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## Immunotherapy with myeloid cells for tolerance induction

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### Abstract

**Purpose of review**—Understanding the interplay between myeloid dendritic cells and T cells under tolerogenic conditions, and whether their interactions induce the development of antigen-specific regulatory T cells (Tregs) is critical to uncover the mechanisms involved in the induction of indefinite allograft survival.

**Recent findings**—Myeloid dendritic cell–T-cell interactions are seminal events that determine the outcome of the immune response, and multiple in-vitro protocols suggest the generation of tolerogenic myeloid dendritic cells that modulate T-cell responses, and determine the outcome of the immune response to an allograft following adoptive transfer. We believe that identifying specific conditions that lead to the generation of tolerogenic myeloid dendritic cells and Tregs are critical for the manipulation the immune response towards the development of transplantation tolerance.

**Summary**—We summarize recent findings regarding specific culture conditions that generate tolerogenic myeloid dendritic cells that induce T-cell hyporesponsiveness and Treg development, and represents a novel immunotherapeutic approach to promote the induction of indefinite graft survival prolongation. The interpretations presented here illustrate that different mechanisms govern the generation tolerogenic myeloid dendritic cells, and we discuss the concomitant therapeutic implications.

### Keywords

immunotherapy; myeloid dendritic cells; regulatory T cells

### Introduction

A major challenge in transplantation is to control the strong immune responses to foreign antigens that are responsible for graft rejection. Although immunosuppressive drugs efficiently inhibit acute graft rejection, a substantial proportion of patients suffer chronic rejection leading to functional loss of the graft. Induction of immunological tolerance constitutes more than just a laboratory solution for the need of lifelong treatment with immunosuppressive drugs for transplant recipients [1], and immunotherapy with myeloid dendritic cells and their precursors represent a promising tool for the establishment of

indefinite allograft survival [2] Over the last years there have been an increasing number of articles that manipulated myeloid dendritic cell precursors to influence the immune response towards tolerance. Some of these articles also investigated the induction of antigen-specific tolerance using particular in-vitro culture conditions that cause T-cell hyporesponsiveness and promote regulatory T-cell (Treg) development. We have reviewed numerous strategies that are available to generate stable myeloid dendritic cells with tolerogenic properties, which include their modification with chemicals [3], cytokines [4], peptides [5]; via gene modification [6], small interfering RNA [7], and immunosuppressive drugs [8], some of which have already given promising results in macaques [9].

## Generation of tolerogenic myeloid cells *in vitro*

### Immunosuppressants

**Rapamycin**—Most of the experimental research with rapamycin conditioned dendritic cells for the induction of transplantation tolerance comes from Angus Thomson laboratory, which initially reported that rapamycin-treated alloantigen-pulsed dendritic cells infused 1 week before transplantation inhibits antigen-specific T-cell responsiveness and prolongs skin graft survival [10]. Similar studies reported indefinite vascularized skin allograft survival in recipient rats treated with antilymphocyte serum and cyclosporine, together with rapamycin-conditioned dendritic cells cultured with donor-derived peptide [11]. Interestingly, T cells from long-surviving grafts in these mice exhibit donor-specific hyporesponsiveness and expression of Foxp3 [11], which is consistent with a recent study which suggests that inhibition of mammalian target of rapamycin (mTOR) signaling during T-cell activation by rapamycin induces foxp3 in synergy with TGF- $\beta$  [12].

**Mycophenolic acid**—Human dendritic cells cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, TNF- $\alpha$ , and mycophenolic acid (MPA) results in the generation of alloantigen-specific and contact-dependent suppressive Foxp3-expressing Tregs that secreted large amounts of IL-10 and TGF- $\beta$ , and have high expression of CD25, glucocorticoid-induced tumor necrosis factor receptor (TNFR) [GITR], CTLA-4, and CD95 [13].

