Harnessing regulatory T cells for the therapy of lupus and other autoimmune diseases

Regulatory T cells (Tregs) maintain immunological homeostasis and prevent autoimmunity. The depletion or functional alteration of Tregs may lead to the development of autoimmune diseases. Tregs consist of different subpopulations of cells, of which CD4+CD25+Foxp3+ cells are the most well characterized. However, CD8 Tregs also constitute a major cell population that has been shown to play an important role in autoimmune diseases. This review will discuss the role of Tregs in autoimmune diseases in general and specifically in systemic lupus erythematosus (SLE). SLE is a multisystem autoimmune disease characterized by the production of autoantibodies against nuclear components and by the deposition of immune complexes in the kidneys as well as in other organs. Abnormalities in Tregs were reported in SLE patients and in animal models of the disease. Current treatment of SLE is based on immunosuppressive drugs that are nonspecific and may cause adverse effects. Therefore, the development of novel, specific, side effect-free therapeutic means that will induce functional Tregs is a most desirable goal. Our group and others have designed and utilized tolerogenic peptides that ameliorate SLE manifestations in murine models. Here, we demonstrate the role of CD4 and CD8 Tregs, as well as the interaction between the two subsets of cells and the mechanism of action of the tolerogenic peptides. We also discuss their therapeutic potential for the treatment of SLE.

Keywords: apoptosis • autoimmune disease • autoreactive T cell • peptide-induced regulatory T cell • regulatory T cell • systemic lupus erythematosus • tolerance induction • tolerogenic peptide

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Breakdown of immunologic self-tolerance that usually controls self and nonself discrimination results in the development of autoimmune diseases. Therefore, the elucidation of regulatory mechanisms is of great importance for protection against the generation of self-directed immune responses and, consequently, the initiation of autoimmune diseases.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by increased production of autoantibodies and systemic clinical manifestations. The disease may involve nearly every organ. T and B cells play a role in the development of SLE. Treatment for SLE, as for most autoimmune diseases, relies on the use of corticosteroids and immunosuppressive drugs that are nonspecific and can cause adverse effects [1]. In spite of intensive efforts, no new treatments have been approved for SLE for over 30 years. Thus, there is an unmet need for novel therapeutic means that will be specific, with improved efficacy and lower toxicity than the currently available therapies for SLE.

A central mechanism of peripheral tolerance involves the active suppression of autoimmune responses by T cells with regulatory capacity [2]. Hundreds of publications discussing regulatory T cells (Tregs) over the past few years have indicated the existence of this unique T-cell lineage with the capacity to regulate autoimmunity. It is now evident that Tregs consist of heterogeneous populations of CD4+ and CD8+ cells. The subset of Tregs that has generated the highest level of interest among researchers is that of CD4+CD25+ cells. These cells consist of thymus-derived, naturally occurring Tregs and peripherally induced Tregs that are similar phenotypically and functionally. CD4 Tregs are decreased in number and/or function in subjects with an active SLE disease; however, functional regulatory cells were shown to be upregulated following treatment and clinical improvement. Similarly, a crucial role has been demonstrated for Tregs in a number of murine models of SLE. Thus, the possibility of therapeutic intervention using Tregs of relevant specificity in SLE is very attractive and the induction of antigen-specific functional Tregs utilizing various approaches is achievable. Therefore, a better understanding of the various developmental pathways and mechanism of action of Tregs is of utmost importance and may lead to the development of novel specific strategies for the treatment of SLE.
Role of T cells in the pathogenesis of lupus & other autoimmune diseases

The concept that many organ-specific autoimmune diseases are T-cell mediated is well established. Thus, Type 1 diabetes mellitus (DM) in patients and in nonobese diabetic (NOD) mice, which are extensively studied as a spontaneous model of the disease, was reported to be T-cell mediated [3,4]. Similarly, multiple sclerosis (MS) is mainly a T-cell-mediated disease, as is its animal model – experimental autoimmune encephalomyelitis (EAE) – which is an inflammatory demyelinating disease mediated by myelin-specific CD4+ Th1 lymphocytes [5–7]. Furthermore, in rheumatoid arthritis and in murine collagen-induced arthritis, which is a widely accepted arthritis model, T cells were reported to play a pivotal role [8–10]. Even in myasthenia gravis (MG) and in experimental autoimmune MG (EAMG), where the attack of specific antibodies on the acetylcholine receptor is the accepted cause of disease, there is ample evidence that T cells play a key role in the etiopathology of the disease in humans and in animals [11–13]. The role of T cells in organ-specific autoimmune diseases has been confirmed and revisited with the discovery of Th17 cells. Th17 cells or their cytokine, IL-17, were demonstrated to be essential in the induction of experimental autoimmune inflammation in animal models of experimental allergic encephalomyelitis [14], collagen-induced arthritis [10], colitis [15] and experimental autoimmune myocarditis [16].

