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REVIEW

Tolerogenic dendritic cells and their applications in transplantation

Haibin Li and Bingyi Shi

In transplantation immunology, the ultimate goal is always to successfully and specifically induce immune tolerance of allografts. Tolerogenic dendritic cells (tol-DCs) with immunoregulatory functions have attracted much attention as they play important roles in inducing and maintaining immune tolerance. Here, we focused on tol-DCs that have the potential to promote immune tolerance after solid-organ transplantation. We focus on their development and interactions with other regulatory cells, and we also explore various tol-DC engineering protocols. Harnessing tol-DCs represents a promising cellular therapy for promoting long-term graft functional survival in transplant recipients that will most likely be achieved in the future.
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INTRODUCTION

Dendritic cells (DCs), which were first described as 'large stellate cells' in 1973, are the most important professional antigen-presenting cells. DC research is one of the hottest areas in immunology research and has recently undergone significant advances.[1–3] DCs are the bridge between innate and adaptive immunity and can induce protective immune responses following stimulation by a variety of stimuli. DCs are a heterogeneous population. Based on their phenotype and function, DCs can be divided into conventional DCs and plasmacytoid DCs (pDCs). Additionally, according to their developmental status, DCs can be divided into immature DCs (imDCs) and mature DCs (mDCs). Many studies have shown that DCs are essential mediators of pro-inflammatory or anti-inflammatory (tolerogenic) responses,[4] which have been ascribed opposing roles in tolerance induction and the silencing of immune responses.[5,6] The subsets of DCs that suppress immune responses were initially termed regulatory DCs (DCregs).[7–9] Currently, DCregs are generally known as tolerogenic DCs (tol-DCs) because of their functions in inducing T cell apoptosis, anergy and regulatory T cells (Tregs).[10]

Currently, patients can successfully acquire immunological tolerance after treatment with nonspecific immunosuppressive drugs. Considering that non-specific immunosuppression often causes serious side effects, such as opportunistic infections, most clinicians and immunologists strive to induce an appropriate graft-specific tolerogenic response. DCs with regulatory or tolerogenic functions have attracted much attention because they play important roles in maintaining immune homeostasis.[11,12] Recent studies have focused on the prospective value of tol-DCs to induce clinical organ graft tolerance and to prolong graft survival.[13–15] Here, we provide an overview of the characteristics of tol-DCs, describe their biological functions and highlight recent advances that may yield DC-based therapeutic opportunities.

THE INDUCTION OF TOL-DCS

Recent studies have shown that tol-DCs promote central or peripheral tolerance through various mechanisms, including the deletion of T cells, the induction of Tregs and anergic T cells,[16] the expression of immunomodulatory molecules (e.g., PD-L1 and -L2, hemeoxygenase 1, HLA-G, CD95L, TNF-related apoptosis-inducing ligand, galectin-1 and DC-SIGN), and the production of immunosuppressive factors including IL-10, TGF-β, indoleamine 2,3-dioxygenase (IDO), IL-27 and NO.[14,17,18] There are two views on the critical inductive factors for the immunologic or tolerogenic functions of tol-DCs: one is that that the stage of differentiation, activation and maturation determines their function, and the other is that tol-DCs encounter environmental factors that determine tolerance induction.[19]

The potential of DCs to induce tolerogenic or inflammatory responses is directly related to their maturation status. T-cell tolerance can be induced by imDCs that express low surface

