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Inhibitory CD8+ T cells in Autoimmune Disease

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Abstract

Rheumatologists have long been focused on developing novel immunotherapeutics to manage such prototypic autoimmune disease as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). The ultimate challenge in immune suppressing patients with RA and SLE has derived from the dilemma that both protective and harmful immune responses result from adaptive immune responses, mediated by highly diverse, antigen-specific T cells and B cells endowed with powerful effector functions and the ability for long-lasting memory. As regulatory/suppressor T cells can suppress immunity against any antigen, including self-antigens, they emerge as an ideal therapeutic target. Several distinct subtypes of $CD8^+$ suppressor cells (Ts) have been described that could find application in treating RA or SLE. In a xenograft model of human synovium, $CD8^+CD28^-CD56^+$ T cells effectively suppressed rheumatoid inflammation. Underlying mechanisms involve conditioning of antigen-presenting cells (APC). Adoptively transferred $CD8^+$ T cells characterized by IL-16 secretion have also exhibited disease-inhibitory effects. In mice with polyarthritis, $CD8^+$ Ts suppressed inflammation by IFN-γ-mediated modulation of the tryptophan metabolism in APC. In SLE animal models, $CD8^+$ Ts induced by a synthetic peptide exerted suppressive activity mainly via the TGFβ-Foxp3-PD1 pathway. $CD8^+$ Ts induced by histone peptides were found to downregulate disease activity by secreting TGFβ. In essence, disease-specific approaches may be necessary to identify $CD8^+$ Ts optimally suited to treat immune dysfunctions in different autoimmune syndromes.

Introduction

Studying autoimmune diseases has been instrumental in deciphering how the immune system functions to protect and to attack the host and how immune-mediated protection and tissue injury are closely interlinked. With most of the autoimmune syndromes being genetically associated to major histocompatibility complex (MHC) class II haplotypes, much of the interest in understanding pathogenic immune responses has been focused on $CD4^+$ T cells. Inappropriate activation of $CD4^+$ T cells by antigen presented in the context of disease-associated human leukocyte antigen (HLA) molecules provided a framework for a general paradigm applied to many of the autoimmune syndromes. It seemed less obvious that autoimmunity may result from a lack of immunosuppression. The concept of T cells suppressing immune responses was introduced by Gershon and Kondo in a research paper describing $CD8^+$ suppressor T cells (Ts) [1]. $CD8^+$ Ts attracted much interest until the mid 1980's but fell into disregard when it was difficult to identify the responsible cell populations and the molecular mechanisms underlying the suppressor activity. However, the appeal for T

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cells with suppressor/regulatory activity was resurrected after a seminal publication by Sakaguchi and colleagues identifying CD25, interleukin (IL)-2 receptor α chain, as a molecular marker of suppressive $CD4^+$ T cells [2]. A large series of studies has now demonstrated the very important role of $CD4^+CD25^+$ regulatory T cells (Tregs) for immune homeostasis and the regulation of autoimmunity [3–5]. Currently, it is believed that regulatory T lymphocytes including natural $CD4^+CD25^+Foxp3^+$ Tregs, adaptive $CD4^+CD25^+Foxp3^+$ Tregs [6], T regulatory 1 (Tr1) cells [7], and T helper (Th) 3 cells [8] are critically involved in the maintenance of immune homeostasis and the prevention of autoimmune diseases. Recent publications have established that the regulatory/suppressor T-lymphocyte family consists not only of the $CD4^+$ T-cell population but also includes $CD8^+$ T-cell populations. Notably, several types of $CD8^+$ Ts with several phenotypes (Fig. 1) seem to exist in humans and experimental animals (Table 1), whose modes of suppressive action can be categorized into three mechanisms: cell-cell contact-mediated suppression, anti-inflammatory cytokine secretion, and cytotoxicity to the target cells.

A. The multiplicity of $CD8^+$ Ts

1. Ts induced by stimulation of $CD8^+$ T cells

A major breakthrough in the field of suppressive T cells was the discovery that a distinct lineage of $CD4^+$ T cells, $CD4^+CD25^+$ T cells, arises in the thymus and is characterized by the expression of the transcription factor Foxp3. However, it is now accepted that Tregs can emerge after stimulation of peripheral T cells. Especially in the human, most inhibitory $CD8^+$ T cells are adaptive, induced after one time or several rounds of stimulation. Our knowledge of the family of induced peripheral regulatory/suppressor T lymphocytes has been growing, but it is currently not understood whether the different types of inducible $CD8^+$ Ts are distinct cell populations, overlapping, or essentially derive from one source. Mostly, they emerge after T-cell receptor (TCR) stimulation and exhibit their downregulatory function by impairing the responsiveness of other T cells. A well-studied type of $CD8^+$ Ts are $CD8^+CD28^-$ T cells which are induced by stimulating peripheral blood mononuclear cells (PBMC) with either allogeneic [9] or xenogeneic stimulator cells [10] or, alternatively, are enriched by multiple rounds of stimulating with antigen-pulsed autologous antigen-presenting cells (APC) [11]. Suciu-Foca and colleagues have studied in detail how such $CD8^+CD28^-$ Ts suppress autologous and heterologous $CD4^+$ T-cell proliferation and have discovered that such Ts function by rendering APC tolerogenic. As the underlying molecular mechanism, these authors identified the induction of receptors that transmit negative signals, specifically upregulation of immunoglobulin-like transcript (ILT) 3 and ILT4 [12]. Alloantigen-induced $CD8^+CD103^+$ Ts have been reported to suppress T-cell proliferation in mixed lymphocyte culture via a cell to cell contact-dependent mechanism [13].

Continuous antigen stimulation in the presence of $CD14^+$ monocytes has emerged as a means to generate $CD8^+CD25^+Foxp3^+$ Ts which then suppress T-cell proliferation by a contact-dependent mechanism [14]. An alternative mode of inducing $CD8^+CD25^+CTLA\text{-}4^+Foxp3^+$ Ts involves TCR stimulation with modified anti-CD3 monoclonal antibodies (mAb); such $CD8^+$ Ts require cell contact to mediate their inhibitory function [15]. Autoreactive $CD8^+CD25^+CTLA\text{-}4^+Foxp3^+$ Ts clones have been isolated from healthy and ankylosing spondylitis patients using autologous LPS-activated dendritic cells (DC); suppression of T-cell proliferation by such autoreactive $CD8^+$ Ts involves a CTLA-4-dependent mechanism [16]. Naïve $CD8^+$ T cells primed with allogeneic CD40 ligand-activated plasmacytoid DC differentiate into $CD8^+$ Ts, which inhibit allospecific proliferation of naïve $CD8^+$ T cells by producing significant amounts of IL-10 [17]. Plasmacytoid DC derived from tumor ascites induce

$CD8^+CCR7^+CD45RO^+$ $IL\text{-}10^+$ suppressor T cells, which suppress myeloid DC-mediated tumor-associated antigen-specific T-cell effector function through IL-10 [18]. Ts induced by activating $CD8^+$ T cells in the presence of both IL-2 and TGFβ downregulate IgM and IgG production from B cells cultured with $CD4^+$ Th cells [19]. Human PBMC stimulated with hepatitis C virus or flu virus-specific peptides give rise to $CD8^+CD103^+CTLA\text{-}4^+Foxp3^+GITR^+$ T cells displaying cell to cell contact-dependent suppressive activity [20]. Bacillus Calmette-Guérin's stimulation results in the differentiation of $CD8^+CD25^+LAG3^+Foxp3^+$ T cells, which suppress T-cell proliferation partly through the secretion of CCL4 [21]. Notably, Dhodapkar and Steinman have provided direct experimental evidence that $CD8^+$ Ts are functional in humans in vivo [22]. In a clinical trial, the authors injected immature DC pulsed with influenza matrix peptide into healthy human subjects. These in vivo stimulation conditions led to antigen-specific silencing of effector T-cell function, and the suppression was mediated by cell-cell contact.