Systemic lupus erythematosus is an autoimmune disease characterized by the presence of an array of antibodies, including antibodies to DNA, nuclear antigens and ribonucleoproteins. The progression of the disease is associated with general clinical manifestations and damage to tissue and organs caused by deposition of immune complexes [1]. Besides intrinsic dysfunctions of B lymphocytes that characterize SLE, T cells were shown to play a central role in the pathogenesis of SLE in mouse models and in patients [17,18]. Numerous abnormalities were shown to affect T-cell responses, the production of T-cell cytokines, and the T–B-cell dialog in lupus. Polyclonal CD4+ T-cell activation is a hallmark of human and murine lupus [19], suggesting a global defect in the maintenance of T-cell tolerance to self. Peripheral T cells in lupus-prone mice have a significantly lower threshold for T-cell activation as compared with T cells of normal mice. This intrinsic T-cell abnormality might ultimately lead to enhanced helper function for B-cell autoimmunity [20]. T cells that are oligoclonal, based upon T-cell receptor (TCR) usage, were shown to infiltrate the kidneys of lupus patients [21]. In addition, expansion of oligoclonal T cells in peripheral blood of SLE patients was demonstrated [22]. In agreement, ablation of CD4 T cells by either thymectomy, genetic deletion of MHC class II molecules or anti-CD4 treatment [23–25] ameliorated disease manifestations in murine disease models. The role of T cells was further demonstrated using a model for the induction of SLE in non-SLE-prone mice. Thus, experimental SLE can be induced in naive BALB/c mice by their immunization with a monoclonal anti-DNA antibody that bears a common idiotype (Id), namely 16/6Id [26]. Nevertheless, the disease could not be induced in BALB/c nude mice [27]. Furthermore, experimental SLE could be induced with cells of 16/6Id-specific lines that were administered into syngeneic mice [28]. Moreover, experimental SLE could be induced by inoculating C57BL/6 (H-2b) mice (a strain resistant to the induction of SLE by immunization with the disease-inducing antibody 16/6Id), with the H-2-matched T-cell line of C3H.SW origin [28], supporting the essential role of T cells in SLE.

The SLE-prone MRL/lpr mice develop a spectrum of disease manifestations that are similar to fulminant human SLE. MRL/lpr mice with B cells, but no circulating antibodies, still developed interstitial nephritis and vasculitis, as well as a glomerular disease. These nonsecret- ing B cells were discussed to be important as antigen-presenting cells (APCs) that activate T cells, suggesting a direct role for the latter cells in causing the tissue damage observed in SLE [29].

The role of CD8 T cells in the pathogenesis of SLE was also demonstrated. Thus, abnormal CD8 T-lymphocyte functions, including inappropriate cytokine production, aberrant cytotoxicity and inhibited suppressor capacity, have been reported in SLE patients [30,31]. Furthermore, depletion of CD8 cells accelerated the development of SLE in mice with an induced experimental SLE by the 16/6Id-bearing anti-DNA monoclonal antibody [32] and in genetically prone (NZB x B x SB)F1 mice [33]. Likewise, CD8 T cells with a C57BL/6 background, which are resistant to the induction of SLE, were shown to be susceptible to the experimental disease [34]. In addition, CD8 cells specific to the 16/6Id were capable of immunomodulating experimental SLE induced by the 16/6Id [35]. Thus, there is no doubt that T cells play a central role in driving the development of lupus, both in mice and in humans.
Tregs in autoimmune diseases & specifically in SLE

Regulation of lymphocyte survival is essential for the maintenance of lymphoid homeostasis, thereby preventing the development of autoimmune diseases. The existence of autoreactive T cells in healthy individuals suggests that peripheral tolerance mechanisms exist to control the response of these cells. Accumulating evidence indicates that not only clonal deletion and anergy but also T-cell-mediated control of self-reactive T cells contributes to the maintenance of immunological self-tolerance. Actually, Tregs are now widely regarded as the primary mediators of peripheral tolerance and dendritic cells (DCs) play a role in their development. Several subtypes of Tregs have been described with distinct phenotypes, cytokine production profiles, and modes of action. Most of these Tregs are CD4+; however, CD8+ Tregs with distinct phenotypes, cytokine production profiles, and modes of action. The efficiency of suppressor capacity, as well as natural killer T cells, have also been reported. In the CD4+ regulatory T-cell compartment, IL-10-producing T-regulatory cell type 1 (T_{reg1}), TGF-β-secreting Th3 cells and CD4+CD25+ cells were reported. This review will focus on CD4+ and on CD8 Tregs.

CD4 Tregs

Extensive work performed both in mice and in humans has documented that CD4+CD25+ T cells are an important subset of Tregs. CD4+CD25+ Tregs (CD4 Tregs) that are naturally occurring and expressing Forkhead winged helix protein-3 (Foxp3) are potent inhibitors of a variety of immune responses. Their depletion or functional alteration leads to the development of autoimmune diseases in otherwise normal animals. Such CD4 Tregs are produced by the normal thymus as a functionally distinct and mature subpopulation of T cells. Genetic defects that affect the development or function of these Tregs may lead to the development of autoimmune and other inflammatory disorders in humans. Induced or adaptive CD4 Tregs represent a second subset of CD4+CD25+ functional suppressive cells. Antigen peptides that are given either in a tolerogenic form or by a tolerogenic route (using injection, oral or nasal administration) may selectively induce the appearance of T cells with the regulatory phenotype and function. These antigen-dependent induced regulatory cells are fully functional following their exposure to antigen tolerogens, as in the case of certain immunomodulatory therapies.

Reduced levels or function of circulating CD4 Tregs were described in patients with numerous autoimmune diseases, such as MS, juvenile idiopathic arthritis, psoriatic arthritis, autoimmune liver disease, MG and Kawasaki disease. Further conflicting data have been reported for rheumatoid arthritis and immune-mediated Type 1 DM. Nevertheless, the status and role of CD4 Tregs in experimental models of autoimmune diseases are much clearer.

Involvement of CD4 Tregs in EAE was demonstrated using various approaches. For example, strains of mice that harbor a monoclonal myelin basic protein (MBP)-specific antibody CD4+ T-cell repertoire spontaneously develop EAE. It has been postulated that the latter has been a result of the failure of CD4 Tregs to utilize the endogenous TCR α-chain in Rag-2-deficient mice. Furthermore, CD4 Tregs were shown to confer significant protection from the development of myelin oligodendrocyte glycoprotein (MOG)35–55- induced EAE. It was demonstrated that myelin proteolipid protein-specific CD4 Tregs confer genetic resistance to the development of EAE and Tregs were also reported to be involved in the resistance of male mice to the development of EAE. By using CD4+ T-cell-driven EAE as a model, it was found that depletion of CD4 Tregs allowed pathology to develop in response to suboptimal T-cell stimulation, thus demonstrating the role of CD4 Tregs in raising the threshold of triggering autoreactive T-cell responses.

