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REVIEW

Leveraging current insights on IL-10-producing dendritic cells for developing effective immunotherapeutic approaches

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Dendritic cells (DC) are professional antigen-presenting cells involved in promoting and controlling immune responses. Different subsets of DC, named tolerogenic (tol)DC, play a critical role in the maintenance of tissue homeostasis and in fostering tolerance. These unique skills make tolDC especially attractive for strategies aimed at re-establishing/inducing tolerance in immune-mediated conditions. The generation of potent tolDC *in vitro* from peripheral blood monocytes has seen remarkable advancements. TolDC modulate T cell dynamics by favoring regulatory T cells (Tregs) and curbing effector/pathogenic T cells. Among the several methods developed for in vitro tolDC generation, IL-10 conditioning has been proven to be the most efficient, as IL-10-modulated tolDC were demonstrated to promote Tregs with the strongest suppressive activities. Investigating the molecular, metabolic, and functional profiles of tolDC uncovers essential pathways that facilitate their immunoregulatory functions. This Review provides an overview of current knowledge on the role of tolDC in health and disease, focusing on IL-10 production, functional characterization of *in vitro* generated tolDC, molecular and metabolic changes occurring in tolDC induced by tolerogenic agents, clinical applications of tolDC-based therapy, and finally new perspectives in the generation of effective tolDC.

Keywords: cell metabolism; cell therapy; dendritic cells; IL-10; T regulatory cells; tolerance; tolerogenic DC

Abbreviations

Ags, antigens; AHR, aryl hydrocarbon receptor; AHRR, aryl hydrocarbon receptor repressor; ARNT, aryl hydrocarbon receptor nuclear translocator; ATDC, autologous tolerogenic dendritic cells; BATF, basic leucine zipper ATF-like transcription factor; CCL2, chemokine ligand 2; CD, celiac disease; cDC, conventional DC; COPD, chronic obstructive pulmonary disease; DC, dendritic cells; $DC^{IL\text{-}10}$, IL-10-engeneered DC; Dexa, dexamethasone; FAO, fatty acid oxidation; GLUT1, glucose transporter 1; GMP, good manufacturing practise; GzB, granzyme B; HSCT, hematopoietic stem cell transplantation; IBD, inflammatory bowel disease; IDO, indoleamine 2,3-dioxygenase; IFN-α, interferon alpha; IFNs, type I interferons; IL-10, Interleukin-10; ILT3, immunoglobulin-like transcript-3; ILT4, immunoglobulin-like transcript-4; LC, langherans cells; LCMV, lymphocytic choriomeningitis virus; moDC, monocyte-derived DC; MS, multiple sclerosis; NMOSD, neuromyelitis myelitis optica spectrum disorder; OXPHOS, oxidative phosphorylation; pDC, plasmacytoid DC; PDL-1, programmed death-ligand 1; PDL-2, programmed death-ligand 2; PGE_2, prostaglandin E_2; RA, retinoic acid; RA, rheumatoid arthritis; RALDH, retinaldehyde dehydrogenase 2; T1D, type 1 diabetes; TCA, tricarboxylic acid; TF, transcription factor; TGF-β, transforming growth factor-beta; TLR, toll like receptor; tolDC, tolerogenic dendritic cells; Tr1 cells, T regulatory type 1 cells; Tregs, regulatory T cells; UCN2, urocortin 2; VDR, VitD3 receptor; VitD3, vitamin D3.

Dendritic cells (DC) are professional antigen-presenting cells (APC) that efficiently uptake, process, and present antigens (Ags) to prime T cells, initiating immune responses. DC are found in tissue that form the interface between the body and the environment (i.e., lung mucosa, skin, and gastrointestinal tract) and are constantly exposed to foreign proteins and pathogens, but also circulate in peripheral blood. Under steady-state conditions, DC show an immature phenotype, characterized by low levels of co-stimulatory molecules, and a poor ability to stimulate effector responses. Upon encountering Ags, DC migrate to secondary lymphoid organs where they mature and interact with T and B cells, shaping the adaptive immune response. In humans, transcriptional profiling and single-cell RNA sequencing analysis led to the definition of three subclasses of DC: conventional DC (cDC1, cDC2, and cDC3), plasmacytoid DC (pDC), and monocyte-derived DC (moDC) [1–5]. cDC1 are identified by the expression of CD11c, CD141 (BDCA-3), CLEC9A (DNGR-1), and XCR1, recognize viral and intracellular Ags, upon activation, secrete type III IFN and IL-12, and cross-present Ags to $CD8^+$ T cells [6–8]. cDC2 express CD11c, CD1c (BDCA-1), and Signal regulatory protein (SIRP)α and are involved in $CD4^+$ T cell activation, priming preferentially T helper(h)1 and Th17 responses [5,9]. Recently, cDC3 express BTLA [2] and it has been reported that they can be distinguished phenotypically in $CD5^+CD163^-CD14^-$ and $CD5^-CD163^+CD14^+$, with the first subsets identified as precursors of inflammatory DC and named cDC3s [10–12]. MoDC express markers associated with monocytes and DC, are identified as $CD14^+CD1c^+CD209^+CD163^+$ cells, rapidly expand during acute infections and synergize with cDC in propagating immune responses [13,14]. pDC are CD11c negative, express CD303 (BDCA-2), CD304 (BDCA-4), and CD123, and play a crucial role in antiviral immunity due to their ability to secrete type I interferons (IFNs) [15,16].

Dendritic cells are not only involved in mounting effective immune responses but also play key roles in maintaining tissue homeostasis and promoting tolerance. A specialized subset of DC, named tolerogenic DC (tolDC), are immature or semi-mature DC with low expression of co-stimulatory molecules and pro-inflammatory cytokines. The enhanced capacity for Ag uptake and processing, coupled with high expression of inhibitory molecules and secretion of immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-β, enable tolDC to orchestrate peripheral immune tolerance by promoting clonal T cell anergy [17], inhibiting the activation and function of effector T cells, inducing the differentiation of regulatory T cells (Tregs), and generating and maintaining an anti-inflammatory microenvironment to sustain immune tolerance [15,18–20]. A better understanding of the mechanisms regulating adaptive immune responses by tolDC and the development of protocols to generate tolDC *in vitro*, opened the possibility of translating tolDC as cell therapy in immune-mediated diseases. Different tolerogenic strategies, including the use of immunosuppressive drugs (e.g., dexamethasone or rapamycin), or pharmacological agents (e.g., Vitamin D3 or A), or cytokines (e.g., IL-10 or TGF-β), have been applied to generate effective human tolDC from peripheral blood monocytes [18,19,21,22]. All of these approaches generate cells characterized by a semi-mature phenotype, and ability to secrete modulatory molecules, modulate T cell responses, and promote Tregs. Functional assays demonstrated that IL-10 treatment is the most effective in promoting tolDC with the ability to induce Tregs with the strongest suppressing activity [23]. Our group contributed to the identification of IL-10 as a key factor for promoting the differentiation of potent tolDC and described a subset of cells, named DC-10, generated from monocytes in the presence of exogenous IL-10 during DC differentiation [24], and, more recently, IL-10-engeneered DC ($DC^{IL\text{-}10}$) generated by lentiviral vector (LV)-mediated IL-10 gene transfer into monocytes during DC differentiation [25]. DC-10 and $DC^{IL\text{-}10}$ secrete IL-10 spontaneously and upon activation, and efficiently promote IL-10-producing T regulatory type 1 (Tr1) cell differentiation [26].

Genomic profiling of *in vitro*-generated tolDC provided some information regarding the molecular mechanisms underlying their tolerogenic phenotype and functions. Metabolic analysis revealed distinct signatures, such as increased oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) and decreased glycolysis, contributing to the tolDC immunosuppressive functions. Production of lactate shapes T cell responses towards tolerance [27,28] and increased expression of genes related to OXPHOS and FAO, with IL-10 blocking the shift to glycolysis and favoring OXPHOS [29], have been associated with tolDC modulatory activities.

With a specific focus on IL-10 production, this Review delves into the multifaceted characteristics of tolDC in health and disease *in vivo*, and of *in vitro* differentiated human tolDC, shedding light on the contributions of IL-10 produced by these cells in modulating T cell responses and promoting Tregs.

In vivo tolerogenic dendritic cell phenotype and functions

Human DC resident in the skin, lung, and gut and circulating in peripheral blood play a key role in mounting immune responses, maintaining tissue homeostasis, and modulating immune responses. We deliver an overview of tissue-resident DC phenotype and functions in healthy and pathological conditions.

