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CD8+ Tregs in Lupus, Autoimmunity, and Beyond

Ravi K Dinesh[a], **Brian J Skaggs**[a], **Antonio La Cava**[a], **Bevra H. Hahn**[a], and **Ram Pyare Singh**[a,*]

[a]Division of Rheumatology, Dept of Medicine at the David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, 90095-1670

Abstract

While $CD4^+CD25^{high}$ regulatory T cells (Tregs) have garnered much attention for their role in the maintenance of immune homeostasis, recent findings have shown that subsets of $CD8^+$ T cells ($CD8^+$ Tregs) display immunoregulatory functions as well. Both $CD4^+$ Tregs and $CD8^+$ Tregs appear impaired in number and/or function in several autoimmune diseases and in experimental animal models of autoimmunity, suggesting the possibility of immunotherapeutic targeting of these cells for improved management of autoimmune conditions. Our group has developed a strategy to induce $CD8^+$ Tregs in autoimmune mice through the use of a tolerogenic self-peptide, and new information has been gained on the phenotype, function and role of induced $CD8^+$ Tregs in autoimmunity. Here we present an overview of the role and mechanisms of action of $CD8^+$ Tregs in autoimmunity, with a special focus on lupus. We also discuss the potential role of $CD8^+$ Tregs in other diseases, including chronic infection and cancer.

Keywords

Autoimmunity; Systemic Lupus Erythematosus; $CD8^+$Tregs; Immune tolerance/ Suppression

*To whom correspondence may be addressed. rpsingh@mednet.ucla.edu TEL: 310 267 5749; FAX: 310 206 8606.

Take-home messages

- The most common $CD8^+$ Treg surface markers reported are CD25 and a lack of CD28; others are Foxp3, CD103, CD122, CTLA-4, and GITR.
- $CD8^+$ Tregs suppress through a variety of mechanisms that include secretion of cytokines, cell-cell contact, induction of anergy in APC, and cytotoxicity.
- Tolerization using peptide therapy has been shown to be successful in alleviating murine lupus. Studies suggest that $CD8^+$ Tregs could be important for the proper function of Tregs.
- The suppressive function of $CD8^+$ Tregs isolated from SLE patients appears impaired.
- Studies on patients with refractory lupus who have undergone hematopoietic stem cell transplantation (HSCT) indicate that the induction of a population of $CD8^+$ Tregs plays a major role in the attenuation of disease.
- The role of induced $CD8^+$ Tregs in a variety of other murine models of autoimmune conditions has been detailed. These Tregs vary in their mechanisms of suppression and markers. Further research on $CD8^+$ Tregs as therapeutic targets is likely to lead to findings with a great potential for translational significance.

1. Introduction

The first mention that $CD8^+$ T cells were capable of suppressing other immune cells was reported in the 1970s [1]. After that, in the 1970s and early 1980s, the immunological research on cells with an inhibitory capacity favoring tolerance focused on $CD8^+$ T cells, but this work fell out of favor due to methodological and technological limitations [2-6]. Renewed interest in regulatory T cells followed the discovery of $CD4^+CD25^+$ Tregs some decades later, as these cells were found to suppress effector T cells and inflammatory cytokines and thus play an important role in immune homeostasis and tolerance [7-11]. Since then, multiple subsets of $CD4^+$ T cells, $CD8^+$ T cells and B cells have been found to manifest immunoregulatory properties [12-16].

For $CD8^+$ Tregs, multiple subsets have been identified and found to suppress through different mechanisms that include direct cell-cell contact with target cells, the secretion of cytokines, and the induction of anergy in antigen presenting cells. Despite significant advances, problems remain in the identification of specific marker(s) that could differentiate $CD8^+$ Tregs from non-regulatory $CD8^+$ T cell subsets. So far, $CD8^+$ Tregs with immunosuppressive properties have been described in pathologies ranging from autoimmunity to cancer and viral infection [16-22]. In particular, $CD8^+$ Tregs have been found impaired in numbers and/or function in patients with autoimmune diseases and in experimental animal models of autoimmunity [23, 24].

Work in our and other laboratories has focused on the induction of regulatory $CD4^+$ and $CD8^+$ T cell populations in lupus and in other autoimmune diseases, and it has been shown that such induction can be beneficial in alleviating disease. The development of autoimmunity is contingent on polygenic predisposition and the presence of environmental factors that can provoke an imbalanced immune response against self-components, with a resulting loss of immune tolerance. Delineating the role and function of immunoregulatory cell populations has the potential to open the door for immunotherapy in autoimmune diseases.

2. Natural and Induced Regulatory T cells

Tregs are often classified as either “natural” or “induced”. Natural Tregs are long-lived and develop in the thymus, while induced Tregs are thought to arise in the periphery following stimulation. Natural $CD8^+$ Tregs include $CD8^+CD25^+$ T cells, which share functional and phenotypic similarities with $CD4^+$ Tregs, expressing surface CTLA-4, GITR and intracellular Foxp3 [25]. $CD8^+CD25^+$ Tregs suppress $CD4^+CD25^-$ T cells in a membrane-bound-TGFβ- and CTLA-4-mediated contact-dependent manner that induces a IL-2Rα downregulation in the target T cells [25]. Another subset of natural $CD8^+$ Tregs are $CD8^+CD122^+$ T cells that have been shown to alleviate experimental autoimmune encephalomyelitis (EAE) upon adoptive transfer [26,27]. Finally, naturally occurring human $CD8^+CXCR3^+$ T cells secrete IL-10 and suppress IFNγ production, in a manner similar to murine $CD8^+CD122^+$ Tregs [28].