Finally, glatiramer acetate, which is used for the treatment of MS patients, was shown to boost the expression of Foxp3 on CD4 Tregs during EAE. Moreover, adoptive transfer of purified Tregs from glatiramer acetate-treated EAE mice resulted in a better effect than the transfer of Tregs from untreated EAE controls.

Similar to the case in MG patients, where a deficiency in CD4 Tregs was shown to favor the development of disease, normalization of CD4 Tregs following immunosuppressive therapy was demonstrated to be of clinical value. Amelioration of EAMG manifestations was also reported to correlate with the development of functional CD4 Tregs. Thus, an improvement in the clinical status of rats with EAMG was reported following treatment with pentoxifylline, which upregulated CD4 Tregs. Furthermore, EAMG induced in C57BL/6 mice was effectively suppressed by GM-CSF treatment that resulted in the expansion of Tregs. In addition, a dual altered peptide ligand (APL), composed of two APLs of two myasthenogenic peptides, was reported to inhibit in vitro and
in vivo MG-associated autoreactive responses. The dual APL upregulated functional CD4 Tregs and depletion of CD4 cells diminished the inhibitory effect of the dual APL [47–49]. In addition, an established EAMG, induced in C57BL/6 mice by Torpedo acetylcholine receptor, was downregulated following treatment with the dual APL via the upregulation of functional CD4 Tregs [50].

The role of CD4 Tregs in autoimmune-prone NOD mice was extensively studied. Although CD4 Tregs undoubtedly play a role in governing the onset and development of diabetes, the issue of the number of CD4 Tregs in the NOD mouse is controversial. Nevertheless, it was demonstrated that small numbers of antigen-specific CD4 Tregs can reverse diabetes after disease onset [51]. Suppression in the NOD model, especially after anti-CD3 therapy, was shown to depend on active immunoregulation that is TGF-β dependent [52]. Furthermore, DC-expanded, islet-specific CD4 Tregs could block diabetes months after initiation of insulitis and restored long-term normoglycemia when given to recent-onset diabetic mice [53]. Thus, the emerging experimental evidence suggests that, as in other models of autoimmune diseases, CD4 Tregs are capable of controlling Type 1 DM at various levels.

CD4 Tregs in SLE

Most reports agree that CD4 Tregs are defective in SLE and especially in patients with active disease. Whereas decreased numbers of CD4 Tregs were determined in some studies of SLE patients, especially during the active phase of the disease, others claim a defective activity of the Tregs that is correlated with downregulation of the expression of Foxp3 [54–57]. Although it is still not clear whether the abnormality of SLE is related to a defective number or function of CD4 Tregs, or both, whether Foxp3 is the exclusive marker of functional Tregs, and what conditions lead to the suppressive function of the CD4 Tregs, the accumulating data suggest that strategies to enhance the function of CD4 Tregs might benefit patients with SLE.

The differences in reports regarding the status of CD4 Tregs in SLE patients may be due to the stage of disease, its activity, various disease manifestations and the influence of therapies. In addition, the use of different surface markers for the definition of Tregs owing to the lack of an absolute specific marker may also account for the differences between observations. Lupus-prone mouse models, which are more homogeneous than SLE patients, allow a more precise examination of CD4 Tregs without the interference of treatment with various immunosuppressive drugs. The involvement of numerical and/or functional modification of Tregs in lupus was demonstrated by the fact that glomerulonephritis accompanied by anti-DNA antibody production was detected in approximately 30% of immunodeficient mice reconstituted with CD25-depleted T cells, indicating that systemic autoimmune diseases might be controlled by CD4 Tregs [58]. In addition, depletion of CD4 Treg cells of nonautoimmune mice with anti-CD25 neutralizing antibody and breaking their tolerance resulted in the production of higher titers of anti-ds/ssDNA-specific antibodies than in the isotype control-treated group, indicating the involvement of CD4 Tregs in the regulatory mechanisms of autoantibody production [59]. Furthermore, (NZB × NZW)F1 and (SWR × NZB)F1 mice, which spontaneously develop a lupus-like disease, were reported to display a lower percentage of CD4 Tregs than BALB/c, DBA/1 and (DBA × NZW)F1 non-SLE-prone mice [60]. Reduced numbers of CD4 Tregs were also determined in mice congenic for the NZM2410 sle1 locus [61]. The reduced number of CD4 Tregs was associated with downregulation in the expression of Foxp3. MRL/lpr mice, in which a strong lupus disease develops, were shown to exhibit a normal percentage of CD4 Tregs and the Foxp3 mRNA and protein expression was not altered. Nevertheless, MRL/lpr CD4 Tregs displayed a reduced capacity to suppress proliferation and proinflammatory cytokine secretion from effector cells [62]. Adoptive transfer of exogenously expanded CD4 Tregs reduced the rate at which (NZB × NZW)F1 mice developed renal disease and improved survival, further supporting the role of these cells in disease regulation [63]. Further suggestions for the role of CD4 Tregs in the regulation of SLE manifestations in animal models are based on the observations that treatment with means that ameliorate lupus is associated with the upregulation of functional CD4 Tregs. Thus, induction of mucosal tolerance by administration of the histone peptide H471–94 for tolerance induction in the lupus-prone (NZB × NZW)F1 mice to levels observed in normal mice [60]. Using low doses (1 µg/mouse) of a nucleosomal histone peptide H471 restored the lower numbers of CD4 Tregs in MRL/lpr mice congenic for the NZM2410 sle1 locus [61]. In addition, the use of different surface markers for the definition of Tregs owing to the lack of an absolute specific marker may also account for the differences between observations. Lupus-prone mouse models, which are more homogeneous than SLE patients, allow a
T cells and autoantibody production. The cells were also effective in suppressing lupus-associated responses upon adoptive transfer in vivo [64]. The histone deacetylase inhibitor trichostatin A decreased inflammatory mediator production, autoantibody production, and indices of disease in (NZB × NZW)F1 mice in association with an increase in CD4 Tregs [65]. Finally, (NZB × NZW)F1 mice can be protected from developing SLE when given high intravenous doses (1 mg/mouse) of a synthetic peptide (pConsensus [pCons]) based on a shared CDR1 framework 2 epitope encoded within the variable heavy chain region of several murine anti-dsDNA immunoglobulins. Treatment with this peptide delays the appearance of autoantibodies in the treated mice and prolongs their survival. Administration of the tolerogenic peptide led to the expansion of peptide-reactive CD4 Tregs that inhibited in vitro the production of anti-dsDNA antibody-producing cells [66,67].

