Published in final edited form as:

Adv Drug Deliv Rev. 2008 January 14; 60(2): 91–105. doi:10.1016/j.addr.2007.08.025.

Advancements in Immune Tolerance

Ping-Ying Pan*, Junko Ozao, Zuping Zhou, and Shu-Hsia Chen*

Department of Gene and Cell Medicine, Mount Sinai School of Medicine, New York, NY, 10029, USA

Abstract

In recent years, considerable attention has been given to immune tolerance and its potential clinical applications for the treatment of cancers and autoimmune diseases, and the prevention of allograft rejection and graft-versus-host diseases. Advances in our understanding of the underlying mechanisms of establishment and maintenance of immune tolerance in various experimental settings and animal models, and in our ability to manipulate the development of various immune tolerogenic cells *in vitro* and *in vivo*, have generated significant momentum for the field of cell-based tolerogenic therapy. This review briefly summarizes the major tolerogenic cell populations and their mechanisms of action, while focusing mainly on potential exploitation of their tolerogenic mechanisms for clinical applications.

Keywords

Immune tolerance; cancer immune therapy; autoimmune; Treg; MDSC; dendritic cells; GVHD

1. Introduction to Immune tolerance

The hallmark of the immune system is the ability to discriminate between self and non-self antigens i.e., it must be able to maintain tolerance to self-antigens yet be able to mount effective immune responses against pathogens and malignant cells. The relationship between tolerance and immunity is dynamic and can be visualized as the two arms of a scale, which must be delicately balanced. A tilt toward either side results in adverse pathophysiological conditions that may lead to disease manifestations, such as infections, malignancies, or autoimmunity. In order to avoid harmful self-reactivity, self-tolerance within the T-cell repertoire is achieved through central and peripheral tolerance. Thymic education constitutes the main process of central tolerance in which the majority of developing T cells with strong self-reactivity are deleted [1–4]. After exiting the thymus, mature T cells are subjected to secondary selection (peripheral tolerance) by which the majority of self-reactive T cells are deleted or rendered anergic [5–7]. However, even under the tight regulation of central and peripheral tolerance, abundant evidence suggests that potentially hazardous self-reactive lymphocytes are present in the periphery of normal individuals [8,9]. Residual self-reactive T cells may remain dormant (i.e., they fail to be activated by self-antigens) due to low avidities of their T cell receptors

*Corresponding authors: Ping-Ying Pan, Ph.D., Mailing address: Department of Gene and Cell Medicine, Box 1496, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, Tel: (212) 659-8254, Fax: (212) 803-6740, Email: ping-ying.pan@mssm.edu, Shu-Hsia Chen, Ph.D., Mailing address: Department of Gene and Cell Medicine, Box 1496, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, Tel: (212) 659-8256, Fax: (212) 803-6740, Email: shu-hsia.chen@mssm.edu.

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(TCRs) for self-antigens, lack of co-stimulation from antigen-presenting cells, or seclusion of self-antigens. In addition to these passive mechanisms, evidence accumulated in the past 15 years indicates that CD4⁺CD25⁺ T regulatory cells (Tregs) play an important role in the maintenance of peripheral self-tolerance as well as down-regulation of various immune responses [10–13].

Although peripheral self-tolerance is essential for proper functioning of the immune system, it can be utilized as an evasive mechanism by which tumor cells avoid recognition and destruction by immune system. On the other hand, disruption of self-tolerance by infections or other mechanisms (genetic abnormality or environmental factors) may contribute to manifestations of autoimmune diseases. Therefore, it is conceivable that novel therapeutic modalities can be devised to enhance anti-tumor responses through prevention or reversion of the immune tolerance associated with advanced malignancies or to suppress autoimmunity and other undesirable immune responses, such as allo-immunity and graft-versus-host diseases in transplantation, by introduction or re-establishment of various tolerogenic mechanisms.

Evidence from animal models has shown that cell-based tolerogenic therapy can prevent or cure transplant rejection or autoimmune diseases and that depletion of Tregs or blockade of tumor associated tolerogenic mechanisms can enhance immune-based cancer therapy. This review will briefly discuss various mechanisms of cell-mediated immune tolerance and will focus on how they can be exploited to prevent immune tolerance in cancer and treat autoimmune diseases, or to establish donor-specific transplantation tolerance.

2. Mechanisms of Cell-Mediated Immune Tolerance

Immune tolerance is a double-edged sword that is vital for the suppression of self-reactivity and the prevention of autoimmune diseases. However, tumor cells, due to their self-derived nature, can exploit the tolerance mechanisms established for self-antigens and evade the immune system. Various immune cell types have been shown to contribute to the establishment and maintenance of immune tolerance in different diseases or experimental settings. These include Tregs, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAM), nature killer T (NKT) cells, and various dendritic cell (DC) subpopulations (Figure 1). These tolerogenic cells, many of which exert their regulatory function through mutual interaction with each other, work in concert to establish an immunosuppressive network that maintains peripheral tolerance and favors tumor growth or transplantation tolerance [14–22].