Most of the reported $CD8^+$ Tregs subtypes are induced. These include suppressor $CD8^+CD103^+$ T cells induced by allostimulation and augmented in cultures with IL-10, IL-4, and TGFβ [29], or $CD8^+CD103^+$ $Foxp3^+$ cells expressing GITR and CTLA-4 and induced by stimulation with viral peptides [30]. One study reported that $CD8^+CD103^-$ T cells could be induced to become regulatory $CD8^+CD103^+$ cells by alloantigen stimulation or treatment with TGFβ *in vitro*; these $CD8^+$ Tregs suppressed in a cell-contact dependent manner that appears to play an important role in liver allograft tolerance [31]. In a study of kidney allograft survival, IL-10-producing $CD8^+$ γδ Tregs were found to be responsible for the increased survival of transplanted mice that received repeated feeding of an oral alloantigen [32]. $CD8^+CD28^-$ Treg cells were also induced by incubation with xenogeneic antigen presenting cells (APCs) [33]

or allogeneic stimulation [34]. Non-antigen-specific $CD8^+CD28^-$ Tregs have also been generated and suppress antigen-specific T lymphocytes and cytotoxic T lymphocytes after culture of PBMCs with GM-CSF, IL-10, IL-2, and autologous monocytes [35,36]. Other types of induced Tregs include $CD8^+CD25^+Foxp3^+$ suppressor T cells that can be induced through co-incubation with $CD14^+$ monocytes and continuous antigen stimulation [37]; $CD8^+CD25^+Foxp3^+CTLA\text{-}4^+$ T cells arising in co-cultures with LPS-activated dendritic cells (DC) [38]; and $CD8^+CD25^+Foxp3^+LAG3^+CCL4^+$ T cells arising upon antigen stimulation with Bacillus Calmette–Guerin [39]. Induced CD8 inhibitory populations also include $CD8^+CCR7^+CD45RO^+$ T cells induced by plasmacytoid DCs from tumor ascites that suppress through IL-10 [40] and $CD8^+CD27^+CD45RA^+$ cells induced in co-cultures with CD40 ligand–activated plasmacytoid DC that suppress through IL-10 [41]. Induction of alloantigen-specific $CD45RO^+CCR7^-$ memory $CD8^+$ Tregs expanded from $CD8^+CD25^-$ T cells *in vitro* through CD40-activated B cells depends on IFNγ, IL-2, IL-4, and CTLA-4; these Tregs are $CD8^{high}$ and express Foxp3, CD25, CD27, CD28, and CD62L [42]. One subset of $CD8^+$ Tregs suppressed T effector cell function in healthy human subjects after injection of immature DC pulsed with influenza matrix peptide [43]. Recently, in a model of Anterior Chamber-Associated Immune Deviation (ACAID), it has been found that ACAID-induced $CD8^+$ Tregs secrete TGFβ and express CD94 and NKG2A [44]. While the role of IL-2 and TGFβ in the induction of $CD4^+CD25^+$ Tregs is well established [45], the methods of induction of $CD8^+$ Tregs are as diverse as the subsets reported. Table 1 lists the $CD8^+$ Tregs currently identified in the literature.

3. Markers of CD8+ Tregs

A specific marker for identification of $CD8^+$ Tregs is still elusive. Many of the markers for subsets of $CD8^+$ Tregs overlap with markers for $CD4^+$ Tregs, e.g. surface CD25 [25,30,38, 39,46] and intracellular Foxp3 [27,30,37,39,46-49]. While Foxp3 expression has been suggested as a unique marker for the identification of both $CD4^+$ Tregs and $CD8^+$ Tregs, the finding that TCR activation also upregulates Foxp3 expression in cells without significant regulatory capacity [10,50-52] diminishes enthusiasm for this idea. It has been argued that non-regulatory $Foxp3^+$ T cells could represent a dormant reservoir with the potential to become regulatory cells after homeostatic expansion [53]. Additionally, Foxp3 is expressed in human and murine non-lymphoid cells [54-56], and in humans non-regulatory cells can display transient upregulation of $Foxp3^+$ [57,58]. It has been debated in the literature whether CD28 is present [47,48] or absent [33,35,59-62] on the surface of $CD8^+$ Tregs. Data from our lab suggest that Foxp3 expression might represent a better indicator of a suppressive phenotype because both $CD28^-$ and $CD28^+$ $CD8^+$ Tregs that express Foxp3 can mediate suppression [47-49].

4. Mechanisms of Suppression

Not unlike their $CD4^+$ Treg counterparts [63], $CD8^+$ Tregs suppress through a variety of mechanisms that include secretion of cytokines, cell-to-cell contact, induction of a tolerogenic phenotype in APCs that can then induce regulatory $CD4^+$ T cells, and cytotoxic activity (Figure 1). In the case of suppression through cytokine secretion, different subsets of $CD8^+$ Tregs are reported to suppress through the secretion of different cytokines. Among the cytokines and chemokines reported to play a suppressive role are IL-10 [26,27,40,41,59,60,64,65], TGFβ [25,47-49,66-68], IFNγ [69], IL-16 [70], and CCL4 [39]. Some $CD8^+$ Treg subsets, in a manner similar to Tregs, can suppress through a cell contact dependent mechanism [29,30,37,46,60]. Additionally, membrane bound TGFβ and CTLA-4 play a role in cell-cell contact dependent mechanisms of $CD8^+$ Treg-mediated suppression [25,38].

$CD8^+CD28^-$ Tregs can also render APCs tolerogenic through the upregulation of inhibitory receptors such as immunoglobulin-like transcript (ILT)-3 and ILT-4 on APCs [71,72]. Tolerogenic APCs can then have an anti-inflammatory function and induce anergy and possible regulatory functions in $CD4^+$ T cells [71,73,74]. One study has shown a $CD8^+CD28^-$-mediated downregulation of the costimulatory ligands CD80 and CD86 on APCs as important in the suppression of $CD4^+$ T cell responses [75], and another study has reported that CD80 and CD86 play an important role in the suppressive activity of $CD8^+CD122^+$ T cells [76]. Finally, another mechanism of suppression for $CD8^+$ Tregs is cytolysis of antigen activated $CD4^+$ Th cells, which is dependent on the expression of the MHC class 1b molecule Qa-1 (HLA-E in humans) [77-80].