**Cyclosporine A**—It has been recently proposed that cyclosporine A increases the production of IL-10 in dendritic cells, and inhibits dendritic cell allostimulatory capacity by up-regulating B7 expression [14].

**Dexamethasone**—Human myeloid dendritic cells cultured with GM-CSF, dexamethasone, and lipopolysaccharide (LPS) produce high levels of IL-10 and reduces Th1 cytokine production [15]. Likewise, dexamethasone-stimulated human monocytes become tolerogenic dendritic cells that stimulate CD4<sup>+</sup> T lymphocytes to become Treg, which inhibit antigen-specific immune responses by secreting IL-10 [16]. In rats, in-vitro generated dendritic cells cultured with GM-CSF, IL-4, and dexamethasone bone marrow cells expand Treg, whereas human blood monocytes cultured with GM-CSF, IL-4, dexamethasone, and LPS induce T-cell anergy [17]. With regards to transplantation, donor hyporesponsive dendritic cells can be generated from rat bone marrow cells with GM-CSF, IL-4, and Flt3L in the presence of dexamethasone and LPS, although they do not prolong fully allogeneic allograft survival [18].

**Prostaglandin**—Dendritic cells generated in the presence of GM-CSF, IL-2, and prostaglandin are more efficient than anti-CD3/CD28 mAbs in expanding Treg and prevent unwanted immune reactions to allografts, since only infusion of dendritic cell-expanded Treg suppresses recipient response to donor alloantigens [19].

## Chemicals

**Vitamin D3**—Vitamin D3 (VD3)-matured dendritic cells are able to convert CD4<sup>+</sup> T cells into IL-10-secreting antigen-specific Tregs that suppress proliferation of effector T cells [20]. When combined with LPS, human monocyte cultured in the presence of GM-CSF, IL-4, VD3, and dexamethasone generates tolerogenic dendritic cells that retain a semimature phenotype with an anti-inflammatory cytokine profile, which is important for optimizing their therapeutic potential [21]. This is of special interest, since some of the critical aspects for immunotherapy with myeloid cells suggest that tolerogenic dendritic cell maturation must be impaired, specially during potential infections or inflammatory episodes following their adoptive transfer [22,23]. With regards to the effects of VD3 on T cells, it has been recently suggested that VD3 induces a transient expression of CTLA-4 and FoxP3 in cultured human CD4<sup>+</sup>CD25<sup>-</sup> T cells, which may be used to modulate the immune response [24].

**Aspirin**—Aspirin-treated human dendritic cells are resistant to maturation and exhibit a reduced expression of co-stimulatory molecules, such as CD40, CD80, and CD86, and increased expression of immunoglobulin-like transcript-3 (ILT3), which induces de-novo generation of Treg and promotes transplantation tolerance [3]. This is consistent with previous data, which suggest that tolerogenic myeloid cells must express low levels of cell-surface coestimulatory molecules to regulate the immune response [25].

**Aryl hydrocarbon receptor**—Recipient-derived mouse bone marrow cells cultured in the presence of GM-CSF, LPS, and an activator of the aryl hydrocarbon receptor, prevents islet allograft rejection by reducing antigen specific T-cell responses in the draining lymph node [26].