2.1 Tregs

Tregs play an important role in the control of immune reactivity against self-antigens and have the ability to inhibit chronic inflammatory responses and to maintain immune tolerance in tumor-bearing hosts [11,12,23]. They were first discovered by S. Sakaguchi more than a decade ago and were described as a minor population of CD4⁺ T cells that co-expressed CD25 [24]. Although no specific surface markers can be associated with Tregs, the forkhead/winged helix transcription factor (*FOXP3*) has been identified as a key regulatory gene for the development and function of Tregs [25–27]. Since these CD4⁺CD25⁺FoxP3⁺ Tregs develop in the thymus and are present in healthy individuals from birth, they are referred to as natural Tregs [28–32]. CD4⁺CD25⁺FoxP3⁺ Tregs can also be derived from mature naïve CD4⁺CD25[−] T cells in various settings of immunity [21,33,34]. In addition to CD4⁺CD25⁺FoxP3⁺ Tregs, multiple varieties of T cell with regulatory activities have been described over the past 10 years. These cells are generated, *in vitro* and *in vivo*, from mature CD4⁺ T cells under specific conditions of antigen stimulation or cytokine exposure [33–37]. The adaptive Tregs can be further classified on the basis of their cytokine profile. Chronic activation of both human and murine CD4⁺ T cells in the presence of interleukin (IL)-10 gives rise to Tr1 cells with low proliferative capacity that produce high levels of IL-10, low levels of IL-2 and no IL-4. [37–39]. Th3 cells,

a regulatory population that produces a high level of TGF- β , are preferentially induced by dendritic cells in the gut and the induction is further enhanced in the presence of IL-4, TGF- β , IL-10, and anti-IL-12 [40–45]. Furthermore, regulatory T cells can be derived from CD8⁺ T cells [46–50]. Interestingly, a subset of CD8⁺CD25⁺ Tregs has also been identified that shares phenotype, functional features, and mechanism of action with CD4⁺CD25⁺ natural Tregs [51,52].

Tregs have been shown to inhibit a variety of immune responses both *in vitro* and *in vivo* [53–72]. Although Tregs require activation by antigen exposure to initiate suppressive functions, the effector (suppression) phase is independent of antigen specificity. Multiple mechanisms of suppression by regulatory T cells have been described, with differences observed between *in vitro* and *in vivo* results and among the various subsets of Tregs. Soluble cytokines, cell-cell contact dependent mechanisms, or both have been shown to contribute to the suppressive activities mediated by Tregs. In many experimental systems, Tregs function *in vivo* by production of the immunosuppressive cytokines IL-10 [73,74] and TGF- β [61,75, 76]. In contrast, Tregs suppress immune responses *in vitro* by a contact dependent mechanism [65,77]. In addition to the direct suppressive mechanisms, Tregs can inhibit immune responses through modulation of dendritic cell function [12,18,78]. More recently, IL-9 has been shown to represent a functional link through which activated Treg recruit and activate mast cells to mediate regional immune suppression in a skin allograft tolerance model [79].

2.2 Myeloid derived suppressor cells

Gr-1⁺CD11b (Mac-1)⁺ MDSCs (also known as immature myeloid cells) are found in a significantly higher number in bone marrow, circulation, and peripheral lymphoid organs of mice and humans during tumor progression [80–89]. They have been shown to contribute to tumor-induced immune dysfunctions through a myriad of mechanisms, including IFN- γ -dependent production of nitric oxide [83,90–92], arginine depletion through IL-4/IL-13-dependent production of arginase [85,89,92–95], and production of reactive oxygen species [96,97]. In addition to the above-mentioned mechanisms, we found that MDSCs induced the development of CD4⁺CD25⁺FoxP3⁺ Tregs *in vivo*, which are anergic and can suppress anti-tumor responses in tumor-bearing mice [21]. More recently, an additional MDSC-mediated suppressive mechanism was described in which MDSCs induce CD8⁺ T-cell tolerance by blocking peptide–MHC–TCR binding through the nitration of the TCR–CD8 complex [98].

2.3 Tumor-associated macrophages

A body of evidence indicates a correlation between an increased number of macrophages in the tumor and poor prognosis in various animal tumor models and in human malignancies [99,100]. TAMs have been shown to exert a negative effect on anti-tumor responses. Circulating monocytes are recruited to the tumor, where they differentiated into macrophages. Moreover, MDSCs isolated from spleens of tumor-bearing mice can reach the tumor site upon adoptive transfer and become F4/80⁺ TAMs characterized by heightened STAT1 phosphorylation and constitutive expression of arginase (*ARG1*) and inducible nitric oxide synthase (*iNOS*) [101]. Interestingly, cross-talk between MDSC and macrophages has recently been demonstrated in which IL-12 secretion by macrophages is suppressed by MDSC through the secretion of IL-10, thereby eliciting a tumor-promoting type 2 (alternatively activated) macrophage response [102].