The Transplant Institute of the People's Liberation Army, The 309th Hospital, Beijing, China
Correspondence: Dr BY Shi, The Transplant Institute of The People's Liberation Army, The 309th Hospital, Beijing 100091, China.
E-mail: shibingyi@medmail.com.cn
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levels of MHC class II and costimulatory molecules, whereas inflammatory T cell immune responses are induced by mature DCs that express higher levels of these antigen presenting and costimulatory molecules.[20] In NOD mice, in which a mimotope peptide can be recognized by the diabetogenic $CD8^+$ T cell clone AI4, peptide was targeted to DCs by the endocytic receptor DEC-205 and antigen-specific tolerance was achieved.[21] However, the group of DCs that present antigen, subsequently activate $CD8^+$ T cells, and then initiate immune reactions belong to the same type of DCs.[22] *In vivo*, it was found that tolerance can be induced by targeting OVA antigen to $CD8^+DEC205^+$ DCs and $CD8^-33D1^+$ DCs. Furthermore, immune reactions can be induced by mature DCs stimulated through CD40L.[23] ImDCs are characterized by increased expression of FcγRII, TLRs, DEC-205, CCR1, CCR2, CCR7, CCR5, CCR6, CXCR1, CXCR2 and PD-L1, decreased expression of MHC-II, and costimulatory molecules, such as CD86, CD40, CD54 and PD-L2.[24–28] These imDCs have been described to have the capacity to induce or expand Tregs. However, some studies have shown that phenotypically mature DCs (high expression of costimulatory molecules) do not always induce Th1 responses but instead promote Tregs.[29]

It was reported that a unique subset of progenitor cells could differentiate into immature DCs after long-term culture, with a $CD11c^{low}CD11b^{high}MHCII^{low}CD86^{low}$ DC phenotype.[30] Subsequent research examined the influence of lymphoid microenvironments on DCs and found that both contact with stromal cells and TGF-β promoted mature DCs, with a $CD11c^{high}CD11b^{low}MHCII^{high}$ DC phenotype, to differentiate into a regulatory DC subset, with a $CD11c^{low}CD11b^{high}$-$MHCII^{low}CD86^{low}$ DC phenotype.[31] Splenic stromal cells, which mimic the secondary lymph organ microenvironment, can drive mDCs to proliferate and differentiate into a novel DCreg subset that is $CD11b^{high}Ia^{low}$ and produces increased amounts of IL-10 but minimal IL-12p70, which inhibits T-cell proliferation.[16,32] Studies have illustrated that the microenvironment of the lung and liver, in addition to the spleen, is also important for the programming of progenitors to differentiate into DCregs *in situ*, which contributes to the maintenance of tolerance.[33] Therefore, it is reasonable to hypothesize that the lymphoid organ microenvironment is important for regulating the development and function of immune cells,[34] especially DCs, whose unique functions largely result from local microenvironmental influences in different organs or tissues.[35] These data highlighted a previously unknown mode of regulation of the T-cell response through antigen-presenting cells.

Moreover, recent studies have also shown that tumor microenvironments can induce the generation of DCregs or tol-DCs. Han *et al.*[36] recently identified a novel subset of human $CD14^+CTLA\text{-}4^+$ DCregs in hepatocellular carcinoma, which suppresses T-cell responses by CTLA-4-dependent IL-10 and IDO production. Mouse $CD11b^{high}Ia^{low}$ DCregs induced by 3LL lung cancer could suppress T-cell responses through arginase I.[37]

The pDCs, which are differentiated from lymphoid progenitor cells in lymphoid organs, have been found to be able to regulate T-cell responses to alloantigens and prolong organ graft survival.[38,39] These regulatory pDCs exhibit a semi-mature phenotype and are more likely than imDCs to induce the generation of Tregs, Meanwhile, pDCs change much less than imDCs *in vivo* after exposure to an inflammatory microenvironment.[40] The concept that pDCs have the potential to promote graft tolerance has emerged recently.[41–43] One potential explanation for this phenomenon is that pDCs can induce IL-10-producing T cells *via* ICOS–ICOSL (B7RP-1) interactions.[44,45] pDCs have been shown to promote the induction of IL-10-secreting Tregs and may prolong heart allograft survival *in vivo*.[46] Furthermore, in a rat model of cardiac allograft tolerance induced by CD40Ig, pDCs accumulated in the graft and spleen, but not the lymph nodes, and induced $CD8^+$ Tregs.[47] Injection of Flt3L-mobilized splenic pDCs of donor origin also significantly prolonged experimental cardiac allograft survival.[48] It is accepted that the balance between the expression of inhibitory PD-L1 and the costimulatory B7-1 (CD80) and/or B7-2 (CD86) ligands by pDCs mediates the outcome of their interactions with T cells.[49] High PD-L1/CD86 ratios on pDCs correlate with increased counts of $CD41^+CD25^{high}Foxp3^+$ Tregs in patients who show immune tolerance to their graft.[50] An additional pathway, involving IDO, was proposed to be essential for human pDC-induced generation of adaptive Tregs.[51] Clinical trials identified tolerogenic properties of pDCs in operationally tolerant pediatric liver allograft recipients and patients on low-dose immunosuppressive therapy.[52] A phase II clinical trial revealed that eight HLA-mismatched living donor renal transplant recipients achieved tolerance after pDC infusion as facilitating cells in a mixed chimerism strategy.[53] It was demonstrated that these facilitating cells promoted the generation of Tregs and prevented GvHD development in mice.[54]

