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Tempering Allorecognition to Induce Transplant Tolerance With Chemically Modified Apoptotic Donor Cells

D. P. McCarthy¹, J. Bryant², J. P. Galvin^{1,2}, S. D. Miller^{1,*}, and X. Luo^{1,2,3,*}

¹Department of Microbiology-Immunology and Interdepartmental Immunobiology Center, Feinberg School of Medicine, Northwestern University, Chicago, IL

²Department of Medicine, Feinberg School of Medicine, Northwestern University, Evanston, IL

³Comprehensive Transplant Center, Feinberg School of Medicine, Northwestern University, Evanston, IL

Abstract

The development of organ transplantation as a therapy for end-stage organ failure is among the most significant achievements of 20th century medicine, but chronic rejection remains a barrier to achieving long-term success. Current therapeutic regimens consist of immunosuppressive drugs that are efficient at delaying rejection but are associated with significant risks such as opportunistic infections, toxicity, and malignancy. Thus, the induction of specific immune tolerance to transplant antigens is the coveted aim of researchers. The use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (ECDI)-treated, autoantigen-coupled syngeneic leukocytes has been developed as a specific immunotherapy in preclinical models of autoimmunity and is currently in a phase II clinical trial for the treatment of multiple sclerosis. In this review, we discuss the use of allogeneic ECDI-treated apoptotic donor leukocytes (allo-ECDI-SP) as a strategy for inducing antigen-specific tolerance in allogeneic transplantation. Allo-ECDI-SP therapy induces long-term systemic immune tolerance to transplant antigens by subverting alloimmune recognition and exploiting apoptotic cell uptake pathways to recapitulate innate mechanisms of peripheral tolerance. Lastly, we discuss potential indications and challenges for transitioning allo-ECDI-SP therapy into clinical practice.

Introduction

Organ transplantation is an invaluable component of therapeutic medicine for the treatment of end-stage disease and organ failure. Estimates for 2010 indicate that over 106 000 solid organ transplants were performed worldwide in nearly 100 countries, revealing how integral this recently emergent field has become to modern medicine (1). Although 1-year graft survival for renal transplantation has improved to roughly 90% over the past 2 decades due to advances in immunosuppression, long-term survival has remained relatively static due to

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*Corresponding authors: Stephen D. Miller, s-d-miller@northwestern.edu and Xunrong Luo, xunrongluo@northwestern.edu.

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chronic rejection. At present, therapy for transplant rejection is limited to chronic immunosuppression that is effective at preventing acute rejection, but is associated with significant risks including opportunistic infections, organ toxicity, metabolic derangement, and malignancy. Thus, developing a therapeutic regimen for transplant rejection that does not compromise the immune system, but can specifically constrain the deleterious response to allogeneic tissue is paramount for the future of transplant medicine. However, the complexity of allelic variation at the HLA loci and the propensity of the immune system for recognizing foreign HLA alleles have made the prospect of antigen-specific tolerance difficult to achieve.

According to a World Health Organization report, over 2500 new HLA alleles were identified between the years 2004 and 2010 (2); conversely, limiting dilution studies have determined that approximately 1–10% of the T cell repertoire can respond “directly” to donor-derived APCs presenting intact foreign peptide/MHC molecules (3,4). Understanding how T cells selected on self-restricted molecules can react to foreign MHC with such vigor has been the subject of intense investigation for decades. The evolutionary bias of TCRs for intra-species MHC molecules, TCR degeneracy, and polyspecificity of the TCR are mechanisms that have been cited as contributing to the high frequency of alloreactive T cells in the T cell repertoire (5). Recent investigation into the nature of alloreactivity has provided evidence that up to 50% of the alloresponse in GvHD is mediated by T cells that have undergone incomplete allelic exclusion and express dual TCRs (6,7). Moreover, increasing evidence suggests that higher primates and humans not previously exposed to primary allografts can harbor existing populations of virus-specific memory T cells that are cross-reactive and provide heterologous immunity to alloantigens (8). Additionally, the processing and representation of allogeneic peptides on endogenous MHC to T cells (indirect allorecognition) further increases the alloresponse by propagating additional cellular and humoral mechanisms. As a consequence of these factors, the reactivity of the T cell repertoire to foreign MHC is on the order of 100–1000 fold greater in magnitude than the T cell response to conventional antigens, and this presents a formidable barrier to the development of antigen-specific tolerance strategies to lead to acceptance of organ transplants.