Skin-resident dendritic cells

Skin DC function as cutaneous sentinels and modulators of T cell responses contributing to skin homeostasis, promoting either inflammation or tolerance [30]. Langherans cells (LC) are skin-resident macrophages that upon Ag encounter differentiate and acquire DC-like functions: the ability to migrate to skin-draining lymph nodes (LN) and to interact with naïve T cells. LC in the epidermis are identified by the co-expression of CD207 (Langerin) and EpCAM and display an immature phenotype with a modest expression of HLA molecules. LC respond to viruses or pathogens that enter the body via the skin and play a pivotal role in the maintenance of skin immune homeostasis [31,32] (Table 1). LC influence adaptive immunity by recruiting/inducing regulatory and conventional T cells: upon maturation, LC promote $CD4^+$ Th17 responses [33–35], while under specific inflammatory conditions, LC mediate immunosuppressive functions by promoting anergic $CD8^+$ T cells and/or depletion of auto-reactive T cell clones, and selectively inducing the activation and proliferation of skin resident Tregs [36]. The contribution of LC in modulating skin inflammatory disease is well recognized. Reduced frequency and functions of LC have been reported in patients with allergic dermatitis [37]. In the pro-inflammatory microenvironment of psoriatic lesions, LC play an active role in sustaining inflammation. Indeed, following toll-like receptor (TLR) activation, LC from psoriatic lesions produce IL-23, sustaining the IL-23/IL-17 pathogenic axis [38,39]. However, LC from these psoriatic lesions also express increased levels of anti-inflammatory mediators (e.g., IDO and PDL-1), thus also suggesting a protective role [38].

A peculiar subset of cDC1 expressing CD141, CD14, and HLA-DR, but not CD1a, called $CD141^+$ dermal (D) DC are present in the dermis; they are characterized by the absence of CD83, display significant levels of co-stimulatory and HLA molecules, and express BATF3, and XCR1 [40].

Plasmacytoid DC are not usually present in normal skin and are slightly increased in patients with atopic dermatitis, but a large proportion of pDC has been reported in cutaneous lupus erythematosus (LE) and in psoriatic lesions [41–43]. pDC accumulated in psoriatic lesions produce IFN-α, which contributes to T cell activation and the development of skin inflammation [42]. In contrast, a subset of pDC expressing granzyme B (GzB) accumulate in LE skin lesions [43] and have been proposed to inhibit T cell proliferation [44,45] (Table 1).

Intestinal resident dendritic cells

Intestinal DC comprising cDC1, cDC2, and pDC are present in lamina propria of small intestine and colon, as well as in Peyer's patches and draining LN. Intestinal DC limit reactivity to the gut microbiota, mediate tolerance to food Ags, and are required for optimal response to intestinal pathogens [46,47]. Crucial for maintaining gut homeostasis and in regulating immune responses are a subset of intestinal DC characterized by the expression of the integrin CD103 (mucosal $CD103^+$ DC) [47]. $CD103^+$ DC are involved in activating Th and innate lymphoid (ILC) cells [48], and in human can be divided based on the expression of SIRPα into different subsets with distinct immunological functions: $SIRP\alpha^+$ $CD103^+$ DC activate Th17 and ILC type 3 (ILC3), and $SIRP\alpha^-$ $CD103^+$ DC induce Th1 responses [49,50]. Intestinal murine $CD103^+$ DC have been shown particularly effective in promoting $Foxp3^+$ Tregs from naïve $CD4^+$ T cells thanks to their ability to secrete TGF-β and retinoic acid (RA) [51,52]. In an experimental model of colitis, the presence of $CD103^+$ DC was positively associated with disease prevention via Treg induction [53]. Murine $CD103^+$ DC isolated from mesenteric LN and from small intestine lamina propria secrete high levels of indoleamine 2,3-dioxygenase (IDO), which controls Ag-specific Foxp3 Treg and Th1/Th17 cell balance and has been proposed to be involved in promoting oral tolerance [54]. In intestinal tissues of patients with active inflammatory bowel disease (IBD) and celiac disease (CD) patients a decreased frequency of $CD103^+$ DC has been reported [55,56]. Moreover, intestinal $CD103^+$ DC from patients with ulcerative colitis promote effector T cells that secrete IFN-γ, IL-13, and IL-17 but no $FOXP3^+$ Tregs [46,57]. The mechanisms underlying the functional defects of $CD103^+$ DC in promoting Tregs has been associated with their ability to secrete high levels of IL-6, IL-12, and TNF-α, and low amounts of RA [46] (Table 1).

A subset of human DC expressing high levels of CD141 and DNGR-1 are present in small intestine lamina propria and Peyer's patches and are important players in regulating T cell responses [58,59]. The

Table 1. Tissue-resident tolerogenic dendritic cells. cDC2, type 2 conventional DC; CLEC9A, C-type lectin domain containing 9A; COPD, chronic obstructive pulmonary disease; GvHD, graft versus host disease; GzB, granzyme B; ICOS-L, inducible T cell co-stimulatory ligand; IDO, indoleamine 2,3-dioxygenase; IFN-α, interferon-alpha; IL-10, interleukin-10; IL-27, interleukin-27; pDC, plasmacytoid DC; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; TGFβ, transforming growth factor β; Th2, T-helper 2; Tr1, regulatory type 1 cells; Treg, T regulatory cells.

Tolerogenic dendritic cell	Surface markers	Mechanisms of regulation	Dysregulation in pathological setting	References
Skin resident DC				
Langherans cells (LC)	CD11c CD1a CD207 EpCAM	IL-10 release, $CD8^+$ T cell anergy, $CD8^+$ T cell depletion, $FOXP3^+$ Treg induction	Allergic dermatitis, psoriasis	[36,78,79,181]
$CD141^+$ dermal DC ($CD141^+$DDC)	CD11c CD1a CD14 CD141	IL-10 release, $CD25^+$ Treg induction, Ag cross-presentation	Skin inflammation in GvHD	[40,81]
pDC GzB^+	CD123 BDCA-2 BDCA-3 GzB	Inhibit T cell proliferation	Cutaneous lupus erythematosus (LE)	[43]
Gut resident DC				
$CD103^+$ DC	CD11c CD103	TGF-β and RA release, IDO expression, $Foxp3^+$ Treg induction	Celiac diseases (CD) inflammatory bowel disease (IBD)	[51,53,54]
$CD141^+DNGR\text{-}1^+$ DC	CD11c CD141 DNGR-1 (CLEC9A)	Ag cross-presentation	N/A	[58,59]
DC-10	CD14 CD16 CD141 CD163	IL-10 release, Tr1 cell induction	Celiac disease (CD)	[65]
Lung-resident DC				
$CD103^+$ DC	CD11c CD103	TGF-β and RA release, RALDH, $Foxp3^+$ Treg induction	N/A	[72,182]
$CD1c^+$ cDC2	CD1c CD1a ICOS-L	IL-10 and IL-27 release, IL-10-producing Treg induction	Impaired respiratory immunity in COPD	[96]
pDC	CD123 BDCA-2 BDCA-3	Inhibition of Th2 responses by IFN-α	Allergy	[76]

expression of DNGR-1 on $CD141^+$ DC endowed them to cross-present Ags, including dead cell-associated Ags known to promote dysfunctional and short-lived T cells [60] (Table 1).

Gut pDC are different from typical circulating pDC. Indeed, the mucosal microenvironment enriched in IL-10 produced by activated DC and macrophages, prostaglandin E_2 (PGE_2) produced by stromal cells, and TGF-β derived from intestinal epithelial cells can prevent the production of type I IFNs by gut pDC [61]. In a pre-clinical model of oral tolerance, dietary Ag presentation by pDC suppressed Ag-specific T cell responses and pDC depletion abrogated tolerance induction. Oral tolerance induced by gut pDC relayed on the induction of $Foxp3^+$ Tregs [62,63] (Table 1). Controversial results on the presence of pDC in gut mucosa of CD patients have been reported. In contrast to the accumulation of pDC observed in mucosa of untreated CD patients [64], we and others reported that pDC are poorly represented in CD gut mucosa and their frequency is comparable in patients with active disease or not [65,66]. In gut mucosa and mesenteric LN of IBD patients, an increased frequency of pDC with the ability to secrete TNF-α and IL-6, but not IFN-α, also upon TLR-9-mediated activation, has been reported [67,68].

