Advances on CD8$^+$ Treg Cells and Their Potential in Transplantation

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Abstract: Although cluster of differentiation (CD)8$^+$ regulatory T (Treg) cells have been in the last 20 years more studied since evidences of their role in tolerance as been demonstrated in transplantation, autoimmune diseases and cancer, their characteristics are still controversial. In this review, we will focus on recent advances on CD8$^+$ Treg cells and description of a role for CD8$^+$ Treg cells in tolerance in both solid organ transplantation and graft-versus-host disease and their potential for clinical trials.

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The Past of CD8$^+$ Regulatory T Cells

Cluster of differentiation (CD)8$^+$ regulatory T (Treg) cells were, historically, the first identified cell subset with a suppressive potential in 1972.[1] However, the lack of a unique marker to identify suppressor T cells, the lack of evidence of an I-J gene within the major histocompatibility complex (MHC) region to govern the suppressor T-cell function, the lack of evidence of any I-J restricted—or not restricted—suppressor factors, and the difficulties to identify functional genes encoding for an antigen-specific TCR contributed to the end of CD8$^+$ Treg cell studies for years.[2]

In contrast, CD4$^+$ Treg cells were defined in 1995 with solid markers and have been shown to be heterogeneous.[3] It has been 20 years now that CD8$^+$ Treg cells have been resurrected.[4] Characteristics of CD8$^+$ Treg cells are still controversial and reproducibility of previously published results by multiple laboratories will be key to reach a proper understanding and consensus. In this review, we will focus on recent advances on CD8$^+$ Treg cells and evidences of a role for CD8$^+$ Treg cells in tolerance in both solid organ transplantation and graft versus host disease (GVHD) and their potential for clinical trials.

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Transcription Factors and Cell Membrane Markers

CD8$^+$ Treg cells have a heterogeneous phenotype, as it is also the case for CD4$^+$ Treg cells which are predominantly defined as Foxp3$^+$ although other subsets of CD4$^+$ Treg cells Foxp3$^-$ do exist, such as Tr1 and Th3 Treg cells. The heterogeneity of CD8$^+$ Treg cell phenotype probably has not only in part biological basis but it also arises from the use in many studies of small panels of markers by the different groups that have studied CD8$^+$ Treg cells. Additionally, it is possible that differences in phenotype among different species likely hide similarities within this heterogeneity. Table 1 summarizes the phenotype of CD8$^+$ Treg cells involved in solid organ transplantation and GVHD in mice, rats, and humans since 2010 but many other publications describe models before this date.

Foxp3 regulates several key genes implicated in CD4$^+$ Treg cell function.[37] In mice with Foxp3 reporter genes, as well as using anti-Foxp3 antibodies, steady-state CD4$^+$ T cells are the predominant cell type that express Foxp3, although expression is also observed in CD8$^+$ T cells.[38] Using antibodies, natural Foxp3$^+$CD8$^+$ Treg cells have also been described by several groups both in rats[25] and humans.[27-29,34,36] Likewise, transgenic Foxp3-green fluorescent protein (GFP) rats not only show expression of Foxp3 predominantly in CD4$^+$ cells but also in CD8$^+$ T cells and in higher proportion than that in mice (30% of Foxp3$^+$ cells are 30% of Foxp3+ cells are CD8+ in rat vs 1-10% in mice)(manuscript in preparation). In mice, rats, and humans, Foxp3 is expressed in induced CD4$^+$ and CD8$^+$ Treg cells in a variety of situations.[38] Therefore, after transplantation and treatment with tolerogenic strategies, it is logical that many studies describe CD8$^+$ Treg cells as Foxp3$^+$-induced Treg cells (Table 1). Nevertheless, demethylation of Treg cell–specific demethylated region sequences of the Foxp3 gene is higher in natural versus induced

TABLE 1.

$CD8^+$ Treg cells in transplantation (since 2010)

Phenotype	Functional characterization	Transplantation model and or generation	References
Mouse			
$CD122^+CD28^+PD\text{-}1^+CD127^-$	IL-10 production, dependent on PD-1 and CD28 signaling.	Skin allotransplantation	5
$CD28^{+/-}$	ND	Skin allotransplantation; treatment with rapamycin in CD4 KO mice	6
$Foxp3^+CD25^+CD103^+CTLA\text{-}4^+CD122^-CD62L^-CD28^-$;	inhibition of T cell activation contact-dependent, not cytotoxic, inhibition of DC maturation and induction of $CD4^+$ $Foxp3^+$ Treg cells through TGFβ	Allogeneic DCs + rapamycin + IL-2 + TGF-β	7
$Foxp3^+CD25^+CD44^+GITR^+CTLA\text{-}4^+PD\text{-}1^+CD103^+$	ND	GVHD; induced $CD8^+$ $Foxp3^+$ Treg cells expand that and suppress more than $CD4^+$ $Foxp3^+$ Treg cells, $CD8^+$ but not $CD4^+$ iTreg cells essential to prevent GVHD	8
$Foxp3^+CD25^+GITR^+CTLA\text{-}4^+CD122^-$	ND	GVHD; in vivo induced $CD8^+$ Treg cells during, TGF-β– and IL-2–dependent	9
$Foxp3^+CD25^+GITR^+CD62L^-\alpha4\beta7^+CD28^+CTLA4^+$ $Helios^-$	low cytotoxicity	GVHD, rapamycin and IL-2 increased expansion	10
$CCR7^+CD44^{high}CD62L^{high}$ central memory;	Production of IFNγ induced NO production by DCs	Lung allotransplantation; CD28/B7 and CD40/CD154 blockade	11
$CD122^+CD25^-IL15Ralpha^{high}$	ND	Pancreatic islet allotransplantation	12
CD11c	ND	$CD8^+$ Treg cell induction by DC cross-presentation through Tmem176b	13
$CD39^+CD73^+CTLA\text{-}4^+$	ND	GVHD; alloreactive $CD8^+$ Treg cells inhibited GVHD moderatly and showed GVL activity whereas alloreactive $CD4^+$ Treg cells inhibited both. Treatment of GVHD and GVL with combined $CD8^+$ and $CD4^+$ Treg cells superior than each cell type independently.	14
$PD1^+CD44^+CD69^+CD122^+CD62L^-CD127^-Foxp3^-$	ND	Liver allotransplantation; gene transfer of donor MHC-I	15
$CD122^+$	ND	Pancreatic islets allotransplantation; Qa-1 deficient mice show accelerated rejection and anti-CD45RB imposed tolerance through $CD8^+$ and/or $CD4^+$ Treg cells	16
$PD\text{-}1^+$	Central memory but not effector memory, interaction with DCs, IFNγ production	Lung allotransplantation; costimulation blockade	17
$CD122^+$;	Inhibition of DC maturation	Skin allotransplantation; emodin treatment,	18
$CD122^+PD1^+FasL^+$	FasL-mediated cytotoxicity of T $CD4^+$	Skin allotransplantation; IL-15 treatment	19
$CD8^+$	Suppression by lysis of activated $CD4^+$ T cells through recognition of Qa-1	Cardiac allotransplantation;	20
Rat			
$CD45RC^{low/-}$	Migration into grafts, pDCs, IFN-γ, IDO	Heart allotransplantation; costimulation blockade using CD40lg	21,22
$CD45RC^{low/-}$	Fibroleukin-2	Heart allotransplantation; costimulation blockade using CD40lg	23
$CD45RC^{low}Foxp3^+$; $IL\text{-}34^+$, macrophages		Heart allotransplantation; costimulation blockade using CD40lg	24
$CD45RC^{low}Foxp3^+$	CD8 and TCR engagement	Heart allotransplantation; donor allopeptides in vitro stimulation and in vivo treatment	25,26
Human and nonhuman primates			
$CD28^-CD25^+FOXP3^+CTLA\text{-}4^+CD62L^-CD127^-perforin^-$	APC- and contact-dependent, cytokine independent	APCs+IL-2 and IL-15	27