5. $CD8^+$ Tregs and Lupus

5.1 Lupus-prone Murine Models

We and others have developed strategies to tolerize lupus-prone mice with either histone-based peptides or peptides based on complementary determining regions of anti-dsDNA antibodies [68,81-85]. We have developed a tolerogenic 15-amino acid peptide – called pConsensus (pCons) – that is based on MHC class I and class II T-cell determinants in the V_H region of murine anti-DNA Ig [83-85]. Our model of SLE utilizes New Zealand black/New Zealand White F1 female (BWF1) mice, which spontaneously develop a disease that clinically and immunologically resembles human systemic lupus erythematosus (SLE). The administration of a high dose of pCons (1 mg i.v.) to BWF1 lupus-prone mice increased survival, delayed the onset of nephritis, decreased $CD4^+$ T cell-mediated secretion of IFNγ, and decreased serum levels of autoantibodies against dsDNA, nucleosomes, and cardiolipin [83,86]. This tolerance, and alleviation of disease, is mediated in large part by pCons-induced $CD4^+$ Tregs [87] and $CD8^+$ Tregs [49]. Initial studies [49] showed that following tolerization with pCons, BWF1 mice have an expansion of $CD8^+$ T cells and developed $CD8^+$ Tregs capable of suppressing anti-DNA IgG production (both *in vivo* and *in vitro*) and $CD4^+$ Th cell production of IFNγ *in vitro* [49]. The $CD8^+$ Treg cells isolated from spleens of tolerized mice suppressed target cells through the secretion of TGFβ. Additionally, silencing of Foxp3 with siRNA abrogated the ability of $CD8^+$ Tregs to suppress anti-DNA antibodies. We later showed that $CD8^+$ T cells from tolerized mice are capable to suppress $CD4^+$ Th cell proliferation from naïve mice *in vitro* [47,48]. Additionally, $CD8^+$ cells (both $CD28^+$ or $CD28^-$) from pCons-treated mice have upregulated Foxp3 expression, and the inhibitory function of tolerized $CD8^+$ T cells lasts for up to eight weeks after tolerization [47]. Interestingly, while both tolerized $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subsets are $Foxp3^+$, the $CD8^+CD28^-$ T cells have greater Foxp3 expression that also lasts for a longer period of time [47]. In either subset, gene-silencing of Foxp3 yields a decline of suppression and TGFβ production [47,48]. Incidentally, some tolerized $CD8^+$ T cells also display an increased expression of the cytotoxic effector molecule granzyme B and are weakly cytotoxic against B cells from nephritic mice [48].

Our most recent work has focused on programmed death 1 (PD-1), a membrane protein, that upon binding to its ligand PD-L1 inhibits positive signaling pathways, causing cell anergy and cytotoxicity [88-91]. We have shown that there is reduced mRNA and protein expression of surface PD-1 in $CD8^+$ T cells from pCons-tolerized mice [48]. Foxp3 expression decreased upon PD-1/PDL-1 blockade in T cells from tolerized mice. However, PD-1/PD-L1 blockade increased the expression Foxp3 in naïve T cells [48]. One possible explanation could be that downregulation of PD-1 is needed for the initial acquisition of suppressive capacity, but the complete elimination of this molecule leads to the disabling of $CD8^+$ Treg suppressive capacity and autoimmunity [48,92]. Therefore, PD-1 expression should be low to intermediate for $CD8^+$ Tregs to retain suppressive function. In support of this possibility, studies of chronic viral infection suggest that high expression of PD-1 is associated with increased rates of apoptosis and anergy along with the inability to be rescued by growth cytokines. In contrast,

cells with low to intermediate expression of PD-1 could be converted to cells capable of cytotoxicity for virus-infected cells [93-100].

Using the BWF1 SLE mouse model, Mozes' group studied Tregs in mice treated with a tolerogenic peptide based on the light chain complementarity determining region 1 (hCDR1) of human anti-dsDNA antibodies [81,101]. Tolerization with hCDR1 induced $CD4^+CD25^{high}$ Tregs and $CD8^+CD28^-$ Tregs in mice, which suppressed lymphocyte proliferation and autoantibody production, increased TGFβ and decreased IFNγ and IL-10 production in cultured splenocytes, and decreased lymphocyte apoptosis with down regulation of caspase-2, -8 and JNK and up-regulation of Bcl-xL [82,101-105]. The $CD8^+$ Tregs appear temporally essential in that model for the suppressive function, Foxp3 upregulation, and the expansion of $CD4^+CD25^+$ Tregs [105], suggesting that there is crosstalk between the two Treg subsets and that $CD8^+$ Tregs are necessary for the optimal function of $CD4^+CD25^{high}$ Tregs.

Kang et al. [68] conducted studies with a histone-derived tolerizing peptide ($H4_{71-94}$) in (SWR X NZB) SNF1 lupus-prone mice. SNF1 mice treated with a biweekly, subcutaneous low dose (1μg) of histone peptides had longer survival, a 50% decrease in anti-dsDNA autoantibodies, and reduced anti-nucleosome and anti-histone autoantibody production after three months of treatment. The attenuation of disease was concomitant with the expansion of $CD4^+CD25^+$ Tregs and $CD8^+$ Tregs. $CD8^+$ Tregs from the spleens of treated mice expressed TGFβ, CD62L, and GITR. The TGFβ secreted from $CD8^+$ Tregs was the mechanism primarily responsible for the observed decrease in autoantibody production.

