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Review

Immune checkpoints and the regulation of tolerogenicity in dendritic cells: Implications for autoimmunity and immunotherapy

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ABSTRACT

The immune system is responsible for defending the host from a large variety of potential pathogens, while simultaneously avoiding immune reactivity towards self-components. Self-tolerance has to be tightly maintained throughout several central and peripheral processes; immune checkpoints are imperative for regulating the immunity/tolerance balance. Dendritic cells (DCs) are specialized cells that capture antigens, and either activate or inhibit antigen-specific T cells. Therefore, they play a key role at inducing and maintaining immune tolerance. DCs that suppress the immune response have been called tolerogenic dendritic cells (tolDCs). Given their potential as a therapy to prevent transplant rejection and autoimmune damage, several strategies are under development to generate tolDCs, in order to avoid activation and expansion of self-reactive T cells. In this article, we summarize the current knowledge relative to the main features of tolDCs, their mechanisms of action and their therapeutic use for autoimmune diseases. Based on the literature reviewed, autologous antigen-specific tolDCs might constitute a promising strategy to suppress autoreactive T cells and reduce detrimental inflammatory processes.

1. Introduction

The immune system must be able to protect host tissues against a broad range of pathogens, without reacting to self-components. Thus, immune homeostasis has to be precisely maintained in a physiological state, through a balance of activating and inhibitory immune signals known as “immune checkpoints”. Dysregulation of such signaling processes has been associated with autoimmunity and chronic inflammation. Along these lines, dendritic cells (DCs) play a key role in the induction and maintenance of immune tolerance.

DCs were first described in the 70s as “large stellate cells” with unusual morphology, movement, and potent capacity for stimulating primary mixed leukocyte reactions [1]. Almost 50 years later, DCs are well-known professional antigen-presenting cells (APCs) that connect the innate and adaptive immune responses. These cells represent a highly heterogeneous population with different morphologies, origins, and immunological functions. DCs patrol their environment, sensing and capturing antigens. Consequently, they play a central role in determining the induction of immunity or tolerance. During the past decades, these cells have been largely studied as activators of the

Abbreviations: DCs, Dendritic cells; APC, Antigen-presenting cell; Treg cells, Regulatory T cell; tolDC, Tolerogenic dendritic cell; iDC, Immature dendritic cell; mDC, Mature dendritic cell; Semi-mDC, Semi-mature dendritic cell; pDC, Plasmacytoid dendritic cells; IDO, Indoleamine-2,3-dioxygenase; CTLA-4, Cytotoxic T lymphocyte associated antigen-4; PD-1, Programmed death-1; FcRs, Fc receptors; HO-1, Heme-oxygenase-1; BM, Bone marrow; GCs, Glucocorticoids; BMDCs, Bone marrow derived-dendritic cells; MoDCs, Monocyte derived-dendritic cells; MS, Multiple sclerosis; EAE, Experimental autoimmune encephalomyelitis; RA, Rheumatoid arthritis; CIA, Collagen induced arthritis; IBD, Inflammatory bowel disease; SLE, Systemic lupus erythematosus; CNS, Central nervous system; Breg, Regulatory B cell; Perf-DCs, Perforin secreting DCs; RALDH1, Retinaldehyde dehydrogenase 1; NAD, Nicotinamide adenine dinucleotide; Teff cell, Effector T cell

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immune response; however, the tolerogenic role of DCs has been gaining interest in recent years. It has been shown that DCs contribute significantly to the induction and maintenance of central and peripheral tolerance. This is evidenced by the general loss of tolerance observed after DC depletion, highlighting the key role of these cells during immune tolerance processes [2].

To promote tolerance, DCs must integrate information from both innate and adaptive immunity. This information can be "passive" in the absence of stimuli, or "active", consisting of anti-inflammatory cytokine sensing or interactions with regulatory T (Treg) cells.

In order to promote immunity DCs engulf, process and present antigens to specific naïve T cells, which consequently become activated through co-stimulatory molecules (CD80 and CD86) and DC-derived cytokines. In contrast, tolerogenic dendritic cells (tolDCs) promote tolerance rather than immunity following the interaction with naïve T cells [3]. The tolDC concept arose from the observation that immature dendritic cells (iDCs) are able to interact with T cells and regulate their function. Accordingly, adoptive transfer of iDCs is associated with prolonged antigen-specific graft survival. Thereupon, a broad range of strategies, protocols and pharmacological agents have emerged to induce and maintain an immature-like state in DCs and promote their tolerogenic potential as tolDCs [4].

Throughout this review, we will summarize the aspects associated to the *in vitro* generation of tolDCs, the mechanisms through which these cells modulate the immune response, and their potential use as immunotherapeutic agents for autoimmune diseases.

2. General features of DCs and tolDCs

DCs exhibit a broad heterogeneity. In mice, they can be classified in a simplified way into four categories: (i) classical or conventional DCs (cDCs), further sub-divided into classical type 1 (cDC1s: $CD8^+/CD103^+$) and classical type 2 (cDC2s: $CD11b^+$ $CD172a^+$), (ii) plasmacytoid dendritic cells (pDCs), (iii) Langerhans cells and (iv) monocyte-derived DCs (MoDCs) [5]. Experiments using mice deficient in different cDCs indicated that cDC1s induce peripheral Treg cells, whereas cDC2s are less efficient at such induction [6,7]. Even though most of the currently available information comes from murine DCs, research has recently focused on the study of subsets of human DCs. Specifically, the distribution of the homologs of murine cDC1 ($CD141^{hi}/CD13^{hi}$) and cDC2 ($Sirp\text{-}\alpha^+/CD1c^+$) was determined in different tissues and age groups [8]. Importantly, monocytes are the most frequently used precursors for DCs generation from human samples (MoDCs), unlike murine DCs usually produced from bone marrow precursors (BMDCs). This fact has made it difficult to effectively address whether murine and human DCs subsets are functionally equivalent [8]. Moreover, $CD141^+$ DCs (homologous to murine $CD8^+$ DCs) do express CCR9 but differ in expression of TLR, presenting different sensory functions. Despite these differences, it has been proposed that both pDCs and a $CD141^+$ subtype (similar to murine pDCs and $CD8^+$ DCs) are involved in tolerance maintenance in humans [9].