## Interleukins/cytokines

Pioneer studies from Austin and colleagues suggested that low doses of GM-CSF in the absence of IL-4 generates allogeneic immature dendritic cells *in vitro* that are resistant to different maturation stimuli, induce T-cell unresponsiveness *in vitro*, and promote alloantigen-specific graft acceptance [27]. Similarly, human monocytes cultured with GM-CSF in the absence of IL-4 generate semimature myeloid cells with low stimulatory activity [28]. Similarly, Torres-Aguilar and colleagues [4] have recently reported different ways of generating tolerogenic dendritic cells from culturing human monocytes with GM-CSF and IL-4 simultaneously with IL-10 ± IL-6 ± TGF-β1, which enhanced the expression of the regulatory molecules, and induced strong antigen-specific anergy in memory T cells. Others have suggested that GM-CSF, IL-4, and IFN-γ human monocytes develop into maturation-resistant dendritic cells that express DC-SIGN, Langerin, and CD123, and promote nonspecific Treg development [29]. With regards to transplantation, alloantigen-activated CD4<sup>+</sup> T cells cultured in the presence of IFN-γ promotes the generation of CD25<sup>+</sup>CD62L<sup>+</sup>Foxp3<sup>+</sup> T cells capable of preventing allograft rejection following adoptive transfer [30,31]. Semimature dendritic cells generated from murine bone marrow progenitors cultured with GM-CSF, IL-4, TNF-γ, and LPS, secrete low levels of IL-6 and IL-12p70, induce effector T-cell hyporesponsiveness *in vitro*, expand Foxp3<sup>+</sup> Treg *in vivo*, prolonging skin allograft survival [32]. Murine bone marrow cells cultured with GM-CSF, IL-10, and LPS generates alternatively activated donor dendritic cells that express high levels of PD-L1, display a reduced alloreactive T-cell-stimulating capacity, and expand Foxp3<sup>+</sup> Treg *in vitro*, prolonging skin allograft survival [33]. More recently, Thomson and colleagues reported that coculture of Treg with immature donor-derived dendritic cells in medium with GM-CSF and IL-4, induces potent alloantigen-specific Treg that prolong more than 80% over 150 days cardiac graft survival in mice when combined with low-dose rapamycin [34], which suggest a feedback loop between myeloid dendritic cells and Tregs

[35]. In rats, immature dendritic cells cultured with GM-CSF and donor cell lysates are able to induce peripheral immune tolerance to hind limbs when combined with rapamycin and antilymphocyte serum [36]. Syngeneic adherent rat bone marrow cells cultured in the presence of GM-CSF express high levels of HO-1, are poor stimulators of allogeneic T cells, and prolonged cardiac allograft survival [37\*].

### Proteins–peptides

**Vasoactive intestinal peptide**—Vasoactive intestinal peptide (VIP) generates anergic T cells by inducing cell cycle arrest and inhibiting cytokine production in allogeneic human T cells. VIP also generates Foxp3-expressing Treg from CD4<sup>+</sup>CD25<sup>-</sup> T cells after allogeneic stimulation, which exerts a protective role in a mouse model of acute graft-versus-host disease (GvHD) [38]. Interestingly, dendritic cell differentiated in the presence of VIP generates IL-10-secreting CD4<sup>+</sup> and CD8<sup>+</sup> T cells [39], and impair allogeneic antigen-specific responses of donor CD4<sup>+</sup> T cells in mice receiving bone marrow transplants by inducing Treg in the graft [40].

**Low-dose peptide**—Turner and colleagues [41] recently reported that both, GM-CSF derived immature dendritic cells, and GM-CSF and IL-4-derived mature dendritic cells presenting low dose of antigen expands Foxp3<sup>+</sup> Treg, which depends on IL-6 production following dendritic cell–T-cell interactions.