Lung-resident dendritic cells

In human lung, cDC1, cDC2, pDC, and CD1a$^+$ epithelial DC have been reported [69]. In pre-clinical models, it has been shown that lung-resident DC have a limited ability to promote Foxp3$^+$ Tregs, especially compared to macrophages [70], and upon allergen exposure, these DC undergo maturation, leave the lung, and migrate to draining LN, where they activate naïve T cells. However, in the absence of inflammatory signals, lung DC migrate to draining LN and promote T cell tolerance upon Ag-exposure [71]. In a pre-clinical model of airway Ag delivery, tolerance is mediated by a subset of CD103$^+$ lung-resident DC that promote Foxp3$^+$ Treg induction via TGF-β, and RA secretion and retinaldehyde dehydrogenase 2 (RALDH) expression [70,72], a process essential for maintaining immune balance in response to allergens and preventing excessive immune reactions.

Plasmacytoid DC are present in the nasal mucosa of allergic patients [73], and after allergen inhalation their number increases [74,75]. Under steady-state conditions, lung pDC have been shown to be essential for inducing tolerance to harmless Ags [76] and preventing the development of allergic diseases by secreting type I IFNs that maintain the Th1/Th2 balance and prevent the shift towards Th2 dominance in the airways [76,77].

IL-10-mediated modulatory functions of tolerogenic dendritic cells *in vivo*

Among the cytokines produced by DC, IL-10 is a key regulatory cytokine limiting and ultimately terminating excessive T cell responses in several tissues. We summarize evidence obtained in pre-clinical models and studying human tissues (skin, lung, and peripheral blood) and cells on the presence and impact of IL-10 produced by resident DC.

The role of IL-10 produced by LC in modulating immune responses in the skin is supported by data in pre-clinical models showing that UVB irradiation of the murine skin leads to CD40L [78] and RANK expression in LC, which upon interaction with keratinocytes expressing RANKL secrete IL-10, driving CD4$^+$ Treg proliferation [79]. Moreover, in pre-clinical model of psoriasis, LC depletion before disease onset had no effect, while LC depletion in diseased mice worsened psoriasis symptoms. In this model, IL-10 produced by LC was responsible for the amelioration of psoriatic inflammation and correlated with upregulation of PDL-1 and with the reduction of pro-inflammatory cytokine release induced by IL-23 [80]. In human skin, CD141$^+$ DDC constitutively produce high levels of IL-10, are less proficient at stimulating allogenic CD4$^+$ T cell proliferation, and induce anergic, allo-specific CD25$^+$ Tregs [40] via a neuropeptide, urocortin 2 (UCN2), upon interaction with its specific receptor (CRHR2), supporting a role of these cells in modulating immune responses in the skin [81] (Table 1).

In the intestine, IL-10 can be produced by leukocytes [82] and epithelial cells [83], contributing to the maintenance of gut homeostasis and modulation of immune responses. The relative importance of IL-10 derived from T cells or DC in the development of intestinal inflammation has been investigated using transfer experiments in wild type and IL-10-deficient mice. These studies demonstrated that IL-10 produced by DC is critical for suppressing pathogenic immune responses to commensal intestinal microbiota [84]. Moreover, IL-10 produced by DC maintains FoxP3 expression in T cells during intestinal inflammation, which prevents colitis in the CD45RB^{high} CD4$^+$ cell transfer model of the disease [85]. In humans, the central role of IL-10 signaling in DC in controlling pathogenic CD4$^+$ T cells is supported by studies of patients with IL10RA deficiency or STAT3 mutations, who are affected by early-onset IBD [86].

Our group identified a specific subset of DC characterized by the ability to secrete IL-10 and to promote Tr1 cells *in vitro* [24]. DC-10 are present in peripheral circulation, in the spleen and LN of healthy subjects [24,87] and accumulate in human decidua in the first trimester of pregnancy [88]. DC-10 can be differentiated *in vitro* in the presence of medium derived from decidualized cells, suggesting that DC-10 are induced locally in the decidua to support fetus-maternal tolerance [89]. In line with this hypothesis, a low frequency of decidual DC-10 have been reported in women with early and late onset of preeclampsia [90]. DC-10 are defective, both in numbers and phenotype, in newly diagnosed T1D patients and in first-degree relatives of T1D patients [91] and in MS patients [92]. Conversely, while DC-10 are present in peripheral blood of subjects with different stages of CD, the presence of DC-10 uniquely characterizes the intestinal mucosa of subjects with positive serology and normal intestinal mucosa (potential-CD), where they can contribute to maintain mucosal health by controlling pathogenic T cell responses [65]. Overall, these lines of evidence indicate that DC-10 plays a role in maintaining tolerance (Table 1).

Lung-resident IL-10-producing DC suppress allergic T cell responses by promoting allergen-specific Tregs [93]. In pre-clinical models, intranasal delivery of Ag

into the respiratory tract has been associated with the induction of Ag unresponsiveness by IL-10 produced by pulmonary DC [94]. A similar mechanism has been reported in allergic patients after allergen-specific immunotherapy, in which allergen is taken up, processed, and presented by lung DC, which, by producing IL-10, promote allergen-specific Tregs [71]. Moreover, IL-10 production can be efficiently induced in DC isolated from respiratory tract of non-atopic, but not of atopic, patients with chronic rhinosinusitis, thus indicating that tissue-specific IL-10-producing DC are involved in modulating allergic responses in human as well [95]. Finally, in patients with chronic obstructive pulmonary disease (COPD), specialized tolDC expressing IL-27, IL-10 and ICOS-L have the unique ability to induce IL-10-producing Tregs, which limit excessive inflammation [96] (Table 1).

Increased IL-10 production by DC has been reported during HIV and HCV, specifically inducing loss of T cell responses, overall leading to persistent viral infection [97–99]. In a pre-clinical model of the persistent lymphocytic choriomeningitis virus (LCMV) infection, the blockade of IL-10 signaling restored the antiviral immune response and resulted in viral clearance by the reduction of $CD8\alpha^-$ DC, the main producers of IL-10 involved in priming IL-10-producing $CD4^+$ T cells that negatively control viral clearance [100]. On the same line, during mycobacterial infection, IL-10 produced by DC plays a dual inhibitory effect on the immune response, both limiting the production of pro-inflammatory cytokines (e.g. IL-12) and downregulating infected DC migratory capacity [101]. Immunization of mice with Ag and cholera toxin modulates DC activation *in vivo* by promoting IL-10 secretion, leading to Ag-specific Tr1 cell induction [102].

Ex vivo-induced tolerogenic DC *in vitro*: induction and functions

A better understanding of the mechanisms underlying immunoregulation by DC has prompted investigators to develop strategies for generating tolDC suitable for therapies in immune-mediated diseases. Human $CD14^+$ peripheral blood cells are driven towards prototypic DC using growth factor and cytokine cocktails, and then "tolerized" with immunosuppressive drugs, pharmacological agents, or anti-inflammatory cytokines [18,19,103]. A summary of the approaches used to generate human tolDC, highlighting their ability to produce IL-10 and their multifaceted functions, is presented here.

Dexamethasone-modulated DC

Dexamethasone (Dexa) is a glucocortoid with a pivotal regulatory role in reshaping the differentiation of monocyte-derived DC [104,105]. Addition of Dexa during the 7-day differentiation of monocyte-derived DC promoted Dexa-DC that secrete IL-10 upon LPS stimulation, and low amounts on IL-12. IL-10 production by these Dexa-DC is induced by phosphorylation of ERK–MAPK induced by LPS. Dexa-DC are resistant to maturation induced by LPS or CD40L and exhibit weak T cell stimulatory activity, which is partially reversed by IL-10 neutralization [105]. Introduction of Dexa on days 3 and 6 of monocyte-derived DC differentiation followed by activation with a cytokine cocktail comprising IL-1β, TNF-α, IL-6, and PGE_2 lead to Dexa-DC with the ability to secrete IL-10 spontaneously and upon activation. These Dexa-DC displayed low stimulatory capacity in primary stimulation and induced Ag-specific T cell anergy. Monocytes from Crohn's disease patients also differentiate into Dexa-DC with the capacity to secrete IL-10 and to induce hypo-proliferative T cell responses [106]. Upon repetitive stimulation of naïve $CD4^+$ T cells, Dexa-DC promote Ag-specific Tregs that suppress T cell responses in a bystander fashion, independent of Ag and IL-10 [107] (Fig. 1).