It is important to note that depletion of different DC types produces alterations in tolerance and homeostasis. Resident DCs contribute to autoreactive T cell elimination and central tolerance maintenance by cross-presentation of antigens in a non-inflammatory environment [10]. Depending on the physiological state, this can result in anergy or Treg cell expansion. Therefore, cross-presentation is involved in both central and peripheral tolerance. The former involves negative selection driven by medullary thymic epithelial cells through the presentation of tissue-specific antigens [11]. On the other hand, peripheral cross-presentation of tissue-specific antigens has been constitutively observed in $CD8^+$ DCs [10,12,13]. Moreover, it is worth highlighting that defects in cross-presentation produce imbalanced inflammatory responses and autoimmunity underscoring the key role of this mechanism in maintaining tolerance [11]. Additionally, DCs can induce autophagy, a process associated with central tolerance maintenance and which has been observed in DC subtypes specialized in cross-presentation [14].

In addition to the four main categories discussed, DCs are classically divided into two major functional and phenotypic stages, *i.e.* mature DCs (mDCs) and immature DCs (iDCs). Maturation is usually associated with the increased expression of co-stimulatory molecules and MHC-II, together with pro-inflammatory cytokine production in response to several stimuli (*i.e.* pro-inflammatory cytokines and microbial products) [3]. In contrast, the immature phenotype is characterized by highly efficient antigen uptake through several mechanisms, such as macropinocytosis, receptor-mediated endocytosis, and phagocytosis. In addition, iDCs show low expression of MHC-II and co-stimulatory molecules, which leads to a suboptimal interaction with T cells and, consequently, to T cell anergy [3]. It has been shown that iDCs are also able to uptake apoptotic components and contribute to Treg cell expansion and peripheral tolerance induction by presenting self-antigens to $CD4^+$ T cells [11].

Commonly, iDCs have been considered responsible for the induction of tolerance, whereas mDCs have been thought to promote immunity. However, it has been postulated that some semi-mature DCs (semi-mDCs) generated *in vitro* are more efficient preventing autoimmunity than iDCs [3,15]. Some of these semi-mDC develop when iDCs are exposed to particular stimuli, such as TNF-α, IFN-γ or E-cadherin disruption [16]. This state is defined as a high expression of MHC-II and co-stimulatory molecules, in the absence of pro-inflammatory cytokines [3], thus tolerance is achieved through the induction of T cell anergy and Treg cell differentiation [16].

It follows that a final way to classify DCs is given by their functional characteristics. Depending on their ability to induce immunogenicity or promote tolerance, we can divide DCs in immunogenic (imDCs) or tolerogenic (tolDCs) respectively (Fig. 1). As reviewed, both iDCs and mDCs have been well characterized [3]. In contrast, the full definition and characterization of tolDCs remain a controversial issue. However, tolDCs can generally be considered active specialized tolerance inducers that promote several mechanisms, including peripheral Treg cells expansion [17]. Accordingly, tolDCs include not only iDCs, but also tolerogenic semi-mDCs [3,16], like those mentioned above. This semi-mature phenotype can be naturally occurring, or pharmacologically and genetically induced, as discussed below. Importantly, naturally occurring tolDCs have been identified *in vivo* in mice [18], and their presence has been associated with a significant increase in graft acceptance during kidney transplantation [19]. However, whether tolDCs correspond to a separate lineage is still under debate as will be discussed below. It is also important to note that, while iDCs exhibit all the regulatory properties of tolDCs, their phenotype is relatively unstable under inflammatory conditions, rendering immunogenic properties. Therefore, iDCs can be thought as being found in the intersection between tolDCs and imDCs, and their function is largely dependent on the context; this hinders their use as therapeutic agents in several disorders [20]. In contrast, activated mDCs represent a clear subset of imDCs.

An interesting question discussed in the literature is whether tolDCs are indeed a specific functional state of DCs, as we pose above, or if they actually represent an independent cell lineage. Supporting the second hypothesis, several DCs subsets have been associated with tolerance maintenance [21]. For example, a subset of cDCs expressing perforin (perf-DCs), which ensure peripheral tolerance by limiting autoreactive T cells, has been identified *in vivo* [22]. Additionally, $CD103^+$ DCs have been involved in mucosal tolerance induction, unlike MoDCs, which have been shown as a dispensable population in this process. On the other hand, evidence supporting the first hypothesis includes the fact that both $CD8^+$ cDCs [23,24] and pDCs [25] have been implicated in tolerance induction, as previously mentioned. Accordingly, $CD8^+$ cDCs were found to express indoleamine-2,3-dioxygenase (IDO) and release TGF-β [26], whereas pDCs have been implicated in oral tolerance induction [27]. Therefore, these DCs have been suggested to be specialized in tolerance maintenance, supporting the separate lineage hypothesis [28]; nevertheless, it can also promote immunity depending on integrated factors, such as the context and stimuli, giving support to the maturation state hypothesis. Further data supporting a functional state shows that, although antigen-conjugated antibodies against DEC-205 induce specific tolerance, this effect is avoided with anti-CD40