### Viral induced molecules

Pioneer studies from Catral and colleagues demonstrated that transfection of donor-derived bone marrow derived dendritic cells with FasL-induced hyporesponsiveness to alloantigen *in vivo* and prolonged 15 days the graft survival of fully mismatched cardiac allografts [42]. In mice, donor-derived dendritic cells transfected with recombinant adenovirus encoding human CTLA4Ig reduces the allogeneic T-cell stimulation *in vitro*, and prolongs cardiac allograft survival for 40 days when injected intravenously [43]. George and colleagues demonstrated that transfecting human dendritic cells with CTLA4 fused to the endoplasmic reticulum retention signal sequence induces antigen-specific anergy in responding T cells by preventing dendritic cell expression of CD80 and CD86 in the cell surface [6]. One of the most promising results uses donor-derived immature dendritic cells transfected to express soluble TNF receptor are resistant to maturation, which are unable to present antigen due to their low phagocytic properties, promote the development of IL-10 regulatory T cells *in vitro*, and induce long-term survival of cardiac allografts of 50% of the grafts when transferred before transplantation without further treatment [44]. The same laboratory, also investigated the induction of tolerance with IL-10 dendritic cell gene transfection, although only 30-day graft survival prolongation was observed [45]. Several studies by Vassalli and colleagues reported that gene transfer of programmed death ligand-1 Ig, indoleamine 2,3-dioxygenase, and soluble IL-1R Ig fusion protein, and IL-18 binding protein to donor dendritic cells attenuates cardiac allograft rejection and prolongs cardiac allograft survival by 1 week [46–48]. More recently, lentiviral vectors have been used to genetically engineer VIP-expressing bone marrow-derived dendritic cell which reduces dendritic cell expression of proinflammatory cytokines and increases their IL-10 production following local delivery [49].

### Immunoglobulins

Human dendritic cells generated *in vitro* in presence of CTLA4-Ig suppress T-cell proliferation by up-regulating the levels of HLA-G5 in plasma of CTLA4-Ig-treated patients, with the concomitant immunosuppressive applications [50].

## Embryonic stem cells

There is also a great interest in manipulating the immune response using myeloid cells derived from stem cell progenitors *in vitro*. Human embryonic stem cells (ESCs) cultured with bone morphogenetic protein-4, GM-CSF, stem cell factor and vascular endothelial growth factor (VEGF) in serum-free media generates hESC-derived monocytic cells that can be further differentiated mature dendritic cells with GM-CSF, TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$  and PGE2 that generates unlimited numbers of immunogenic dendritic cells [51]. Chen and colleagues have recently reported a protocol to generate myeloid-derived suppressive cells from murine embryonic stem cells in a three-step differentiation strategy which generated embryonic bodies from HoxB4-transduced ESCs cultured with c-kit ligand conditioned medium, IL-6, and WEHI-3, followed by c-kit ligand conditioned medium, thrombopoietin, VEGF, and Flt-3L. The resulting myeloid-derived suppressor cells exhibited a strong suppressive capacity *in vitro*, and was able to induce indefinite allograft survival of allogeneic bone marrow transplants [52\*].

## Generation of tolerogenic myeloid cells *in vivo*

One potential risk of immunotherapy with tolerogenic dendritic cells generated *in vitro* is that they may switch to a T-cell-activating phenotype when encountering inflammatory signals *in vivo*, since the local microenvironment plays an important role in the modulation of dendritic cells [53]. Complex myeloid dendritic cell–T-cell interactions occur in defined micro-anatomic domains within secondary lymphoid organs that lead either to successful T-cell priming or T-cell unresponsiveness, due to regulatory mechanisms that include anergy, deletion, or induction of Treg. Here we summarize recent data regarding the *in-vivo* induction of tolerogenic dendritic cells.

## Chemicals

**Vitamin D3**—*In-vivo* administration of VD3 prevents dendritic cell maturation independently of Toll like receptor (TLR) stimulation [54], and topically applied VD3 increases the suppressive capacity of Foxp3<sup>+</sup> Treg in the draining lymph nodes [55]. Vitamin D analogs also prevent antigen-specific priming of alloreactive CD8<sup>+</sup> T cells and expands antigen-specific Foxp3<sup>+</sup> Treg following immunization [56]. In transplantation, pioneer studies of Deluca and colleagues demonstrated that *in-vivo* administration of VD3 markedly increases allograft survival in both murine and rat vascularized and nonvascularized transplant models [57]. A possible explanation for this is that activation of VD3 receptor reprograms dendritic cell maturation to differentiate them into tolerogenic cells [58], which can be used *in vivo* to induce Treg-dependent antigen-specific transplantation tolerance to murine islet allografts [59].

**Aryl hydrocarbon receptor**—*In-vivo* activation of aryl hydrocarbon receptor induces antigen-specific long-term islet allograft acceptance by promoting Treg survival and function [26].