Nonantigen-specific $CD8^+$ Ts have also been reported [23,24]. This type of $CD8^+$ Ts can be generated in vitro without TCR stimulation from $CD8^+CD28^-$ T cells, but not $CD8^+CD28^+$ T cells when cultured with autologous monocytes, GM-CSF, and IL-2 or IL-10 and IL-2. Functionally, these $CD8^+$ Ts are capable of inhibiting the proliferation of antigen-specific T lymphocytes as well as the antigen-specific cytotoxicity of cytotoxic T lymphocytes through IL-10 secretion.

2. Natural $CD8^+$ suppressor T cells

   Naturally occurring Tregs constitute an endogenous long-lived population of T cells that develop in the thymus. They are believed to have specificity for self antigens and are poised to prevent autoimmunity. As an overriding theme, $CD8^+CD25^+CTLA\text{-}4^+GITR^+Foxp3^+$ T thymocytes have similarities with natural $CD4^+CD25^+$ regulatory thymocytes [25]. Like $CD4^+CD25^+$ regulatory thymocytes, $CD8^+CD25^+$ thymocytes restrain the proliferative expansion of autologous $CD4^+CD25^-$ T cells in a contact-dependent manner, which can be reversed by a mixture of anti-CTLA-4 and anti-TGFβ antibodies (Ab). In mice, $CD8^+CD122^+$ T cells have received special attention. As naturally occurring $CD8^+$ Ts, they are regarded as a functional T-cell subset that impacts immunity through the release of the anti-inflammatory cytokine IL-10 [26]. Mice genetically deficient for CD122, the IL-2/IL-15 receptor β chain, spontaneously develop severe hyperimmunity by expanding abnormally activated T cells [27]. When CD122-deficient neonates receive adoptively transferred $CD8^+CD122^+$ T cells, the development of these abnormal T-cell populations is totally prevented [28]. Furthermore, adoptive transfer of $CD8^+CD122^+$ Ts ameliorates established experimental autoimmune encephalomyelitis (EAE) [29]. However, so far there has been no report on human $CD8^+CD122^+$ Ts.

3. Inhibitory $CD8^+$ T cells recognizing MHC class Ib molecules on $CD4^+$ T cells

   The original observation that the interaction between $CD8^+$ and $CD4^+$ T cells resulted in inhibition if activated $CD4^+$ T cells expressed the MHC class 1b molecule Qa-1 supported the notion that a third class of $CD8^+$ suppressor cells may exist that communicates through defined restriction elements. The equivalent to Qa-1 in humans is the HLA-E molecule [30–33]. This class of MHC class Ib-restricted $CD8^+$ Ts downregulates disease activity in multiple sclerosis (MS) [34,35] and EAE [36–38] at least in part by killing antigen-activated autologous $CD4^+$ T cells expressing the HLA-E/Qa-1-peptide complex as reviewed elsewhere in this issue.

In essence, it appears that nature relies on a multitude of approaches to inhibit unwanted immune responses. As an overarching theme, $CD8^+$ Ts suppress by directly killing immune

cells, an elegant mode of disrupting an immune response, by mediating negative signals, or by coopting other cells to elaborate suppressive molecules. TGFβ and IL-10 represent important players in immunosuppressive regulation (Fig. 2). Altering metabolic activities, such as inducing tryptophan catabolism through the induction of indoleamine 2,3-dioxygenase (IDO) provides a wide field for immunosuppressive impact with $CD8^+$ Ts emerging as important players [39].

B. Inhibitory CD8 T^+ cell in autoimmune syndromes –Potential new therapeutic targets

Obviously, the great promise is that regulatory/suppressor T cells can eventually be therapeutically used to ameliorate chronic inflammatory autoimmune disease, including prototypic rheumatic autoimmune disease, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Data in murine models suggest that Tregs can be induced by essentially any antigen. Additionally, in experimental systems, antigen-specific Tregs can prevent as well as reverse already established autoimmunity. Much less is known about the potential role that regulatory/suppressor T cells have in human autoimmune disease. We will focus the rest of this review on $CD8^+$ Ts and summarize the current information we have for human autoimmune syndromes and for autoimmune models in experimental animals, with a special emphasis on rheumatic and connective tissue disease.

1. Rheumatoid arthritis and murine models of rheumatoid-like polyarthritis

   RA is a quintessential immune-mediated disease which targets small and large joints in a symmetrical pattern. Affected patients are disabled by pain and stiffness, and eventually suffer from irreversible loss of joint function and mobility. The systemic inflammatory response is associated with extraarticular manifestations, such as rheumatoid lung and nerve disease and rheumatoid nodule formation. The recent recognition that RA leads to premature atherosclerotic disease has markedly broadened the spectrum of disease manifestations and the challenges in managing this chronic disorder [40]. In the joint, the underlying pathology is that of dense inflammatory infiltrates accumulating in the synovial membrane. T cells and B cells acquire a highly sophisticated lymphoid organization, at times resulting in the formation of ectopic germinal centers [41–43]. Besides lymphocytes, inflammatory and tissue destructive networks involve a series of immune cell types, such as macrophages, DC, and mast cells. Non-immune resident cells, particularly synovial fibroblasts, endothelial cells, and osteoclasts contribute critically to the immunopathogenesis, specifically by forming tissue invasive pannus, cartilage, and bone erosions [44,45]. Over the last decade, cytokines, in particular TNFα, have emerged as excellent targets for therapeutic interventions. Other successful treatment strategies include depletion of B cells and disrupting costimulatory pathways [46, 47].