Zheng et al. explored the role of $CD8^+$ and $CD4^+$ Tregs in murine Stimulatory Graft-vs. -Host Disease (SGVHD), a lupus-like disease [66]. SGVHD was induced in (DBA/2 × C57BL/6) F1 mice following the injection of T cells from DBA/2 mice. $CD4^+$ and $CD8^+$ T cells with inhibitory capacity favored an increased survival of mice and required IL-2 and TGFβ. The transfer of TGFβ-primed cells to diseased animals with high anti-DNA Ab levels increased survival and decreased anti-DNA autoantibodies and proteinuria in host mice.

Of note, many studies in lupus-prone mice share numerous similarities in their findings on induced $CD8^+$ Tregs despite the differences in the type of model used and the method of induction. Many times the suppression was mediated by TGFβ and decreased IFNγ, and the induced regulatory populations suppressed proliferation of responder $CD4^+CD25^-$ T cells *in vitro*. In the case of Foxp3 expression, our group [47-49] and Mozes' group [105] found that Foxp3 expression was upregulated in $CD8^+$ T cells following treatment with tolerizing peptides that were based on anti-dsDNA IgG. However, Kang et al. [68], found that only $CD4^+CD25^+$ T cells and not $CD8^+$ Tregs contained upregulated Foxp3 following tolerization with peptides based on histone sequences. While groups have defined $CD8^+$ Tregs as cells lacking surface CD28 (104), our studies [47] and Kang et al. [68] suggest that both $CD28^+$ and $CD28^-$ T cells can mediate suppression.

5.2 In Human SLE

Studies on T cells from lupus patients have shown conflicting results on the possible impairment of the regulatory compartment in SLE. Several studies have shown that $CD4^+CD25^{high}$ Tregs are reduced in number and/or have impaired function in SLE patients compared to controls [9,15,106,107], while other studies have shown the contrary [108,109]. Treatment of PBMCs isolated from SLE patients with anti-DNA IgG peptides has been shown to increase the number and suppressive function of $CD4^+CD25^{high}$ Tregs *in vitro* [110,111]. Similarly, some studies on $CD8^+$ Tregs in lupus patients have reported defective and/or reduced numbers [112,113], while another study found no difference in the number of $CD8^+$ Tregs in SLE patients [114]. Tulunay et al. [113] found that $CD8^+CD28^-$ T cells from healthy patients had higher mRNA expression of TGFβ when compared to $CD8^+$ $CD28^+$ T cells.

Filaci et al. [112] generated $CD8^+$ Tregs following incubation of purified $CD8^+$ T from SLE patients and healthy patients with IL-2 and granulocyte monocyte colony stimulating factor (GM-CSF). While there were no major phenotypic differences between $CD8^+$ Tregs from each group, $CD8^+$ Tregs from SLE patients with active disease showed no ability to suppress, while $CD8^+$ Tregs from patients in remission had a suppressive capacity similar to healthy controls. There were no differences in IL-10, IL-4, IFNγ, or TGFβ production between $CD8^+$ Tregs from subjects with and without disease, but $CD8^+$ Tregs from SLE patients produced lower levels of IL-6 and higher levels of IL-12 compared to healthy subjects. Regulatory function was dependent on IFNγ and IL-6. The authors hypothesized that the dysfunction of $CD8^+$ Tregs in SLE patients was possibly due to an imbalance between inhibitory (IL-6) and stimulatory (IL-12) cytokines.

In a recent study on the effects of autologous hematopoietic stem cell transplantation (HSCT) in refractory lupus patients, Zhang et al. [115] showed that post-transplant patients who improved had a definable difference in T cell function from non-responders. Specifically, there was an abrogation of pathogenic T cell activity (as indicated by a reduced IFNγ response following culture with histone peptides) and an increased IL-13 response. Importantly, there was also an increase in the number of $CD4^+CD25^{high}Foxp3^+$ T cells, $CD8^+Foxp3^+$ T cells, and $CD8^+CD103^+$ T cells. In short-term cell lines from post-transplant patients, $CD8^+Foxp3^+$ T cells had increased expression of Latency Associated Peptide (LAP), CD103, PD-1, PD-L1, and CTLA4. $CD8^+$ Tregs from post-transplant patients suppressed through the secretion of TGFβ and had a stronger suppressive function on the proliferation of $CD4^+CD25^-$ cells than $CD4^+CD25^+$ Tregs from patients. Suppression was contact-independent and both non-specific and autoantigen specific.

6. $CD8^+$ T Cells in Other Autoimmune Diseases

6.1 Rheumatoid Arthritis

Using a chimera model of RA (where synovial tissues from RA patients are grafted onto severe combined immunodeficiency (SCID) mice), Klimiuk et al. [70] found a marked reduction in lesional immune activity and tissue production of IFNγ, IL-1β, and TNF-α following adoptive transfer of autologous synovial tissue $CD8^+$ T cells into human synovium-SCID chimeras. Blocking experiments and *in vitro* culture experiments revealed that these $CD8^+$ Tregs suppressed through the secretion of IL-16. Davila et al. [75], working in a similar model, generated $CD8^+$ $CD28^-$ $CD56^+$ T cells with a suppressive capacity by stimulating PBMCs of healthy $HLA\text{-}A2^-$ donors with irradiated HLA-A2 myelomonocytic THP-1 cells. The $CD8^+CD28^-$ T cells suppressed in a contact-dependent fashion, and not through cytokine secretion. The adoptive transfer of $CD8^+CD28^-CD56^+$ T cells resulted in decreases in CXCL10 and Mig chemokines and transcription of IFNγ and TNF-α. Additionally, the $CD8^+$ Tregs induced tolerogenic cells by down regulating the costimulatory molecules CD80 and CD86 on APCs and synovial fibroblasts, respectively.