Costimulation blockade strategies

The 1990s and first half of the following decade saw costimulation blockade emerge at the forefront of experimental strategies designed to induce transplant tolerance. T cell activation requires engagement of the TCR by cognate peptide/MHC in the presence of APC-derived costimulatory molecules, and signaling through the CD28/CD80/CD86 axis is the quintessential costimulatory pathway involved in T cell activation. Engagement of the TCR in the absence of CD28-mediated costimulation renders T cells anergic and functionally hyporesponsive to subsequent stimulation (9). Thus, multiple experimental strategies have attempted to exploit the two-signal hypothesis of T cell activation by depriving T cells of costimulatory signals following transplantation. CTLA-4 is a natural receptor for CD80 and CD86 that antagonizes T cell activation by limiting CD28 stimulation and delivering negative signals to the T cell. In spite of showing initial promise in laboratory settings, tolerance protocols using the fusion protein CTLA-4(Ig) has met with unexpected

difficulties in clinical translation. Treatment with the CTLA-4(Ig) fusion protein Belatacept in the setting of renal transplantation was successful at promoting 1-year graft survival and superior renal function, but was also associated with a higher frequency of acute rejection, malignancy, and CNS posttransplant lymphoproliferative disorder when compared to cyclosporine in a Phase III clinical trial (10). CD154 is a potent T cell-derived signaling molecule that interacts with its receptor CD40 on APCs to induce APC activation and the expression of IL-12 and costimulatory molecules CD80/CD86 (11). MR1, an anti-murine CD154 antibody has been used in preclinical studies to promote transplant tolerance with great efficacy, especially when used in combination with donor-specific transfusion (DST). This tolerance occurs through a number of mechanisms, including T cell anergy and deletion through targeting the indirect antigen presentation pathway by phagocytosis of the infused donor cells (12–15). Surprisingly, the translation of this therapy into clinical settings was abruptly ended by the development of thrombotic events, due to the unexpected expression of CD154 on platelets in higher primates (16,17). Costimulatory blockade may inadvertently increase the likelihood of acute rejection as previously mentioned. This may be due to the low reliance of memory T cells on co-stimulation, their cross-reactivity for alloantigens, and a reduction in CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (T_{REGS}) as a consequence of their dependence on co-stimulation (8,18–20). Since the level of intra-graft Foxp3 expression is associated with a superior prognosis for graft survival, a better understanding of the disparity in the timing and requirement for co-stimulation in different T cell populations is needed to minimize complications (21).

Cell-based immunotherapy

As a result of these shortcomings, cell-based immunotherapy has reemerged at the forefront of experimental tolerance protocols, such as mixed hematopoietic chimerism (22) and the adoptive transfer of *ex vivo* expanded, donor-specific T_{REGS} (23). A third form of cell-based tolerance that has proven successful in experimental settings is the use of drug conditioned (24) or chemically modified allogeneic APCs (25). This form of tolerance predates costimulation blockade and other forms of cell-based immunotherapy by many decades, dating back to the hapten-drug studies of the late 1920s (26), which demonstrated that chemical haptens coupled to the cell membrane of leukocytes could be used to prevent hapten-induced contact dermatitis in an antigen specific manner (27,28). The induction of regulatory cells and the clonal inhibition of cellular and humoral immunity were demonstrated to be the mechanisms responsible for tolerance induced by coupled-cell administration (25). Many strategies employing drug-conditioned APCs, such as rapamycin-conditioned DCs (29) and vitamin D3-treated DCs (30), attempt to recapitulate these mechanisms by modifying the DC phenotype to favor the induction of transplant tolerance. However, a number of recent publications have called into question the ability of drug-modified DC protocols to induce transplant tolerance, instead suggesting that these strategies risk enhancing alloimmune responses and may promote graft rejection (31,32).

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (ECDI) is a hygroscopic, water-soluble chemical that has been used extensively by biochemists as a cross-linker for peptide synthesis and conjugation. Introduction of ECDI in a reaction mixture with peptides or proteins activates free carboxyl groups, catalyzing the formation of covalent peptide bonds