$LAG3^+Foxp3^+CTLA\text{-}4^-$	ND	Allogeneic pDCs; inhibition of T cells, including memory T cells	28
$CD8^{high}CD25^+FOXP3^+$	CTLA4-dependent	GVHD in immune humanized NSG mice; CD40-stimulated;allogeneic B cells	29
$CD28^-$	ND	Multiple MLRs; with belatacept	30
$CD28^-$	↑ BCL6 transcriptional repressor and ↓ inflammatory miR-21, miR-30b, miR-146a, and miR-155	Multiples MLRs; with ILT3.Fc	31
$CD28^-$	IL-10, TGF-β, cytotoxicity against $CD4^+$	Allogeneic MSCs	32
$CD8^{low}CD28^-$	Cell contact and TGF-β-dependent	soluble peptides (tyrosinase and hepatitis B)/HLA-A2 dimers	33
$CD8^{high}FOXP3^+CD103^+CD62L^+CCR7^+$	ND	GVHD in immune humanized NSG mice; suppressive monocytes	34
$CD8^+CD28^-$	ND	Liver allotransplantation	35
$CD45RC^{low}Foxp3^+GITR^+IL\text{-}34^+IL\text{-}10^+TGFβ^+IFNγ^{int}$	No cytotoxicity, contact-dependent and IFNγ, IL-34, and TGFβ-dependent	GVHD in immune humanized NSG mice; Expansion with anti-CD3, anti-CD28, high-dose IL-2 and IL-15. Beneficial effect of addition of rapamycin on expansion fold and suppression.	36

ND, not described.

$CD4^+$ Treg cells,[39] and this was also the case in human-induced alloreactive $CD8^+CD45RC^{low/-}$ Treg cells as compared with $CD8^+CD45RC^{high}$ non–Treg cells.[36] At the same time, and as for $CD4^+$ Treg cells, $Foxp3^-CD8^+$ Treg cells have been described in all species analyzed and in many pathophysiological situations, such as cancer, infectious disease, autoimmunity as well as organ transplantation and GVHD (Table 1). In conclusion, for $CD8^+$ Treg cells as for $CD4^+$ Treg cells, Foxp3 is an important marker of a subset of $CD8^+$ Treg cells that needs to be analyzed in any new transplantation tolerance model but its absence does not preclude the existence of $CD8^+$ Treg cells. Helios is a transcription factor that has been described as predominantly expressed by thymus-derived $CD4^+Foxp3^+$ Treg cells but not in peripherally induced $CD4^+$ Treg cells[40] although this finding has been questioned.[41] Helios controls at least some regulatory functions and survival in both $CD4^+$ and $CD8^+$ Treg cells.[42,43] $CD8^+$ Treg cells in mouse GVHD models were $Helios^{-}$[8,10] (Table 1).

As for effector cells, $CD4^+$ and $CD8^+$ Treg cells have been divided into naive, central memory, and effector memory subpopulations.[36,44] Several of the transplantation models show a phenotype of $CD8^+$ Treg cells as effector memory cells $CD62L^-$, $CD122^-$, $CD28^-$[7,9,15,27,31-33,35,36] or central memory $CD44^{high}$ chemokine (C-C motif) receptor (CCR) 7^+CD62L^+,[11,34,36] but others describe more naive $CD122^+$ and/or $CD28^+$ $CD8^+$ Treg cells[5,10,12,16,18,19] (Table 1). As far as for the CD45 isoforms as markers of naive or differentiated cells, it has been traditionally accepted that RA^+RO^- cells are naive, whereas RA^-RO^+ are memory, but it has also been shown that $CD45RA^+$ cells may arise from RA^- after stimulation and thus the expression of RA depends on the elapse time since the last antigen activation.[45] For human $CD4^+Foxp3^+$ Treg cells, there are 2 populations, $CD45RA^+$ or RA^-, with different regulatory and proliferation potential.[46,47] Human $CD8^+Foxp3^+$ Treg cells can also be divided in 2 populations of $CD45RA^+$ or $CD45RA^-$ cells,[36] but their suppressive role has not been analyzed. CD45RB is expressed by all human $CD4^+$ and $CD8^+$ Treg cells.[36] Finally, the $CD45RC^{low/-}$ fraction of T cells includes $CD4^+$ $Foxp3^+$ and $CD8^+$ $Foxp3^+$ Treg cells in rat and human models of transplantation or GVHD, whereas effector cells or their precursors reside in the $CD45RC^{high}$ fraction.[21,25,36,48-50]

In several transplantation models, $CD8^+$ Treg cells have been shown to express markers of exhaustion, such as lymphocyte-activation gene 3 (LAG3), PD-1, cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related (GITR)[5,8,10,15,17,19,27-29] (Table 1), but that can also be activation markers, exhaustion being defined by several other characteristics.[51]

In the future, the use in mouse, rat, and human cells of large panels of antibodies directed against intracellular and cell membrane molecules using new high-density techniques, such as mass cytometry or new cytofluorimetry technologies, as well as single cell transcriptomic analysis, would allow to better define common or different subsets that would then need to be confirmed in suppressive assays.