Vitamin D3-modulated DC

1α,25-Dihydroxy VitD3, the active metabolite of VitD3, is widely recognized as a potent natural regulator of both innate and adaptive immune responses. Different protocols to generate VitD3-DC have been reported; indeed, VitD3 was added at different time points during monocyte-derived DC differentiation (a) during 7-day differentiation [108–110]; (b) at days 0, 3, and 6 [111]; (c) at days 0, 3 and 6, and then cells are stimulated by a cocktail of IL-1β, IL-6, TNF-α, and PGE_2 [28,112,113]; or (d) on day 5 [81]. Independently of the protocol used, VitD3-DC secrete IL-10 spontaneously, which increased upon stimulation with LPS or CD40L, and also secrete low amounts of IL-12 and IL-23 [108,112,114]. VitD3-DC also secrete chemokine ligand 2 (CCL2), TGF-β and low levels of TNF-α [112,115]. VitD3-DC display a semi-mature phenotype, with lower expression of CD86 and HLA-DR compared to their DC counterpart. Moreover, VitD3-DC upregulate inhibitory molecules such as programmed death-ligand 1 (PDL-1), PDL-2, and immunoglobulin-like transcript (ILT)-3 and ILT-4 [116]. VitD3-DC display low stimulatory activity, induce apoptosis of effector T cells [111], and inhibit Ag-specific T cell proliferation, which is associated with

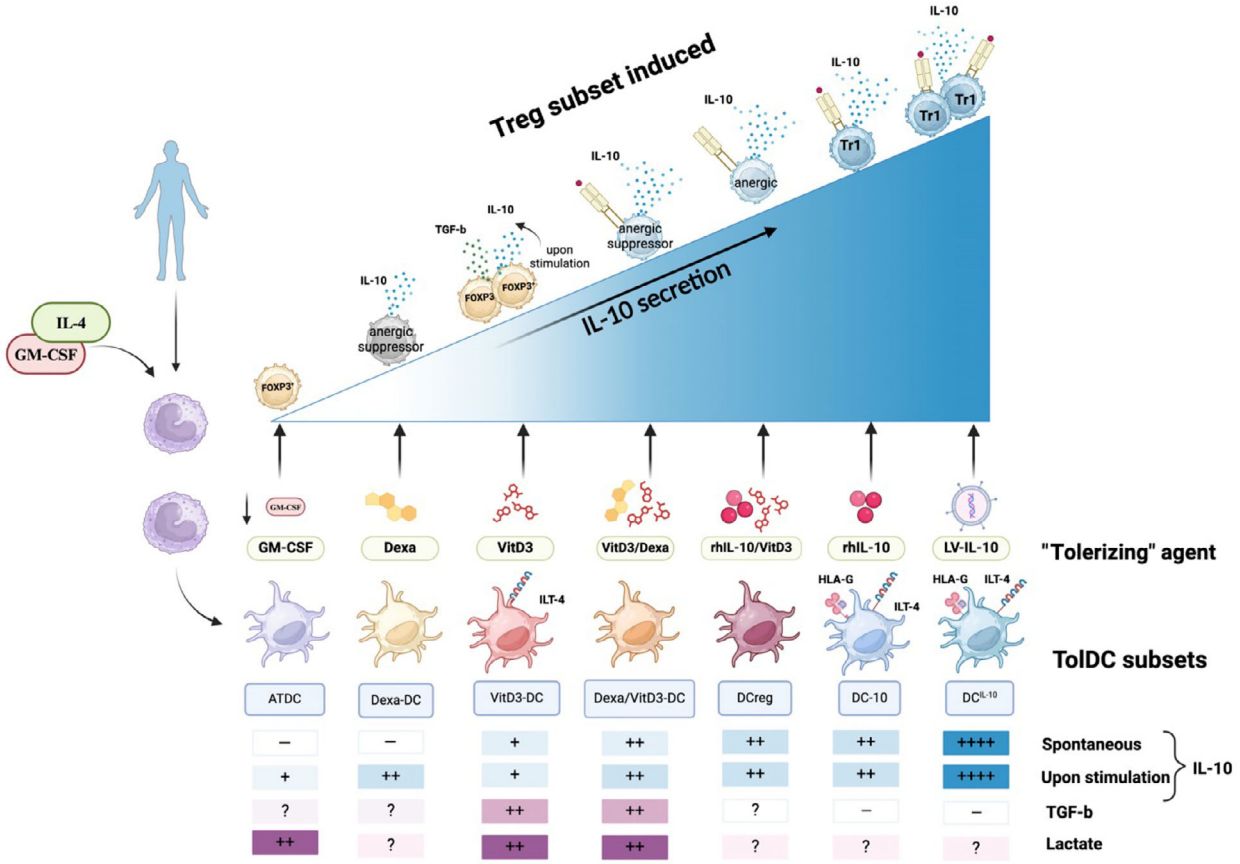

Fig. 1. Tolerogenic dendritic cells and their ability to promote T regulatory cells. Different tolDC subsets generated with the indicated "tolerizing" agent are presented. The levels of IL-10 production, spontaneous and upon stimulation, of TGF-β expression/secretion and the production of lactate by specific tolDC subsets are depicted. TolDC promote different subsets of $CD4^+$ T cells. ATCD generated with low doses of GM-CSF induce $FOXP3^+$ regulatory T cells (FOXP3) [27]. Repetitive stimulation of $CD4^+$ T cells with Dexa-DC generated by the addition of Dexa during the 7-day differentiation of monocyte-derived DC promote anergic/suppressor cells able to produce IL-10 [107]. Repetitive stimulations with VitD3-DC generated by addition of VitD3 during the 6-day differentiation of monocyte-derived DC and matured with CD40L and pulsed with proinsulin peptide (C19-A3) induced $FOXP3^+$ Tregs spontaneously expressing TGF-β and IL-10 upon Ag-specific stimulation [117]. Dexa/VitD3-DC generated with addition of Dexa on day 3 and addition of Dexa and VitD3 and activation with MPLA on day 6 promote anergic/suppressor IL-10-secreting T cells [123,126]. DCreg generated with addition of VitD3 on day 0 and 4 and IL-10 on day 4 of the 7-day monocyte-derived DC induce anergic IL-10-secreting T cells [138]. DC-10 generated with addition of IL-10 during the 7-day differentiation of monocyte-derived DC [24] or by LV-IL-10-mediated gene transfer into monocytes before differentiation into DC [25] promote allo/Ag-specific Tr1 cells. A correlation between higher IL-10 production and ability to promote Tr1 cells is presented. IL-10: (–) = no secretion, (+) = < 500 pg·mL^{-1}, (++) = 500–1000 pg·mL^{-1}, (+++) > 10 ng·mL^{-1}. TGF-β: (?) = unknown, (++) = > 200 pg·mL^{-1} or membrane-bound expression of LAP-TGF-β. Lactate: (?) = unknown, (++) = > 10 nM.

a decrease in the relative prevalence of IFN-γ-producing T cells, and a significant down-modulation of genes involved in cell cycle and cell response to pro-inflammatory stimuli [113]. VitD3-DC upon repetitive stimulation of naïve $CD4^+$ T cells, promote the induction of Ag-specific Tregs expressing FOXP3, PD-1, and membrane-bound TGF-β and upregulated IL-10 and CTLA-4 after stimulation with the cognate Ag [117] (Fig. 1), which suppresses Ag-specific T cell responses via linked suppression in a cell-to-cell contact dependent manner independently of Ag and IL-10 [107,117]. VitD3-DC significantly influences the migratory properties of DC towards inflamed tissues by upregulating the chemokine receptor CXCR3 [118]. VitD3-DC derived from monocytes from patients with relapsing–remitting MS share similar properties with those differentiated from healthy subjects, including a semi-mature phenotype, an anti-inflammatory profile, a reduced capacity to induce allogeneic T cell proliferation, ability to promote Ag-specific T cell unresponsiveness, and

resistance to maturation [21,112,114,119,120]. A subset of VitD3-DC expressing high levels of CD141, named VitD3-CD141^{hi}, secrete IL-10, are phenotypically and functionally superimposable to CD141^{+} DDC, and display low stimulatory capacity and induce tolerance in humanized mouse models of disease [81].