## Interleukins/cytokines

**GM-CSF:** *In-vivo* administration of mouse GM-CSF promoted the development of CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid-derived suppressor cells that prevent CD8<sup>+</sup> T-cell-mediated immune response [60]. Interestingly, GM-CSF promotes the expansion of specific myeloid derived suppressor cell (MDSC) subsets in the spleen of tumor-bearing mice that were responsible for tolerance [61].

## Proteins–peptides

Delivering antigens specifically to DEC205 targets MHC class I T-cell responses, whereas targeting dendritic cells via 33D1 preferentially modulates MHC class II T-cell responses [62]. Lechler and colleagues have recently conjugated the 33D1 mAb with the K<sup>d</sup>, which deletes antigen-specific T cells, promotes Foxp3 Treg development, and induces indefinite skin graft survival when combined with anti-CD8 mAb [63\*].

## Conclusion

There is a growing interest in taking dendritic cells into medicine [2]. The international Society for Dendritic Cell and Vaccine Science has recently been created (<http://www.dc-vaccine.org/>), and the next international symposium on dendritic cells will focus on the importance of developing dendritic cell vaccines. Dendritic cell immunotherapy in transplantation utilizes dendritic cells matured *in vitro* under specific culture conditions that are injected intravenously later on as tolerogenic dendritic cells. This approach may not give satisfactory results in transplantation simply because myeloid dendritic cells are poorly specialized in migrating to the lymph nodes via high endothelial venules (HEVs) (reviewed in [64]). This is of special interest, since Lakkis and colleagues [65] reported 10 years ago, that the immune response to transplant antigens leading to graft rejection can be triggered in the spleen and the lymph nodes. Therefore, we think that immunotherapy with dendritic cells to induce antigen-specific transplantation must consider that tolerogenic dendritic cells need to migrate the peripheral sites where antigen-specific T cells proliferate, namely the spleen and the lymph nodes [66]. For nonvascularized skin transplants, we would like to propose injections of *ex vivo*-matured tolerogenic dendritic cells in the skin, rather than into blood, to augment the tolerogenic responses in specific skin draining lymph nodes. For vascularized cardiac transplants, we would like to propose immunotherapy with blood-circulating cells, such as CD8<sup>+</sup>DC, monocytes, and plasmacytoid dendritic cells (pDC), which potentially control the immune response in the recipients' lymph nodes and the spleen. This is of special interest, since CD8<sup>+</sup>DC [67], monocytes [68], and pDC [69] have been suggested to participate in Treg development, and novel approaches that target specific dendritic cell subsets *in vivo* to promote indefinite skin allograft survival [63\*]. Additionally, it is possible that HEVs may need to be activated locally [70], or systemically [71,72] to ensure efficient migration of specific dendritic cell subsets and their precursors to the lymph nodes for successful immunotherapy, bearing in mind that these activators may affect the release of potentially nonregulatory cytokines such as IL-6.

We also believe that a combination of donor and recipient dendritic cells may be necessary to achieve indefinite allograft survival in transplantation. Acute rejection is mediated by CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes that recognize transplant antigens through the direct pathway of allorecognition, whereas chronic rejection is mediated by CD4<sup>+</sup>T cells that recognize transplant antigens through the indirect pathway of allorecognition [73,74]. In this respect, Treg stimulated through both, the direct and indirect pathways of allorecognition prevent acute and chronic rejection in recipient mice preconditioned with sublethal irradiation following adoptive transfer [75], which suggest the potential use of Treg for future cell-based immunotherapy in transplantation [76]. Therefore, it seems reasonable to think that that a combination of donor dendritic cells that induce direct T-cell hyporesponsiveness, and recipient dendritic cells that induce indirect T-cell hyporesponsiveness and Treg development are both necessary for the induction of transplantation tolerance using dendritic cell immunotherapy.

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