Mechanisms of Action

$CD8^+$ Treg Cells Induce Tolerogenic Antigen-presenting Cells Through cell Membrane Molecule Interactions

Several studies support a role for CTLA-4 in $CD8^+$ Treg cell suppressive function (Figure 1). For example, clonally

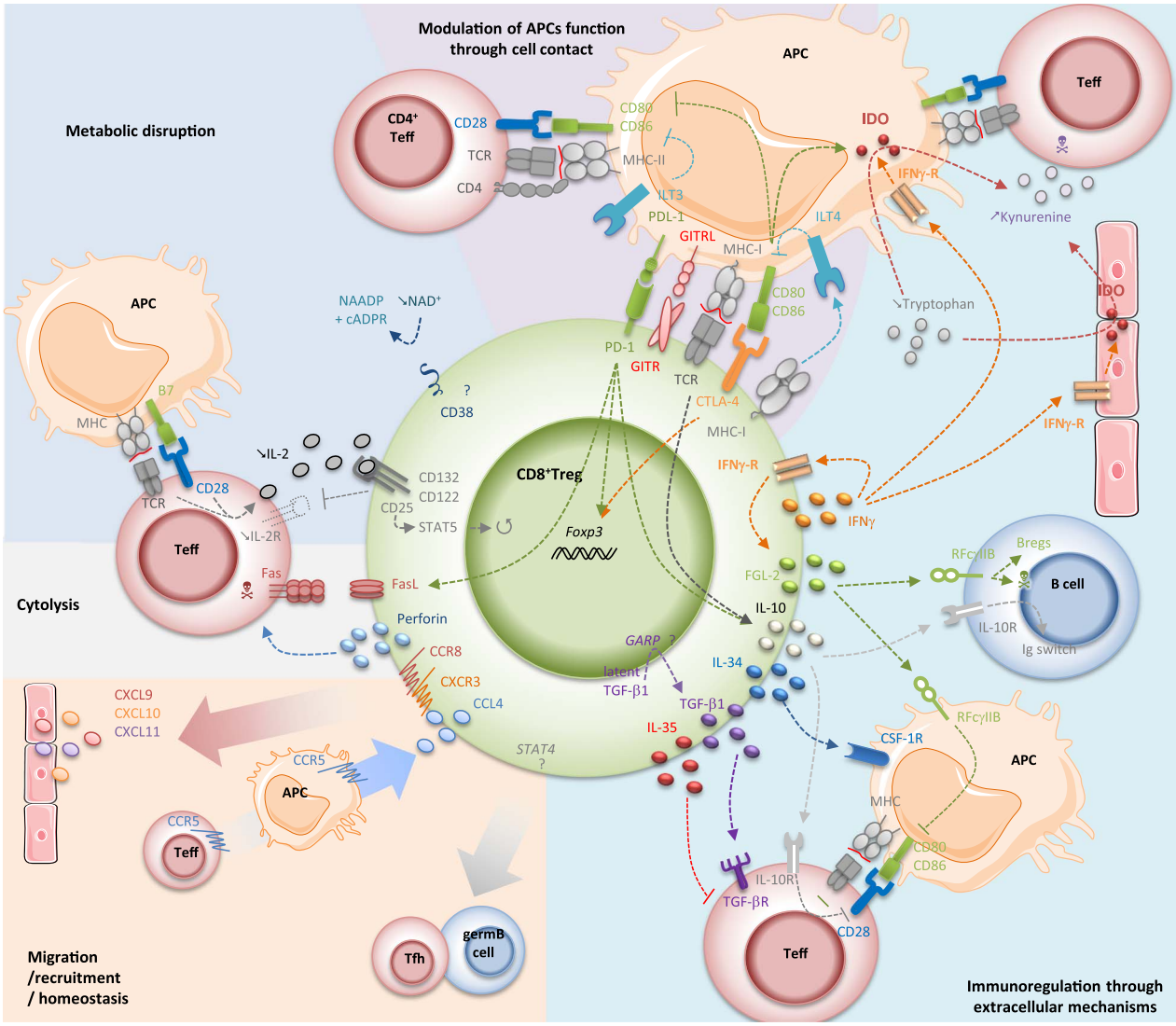

FIGURE 1. Schematic illustrating described mechanisms of action of CD8$^+$ Treg cells in transplantation.

expanded HLA-restricted Foxp3$^+$CD8$^+$ Treg cells were dependent on CTLA-4 expression to suppress effector T-cell responses in vitro.[52] Moreover, preincubation of CD8^{hi} Treg cells with anti-CTLA-4 blocking monoclonal antibody (mAb) abolished the protection of humanized mice from acute GVHD induced by PBMC injection.[29] Mechanisms of action of CTLA-4 were previously deciphered in studies focused on CD4$^+$ Treg cells. Wing et al[53] highlighted that interaction of CTLA-4 with B7 molecules expressed on DCs decreases expression of costimulatory molecules, leading to weak antigen presentation to effector T cells. CTLA-4/CD80 interaction also promotes formation of Treg cell immune synapses, Treg cell activation, and Foxp3 expression.[54-56] Furthermore, CTLA-4/B7 interaction promotes the expression of the immunoregulatory molecule indoleamine 2,3-dioxygenase (IDO) in DCs, which impairs effector T-cell proliferation through deprivation of tryptophan and generation of proapoptotic metabolites[57] and conversion of naive T cells into Treg cells,[58] and IDO has also been described to generate CTLA-4$^+$ CD8$^+$ Treg cells.[28] In contrast, although the role of LAG3 in CD4$^+$ Treg cell suppressive function has been well described using blocking mAb and deficient animals,[59] and although LAG3 is expressed on CD8$^+$ Treg cell subsets,[60] its role in CD8$^+$ Treg cell function requires further investigation. Indeed, LAG3 blockade did not abrogate CD8$^+$CTLA-4$^+$Foxp3$^+$ plasmacytoid dendritic cell (pDC)-induced Treg cell–mediated suppression,[28] but a compensatory upregulation of other checkpoint pathways should be considered.[61] Nevertheless, transfer of LAG3-deficient CD4$^+$ T cells, but not LAG3-deficient CD8$^+$ T cells, exacerbated the GVHD, suggesting a preferential role for LAG3 in CD4$^+$ Treg cells in this model.[62]

Suciu-Foca's team[63,64] showed the major role of the inhibitory receptors immunoglobulin-like transcript (ILT)3 (unknown ligand) and ILT4 (ligand MHC-I) expressed by DCs in suppressive activity exerted by CD8$^+$CD28$^-$ Treg cells on CD4$^+$ T-cell responses in vitro, by blocking these molecules in a coculture assay. Moreover, they reported that CD8$^+$CD28$^-$ T cells from heart transplanted patients in quiescence inhibited CD40L-triggered upregulation of CD86 on donor antigen-presenting cells (APCs) in contrast to Treg cells from rejecting patients.[63] Indeed, CD8$^+$CD28$^-$Treg cells can upregulate the expression of ILT3 and ILT4 on APCs that induce a downregulation of costimulatory (CD80/CD86)

and adhesion (CD54/CD58) molecules, therefore rendering them tolerogenic.[63,65] Reciprocally, ILT3-Fc soluble form confers immunosuppressive properties to $CD8^+$ T cells.[31,66]

$CD8^+Foxp3^+$ Treg cells have a higher expression of GITR than $Foxp3^-$ cells,[67] and GITR is exclusively expressed in thymic $CD25^+$, but not in $CD25^-$ $CD8^+Foxp3^+$ T cells.[68] To investigate the role of GITR in Treg cell function, the authors used a blocking mAb that did not abrogate the suppressive effect of $CD8^+Foxp3^+$ Treg cells on effector T cell proliferation in vitro.[68] However, the role of GITR for mouse $CD4^+$ Treg cell interaction with APCs was highlighted using agonist mAb,[69] and this approach has not yet been tested with $CD8^+$ Treg cells. We sorted human $CD8^+CD45RC^{low}$ nTreg cells based on GITR expression and observed that $GITR^+$ Treg cells were more efficient than $GITR^-$ cells to suppress Teff proliferation stimulated by allogeneic APCs in vitro. Our results suggest a role for GITR to interact with allogeneic APCs.[36]