between the active carboxyl group and primary amines. Studies comparing ECDI with other cross-linking agents have demonstrated the superiority of ECDI as a tissue fixative for immunohistochemistry and as a cross-linking agent for conjugating peptides to cellular membranes (33,34). When compared to other cross-linking agents, ECDI-treated cells demonstrated better viability when maintained at 4°C. However, within hours of *in vivo* administration ECDI-treated leukocytes undergo rapid apoptosis, although the precise mechanism of how this occurs remains unclear (35). Thus, the association of protein and peptide antigens (Ag) by covalent linkage to apoptotic leukocytes promotes a highly efficacious method for inducing Ag-specific immune tolerance in the context of autoimmune and allergic disease (36). Phagocytosis and representation of antigen covalently linked to the apoptotic ECDI-treated leukocyte appears to be the dominant mechanism by which T cells recognize the associated antigen, since antigen-conjugated allogeneic splenic leukocytes or red blood cells were able to induce tolerance as effectively as antigen-conjugated syngeneic leukocytes in a model of PLP₁₃₉₋₁₅₁-induced EAE (35). Thus, ECDI-treated Ag-coupled leukocytes have the potential to regulate T cell responses to cryptic or undetermined peptide sequences within a protein antigen, and multiple antigens can be simultaneously targeted (37,38). This approach is useful for modulating multi-determinant T cell responses as in the context of autoimmune epitope spreading or allogeneic transplant rejection. Intravenous infusion of ECDI-treated Ag-coupled leukocytes can be used in a therapeutic application without inducing anaphylaxis (39), contrary to other antigen-specific strategies such as soluble peptide tolerance and altered peptide ligand therapy (40,41). In agreement with these pre-clinical studies, a human phase I clinical trial utilizing autologous patient peripheral blood leukocytes ECDI-coupled with a cocktail of encephalitogenic peptides has shown promising results indicating that this approach may be safe and efficacious as an immunotherapy for multiple sclerosis (42).

In the context of allogeneic transplantation, ECDI-treated apoptotic leukocyte treatment represents a promising therapy for the prevention of allograft rejection. As allogeneic leukocytes express donor antigens directly on the cell surface, tolerance can be induced to a full spectrum of allogeneic MHC and minor antigens by directly fixing the membrane with ECDI prior to *i.v.* infusion. Using this method, our labs have demonstrated that intravenous infusion of ECDI-treated apoptotic allogeneic splenocytes from the transplant donor into transplant recipients at one week before and 24 h after the transplant will induce long-lasting tolerance for the survival of minor antigen mismatched skin grafts, full-MHC mismatched heart allografts, and full-MHC mismatched pancreatic islets for the restoration of euglycemia in a model of streptozotocin-induced diabetes (43–46). This therapy is allo-specific (46). This approach is also flexible since B cells of donor origin can be expanded *in vitro* and subsequently treated with ECDI to induce tolerance. Additionally, the lysate from allogeneic donor cells can be conjugated to syngeneic splenocytes with ECDI to induce tolerance with no loss of efficacy (47). The potency of ECDI-treated allogeneic leukocytes is dependent upon their exploitation of apoptosis pathways to reshape the peripheral T cell repertoire by several mechanisms including energy, deletion, and the induction of regulatory elements such as the expansion of Foxp3⁺ T_{REGS} (46). Acting in concert, these mechanisms lead to effective induction of tolerance and long-term maintenance of tolerance. Furthermore, while antigen-coupled ECDI-treated leukocytes effectively restrain the

function of naive T cells, emerging evidence suggest that they also down-regulate the function of memory T cells. In the autoimmune EAE model, antigen-coupled ECDI-treated leukocytes prevent adoptive transfer EAE induced by previously activated/memory autoreactive T cells (38). We are currently conducting similar experiments using pre-sensitized alloimmune transplant models, also demonstrating potential protective effect of ECDI-treated donor leukocytes in such recipients. Therefore, the potential of ECDI-treated donor leukocytes to constrain both naive and memory T cells may significantly enhance its application in clinical transplantation where pre-sensitization is frequently encountered.