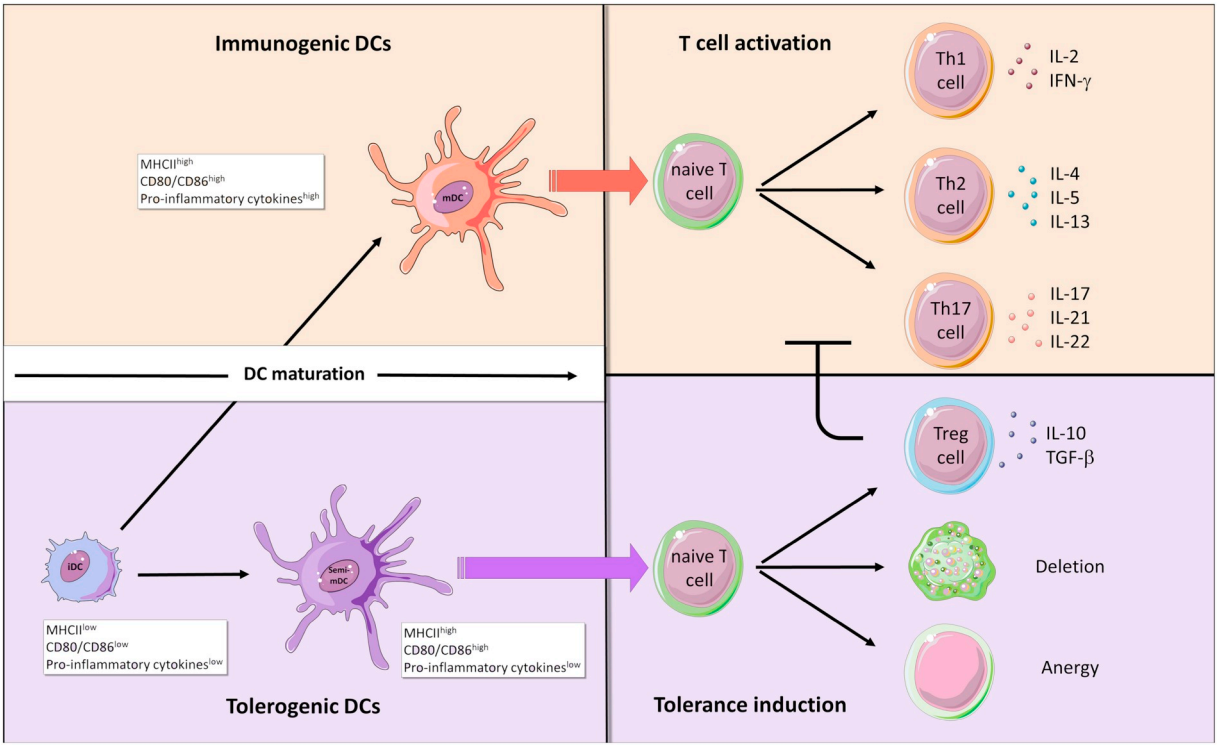

Fig. 1. Dendritic cell (DC) function and maturation state: The two functional classes of dendritic cells are showed. Above, immunogenic DCs (imDCs) are represented by cells with a mature phenotype (mDCs) indicated by high expression of MHC-II, co-stimulatory molecules CD80/ CD86, and pro-inflammatory cytokines production. Immunogenic DCs interacting with a naïve T cell induce different subsets T helper (Th1, Th2 or Th17) profile. Below, the non-immunogenic type is represented by tolerogenic DCs (tolDCs), which can induce and maintain tolerance. This category encompasses both immature (iDCs) and semi-mature DCs (semi-mDCs). iDCs are characterized by low expression of MHC-II, co-stimulatory molecules CD80/CD86 and pro-inflammatory cytokines production. In contrast, semi-mDCs present high expression of MHC-II and co-stimulatory molecules, but with low production of pro-inflammatory cytokines. Importantly, iDCs can potentially transition towards an immunogenic profile depending on the context and conditions, or if full maturation is induced. When tolerogenic DCs present antigens to naïve T cells it can be induced deletion, anergy or Treg cell differentiation which also suppress Teff cells functions.

antibodies, which activate DCs [29,30]. Moreover, as previously mentioned, a semi-mature state induced without inflammatory signals produces functionally tolerogenic DCs with a more stable phenotype than iDCs [3]. Therefore, although a transcriptional analysis of tolDCs would be necessary to better understand the nature of these cells, the summarized information strengthens the idea that tolDCs represent a functional state of DCs instead of a separate lineage.

Importantly, tolDCs can modulate the intensity and duration of the immune response, partly due to a particular set of co-inhibitory and co-stimulatory immune modulators known as immune checkpoints. The balance between these regulatory molecules is critical for the maintenance of immune homeostasis, particularly the preservation of self-tolerance. The importance of immune checkpoints in autoimmunity is highlighted in part by the reported effect of their inhibitors in the development and exacerbation of autoimmune diseases after cancer treatment with these drugs [31]. Also, the failure of immune checkpoints has been described in several autoimmune conditions such as inflammatory myopathies with the involvement of autoimmune features [32], diabetes, multiple sclerosis and systemic lupus erythematosus (SLE) [33].

To simplify, from here on we will use the term tolDCs in reference to those DCs that promote tolerance instead of immunity, except in those cases in which it is relevant to clarify the maturation state, in order to differentiate them from imDCs. Below, the main characteristics associated with tolDCs will be summarized, with particular emphasis on immune checkpoints.

2.1. Cell surface receptors

The ability of DCs to prime T cell activation and expansion depends, at least in part, on the interaction between several molecules on the surface of both cells. These interactions occur within a complex contact area known as the immunological synapse. Here, T cell receptor engagement is the main event, and other surface molecules interact to produce co-stimulatory signals. While many co-stimulatory molecules are involved in this synapsis, interactions between CD80/CD86 on DCs and CD28 on T cells have become the archetype of co-stimulation [34]. The integration of all these signals leads to T cell activation; therefore, reduced levels of CD80/CD86 on the surface of DCs are frequently associated with a tolerogenic profile. It is important to highlight that both CD28 and CD80/CD86 are considered immune checkpoints involved in DC activation.

CD40 and its ligand (CD40L) are other immune checkpoints relevant to DCs. CD40 signaling induces upregulation of MHC-II and CD80/CD86 in DCs, optimizing their ability to activate T cells. Conflicting results show that DCs stimulated with CD40L have a reduced stimulatory capacity and high production of anti-inflammatory cytokines (*i.e.* IL-10), indicating that a tolerogenic state can be induced by CD40 signaling [35]. This inconsistency might be because CD40 and CD40L are detected on both DCs and activated T cells, generating the possibility of bidirectional interactions. Indeed, CD40-CD40L interactions between DCs and T cells have been shown to produce reciprocal effects [36]. In addition, signaling by this ligand-receptor pair, the expression levels of CD40 are also relevant for the maturation and function of DCs. Consistently, tolDCs induced by vitamin D3 express low levels of CD40 and CD80/CD86 [37]. Therefore, expression and signaling of CD40 on DCs might influence the maturation and induction of the tolerogenic state. This is due to alterations in their capability of interacting with T cells through other co-stimulatory receptors and complex bidirectional stimulation processes. Interestingly, it has been reported that SLE patients display an increased expression of the co-

stimulatory molecules CD40 and CD86, as compared to healthy donors [38].

Programmed cell death-1 (PD-1) and the cytotoxic T lymphocyte associated antigen-4 (CTLA-4), are well-established immune checkpoints and co-inhibitory regulators. Although both CTLA-4 and PD-1 receptors are mainly expressed in T cells and Treg cells they are also detected in a subset of tolDCs [39]. Importantly, a new subset of DCs $CD14^+$ $CTLA\text{-}4^+$ and $PD\text{-}1^+$ that suppress $CD4^+$ T cells has been recently described in hepatocellular carcinoma [39].