PD-1 is expressed not only by exhausted T cells but also by activated $CD4^+$ and $CD8^+$ T cells, including Treg cells.[70] PD-1 was shown to be not only a marker to distinguish memory ($PD\text{-}1^-$) from $CD8^+CD122^+PD\text{-}1^+$ Treg cells but also blocking PD-1 abrogated suppression on Teff proliferation in vitro.[5] Indeed, Dai et al[5] showed that PD-1/PD-L1 interaction between Treg cells and APCs is required for interleukin (IL)-10 production and suppression. $CD8^+CD122^+PD\text{-}1^+$ acted through FasL expression and induced T-cell apoptosis in a Fas/FasL and IL-10–dependent manner in vitro and in a skin transplantation model. Administration of IL-15 in vivo was shown to expand adoptively transferred $CD122^+PD\text{-}1^+$ $CD8^+$ Treg cells in vivo.[19] In addition, PD-L1 has a pivotal role in the development, maintenance, and motility of $CD4^+$ Treg cells and PD-L1 enhances and sustains Foxp3 expression and Treg cell suppressive function.[54,71] The use of agonistic anti-PD-1 antibodies or of PD-L1-Fc in transplantation could induce tolerance.

$CD8^+$ Treg Cells Can Induce Suppression Through Extracellular Mechanisms

Several ILs have been shown important in $CD8^+$ Treg cell suppressive properties (Figure 1). IL-10 plays a major regulatory role on inflammatory responses. In mice, IL-10 is involved in both $CD4^+$ and $CD8^+$ Treg cell suppressive activity and is responsible for the higher suppressive function of $CD8^+CD122^+$ Treg cells than $CD4^+$ Treg cells, concomitantly with IL-10 expression levels reported in these cells.[12] IL-10 is expressed after direct recognition of conventional T cells by $CD8^+CD122^+$ Treg cells through MHC class I/ $\alpha\beta$TCR interaction[72] and dependently on CD28 and PD-1 signaling.[5] Their human counterpart $CD8^+CXCR3^+$ Treg cells showed similar properties.[73] Mesenchymal stromal cells have been shown to enhance the regulatory function of $CD8^+CD28^-$ Treg cells by upregulating the expression of IL-10 and FasL.[32] IL-10 is also important for intestinal $CD8\alpha\alpha$ T cells to maintain gut integrity.[74] Concerning $CD8^+CD45RC^{low}$ Treg cells, we have reported a higher expression of IL-10 in rat $CD8^+CD45RC^{low}$ Treg cells as compared with $CD8^+CD45RC^{high}$ Teff,[21] and more recently, we demonstrated a higher suppressive activity in IL-10–expressing human $CD8^+CD45RC^{low}$ Treg cells.[36] IL-10 has been described to act through inhibition of CD28 tyrosine phosphorylation and signaling pathway[75] and seems important for Foxp3 expression because mice lacking IL-10 failed to maintain Foxp3 expression in $CD4^+$ Treg cells resulting in impaired regulatory activity.[76]

We recently reported a role for a newly identified cytokine, IL-34, in both $CD4^+$ and $CD8^+$ Treg cell function.[24] Only $CD8^+$ and $CD4^+$ Treg cells express IL-34. By blocking IL-34 with antibodies in a coculture suppressive assay, we showed that IL-34 is involved in the suppressive activity of CD40Ig-induced $CD8^+CD45RC^{low}$ Treg cells in a rat transplantation heart model.[21,24,77] Similarly, blocking of IL-34 partially abrogated suppression mediated by both $CD4^+$ and $CD8^+CD45RC^{low}$ human Treg cells.[24,77] We also demonstrated IL-34 capacity to induce $CD4^+$ and $CD8^+$ Treg cells as a feedback loop, in vivo and in vitro, through monocyte polarization toward M2-type macrophages to protect allograft from acute and chronic rejections.[24,77,78]

In contrast to IL-34–specific expression by $Foxp3^+$ Treg cells, expression of IL-35 is not restricted to regulatory cells,[79] but blocking of IL-35 with mAb reversed antigen-specific $CD8^+CTLA\text{-}4^+$ Treg cell–mediated suppression in a trans-vivo DTH assay.[80] The exact role of IL-35 in transplantation has not yet been investigated.

IFN-γ immunoregulatory properties have been now commonly accepted, produced by both $CD4^+$ and $CD8^+$ Treg cells and acting on APCs and endothelial cells.[81,82] Several studies support evidences for a role of IFN-γ in $CD8^+$ Treg cell suppressive function. Like IL-10, IFN-γ is required for $CD8^+CD127^-CD25^{hi}FoxP3^{hi}$-induced Treg cells to suppress.[83] IFNy is also required for the generation and the function of $CD8^+$ Treg cells induced by the introduction of antigens in the immune-privileged anterior chamber of the eye. Indeed, *Ifn-*$\gamma^{-/-}$ mice or mice treated with anti-IFN-γ mAb before injection of an alloantigen failed to develop anterior chamber-associated immune deviation.[84] IFN-γ can induce other immunoregulatory mediators to control immune responses. For example, $CD8^+CD11c^+$ induced Treg cells control autoimmune diseases through secretion of IFN-γ that induces IDO expression in DCs.[85] In addition, our group has shown that CD40Ig-induced $CD8^+CD45RC^{low}$ Treg cells secrete IFN-γ to induce IDO expression in pDCs and endothelial cells.[21,22] IFN-γ can also promote fibrinogen-like protein 2 (FGL2) expression by these Treg cells as a feedback loop.[22,23]

Similarly, TGF-β is important for the suppression mediated by $CD8^+CD122^+$ Treg cells. Indeed, blocking TGF-β1 reverted suppression exerted by $CD8^+CD122^+$ Treg cells on T-cell proliferation in an APC-independent mechanism.[86] Moreover, TGF-β participates in the regulation of the encephalitogenic $CD4^+$ T cells by $CD8^+CD122^+$ Treg cells, as well as IL-10 and IFN-γ.[87] Similar experiments of blocking TGF-β in coculture assays have been used to prove its involvement in suppressive function of peptide-specific $CD8^+$ iTreg cells,[88] human corneal endothelial cell-induced $Foxp3^+CD25^+CD8^+$ Treg cells[89] and $CD8^+CD45RC^{low}$ human Treg cells,[36] but not of $CD8^+CD45RA^+CCR7^+Foxp3^+$ Treg cells.[90] Glycoprotein-A repetitions predominant protein a transmembrane protein that binds latent-TGF-β1 form on cell surface to mediate TGF-β1 release was also detected at mRNA level in $CD8^+CD25^+$ Treg cells,[91] but its exact role in $CD8^+$ Treg cell function and in transplantation has not yet been examined. The immunoregulatory properties of the soluble form of FGL2 have been first highlighted in $CD4^+$ Treg cells.[92-95] FGL2 expression was then reported in memory $CD8^+$ T cells,[96] in $CD8\alpha\alpha$ Treg cells,[97] and we demonstrated its involvement in rat CD40Ig-induced $CD8^+CD45RC^{low}$ Treg cell function.[22,23] FGL2 links to FcγRIIB receptor to inhibit bone

marrow–derived dendritic cell maturation, induce B-cell apoptosis[98] or generate regulatory B (Breg) cells.[23]