### CD8 Tregs

In addition to CD4 Tregs, CD8 Tregs have also been demonstrated to play an important role in autoimmune diseases. Some subsets of CD8 Tregs have been suggested to suppress immunity in a nonspecific manner (e.g., CD8+CD25+, CD8+CD122+, CD45RChigh and CD8-defined Tregs). By contrast, antigen-specific CD8 Tregs are primed to foreign or self-antigens and downregulate specifically the relevant immune responses. These CD8 Tregs include CD8+CD28- Tregs that frequently express Foxp3, CD8+CD75+ Tregs, CD8+CD45RC+ T cells, and TCR peptide-specific CD8aa Tregs [68,69]. In humans, CD8 dysfunction has been implicated in various autoimmune diseases including inflammatory bowel disease, Type 1 DM, rheumatoid arthritis and MS [70-72]. In MS, it was further demonstrated that the beneficial effects of immunotherapy with glatiramer acetate were at least partially due to the induction of CD8 Tregs [73].

CD8 Tregs have been associated with disease protection and recovery from EAE in rodents. For example, CD8+ T-cell depletion rendered the resistant CD28-deficient mice susceptible to EAE [74]. In the model of MOG35-55-induced EAE, the depletion of CD8+ T cells could enhance EAE [75]. Moreover, it was reported that MBP-specific tolerogenic APCs halt the progression of EAE via the induction of CD8 Tregs that suppress MBP-specific autoimmunity [76]. In addition, MHC class Iβ-restricted CD8+ T cells downregulated EAE disease activity and CD8+CD122+ Tregs reduced the signs of EAE, especially in the recovery phase [77]. In a study of a mouse model for inflammatory bowel disease, CD8+CD28- Tregs isolated from the spleen or gut prevented the development of the disease induced by transfer of colitogenic T cells into immunodeficient hosts [78]. In a model of EAMG, it was demonstrated that CD8+CD28- Tregs were involved in the suppression of EAMG-associated responses by a dual APL. The ability of the dual APL to inhibit the proliferation of T cells specific to Torpedo acetylcholine receptor was abrogated in CD8+ immunized mice. Furthermore, the dual APL did not suppress the production of the pathogenic cytokine IFN-γ in CD8-knockout mice that were immunized with the EAMG-inducing Torpedo acetylcholine receptor, suggesting that CD8 Tregs play an important role in the mechanism of action by which the dual APL ameliorates EAMG-associated autoreactive responses [79].

### CD8 Tregs in SLE

The information regarding CD8 Tregs in human lupus is limited. In addition to the observation that the functional activity of CD8+ suppressor cells is impaired in SLE patients with active disease and that the defective function could be related to abnormal cytokine secretion of these cells, there is a contrasting report that CD8+CD28- T cells are not quantitatively reduced in patients compared with controls [80,81].

Regarding murine models of lupus, the Hahn group demonstrated that after treatment of (NZB × NZW)F1 mice with their tolerogenic peptide, namely pCons, the number of CD8+ inhibitory cells increased in addition to CD4 Tregs [82]. The CD8 inhibitory cells were of both CD28+ and CD28- phenotypes. Both subsets of CD8 Tregs expressed elevated levels of Foxp3 and their suppressive activity could be fully inhibited by blockade of Foxp3 using the small interfering RNA technology [82]. Nevertheless, the expression of Foxp3 was significantly higher and persistent in the CD8+CD28- cells [82], suggesting that a predominant regulatory activity exists for the CD28- cell compartment. Secretion of TGF-β increased in both CD28+ and CD28- subsets of CD8 inhibitory cells as well. CD8 Tregs could directly suppress proliferation of both T and B syngeneic cells, and they were resistant to apoptosis at least partially via the upregulated expression of Bcl-2 [81]. The emergence of CD8+ cells was demonstrated to be associated with the downregulation of surface expression of programmed death (PD)-1 [83].
Thus, the suppressive capacity of the pCons-induced CD8+ cells depended on the intracellular expression of Foxp3 and on the alteration of PD-1 on the cell surface. Furthermore, induction of tolerance in the lupus-prone (SWR × NZB)F1 mice by the injection of very low doses (1 µg/mouse) of a nucleosomal histone peptide H4<sub>71–94</sub> induced CD8 Tregs in addition to CD4 Tregs. The CD8 Tregs required TGF-β for immunosuppression and were effective in suppressing lupus autoimmune responses and were adoptive transfer in vivo. In vitro, these cells suppressed IFN-γ responses of pathogenic lupus T cells to nucleosomal epitopes and reduced autoantibody production by inhibiting nucleosome-stimulated T-cell help to nuclear autoantigen-specific B cells [64]. The substantial type of APCs that played a role in the tolerance induction by H4<sub>71–94</sub> was determined to be plasmacytoid DCS [64]. Thus, the evidence indicated that both CD4 and CD8 Tregs play an important role in the amelioration of SLE manifestations in patients as well as in murine models of the disease.