   Immunoregulatory functions of $CD8^+$ Ts have been investigated in a chimera model of human RA. In this model, synovial tissues from RA patients are engrafted into severe combined immunodeficiency (SCID) mice and defined T-cell populations are adoptively transferred. Immuno-stimulatory and inhibitory effects are measured by analyzing T-cell and macrophage function in the explanted grafts. Davila et al. [48] have approached the topic of anti-inflammatory $CD8^+$ T cells by generating $CD8^+CD28^-CD56^+$ T cells. The induction protocol involved stimulating PBMC from HLA-A2 negative donors with irradiated HLA-$A2^+$ myelomonocytic THP-1 cells. Such $CD8^+CD28^-CD56^+$ T cells suppressed both autologous and heterologous T-cell responses. Subsequently, the authors isolated $CD8^+CD28^-CD56^+$ T-cell clones from synovial tissues of RA patients, expanded them in vitro, and adoptively transferred them into nonobese diabetic (NOD)-SCID mice engrafted with RA synovial tissues. In the synovial lesions, $CD8^+CD28^-CD56^+$ T cells displayed strong anti-

inflammatory activities, whereas $CD8^+CD28^+CD56^-$ T-cell clones had proinflammatory function. Specifically, $CD8^+CD28^-CD56^+$ Ts inhibited production of IFNγ, TNFα, CXCL10, and CXCL9 in autologous and HLA class I-matched heterologous synovitis. Adoptively transferred $CD8^+$ Ts downregulated the costimulatory molecules CD80 and CD86 on synovial fibroblasts, suggesting that immunosuppression in the tissue lesions was predominantly mediated by altering APC function. $CD8^+CD28^-CD56^+$ Ts failed to upregulate ILT3 and ILT4, both in vitro as well as in vivo. Another $CD8^+$ Ts population has been demonstrated to exhibit disease-suppressing activities in RA synovial lesions. Klimiuk et al. [49] have reported that IL-16-producing $CD8^+$ T cells had profound disease-suppressing effects when adoptively transferred into human synovium-SCID chimeras. In contrast to $CD4^+$ T cells which amplified the tissue production of pro-inflammatory cytokines, IL-16-secreting $CD8^+$ T cells successfully disrupted inflammatory networks in the synovial implants. Activation of the $CD8^+$ Ts was accompanied by the induction of IL-16 mRNA. Adoptive transfer of the $CD8^+$ Ts enhanced IL-16 transcription in the synovial grafts. Administration of anti-IL-16 Ab partially reversed T cell-mediated suppression of tissue IFN-γ production. Partial reversal suggests either incomplete neutralization of tissue IL-16 or the contribution of another mediator. Daily treatment with recombinant IL-16 suppressed IFN-γ, TNF-α, and IL-1β expression in synovium-SCID mouse chimeras. Therefore, IL-16-producing $CD8^+$ T cells residing in the RA synovium appear to exert anti-inflammatory activity typically assigned to Tregs. As IL-16 is a natural ligand for the CD4 molecule, the anti-inflammatory effect of IL-16 may be mediated by $CD4^+$ T cells. Possible mechanisms include induction of energy in $CD4^+$ effector-T cells [50,51]. Alternatively, it has been proposed that IL-16 could have a role in enriching $CD4^+CD25^+Foxp3^+$ Tregs in the tissue (Fig. 3) [52].

In murine collagen type II (CII)-induced arthritis (CIA), a model frequently utilized to study innate and adaptive immune responses causing destructive polyarthritis, $CD11c^+CD8^+$ T cells seems to function as effective Ts. Seo et al. [53] found that administration of agonistic anti-4-1 BB mAb inhibits CIA development and improves even established CIA. Anti-4-1 BB mAb administration suppressed serum antibodies to CII in CIA mice and $CD4^+$ T-cell recall responses to CII in in vitro proliferation assays. Injection of anti-4-1 BB mAb caused a massive and CII-dependent clonal expansion of $CD11c^+CD8^+$ T cells and was associated with the accumulation of IDO in $CD11b^+$ monocytes and $CD11c^+$ DC in the spleen and lymph nodes. $CD11c^+CD8^+$ T cells produced substantial amounts of IFNγ but not TGFβ, IL-4, or IL-10. Adoptive transfer of $CD11c^+CD8^+$ T cells collected from CII, complete Freund's adjuvant (CFA), and anti-4-1 BB-treated mice suppressed CII-specific CD4 + T-cell recall responses and disrupted the development of CIA. The effect of anti-4-1 BB administration in CIA was reversed by anti-IFNγ mAb or co-administration of the IDO inhibitor 1-methyltryptophan. These data suggest that IFNγ-producing $CD11c^+CD8^+$ T cells are selectively expanded by triggering the costimulatory receptor 4-1 BB. Such $CD8^+$ T cells function to suppress antigen-specific $CD4^+$ T cells and ameliorate CIA through an IDO-dependent mechanism.

2. Systemic lupus erythematosus and murine models of tissue inflammation initiated by autoantibodies to DNA and RNA.

SLE is often referred to as a classical systemic autoimmune syndrome. Almost any organ system can be affected by chronic inflammatory attack. Although the etiology remains elusive, several major immune defects have been identified in SLE patients which play an important role in diagnosing the syndrome. Specifically, SLE patients form autoantibodies to a broad range of nuclear antigens. Autoantibodies to DNA and

RNA are now considered pathogenically relevant in driving innate immune responses with a bias towards overproduction of type I interferons [54]. Defective clearance of apoptotic cells has also been considered a critical disease mechanism [55]. Many aspects of SLE are explained by the deposition of immune complexes that initiate vascular damage and tissue inflammation. SLE, like RA, is a risk factor for accelerated atherosclerosis; underlying pathomechanisms are not understood [56]. With no new therapy approved for SLE in several decades, Tregs hold great hope for a therapeutic innovation.

Filaci et al. [57] have generated $CD8^+$ Ts in vitro by incubating purified $CD8^+$ T cells and autologous monocytes from SLE patients and healthy subjects with IL-2 and GM-CSF. They assessed the suppressive activity of induced $CD8^+$ Ts by adding them to anti-CD3-stimulated autologous PBMC proliferation assays. Although the phenotypes of $CD8^+$ Ts from healthy controls, those from SLE patients in remission, and those from patients with active SLE were comparable, only $CD8^+$ Ts from patients with SLE in remission and healthy controls functioned as effective suppressors. $CD8^+$ T cells from patients with active SLE failed to suppress committed $CD4^+$ T cells. Such functionally defective $CD8^+$ Ts produced lower levels of IL-6 and higher levels of IL-12 compared with healthy controls. IL-4, IL-10, IFNγ, and TGFβ concentrations were comparable between SLE patients and healthy subjects. Adding anti-INFγ mAb to the cultures and pretreating $CD8^+$ Ts from healthy donors with IL-6 antisense oligonucleotides counteracted the inhibitory activity. Recombinant IL-6 inhibited anti-CD3 mAb-induced PBMC proliferation, whereas IL-12 stimulated T-cell expansion. However, an alternative mechanism may explain the functional differences between the impaired $CD8^+$ Ts isolated from active SLE patients and controls. Notably, $CD8^+$ Ts from patients with SLE in remission also produced lower levels of IL-6 than healthy controls, had comparable levels of IL-12 to patients with active SLE, and IFNγ production for all experimental groups was essentially indistinguishable.