CTLA-4 on T cells competes with the CD28 receptor for the binding to CD80 and CD86 ligands. It has been shown that DCs can inhibit T cell function through the activation of this molecule. Previous studies have also shown that CTLA-4 in Treg cells interacts with CD80/CD86, inducing a tolerogenic profile in DCs (IL-10 and IDO production) [40]. Interestingly, some tolDCs associated to carcinomas express both CD86 and CTLA-4, and it has been suggested that these cells might be able to induce tolerance not only in T cells but also in other DCs by CTLA-4/CD86 engagement [39].

As previously mentioned, tolDCs inhibit $CD8^+$ T cell activation; however, peripheral clonal deletion can only occur when the presence of antigens is persistent. Additionally, $PD\text{-}1^+$ DCs are especially able to suppress $CD8^+$ T cell function and proliferation, showed by a decrease in IL-2 and IFN-γ production [41], although the same is not necessarily true for $CD4^+$ T cells. Consistently with this notion, T cells activated with PD-1-deficient DCs display an increased antigen-specific $CD8^+$ T cell proliferation. Moreover, PD1 can expand inducible Treg cells (iTreg cells) by inducing Foxp3 expression in T cells and these iTreg cells can induce T cell-unresponsiveness dependent of the presence of the transcription factor Hopx [42].

Additionally, increased expression of inhibitory molecules, such as PDLs (B7−Hs), immunoglobulin like transcript 3 (ILT3) and ILT4, and Fas ligand (FasL), on the surface of DCs is also associated with a tolerogenic phenotype [43]. The B7 co-inhibitory molecules (PDL-1, PDL-2, B7-H3 and B7-H4) are cell-surface protein ligands that bind to receptors in lymphocytes and have a suppressive effect on T cell activation [44]. Thus, their increased expression on DC membranes, and their production as soluble molecules, render a tolerogenic profile in DCs [44]. PDL-1 and PDL-2 interact with PD-1 on T cells, driving them to anergy or functional inactivation and, consequently, favoring the induction and maintenance of tolerance [45]. Moreover, the high expression of ILT3/4 contributes to antigen-specific unresponsiveness in T $CD4^+$ cells [46]. Overall, these molecules have been considered markers for tolDCs.

Fc receptors (or FcRs) are tightly regulated receptors expressed in most innate immune cells, as well as B cells. FcRs are functionally classified as inhibitory or activating receptors. These receptors have a differential expression of patterns on various APCs. Relative expression of inhibitory and activating FcγRs can influence DC function, including their ability to eliminate pathogens and modulate T cell response. Both inhibitory FcγRIIb and activating FcγR mediate immune complex capture and promote antigen presentation to both $CD4^+$ and $CD8^+$ T cells. However, inhibitory FcγRIIb is mainly expressed in pDCs and cDCs and have the ability to counteract the effect of activating FcγRs [47]. This is evidenced by the exacerbated T cell immune response observed in FcγRIIb-deficient mice [48]. Thus, this inhibitory receptor could be fundamental for the maintenance of tolerance toward self-antigens, such as those derived from uncleared apoptotic cells. In addition, downregulation of FcγRIIb observed after DC maturation, produces a reduction of the immune activation threshold, highlighting the critical role of this receptor as a checkpoint in the process of antigen-presentation and T cell priming. The importance of this immune checkpoint is evidenced by the tight regulation to which it is subjected: Th2 cytokines produce an increased expression of FcγRIIb and it is downregulated by Th1 cytokines. The opposite effect is observed in regard to activating FcγRs. As a whole, these findings suggest that cytokines affect the expression of inhibitory and activating FcγRs, modulating the immune activation threshold of DCs [49].

Finally, the previously mentioned DEC-205 is a known receptor of apoptotic and necrotic components mainly present on DCs, which facilitates endocytosis. It is also an important factor for efficient antigen presentation to $CD8^+$ and $CD4^+$ T cells by MHC-I and MHC-II. Consequently, anti-CD205 antibodies conjugated with specific antigens have been used as therapeutic agents, since antigen delivery *via* DEC-205 in the absence of inflammatory stimuli leads to tolerance [29].

2.2. Cytokine production

Immunogenic DCs provide stimulation through antigen-presentation (signal 1), co-stimulatory molecules (signal 2), and also through pro-inflammatory cytokine production (signal 3); these latter allow them to modulate the polarization of T cell responses to different profiles (Th1, Th2, Th17 or Treg). Thus, it has been observed that T cell stimulation by DCs through signals 1 and 2 in the absence of signal 3 can induce IL-10-producing Treg cells [3,50]. Similar to mDCs, some semi-mature tolDCs express high levels of MHC-II, but produce low levels of pro-inflammatory cytokines and high levels of anti-inflammatory cytokines [3,51]. These anti-inflammatory cytokines, such as IL-10, inhibit the synthesis of pro-inflammatory cytokines (IFN-γ, IL-12 and TNF-α); meanwhile, TGF-β stimulates Treg cells. Thus, DCs produced under IL-10 conditions display a tolerogenic profile [52] and are able to induce Treg cells [53].

In summary, in addition to expressing characteristic inhibitory cell surface receptors, tolDCs also exhibit a particular pattern of cytokine secretion. Therefore, some authors have suggested to define tolDCs as $MHC\text{-}II^{high}$ pro-inflammatory $cytokines^{low}$, whereas immunogenic DCs should be defined as $MHC\text{-}II^{high}$ pro-inflammatory $cytokines^{high}$. Thus, tolDCs produce anti-inflammatory cytokines and express inhibitory molecules, which promote immune tolerance [3].

2.3. Molecules associated with signaling pathways

It has been reported that DCs with high expression of IDO, suppress T cell responses and promote tolerance. IDO is an intracellular enzyme that catalyzes the conversion of tryptophan to kynurenine, increasing Kyn pathway metabolites that ultimately lead to suppression of the immune response [54].