Dexamethasone/vitamin D3-modulated DC

The production of Dexa/VitD3-DC involves a stepwise process that harnesses the synergistic effects of both Dexa and VitD3 [121,122]. To generate Dexa/VitD3-DC monocytes are exposed to Dexa on day 3, and then treated with Dexa and VitD3 and activated with a TLR-4 agonist on day 6 [121,123,124]. TLR-4-mediated activation is necessary to stimulate the migratory activity and Ag presentation of Dexa/VitD3-DC while maintaining their tolerogenic characteristics. LPS-activated Dexa/VitD3-DC demonstrate CCR7-dependent migration towards T cell areas in secondary LNs, whereas non-activated Dexa/VitD3-DC express lower levels of CCR7 and exhibit limited migratory capacity [125]. Dexa/VitD3-DC produce IL-10 and lower levels of pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-23, and TNF-α) and undetected IL-12 [124,125]. In addition, Dexa/VitD3-DC express LAP-TGF-β, which is involved in their ability to regulate CD4^{+} T cell responses [126]. Dexa/VitD3-DC display a phenotype with reduced expression of CD80, CD86, and CD40. Consistent with the semi-mature phenotype, Dexa/VitD3-DC poorly induce CD4^{+} T cell proliferation and promote T cell anergy [124,125] (Fig. 1).

IL-10-modulated dendritic cells

IL-10-modulated DC are generated from monocyte-derived DC differentiated with IL-4 and GM-CSF and treated either with IL-10 and a maturation stimulus of day 5 (IL-10-DC) [127–129] or during 7-day culture (DC-10) [24]. Comparative analysis of the cytokine production profile of IL-10-DC and DC-10 demonstrated that both cell types spontaneously secrete IL-10 but not IL-12, whereas IL-10-DC secrete larger amounts of TNF-α compared with DC-10. Upon activation, IL-10-DC and DC-10 retain the ability to secrete IL-10, but IL-10-DC produce significantly greater amounts of IL-12 and TNF-α compared to DC-10 [129]. IL-10-DC express intermediate levels of CD80 and CD86, and ILT-3/ILT-4 at high levels, are a mixed population of cells expressing intermediate levels of HLA-DR and CD14 that can be segregated into CD83^{high}CCR7^{+} and CD83^{low}CCR7^{-} cells with different migratory capacities [127,130]. IL-10-DC display low stimulatory activity and promote anergic suppressor T cells [127,128,130]. DC-10 is a homogeneous population of cells characterized by the co-expression of CD14, CD16, CD141, CD163 [87], and of CD80, CD86, HLA-DR, HLA-G and ILT3/ILT4 [24]. DC-10 induce allo-specific CD4^{+} anergic Tr1 cells via the IL-10-dependent ILT4/HLA-G pathway [24,131] (Fig. 1). Allergen-pulsed DC-10 promote T cell hypo-responsiveness and the differentiation of allergen-specific Tr1 cells *in vitro* [129,132]. The pivotal role of DC-10 in promoting allo-specific anergic T cells [133], containing already differentiated allo-specific Tr1 cells [24], prompted the use of DC-10 to differentiate Tr1 cells for cell-based approaches in the context of hematopoietic stem cell transplantation (HSCT) for hematological malignancies [134]. In a GMP-compliant protocol, patient-derived DC-10 are co-cultured with purified donor-derived CD4^{+} T cells in the presence of exogenous IL-10 to generate alloAg-specific Tr1 cells, named T-allo10 cells, that underwent clinical evaluation in patients with hematological malignancies receiving an HLA–mismatched HSCT (Clinicalgov identifier NCT03198234 [135]).

Recently, our group developed an efficient protocol to genetically engineer monocyte-derived DC to over-express IL-10 (DC$^{IL\text{-}10}$) using a bidirectional lentiviral vector (LV) encoding for human IL-10 and the marker gene ΔNGFR [25]. DC$^{IL\text{-}10}$ are characterized by the ability to secrete supra-physiological levels of IL-10 in the absence of IL-12 and TNF-α, and acquire the DC-10 characteristic phenotype [24] expressing CD11c, CD14, CD16, CD141, CD163, ILT-4, and HLA-G molecules, and are phenotypically and functionally stable upon activation *in vitro*. DC$^{IL\text{-}10}$ modulate allo-specific CD4^{+} T cell responses, induce allo-specific Tr1 cells [25] (Fig. 1), prevent CD8^{+} T cell cytotoxicity, and promote allo-specific anergic CD8^{+} T cells [136]. The LV platform has been implemented by developing LVs co-encoding IL-10 and specific peptides fused to the MHC class II invariant chain to generate Ag-specific IL-10 engineered DC (DC$^{IL\text{-}10/Ag}$). DC$^{IL\text{-}10/Ag}$ secrete supra-physiological levels of IL-10 and low amounts of pro-inflammatory cytokines and display a semi-mature phenotype. Using immunodominant peptides (e.g., insulin or gliadin peptides), it has been shown that DC$^{IL\text{-}10/Ag}$ efficiently inhibit Ag-specific CD4^{+} and CD8^{+} T cell responses *in vitro*, promote *bona fide* Ag-specific Tr1 in healthy subject and patient cells, and prevent T1D development in NOD mice [137]. The highly versatile LV platform allows the generation of sets of LVs co-encoding for IL-10 and autoAg peptide/s which promoting stable expression and presentation of encoded peptide/s to both CD4^{+}

and $CD8^+$ T cells lead to a broad Ag-specific immunological unresponsiveness; induction of Ag-specific Tr1 cells from naïve $CD4^+$ T cells and the conversion of pathogenic $CD4^+$ T cells into Ag-specific Tr1 cells; and (the induction of exhausted Ag-specific pathogenic $CD8^+$ T cells; *in vivo* injection of engineered DC does not interfere with protective immunity against pathogens [137]. Therefore, the LV-IL-10/Ag platform represents an innovative tool to generate IL-10-producting tolDC in a personalized manner suitable for restoration and/or induction of Ag-specific tolerance.

Vitamin D3/IL-10 dendritic cells

The generation of VitD3/IL-10-DC, named DCreg, involves addition of VitD3 on day 1 and 5 of monocyte-derived DC differentiation, followed by addition of IL-10 and maturation cocktail containing IL-1β, IL-6, TNF-α, and PGE_2 on day 5. DCreg secrete IL-10 but not IL-12 or TNF-α, and upon LPS stimulation, maintain their cytokine production profile [138,139]. DCreg display a semi-mature phenotype with low expression of CD80, CD86 and CD40, but significant levels of PDL-1, crucial for dampening T cell stimulatory activity, and maintain a high PDL-1 : CD86 ratio even after stimulation [138]. DCreg poorly stimulate allogeneic T cells in primary response, with T cells stimulated with DCreg secreting limited amounts of IFN-γ, IL-17, IL-4 and granzyme B [138]. DCreg failed to induce or only induced limited allogeneic $CD4^+$ and $CD8^+$ T cell proliferation [140]. In MLR, the only cytokine present in DCreg cultures is IL-10 [138] (Fig. 1).

Autologous tolerogenic dendritic cells

Human autologous tolerogenic dendritic cells (ATDC) are monocyte-derived DC differentiated in the presence of GM-CSF at low concentrations, which secrete IL-10 and limited levels of IL-12 upon stimulation with LPS and IFN-γ, display an immature phenotype characterized by low expression of co-stimulatory (CD80, CD86 and CD40) and HLA-DR molecules, poorly stimulate allogeneic T cells, and suppress $CD4^+$ T cell proliferation in co-culture with mature DC. The inhibitory effect on T cell proliferation involves a reduction in IFN-γ- and IL-17-producing T cells, coupled with $FOXP3^+$ Treg expansion via lactate production [27] (Fig. 1). GMP-compliant ATDC generated from end-stage renal disease patients and healthy controls display the same tolerogenic phenotype, resistance to maturation, and ability to modulate T cell responses [141].

Molecular and metabolic pathways underlying IL-10-mediated tolerogenic dendritic cell functions

The expression of IL-10 is modulated by epigenetic mechanisms, including chromatin remodeling, 3D chromatin loops, histone modification, and DNA methylation [142–145]. Moreover, IL-10 controls gene expression by promoting changes in the chromatin accessibility in intestinal macrophages during bacterial infections [146], by inhibiting the recruitment of specific factors to enhancer regions in adipocytes [147]; or by selectively regulating the expression of specific transcriptional repressors in macrophages [148]. Cellular metabolism represents one of the critical mechanisms involved in determining the immunogenic or tolerogenic DC fate. We present an overview of the molecular modification associated with *in vitro* differentiated tolDC, underlining the critical role of IL-10 in imprinting their tolerogenic function and on metabolic changes described thus far in *in vitro* differentiated tolDC.