Recruitment of regulatory cells on the inflammatory site is key to control immune responses. For example, by secreting CCL4, $CD8^+CD25^+Foxp3^+LAG3^+$ Treg cells may recruit other T cells, DCs, and macrophages expressing CCR5 on the inflammatory site.[60] In addition, CCL4 has immunosuppressive properties, and it is crucial for these Treg cells to suppress since blocking Abs reverted most of inhibition of Teff proliferation in vitro.[60] Finally, the expression of CXCR3 by human $CD8^+$ Treg cells and mice $CD8^+CD122^+$ Treg cells suggests their own recruitment along gradient of monokines induced by IFN-γ (CXCL9, 10, and 11) toward the inflammatory site to control the immune reaction.[73] Similarly, the expression of CCR8 by thymic $CD8^+CD25^+Foxp3^+$ Treg cells could have a migratory role.[68]

Treg Cells Can Selectively Kill Teff Through Cytolysis Mechanisms

Perforin is used by some $CD8^+$ Treg cells to induce cytolysis of target cells (Figure 1). For example, Qa1-restricted $CD8^+$ Treg cell suppressive function was dependent on perforin expression as $Perf^{-/-}$ Treg cells could not inhibit proliferation of effector T cells in vitro.[99,100] Fas/FasL interaction, associated to autoimmune induced cell death,[101] is another mechanism used by $CD8^+$ Treg cells to suppress immune responses. $CD8^+CD122^+PD\text{-}1^+$ Treg cells induce apoptosis through this mechanism because $Fasl^{-/-}$Treg cells or $Fas^{-/-}$ Teff did not result in target cell apoptosis.[19,102] The Fas-mediated cytolysis can be processed in an Ag-independent manner by $CD11c^{high}CD8^+CD122^{hi}$ Treg cells, acting dependently on CD11c ligation,[103] on activated but not on resting $CD4^+$ T cells.[4] $CD8^+CD28^-$ Treg cells in drug free kidney transplanted tolerant patients express high levels of perforin and granzyme A but no degranulation was observed in contact with donor HLA-I antigens in vitro.[104] In addition, these Treg cells are less sensitive to perforin and granzyme A–mediated apoptosis.[104] Similar observations were done regarding Fas/FasL apoptosis induction. Murine $CD8^+$ iTreg cells (induced by TGF-β-treated APCs) act directly on $CD4^+$ Teff cells through a Fas-mediated mechanism and not on APCs presenting the antigen,[105] and murine Ag-activated $CD8^+$ T cells are also resistant to Fas-mediated apoptosis.[4]

Regarding other $CD8^+$ Treg cell subsets, neither $CD8^+CD103^+$ nor $CD8^+CD45RC^{low}$ Treg cells use cytolysis mechanisms to suppress.[106,107] Our group confirmed that human $CD8^+CD45RC^{low}$ nTreg cells, as for rat $CD8^+CD45RC^{low}$, did not induce apoptosis of allogeneic APCs or allostimulated autologous Teff.[21,36]

Metabolic Disruption

Although IL-2 is required for Teff to proliferate and develop immune responses, $CD8^+$ Treg cells are reported to act on IL-2 availability and sensitivity (Figure 1). First, high expression of subunits of IL-2R, CD122 and/or CD25, allows $CD8^+$ Treg cells to impoverish Teff environment by consumption while expanding themselves. CD122 is largely expressed by human $CD8^+$ T cells, including both $CD8^+$ Treg cells and memory $CD8^+$ T cells.[108] Expression of CD122 or CD25 confers $CD8^+$ Treg cells a high susceptibility to low dose of IL-2.[108,109] Indeed, expansion of $CD8^+Foxp3^+$ Treg cells can be induced by IL-2/IL-2 mAb complexes[110] or induction of signal transducer and activator of transcription 5 (STAT5), which is involved in IL-2R signaling.[111] In addition, $CD8^+CD28^-$ Treg cells are reported to decrease the production of IL-2 by Teff in response to APCs.[112] Finally, $CD8^+CD25^+$ Treg cells are able to downregulate IL-2R expression by Teff and therefore to inhibit their proliferation.[113] In the context of allogeneic response, $CD8^+CD45RC^{low}$ Treg cells upregulate CD25 expression both in rat and in human but addition of exogenous IL-2 does not seem to revert Treg cell–mediated suppression on alloimmune responses in vitro.[25,36]

CD38 is a nucleotide metabolizing ectoenzyme that is able to not only transform nicotinamide adenosine diphosphate ribose into ADPribose and cADP-ribose, but also hydrolyze cADP-ribose into ADPribose.[114] $CD4^+$ Treg cells and Breg cells express CD38.[115] Although the exact role of the enzyme activity of CD38 on immune responses is not clear, genetic inactivation or anti-CD38 mAb inhibits several immune responses.[114] $CD8^+$ Treg cells have been defined as being $CD38^{high}$ and human $CD8^+CD45RC^{low}$ Treg cells express CD38 although the functional role of CD38 in suppression and in transplantation was not described.[36,116]

Regulation of T Follicular Helper Cells and Germinal B Cells

Evidences for a role of $CD8^+$ Treg cells in the regulation of T follicular helper (Tfh) cells have been more recently provided by different studies (Figure 1). In a model of tumor growth in Qa-1 mutant mice, impaired Qa-1-restricted $CD8^+$ Treg cells could not suppress, tumor growth was reduced, and this reduction was associated with enhanced expansion of Tfh cells and germinal B cells.[117] However, these mice did not generate excessive Tfh cell response, and alloantibody production and tolerance induction by anti-CD45RB mAb was not impaired.[16] This regulation of Tfh cells by $CD8^+$ Treg cells has been attributed to STAT4.[118] In $Stat^{-/-}Ldlr^{-/-}$ insulin resistant mice, $CD8^+$ Treg cells suppressed Tfh cell and germinal B-cell development upon immunization or adaptive transfer.[118]

The Role of Alloantigen Recognition in $CD8^+$Treg Cell–mediated Suppression

Several studies demonstrated that antigen-specific $CD4^+$ Treg cells have a higher suppressive activity compared to polyclonal Treg cells.[119-122] Based on cell therapy processes, the effect induced by 1.5×10^8 to 1×10^9 allogeneic $CD4^+$ Treg cells would be equivalent to the effect induced by 5×10^9 polyclonal Treg cells[123] with lower nonspecific drawbacks.