Characterization & mechanisms of action of CD4 Tregs

Many of the cell markers that can be determined for CD4 Tregs are associated with the activation status of the cells in both mice and humans and therefore cannot be used solely to differentiate between activated CD4 cells and CD4 Tregs. In the mouse, the IL-2 receptor α-chain (CD25), which is upregulated in T cells following activation, is a typical marker of CD4 Tregs [84]. The expression of cytotoxic T-lymphocyte-associated antigen (CTLA)-4 is another example of a typical regulatory molecule that is upregulated in nonregulatory cells following their activation. Attempts to improve the definition of T cells with regulatory activity resulted in their allocation within subsets of cells that express memory-related markers such as CD45RB<sup>hi</sup> and CD62L<sup>lo</sup><sup>hi</sup>. The discovery of Foxp3 to be expressed specifically in CD4 Tregs enabled a most reliable differentiation between activated T cells and cells that function as suppressors/regulators [85,86]. Neuropilin-1 is a coreceptor to a tyrosine kinase receptor that is constitutively expressed on the surface of CD4 Tregs independently of their activation status, in contrast to nonregulatory T cells whose expression is downregulated following activation of the cell [87]. In addition, in this case, only CD4 cells that highly expressed neuropilin-1 exhibited high levels of expression of Foxp3. Those molecules that mostly participate in the immune regulation mediated by inducible (adaptive) CD4 Tregs are discussed below.

Foxp3 is a key transcription factor that is required for the development, maintenance and function of Tregs, and is sufficient to confer regulatory activity on naive T cells [85,86]. Foxp3 is considered to be a master gene because it controls the expression of multiple genes that mediate the regulatory activity of Tregs [88]. Furthermore, Foxp3 plays a major role in the regulation of immune responses only when it is expressed in T cells [89].

The best defined inhibitory receptor is CTLA-4 [90]. It is expressed constitutively in Tregs, but it is further upregulated by cell stimulation, thus contributing to the function of Tregs [91,92]. CTLA-4 in Tregs leads to the down-regulation of CD80 and CD86 on DCs, which in turn attenuates the activation of effector T cells [93–96]. Mechanisms that explain the suppressed activity of effector cells by CTLA-4 involve an increase in the activation threshold of T cells [97], and the induction of indoleamine 2,3-dioxygenase [98]. Deficiency in CTLA-4 [99,100] or deficiency in B7 molecules (CD80 or CD86) [101] was shown to interrupt this suppressive process. Although Tregs from CTLA-4-deficient mice were reported to develop and function normally through compensatory upregulated production of TGF-β [102], it has been recently shown that CTLA-4-deficient Tregs have diminished suppressive capacity that could not be overcome even when compensatory suppressive mechanisms became more active [103]. Furthermore, the ability of Tregs to control the differentiation of activated T cells into IFN-γ-producing effector cells [104] was abrogated by Tregs lacking the expression of CTLA-4 [103]. Hence, CTLA-4 is a critically required molecule for Tregs activity.

TGF-β is an additional essential molecule that is important for maintaining the expression of Foxp3 in Tregs and is central for the suppressive capabilities of these cells [105–107]. However, the downstream signaling through which TGF-β mediates the expression of Foxp3 is unclear. The suppressive effects of TGF-β can be transferred to effector T cells through soluble forms of this cytokine, or through direct contact with Tregs, which display TGF-β on their surface [108]. When cell-to-cell contact takes place, TGF-β molecules on the surface of Tregs are triggered to aggregate through signals originating from CTLA-4 [108]. T cells that cannot respond to TGF-β thus escape control by Tregs [107] and, as a consequence, generalized autoimmunity is developed in vivo [109].

PD-1 is a cell-surface inhibitory receptor [90]. It is upregulated on activated T cells (and B cells), and the concurrent engagement of PD-1 and
Tregs expressing TCR that is specific for an islet antigen were shown to be efficient in suppressing and reversing early onset of diabetes in NOD mice, whereas polyclonal Tregs were considerably less effective [51,122,123]. Similar effects were determined in mice that develop EAE spontaneously, in which the development of the disease was prevented following the adoptive transfer of CD4 Tregs whose TCRs were specific for MBP, in contrast to nonspecific Tregs that were ineffective [124]. Similarly, CD4 Tregs from healthy, prediseased NZM mice were not capable of improving glomerulonephritis and sialoadenitis developed spontaneously in the mice [125]. More recently, the notion that CD4 Tregs are disease specific was also demonstrated in mice that were thymectomized on day 3 by showing that the development of autoimmune ovarian disease could be suppressed by CD4 Tregs from the ovarian lymph nodes, but not by CD4 Tregs from nonovarian lymph nodes [126]. The results of these studies suggest that Tregs are antigen specific, and therefore therapeutic manipulations aimed at inducing functional suppressive Tregs should be carried out in the context of an antigen-specific driven process in order to enable the application of a controlled and an efficient function of the newly generated suppressive cells.