The epigenetic and genetic landscape of *in vitro* differentiated tolDC

Comparative transcriptional profiles indicated that depending on the "tolerizing" agent used to differentiate *in vitro* tolDC, different genetic signatures are induced; nevertheless, gene set enrichment analysis reveals that Dexa-DC, VitD3-DC, and DC-10 share several immune-regulatory and anti-inflammatory pathways [87,149]. To better outline the molecular mechanism underlying the induction of tolerogenic features in tolDC, a combination of transcriptional profiles and the epigenetic landscape has been applied. During VitD3-DC differentiation, VitD3 receptor (VDR) translocates to the nucleus after ligand recognition and acts as a transcription factor (TF) controlling the expression of a set of immune genes [150] and promoting DNA demethylation, and consequently transcriptional activation of genes associated with the acquisition of the tolerogenic properties of DC [151]. Our group recently performed chromatin and transcriptomic studies of DC-10, demonstrating that the Aryl Hydrocarbon Receptor (AHR) pathway is activated downstream of IL-10 during DC-10 differentiation [92]. AHR is a ligand-activated TF, which is widely expressed in the body and evolutionarily conserved [152]. In the absence of a ligand, AHR resides in the cytoplasm in an inactive complex, but with high affinity for its ligands [153]. Upon binding to an agonist, AHR translocates to the nucleus, where it binds

and heterodimerizes with ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator, also known as HIF1β) to modulate gene transcription [92,153]. AHR-driven gene expression is negatively regulated by AHR repressor (AHRR), which competes with the AHR-ligand complex for interaction with ARNT [154], and by hypoxia-inducible factor 1α (HIF1α) [155]. IL-10-mediated activation of AHR is required for the establishment of a set of genes, named DC-10 core genes, which are critically involved in the establishment of the tolerogenic functions in DC-10 [92]. The AHR/IL-10 pathway is involved in Tr1 and regulatory B (Breg) cell differentiation, with AHR activity required for IL-10 production [156–158]. During Tr1 cell differentiation induced by IL-27, the expression of AHR and c-Maf is upregulated, and upon activation AHR/c-Maf complex promotes the transactivation of IL-10 and IL-21 promoters, leading to cytokine release [156,157]. Similarly, in Bregs, AHR directly binds and regulates the expression of IL-10, supporting Breg phenotype and restraining the transcription of pro-inflammatory mediators [158]. Conversely, during DC-10 differentiation, AHR activation is induced by IL-10 [92], and the IL-10/AHR pathway promotes a unique epigenetic imprinting in monocytes allowing the activation and transcription of specific genes associated with DC-10 tolerogenic activity. AHR inhibition during DC-10 differentiation severely impairs chromatin accessibility but not DC-10-specific enhancer accessibility, thus indicating that AHR acts as TF, and it does not play a primary role in establishing IL-10 chromatin remodeling during DC-10 differentiation.

Besides the "master regulator" TF, a set of "pioneering" TFs are required to shape the epigenetic landscape that regulates the accessibility of transcriptional regulators [159,160]. Several pioneering TFs have been reported as key regulators of chromatin accessibility and gene expression patterns in the differentiation of mouse and human Tregs [161,162]. Specifically, Basic Leucine zipper ATF-like transcription factor (BATF) was reported to be essential for restructuring the genomic landscape, rendering the chromatin accessible to TFs required for Tr1 cell development downstream of IL-27 and for FOXP3 Tregs induced by IL-33 [162–164]. BATF expression is induced by IL-10 in monocytes at early time points during DC-10 differentiation, and BATF binding sites are significantly enriched at DC-10 enhancers and are maintained upon AHR inhibition during DC-10 differentiation, thus suggesting that BATF might act as pioneering TFs regulating chromatic accessibility upstream of AHR [92]. Investigation is ongoing to better highlight the role of BATF in promoting chromatic accessibility during IL-10-mediated induction of DC-10.

Metabolic mechanisms associated with *in vitro* differentiated/generated tolerogenic DC

Dendritic cells are characterized by aerobic glycolysis, which supports energetic demands during Ag uptake and processing, and migration to secondary lymphoid organs [165]. DC also engage FAO and OXPHOS, which contribute to the production of metabolites and signaling molecules that modulate DC phenotype and immune properties [29,166]. At the immature state, OXPHOS and FAO are the main energy sources for DC; conversely, DC maturation induced by LPS is associated with a metabolic switch towards aerobic glycolysis and a downregulation of OXPHOS gene expression, a phenomenon similar to the Warburg effect, in which the tricarboxylic acid (TCA) cycle is decreased, and lactate production is increased [167–169] (Fig. 2). VitD3 and Dexa have been shown to limit the glycolytic switch, rendering tolDC less dependent on glucose for survival and function, and less sensitive to death by nutrient starvation [170]. VitD3 alone or in combination with Dexa promotes the upregulation of genes associated with both glucose metabolism, TCA, and OXPHOS [168,170,171] (Fig. 2). Early transcriptional reprogramming of metabolic pathways is induced by VitD3 during monocyte-derived DC differentiation, which promote OXPHOS, and it is also associated with increased aerobic glycolysis. This mechanism allows VitD3-DC to secrete increased levels of lactate, compared to immature DC, which is associated with increased glucose uptake rates (Fig. 2). In this context, the PI3K/Akt/mTOR pathway was shown to be central in inducing and maintaining the tolerogenic properties of VitD3-DC. Thus, glycolysis and the PI3K/Akt/mTOR pathway are central regulators of the tolerogenic activities of VitD3-DC [171]. Similarly, protein expression and functional analyses of VitD3/Dexa-DC demonstrated a stable OXPHOS program with the generation of reactive oxygen species and superoxide, and of mitochondrial respiration, associated with an overall higher glycolytic capacity and reserve compared with mature DC [168] (Fig. 2). HIF-1α and mTOR are central regulators of the metabolic switch in DC [167]. mTOR functions as a master regulator of glucose metabolism [172], able to induce glycolysis through the activation of HIF-1α, which decreases OXPHOS levels, increasing glycolysis [165]. Genes associated with glycolysis and mTOR signaling characterize ATDC, and functional analysis demonstrated that ATDC are highly glycolytic, showing high