Apoptosis and innate immune recognition of allogeneic ECDI-treated leukocytes

Apoptosis has been largely implicated in the maintenance of peripheral tolerance, and defects in apoptotic clearance have been demonstrated to have significant consequences on immune homeostasis (48,49). Initial reports of the apoptosis-inducing consequence of ECDI demonstrated a 30–35% incidence of apoptosis in ECDI-treated allogeneic DCs cultured for 24 h *in vitro* (50), while subsequent experiments *in vivo* have revealed that the majority of ECDI-treated Ag-coupled syngeneic splenic leukocytes are fragmented within 3 h of tail vein injection (51). When donor ECDI-treated splenocytes pre-labeled with PKH-67 were administered to recipients, the majority of donor cells were rapidly internalized by recipient MHC class II expressing splenocytes (52). Studies from the EAE model using fluorescently labeled cells have shown that following i.v. infusion of ECDI-treated Ag-coupled leukocytes, the cells localize predominantly in the lungs, liver, and spleen within 1 h and are almost completely fragmented at 3 h post i.v. infusion (51). These apoptotic fragments were primarily associated with F4/80⁺ macrophages in the splenic marginal zone (51), an area between the red pulp and lymphoid follicles that positions marginal zone APCs to capture blood borne matter and represent Ags to lymphocytes in the white pulp (53). Marginal zone macrophages (MZM) are a population of professional APCs specialized in their ability to capture and clear cellular debris from the blood due to the expression of various scavenger receptors that recognize particulate antigens (54), polyanionic molecules (55), oxidized low-density lipoproteins (56), and dying cells (57). Targeted deletion of specific scavenger receptors such as DC-SIGN/SIGN-R1, SR-A, MARCO, and CD68 has been shown to augment autoantibody production in mouse strains susceptible to lupus (58,59), while the depletion of MZMs abrogates the apoptotic cell-induced upregulation of TGF- β and increases the expression of proinflammatory cytokines, antigen-specific T cell proliferation (59), and resistance to tolerance induction in a model of MOG-induced EAE (60). In spite of the association between the apoptotic cells and MZMs, genetic deletion of MARCO had no affect on tolerance induced by ECDI-treated, antigen-coupled leukocytes (61), nor did the depletion of splenic macrophages by clodronate liposomes (52). Upon further investigation, splenic DCs appear to be the critical APC population involved in mediating tolerance to allo-ECDI-SP since the administration of diphtheria toxin to DTR-CD11c transgenic mice at the time of allogeneic ECDI-treated splenocyte infusion prevented the establishment of tolerance (52). Although these data appear to be in conflict with observations regarding the role of splenic macrophages in ECDI-treated antigen-coupled cell therapy, MZMs have been reported to acquire and transfer antigen to splenic CD8 α ⁺ DCs for presentation to T cells

(62); similarly, a recent publication by Mellor and colleagues provides evidence that antigen-bearing, CD11c⁺ CD8⁺ CD103⁺ marginal zone DCs are recruited to the follicles by metallophilic MZMs in a CCL22-dependent manner following phagocytosis of apoptotic cells (63). Thus, the phagocytosis of apoptotic cells by MZMs may facilitate presentation of antigens by DCs to the T cells in the splenic lymphoid follicles, thereby providing a conciliatory mechanism for our observations regarding these splenic populations. Nonetheless, the spleen has been demonstrated to be required for tolerance induction mediated by ECDI-treated, antigen-coupled leukocytes since splenectomized mice were not protected from PLP₁₃₉₋₁₅₁ induced EAE following the administration of PLP₁₃₉-coupled splenocytes (51).

Cytokines and negative costimulation in ECDI-treated cell tolerance

The unresponsiveness induced by ECDI-treated Ag-coupled leukocytes has been attributed to T cell anergy and deletion. CD4⁺ T cells receiving cognate signals via the TCR in the absence of APC-derived costimulation fail to sustain IL-2 production and become anergic to secondary stimulation (9,64). Experiments from autoimmune models have demonstrated that tolerance mediated by ECDI-treated Ag-coupled leukocytes is dependent upon low APC expression of CD80 and CD86 which favors binding to CTLA-4 over CD28 on T cells, and blockade of CTLA-4 signaling at the time of tolerization inhibited unresponsiveness in the EAE model of MS (65). Conversely, the PD-1/PD-L1/PD-L2 pathway has been strongly implicated as a target for immunotherapy in tolerance models due to its role in T cell exhaustion and anergy (66). Expression of the PD-1 receptor is induced on T cells following activation where it binds to its ligands PD-L1 and PD-L2 to negatively regulate T cell function. PD-L1 can also bind to CD80 and prevent signaling to CD28 (67), and antibody blockade against either PD-1 or PD-L1 can abrogate transplant tolerance (68) and the protection mediated by ECDI-treated Ag-coupled leukocytes in a model of type 1 diabetes (69). How administration of ECDI-treated Ag-coupled leukocytes establishes an environment wherein negative costimulation is the favored outcome may be a consequence of the immunoregulatory cytokine milieu induced by recognition of apoptotic debris.