New Zealand Black (NZB)/New Zealand White (NZW) F1 female (BWF1) mice spontaneously develop autoantibodies, including anti-DNA antibodies and have been widely used as a model to study immunoregulatory defects in SLE. Hahn and colleagues [58] have made the important observation that high dose administration of an artificial synthetic peptide based on an amino acid sequence containing MHC class I and MHC class II determinants in the V_H region of Ig anti-DNA called pConsensus (pCons, FIEWNKLRFRQGLEW) to either young or diseased mice results in decreased serum levels of autoantibodies and prolonged survival. The mechanism of this protection from disease involves multiple mechanisms, including anergy induction in $CD4^+$ Th cells [59], induction of $CD4^+CD25^+$ Tregs [59], and induction of $CD8^+$ Ts [60–62]. $CD8^+$ T cells from pCons-treated mice suppressed anti-dsDNA Ab production in vivo [60]. Both $CD8^+CD28^+$ and $CD8^+CD28^-$ T-cell populations from the spleen of pCons-injected mice suppressed production of anti-DNA antibodies [60] and inhibited the proliferation of $CD4^+$ Th cells in vitro [62]. Naïve $CD8^+$ T cells lacked these functional activities. $CD8^+$ Ts induced in this system succeeded in suppressing CFSE-labeled B-cell proliferation [61]. Characterization of the $CD8^+$ Ts revealed expression of Foxp3 and TGFβ, whose mRNA were further induced by secondary polyclonal stimulation with anti-CD3 and anti-CD28 mAb. Anti-TGFβ Ab addition abrogated suppression of anti-DNA Ig production [60] and the proliferation of naïve $CD4^+$ T and B cells [61]. Based on transwell experiments, the $CD8^+$ Ts did not require cell-cell contact for their regulatory interference [61]. In vitro addition of recombinant TGFβ induced Foxp3 expression in a dose-dependent manner. Small interfering (si) RNA of Foxp3 treatment of the $CD8^+$ Ts abrogated the ability to inhibit anti-DNA production, the proliferation of $CD4^+$ T cells, and TGFβ

secretion [62]. Foxp3 and TGFβ may regulate the expression of each other, creating an autocrine loop in the induction and maintenance of Ts. However, it is likely that TGFβ secretion is not the only factor that directly suppresses autoreactivity in this experimental model, because naïve $CD4^+$ T cells that give help to B cells secrete almost the same quantities of TGFβ [62]. Notably, blocking PD1/PDL1 with anti-PD1 or anti-PDL1 mAb abrogated the suppressive activity of the $CD8^+$ Ts [61]. $CD8^+CD28^-$T cells from pCons-treated mice could kill B cells from nephritic animals, which expressed B-cell receptors with anti-DNA specificity. One cytotoxic effector molecule, granzyme B, was increased in pCons-treated $CD8^+$ T cells compared with naïve $CD8^+$ T cells [61]. In summary, pCons-induced $CD8^+$ Ts may utilize diverse strategies to counteract disease-inducing immunity, most significantly utilizing the TGFβ/Foxp3/PD1 axis as well as cytotoxicity.

Kang et al. [63] have reported similar results to the above data; by administering a very low-dose of nucleosomal histone peptide autoepitopes to lupus prone (SWR × NZB) F1 (SNF1) mice, the authors were able to reduce autoantibody levels and extend life span, mostly by deferring nephritis. Mechanistic studies demonstrated that this low-dose peptide therapy induced $CD8^+$ Ts as well as $CD4^+CD25^+$ Tregs. Splenic $CD8^+$ T cells from peptide-tolerized mice were positive for the surface expression of TGFβ, CD62L, and GITR, typical markers of mature Tregs. The generated $CD8^+$ Ts suppressed the production of INFγ and IgG autoantibodies to dsDNA, ssDNA, and anti-nucleosome. Adoptive transfer of the $CD8^+$ Ts from low-dose peptide-tolerized SNF1 mice significantly suppressed IgG autoantibodies to dsDNA, ssDNA, nucleosomes, and histone in SNF1 mice immunized with pathogenic H1′22–42 peptide. In a helper-suppression assay with $CD4^+$ Th and B cells, the suppression of IgG autoantibody production by $CD8^+$ Ts was almost completely abrogated by the addition of anti-TGFβ Ab. Upon antigen-specific or anti-CD3 stimulation, the $CD8^+$ Ts released increased amounts of total TGFβ. Furthermore, in transwell experiments, $CD8^+$ Ts placed in the upper chamber suppressed IgG autoantibody production from $CD4^+$ Th cells and B cells cocultured in the lower chamber. These data indicate that soluble TGFβ from the $CD8^+$ Ts mediated the immunosuppression, at least in the in vitro helper-suppression assay.

Zheng et al. [64] have generated $CD4^+$ and $CD8^+$ suppressive T cells ex vivo with the intention to explore their therapeutic efficacy in a murine model of stimulatory graft-vs-host disease (sGVHD) associated with a lupus-like syndrome. Induction of DBA/2 T cells with irradiated APC from C57BL/6 in the presence of TGFβ and IL-2 induces $CD4^+$ and $CD8^+$ suppressive T cells. Injection of DBA/2 T cells into (DBA/2 × C57BL/6) F1 mice causes sGVHD. Adoptive transfer of ex vivo-generated suppressive T cells could essentially prevent or ameliorate sGVHD. Both coadministration of suppressive and stimulatory T cells together and injection of suppressive T cells two weeks after disease induction had beneficial therapeutic effects. Unfortunately, the authors did not assess $CD4^+$ and $CD8^+$ suppressive T-cell populations separately in the in vivo experiments. The same authors also showed that the ex vivo-generated $CD8^+$ T cells suppressed naïve DBA/2 T-cell proliferation in culture with irradiated B6 splenic APC. In this in vitro culture, neutralizing anti-TGFβ Ab partially reversed inhibitory effects, opening the possibility that additional pathways of immunosuppression were functional in this experimental system.

3. Inflammatory bowel disease

Like most autoimmune syndromes, inflammatory bowel diseases (IBD) are considered to represent sequel of aberrant immune responses in genetically predisposed hosts. For Crohn's disease (CD) and ulcerative colitis (UC) gut luminal antigens, in particular the anaerobic microbiota of the distal ileum and colon, have

attracted great attention, and the current paradigm proposes that interactions of such bacteria with the host's epithelial cells and the mucosal immune system eventually result in continuous microbial antigenic stimulation and associated tissue damage [65]. Formerly categorized as Th1- and Th2-mediated diseases, CD and UC are currently re-investigated for the contribution of the IL-23/Th17 axis [66]. Regulatory T cells are believed to be crucial in adjusting response thresholds to microbial antigen as well as modulating tissue-damaging immune reactions and as such are regarded as promising new therapeutic targets [67].

Limited information is available about the phenotypic and functional characteristics of such regulatory/suppressor T cells in humans. There is, however, evidence that T cells from the $CD8^+$ Ts family may warrant further exploration. Allez et al. [68] have generated $CD8^+$ Ts by stimulating peripheral blood T cells with irradiated allogeneic intestinal epithelial cells (IEC). Phenotypic markers of such $CD8^+$ Ts included CD101 and CD103; functionally, they suppressed Ig production by pokeweed mitogen (PWM)-stimulated PBMC and the proliferation of $CD4^+$ T cells in an unrelated mixed lymphocyte reaction, whereas $CD8^+CD101^-CD103^-$ T cells did not. The same group [69] has shown that lamina propria (LP) $CD8^+$ T cells derived from normal controls possess regulatory activity, whereas both unfractionated LP lymphocytes and purified LP $CD4^+$ T cells do not. Either LP $CD8^+CD28^+$ or $CD8^+CD28^-$ T cells inhibit Ig production by PWM-stimulated PBMC in a cell contact-dependent manner. LP $CD8^+$ Ts express the $CD101^+$, $CD103^+$, and $CD45RO^+$ phenotype. Whereas $CD8^+$ T cells isolated from non-inflamed mucosa displayed suppressive capabilities, LP $CD8^+$ T cells derived from CD and UC specimens left Ig production unchanged, suggesting a deficiency of $CD8^+$ Ts in IBD. Previous studies have indicated that $CD8^+$ Ts expandable by IEC stimulation in vitro may be enriched in the TCR $V\beta5.1^+$ subset [70]. Interestingly, the frequency of $V\beta5.1^+$ $CD8^+$ T cells was diminished amongst IBD LP when compared to normal controls [69].