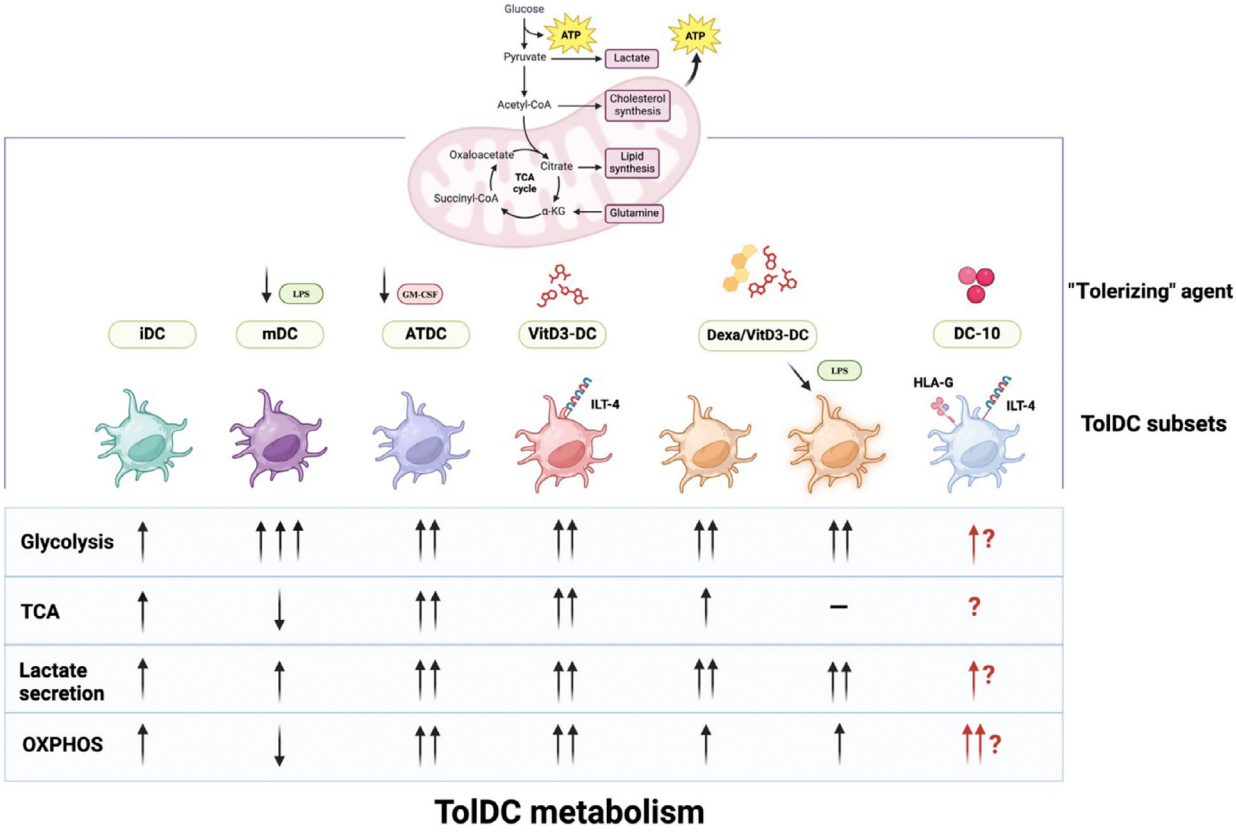

Fig. 2. Metabolic profile of tolerogenic dendritic cells. TolDC subsets generated with different tolerizing agents and their metabolic profile are presented. Immature DC (iDC) are characterized by basal levels of glycolysis, TCA, lactate production and OXPHOS. Matured DC (LPS-stimulated DC – mDC) exhibit high glycolysis, low levels of TCA and OXPHOS compared to iDC and maintain basal levels of lactate production [29]. ATDC display medium levels of glycolysis, produce high levels of lactate, and medium levels of OXPHOS and TCA [27]. VitD3-DC are characterized by medium levels of glycolysis, TCA, lactate production, and OXPHOS [171]. Dexa/VitD3-DC, left unstimulated or LPS-stimulated, display medium levels of glycolysis, low levels of TCA, medium levels of lactate production, and low levels of OXPHOS [168]. Due to the effect of IL-10 on limiting the complete metabolic switch in myeloid cells, DC-10 might display low levels of glycolysis, lactate production and medium levels of OXPHOS. ATDC, autologous tolerogenic dendritic cells; Dexa/VitD3-DC, vitamin D3/dexamethasone dendritic cells; iDC, immature DC; mDC, mature DC; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid cycle; VitD3-DC, vitamin D3-dendritic cells. Basal range = ↑; Medium range = ↑↑; High range = ↑↑↑; Low range = ↓; No data available: –; Hypothesis = ?

glucose uptake that is efficiently converted into lactate, which is involved in their tolerogenic activities [27]. The role of lactate in modulating immune responses by VitD3-DC have been recently reported. CD115 signaling in VitD3-DC promotes metabolic reprogramming, leading to enhanced glycolysis and glucose consumption and production of lactate [28] (Fig. 2). IL-10 limits the complete metabolic shift to aerobic glycolysis in monocyte-derived DC by antagonizing TLR-mediated induction of glycolysis driven by PI3K/Akt signaling [169] and glucose uptake, while increasing mitophagy and OXPHOS [173]. IL-10 induces downregulation of the translocation of glucose transporter 1 (GLUT1) from intracellular vesicles to the cell surface, overall limiting glucose uptake by myeloid cells and suppressing mTOR activity through the induction of the mTOR inhibitor, DDIT4 [173]. Compared with DC, DC-10 express significantly higher levels of DDIT4, which by inhibiting mTOR, allows AHR nuclear translocation leading to DC-10 gene core expression [92]. It can be hypothesized that DDIT4 might modulate their metabolism by inhibiting the mTOR pathway in DC-10.

The recognized conclusion that OXPHOS is associated with tolerogenic phenotypes, while glycolysis is associated with pro-inflammatory phenotypes in DC is partially correct. Based on the published evidence, VitD3, Dexa, and low doses of GM-CSF induce both

OXPHOS and glycolysis, but the latter at levels different to those observed in fully activated and mature DC. These metabolic changes result in the co-existence of OXPHOS, providing energy, and a high glycolysis rate, which maximize the conversion of glucose in lactate (Fig. 2). Based on the evidence in myeloid cells, DC-10 might display different metabolic reprogramming with increased OXPHOS and limited glucose uptake and thus limited glycolysis, potentially promoting lactate at low levels compared to other tolDC (Fig. 2). These differences might reflect the tolerogenic activities of IL-10-induced DC. Further studies are warranted to better define the metabolic changes induced by IL-10 in tolDC, and specifically in DC-10.

Translational application of *in vitro*-derived tolerogenic dendritic cells

In this section, we summarize results obtained in clinical settings in which the above described *in vitro* differentiated tolDC have been administered to patients, and discuss limitations and challenges to be considered to routinely use this approach in clinical practice.

Clinical application of TolDC-based therapy

In the context of autoimmune diseases, several subsets of *in vitro* differentiated tolDC have been tested as cell-based therapy, overall indicating the feasibility and safety of the approach; in addition, some short-term clinical benefits have been observed (Table 2).

In patients with MS and neuromyelitis optica spectrum disorder (NMOSD), intravenous injections of Dexa-DC loaded with a pool of seven myelin peptides and an aquaporin-4 peptide, respectively, stabilized clinical symptoms in terms of relapse, disability, and imaging. Immunological assessment revealed the induction of Ag-specific IL-10-producing Tr1 cells in peripheral blood at 12 weeks post-injection ([174]; ClinicalTrials.gov identifier: NCT02283671). Based on these preliminary data, a phase II clinical trial administering myelin-specific Dexa-DC in combination with immunomodulatory drug is ongoing in MS patients (TolDecCOMBINEM; ClinicalTrials.gov identifier: NCT04530318). In MS patients with active disease, autologous VitD3-DC loaded with a pool of seven myelin peptides were injected intradermally (MS-tolDCs) (ClinicalTrials.gov identifier NCT02618902) or intranodally (TOLERVIT-MS) (ClinicalTrials.gov identifier NCT02903537) [119,175]. Preliminary results indicated that both delivery routes are safe, feasible, and well-tolerated [20].

In rheumatoid arthritis (RA) patients, a single intra-articular administration of un-pulsed Dexa-DC (TolDCfoRA, ClinicalTrials.gov identifier: NCT03337165), or Dexa/VitD3-DC pulsed with a cocktail of citrullinated Ags (AutoDECRA; ClinicalTrials.gov Identifier: NCT01352858) was safe, feasible and well-tolerated. Treatment with Dexa/VitD3-DC stabilized knee symptoms in two patients who received the higher DC dose [176]. A phase I/II clinical trial with Dexa/VitD3-DC loaded with B29-HSP70 peptide (TOLERANT, ClinicalTrials.gov Identifier: NCT05251870) intranodally administered to RA patients is ongoing [20].

In T1D patients, intradermal administration of VitD3/Dexa-DC loaded with the proinsulin peptide C19-A3 (PIpepTolDCs, D-Sense trial; ClinicalTrials.gov Identifier: NCT04590872) stabilized β-cell function and diabetic control during the 6 months of monitoring. Immunological assessment revealed reduction of Ag-specific T cell proliferation and IFN-γ production, as well as increased IL-10 secretion in some treated patients [177].

In the context of allo-transplantation, a single intravenous infusion of donor-derived DCreg in adult living donor liver transplant recipients (ClinicalTrials.gov Identifier: NCT03164265) promoted transient increases in the expression of HLA, PDL-1 and other immunomodulatory molecules in circulating small extracellular vesicles, increases in the expression of donor HLA on recipient APC, and changes in $CD8^+$ T memory and Treg populations [140]. Follow-up of patients at 12 months after transplantation and DCreg treatment in comparison with matched standard-of-care transplanted patients confirmed the lower frequency of effector $CD8^+$ T cells expressing T-bet and Eomes, reduced proportion of $CD16^{bright}$ NK cells, increased frequency of $CD141^+CD163^+$ DC, and reduced allo-specific IFN-γ production by T cells [178]. A single intravenous infusion of ATDC into patients one day before transplantation in conjunction with standard immunosuppression (MMF and Tacrolimus) in patients undergoing kidney transplantation from living donors (ClinicalTrials.gov Identifier: NCT02252055) under the umbrella of The One Study (http://www.onestudy.org/) led to 100% graft survival at 3 years post-transplantation. MMF was successfully reduced/-stopped in five out of nice ATCD-treated patients. Immuno-monitoring of ATDC-treated patients revealed reduction of the frequency of activated $CD8^+$ T cells compared to control transplanted and treated with standard-of-care immunosuppressive protocol, and an increase at early time point after transplantation of $FOXP3^+$ T cells [179].