IL-10 is a regulatory cytokine that has a non-redundant role in immune homeostasis and inflammation (70,71). Early studies on IL-10 reported an inhibitory effect of this cytokine on the expression of CD80 by macrophages, without affecting MHC class II presentation (72), and IL-10 has also been shown to induce expression of the negative costimulatory ligand PD-L1 in a STAT3-dependent manner (73). In agreement with these studies, splenic macrophages were shown to express IL-10 following infusion of ECDI-treated Ag-coupled leukocytes, and both MZM and splenic CD8α⁺ DCs uptaking apoptotic debris demonstrated an IL-10 dependent increase in their expression of PD-L1 with no significant upregulation of CD40 or CD80 (51,52). Moreover, the inhibition of either IL-10 or PD-L1 in mice administered ECDI-treated leukocytes prevented tolerance in the context of both autoimmunity and allogeneic islet transplantation (46,51,52). Thus antigen is presented to T cells specific for cross-presented alloantigens (indirect allospecificity) in the context of low costimulation and the provision of inhibitory signals. Surprisingly, experiments examining T cells of indirect allospecificity showed significant activation after allo-ECDI-SP treatment. The administration of ECDI-treated BALB/c-SP induced TCR transgenic TEa CD4⁺ T cells

(specific for a BALB/c I-E α^d peptide complexed with the B6 MHCII I-A b molecule) to undergo robust proliferation and produce IFN- γ . Following transplantation of the allogeneic islets and a second infusion of allo-ECDI-SP (52), these TEa T cells were later depleted by IFN- γ -dependent mechanisms, a finding that is consistent with a requirement for T cell deletion in the establishment of transplant tolerance (74). IL-10 and IFN- γ have been reported to condition DCs to express lower TNF- α and IL-12p40 while increasing levels of indoleamine 2,3 dioxygenase (IDO) (75). Additionally, both IDO and IFN- γ are critical for transplant tolerance mediated by allo-ECDI-SP, and recent experiments have elucidated splenic myeloid derived suppressor cells as a source of IDO and IFN- γ in the splenic environment following treatment (45,52). In lieu of the silencing effect of allo-ECDI-SP on the indirect allorecognition pathway, graft reactive B cells do not undergo class switching to produce alloantibodies, and effector cell infiltration is largely reduced in the allograft of tolerized recipients (45). This suppression of the indirect CD4 $^+$ T cell response may be the most critical outcome of allo-ECDI-SP therapy, confirming reports that the indirect alloresponse rapidly becomes the dominant pathway involved in graft rejection (76).

Modulation of the direct allorecognition pathway can occur by T cells directly interacting with allo-ECDI-SP (50), and this is in agreement with prior reports that T cells with cognate TCRs could be tolerized by directly engaging the peptide-MHC molecules. Indeed, antibody blockade of MHC molecules on ECDI-treated, antigen-coupled leukocytes prevented the induction of T cell unresponsiveness in an *in vitro* culture system (9). As treatment of cells with ECDI prevents the expression of costimulatory molecules CD40, CD80, and CD86 (77), presentation by ECDI-treated-SP results in the provision of TCR signals in the absence of costimulation. Experiments utilizing allo-ECDI-SP cultured with allogeneic T cells demonstrated a 30–50% reduction in cluster formation concomitant with impaired T cell proliferation and IFN- γ production (50,64). Thus, for CD4 $^+$ T cells from the direct allorecognition pathway, intravenous infusion of allo-ECDI-SP may directly engage these cells and subsequently induce their anergy. Although one potential advantage of targeting T cells from the direct allorecognition pathway is the inactivation of graft-reactive CD8 $^+$ T cells, both *in vitro* and *in vivo* evidence support an argument for unresponsiveness in the CD8 $^+$ T cell compartment being mediated indirectly through the CD4 $^+$ T cells. In a culture assay examining the lytic ability of cytotoxic T cells following encounter with allo-ECDI-SP, cytotoxic T cells lysed stimulator cells in a secondary MLR if CD4 $^+$ T cells were absent from the MLR, but lysis was reduced if CD4 $^+$ T cells previously cultured with allo-ECDI-SP were added to the MLR (78). In an H-Y model of skin graft rejection, tolerance to the MHC class-I restricted H-Y antigens failed to protect the graft from rejection while tolerance to the MHC class-II restricted epitope Dby prevented graft rejection and limited the activation, proliferation, and function of H-Y specific CD8 $^+$ T cells (79). Interestingly, a recent publication by Pettigrew and colleagues provides evidence for the involvement of CD4 $^+$ T cells of the indirect lineage in providing T cell help to directly alloreactive CD8 $^+$ T cells. The authors found that recipient dendritic cells could present processed and intact donor allogeneic MHC to activate CD8 $^+$ T cells of direct specificity, but only if the recipient DCs also expressed recipient MHC class II (80). Therefore, inhibition of the direct and indirect CD4 $^+$ T cell response to alloantigens by ECDI-SP treatment may be sufficient to limit CD8 $^+$ T cell activation in the context of allogeneic transplantation. Additionally, active