Similarly, studies support the importance of antigen specificity for $CD8^+$ Treg cells to efficiently suppress through direct lysis of target cells or through inhibition of APC maturation. For example, the recognition of Qa1-peptide on activated mice $CD4^+$ T cells by the TCR of $CD8^+$ Treg cells is determinant for $CD8^+$ Treg cells to induce IFN-mediated direct lysis of effector memory $CD4^+$ T cells.[20] Also, specific allorecognition of donor DCs is required for noncytotoxic $CD8^+Foxp3^+$ Treg cells to induce suppression through inhibition of DC maturation.[7] Furthermore, conferring antigen specificity to murine polyclonal $CD8^+25^+$ Treg cells by using OVA-specific exosomal MHC complexes increased the inhibition of effector T-cell responses through direct perforin-mediated apoptosis induction and prevention of DC maturation.[100] Finally, antigen-specific $CD8^+$ Treg

cells can induce regulatory properties in alloreactive T cells in vivo based on their capacity of infectious tolerance.[124] Altogether, interaction with MHC-peptide on APC or on T cells is important for $CD8^+$ Treg cells to induce tolerance.

One obvious advantage of $CD8^+$ Treg cells compared with $CD4^+$ Treg cells is the persistence of donor MHC-I presentation over time. Indeed, the short life of donor APCs for direct alloreactive $CD4^+$ Treg cells activation and low occurrence of indirect alloreactive Treg cells are limitations for $CD4^+$ Treg cells cell therapy. By contrast, direct presentation to alloreactive $CD8^+$ Treg cells by $MHC\text{-}I^+$ graft cells is still effective at long term. Moreover, our group has shown that indirect presentation of donor peptide to rat $CD8^+$ Treg cells is even more efficient than direct recognition of donor cells to inhibit effector T-cell alloresponse.[25]

Therefore, therapeutic strategies converge to expand antigen-specific $CD8^+$ Treg cells. Indeed, in vivo administration of donor MHC or donor-derived peptide to the recipient before the graft expanded allospecific preexisting $CD8^+$ Treg cells that protected islet graft from rejection in mice and induced donor specific cardiac allograft tolerance in rat.[25] Proof of concept in a humanized mice model of GVHD showed that ex vivo expansion of naive precursors $CD8^{hi}$ Treg cells with donor APCs allowed to control GVHD through a CTLA4-dependent mechanism.[29] Finally, our preliminary results also showed that lower dose of donor specific HLA-A2 chimeric antigen receptor $(CAR)^+$ $CD8^+$ Treg cells than polyclonal ones would be sufficient to delay skin graft rejection in a humanized mice model (unpublished data).

Synergistic Roles of $CD4^+$ and $CD8^+$ Treg Cells to Suppress

We and others have demonstrated that $CD4^+$ and $CD8^+$ Treg cells synergize to induce tolerance in murine models. Heinrichs et al[14] demonstrated the beneficial effect of combining $CD4^+$ and $CD8^+$ Treg cells in a model of GVHD/GVL in mice. Indeed, $CD4^+$ and $CD8^+$ Treg cells were not able to control GVHD development separately but were efficient when combined. Moreover, addition of $CD8^+$ Treg cells allowed preserving the GVL effect that was abrogated by $CD4^+$ Treg cells alone. Similarly, in a model of tolerance to the cardiac allograft in rat induced by IL-34 treatment, we observed that adaptive transfer of IL-34–induced $CD4^+$ or $CD8^+$ Treg cells in a newly grafted recipient induced tolerance in 50% animals whereas transfer of total T cells protected all animals from graft rejection, suggesting a synergy between the $CD4^+$ and $CD8^+$ Treg cell populations.[24]

Suppression mechanisms used by $CD4^+$ and $CD8^+$ Treg cells are complementary to act on target cell panel. Whereas naive effector T cell response can be suppressed by $CD4^+$ Treg cells, memory T cells responses cannot.[125] Long et al[20] demonstrated that addition of $CD8^+$ Treg cells allowed to efficiently suppress memory effector T-cell responses. In addition, it is likely that the diversity of immunoregulatory cytokines produced by $CD4^+$ and $CD8^+$ Treg cells combined to diverse contact-mediated tolerogenic APCs, and anergic effector T cells create a propitious milieu for the recruitment and conversion of other regulatory cell types as an "infectious" tolerance.[124] For example, TGF-beta secreted by $CD8^+$ Treg cells is used for $CD4^+$ Treg cell expansion in cell therapy processes,[126] IL-10 is important for Foxp3 expression in $CD4^+$ Treg cells,[76] and we have shown that rat $CD8^+CD45RC^{low}$ Treg cells secrete IL-34 that induce Mreg, inducing in turn $CD4^+$ and $CD8^+$ Treg cells,[24] as well as FGL-2 that induce Breg cells,[23] and IFNγ that induce IDO expression in tolerogenic DCs.[21] Finally, the presence of alloantigen required for Treg cell generation and persistence is different and complementary regarding the life span and allogenicity of donor MHC-I and MHC-II.

Therefore, therapeutic strategies aiming to induce both $CD4^+$ and $CD8^+$ Treg cells are promising. Indeed, we recently demonstrated the induction of tolerance to the allograft in a rat model and in a model of GVHD in humanized mice as a proof of concept by a short-term depletion of $CD45RC^{high}$ effector T cells promoting the long-term expansion of both $CD4^+$ and $CD8^+$ Treg cells in vivo.[26] In addition, we have shown that IL-34 treatment of the recipient induced simultaneously $CD4^+CD25^+$ and $CD8^+CD45RC^{low}$ Treg cells in a rat cardiac allograft model, and that both Treg cells were able of infectious tolerance when adaptively transferred in a newly untreated and grafted recipient.[24]

$CD8^+$ Treg Cells, a Promising Therapeutic in Transplantation

Clinical trials with Treg cells or Treg cells inducing regimens have started in the last years,[127,128] but to date, there are no clinical trials embarking $CD8^+$ Treg cells despite their potential that they have either alone or associated to other regulatory populations. Indeed, the possibility of synergy between $CD4^+$ and $CD8^+$ Treg cells has been shown[14] and should be considered to be applied more widely, because each cell type could use different but complementary mechanisms of action and most of all MHC-I– or MHC-II–dependent activation for $CD8^+$ and $CD4^+$ Treg cells, respectively, is an important difference.