Guided by the clinical observation that blockade of TNF is highly successful in inducing and even maintaining remission in some IBD patients, animal models have been developed that utilize TNF overexpression to induce disease. Deletion of a 69 bp stretch within the AU-rich region (ARE) of the TNF gene heightens mRNA stability and increased TNF protein synthesis. TNF overexpression in TNFΔARE mice leads to the development of transmural CD-like chronic inflammation in the terminal ileum [71]. Ho et al. [72] have examined CD8 Ts function in such mice and have found that $CD8^+CD44^-CD103^+$ T cells produce TGFβ, but not IL-10 or IFNγ $CD8^+CD44^-CD103^+$ T cells derived from TNFΔARE mice downregulate the proliferation of $CD4^+$ T cells in culture with APC and anti-CD3 compared to $CD8^+CD44^-CD103^+$ T cells isolated from wild-type mice. Adoptive transfer of $CD4^+$ T cells from TNFΔARE mice into immunodeficient $RAG^{-/-}$ mice induces ileitis but co-transfer of $CD8^+CD44^-CD103^+$ Ts from wild type mice as well as TNFΔARE mice attenuates the ileitis histology. An alternate experimental model mimicking IBD utilizes injection of $CD4^+CD45RB^{high}$ cells into syngeneic RAG-2-deficient mice to generate gut mucosal inflammation. In this model, Ménager-Marcq et al. [73] have demonstrated that $CD8^+CD28^-$ but not $CD8^+CD28^+$ T cells freshly isolated from the spleen or gut efficiently prevent the development of colitis as assessed by body weight and histology. $CD8^+CD28^-$ T cells derived from IL-10-deficient mice lack the functional ability to avert colitis. Moreover, the inflammation induced by $CD4^+CD45RB^{high}$ cells derived from mice transgenic for the dominant negative form of the TGFβ receptor II (dnTbRII-Tg) was resistant to the inhibitory effect transmitted by $CD8^+CD28^-$ Ts. In this study, the in vitro behavior of $CD8^+CD28^-$ Ts was different from that seen in in vivo experiments. In allogenic mixed lymphocyte cultures, $CD8^+CD28^-$ Ts from wild-type mice inhibited

proliferation and IFNγ production by $CD4^+$ T cells. If MHC-deficient APC plus anti-CD3e antibody were applied to drive $CD4^+$ T-cell proliferation, wild type, but also IL-10-deficient, Ts were effective inhibitors. Proliferation of dnTbRII-Tg CD4 responder T cells was also significantly inhibited by wild-type Ts, although suppression of T-cell proliferation was less efficient than in the presence of IL-10 or TGFβ signaling. These cytokines may play a minor role in in vitro suppressor assay in contrast to the in vivo experiments.

4. Other autoimmune diseases

   Graves' disease (GD) is a thyroid-specific autoimmune disease in which the autoantigen is known. Patients with GD built stimulatory autoantibodies against the thyroid stimulating hormone receptor (TSHR) [74]. Clinical consequences of this aberrant immune response include hyperthyroidism. In a murine model of autoimmune thyroid disease induced through an adenovirus expressing TSHR, Saitoh et al. [75] have explored the functional contribution of $CD8^+CD122^+$ Ts. These authors have also examined how $CD4^+CD25^+$ Tregs affect disease induction and disease course by depleting such regulatory cells through administration of anti-CD25 mAb. In both C57BL/6 and BALB/c mice, intramuscular injection of adenovirus expressing the human TSHR-A subunit induces serum T4 and anti-TSHR elevation (indicators for hyperthyroidism). Antibody-mediated depletion of $CD8^+CD122^+$ T cells promptly increases the incidence of hyperthyroidism indicating an active role of Tregs in controlling anti-TSHR immune responses.

Myasthenia gravis (MG) is an antibody-dependent autoimmune syndrome in which autoantibodies against the acetylcholine receptor (AChR) of skeletal muscles disrupt neurotransmission and leave affected patients with muscle weakness and excessive fatigue. T cells have been implicated as master regulators in the immune pathogenesis. An experimental model of autoimmune MG (EAMG) exists in which mice are rendered sick by immunizing against AChR. [76]. Ben-David et al. [77] have induced EAMG by hyperimmunizing mice with Torpedo AChR (TAChR). Autoimmunity can be abrogated if mice receive an altered peptide ligand (APL) composed of two tandemly arranged single amino acid analogs of myathenogenic peptide. Lymph nodes (LN) from EAMG mice contain T cells that proliferate and secrete IFNγ when restimulated with the antigen. On the other hand, LN cells from mice treated with APL concomitant with TAChR immunization have significantly lower proliferative activity and IFNγ secretion. In $CD8^{-/-}$ mice, the suppressive effects of the dual APL are abolished while $CD4^+CD25^+Foxp3^+$ Tregs are induced to the same extent as in wild-type mice. Furthermore, approximately 90% of $CD8^+$ T cells from LN in the dual APL-treated mice are $CD28^-$ cells, and 28% of $CD8^+CD28^-$ T cells are $Foxp3^+$ cells. Therefore, $CD8^+CD28^-Foxp3^+$ T cells may well have a regulatory role for EAMG-associated autoimmune responses.

Summary and Outlook

A wide variety of inhibitory T-cell populations appear to exist within the $CD8^+$ T-cell compartment. Means of induction (natural versus adaptive/induced), phenotypes ($CD28^+$/$CD28^-$), and suppressive mechanisms (cell-cell contact, anti-inflammatory cytokine secretion, or killing) are highly diverse (Table 1). Whether this diversity reflects heterogeneity of cell populations or results from an intricate interplay of inhibitory $CD8^+$ T cells with a unique microenvironment remains to be resolved. Surprisingly, similarities between $CD4^+$ Tregs (natural $CD4^+CD25^+Foxp3^+$ Tregs versus adaptive/induced $CD4^+CD25^+Foxp3^+$ Tregs, Tr1, and Th3) are broad, which brings into question how CD4 and CD8 receptors finally contribute to the immunoregulatory potential of these cell populations. A barrier in investigating $CD8^+$ Ts stems from the lack of a specific cell surface marker, again an issue reminiscent of