suppression by T_{REGS} may also limit the activation of graft-reactive T cells (81). Although the study by Corlett et al (78) did not examine the phenotype of the CD4⁺ T cells that inhibit CD8⁺ T cell activation following exposure to allo-ECDI-SP, CD4⁺ CD25⁻ T cells isolated from human PBLs and co-cultured with allo-ECDI-SP were induced to express a Foxp3⁺ T_{REG} phenotype and demonstrated suppressive function when added to a primary MLR. Thus, tolerance in the CD8⁺ compartment following allo-ECDI-SP therapy may be mediated by active regulation involving Foxp3⁺ T_{REGS} in addition to the absence of functional CD4⁺ T helper responses, possibly as a consequence of altered CD154 expression (79).

Regulatory cells in ECDI-treated cell tolerance

Regulatory T cells

T_{REGS} are critical to the induction and maintenance of peripheral immune tolerance, and the expansion of this population has significant potential to mediate tolerance to allogeneic tissue (82). Recent insight into the biology of T_{REGS} suggest that the establishment of transplant tolerance depends on the T_{REG} homing to graft draining lymph nodes and the allogeneic tissue itself where T_{REGS} can suppress the activation of naïve conventional T cells (T_{CONV}) and the function of effector T cells, respectively (83). *In vivo*, T_{REGS} have been shown to be critical for the induction of transplant tolerance mediated by allo-ECDI-SP, and are preferentially expanded in frequency in the secondary lymphoid organs and grafts of tolerized transplant recipients. In a model of allogeneic islet transplantation, CD25 depletion at the time of allo-ECDI-SP treatment prevented the establishment of tolerance to the islet grafts, although CD25 depletion during long-term tolerance maintenance did not have a lasting detrimental effect on graft retention (46). Whether or not these T_{REGS} are expanded from an existing pool of natural T_{REGS} or derived from T_{CONV} has not been thoroughly examined, but CD4⁺ T cells isolated from human PBLs can be induced to express Foxp3 during culture with allo-ECDI-treated PBLs. These T_{REGS} exhibited decreased levels of classic T_{REG} activation markers such as CTLA-4 and GITR, arguing for their induction from T_{CONV} precursors rather than expansion from nT_{REGS} (77). Furthermore, the requirement for TGF-β at the time of tolerance induction to allo-ECDI-SP supports the conversion of T_{REGS} from T_{CONV} cells. Little is known regarding the specificity of the T_{REG} response mediated by allo-ECDI-SP. Although T_{REGS} were previously thought to mediate suppression in a non-specific bystander manner, transgene expression of indirect alloreactivity by TCR DNA transfer into T_{REGS} has been shown to favor transplant tolerance (84), and a recent publication by the Rudensky lab has reported that continued expression of the TCR is critical for T_{REG} mediated suppression (85), thereby supporting an argument for the importance of antigenspecificity in T_{REG} function. In spite of the observations that T_{REGS} can be induced by direct stimulation with allo-ECDI-SP using human PBLs, in the murine model, 4C Tg CD4⁺ T cells displaying a TCR specific for allogeneic MHC (I-A^d) were not observed to upregulate FoxP3 expression during tolerance induction by allo-ECDI-SP, nor were TEa CD4⁺ T cells of indirect specificity (52). However, studies from the EAE model have demonstrated that a transferred population of CD4⁺ CD25⁺ splenocytes derived from PLP₁₃₉₋₁₅₁-SP treated donors conferred better protection from PLP₁₃₉₋₁₅₁ mediated EAE disease when compared to CD4⁺ CD25⁺ splenocytes derived from OVA₃₂₃₋₃₃₉-SP treated donors (51), consistent with a role for

antigen-specificity in T_{REG} mediated suppression. Therefore, additional untested donor-reactive TCR specificities may better support T_{REG} induction or expansion. Nonetheless, significant questions remain regarding the biology of T_{REGS}, their specificity, and the mechanism of their contribution to ECDI-treated SP tolerance therapy.