Ex Vivo Expanded $CD8^+$ Treg Cells and Cell Therapy

$CD8^+$ Treg cells have demonstrated a great capacity to expand ex vivo to be used to suppress alloimmune responses through adaptive cell transfer. In a major mismatch and haploidentical murine model of hematopoietic stem cell transplantation, it has been demonstrated that alloreactive expanded $CD4^+$ Treg cells efficiently prevented the GVHD but abrogated the GVL effect, and alloreactive expanded $CD8^+$ Treg cells less efficiently prevented the GVHD but spared the GVL effect. They showed that a combination of both $CD4^+$ and $CD8^+$ Treg cells efficiently achieved the optimal goal in bone marrow transplanted mice by preventing the GVHD and preserving the GVL effect.[14]

Expansion of naive human $CCR7^+CD8^+$ Treg cells in the presence of low-dose anti-CD3 and IL-15 upregulates expression of Foxp3, CD25, and CD103 and their ability to suppress in a contact-dependent manner $CD4^+$ effector T-cell response.[90] Naive $CD8^+$ T cells expanded in the presence of donor bone marrow–derived dendritic cells, TGF-β1, retinoic acid, and IL-2 gave rise to antigen-specific $CD25^+Foxp3^+CD8^+$ Treg cells able to inhibit full MHC-mismatch skin allograft in mice in a contact-dependent manner.[7] In a model of antigen-specific TCR transgenic mice and islet allograft, bystander central memory, and not effector memory, $CD8^+$ Treg cells were potent suppressors through TGF-β upon adaptive cell transfer.[129] Adaptive transfer of $CD122^+PD\text{-}1^+CD8^+$ Treg cells in the presence of low-dose IL-15 resulted in significant expansion and protection of skin allograft in mice dependent

on their expression of Fas ligand, IL-10 production, and PD-1 signaling was required.[19,130] Moreover, CD122$^+$PD-1$^+$CD8$^+$ Treg cells were more efficient at inhibiting allograft rejection of pancreatic islets than CD4$^+$CD25$^+$ Treg cells in the presence of IL-15.[12] Human CD25$^+$Foxp3$^+$TNFR2$^+$PD-L1$^+$CD8$^+$ Treg cells expanded 1 week ex vivo with anti-CD3/28 beads and IL-2 or TGF-β1 have strong protective properties in xeno-GVHD model in immunodeficient mice.[131] Human CD8$^+$CD45RC^{low} Treg cells could be efficiently expanded using high-dose IL-2 and IL-15 combination to a subset expressing high level of Foxp3, IL-34, IL-10, TGF-β, and intermediate level of IFNγ that subsequently efficiently inhibited human skin graft rejection and GVHD in humanized NOD scid gamma (NSG) mice in vivo upon adaptive cell transfer.[36]

This beneficial effect of the cytokines ex vivo suggests also that they could be beneficial in vivo if given to the recipient concomitantly to the Treg cell therapy (cf below). It is possible, as for CD4$^+$ Treg cells, that different subsets have different proliferation capacity and that naive CD45RA$^+$CD8$^+$ Treg cells expand more than memory CD45RA$^-$CD8$^+$ Treg cells, but this has not yet been investigated. In addition, the recent description of the existence of stem cell memory CD4$^+$ Treg cells with renewal capacity leads to the question of the existence of such subset within CD8$^+$ Treg cells that could give a tremendous advantage for cell therapy in the context of organ transplantation, but this also requires further investigations.

In Vivo CD8$^+$ Treg Cells Activation and Expansion

There are now numerous animal models that demonstrate that CD8$^+$ Treg cells can be induced in vivo using different strategies and the key role of those induced CD8$^+$ Treg cells in controlling transplant rejection or autoimmune reactions of the host.[21,24,25,132,133] Indeed, CD8$^+$CD28^{low} Treg cells in mice are naturally present in the periphery, emerging from the thymus, under the influence of AIRE, the Autoimmune regulator.[134,135] Thus, most of these strategies aim to tip the balance between effector and regulatory cells. Indeed, for example, it has been shown that some strategies targeting effector T cells, with more or less efficacy, leave a niche for regulatory Treg cell homeostatic proliferation to suppress the immune reactions.[26,136-138]

We have shown that in a model of cardiac allograft transplantation in the rat, CD8$^+$ Treg cells have antigen-specific properties and are expanded in number and function in the presence of a donor allograft and a tolerogenic regimen, such as CD40Ig, a molecule blocking costimulatory interaction and activation.[21] Using CD8$^+$ Treg cells properties, such as antigen specificity (peptides derived from MHC class II molecules of the donor were identified) or suppressive cytokine acting on allogeneic macrophages (IL-34), we have successfully expanded CD8$^+$ Treg cells in number and function (and with a specificity to the donor) and have been able to inhibit allograft rejection and induce tolerance (no lesions of chronic rejection which in such a stringent model is very difficult to induce) for several generations upon adaptive CD8$^+$ Treg cell transfer to newly grafted recipients.[24,25]

We recently demonstrated that approaches targeting the CD45 molecule using monoclonal depleting antibodies in rat model of transplantation can deplete CD4$^+$CD45RC^{high} and CD8$^+$CD45RC^{high} that have been shown to trigger organ rejection while sparing CD4$^+$CD45RC^{low} and CD8$^+$CD45RC^{low} Treg cells.[21,26,49,107,139,140] Although the anti-CD45RB treatment has been shown to increase CD122$^+$CD8$^+$ anti-Qa1 Treg cells splenic percentage and proliferation in mice, transplant tolerance induction by anti-CD45RB was not dependent on CD8$^+$ Treg cells because Qa-1-deficient recipients remain susceptible to the treatment.[16] Anti-CD3 OKT3 mAb was shown to prevent GVHD after in vivo administration within 48 hours or in vitro incubation of the cells before injection in humanized mice.[141] Modified OKT3-γ1(Ala-Ala2) antibodies administration in human patients resulted in CD8$^+$CD25$^+$ Treg cell expansion (of Foxp3$^+$ phenotype),[142] but this was not observed in mice.[137] Anti-CD3 mAb was also shown to induce in vitro CD8$^+$Foxp3$^+$ Treg cells from PBMCs of patients with rheumatoid arthritis, supported by p38 phosphorylation and by monocytes expressing TNFα and CD86.[143] Immunization of mice with antigen and anti–4-1BB (CD137) mAb generated antigen-specific CD8$^+$ Treg cells efficiently inhibiting CD4$^+$ effector T-cell responses in an IFNγ and TGFβ-dependent manner.[88]

Douillard et al[144] showed for the first time that donor-specific blood transfusion induced donor-specific CD8$^+$ Treg cells that infiltrated the graft in a rat model of cardiac transplantation tolerance. These cells displayed a predominant tVbeta18-Dbeta1-Jbeta2.7 TCR rearrangement. Treatment with a depleting anti-CD8 mAb or anti-TCR Vbeta18-Dbeta1-Jbeta2.7 DNA vaccination resulted in abrogation of allograft tolerance.[144,145] Liu et al showed that multiple donor blood transfusions could indeed induce CD8$^+$ Treg cells expressing Foxp3 in a different model of allo-incompatibility in rat.[132] CD8$^+$ Treg cells can also be expanded by disease-relevant peptide-major histocompatibility complexes coated on nanoparticules to prevent the disease. The group of Santamaria has shown that the use of nanoparticules coated with MHC-I loaded with specific peptides induced memory CD44$_+$CD122$_+$CD8$_+$ Treg cells that inhibited T1D and colitis acting in an IFNγ-, IDO-, and perforin-dependent manner.[146,147] This approach could be used with alloantigens to increase CD8$^+$ Treg cells in transplantation. A single 16 amino acid donor-derived peptide has been shown to induce heart transplant tolerance in fully incompatible rats when given during 28 days, starting 7 days before transplantation without any other treatment.[25] adenoassociated virus-mediated expression of donor MHC molecule in a model of liver transplantation in mice efficiently promoted the emergence of a subset of allospecific CD8$^+$ Treg cells.[15]