unequivocally defining $CD4^+$ Tregs. Essentially, identification of inhibitory $CD8^+$ T cells requires a functional suppressor assay. CD25, CTLA-4, GITR, LAG3, CD103, and CD122 are all expressed by activated effector T cells. Lack of CD28 expression also cannot be used as a marker to detect $CD8^+$ Ts because $CD8^+CD28^-$ T cells contain cytotoxic cells [78] and a subset of $CD8^+CD28^+$ cells also has suppressor activity [14,21,61,69]. CD56 is expressed on senescent cytotoxic T cells [79,80]. Although the essential role of Foxp3 expression for the acquisition of regulatory function in $CD4^+CD25^+Foxp3^+$ Tregs has been demonstrated in mice, this molecule is less reliable in humans. TCR activation transiently induces Foxp3 expression on both $CD4^+$ and $CD8^+$ T cells and such $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ cells lack immunosuppressive function [81,82]. These problems have created a formidable challenge in monitoring inhibitory T-cell populations in humans and deciphering their possible contribution to autoimmunity. Also, efforts of replenishing $CD8^+$ Ts in patients deficient for such protective cell populations are limited by the inability to unequivocally identify the relevant cells. Additionally, we still have limited data on the significance of $CD8^+$ Ts in the spectrum of human autoimmune syndromes. Based on what we know currently on the diversity of $CD8^+$ Ts populations, a significant heterogeneity for different disease entities can be expected.

Here, similarities with $CD4^+$ Tregs may be very helpful. As the field of $CD4^+$ Tregs is rapidly expanding and more and more information is available on how such Tregs affect immune homeostasis and immune-mediated disease, some of the rules may be transferable to the $CD8^+$ T-cell compartment.

It is currently unknown whether $CD4^+$ Tregs and $CD8^+$ Ts have similar or distinct roles in regulating immune responses in vivo. Indeed, recent evidence suggests that the two classes of regulatory/suppressive T cells are functionally connected, adding to the versatility of regulatory T cells as targets of novel immunomodulatory therapies. Vlad et al. [83] have proposed that the potential of antigen-specific MHC class I-restricted $CD8^+CD28^-$ Ts to render DC tolerogenic translates into the induction of $CD4^+$ Tregs, making a direct interlink between the immunosuppressive capability of $CD8^+$ and $CD4^+$ T cells. One of the intriguing questions arising from the growing field of suppressive T cells relates to the seeming heterogeneity of regulatory/suppressor T-cell populations. A multitude of $CD4^+$ and $CD8^+$ subpopulations have now been described, all of which can effectively disrupt immune responses, including the pathogenic immune responses causing autoimmune disease. The provocative proposal has been made that any T cells may be able to acquire suppressive functions [84]. In that model, the microenvironment in which immune recognition occurs and in which T cells differentiate would possibly be the shaping force. T cells with anti-inflammatory capabilities are, undoubtedly, ideal targets to redirect immune responses, and their therapeutic potential needs further exploration before inhibitory $CD8^+$ T cells can be exploited clinically. Cell-based immunotherapeutics are a demanding approach. Thus, ultimately we need to understand the molecular mechanisms that inhibitory $CD8^+$ T cells use to elegantly interrupt immune responses and utilize that knowledge to design novel therapeutic strategies.

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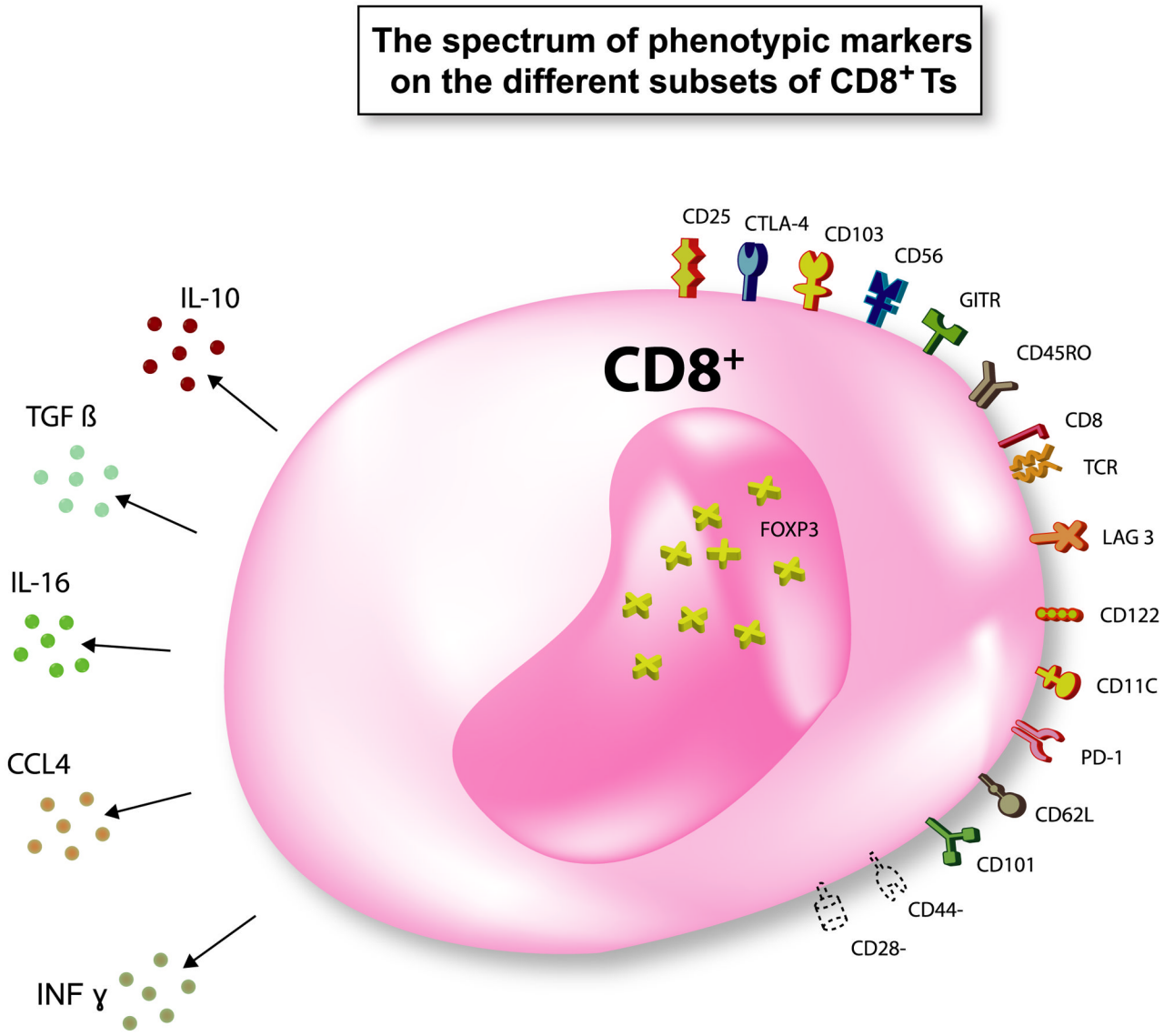

Figure 1. The spectrum of phenotypic markers on the different subsets of CD8$^+$ suppressor T cells (Ts)

Various cell surface markers, cytokines, and Foxp3 have been reported to be expressed by CD8$^+$ Ts. Not all cell surface markers are expressed on one subset of CD8$^+$ Ts. Not all cytokines are produced by one particular CD8$^+$ Ts subset. Foxp3 expression was not determined in some CD8$^+$ Ts.