INTERACTIONS BETWEEN TOL-DCS AND OTHER IMMUNE CELLS

tol-DCs and Tregs

DCs are capable of educating naive T cells to develop into a wide variety of effector cell types, ranging from immunogenic $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells to tolerogenic Tregs. Among the three fundamental signals needed for T-cell activation, signal II, mediated by B7 family molecules, is crucial for the expansion of antigen-specific T cells and determines the quality of ensuing T-cell responses, ultimately promoting either an immune response or tolerance.[55] Tol-DCs play a significant role in the maintenance of peripheral tolerance against self-antigen. Many studies have demonstrated the critical functions of IL-10, TGF-β, IDO and RA in the induction of Tregs by DCs.[56–58] Exposure to a low dose of allergens induces $CD4^+CD25^+$ Tregs that make contact with $CD11c^+$ DCs *via* gap junctions and are induced by them to exert tolerogenic functions. Accordingly, antigen-specific $CD8^+$ Tregs responses are induced by tol-DCs and they inhibit contact hypersensitivity.[59,60] A unique subset of $CD11b^{high}Ia^{low}$ DCregs can regulate immune responses by negative feedback. These DCregs express high levels of Fas, which can be induced by endothelial stromal cell-derived TGF-β *via* ERK activation. Fas ligand (FasL) can

promote DCregs to inhibit $CD4^+$ T-cell proliferation and produce IL-10 and IP-10 *via* ERK-mediated inactivation of GSK-3 and the subsequent upregulation of β-catenin. Interestingly, activated T cells could promote DCregs to secrete more IL-10 and IP-10 in part through FasL interactions.[61]

While tol-DCs drive the differentiation of Tregs to control immune responses, Tregs also modulate the phenotype and function of DCs.[62] IL-10-producing Tregs can inhibit DC maturation.[63] Furthermore, following depletion of $Foxp3^+$ Tregs, DCs that lack of the expression of MHC-II molecules were not able to make cognate interactions with $CD4^+$ T cells, indicating the critical suppressive role of $Foxp3^+$ Tregs that maintains DCs in a tolerogenic state.[64] In the immune tolerance model induced by apoptotic cell administration, tol-DCs promoted the expansion of Tregs *via* PD-L1 expression on their surface, and Tregs facilitated maintenance of a tolerogenic state by tol-DCs *via* IL-10 and TGF-β.[65] Interestingly, different subsets of Tregs require different costimulatory molecule interactions from DCs. For example, strong B7 costimulation is required to maintain the level of natural Tregs, but absent or weak B7 costimulation is required to induce $Foxp3^+$ iTregs. This issue was well reviewed by Pletinckx *et al.*[66] These findings highlight that reciprocal interactions between tol-DCs and Tregs are essential for the induction and maintenance of immune tolerance.

tol-DCs and regulatory B cells (Bregs)