2.4 Natural killer T cells

NKT cells express not only TCRs but also NK receptors [103–105]. Unlike conventional T cells, NKT cells recognize glycolipids presented by CD1d through the use of a unique invariant TCR [103,106–113]. The roles of NKT cells in tolerance have been described in transplantation [114–118], autoimmune diseases [119–127], and anti-tumor responses [128,129]. Various

NKT-mediated suppressive mechanisms have been described, depending on the model system used. Upon activation, NKT cells produce RANTES that is required for the recruitment of F4/80⁺ antigen presenting cells and generation of CD8⁺ Tregs in tolerance induction in anterior chamber-associated immune deviation [130]. In a mouse model in which tumors show a growth-regression-recurrence pattern, CD4⁺ NKT cells produce IL-13 to down-regulate tumor immunosurveillance [128]. Furthermore, IL-13 produced by NKT cells can activate MDSCs to produce TGF- β , which in turn suppresses CD8⁺ cytotoxic T lymphocytes [131].

2.5 Dendritic cells

DCs constitute a heterogeneous population of bone-marrow-derived professional antigen-presenting cells. Three subsets of CD11c⁺ DCs (CD8 α ⁺ DCs, CD4⁺CD8⁻ DCs, and CD4⁻CD8⁻ DCs) have been identified in spleen and lymph nodes of mice [132] whereas human DC subsets include myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [133–135]. In recent years, a murine counterpart of pDCs has been identified, which do not express T-, B-, or myeloid markers and exhibit plasma-cell-like morphology [133,136].

DCs have the potential to induce immunity or tolerance, depending on the state of activation, activation signals, and cytokine milieu [137,138]. Because of the dual functionality (induction of immunity or tolerance), they have been the focus of immune-based therapies for the treatment of tumor and induction of immune tolerance in autoimmune diseases and transplantation. Immature DCs (iDCs) fail to deliver co-stimulatory signals during T-cell activation, resulting in the induction of T-cell tolerance [138,139]. The immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO), which catalyzes the rate-limiting step of tryptophan degradation, contributes to immune tolerance in various experimental settings [140–146]. IDO-expressing DCs are able to suppress T-cell proliferation and promote T-cell apoptosis [147–151].

Various DC subsets have been linked to Treg development. Thymic DC have been implicated in the development of natural Tregs [152]. *In-vitro*-derived immature myeloid DCs (imDC) induce FoxP3⁺ Tregs [153] and Tr1 cells [154]. Furthermore, alloantigen-loaded pDCs isolated from tolerant hosts can mediate the development of alloantigen-specific CD4⁺CD25⁺FoxP3⁺ Tregs in transplant tolerance induced by the combination therapy of donor-specific transfusion and anti-CD40L antibody [16]. Recently, a functionally specialized population of CD103⁺ DCs isolated from the mesenteric lymph nodes and from the lamina propria of normal mice has been shown to promote the conversion of naive T cells into Foxp3⁺ Tregs [155,156]. This conversion process is dependent on TGF- β and retinoic acid. Finally, pertinent to cell-based tolerogenic therapies, is the observation that tolerogenic DCs can be generated *in vitro* in the presence of IL-10, TGF- β , prostaglandin E2, vitamin D3, or Rapamycin. These *in vitro* generated tolerogenic DCs can mediate immune suppression and induce immune tolerance via multiple mechanisms, such as down-regulation of co-stimulatory molecules, alteration of T helper cell polarization, and expansion or *de novo* generation of Tregs [157–160].

3. Prevention of Immune Tolerance in Cancer

Immune tolerance is an important factor to be considered when attempting to devise strategies for immune-based cancer therapies. Cancer cells, tumor-infiltrating lymphocytes, fibroblasts, MDSCs, iDCs and stromal cells all partake in a complex interplay in the tumor-bearing host to produce a unique tumor microenvironment. This tumor microenvironment is very dynamic, with several cell types interacting with one another, producing proinflammatory and tolerogenic cytokines, which further the tumor's ability to induce immune tolerance [161, 162]. In addition, the tumor cell itself can produce various tumor factors and chemokines, which recruit and expand immune suppressive cells, such as Tregs, MDSCs, and iDCs [92,

163]. In this type of environment, most immune therapies lack the ability to overcome the forces of immune tolerance, which evolve as soon as the tumor begins to grow. Strategies to overcome this active immune suppression by focusing on the following major cellular targets are currently under consideration with the ultimate goal of achieving successful immune therapy.

3.1 Tregs as a major target

Several cellular targets have been under investigation in order to identify how best to disrupt tumor-induced immune tolerance. One such major target is the Treg [164]. The peripheral inducible Treg are a major subtype of Treg found at the tumor site, playing a key role in immune suppression in cancer, in contrast to the natural Treg that controls autoimmune diseases [12, 165,166]. Studies have found suppression of tumor growth [71], enhancement of antitumor vaccine activity [69], and increased immune response [70] in Treg depleted mice and in humans.