Seo et al. [69] showed that treatment with anti-4-1BB monoclonal antibody reduced serum antibodies to collagen type II and $CD4^+$ T cell recall responses to collagen type II in a murine collage type II (CII)-induced arthritis (CIA) disease model. Treatment with the antibody was followed by the induction of antigen-dependent $CD8^+CD11c^+$ T cells that secreted IFNγ but not TGFβ, IL-10, or IL-4. The secretion of IFNγ by these $CD8^+CD11c^+$ cells increased indoleamine 2,3-dioxygenase (IDO) in $CD11b^+$ monocytes and $CD11c^+$ DC, and the accumulation of IDO in these cells led to subsequent suppression of antigen-specific $CD4^+$ T cells in the Th1 cell-mediated model of CIA.

More recently, Notley et al. [116] showed that CIA mice treated with a single injection of anti-CD3 antibodies experienced a marked decrease in disease severity as well as reduced joint

damage and inflammation concomitantly with an expansion of $CD8^+$ T cells in lymph nodes and increased percentages of peripheral $CD4^+CD25^+Foxp3^+$ Tregs and $CD8^+CD25^+Foxp3^+$ Tregs. Of note, the $CD4^+CD25^+Foxp3^+$ Tregs from treated mice did not display any differential ability to suppress effector T cells, IFNγ production or IL-17 production, while induced $CD8^+CD25^+Foxp3^+$ T cells in the anti-CD3 treated mice were capable of suppressing effector T cells, IFNγ production, and IL-17 production *in vitro*.

6.2 Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease that leads to the demyelination of the central nervous system (CNS) and serves as an experimental model for human multiple sclerosis (MS) [117]. Working in a model of EAE induced by the MOG_{35-55} peptide, Lee et al. [118] found that depletion of $CD122^+$ cells in EAE mice caused an increase in clinical measures of EAE, an increase in IFNγ production, and a decrease in IL-10 production. $CD8^+CD122^+$ cells from naïve mice were capable of suppressing $CD4^+CD25^-$-mediated production of IFNγ *in vitro*, and adoptive transfer of $CD8^+CD122^+$ Tregs to EAE mice alleviated disease, increased IL-10 production, and suppressed inflammatory cytokine production and CNS infiltration. Zozulya et al. [119] have also explored the role of $CD8^+CD122^+$ T cells in the alleviation of EAE. It was known that treatment of mice with DC loaded with self peptide lead to an acceleration and exacerbation of disease that was paralleled by an accumulation of $CD4^+$ and $CD8^+$ T cells in the CNS [120]. However, treating mice with DC deficient for PD-L1 lead to amelioration of EAE concomitantly with the recruitment of $CD8^+CD122^+$ Tregs into the CNS, restating the link between PD-L1 and DC with the recruitment of $CD8^+CD122^+$ Tregs [119].

Chen et al. [121] described the existence of $CD8^+$ Tregs that express the latency-associated peptide (LAP) on their cell surface and suppress in a TGFβ dependent manner *in vivo* in EAE. $CD8^+LAP^+$ cells from naïve mice were able to suppress $CD4^+CD25^-LAP^-$ T cells *in vitro* and alleviated EAE *in vivo*. While only a small percentage of $CD8^+LAP^+$ cells expressed Foxp3, the overall expression of Foxp3 in $CD8^+LAP^+$ T cells was higher than in $CD8^+LAP3^-$ T cells. In addition, $CD8^+LAP^+$ cells had increased expression of CTLA-4 and decreased expression of CD45 and CD25 when compared to $CD8^+$LAP- cells.

Tennakoon et al. [79] showed that MS patients treated with Glatiramer Acetate (GA), a synthetic peptide shown to be effective in reducing the number of new lesions in relapsing remitting MS, have an expansion of GA-specific $CD8^+$ T cells with suppressive capacity, as compared to untreated patients. These $CD8^+$ Tregs from GA-treated patients suppressed $CD4^+$ T cells in a contact-dependent manner, expressed perforin, and exhibited HLA-E-restricted cytotoxicity.

6.3 Inflammatory Bowel Disease (IBD)

Intestinal Bowel Disease (IBD) describes a group of inflammatory diseases that afflict the small intestine and large intestine, including Crohn's disease (CD) and ulcerative colitis (UC). Menager-Marcq et al. [60] studied $CD8^+$ Tregs in a murine experimental model of IBD-like disease generated by the injection of $CD4^+$ $CD45RB^{high}$ cells into RAG-2 deficient mice. $CD8^+CD28^-$ T cells from naïve mice prevented disease upon adoptive transfer. Interestingly, neither $CD8^+CD28^+$ T cells from unmanipulated mice nor $CD8^+CD28^-$ T cells from IL-10-deficient mice had any suppressive effect in this model. Moreover, when disease was induced with $CD4^+CD45RB^{high}$ T cells from mice without the ability to respond to TGFβ, $CD8^+CD28^-$ T cells were unable to attenuate disease, restating the importance of TGFβ in the mechanisms of $CD8^+CD28^-$ Treg-mediated suppression. Ho et al. [67] used a mouse model of Crohn's Disease that featured over expression of tumor necrosis factor to show that $CD8^+$ Tregs expressing $CD44^-CD103^+$ secreted TGFβ, inhibited *in vitro* $CD4^+$ T cell proliferation, and

attenuated ileitis in immunodeficient $RAG^{-/-}$ mice upon adoptive transfer. These Tregs did not secrete IL-10 or IFNγ.

6.4 Myasthenia Gravis

Mysathenia Gravis (MG) is an autoimmune disease in which weakness and fatigue are caused by the disruption of neurotransmission due to autoantibodies against the acetylcholine receptor (AchR). Ben-David et al. [122] found that mice with experimental autoimmune MG (EAMG) treated with a dual altered peptide ligand (made of two tandemly arranged single amino acid analogs of two myasthenogenic peptides) decreased the clinical manifestations of EAMG, MG-lymph node T cell proliferation, and IFNγ secretion concomitantly with the induction of Foxp3-expressing $CD8^+CD28^-$ T cells. Experiments with CD8 knockout mice suggested that $CD8^+$ Tregs were necessary for the decreased lymph node T cell proliferation and the secretion of IFNγ.