Constitutive IDO expression is observed in tissues where immune tolerance is necessary to maintain homeostases, such as intestine and placenta. In fact, the immunomodulatory role of IDO expression was described in cells from the fetal-maternal interface, where it prevents T cell-mediated rejection. Interestingly, IDO is usually detected in APCs, including a subset of $CD123^+$ $CCR6^+$ MoDCs, $CD8^+$ DCs, $CD19^+$ pDCs, regulatory B cells (Breg), and activated monocytes and macrophages [55]. In addition, IDO^+ DCs generated *in vitro* have $CD14^-$, $CD83^+$, $CD80^+$, $CD86^{high}$, $HLA\text{-}DR^{high}$ phenotype associated with mature cells. Interestingly, $CD8^+$ DCs expressing IDO induce tolerogenic conditions not only in naïve T cells but also in previously stimulated DCs [56].

Additional information is provided by IDO suppression, which prevents suppressive immune responses and Treg cell expansion [57]. In fact, there is considerable evidence suggesting that subsets of DCs can induce both $CD4^+CD25^+$ and $CD8^+$ Treg cells. In addition, IDO can induce apoptosis, Fas-mediated cell cycle arrest, and clonal anergy of T cells; furthermore, it inhibits antigen-specific T cell responses. Similar to IDO, retinaldehyde dehydrogenase 1 (RALDH1) expression has also been associated with suppressive profiles in DCs [58].

Another important enzyme present in tolDCs is heme-oxygenase-1 (HO-1), this enzyme catalyzes the degradation of the heme group and has been described as an anti-inflammatory and immunosuppressive molecule. Accordingly, HO-1 expression impedes DC maturation and is involved in T cell responses inhibition [59]. Interestingly, some HO-1 inducers (such as CoPP) increase both, HO-1 and IDO expression. This same positive correlation is observed for HO-1 inhibitors, suggesting

potential regulatory crosstalk between the HO-1 and IDO pathways [60]. Interestingly, it has been reported that monocytes from SLE patients display reduced expression of HO-1 when compared to healthy controls [61].

Finally, the production of inducible NO synthase (iNOS) and arginine has been associated with tolerogenic profiles in DCs. tolDCs have increased arginase-1 activity, and tolerogenicity is reverted by inhibitors of the said enzyme [62]. Additionally, tolDCs show increased iNOS expression, and DCs from $iNOS^{-/-}$ mice display a loss of tolerogenic function [62]. Furthermore, these mice present an increased susceptibility to earlier development of more severe experimental autoimmune encephalomyelitis, highlighting the importance of these molecules in tolDC performance.

3. Induction of tolerogenicity in dendritic cells

Immunogenic APCs, such as mDCs, work in activating and pro-inflammatory contexts. In contrast, iDCs establish a framework of low stimulation and anti-inflammatory cytokines. By presenting autoantigens to $CD4^+$ T cells in this second context, tolDCs are able to induce tolerance. The mechanisms through which tolDCs can induce and maintain self-tolerance are more specifically associated with the particular characteristics that distinguish this subset of DCs from imDCs. As shown above, these include cell surface receptors, cytokine production and enzyme expression. In conjunction, these pathways define how tolDCs are able to interact with other immune cells to generate a response.

Because of their potential therapeutic importance, the induction of a tolerogenic profile in DCs has been intensely studied and many pharmacological agents which promote a tolerogenic phenotype in these cells have been identified. Taking into account the differences between the various phenotypes of each type of DC, it is important to note that the induction of tolDCs, and their phenotypic and functional characteristics, are usually studied using murine bone marrow-derived-DCs (BMDCs) or human MoDCs. Because the immunological synapse is one of the most important events to determine the outcome of the DC-T cell interaction, different strategies have been developed to induce tolerance rather than immunity through the alteration of the capacity of DCs to establish an adequate communication with T cells. The list of compounds able to “program” DC function is continually growing. Such molecules and other tools are used to evaluate the molecular mechanisms, and to study of the effectiveness of the tolDCs generated in experimental models.

It is important to note that the dual function of DCs (tolerance and immunity) can be a double-edged sword, and stability of the tolerogenic phenotype is a central issue. Because of their plasticity, several environmental stimuli can induce reprogramming in DCs, promoting immunity instead of tolerance [63]. Some subsets of DCs are more prone to revert their phenotype *in vivo*, such as iDCs and semi-mDCs [3]. This must be especially considered when tolDCs are used to target autoimmune diseases characterized by chronic inflammation since they could exacerbate autoimmunity [64]. Thus, methods to promote or induce tolDCs with a robust and stable tolerogenic phenotype are essential for the treatment of autoimmune/inflammatory diseases. Some of these approaches are described below.

3.1. Cytokines

Several cytokines are known to induce the differentiation of BMDCs and MoDCs towards a tolerogenic state *in vitro*; such as IL-10 and TGF-β, while the IFN-γ-effect remains controversial. DCs treated with IL-10 show a reduced ability to produce pro-inflammatory cytokines and regulate T cell responses [65]. Moreover, IL-10-treated DCs induce anergy of $CD4^+$ and $CD8^+$ T cells *in vitro* [66] and inhibits BMDC maturation [67]. On the other hand, TGF-β modulates co-stimulatory molecules expression and pro-inflammatory cytokine production by BMDCs [68]. In addition, MoDCs treated with IFN-γ are unable to present antigens to T cells efficiently; further, such DCs are capable of inducing the expression of Foxp3 in naïve $CD4^+$ T cells [69]. Importantly, the IFN-γ effect is highly dependent on dose and length of treatment. Some evidence suggests that high doses of IFN-γ suppress MoDCs maturation. In contrast, other studies have shown that high amounts of IFN-γ induce maturation, while low IFN-γ amounts promote a tolerogenic profile. Taken together, these results suggest a need for more detailed protocols regarding doses and exposure times for the differentiation and modulation of DCs.

3.2. Pharmacological agents

The growing information regarding DC biology has triggered the development of new pharmacological agents designed to interfere with some immune checkpoints, such as differentiation, expansion, migration, maturation, as well as antigen uptake, processing and presentation.