Therefore, one strategy to decrease immune tolerance has been to eliminate Tregs in patients. An IL-2 diphtheria toxin fusion protein ONTAK (Seregan/Ligand pharmaceuticals, San Diego, USA) has been developed that can deplete Tregs. In addition, CD25⁺ Treg depletion by specific antibodies has been explored. Mahnke et al. has tested ONTAK in patients and has found that treatment of ONTAK is able to deplete Treg in the peripheral blood (while maintaining other cell populations), is well tolerated, and increases circulating peptide-specific CD8⁺ cells in about 90% of vaccinated melanoma patients [167]. Another group has studied the combination effects of ONTAK with a recombinant poxviral vaccine in a murine model. The results showed that injection of ONTAK one day prior to vaccination enhanced antigen-specific T-cell responses above the levels induced by vaccination alone and thus showing an advantage to combination therapy [168]. It remains to be seen whether this treatment will have similar and long-term effects in humans.

Other innovative strategies to decrease the Treg population have been to target Treg function. Wang et al. [169] have been pursuing a strategy to use Poly G oligonucleotides to reverse suppression mediated by Treg via TLR8 and MyD88 pathways, since they discovered that TLR8 reversed the suppressive function of CD4⁺ Tregs. The length of time that poly G needs to be used so that Treg suppressive functions can be continuously blocked remains a critical issue and requires further investigation and development. Finally, the other promising approach to overcome immune tolerance is to identify which major cell type targets are involved in the development of Treg and control their function.

3.2 MDSC-mediated T-cell tolerance

Our laboratory has shown that a subset of MDSCs found in tumor-bearing mice and patients can induce Treg *in vitro* and in tumor-bearing mice [21]. The MDSCs is an aberrant cell population, which is prevalent in the spleen, bone marrow, peripheral blood and tumor of tumor-bearing mice and in human cancer patients [80,170]. They are characterized by being Gr-1⁺, CD11b (Mac-1)⁺, and CD115⁺ [21]. MDSCs can suppress T-cell responses through multiple mechanisms as described above. In addition, when MDSC are cocultured with tumor specific activated T cells *in vitro* or injected into tumor bearing mice *in vivo*, we have found that MDSC can induce tumor specific Treg development by secretion of IL-10 and TGF- β , which depends on IFN γ signaling pathway. In addition, there is evidence that MDSC can migrate to the tumor site and induce anergy in the tumor microenvironment and may differentiate at this stage into TAMs [92] and participate in tumor angiogenesis [171,172].

Several strategies have been employed to block the MDSC suppressive and Treg inducing function. One strategy has been to deplete MDSC *in vivo*; however, no specific marker for

MDSC depletion is currently available. Gr-1 has been used as a marker to deplete this population in mice [173]. However, this strategy may not be suitable for clinical application due to its lack of specific markers for human MDSC. Nonetheless, various groups have shown that depletion of MDSC will improve tumor killing and decrease immune tolerance in animal models [174].

Other strategies have included attempts to limit the accumulation of MDSC in the tumor-bearing host. Our laboratory has found that tumor-derived stem cell factor (SCF) appears to be critical in promoting MDSC expansion and accumulation. Mice with SCF-silenced tumor cells have a significant decreased number of MDSCs and a less tolerogenic tumor microenvironment is associated with SCF silencing in tumor cells. In addition, treatment of tumor-bearing mice with anti-ckit (SCF receptor) blocking monoclonal antibodies in conjunction with immune-based therapy can significantly improve the long-term survival rate of large tumor-bearing mice (Blood in revision). Small molecule inhibitors that block ckit signaling are currently available for the treatment of gastrointestinal stromal tumors [175, 176] and might be useful for the purpose of reversing MDSC mediated immune suppression.

Other pharmaceutical small molecule drugs also provide an important strategy to block the function of MDSC. Bronte et al. have shown that inhibition of phosphodiesterase-5 appears to augment endogenous antitumor immunity by reducing MDSC function [177]. They showed that treatment of sildenafil reduced the MDSC-mediated suppressive machinery of arginase 1 and nitric oxide, enhanced intratumoral T cell infiltration and activation, and reduced tumor outgrowth in tumor-bearing mice. Other studies show that aspirin derivatives may also reduce MDSC function [90].

Since MDSCs exhibit an immature phenotype of the myeloid lineage, strategies to promote differentiation of this cell population may also prevent Treg development and MDSC suppressive function. MDSC can differentiate into monocyte/macrophages and dendritic cells under specific culture conditions [178,179]. Our laboratory has found that engagement of co-stimulatory molecule CD40 can prevent Treg induction by MDSCs, indicating that CD40 is required for Treg induction (manuscript in preparation). Other groups have also studied the use of ATRA [180] as well as other cytokine regimens including IFN- γ + TNF (tumor necrosis factor) regimens to further differentiate MDSCs [181]. These strategies appear to be promising in animal models; however, further translation and proof of their ability to overcome immune tolerance and thus improve the immune therapeutic outcome in cancer patients needs to be thoroughly investigated.