6.5 Autoimmune Diabetes

Evidence for a role for $CD8^+$ Tregs in Non Obese Diabetic (NOD) mice has been available for more than a decade. Harrison et al. [123] found that treatment of NOD mice with aerosol insulin after onset of clinical disease alleviated the disease concomitantly with the induction of $CD8^+$ γδ T cells with regulatory properties. Wang et al. [124] found that adoptive transfer of LPS-stimulated splenocytes retrovirally transduced with a IgG fusion construct of glutamate decarboxylate 65 (GAD) had an alleviation of disease paralleled by the rise of $CD4^+$ Tregs and $CD8^+Foxp3^+$ Tregs. In vivo transfer of retrovirally-transduced splenocytes that were either depleted of $CD8^+$ T cells or $CD8^+CD25^+$ T cells led to a decrease in $CD8^+Foxp3^+$ Tregs as well as a worsening of disease.

Bisikirska et al. [46] showed that type 1 diabetes patients treated with an anti-CD3 mAb had an attenuation of disease that corresponded with an increase in the number of $CD8^+CD25^+$ T cells and an expansion of the $CD8^+$ T cell compartment. Similar results were found in *in vitro* culture experiments that showed an expansion of $CD8^+$ T cells as well as the development of $CD8^+$ Tregs that expressed GITR and Foxp3 and suppressed $CD4^+$ T helper proliferation and antigen-specific responses.

7. CD8 Tregs in Cancer and Infectious Disease

The induction of $CD4^+$ Tregs and $CD8^+$ Tregs might be beneficial in attenuating autoimmunity and transplantation, but the same is not the case in cancer and chronic infection. In these diseases, induction of $CD8^+$ Tregs may not be beneficial and can instead be harmful to the host.

7.1 $CD8^+$ Tregs and infectious disease

The role of $CD8^+$ Tregs in infectious diseases is complex. While the suppression of immune responses can be harmful to the host by allowing for pathogenic persistence, regulatory T cell activity can limit tissue damage and pernicious inflammation. Chronic viral infection is a result of viral persistence resulting from inappropriate virus specific T-cell responses. $CD8^+$ Tregs mediate the persistence of infection by suppressing cytotoxic T cell responses through the secretion of anti-inflammatory cytokines [125-127]. $CD8^+$ Tregs subsets play a role in the persistence of infectious diseases such as Hepatitis C (HCV) [30,128,129], human immunodeficiency virus (HIV) [130-134] and Herpes Simplex Virus (HSV-2) infection [135]. It has been hypothesized that $CD8^+$ Tregs can both play an important role in viral persistence and serve an important function in protection from pathogenic tissue damage due to overwhelming anti-viral cytotoxic T cell responses [18]. Studies on PBMCs from HIV patients found increased IL-10-secreting $CD8^+$ Tregs that decreased the $CD8^+$ T cytotoxic

function in patients with progressive or active disease as compared to patients with non-progressing disease [130]. Stimulation with HIV antigens resulted in increased numbers of IL-10-producing $CD8^+$ T cells that suppressed through a cell-cell contact-dependent mechanism [130,131]. Another study on HIV patients found that PBMCs stimulated with HIV antigens increased TGFβ-producing $CD8^+$ Tregs that suppressed IFNγ production [136]. In animal models of Simian Immunodeficiency Virus (SIV), increased $CD4^+$ Tregs and $CD8^+CD25^+$ Tregs following infection have also been reported [133,134].

Furthermore, studies on PBMCs from HCV patients stimulated with HCV antigens found an IL-10-dependent induction of $CD8^+CD25^+$ Tregs expressing GITR and CTLA-4 that were capable to suppress IFNγ production in a contact-dependent fashion [30]. Of note, in many of these instances, IL-10 has a key role in allowing viral persistence through the suppression of anti-viral immune responses, and the blockade of IL-10 results in the increase of $CD8^+$ T cell production of IFNγ [128] and viral clearance [137,138].

7.2 $CD8^+$ Tregs and Cancer

While much work has been done on the role of $CD4^+CD25^+$ Tregs in cancer [139-142], the role of $CD8^+$ Tregs has only recently started to garner attention [143,144]. $CD8^+$ Tregs appear to be of detriment in cancer, as they may allow for the suppression of anti-tumor immunity that can lead to tumor growth [144]. Both $CD4^+$ Tregs and $CD8^+$ Tregs are found at higher percentages in tumor microenvironments [144-147]. Tumors secrete cytokines that create a milieu favorable for the recruitment and induction of $CD8^+$ Tregs [61,62]. Furthermore, plasmacytoid DC induce $CD8^+$ Tregs in human tumors [40]. $CD8^+CD28^-$ Tregs found among Tumor Infiltrating Lymphocytes have an increased functionality and suppressor activity that appears correlated with advanced stages of the disease [61], and in human colorectal cancer and prostate cancer, increased numbers of $CD8^+CD25^+Foxp3^+$ Tregs can better suppress effector T cells in the tumor microenvironment compared to regular tissue [62,145]. Interestingly, $CD8^+CD25^+$ T cells in prostate tumors can have their suppressive function silenced by using Toll-like Receptor 8 ligands [62].

8. Concluding Remarks

A multiplicity of different $CD8^+$ Tregs subsets appears to have different mechanisms of suppression, markers, and modalities of induction. This diversity is one of the field's most salient characteristics because there is so far no single molecule that can specifically identify $CD8^+$ Tregs, either naturally occurring or induced. On the other hand, the differences among subsets could be more than superficial and instead result from unique characteristics of the model studied and/or the methods of induction. Further research on surface markers, mRNA expression, and cytokine secretion is needed to identify unique and divergent characteristics of the multiple $CD8^+$ Treg subtypes. For now, the reemergence of $CD8^+$ Tregs in recent years after their original discovery over three decades ago has created an exciting new field of investigation. Of note, it has become clearer that $CD4^+$ and $CD8^+$ Tregs have overlapping and independent inhibitory roles. *In vivo* and *in vitro* experiments in murine lupus [105], murine MG [122], and human SLE [115] seem to suggest that $CD8^+$ Tregs might be necessary for the proper function of Tregs, indicating that a crosstalk between $CD4^+$ Tregs and $CD8^+$ Tregs might be in play in the mechanisms of immune tolerance and that bidirectional interactions between T cells and DC are responsible for the functionality of regulatory T cell populations [148].