Table 2. Clinical trials using tolDC treatment in immune-mediated diseases.

Tolerogenic DC					
Tolerizing agent and Ags		Route/frequency/dose	No. of patients/disease	Outcome	Number/phase/reference
Dexa-TolDC	Dexa	Intraperitoneal Single 3 biweekly dose escalating	9 refractory Crohn's disease patients	No clear signal of clinical efficacy	EudraCT number 2007-003469-42 Phase I completed [183]
	Dexa 7 myelin or 1 AQP4 peptides	Intravenous 3 biweekly dose escalating	8 RRMS/progressive MS patients 4 NMOSD patients	Tr1 cells at week 12 (compared with baseline)	NCT02283671; TolDec-EM-NMO Phase Ib completed [174]
	Dexa 7 myelin peptides	NR 3 biweekly dose with immunomodulant	45 RRMS patients	Ongoing	NCT04530318; TolDecCOMBINEM Phase II recruiting
	Dexa/IFNα/GM-CSF	Intra-articular Single dose escalating	12 RA patients	Potential for long-term efficiency	NCT03337165; TolDCfoRA Phase I completed [20]
VitD3-DC	VitD3 7 myelin peptides	Intradermal 4 biweekly and 2 monthly dose escalating	9 patients with active MS	NR	NCT02618902; MS-tolDCs Phase I active, not recruiting
	VitD3 7 myelin peptides	Intranodal 4 biweekly and 2 monthly dose escalating	12 patients with active MS	Ongoing	NCT02903537; TOLERVIT-MS Phase I recruiting
Dexa/VitD3-DC	Dexa/VitD3 Autologous synovial fluid	Intra-articular Single dose escalating	9 RA patients with inflammatory arthritis	Knee symptoms stabilized in two patients but no clinical or immunomodulatory effects	NCT01352858; AuToDeCRA Phase II completed [173]
	Dexa/VitD3 B29-peptide of HSP70	Intranodal Two monthly dose escalating	18 RA patients	Ongoing	NCT05251870; TOLERANT Phase I/II recruiting
	Dexa/VitD3 Proinsulin Peptide (C19-A3)	Intradermal Two (day 0 and 28) dose escalating	9 T1D patients	β-cell function and diabetic control remained stable during the 6 months of monitoring.	NCT04590872; PIpepTolDC Phase I completed [177]
DCreg	VitD3/IL-10 Donor-derived monocytes	Intravenous Single dose	16 patients underwent kidney transplantation from living donors	Reduced effector $CD8^+$ T and NK cells, increase in $CD141^+CD163^+$ DC at 12 months	NCT03164265; DCreg Phase I/IIa active/not recruiting [178]
ATDC	Low GM-CSF Patient-derived monocytes	Intravenous Single dose	11 patients underwent kidney transplantation from living donors	Mycophenolate was reduced/stopped in five patients. Increased Foxp3 expression.	NCT02252055; ATDC Phase I completed [179]

Dexa, dexamethasone; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorders; RA, rheumatoid arthritis; RRMS, relapsing remitting multiple sclerosis; T1D, type 1 diabetes; VitD3, vitamin D3.

Limitations and challenges of using tolDC-based therapy

Results obtained in patients treated with tolDC have attracted widespread interest among researchers and clinicians. However, to routinely use this approach in clinical practice, several challenges (i.e. the optimal dose for efficacy, route of administration and migratory ability, stability of infused cells) should be considered.

Dose escalation studies have been performed to identify the tolerated dose. In all clinical trials performed, the number of cells administered were well-tolerated, although either no clinical improvement of the disease or limited effects were observed. To reach the optimal dose for efficacy, multiple tolDC injections were used, and the results, although in a limited number of patients, suggest that repetitive administration of tolDC might be effective in modulating immune responses, and promoting Treg cells [174,177]. Thus, scale-up production of tolDC is required to meet clinical demand. In one clinical trial, it was reported that due to technical limitations in producing the target highest dose of tolDC, patients received the maximum yield available from the cultures [174]. The scalability of tolDC production is also closely related to the cell source and the preparation of cells from patients' monocytes, which may vary significantly in number, quality, and the ability to efficiently convert into tolDC [92,149,180]. Efforts are ongoing to optimize apheresis collection to improve monocyte yield and fitness to ensure the differentiation of effective tolDC from MS in the context of Horizon Europe – Health Cluster 2022, the IMMUTOL project (https://immutol-horizon.eu/).

The timing and the schedule of tolDC administration is another important issue to consider. In most autoimmune settings, the clinical symptoms and the breakdown of tolerance do not occur simultaneously, and thus tolDC administration in the early onset of the disease might be beneficial. However, tolDC-based therapy entered the clinical arena quite recently; therefore, for safety reasons patients either refractory to conventional treatments or patients in stable disease after conventional treatment have been recruited and treated. This poses an extra consideration in terms of efficacy results, since the more established the disease and the inflammation, the longer the timeframe needed to evaluate the potential therapeutic effects. However, only short-term evaluations of efficacy have been performed (Table 2). Thus, long-term follow up of treated patients, and ongoing or planned phase II clinical trials will better define the efficacy of tolDC-based therapy.

Another important aspect linked to efficacy of the treatments is the ability of tolDC to reach the disease-affected organs or relevant LNs once injected *in vivo*. For this reason, different routes of tolDC administration (i.e. intravenous, intradermal intranodal, intra-articular) have been used (Table 2) [20]. However, based on the limited number of patients enrolled in the studies it is difficult to draw conclusions for the selection of the optimal route of administration for clinical efficacy. We cannot exclude that depending on the disease, a specific route of administration is needed for optimal clinical effects. Importantly, the ability of tolDC to secrete modulatory molecules (i.e. IL-10, TGF-b, lactate) might be beneficial locally in the target organ to dampen inflammation and directly inhibit pathogenic responses [20]. However, to exert these effects, tolDC should be stable, since in a pro-inflammatory environment they can convert into immunogenic DC, exacerbating the disease. For this reason, assessing the stability of *ex vivo* generated tolDC during the development of effective tolerogenic cells [25,119,121] or stabilized tolDC by specific treatment during differentiation is critically important.

Addressing these multifaceted challenges is crucial for advancing tolDC-based therapy into a viable and effective treatment option for immune-mediated diseases.

Conclusions and future perspectives

Different tolerizing strategies have shown potency at promoting tolDC applied as immunotherapy in immune-mediated diseases. Clinical trials demonstrated the safety and feasibility of the approach with some indication of regulation of pathogenic immune responses, but evidence of tolerance induction is still under investigation. Depending on the agent used for tolDC generation, cells acquire common features, including induction of hypo-responsiveness in T cells, but differ in their ability to prime $CD4^+$ T cells towards different phenotypes and functions. A literature assessment focusing on IL-10 secretion, expression of specific markers, and functional features revealed that tolDC depending on the treatment used for their induction promote $CD4^+$ T cell anergy, induction/expansion of $FOXP3^+$ Tregs, and/or generation of IL-10-producing T or Tr1 cells (Fig. 1). Based on this survey, it can be postulated that the ability to promote $FOXP3^+$ Tregs is correlated with the levels of lactate and/or TGF-β produced by tolDC; conversely to efficiently promote IL-10-producing T cells and Ag-specific Tr1 cells, tolDC need to secrete IL-10 at high levels and express tolerogenic molecules, such as HLA-G and ILT-4 [24,25]. However, these latter features

render tolDC inefficient in promoting FOXP3$^+$ Tregs [24]. To differentiate tolDC able to restore tolerance via multiple Treg pools, one of the possible approaches is combination treatments, assessing synergistic effects of combining IL-10-modulated DC with other immunomodulatory agents, as was proposed for DCreg [138,139]. In the context of Horizon Europe – Health Cluster 2022, the IMMUTOL project aims at developing an improved version of VitD3-DC consisting of VitD3-DC genetically engineered to overexpress IL-10 and Ag (https://immutol-horizon.eu/).

Growing evidence has established that distinct molecular programs and metabolic reprogramming act as a regulatory switch in determining the diversity of tolDC. A better understanding of the specific molecular events triggered by tolerizing agent signaling and the likely intersection between gene signature and metabolic pathways will help design new targeted therapies to restore/modulate immune tolerance.

Moving forward, continued research efforts aimed at refining and optimizing these therapeutic approaches are essential to validate their clinical applicability across a spectrum of immune-related conditions.

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