Chapter 5

T Regulatory Cells in Systemic Lupus Erythematosus: Current Knowledge and Future Prospects

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Additional information is available at the end of the chapter

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Abstract

Systemic lupus erythematosus (SLE) is one of the most diverse autoimmune diseases, regarding clinical manifestations and therapeutic management. Visceral involvement is often and is generally associated with increased mortality and/or permanent disability. Thus, a reliable assessment of disease activity is required in order to follow-up disease activity and apply appropriate therapy. Several serological indexes have been studied due to their competence in assessing disease activity in SLE. Apart from conventional and currently assessed serological indexes, regulatory T cells (Tregs), a CD4+ cellular population of the acquired immune compartment with homeostatic phenotype, are currently under intense investigation in SLE. In this chapter, Tregs ontogenesis and subpopulations are discussed focusing on their implications in immunopathophysiology of SLE. The authors present data indicating that this CD4+ population is highly associated with disease activity and response to treatment, concluding that Tregs are a promising biomarker in SLE. Future prospective includes Tregs implication in SLE therapeutic interventions.

Keywords: regulatory T cells, systemic lupus erythematosus, SLE immunopathphysiology, Treg therapy

1. Introduction

Systemic lupus erythematosus represents the prototype of autoimmune diseases and is characterized by an unparalleled variety of clinical and laboratory manifestations. From a pathogenetic perspective, a breakdown of immune tolerance will lead to the proliferation and functional differentiation of certain effector cells of the innate and adaptive immunity, such as

neutrophils, dendritic cells (DCs), macrophages, and auto-reactive lymphocytes [1, 2]. The net result will be the production of pro-inflammatory cytokines and autoantibodies, formation of immune-complexes and, eventually, tissue damage driven by the deposition of these complexes onto certain tissues and the activation of the complement cascade; other mechanisms have also been described, such as autoantibody- and cell-mediated toxicity. Tissue damage will, in turn, provide the substrate for neo-epitope formation or the revealing of cryptic epitopes; this will further amplify the autoimmune response. Given the clinical diversity of SLE, several studies investigating the molecular mechanisms of the disease have yielded contradictory findings regarding multiple cellular subpopulations or soluble mediators. These findings seem to be influenced by disease duration, global disease activity, therapeutic variables, and other confounders [2]. Among them, an impairment of the mechanisms of the peripheral immune tolerance, mainly represented by the T regulatory cells (Tregs), has been universally documented in SLE and considered to be a crucial factor in disease pathogenesis.

1.1. T regulatory cells

Tregs represent a subpopulation of the CD4+ T lymphocytes which were first described in the 1970s [3] as suppressor cells since their primary function was the suppression of the immune response [4]. At that time, the term 'infectious tolerance' was introduced to describe the acquisition of suppressive capacity of non-suppressor cells from Tregs with an, as yet, unknown mechanism [5]. The study of this cellular subpopulation was initially abandoned due to technical difficulties with regard to the isolation and analysis of these cells because of the lack of specific surface markers [6, 7].

Research interest in suppressor T cells re-emerged in 1995, when Sakaguchi et al. described the intense expression of the α chain of the IL-2 receptor (IL-2Rα, CD25) on their surface [8]. These cells were then called regulatory T cells since their function was the multifaceted regulation of the immune response and the maintenance of immune homeostasis [9]. During the next few years, several investigators showed that these cells are characterized by a unique functional phenotype, which is marked, not only by the over-expression of the CD25, but also from decreased responsiveness after polyclonal stimulation of their T cell receptor (TCR) [10, 11]. These studies suggested that their regulatory/suppressive capacity against the effector T cells was irrespective of the antigen that generated the initial activation of the effector cells (non-antigen specific and, thus, non-MHC restricted).

The demonstration of the high surface expression of the CD25 molecule led to the characterization and distinction of Tregs from other subsets of T lymphocytes, as well as to the discovery of their thymic origin and initial functional differentiation [12]. However, it was later shown that CD25 is not exclusively expressed in Tregs. Other recently activated T lymphocytes and all T cells with regulatory function *in vitro* were also expressing this molecule [13]. As might be expected, Tregs do express the highest levels of CD25 ($CD25^{high}$) as compared to the conventional CD4+ T cells, in which its expression is transient and of low intensity. Based on flow cytometric analysis, it has been shown that, among CD4+CD25+ cells, only those at the upper 2% of CD25 expression possess suppressive capacity [14].