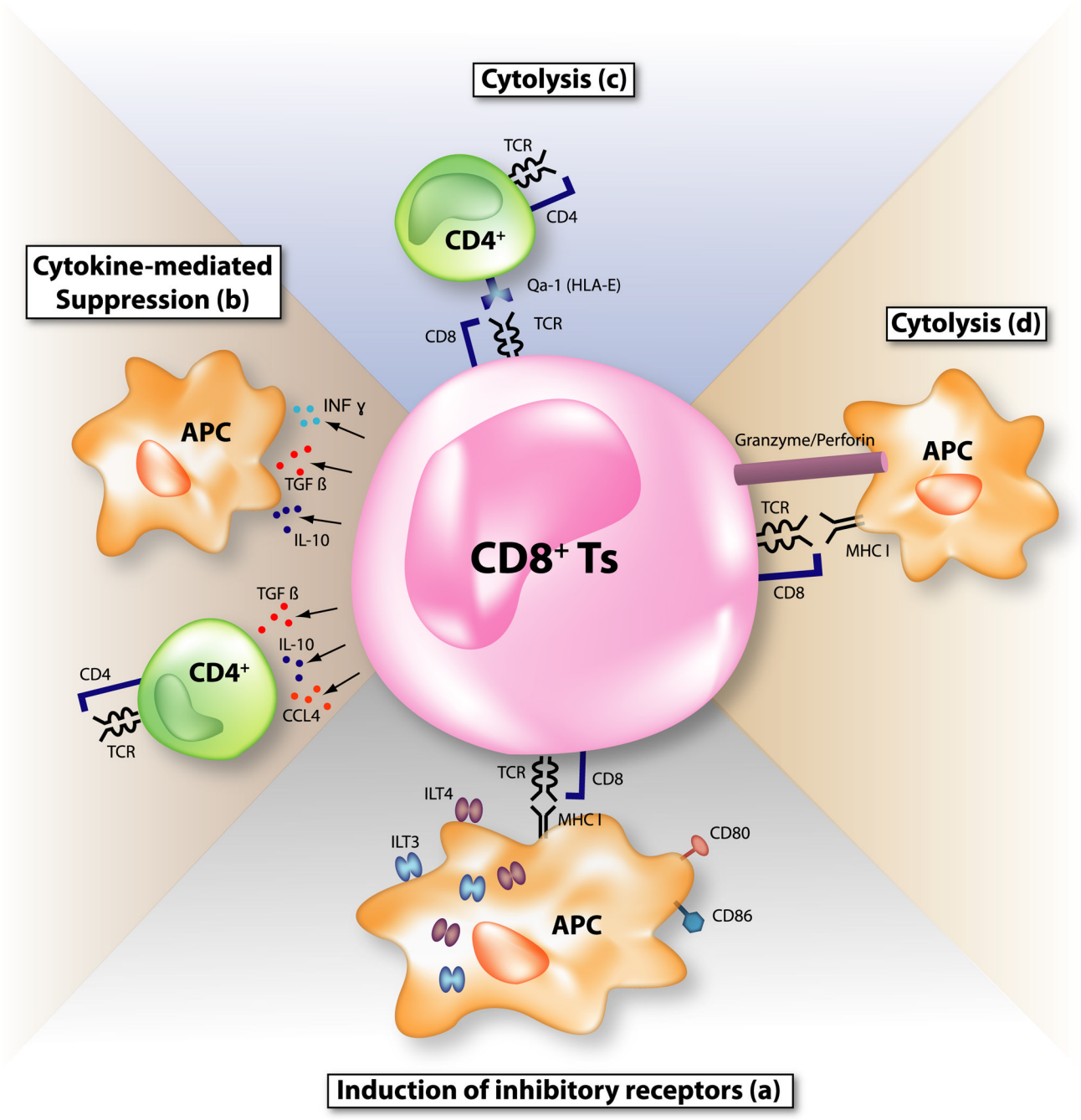

Figure 2. Possible mechanisms of suppression by CD8$^+$ suppressor T cells (Ts)

Various suppression mechanisms by CD8$^+$ Ts have been proposed so far. (a) CD8$^+$CD28$^-$Foxp3$^+$ Ts upregulate inhibitory receptors, immunoglobulin-like transcript (ILT) 3 and ILT4 on antigen-presenting cells (APC), rendering APC tolerogenic. Costimulatory molecules on APC, CD80, and CD86 are also downregulated. (b) Immunosuppressive cytokines such as interleukin (IL)-10, TGFβ, IFNγ, and CCL4 are secreted by CD8$^+$ Ts. (c) Major histocompatibility complex (MHC) class Ib molecule (Qa-1 in mice and HLA-E in humans)-restricted CD8$^+$ Ts kill activated effector CD4$^+$ T cells and dampen immune reactions. (d) CD8$^+$ cytotoxic lymphocytes (CTL) work as suppressive T cells. When CTL encounter antigen-bearing APC, CTL may kill APC and attenuate immune responses.