Presently, a major problem in the study of $CD8^+$ Treg is the lack of a specific marker that unequivocally defines these cells as suppressors —a problem that also exists to a lesser degree for $CD4^+$ Tregs. Some of the best-described subsets of $CD8^+$ Tregs include induced and natural $CD8^+CD28^-$ T cells and $CD8^+CD25^+$ T cells, as well as naturally occurring $CD8^+CD122^+$ T

cells. Foxp3 expression has been looked at as a possible marker but it appears that different subsets may have not only diverse sets of markers but also very diverse methods of suppression. Some $CD8^+$ Tregs can suppress through cell-cell contact dependent mechanisms and others by secretion of cytokines/chemokines such as IL-10, TGFβ, and CCL4. Other mechanisms of suppression include the induction of tolerogenic APC or the exertion of cytotoxicity on target cells. It is not clear whether the different subsets may be completely distinct from each other or whether they may derive from a common precursor.

Here we outline the important consideration that $CD8^+$ Tregs, along with $CD4^+$ Tregs, play crucial roles in autoimmune diseases, infectious diseases, and cancer. The opposing nature of autoimmunity and proliferative diseases such as cancer suggest that therapies designed to target these cells and exploit their suppressive characteristics should be carefully designed to obtain clinical benefits. Considering that work in experimental models has shown that antigen-specific Tregs can prevent, attenuate, or reverse the course of autoimmune disease, further research is now required to finely tune the current information towards acquisition of the specific roles and function of $CD8^+$ Tregs for their use in settings with translational significance to humans.

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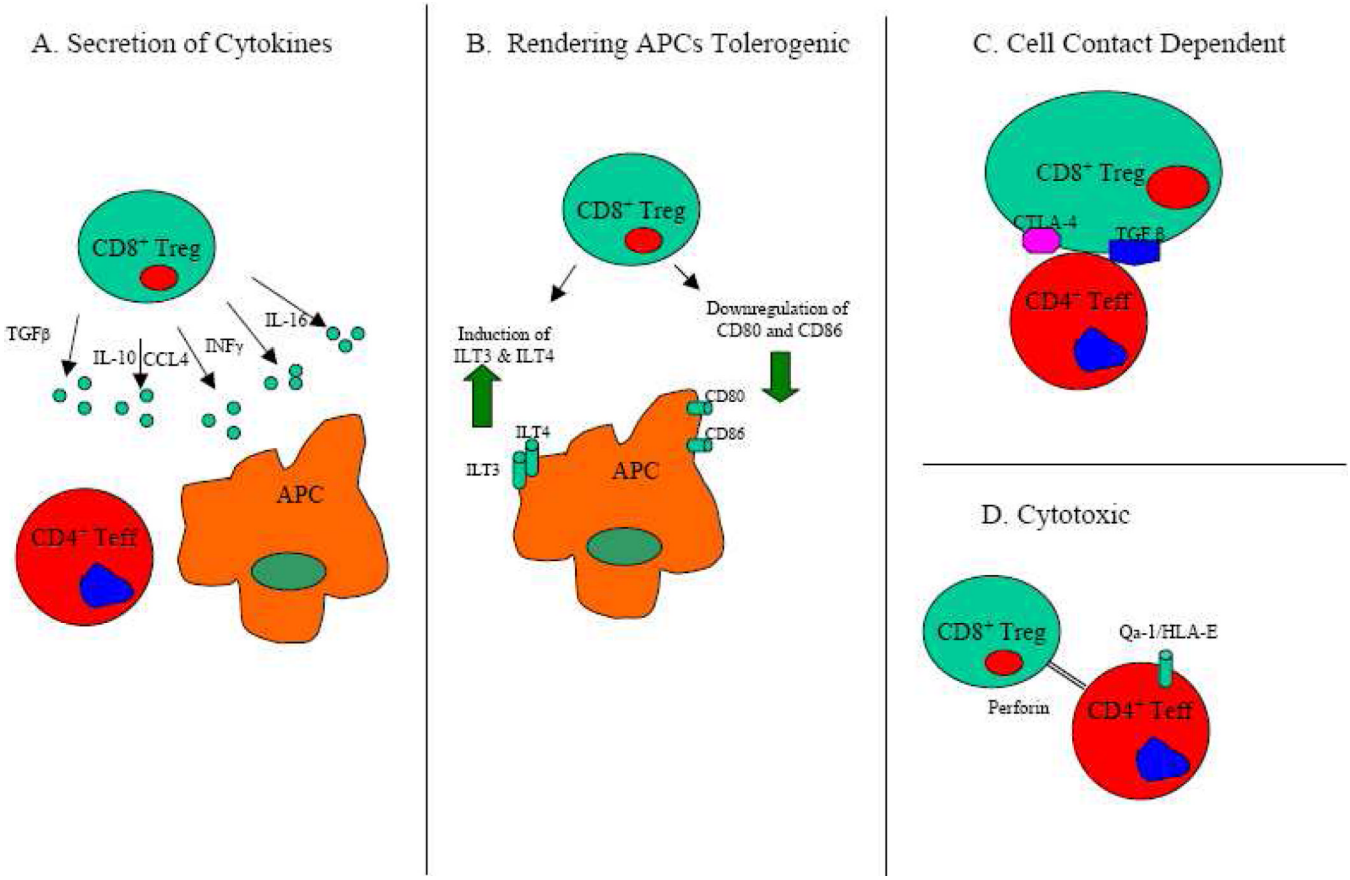

Figure 1. Mechanisms of Suppression of CD8$^+$ Tregs

A) CD8$^+$ Tregs secrete cytokines/chemokines such as IFNγ, TGFβ, IL-16, IL-10, and CCL4 that suppress immune responses. B) CD8$^+$ Tregs render APCs tolerogenic by downregulating stimulatory molecules, such as CD80 and CD86, and upregulating inhibitory receptors, such as ILT3 and ILT4. C) CD8$^+$ Tregs can suppress in a contact-dependent fashion that may be dependent on surface expression of molecules such as membrane-bound TGFβ or CTLA-4. D) MHC Class I restricted CD8$^+$ Tregs are capable to kill activated CD4$^+$ T effectors that express Qa-1/HLA-E.