3.3 Dendritic cell as a target for intervention of tumor-associated tolerance

imDC and pDCs appear to play a pivotal role in the maintenance of tumor-induced immune tolerance. Fully mature dendritic cells can aid in antigen presentation and immune activation, thereby increasing the immune response against cancer. However, in the tumor microenvironment, dendritic cells are unable to fully mature and retain immature phenotype, which confers tolerogenic function and may contribute to tumor-induced immune tolerance. pDCs can also mediate immune tolerance in this setting. Thus, several groups are investigating the utilization of dendritic cell biology to develop strategies to minimize the role of immune tolerance in cancer patients.

In the tumor setting, iDCs secrete TGF- β causing proliferation of CD4⁺CD25⁺Foxp3⁺ Treg cells [182]. Furthermore, various cytokines, e.g. IL-10 and TGF- β , and co-stimulatory molecules in the tumor microenvironment appear to potentiate the immune tolerance inducing ability of dendritic cells. Steinbrink et al. showed that IL-10-treated human dendritic cells can induce a melanoma-antigen-specific anergy in CD8⁺ T cells, resulting in their failure to lyse

tumor cells [183]. Therefore, iDC can be a possible cellular target for dendritic cell based immune therapies to break immune tolerance.

DCs can be used to suppress Treg function, especially through the microbe activated TLR pathway. This may explain why LPS-induced mature DCs can break Treg mediated suppression by ameliorating their suppressive functions against proliferation and cytokine production [179]. Furthermore, Chaperot et al. recently found that pDC could acquire cytotoxic functions during the early phases of infection or after activation with TLR7 or TLR9 agonists, suggesting a possible role for TLR agonists in pDC-based cancer therapy [184]. The ability to stimulate alternative pathways in DC activation in the tumor-bearing host may represent yet another cellular targeting strategy that could help overcome tumor-induced immune tolerance.

IDO catabolizes the essential amino acid tryptophan and has been found to be an immune function regulator by suppression of T-cell effector function. IDO is expressed in the tumor microenvironment by both the tumor cells themselves and the surrounding stromal tissue [185]. In addition, pDC found in the tumor-draining lymph node also express IDO, providing a possible mechanism for tumor escape. Munn et al. found that IDO-competent DC subsets acquired potent and dominant T cell suppressive properties after IDO induction with CTLA-4 and blocked the ability of T cells to respond to other stimulatory DCs in the same cultures [186]. Small molecule inhibitors of IDO also show promise as an adjunctive therapy and are currently under investigation [187]. Strategies to inhibit the IDO pathway not only in DCs but also in the host tumor microenvironment may provide another promising approach for breaking tumor-induced immune tolerance.

In conclusion, it is imperative to continue to study these tolerogenic cell types and their interaction as targets of cell-based therapies, in order to overcome immune tolerance in the tumor-bearing host. Thus, by continuing to delineate the critical mechanisms involved in immune tolerance, the chance for successful cell-based immune and biological therapies may become a reality. There is evidence to suggest that, in established large tumors, targeting only one cell type such as Treg cells will not yield efficacious results unless this type of monotherapy is undertaken in early disease [188]. In advanced malignancy, it will most likely be important to undertake a rigorous multi-facet targeted approach in order to completely overcome immune tolerance, increase tumor killing, and have a significant effect on prolonging disease-free, long-term survival in cancer patients.

4. Cell-based tolerogenic therapy

4.1 Treg mediated cell therapy

Treg are important in the maintenance of regulatory mechanisms that keep the T cell response in check in order to prevent autoimmune disease. The potential use of cell-based therapies targeting Treg for several important autoimmune diseases is currently under investigation by a number of research groups. For type I diabetes, this strategy uses a systemic immune modulator, anti-CD3 ϵ -specific antibody, to target Treg. CD4⁺CD25⁺ Treg appear to control autoimmunity in the non-obese diabetic mouse (NOD), a spontaneous animal model of type I diabetes. Studies have shown that combination therapy of anti-CD3 ϵ -specific antibody and intranasal application of proinsulin peptide appears to reverse recent onset diabetes in two separate murine models of diabetes [189]. In addition, this group showed the expansion of insulin-specific Tregs that produce IL-10, TGF- β , and IL-4. Thus, they were able to demonstrate that combining a systemic immune modulator with antigen-specific Treg induction is more efficacious in reversing the onset of diabetes. Furthermore, the manner in which anti-CD3 treatment reverses diabetes by inducing Tregs that function in a TGF- β -dependent manner in NOD mice is similar to the function of adaptive Treg. Their study showed a subset of FoxP3⁺ cells present within the CD4⁺CD25^{low} lymphocyte subset suppresses T

cell immunity in spontaneously diabetic NOD mice in a TGF- β -dependent manner [190]. These TGF- β -dependent adaptive CD4⁺CD25^{low} T cells appear to be induced from peripheral CD4⁺CD25⁻ T lymphocytes by anti-CD3 immunotherapy. This strategy of inducing immune tolerance is unique in that while transient effects of the anti-CD3 antibody, such as antigenic modulation of the TCR-CD3 complex, the induction of apoptosis that preferentially affects activated T cells, and the induction of anergy in T cells, exists [191], the longterm effect of this biologically based therapy appears to be the induction of adaptive Tregs.