Recently, it has been shown that DCs can induce Bregs, a newly defined cell population with a regulatory function, underlining the suitability of DCs for tolerance-inducing strategies.[67,68] Conventional DC-derived IL-12 induces naive follicular B cells to differentiate into IFN-γ- and IL-12-producing effector B cells.[69] Additionally, pDCs regulate B-cell growth, differentiation and immunoglobulin secretion.[70] Furthermore, DCregs can induce splenic B cells to differentiate into IL-10-producing Bregs with a unique $CD19^{high}Fc\gamma IIb^{high}$ phenotype, which demonstrated a new way for DCregs to dampen immune responses by programming B cells into Bregs.[71]

ENGINEERED TOL-DCS

Different approaches have been tested to induce Tregs and to induce tol-DCs with an immature phenotype and an anti-inflammatory cytokine profile. A tolerogenic state in DCs can be induced using several pharmacological agents, such as cyclosporine A, rapamycin, dexamethasone, vitamin A, vitamin D or other cytokines and growth factors.[72–74] These different tol-DCs may share certain features, such as a semimature phenotype and the ability to suppress alloreactive responses in the mixed leukocyte reaction. Tol-DCs differ in phenotype and the regulatory mechanisms that they employ to modulate the immune system.[75]

Genetically engineered tol-DCs

Recently, the insertion of exogenous DNA to enhance tol-DC function has been investigated as a therapeutic possibility for treating autoimmune diseases. 'Killer' DCs, obtained by transfection with DNA encoding FasL or TNF-related apoptosis-inducing ligand, efficiently induce T-cell apoptosis and prevent the rejection of heart grafts in animal models.[76,77] Overexpression of inhibitory molecules, including IL-10, TGF-β, CTLA-4 and SOCS1, enable tol-DCs to more efficiently induce Tregs.[78] Alternatively, many other efforts have been made to generate tol-DCs, such as knocking out or silencing pro-inflammatory molecules or cytokines, including IL-12 and NF-κB,[79] resulting in prolonged survival of cardiac allografts in mice. Utilizing RNA interference directed at IL-12p35 to generate IL-12-silenced DCs, prolonged survival of intestinal allografts was achieved in rats.[80] Recently, clinically applicable protocols have been improved based on some clinical trials.[81]

Photopheresis (extracorporeal photochemotherapy)

Tolerogenic effects induced by extracorporeal photochemotherapy have been described for over 30 years and they had been demonstrated to be a powerful additional therapy to prevent and treat graft-rejection in transplantation.[82] Photopheresis is an atoxic immunomodulatory apheresis-based treatment without generalized immunosuppressive action. Rather, it is directed at suppressing donor-specific T-cell clones. Prolonged cardiac allograft survival was obtained using a method to induce apoptosis by UV-B[83] that caused minimal tissue damage in the long-term allografts and induced the infiltration of small foci of leukocytes, composed mainly of $CD4^+Foxp3^+$ T cells. These findings indicated that donor apoptotic cell therapy promoted the generation or expansion of donor-specific Tregs in an experimental model.[84]

Our previous study showed that after coculture with donor psoralen plus ultraviolet A-treated spleen lymphocytes (PUVA-SP), significantly more IL-10 and IFN-γ were secreted by recipient DCs.[85] After the infusion of PUVA-SP DCs, serum levels of IL-2 and IFN-γ in cardiac allograft recipients significantly decreased, but the serum levels of IL-10 increased. The allograft of the PUVA-SP DCs group survived much longer than those of the control group with a lower rejection grade. Thus, we concluded that PUVA-SP DC should be a novel negative immunoregulatory tool in the clinic because it could mediate Th2 immune deviation and reduce allograft rejection. A follow-up study demonstrated that recipients undergoing photopheresis therapy had longer functioning graft survival, suggesting that the photopheresis treatment contributed to the expected results.[86] A large single-center experience with extracorporeal photochemotherapy for bronchiolitis obliterans syndrome and recurrent acute rejection after lung transplantation resulted in a reduced rate of lung function decline from bronchiolitis obliterans syndrome and optimistic tolerance.[87]