**Requirements for peripheral generation of antigen-specific Tregs**

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<td>Peripheral generation of Tregs occurs in response to an exposure to antigens peptides that are not necessarily presented in the thymus [127]. The antigen-specific Tregs are critical for prevailing organ-specific tolerance [121]. They can be found in the draining lymphoid tissues of the affected organ where self-antigens originating from that organ can constantly be presented to the Tregs [104,128]. The antigen that would be recognized specifically by an appropriate TCR determines the specificity of the target cells in the periphery. However, the amount of the antigen and its mode of administration are both important determinants. Thus, the development of Tregs can be carried out following the administration of small amounts of an antigen, under tolerogenic and not immunogenic conditions [129–132].</td>
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<td>CD4 Tregs possess TCRs of an increased avidity for self-peptides in comparison with CD4+CD25+ cells [117]. The analysis of the TCRs of Tregs revealed a massive diversity of repertoires [118,119], and it has been suggested that Tregs selectively affect effector T cells whose TCRs are for the same antigen [120]. The specific activity of Tregs was demonstrated in different animal models for autoimmune diseases. For instance, CD4 cells originating from thyroid-ablated rats were incapable of protecting against the induction of autoimmune thyroiditis in the recipient mice, hence, exemplifying the significance of organ-derived antigens for potent antigen-specific CD4 Tregs [122]. Furthermore,</td>
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TCR with the same APC causes the termination of TCR signaling [116]. The PD-1 molecule is therefore essential for protecting against autoimmunity [111]. The interactions *per se* between PD-1 and its ligand were not required for Tregs to enable the induction of tolerance and prevention of autoimmune diabetes [112]. Indeed, normal ranges of expression of Foxp3 could be determined even in PD-1-knockout mice, although Tregs from these mice lacked functional suppression [113]. Rather, the inhibition of PD-1 signaling in naive T cells was shown to increase the expression of Foxp3 [84]. By contrast, the expression of PD-1 was reported to induce autoimmune diseases [114]. However, since CD25 is not as useful in humans as in the mouse in defining Tregs, and the correlation between expression of Foxp3 and regulatory function is controversial in humans as well, other markers such as the cell surface α-chain of IL-7 receptor (e.g., CD127) are recommended for proper definition of Tregs [115].

Most of the above markers are used for the definition of human CD4 Tregs as well. However, since CD25 is not as useful in humans as in the mouse in defining Tregs, and the correlation between expression of Foxp3 and regulatory function is controversial in humans as well, other markers such as the cell surface α-chain of IL-7 receptor (e.g., CD127) are recommended for proper definition of Tregs [115].
APCs to promote the induction of Foxp3 in T cells is based essentially on the interactions between cognate T cells and APCs, and not on the production of soluble factors [132]. Among the types of APCs that possess properties of antigen presentation, B cells and DCs are the most studied candidates in association with the induction of antigen-specific Tregs [132,133]. The type of APC to be involved in the induction process of CD4 Tregs is suggested to be determined by the antigen dosing. Thus, DCs are the main APCs to mediate the induction of CD4 Tregs when very small amounts of the tolerogenic peptide are used [133], whereas the usage of increased amounts of antigen would favor the participation of B cells as the main APCs [132,133].

Costimulation

Whereas B7:CD28 costimulation was reported as essential for the development of Tregs in the thymus [134–137], costimulation is not required, and in fact, might hinder the induction of Tregs in the periphery [131,132,138]. Thus, tolerogenic administration of an antigen under repressed expression of CD28 resulted in upregulated expression of Foxp3, unlike the case of enhanced expression of B7 that led to inhibited expression of Foxp3 [139]. The inhibition of CD28 costimulation may favor the induction of Tregs, at least in part because it leads to downregulated levels of activator protein-1, a molecule interacting with the nuclear factor of activated T cells (NFAT) [140]. Moreover, the reduced expression of activator protein-1 may result in NFAT being more available for interaction and complex formation with Foxp3. As previously reported, such Foxp3–NFAT complexes are required for the induction of Tregs both in the periphery [138,142] and in the thymus [103], at least partly owing to its upregulating effects on the production of TGF-β [143].

Cytokines

The peripheral induction of Tregs [106,144], as opposed to the development of naturally occurring Tregs [145], depends on the presence of TGF-β. TGF-β signaling is required for de novo expression of Foxp3 [131]. However, it is not clear whether TGF-β affects the induction of Foxp3 directly through a Smad-signaling pathway or indirectly by its ability to impair the proliferation of T cells, which then might facilitate the expression of Foxp3 by peripheral T cells. TGF-β signaling is required for the suppressive capacity of Tregs, and for the in vivo expansion of Tregs [146]. Recently, it has been shown that TGF-β increases the amounts of acetylated Foxp3 protein binding to active chromatin sites, suggesting that TGF-β may be involved in the prolongation of the half-life of Foxp3 RNA species, and/or the phosphorylation of the chromatin-bound Foxp3, which may enable cellular compartment transitions for other transcription factors [147]. IL-2, in addition to TGF-β, is required for the peripheral induction of Tregs [148,149]. Nevertheless, in certain settings of CD4 Tregs induction, it was demonstrated that the levels of IL-2 were already satisfactory without requiring an exogenous addition of IL-2 [133,150]. It is noteworthy that the development of naturally occurring Tregs in the thymus was shown to be IL-2 independent, although IL-2 signaling was required for the homeostasis maintenance of Tregs [151,152].

Effect T lymphocytes

Effector T cells were recently suggested to play a role in the suppression induced by Tregs because their presence might potentiate Tregs for inhibitory capacity. This concept was considered after demonstrating differences in the molecular signature of Tregs, depending on the presence or absence of effector cells [153]. Thus, it was demonstrated that regulatory mediators (e.g., IL-35) could be upregulated by Tregs only in the presence of effector T cells [154].