The results of these preclinical studies have been translated into clinical applications. Several phase I and II trials have been conducted with two different humanized FcR-non-binding antibodies specific for human CD3 in the US and Europe [192,193]. In one trial, administration of hOKT3 γ 1(Ala-Ala) (teplizumab) for 2 weeks at the time of diabetes onset delayed disease progression for more than 1 year in most of the patients in the trial [194]. CD4⁺ and CD8⁺ Treg populations were induced after drug treatment. The European study using showed a significant decrease in the insulin requirement of treated patients for at least 18 months after one course of daily 6mg FcR-non-binding CD3-specific antibody ChAglyCD3 treatment as compared to placebo controls [195]. In addition, the strategy of combining a systemic immune modulator with antigen-specific Treg induction is actively being pursued in humans trials.

Another disease that much attention has been focused on is an iatrogenic disease, graft versus host disease, which occurs after bone marrow transplantation and is the major cause of morbidity and mortality in those patients. Studies have found that Tregs expressing a high level of L-selectin profoundly inhibited GVHD (graft-versus-host disease), and that these Tregs interfere with the activation and expansion of GVHD effector T cells in secondary lymphoid organs and significantly increased donor bone marrow engraftment in sublethally irradiated mice [196]. This study was one of the first to demonstrate the potential of Treg based therapy for GVHD in a murine model. In addition, a separate study by the same group showed that naïve or *ex vivo*-expanded third-party Treg cells could effectively enhance engraftment of T cell-depleted bone marrow allograft, exhibiting reactivity *in vitro* and *in vivo* similar to that found for donor-type Treg cells. This raises the possibility for third-party Tregs to be prepared *ex vivo* for therapeutic purposes [197].

While strategies promoting the tolerogenic effects of Treg to treat autoimmune disease appear to be promising, there are several barriers that must be overcome in order for these strategies to be successful. One major barrier is the importance of establishing antigen specificity. In animal models antigen-specific regulatory T cells are more effective; however, these are difficult to isolate from humans and can only be expanded *in vitro* using rapamycin, TGF- β or other pharmacological agents. In terms of administering antigen either through the mucosa or by targeting DCs in a non-activating manner in order to induce antigen-specific Treg cells, a reproducible and reliable dose of antigen will be very difficult to establish. While in animal models, it appears that the TGF- β -dependent adaptive CD4⁺CD25^{low} T cells are an important subset to be induced, there appears to be different subsets of human Treg and it will be important to determine which subset(s) should be expanded and used in cell based therapies. In addition, since Foxp3 is transiently expressed by activated T cells in humans, it will be necessary to improve upon the existing cell type specific surface markers in order to target and isolate Tregs efficiently and reproducibly [198]. If these obstacles can be overcome, Treg adoptive transfer and induction therapies may become a reality in the near future.

4.2 Strategies to use MDSC to modulate antigen specific immune tolerance

Since MDSC can suppress CD8⁺ CTL [98], Th1 [83,90–92], and Th2 [85,89,92–95] responses through various mechanisms and establish immune tolerance by induction of Treg development, the tolerogenic activity of MDSC can be exploited to induce T cell tolerance and suppress autoimmune or allo-immune responses. Studies have shown that gonipointin-1

(G-CSF/Flit-3 ligand molecule) and G-CSF expanded GM (granulocyte-monocyte) precursor cells promoted transplant tolerance yet preserved the graft-versus-leukemia effects and generated IL-10 secreting Treg cells [199]. Interestingly, when used alone, G-CSF can achieve similar effects. Low-density granulocytes (LDG) isolated from G-CSF-treated bone marrow or spleen cells can confer full protection from GVHD [200]. Furthermore, myeloid cells with suppressive function can be obtained from bone marrow culture in the presence of a low dose of GM-CSF. These suppressive myeloid cells can prolong allogeneic heart transplantation [201] and were identified, in a subsequent study, to be Gr-1⁺CD11b⁺ MDSCs [202]. Perturbation of cytokine signaling can result in an increase of MDSC. For instance, SH2-containing inositol phosphatase (SHIP) is a negative regulator of the signaling pathway for various cytokines [203]. Expansion and accumulation of a myeloid population that resembles MDSCs phenotypically and functionally, was observed in the peripheral lymphoid tissue of SHIP deficient mice [204]. SHIP deficient mice are also significantly compromised in their ability to prime an allogeneic T cell immune response [205]. More interestingly, targeting SHIP deficiency using an inducible system can induce the expansion and accumulation of MDSCs and abrogate an acute GVHD response, thereby improving the survival rate of recipient mice [206]. Although a correlation between an increase in MDSCs and the suppression of GVHD has been established in this inducible SHIP knockout (KO) model, it is yet to be determined whether or not protection from GVHD is mediated directly and solely by MDSCs.