Cytokines and other molecules that induce tol-DCs

Cytokines play an important role in the generation of tol-DCs. DCs, for example, after culture in the presence of G-CSF, induce Tregs through IL-10 and TGF-β production.[88] IL-10-treated DCs induce antigen-specific T-cell anergy, whereas very low doses of GM-CSF lead to the development of imDCs that induce allo-antigen-specific T-cell unresponsiveness.[89,90] Recently, the clinical applicability of human tol-DCs incubated

with IL-10, TGF-β, dexamethasone, vitamin D3 and rapamycin was studied.[91] IL-10-treated tol-DCs were found to be the optimal tol-DCs for functional Treg induction. Additionally, negative regulators of DCs, Zbtb46 and Btbd4 were found to be TLR-responsive, classical DC-specific transcriptional repressors that were partially responsible for preventing classical DC maturation at the steady state.[92] FOXO3 is also a promising molecular candidate for future DC-based tolerance-inducing strategies.[93]

There are also other methods to induce DCs to become tol-DCs. *Escherichia coli*-activated $CD1c^+$ DCs suppressed allogeneic T-cell proliferation *via* IL-10.[94] These $CD1c^+$ DCs were characterized by low levels of production of TNF-α, IL-6 and IL-12, but high levels of production of the anti-inflammatory cytokine IL-10, and expression of the regulatory molecules IDO and soluble CD25. DCs conditioned by total coumarins of Urtica dentata Hand, a traditional herbal medicine, were maturation-resistant and expressed much lower MHCII (I-Ak) and CD86.[95] Total coumarin-conditioned DCs induced the production of alloantigen-specific Tregs, and the upregulation of PD-L1 and the downregulation of TLR4 were involved. MD-3, a unique mAb against intercellular adhesion molecule 1, has been used to induce the differentiation of imDCs into semimature DCs both *in vitro* and *in vivo*.[96] Antigen-specific T-cell tolerance of porcine islet xenografts was induced in diabetic humanized mice and Rhesus monkeys by tol-DCs through the intercellular adhesion molecule 1 pathway.

Immunosuppressive agents modify tol-DCs

As we have known, immunosuppressive drugs can influence all stages of DCs, including differentiation, antigen uptake, phenotypic maturation and cytokine secretion.[97] Immunosuppressive drugs, such as rapamycin, cyclosporine A, tacrolimus and LF15-0195, can condition DCs to promote the long-term engraftment of vascularized skin allografts with an associated expansion of $CD4^+Foxp3^+$ Tregs in rats and resistance to maturation in response to a proinflammatory stimulus by blocking NF-κB signaling.[98,99] Some studies have clearly demonstrated that anti-inflammatory agents, such as aspirin, can induce tol-DCs to effectively induce $Foxp3^+$ Tregs. Dexamethasone affected DC differentiation by downregulating their capacity to secrete IL-12p70, leading to the induction of IL-10-secreting Tregs. Tol-DCs modulated by vitamin D could act as a promising tool for tolerance induction in the clinic.[77] These tol-DCs showed a stable maturation-resistant semimature phenotype and were proficient in exerting immunoregulatory functions, inducing the induction of increased apoptosis of effector T cells and antigen-specific Tregs.

Alternatively, the inhibition of DC migration into tissues and secondary lymphoid organs is also an efficient way to induce immunosuppression and tolerance in transplantation. Triptolide, a potent immunosuppressive drug capable of prolonging allograft survival, impairs DC migration *in vitro* and *in vivo* by inhibiting CCR7 and COX-2 expression.[100] cyclosporine A, another immunosuppressive drug, also has been found to inhibit DC migration by regulating chemokine and COX-2 expression, thus inhibiting immune responses.[101]