Role of Tregs in the mechanism of action of the tolerogenic peptide hCDR1, which ameliorates SLE

In an attempt to develop specific therapeutic means for SLE, our group designed and synthesized a tolerogenic 19-amino acid peptide, namely hCDR1 [155], based on the sequence of the heavy chain complementarity determining region (CDR)1 of the human anti-DNA monoclonal antibody that bears the major idiotype, 16/6Id [156]. hCDR1 was tested for its ability to ameliorate established SLE manifestations in spontaneous and induced models of SLE in mice. Administration of small doses of hCDR1 (25–50 µg/mouse) subcutaneously [157], once a week for 10–14 weeks resulted in a significant amelioration of the serological (e.g., reduced autoantibody production) and renal (e.g., diminished proteinuria levels and decreased immune complex depositions in the glomeruli) manifestations that developed either in the SLE-prone (NZB × NZW)F1 mice or in BALB/c mice that were induced with experimental SLE with the disease-inducing anti-DNA antibody that
bears the 16/6Id [157]. Furthermore, hCDR1 downregulated disease manifestations associated with CNS lupus, as evidenced by reduced brain pathology and improved behavior parameters (e.g., anxiety and memory) [Lapper S et al., Unpublished Observations]. Similarly, severe combined immunodeficient (SCID) mice that were engrafted with peripheral blood lymphocytes from SLE patients and developed a SLE-like disease benefited from treatment with the tolerogenic peptide hCDR1 [158]. The beneficial effects of hCDR1 were associated with downregulated expression and secretion of the proinflammatory and pathogenic cytokines (i.e., IL-1β, TNF-α, IFN-γ and IL-10) and with upregulation of the immunosuppressive cytokine TGF-β [157]. Note that the weekly tolerogenic administrations of hCDR1 did not result in the production of peptide-specific antibodies.

Analyses of the cell populations obtained from mice with SLE manifestations revealed that in the T-cell compartment, most of the cells were characterized by activated/memory cells with only a small fraction of cells having the naive phenotype [159]. The rates of apoptosis in the activated T cells were significantly higher in comparison with those of the healthy controls. The downregulated expression of Bcl-xL, together with upregulated expression and function of Fas ligand (FasL), caspase 8 and caspase 3 suggested that activation-induced cell death (AICD) was dominant in the T cells of the SLE-affected mice [160,161].

The beneficial effects of treatment with hCDR1 on SLE manifestations could be adoptively transferred. Thus, spleen-derived cells from hCDR1-treated, young, free-of-disease (NZB × NZW)F1 donors efficiently downregulated lupus manifestations in the old, SLE-affected recipients [162]. Studies performed in our laboratory showed that the transferred cells contained subsets of cells with inhibitory capabilities, with CD4 Tregs being the major constituent of this population. Treatment of SLE-affected mice with hCDR1 upregulated CD4 Tregs by 30–40% in comparison with cells of either vehicle or control peptide-treated mice [163]. The hCDR1-induced CD4 Tregs were found to express CD45RBlow, CTLA-4, TGF-β and Foxp3. Furthermore, we showed that the transfer of non-hCDR1-induced Tregs (i.e., Tregs that were enriched from healthy, vehicle-treated mice) into SLE-affected (NZB × NZW)F1 mice did not beneficially affect the disease manifestations. By contrast, the adoptive transfer of enriched hCDR1-induced CD4 Tregs into the diseased mice resulted in significant beneficial responses. The latter effects were equivalent to those achieved following the transfer of 20-fold higher numbers of whole spleen-derived cells from hCDR1-treated mice [163]. Assessment of the in vitro suppressive effects of hCDR1-induced Tregs on effector T cells from the lupus-prone mice confirmed that the Tregs were highly potent even at a 1:100 Treg:T-effector-cell ratio [163]. It is noteworthy that hCDR1-induced CD4 Tregs express higher levels of TGF-β, CTLA-4 and Bcl-xL, in addition to intensified Foxp3. The efficient inhibitory activity of hCDR1-induced CD4 Tregs, together with the demonstration that Tregs originating from a control peptide, the vehicle-treated mice, or from unmanipulated mice, did not have a significant clinical effect on mice with established lupus, suggest that the hCDR1-induced CD4 Tregs were specific to SLE. This notion was further supported by experiments showing that the proliferation of cells from mice with experimental SLE could be inhibited following the transfer of cells from mice that were pretreated with hCDR1, but not from mice that were pre-treated with the dual APL, which was shown to inhibit MG-associated manifestations [47–50].

Development of hCDR1-induced CD4 Tregs in the diseased mice involves interaction with an additional kind of Treg, namely CD8 Tregs. Treatment with hCDR1 led to an upregulation of CD8 cells in association with repressed expression of the CD28 costimulatory molecule, an effect shown to be mediated by CTLA-4 [159,164]. The expression of Foxp3 was confined mainly to the CD8+ CD28+ subset of cells [164]. The induced CD8 Tregs from hCDR1-treated mice inhibited in vitro antigen-specific cell proliferation and the secretion of pathogenic cytokines [164]. However, the in vivo effects of hCDR1-induced CD8 Tregs were quite limited and significantly less prominent than those of hCDR1-induced CD4 Tregs. Nevertheless, in vivo depletion of CD8+ cells diminished the clinical improvement following treatment with hCDR1. It was demonstrated that in the absence of CD8 Tregs, CD4 Tregs were unable to expand in the hCDR1-treated mice, and the expression of Foxp3 was reduced, thereby interfering further with the suppressive function of CD4 Tregs. However, CD8 cells from hCDR1-treated mice that were adoptively transferred into SLE-affected mice led to upregulation of CD4 Tregs with intensified Foxp3 expression in the recipient mice. Therefore, our data suggest that CD8
Tregs are required for the optimal expansion and function of hCDR1-induced CD4 Tregs that ameliorate lupus manifestations [164].

As previously mentioned, T cells of the SLE-afflicted mice exhibited increased rates of apoptosis; however, treatment with hCDR1 reduced the rates of apoptosis in the effector T cells [160,161]. Several signaling pathways for apoptosis were affected by hCDR1.