Myeloid-derived suppressor cells (MDSCs)

MDSCs are a heterogeneous population of activated but immature myeloid cells that are identified by their co-expression of Gr1 and CD11b. First described in cancer, these cells are potent inhibitors of T cell proliferation and function, and are known to suppress immune function under a number of inflammatory conditions (86). These cells are activated by a number of factors including TGF- β and IFN- γ , both of which are required for tolerance by allo-ECDI-SP. Treatment with allo-ECDI-SP induced a splenic population of MDSCs that produced significant levels of IFN- γ -dependent IDO and suppressed CD8⁺ T cell proliferation when compared to control mice. Moreover, MDSCs were found to be present in the cardiac allograft and suppressed the infiltration of CD8⁺ T cells and other effectors. MDSCs present in the graft were also found to produce IL-10 and to recruit T_{REGS} in a CCL4-dependent manner, and depletion of MDSCs restored CD8⁺ T cell infiltration and graft rejection (87). Thus, a critical role for MDSCs in allo-ECDI-SP therapy may be the recruitment of T_{REGS} into the graft, which subsequently suppress graft infiltration by CD8⁺ T cells. This argument is consistent with an early requirement for T_{REGS} following tolerance induction, since the direct CD8⁺ T cell response is activated by passenger leukocytes from the graft that do not persist long-term.

Based on the above-described mechanisms involved in tolerance induced allo-ECDI-SP, there are several potential advantages of this strategy over other forms of cell-based therapies such as DST: (1) unlike DST, allo-ECDI-SP in principle does not require concomitant co-stimulation blockade, as the allo-ECDI-SP uniquely lack the ability to provide adequate co-stimulation signals themselves to the interacting T cells (52); (2) DST carries a potential risk for recipient sensitization, especially in recipients with preexisting alloimmunity (88); in contrast, allo-ECDI-SP does not display a similar risk of sensitization, and may in fact provide further graft protection in recipients with preexisting alloimmunity (Luo, unpublished data). Lastly, our unpublished work showed that in direct comparison with other methods of inducing cell death such as γ -irradiation or paraformaldehyde fixation, ECDI treatment is significantly more efficient for inducing tolerance, likely due to its ability to arrest the treated cells in the apoptotic stage rather than allowing them to progress to the necrotic stage which counters tolerance efficacy.

Conclusions

In summary, ECDI-treated apoptotic donor cells are highly effective in tempering allo-specific immune responses via a multitude of mechanisms employing negative costimulation, inhibitory cytokines, and regulatory cell populations (Figure 1). The efficacy of this form of donor cell-based immunotherapy in allo-sensitized recipients warrants further detailed investigation. Ongoing studies in nonhuman primates will undoubtedly inform future clinical trial design using this form of negative donor vaccination for transplant tolerance induction in humans. Lastly, establishing a source for unlimited production of

donor antigens and an immune modulatory cell-free synthetic particle delivery system of donor antigens will likely significantly streamline the manufacturing of clinical-grade negative donor vaccines, and thus provide an appropriate potential path for moving this therapy toward clinical translation.

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Abbreviations

Ag	antigen
Allo-ECDI-SP	allogeneic ECDI-treated splenocytes/leukocytes
APC	antigen presenting cell
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DST	donor specific transfusion
EAE	experimental autoimmune encephalomyelitis
ECDI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ECDI-SP	ECDI-treated splenocytes/leukocytes
GvHD	graft versus host disease
HLA	human leukocyte antigen
IDO	indoleamine 2,3 dioxygenase
MDSC	myeloid derived suppressor cell
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MS	multiple sclerosis
MZM	marginal zone macrophage
OVA323-339	ovalbumin protein peptide sequence 323–339
PBL	peripheral blood leukocyte
PD-1	programmed cell death 1
PD-L1/2	programmed death ligand 1/2
PLP139-151	proteolipid protein peptide sequence 139–151
TCONV	conventional T cells
TCR	T cell receptor
T_{REG}	regulatory T cell