TRANSLATING TOL-DCS FROM BENCH TO BEDSIDE

Although much knowledge has been gained regarding the origins, phenotypes and functions of animal tol-DCs subsets, it remains a challenge to translate this knowledge to the human immune system and to reveal the relevant biological significance of these cells in organ transplantation. Because of the differences in the markers for DC subsets between mice and humans, it is extremely difficult to address whether there are functional equivalents between mouse and human tol-DCs subsets. Initial studies of DCs in human blood revealed that $CD141^+CD1c^+$ DCs are equivalent to the mouse lymphoid resident $CD8^+$ DCs.[94] However, evidence for the immunosuppressive function of tol-DCs in humans has been limited to the use monocyte-derived DCs. Nevertheless, some achievements have been made in identifying the factors that modulate organ-specific human DCs, as well as the underlying mechanisms for the negative regulation of the T-cell response by these tolerogenic cells.[102–104] Identifying human tol-DCs with similar functions to mouse tol-DCs will significantly advance the translation of immunological discoveries generated in mouse models into the clinic.[105]

Translating laboratory protocols to the bedside is challenging because several issues related to therapeutic tol-DCs must be considered. One such issue is the identification of a maturation-resistant phenotype of tol-DCs. For example, while $CD8\alpha^+$ DCs, the mouse equivalents of human myeloid DCs, could play immunoregulatory roles by inducing T cell apoptosis *via* expression of FasL, other studies have demonstrated that these $CD8\alpha^+$ DCs can produce high amounts of IL-12 and are able to stimulate $CD8^+$ CTLs.[106,107] Hence, it is not sufficient to identify tol-DCs based only on their phenotype; in addition, their stability and tolerogenic effects need to also be carefully considered. Second, the ability of tol-DCs to regulate the immune response must last for a sufficient amount of time. However, clinical studies of tol-DCs in transplantation remain rare. The *One Study*, an ongoing clinical trial founded by the European Union, is the first study to evaluate immunomodulatory cellular therapy in SOT. It is a multicenter phase I/II clinical trial, which will evaluate the safety and feasibility of various types of cell therapy, including expanded tol-DCs, in living-donor kidney transplantation. All centers will utilize a common adjunctive immunosuppressive protocol to provide a true comparison of the various cellular therapies. Control patients will be transplanted in 2013 and cell therapy groups will begin treatment in 2014, providing a follow-up period of 12 months.[108]

CONCLUSIONS

DCs are generally thought to act as a bridge that connects innate and adaptive immunity. DCs have also been shown to be involved in immune regulation, and imDCs, which lack expression of B7 molecules, were found to be important in inducing allo-graft tolerance. However, imDCs are not the only

subset of DCs that can mediate graft tolerance. The pDCs are a unique DC subset that can induce Tregs, thereby mediating tolerance. Tol-DCs can suppress immune responses, which highlights the potential utility of cell-based therapy in organ transplantation. Tol-DCs can promote allograft-specific tolerance through various mechanisms, and their versatile functions are both mediated by different stages of maturation and the microenvironment. Generating a regulatory immune cell network, which includes tol-DCs, can be amplified by positive feedback and is critical for maintaining a tolerogenic environment.

As described above, Tol-DCs can interact with Tregs through a feedback regulatory mechanism that involves several molecules. Tol-DCs also can downregulate immune responses by inducing Bregs. Furthermore, other data have revealed the interactions between tol-DCs and regulatory natural killer cells, natural killer T cells and macrophages, which we do not discuss in this review. Different approaches to induce tol-DCs have been approved, for which there is much optimism. There is no doubt that tol-DCs will be used clinically in the future, which could greatly benefit the survival and quality of life of transplanted recipients. However, some unfortunate safety-relevant problems remain to be faced. For example, the immature phenotype of tol-DCs is not stable, as they can produce pro-inflammatory cytokines upon encounter with a secondary stimulus, or can even mature and then act as antigen-presenting cells to initiate immune rejection. It is important to identify how to prevent DCs from acquiring pro-inflammatory abilities to offer a safe therapeutic option in humans. Therefore, many obstacles must be overcome before immunoregulatory tol-DCs are widely used in the clinic.

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