Maturation is a process through which DCs change their functional phenotype and is triggered by several environmental stimuli, such as cytokines, pathogens, factors released by necrotic cells and effector T cells (Teff cells) expressing CD40L (Fig. 2). Several agents are known to interfere with the phenotypic and functional maturation of DCs. For example, the immunosuppressive effect of glucocorticosteroids (GCs) has long been described and these drugs are frequently prescribed for inflammatory disorders and autoimmune diseases. GCs inhibit DC *in vitro* differentiation and reduce DC *in vivo* numbers; however, the effect produced is dependent on the DCs maturation stage [70]. GCs not only prevent LPS/CD40L-induced maturation, but also increase IL-10 and reduce pro-inflammatory cytokine production [71]. Vitamin D3 and its analogs inhibit DC maturation increasing production of IL-10 and CTLA-4, while reducing expression of co-stimulatory molecules (CD80, CD86, CD40, CD40L) and secretion of IL-12 [72]. Mycophenolate mofetil (MMF) and rapamycin are other drugs with known inhibitory effects on differentiation and pro-inflammatory cytokine secretion [73,74]. mTOR proteins are mammalian targets of rapamycin, which acts as a downstream inhibitor of activation and proliferation pathways [74]. Finally, DC maturation is similarly affected by other anti-inflammatory drugs, such as acetylsalicylic acid, butyric acid and acetyl-l-cysteine. Additionally, CoPP, an HO-1 expression inducer, is capable of preventing DC maturation. DC maturation is tightly linked to transcription factor NF-κB; therefore, depletion of this molecule may promote the induction of a tolerogenic phenotype in DCs. Consistently with this notion, silencing or reducing NF-κB activity can work as a strategy to suppress DC maturation [75]. Some pharmaceutical agents, such as rosiglitazone, hamper DC maturation through the inhibition of NF-κB, preventing T cell activation and inducing antigen-specific tolerance [76].

Pharmacological inhibition of DC differentiation can be achieved with various immunosuppressive drugs, such as GCs, vitamin D3, rapamycin and butyrate. This last drug is a molecule released through the fermentation of gut microbiota, and its anti-inflammatory effect has been related to the down-regulation of several genes involved in inflammatory responses [77]. Accordingly, diets designed to induce a high release of butyrate through microbiota fermentation protect against autoimmunity [78]. It has been observed that butyrate blocks differentiation of DCs from their bone marrow precursors. It also inhibits histone deacetylates, reducing PU.1 and RelB transcription factors [58]. Additionally, butyrate-treated DCs have been reported to increase expression of RALDH and IDO, and to prime naïve $CD4^+$ T cells to differentiate towards IL-10-producing Treg cells [58]. Moreover, butyrate and retinoic acid have synergic regulatory effects on mucosal DCs, increasing CD103 and α4β7 gut-homing expression molecules. Together, these effects have been related to the immunomodulatory function of bacterial fermentation of non-digestible polysaccharides by gut microbiota.

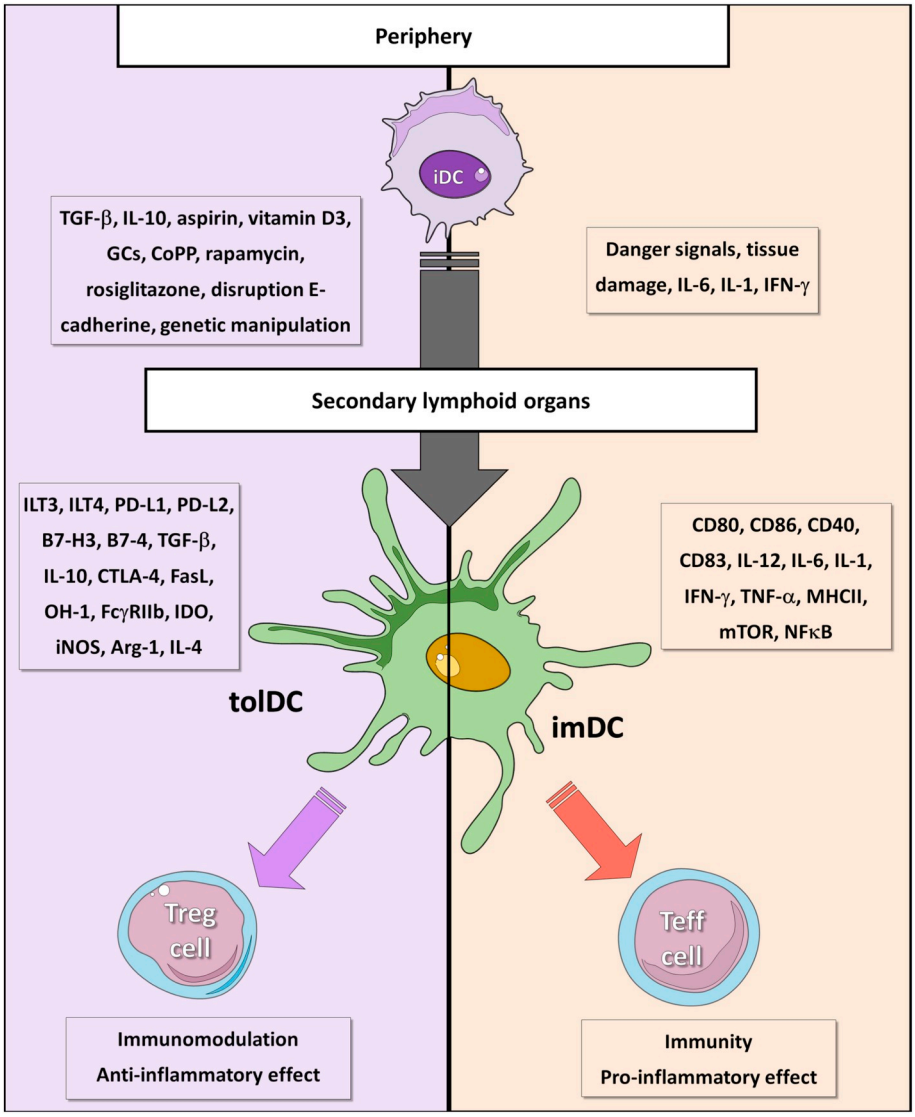

Fig. 2. Dendritic cell (DC) phenotypes: In response to integrated environmental stimuli (*e.g.* pathogens, drugs, injury), immature dendritic cells (iDCs) in the periphery modulate their phenotype towards a tolerogenic or stimulatory profile and migrate to secondary lymphoid organs. Immunogenic dendritic cells (imDCs), are produced in response to danger signals and up-regulate the expression of MHC-II, co-stimulatory molecules and pro-inflammatory factors. On the other hand, tolerogenicity is maintained in response to anti-inflammatory cytokines secretion, and can also be generated experimentally through genetic manipulation or drug exposure. tolDCs express inhibitory member receptors and produce anti-inflammatory cytokines, which allow these cells immunomodulatory properties. As a consequence of their contrasting phenotypes, imDCs interact with naïve T cells to promote effector T cell (Teff cell) expansion, whereas tolDCs induce and expand Treg cells.