Table 1

CD8$^+$ Tregs Subtypes

Table 1: CD8$^+$ Tregs Subtypes				
Subset	**Natural/Induced**	**Cells/Model Studied**	**Mechanism of Suppression**	**Citation**
CD8$^+$ CD28$^-$	Natural	Human cancer cells	IL-10 secretion	[61]
CD8$^+$CCR7$^+$ CD45RO$^+$	Induced	Human ovarian cancer	IL-10 secretion	[40]
CD8$^+$ CD28$^-$ Foxp3$^+$	Natural	Human prostate cancer	Cell-cell contact dependent	[62]
CD8$^+$ TGFβ$^+$ CD25$^-$ Foxp3$^-$	Induced	Human HIV patient PBMC	Secretion of TGFβ	[136]
CD8$^+$ IL10$^+$ CD25$^-$ Foxp3$^-$	Induced	Human HIV patient PBMC	Secretion of IL-10	[130-132]
CD8$^+$ CD28$^-$	Induced	Human lupus, lancer, HIV, Hashimoto's thyroiditis, and Graves' Disease patients	IL-10 secretion	[35,36,112]
CD8$^+$CD103^{high} LAP^{high} Foxp3$^+$	Induced	Human lupus	TGFβ secretion	[149]
CD8$^+$ CD25$^+$ Foxp3$^+$ LAG3$^+$	Induced	Human MTB patients and healthy donors	Secretion of CCL4	[39]
CD8$^+$CD25$^+$ D69$^+$ CTLA-4$^+$ Foxp3$^+$	Induced	Human PBMC from AS patients	CTLA-4 contact dependent	[38]
CD8$^+$CTLA4$^+$Foxp3$^+$ GITR$^+$	Induced	Human PBMC from HCV patients	Cell-cell contact dependent	[30]
CD8$^+$ CD28$^-$	Induced	Human PBMC from healthy honors	Induction of ILT3/ILT4 on APC	[33]
CD8$^+$ CD103$^+$	Induced	Human PBMC from healthy donors	Cell-cell contact dependent	[29]
CD8$^+$ CD25$^+$ CD28$^+$ Foxp3$^+$	Induced	Human PBMC from healthy donors	Cell-cell contact dependent	[37]
CD8$^+$ IL-10$^+$	Induced	Human PBMC from healthy donors	Secretion of IL-10	[41]
CD8$^+$ CD25$^+$ CTLA-4$^+$ Foxp3$^+$	Induced	Human PBMC from Type 1 diabetes and healthy patients	Cell-cell contact dependent	[46]
CD8$^+$ CD25$^+$ CTLA$^-$4$^+$ Foxp3$^+$ GITR$^+$	Natural	Human postnatal thymus specimens	TGFβ and CTLA-4 contact dependent	[25]
CD8$^+$ CD28$^-$ CD56$^+$	Induced	Human synovial tissue from RA Patients/ Human synovium-SCID mouse chimeras	Down-regulation of CD80 and CD86 on synovial fibroblasts	[75]
CD8$^+$ IL-16$^+$	Induced	Human synovium-SCID mouse chimeras	Secretion of IL-16	[70]
CD8$^+$ CD28$^-$	Induced	IBD induced by injection of CD4$^+$CD45RB^{high} cells into (RAG)-deficient mice	Secretion of IL-10,	[60]
CD8$^+$ Foxp3$^+$ PD-1$^+$	Induced	BWF1 lupus-prone Mice	Secretion of TGFβ	[47-49]
CD8$^+$ CD25$^+$ Foxp3$^+$	Natural	Cynomolgus Macaques and African Green Monkey SIV PBMC	Not described	[133,134]
CD8$^+$ CD28$^-$ Foxp3$^+$	Induced	Experimental murine model of Autoimmune Myasthenia Gravis	Reduction of IFNγ	[122]
CD8$^+$ CD11c$^+$	Induced	Murine CII-induced arthritis	IFNγ mediated induction of IDO in CD11$^+$b monocytes and CD11c$^+$ dendritic cells	[69]
CD8$^+$	Induced	Murine stimulatory graft vs. host Disease	Partly TGFβ dependent	[66]
CD8$^+$ CD122$^+$	Natural	Normal mice and CD122 deficient mice	Secretion of IL-10	[26,27,64, 118,119, 150]
CD8$^+$CD62L$^+$ TGFβ$^+$ GITR$^+$	Induced	SNF1 lupus-prone mice	Secretion of TGFβ	[68]
CD8$^+$ NKG2A$^+$ CD90$^+$	Induced	ACAID mice	Secretion of TGFβ	[44]

Table 1: $CD8^+$ Tregs Subtypes

Subset	Natural/Induced	Cells/Model Studied	Mechanism of Suppression	Citation
$CD8^+$ LAP^+	Natural	Experimental Autoimmune Encaphomyelitis	TGFβ and IFNγ Dependent	[121]
$CD8^+$ $CD45RO^+$ $CD101^+$ $CD103^+$	Induced	TNF ARE mice	Cell-cell contact dependent	[67]