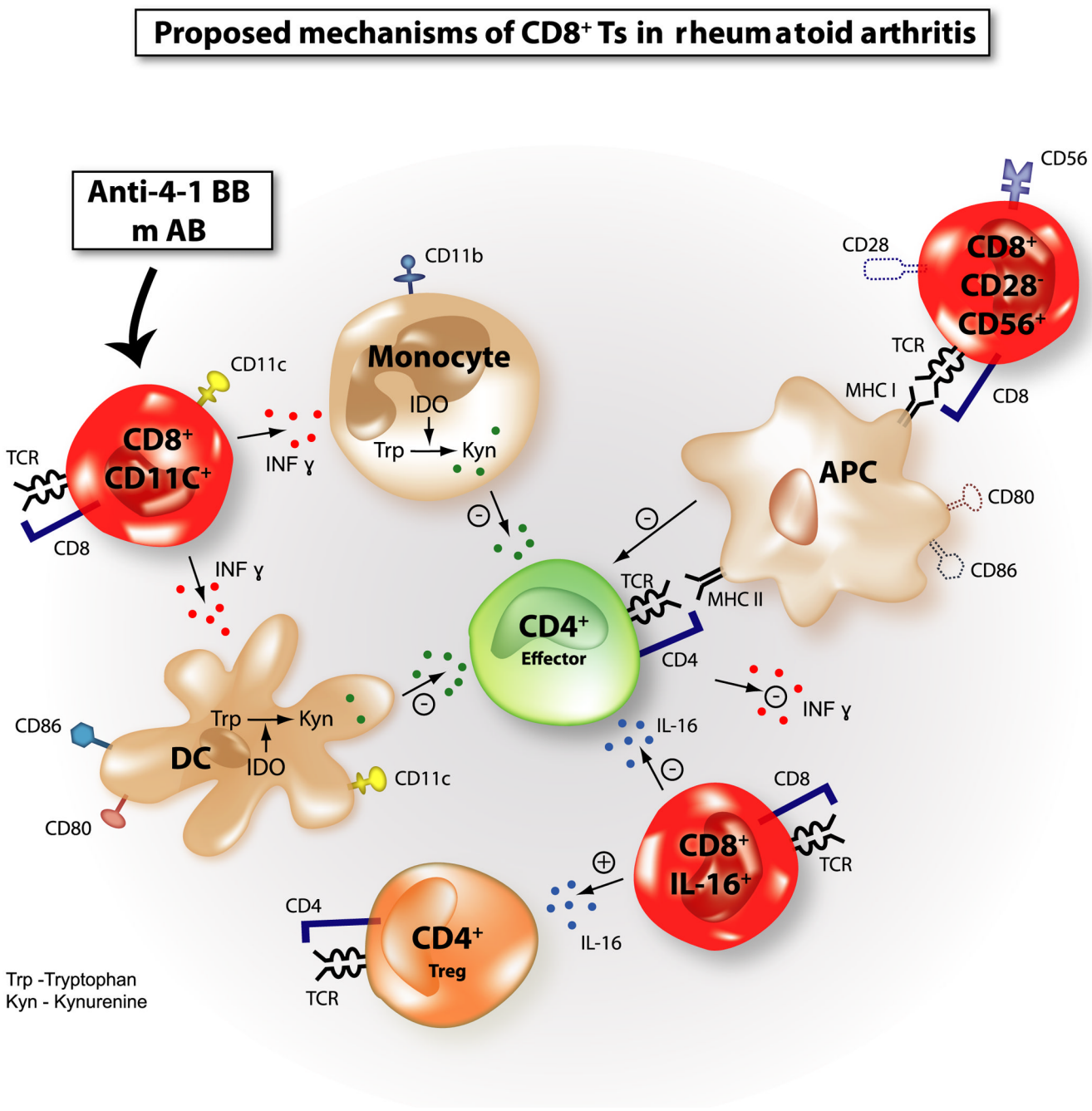

Figure 3. Proposed mechanisms of CD8$^+$ suppressor T cells (Ts) in rheumatoid arthritis (RA)

$CD8^+CD28^-CD56^+$ Ts transferred into SCID-chimera mice transplanted with synovial tissues from RA patients suppress the tissue inflammation measured by proinflammatory cytokines and chemokine expression, downmodulating the expression of costimulatory molecules CD80 and CD86 on antigen-presenting cells (APC) and synovial fibroblast-like cells (FLS). Interleukin (IL)-16-secreting $CD8^+$ Ts suppress the tissue inflammation in the same SCID-chimera model. IL-16 is a natural ligand of the CD4 molecule and induces $CD4^+$ T-cell anergy. IL-16 also may induce or recruit $CD4^+CD25^+Foxp3^+$ Tregs in the tissue. In a mice collagen-induced arthritis model (CIA), $CD8^+CD11c^+$ Ts induced by the administration of anti-4-1 BB monoclonal antibodies (mAb) secrete IFNγ and induce indoleamine 2,3-dioxygenase (IDO) in

$CD11b^+$ monocytes and $CD11c^+$ dendritic cells (DC). IDO catabolizes tryptophan (Trp) into its catabolite such as kynurenine (Kyn). Depletion of Trp and generated Kyn exert immunosuppressive effects on effector $CD4^+$ T cells. Abbreviations: MHC = major histocompatibility complex; T-cell receptor = TCR.

Table 1

Known subtypes of inhibitory $CD8^+$ T cells

Described CD8+ suppressor T cell	Species	Natural or Adaptive	Induction	Mechanism of suppression	Reference
$CD8^+CD28^-Foxp3^+$	human	adaptive	Allogeneic, xenogeneic, or antigen-pulsed autologous APC	Induction of ILT3 and ILT4 on APC	[10–12]
$CD8^+CD103^+$	human	adaptive	Alloantigen	Cell contact dependent	[13]
$CD8^+CD25^+$ $CD28^+Foxp3^+$	human	adaptive	Staphylococcal Enterotoxin B or Anti-CD3	Cell contact dependent	[14]
$CD8^+CD25^+CTLA\text{-}4^+Foxp3^+$	human	adaptive	Modified anti -CD3	Cell contact dependent	[15]
$CD8^+CD25^+CD28^-CTLA\text{-}4^+Foxp3^+$	human	adaptive	Autologous LPS-activated DC	CTLA-4 dependent	[16]
$CD8^+IL\text{-}10^+$	human	adaptive	CD40 ligand-activated plasmacytoid DC	IL-10 secretion	[17]
$CD8^+CCR7^+CD45RO^+IL\text{-}10^+$	human	adaptive	Tumor-ascites derived plasmacytoid DC	IL-10 secretion	[18]
$CD8^+$	human	adaptive	IL-2 and TGFβ		[19]
$CD8^+CD103^+CTLA\text{-}4^+Foxp3^+GITR^+$	human	adaptive	Virus peptide-pulsed APC	Cell contact dependent	[20]
$CD8^+CD25^+Foxp3^+LAG3^+$	human	adaptive	Bacillus Calmette-Guérin's stimulation	CCL4 secretion	[21]
Nonantigen-specific $CD8^+CD28^-$	human	adaptive	Monocytes, GM-CSF, and IL-2 or IL-10 and IL-2	IL-10 secretion	[23,24,55]
$CD8^+CD25^+CTLA\text{-}4^+GITR^+Foxp3^+$	human	natural		TGFβ- and CTLA-4-dependent	[25]
$CD8^+CD28^-CD56^+$	human	adaptive	Allogeneic APC	Downregulation of CD80 and CD86 expression on APC	[48]
$CD8^+IL\text{-}16^+$	human		in RA synovium	IL-16 secretion	[49]
$CD8^+CD122^+$	mouse	natural		IL-10 secretion	[26–28,75]
$CD8^+CD11c^+$	mouse	adaptive	Anti-4-1 BB mAb	IFNγ-mediated induction of IDO in monocytes and DC	[53]
$CD8^+Foxp3^+PD\text{-}1^+TGF\beta^+$	mouse	adaptive	Antigen specific stimulation	Major mechanism: TGFβ-Foxp3-PD1 axis; Minor mechanism: Cytotoxicity via Granzyme B	[60–62]
$CD8^+CD62L^+GITR^+TGF\beta^+$	mouse	adaptive	Antigen-specific stimulation	TGFβ secretion	[63]
$CD8^+$	mouse	adaptive	IL-2 and TGFβ	Partially TGFβ dependent	[64]
$CD8^+CD45RO^+CD101^+CD103^+$	mouse	adaptive	Allogeneic intestinal epithelial cells	Cell contact dependent	[68,69]
$CD8^+CD44^-CD103^+$	mouse	adaptive	in TNF ΔARE mice	TGFβ secretion	[72]
$CD8^+CD28^-$	mouse	adaptive		in vitro: cell contact dependent in vivo: IL-10 and TGFβ dependent	[73]
$CD8^+CD28^-$ $Foxp3^+$	mouse	adaptive	Antigen-specific stimulation		[77]

Abbreviations: APC = antigen presenting cells; ILT = immunoglobulin-like transcript; DC = dendritic cells; RA = rheumatoid arthritis; IDO = indoleamine 2,3-dioxygenase; ARE = AU-rich region