On the other hand, the use of the nucleotide nicotinamide adenine dinucleotide (NAD) precursor as inductors of IDO has been evaluated to treat autoimmune diseases with promising results [79]. Importantly, combined treatments seem to be more effective inducing tolDCs than individual strategies [80].

Another strategy to protect against autoimmunity is to block the migration of reactive mDCs from BM, in order to avoid their contact with T cells in secondary lymphoid organs. Usually, mDC migration from BM towards lymphoid organs is a strictly controlled process directed by chemokines and their receptors in DCs. iDCs express receptors (CCR1, CCR2, CCR5 and CXCR1) which promote a different migration pattern towards non-lymphoid organs [81]. Maturation down-regulates said receptors while increasing CCR7 expression, thus suppressing migration towards secondary lymphoid organs. Therefore, the upregulation of CCR7 in iDCs favors the induction of Treg cells and is an exciting approach to enhance the potential of DCs as immunotherapeutic targets. Certain drugs that affect DCs migration include GCs, vitamin D3 and antagonists of multi-drug resistance protein 1 (MDR1) [82]. In addition, it has been shown that tolDCs overexpressing CCR5 protein through electroporating CCR5 mRNA, migrate towards inflamed locations, allowing localized immunomodulation [83].

As previously mentioned, DCs are specialized APCs with highly efficient machinery to uptake and process antigens through tightly regulated pathways. Thus, iDCs display robust antigen processing capacities, which are gradually reduced during maturation. Subsequently, mDCs are less competent antigen-presenters, but more efficient at stimulating T cells. Thus, disrupting antigen presentation by affecting antigen uptake capacities is a strategy to induce a tolerogenic phenotype. Drugs such as rapamycin can also be used for this purpose since it targets antigen uptake. In addition, pharmacological manipulation of endosomal activity can be achieved with hydroxychloroquine, an antimalarial agent that prevents the acidification of lysosomes, impairing antigen processing and the consequent presentation by MHC-II molecules [84].

3.3. Genetic manipulation and gene silencing

Genetic manipulation of DCs has allowed the upregulation of a large variety of immunosuppressive elements, along with the downregulation of pro-inflammatory factors. Overall, this approach can lead to the induction of tolDCs. Some studies have focused on the constitutive expression of immunosuppressive elements, such as IL-4, IL-10, CTLA-4, TGF-β or IDO [85–89]. In parallel, others aim to upregulate apoptosis-inducing elements, such as FasL and TRAIL [90]. Conversely, other strategies have focused on downregulating pro-inflammatory elements, such as CD80/CD86 and IL-12 [91,92].

The most common genetic manipulation strategy for tolDCs generation is the suppression of NF-κB to induce a stable immature state

[75]. Similarly, genetic interference in RelB (the primary NF-κB protein involved in DC maturation) with iRNAs produces a tolerogenic profile, shown by low MHC-II and CD80 expression, and prevents allograft rejection in heart transplantation in mice [93].

4. Therapeutic applications of tolDCs in autoimmunity

The information discussed above underscores the functional plasticity exhibited by tolDCs, as well as their central role in homeostatic maintenance and regulation of inflammation. The challenge now is how to translate results obtained *in vitro* or murine models into applied solutions for patients suffering from immune-mediated diseases. It is important to mention that tolDCs have been widely used as cell-mediated therapeutic agents for the treatment of different pathologies, as well as immunosuppressive therapy in organ transplant. However, these uses will not be addressed in this document, since their application for autoimmune disease is the primary concern.

tolDCs have been subject of intense study throughout the last decade and, although several clinical trials have been deemed safe, the *in vivo* efficacy in autoimmune diseases is still under evaluation. Additionally, the use of drugs to manipulate immune checkpoints has been associated with adverse effects [31,94]; however, the use of DCs loaded with specific antigens result in an improved therapy avoiding the general immune suppression. Thus, the selection of the appropriate antigen to induce specific tolerance in autoimmune diseases remains a central issue, especially taking into account that usually autoimmunity target multiple epitopes and the associated antigen in many conditions remains to be determined. Thus, the evaluation of tolDCs loaded with a single antigen could incorrectly direct tolerance induction, indicating that another or other antigens are more relevant in said condition, and masking the tolerogenic function of the therapy [95]. Even thus, the results obtained to date suggest a potential beneficial use of tolDCs as therapeutic agents using unload tolDCs, although the general immune suppression should be monitored in that case to avoid the impairment of host immune response against cancer and pathogens.

4.1. Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease characterized by chronic inflammation of the central nervous system (CNS). Pre-clinical trials conducted in the murine experimental autoimmune encephalomyelitis (EAE) model show that repetitive injections of BMDCs treated with TGF-β prevent disease induction [50]. Similarly, TGF-β-treated APCs show therapeutic potential, evidenced by the amelioration of EAE in rats [96]. In addition, the pharmacological inhibition of NF-κB suppresses DC maturation and thus prevents EAE onset in mice [97]. Finally, rats and mice with EAE treated with IFN-γ-generated tolDCs exhibited diminished clinical manifestations, showed by a reduction of macrophage and $CD4^+$ T cell infiltration [98].

A phase I study established the safety of using tolDCs generated through treatment with dexamethasone and loaded with a mix of disease-relevant peptides [99]. Additionally, another phase I study showed that the transference of tolDCs generated through treatment with vitamin D3 induces a stable antigen-specific T cell hypo-responsiveness in relapsing-remitting MS patients [100]. Finally, three clinical trials are currently registered at ClinicalTrials.gov (NCT02283671, NCT02903537 and NCT02618902) in recruiting status.

4.2. Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disorder typically characterized by synovitis and progressive cartilage destruction. A recent study indicates that various DC types have differential functions in RA patients. For example, pDCs seem inclined towards tolerogenic functions [101]. Preceding studies in humans, research was conducted in mice using the collagen-induced arthritis (CIA) model, which resembles some major features of human RA. In CIA mice, adoptive transfer of tolDCs inhibited the severity and progression of the condition through an increase in the production of anti-inflammatory cytokines and the expansion of Treg cells [102].

