirmed that administration of these tolDCs was safe, without any side effects, and led to an increased frequency of a regulatory B cell population in the peripheral blood of patients [146].

Currently, two further phase I clinical trials with tolDCs have been conducted, both in RA patients. The first exploratory study was carried out by Thomas and colleagues, using monocyte-derived tolDCs modulated with BAY11–7082, an inhibitor of nuclear factor kappa-light chain enhancer of activated B cell (NF-κB) signaling, and pulsed with a mixture of four citrullinated peptides, termed Rheumavax [170]. These tolDCs were administered intradermally to RA patients presenting "shared epitope" containing risk alleles and anti-citrullinated peptide antibodies [170]. Besides confirming the safety of a single injection of tolDCs, the authors demonstrated that this approach reduced serum levels of the inflammatory cytokine IL-15 as well as the proportion of effector T cells while increasing the Treg/T effector cell ratio [170].

The results of the second clinical trial "AuToDeCRA," designed by Hilkens and co-workers, were published just now. Autologous monocyte-derived tolDCs modulated with dex, vitD3, and MPLA [81,171], and pulsed with synovial fluid were injected into inflamed joints of RA patients. Although this approach appeared safe and acceptable, no clinical or immunomodulatory effects were detected. Interestingly, two patients (of nine), who received a high dose of tolDCs showed, stabilization of knee symptoms.

Translation of findings, obtained by *in vitro* studies or in animal models of autoimmune diseases, into clinical application has just started. There is still a long way to find the optimal dose, route of administration, and number of required booster injections. Finally, there is an urgent need to find appropriate autoantigens for pulsing of tolDCs.

6. Concluding remarks

TolDCs control immune responses through modulation of T and B cell subsets and function in an antigen-specific manner. Thus, tolDCs are attractive targets of therapeutic approaches to (re-) establish tolerance in autoimmune and inflammatory disorders. Progress has been achieved in developing protocols for the *in vitro* differentiation of monocyte-derived tolDCs for therapeutic purposes. According to the experimental procedure and compound used to induce tolerogenic features, and depending on the targeted lymphocyte population, tolDCs exert distinct mechanisms of regulation, including induction of anergy, conversion to a regulatory phenotype, and antigen-specific deletion. Since each autoimmune disorder involves alterations of a particular lymphocyte subset and function, it has to be carefully evaluated which type of tolDCs might be the most appropriate to obtain the desired outcome. Promising results of *in vitro* assays using patient-derived tolDCs and animal models of autoimmune diseases have opened the way for first clinical trials with tolDCs. Although there is still much work left to transfer tolDCs from bench to bedside, it seems that tolDCs have the potential to be the future treatment for autoimmune diseases.

Take-home messages

- TolDCs are able to modulate adaptive responses by controlling the fate of T and B cells.
- Depending on the modulating agent used for their generation, tolDCs induce anergy or deletion of effector T cells or promote either IL-10 secreting Tr1 or $FoxP3^+$ Treg
- TolDCs prevent or improve disease *in vivo* in animal models of autoimmune MS, RA, and T1D.
- First clinical trials using TolDCs in patients with T1D and RA confirm safety and point to immunoregulatory effects.

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