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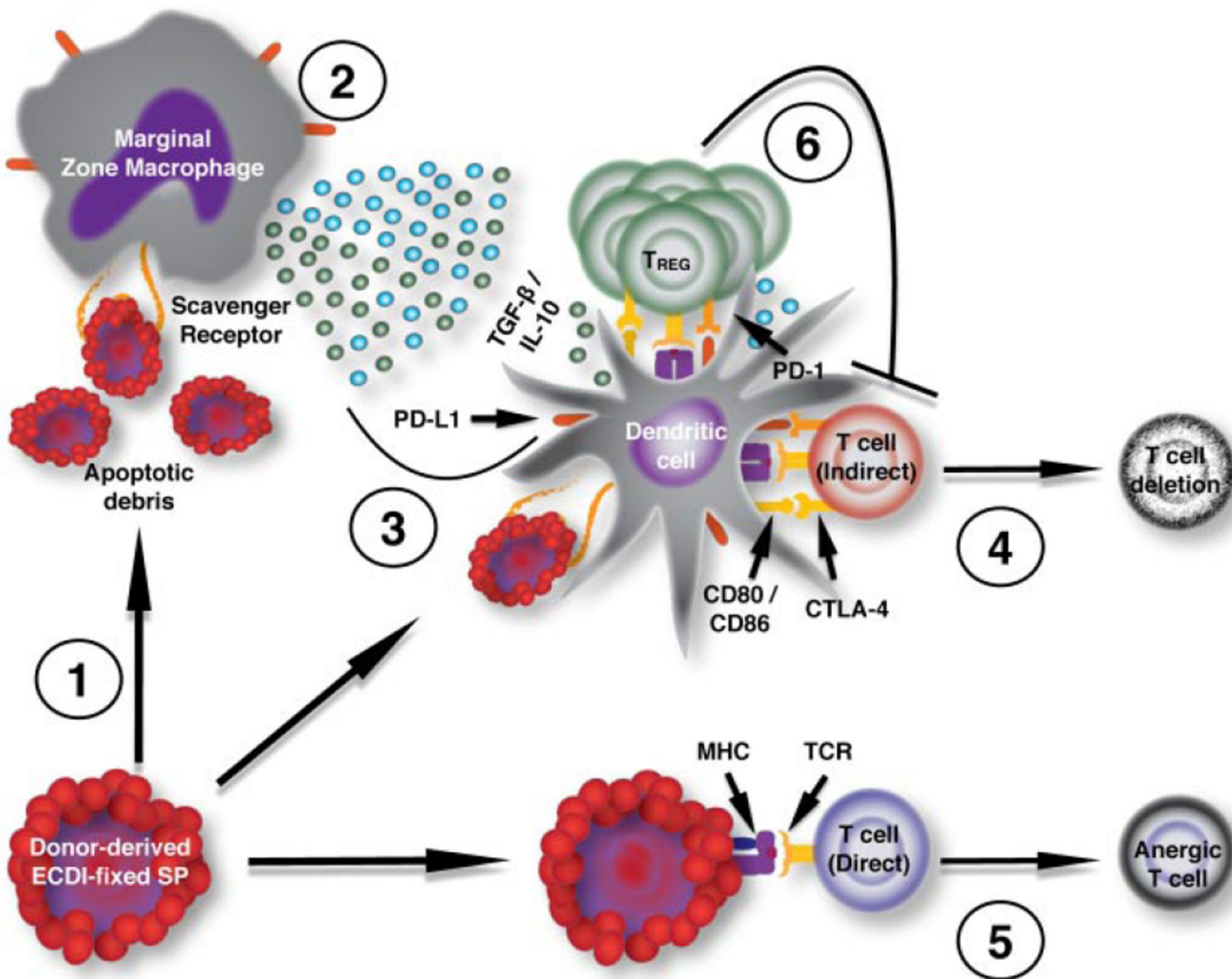


Figure 1. Proposed mechanisms of Allo-ECIDI-SP tolerance

Innate immune responses are required for allo-ECIDI-SP tolerance induction. The splenic marginal zone is the primary interface between the splenic non-lymphoid compartment and the lymphoid compartment. It is composed of B cells and macrophages important for capturing exogenous Ags and debris, which may be processed for subsequent presentation to T cells in T cell zones. For efficient tolerance, allo-ECIDI-SP must be delivered (1) via i.v. administration. Once within the marginal sinus, the ECIDI-SP rapidly degrade via apoptotic pathways (2), with debris and cells recognized and rapidly taken up via scavenger receptors on MZMs and DCs either directly from the marginal zone sinus or via membrane transfer. The uptake of allo-ECIDI-SP triggers the production and secretion of soluble mediators including IL-10 and TGF-β, which have multifarious functions including the regulation of costimulatory molecules, such as PD-L1, on APCs (3). The immunoregulatory milieu provided by the MZM response to the apoptotic allo-ECIDI-SP conditions DCs to present antigen to T cells in the context of low CD80/CD86 expression and increased PD-L1 expression, thereby favoring costimulation through the inhibitory receptors such as CTLA-4 and PD-1 (4). T cells of the indirect allorecognition pathway recognizing cognate

peptide/MHC ligands on host APCs undergo deletion in this context (4), while T cells directly engaging peptide/MHC ligands on allo-ECDI-SP become anergic (5). Regulatory T cells of the Foxp3⁺ lineage expand in the presence of TGF- β to inhibit further priming in the secondary lymphoid organs and effector responses in the transplanted tissue (6).

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