In two phase I clinical trials, the use of tolDCs treated with NF-κB inhibitors, or dexamethasone and vitamin D3, did not show major secondary effects in RA patients [103,104]. Additionally, these tolDCs showed a stable immunosuppressive phenotype resistant to pro-inflammatory stimuli [105]. Importantly, these studies were designed to evaluate the safety of tolDCs in RA patients, but a reduced proportion of T effector cells and an increase in Treg cells was also observed, indicating promising effects [104]. No less important is mentioning the antigen selected in these clinical trials which were citrullinated peptides in NF-κB generated tolDCs or autologous synovial fluid in vitamin D3 tolDCs trial [103,104].

Finally, another clinical trial is currently in recruiting status (NCT03337165) with the aim to evaluate the safety of a single intra-articular autologous transference of tolDCs generated with (IFN-α) and dexamethasone in RA patients.

4.3. Type I diabetes

Type I diabetes is a disease characterized by progressive loss of pancreatic cells and insulin production, mediated by an autoreactive immune response. Importantly, current treatments fail to counteract the autoimmune basis of this pathology. The effect of tolDC transfer was first studied in a non-obese diabetic (NOD) mouse strain prone to develop type I diabetes. tolDCs generated by down-regulation of CD40, CD80 and CD86 incite a reduced prevalence of the disease, mediated by the activation of Treg cells [106]. Additionally, in another study, tolDCs transferred to NOD mice resulted in an increase in Breg cell frequency and a tendency to rescue insulin production capacity in remaining islets [107].

Tolerogenic DCs generated *in vitro* by antisense targeting CD40, CD80 and CD86 and without antigens were evaluated as a treatment for type I diabetes in a phase I study, showing positive results concerning safety [108]. In addition, a new Phase II clinical is registered in ClinicalTrials.gov although still is not yet recruiting where DCs will be obtained by leukapheresis, tolerized *in vitro* with antisense oligonucleotides targeting the primary transcripts of CD40, CD80, and CD86 and transferred by intradermal injection (NCT02354911).

4.4. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is constituted by a set of inflammatory conditions with unknown etiology and characterized by chronic inflammation in the digestive system; it includes ulcerative colitis and Crohn's disease. In mice, a single injection of murine tolDCs pulsed with enterobacterial extract was able to reduce the severity of colitis, through the upregulation of IL-10 and IL-13 [109]. Similarly, mice with DSS-induced IBD or TNBS-induced colitis transferred with tolDCs overexpressing TGF-β showed a decrease in disease severity through the activation of Treg cells [110].

Very few studies evaluate the use of tolDCs in these pathological conditions. However, a phase I study tested the safety of tolDCs generated with dexamethasone, vitamin A, cytokines and prostaglandin E2 for Crohn's disease treatment with beneficial results. In this study, where tolDCs were not loaded with an antigen, patients showed an increase in Treg cells with an improvement in disease severity [111]. Additionally, a Phase I clinical trial is currently in recruiting state with the aim of evaluating the safety and efficacy of intralesional autologous transference of tolDCs in refractory Crohn's disease patients (NCT02622763).

4.5. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease typically characterized by the abundance of self-antibodies, which results in pathologies associated with immune complex formation and deposition.

This is followed by the activation of complement and innate immune cells, which trigger systemic tissue inflammation. Besides, in SLE patients have been reported increased levels of oxidative stress markers and apoptosis, for example, intracellular glutathione and their checkpoints were suggested to be involved in lymphocyte proliferation [112].

Different therapies have been examined to fight the damage caused by SLE. In humans, GCs have been widely used not only to treat SLE patients but also to generate tolDCs for cell transfer assays. Thus, dexamethasone and rosiglitazone have been employed in preclinical studies aiming to generate tolDCs [80]. Even though the benefits of using tolDCs as cellular therapy have been mostly established, little information exists about the potential advantages of tolDCs loaded with specific antigens in SLE and currently there are not registered clinical trials to evaluate tolDCs in SLE patients. In one study, autologous apoptotic cells from SLE patients were used to pulse tolDCs, generating a stable tolerogenic profile with low levels of co-stimulatory molecules, lower levels of IL-6 and IL-12p70, and ability to modulate the activation of $CD4^+$ T cells [80]. Given that the apoptotic cells used in this study contained autoantigens that are a hallmark of SLE (*i.e.* double-stranded DNA, ribonucleoproteins, histones), these results suggest that autoantigen-specificity of tolDCs might be valuable for their therapeutic use.

The beneficial effect of rosiglitazone (a known NF-κB inhibitor), was also observed in FcγRIIb-deficient mice, a murine model of spontaneous SLE. Administration of this drug reduced the levels of CD40 and CD86 in isolated splenic $CD11c^+$ DCs, prevented kidney damage and reduced the production of autoantibodies [113]. On the other hand, BMDCs from lupus mice have been efficiently used to produce tolDCs by silencing of RelB with a shRNA, suggesting a possible cell-targeted therapy based on their immunomodulatory properties.

5. Conclusions and perspectives

In this review, we have discussed the main phenotypic and functional characteristics of tolDCs. Furthermore, current strategies for *in vitro* generation of tolDCs are detailed above, along with possible mechanisms involved in the induction of tolerance by tolDCs.

We have highlighted the lack of a clear and undisputed definition of tolDCs. Based on the analyzed literature, it is likely that immune checkpoints might constitute a novel and important indicator of the tolerogenic state of DCs, and should be further investigated to understand the functional and phenotypic differences they trigger between subsets of DCs.

Finally, we have reviewed the use of tolDCs for the treatment of autoimmune diseases. Checkpoint-based immunotherapies have gained interest in the latest years; however, they have been associated with adverse effects because of its lack of specificity, and induce global immunosuppression related to susceptibility to cancer and infections. As opposed, promising pre-clinical and clinical data suggest that autologous antigen-specific tolDCs comprise a novel and promising strategy for targeting autoimmunity. Importantly, we suggest that immune checkpoints might be relevant targets for immunotherapy in the context of DC-induced tolerogenicity.

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Author contributions

SCF, AML and MJAL wrote the manuscript. AML proofread the manuscript and corrected language use. SCF constructed the figures. JEV, JMPO and AK supervised the work and performed critical revision of the manuscript. All authors revised and approved the manuscript. AMK is a Helen C. Levitt Visiting Professor at Department of Microbiology and Immunology, University of Iowa.

Conflicts of interest

"The authors declare no conflict of interest".

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