Currently, MDSC can only be isolated from tumor-bearing or SHIP deficient mice. Therefore, it will be necessary to derive MDSCs *in vitro* for clinical applications. Findings from an inducible SHIP KO system suggest a potentially useful means by which to derive MDSC *in vitro* and warrants further study to test whether SHIP targeting can favor the generation of MDSC from hematopoietic stem cells *in vitro* and whether *in-vitro*-derived MDSCs can selectively suppress ongoing T cell responses. Our laboratory has been testing this hypothesis in proof-of-principle studies using MDSCs derived from tumor bearing mice to induce immune tolerance in animal models of GVHD and type 1 autoimmune diabetes (T1D). Our results indicate that adoptive transfer of purified MDSCs can prevent GVHD, facilitate the establishment of chimerism in recipient mice, and improve long-term survival to more than 120 days. Furthermore, adoptive transfer of MDSCs can prevent the onset of T1D in Ins-HA transgenic mouse model. More importantly, antigen specific Treg have been established with MHC class II restriction in the long-term survival animals (unpublished results). These results support the importance and multiple roles of MDSCs in immune tolerance. Therefore, MDSC may be utilized as an efficient, tolerogenic cell for the establishment of antigen-specific immune tolerance in autoimmune diseases or in allograft transplantation and GVHD. The challenge to using MDSCs for the establishment of immune tolerance for clinical applications remains the necessity to devise a means for reproducible and efficient *in vitro* generation of MDSC with high tolerogenic activities.

4.3 Dendritic cell mediated immune tolerance

Since dendritic cells play a central role in the induction of both immunity and tolerance, it is plausible that modulation of DC plasticity can be used for the induction of tolerance in transplantation and autoimmune diseases. Several reports have used various approaches to manipulate dendritic cells, e.g. *in vitro* generation of altered DC in the presence of cytokines (GM-CSF, IL-10, or TGF- β) [158]; genetic engineering of DC, e.g. transgenic expression of IL-10, TGF- β , or IDO [207–209] or siRNA silencing of RelB or IL-12 gene expression [210, 211]; or the use of pharmacological mediators, e.g. the anti-inflammatory drugs rapamycin [212], cyclosporine [213], or 1 α ,25-dihydroxyvitamin D3 [214], prostaglandin E2 [215], or ligands for the inhibitory immunoglobulin-like transcript 4 (ILT4) receptor [216]. The use of cytokines, such as G-CSF, GM-CSF or Flit ligand, for the expansion of tolerogenic DC and, potentially, other myeloid cells to prevent allo-graft rejection has generated a certain level of

success. However, the efficacy varied due to the generation of multiple cell types within the culture, depending on the dose of cytokine and/or the duration of cytokine stimulation [202]. Furthermore, the use of purified cell populations vs. bulk populations also contributed to the variations or discrepancies in treatment efficacy. Other studies have used rapamycin or compounds that inhibit DC maturation to render DC tolerogenic [212]. Upon adoptive transfer, the resulting tolerogenic DC when pulsed with cell-free lysate from donor splenocytes prolonged cardiac allograft or suppressed GVHD. Tolerogenic DCs can also be generated *in vitro* by stimulation of bone-marrow-derived DCs with GM-CSF, IL-10, and TGF- β followed by transient incubation with LPS. DC generated by this approach prevented GVHD and retained graft-versus-leukemia activity [158]. Muller's group has shown that IDO production in IDO⁺CD19⁺ DC, which can induce T cell anergy and tolerance, can be amplified by Treg engagement or TLR9 ligation [217]. However, it remains to be determined whether IDO-expressing DC can persist and maintain its tolerogenic activity sufficiently to improve the survival outcome of organs transplanted into recipient hosts. In addition, the co-stimulatory molecule RANKL (receptor activator of NF- κ B ligand) on DC may also play a role in maintaining Treg homeostasis in the periphery. Over-expression of RANKL in keratinocytes resulted in functional alterations of epidermal dendritic cells and systemic increases in the regulatory CD4⁺CD25⁺ T cell population [218]. Thus, epidermal RANKL expression can affect the functional activity of dendritic cells, and, as a result, increase the number of peripheral CD4⁺CD25⁺ regulatory T cells under inflammatory conditions.

A number of conditionally modified DC-based cell therapies have shown promise in various preclinical animal models. However, several issues need to be carefully addressed in order to move these therapies forward for clinical applications. These include maintaining the stability of the phenotype (immaturity) and tolerogenic functions, as well as identifying the most potent subtype of *in-vitro*-conditioned DC, the optimal dose for the induction of tolerance, and the appropriate antigen specificity.