The c-Jun NH$_2$-terminal kinase (JNK) of the p21Ras/MAP kinase pathway was highly expressed in T cells of the diseased mice and treatment with hCDR1 downregulated its activation [165]. Previous reports indicated that high activity of JNK kinase promotes Th1 effector cell maturation [166,167]; therefore, its reduced activity following treatment with hCDR1 may contribute to the downregulation of the pathogenic cytokine IFN-γ. The Fas signaling pathway was also affected by treatment with hCDR1. We found that cells originating from mice with established manifestations of SLE expressed high levels of FasL [161]. The ligation of FasL to its receptor on the affected cells is known to initiate a program of apoptosis that would be mediated via the activation of caspases 8 and 3 [168]. We found that CD4 Tregs from hCDR1-treated mice repressed Fas signaling via the downregulation of the expression of FasL in a CTLA-4-dependent manner, and diminished the activity of caspases 8 and 3 and upregulated the expression of the survival molecule Bcl-xL [159–161]. The suppressive effect on the expression of FasL played a role in restoring the normal profile of the pathogenic cytokines of the diseased mice [161]. It was further demonstrated that the expression of FasL in SLE-afflicted mice had a pathogenic role in kidney disease, which could be controlled following neutralization of FasL [169]. Moreover, inhibition of the activity of caspases in the hCDR1-treated mice could by itself lead to the clinical amelioration observed in our experiments because the mere inhibition of pan-caspase activity (using ZVAD-fmk) in a murine model of lupus was shown to be clinically effective [170].

Bcl-xL was shown to play several roles in lymphocytes from mice with established lupus. As a molecule with an antiapoptotic effect, the low expression of Bcl-xL in T cells of the diseased mice contributed to the increased rates of apoptosis combined with the enhanced activity of the proapoptotic Fas and p21Ras signaling pathways [160,161,165]. Following the administration of hCDR1 to the SLE-afflicted mice, the expression of Bcl-xL in T cells was upregulated. Moreover, the expression of Bcl-xL was enhanced in both the effector T cells and the CD4 Tregs of the tolerated mice. As a result, the rate of apoptosis was downregulated. It is hypothesized that intra-cellular material of apoptotic cells whose levels are frequent and sustained, may be the source of the self-antigens that provoked the autoimmune-associated responses in SLE [171,172]. Hence, the ability of hCDR1 to downregulate the rate of
apoptosis contributes to diminishing at least some of the provoking antigens that drive the immune response and possibly also to a better clearance of apoptotic debris [173]. Furthermore, the induced expression of Bcl-xL led to reducing the activation state of T cells and to downregulated secretion of IFN-γ and IL-10. Importantly, the hCDR1-induced CD4 Tregs elicited the expression of Bcl-xL in the effector T cells [160].

Antigen-presenting cells such as DCs and B cells are candidates for the presentation of hCDR1 to T cells. However, we found that B cells of hCDR1-treated mice exhibited increasing rates of apoptosis, possibly owing to down-regulation of the survival molecule Bcl-xL [160]. Furthermore, treatment with hCDR1 led to reduced numbers of DCs, favoring an immature phenotype of the remaining DCs, as manifested by the reduced expression of MHC class II and B7 molecules [174]. Indeed, immature DCs were previously reported to play a role in the induction of tolerance via their potent ability to induce CD4 Tregs [133]. The effects of hCDR1 on DCs were mediated at least partially by the immunosuppressive cytokine TGF-β [174], which was shown to be upregulated in T cells following treatment with hCDR1 [163].

In summary, mice with established manifestations of lupus that are treated with the tolerogenic peptide hCDR1 were shown to clinically improve in many aspects of the disease. As illustrated in Figure 1, a multistep process leading to better regulation of the immune response is initiated and orchestrated after the induction of functional Tregs. Once hCDR1-induced CD8 and CD4 Tregs develop, the increased state of activation and the elevated rates of apoptosis that typically characterize lupus-derived lymphocytes are ultimately downregulated. Hence, treatment with hCDR1 enables breaking a vicious cycle that occurred...
during the progression of the disease, where the decreased rates of apoptosis contribute to diminishing the perpetuating antigen inoculum and, as a result, the cells are less activated.

**Conclusions & future perspective**

The emerging data from multiple studies that were performed in SLE patients and especially in murine models of lupus are consistent with the view that decreased numbers and/or function of Tregs contribute to the pathogenesis of SLE. Therefore, therapeutic means that will induce functional Tregs of relevant specificities are likely to restore normal immune homeostasis in patients and to protect them from further development of pathogenic responses and tissue destruction. The coexistence of several subpopulations of Tregs has been reported and, as discussed above, an interaction between some of these subsets of Tregs (e.g., CD8+ and CD4+ Tregs) might be required for the optimal immunoregulatory function of the Tregs. Therefore, novel, specific therapeutic means that ‘chronically’ upregulate functional Tregs of the required subsets might be more effective in ameliorating the autoimmune disease than the administration of an expanded specific population of Tregs. Tolerogenic peptides, such as those described in this review that ameliorate the clinical manifestations in murine models of lupus via upregulation of CD4+ and CD8+ Tregs, are potential candidates for more specific and safer therapies that are needed for SLE. It is hoped that the encouraging information gained from numerous experiments performed in various animal models will serve as the basis for well-designed clinical trials aimed at harnessing Tregs for treatment of lupus and other autoimmune diseases.

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Harnessing regulatory T cells for the therapy of lupus & other autoimmune diseases


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* Recent article demonstrating the crucial role of cytotoxic T lymphocyte antigen-4 in the function of CD4 Tregs.
** Reports the essential link between TGF-β and maintenance and function of peripheral CD4 Tregs.
Optimal conditions for the induction of functional antigen-specific CD4 Tregs.


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* Requirement of CD8 Tregs for the induction of functional CD4 Tregs.