Low-dose IL-2 administration in mice and human demonstrated a high sensitivity of CD25$^+$Foxp3$^+$CD8$^+$ Treg cells to IL-2 for expansion and activation and suggested some similarities to CD4$^+$ Treg cells.[109] In nonhuman primates, low-dose IL-2 administration led to expansion of CD45RA$^-$Foxp3^{high}CD8$^+$ Treg cells.[148] In mice, Foxp3$^+$CD8$^+$ Treg cells were massively and specifically expanded by rapamycin and IL-2 antibody complexes to prevent GVHD.[10] In contrast, Hirakawa et al[149] showed that low-dose IL-2 administration did not induce CD8$^+$Foxp3$^+$ Treg cells but rather subsets of CD4$^+$ Treg cells and NK cells. Costimulation blockade with CTLA4Ig in a mice model of lung transplantation acceptance was associated and dependent on central memory CD44^{high}CD62L^{high}CCR7$^+$CD8$^+$ Treg cells infiltrating the graft and producing IFNγ.[11] Dexamethasone, a potent depleting treatment, efficiently expanded CD8$^+$ Treg cells while

decreasing CTLs and conferred a protective role for $CD8^+$ cells in trombocytopenia.[150]

$CD8^+$ Treg cells can also be induced in vitro or in vivo under the influence of other cell subsets, such as dendritic cells. In a primate renal allograft model, prolonged allograft survival was associated with antigen-specific eomesodermin^{low}CTLA4^{high}$CD8^+$ memory Treg cells that were induced by CTLA4Ig blockade and regulatory DC infusion.[133] Plasmacytoid dendritic cells have significant regulatory potential and CD40L-activated pDCs or tumor-associated pDCs have been shown to induce IL-10 producing $CD8^+$ Treg cells in human.[151,152] In mice, treatment with autologous tolerogenic DCs and anti-CD3 in a model of skin transplantation prolonged graft survival, and this was associated with the accumulation in draining lymph nodes of $CD8^+CD11c^+$ T cells and transfer of these cells prolonged graft survival in naive recipients.[13] Human monocyte-derived DCs reduced GVHD in an immune humanized mouse model of GVHD, and this was mediated by the induction of $CD8^{high}CD103^+Foxp3^+$ Treg cells.[34]

Some Immunosuppressive Agents "do no harm"to $CD8^+$ Treg Cells

Immunosuppressive agents have made possible and considerably enhanced allograft survival in human in the short-medium term. Unfortunately, in the long term, they have modestly improved the survival of the graft and the development of chronic rejection lesions, and they also lead to dramatic secondary effects for the patient. Next to that, regulatory cells used for cell therapy in transplantation will have to be combined with these immunosuppressive agents before they become a clinical reality. Zhang et al[153] have shown that rapamycin improved Foxp3 stability in murine-induced $CD8^+$ Treg cells (to a lesser extent than in $CD4^+$ Treg cells). Rapamycin has also been shown to increase the numbers of $CD103^+CD8^+$ Treg cells using human PBMCs; in contrast, cyclosporine A had no effect and prednisolone was deleterious.[154] In kidney transplant patients treated with rapamycin, $CD28^-CD8^+$ Treg cells were increased in the blood 2 years after transplantation.[155] In mice, $Foxp3^+CD8^+$ Treg cells were inhibited by cyclosporine while they were massively and specifically expanded by rapamycin and IL-2 antibody complexes to prevent GVHD.[10] In vitro expansion in the presence of rapamycin has been shown beneficial for $CD4^+CD25^+Foxp3^+$ Treg cells,[156] and we recently showed that it improves both expansion fold and suppression capacity of human $CD8^+CD45RC^{low}$ Treg cells.[36] In contrast, we observed a strikingly deleterious effect of mycophenolate mofetil, a selective inhibitor of the de novo pathway for purine synthesis used exclusively by T and B cells, which inhibited the expansion of the $CD8^+CD45RC^{low}$ Treg cells and decreased suppression. Other immunosuppressors that we tested (methylprednisolone, tacrolimus, cyclosporine A) did not alter $CD8^+$ Treg cell function and expansion.

Future Directions and Challenges

More work is needed to identify specific surface marker (s) that will help isolate and study $CD8^+$ Treg cells as well as to understand the role of Foxp3 for these cells. In the quest of a good marker, the application of single cell technologies to understand T cell heterogeneity will be of high value. As suggested by the literature, it is probable that there are, as it is the case for $CD4^+$ Treg cells, $Foxp3^+$ and $Foxp3^-$ $CD8^+$ regulatory cells. The origin and stability of $CD8^+$ Treg cells is also a current debate, and we must take into consideration the potential plasticity of those cells, as shown by the fact that several protocols have been suggested to maintain $CD8^+$ Treg cell phenotype and increase suppressive potential and number. Recently, the world of cell therapy was shaken by the tremendous results obtained in cancer with the CAR $CD4^+$ and $CD8^+$ T effector cells. These CARs have the advantages of redirecting the specificity of the cells to the target (in passing, boosting their capacity) thus focusing their number and activity where they should. Very recently, 3 groups showed that anti–HLA-A2–specific CAR-$CD4^+$ Treg cells in xeno-GVHD and skin transplantation in immunodeficient mice humanized with human PBMCs displayed increased suppressive capacity compared to control $CD4^+$ Treg cells.[157-159] To date, there are no publications describing CAR-$CD8^+$ Treg cells. This new technology opens several questions and opportunities. What is the potential of CAR-$CD8^+$ Treg cells? Will allograft-redirected CAR-$CD8^+$ Treg cells last long? As in the cancer field,[160] could allograft-redirected CAR-$CD8^+$ Treg cells synergize with CAR-$CD4^+$ Treg cells? The use of such tools could bypass the need for driving antigen specificity in ex vivo expansion protocol to boost the efficacy of the $CD8^+$ Treg cells[25,161]; however, we may still be too early to fully understand whether CAR stimulation will results in similar Treg cell activation and behavior as compared with Treg cells stimulated with donor antigens[122] and high-density single-cell technologies will be also of great interest here. The differences in their mechanisms of action and potential synergy of $CD8^+$ and $CD4^+$ Treg cells are another intriguing possibility that will need to be fully explored in the future.

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