4.4. Mesenchymal stem cells

In addition to the “traditional” tolerogenic cells (such as Treg, DC, and MDSC), mesenchymal stem cells also have potential clinical application in the induction of immune tolerance. Mesenchymal stem cells constitute a rare population in the bone marrow that is capable of differentiating into a variety of cell types, tissues, and organs [219,220]. Due to their multipotent capacity, mesenchymal stem cells are considered to be one of the key players in osteogenesis and hematopoiesis by forming a supportive niche. However, recent studies have clearly shown that mesenchymal stem cells can also exert immunoregulatory functions both *in vitro* and *in vivo*. *Ex-vivo*-expanded mesenchymal stem cells from MHC mismatched mice can suppress a wide range of immune responses *in vitro* via multiple mechanisms, e.g. production of nitric oxide, prostaglandin E₂, IDO, IL-10, TGF- β , and induction of Tregs [221]. The unique property of immune suppression by mesenchymal stem cells has been further substantiated by *in vivo* studies. Mesenchymal stem cells can prolong the survival of MHC-mismatched skin grafts in baboons [222], enhance the engraftment of allogeneic bone marrow transplants in mice [223], limit or cure GVHD after allogeneic HSC transplantation in humans [224,225], and ameliorate or prevent autoimmune disorders, such as experimental autoimmune encephalomyelitis [226], systemic lupus erythematosus [227], and collagen-induced arthritis [228]. Taken together, these properties indicate that MSC may provide an additional promising option for cell-based tolerogenic therapy.

5. Concluding Remarks

In recent years, a growing body of knowledge has been obtained on the mechanisms underlying immune tolerance mediated by various tolerogenic cells and on the means for *in vitro* manipulation, expansion, and generation of these cells, dendritic cells and Tregs in particular.

A major challenge in this emerging field of cell-based tolerogenic therapy is how best to exploit the accumulated knowledge to improve the outcome of clinical applications. Tolerogenic cells can be used for the treatment of various disorders caused by undesirable or heightened immune responses, such as chronic inflammatory diseases, autoimmune diseases, etc. Furthermore, cell-based tolerogenic therapy can complement stem-cell-based therapy to suppress auto-, allo- and graft-versus-host responses. Conversely, reversion of immune tolerance associated with malignancies and chronic infections can enhance the efficacy of immune-based therapies. However, caution must be exercised when manipulating the state of immune tolerance. Non-specific or uncontrolled immune tolerance may render patients susceptible to opportunistic infections whereas systemic reversion of immune tolerance in cancer patients may result in autoimmunity.

The potential of cell-based therapies, including tolerogenic therapy and cell replacement therapy, is limited by a number of factors. Regulatory issues and high cost aside, a major challenge is the ability to generate a reproducible, GMP (good manufacturing practice)-quality cell product so that the therapeutic outcomes from clinical trials at different academic centers can be properly compared and evaluated. Another factor to be considered is the availability of a large quantity of autologous cells. Recent advances in the generation of pluripotent stem cells by nuclear transplantation into fibroblasts should enable the large-scale production of autologous tolerogenic cells [229,230]. An added advantage for the use of stem cells to produce tolerogenic cells is the ability to genetically engineer stem cells to inducibly turn on or off the expression of an array of genes in order to obtain tolerogenicity. Finally, the means by which the tolerogenic phenotype can be maintained during *in vitro* production and after adoptive transfer should also be addressed.

Acknowledgements

In the interest of space limitation, many important studies were regrettably omitted, and we apologized for any such oversight. We thank Ms. Macria Meseck for editing of the manuscript. The research work in our laboratory is supported in part by grants from the NCI, NIDDK, Sharp Foundation, and Black Family Stem Cell Foundation to Shu-Hsia Chen, grant support from Susan G. Komen Breast Cancer Foundation to Ping-Ying Pan, and NIH training grant T32CA078207-06 fellowship to Junko Ozao.

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Figure 1.

Cellular targets in the modulation of immune tolerance. Various cytokines and tumor factors secreted by tumor cells induce the development and accumulation of myeloid-derived suppressor cells (MDSCs). The MDSCs suppress Th1 and Th2 responses through IFN- γ dependent nitric oxide (NO) production and IL-4/IL-13 dependent expression of arginase 1, respectively. Natural killer T cells can also contribute to the stimulation of MDSCs by secreting IL-13. Furthermore, MDSCs can induce T regulatory cells (Treg) in tumor-bearing mice. Other cell types including immature myeloid dendritic cells (imDC) and plasmacytoid dendritic cells (pDC) have also been shown to induce Treg in various experimental settings. Furthermore, MDSC can migrate to the tumor site and become tumor-associated macrophages (TAM) and can also differentiate into imDC. All of these cell types including MDSC, Treg, imDC, pDC, and TAM have a suppressive effect on anti-tumor immunity, hence constituting important cellular targets in the reversion of immune tolerance in the setting of cancer. Conversely, the tolerogenic properties of both MDSC and Treg can potentially be exploited and used to suppress autoimmunity, allo-responses, and graft-versus-host-disease (GVHD) in clinical applications.