In 2001, the gene FOXP3 (Forkhead Box P3) was discovered in mice; its mutations were leading to the spontaneous development of autoimmune phenomena [15]. Mutations of the human

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FOXP3 have been associated with two distinct systemic autoimmune syndromes, namely the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) and XLAAD (X-linked, autoimmunity, allergy, dysregulation) [16–18]. In 2003, it was proven that FOXP3 is the master regulator for the functional differentiation of Tregs and is required for their proliferation [19]. It is found in the X chromosome (Xq11.23-Xq13.3) and consists of 11 exons that code a 48-kDa protein with 431 amino acids [18]. FOXP3 is mainly expressed in the T lymphocytes (mainly those that bear the αβTCR), whereas it is hardly detectable in B cells, γδ T cells, NK, macrophages, and dendritic cells. It is considered the lineage-specification factor of the natural T regulatory cells (nTregs).

The respective transcription factor FOXP3 is highly expressed in the CD4+CD25^{high} T cells, while its early activation in the naive T cells drives their differentiation toward a regulatory phenotype. This is particularly detected under inflammatory conditions where CD4+CD25- T cells overexpress FOXP3, which in turn leads to the increased surface expression of other molecules, such as CTLA-4 (cytotoxic T lymphocyte-associated antigen 4, CD152) and GITR (glucocorticoid-induced TNF receptor-a family-related protein) [20, 21]. These cells now possess suppressive capacity; they secrete less IL-2 and proliferate slowly.

Further research revealed that, like CD25, the expression of FOXP3 is not confined to naturally occurring Tregs; actually, it could be induced in recently activated cells (in low intensity) and CD4+ T cells that acquire suppressive properties afterward [22]. However, based on its critical importance, all cells bearing the FOXP3 key regulator are considered to be regulatory in function. FOXP3+ Tregs are divided in natural and inducible cells, according to their origin (thymus and/or periphery, respectively). The most well-studied subgroups of the inducible Tregs (iTregs) are the Tr1, Th3, and CD8+ Tregs (**Figure 1**).

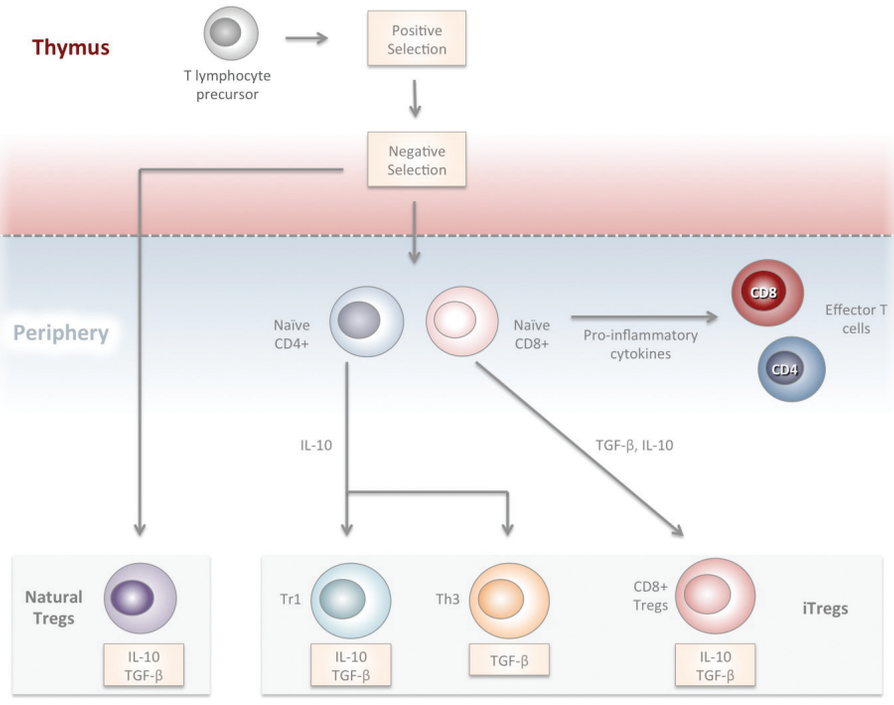

Figure 1. Natural (thymus derived) and inducible (peripheral) Tregs.

1.2. Natural Tregs (nTregs)

Thymic-derived Tregs or natural Tregs are characterized by the CD4+CD25^{high}FOXP3+ phenotype and range between 1 and 3% of the peripheral CD4+ T lymphocytes [13, 23]. They are considered to maintain an anergic state (based on the findings of decreased responsiveness to antigen stimulation and limited proliferation capacity), nTregs have remarkable proliferative potential, both *in vitro* and *in vivo*, upon antigen stimulation in the presence of dendritic cells [24]. Reciprocally, Tregs are able to induce tolerogenic DCs, further complicating their interactions with these cells [7, 12].

nTregs express the same αβTCR as the conventional T lymphocytes but they comprise a distinct clone [25]. They derive from pluripotent stem cells and differentiate in the thymic cortex through a positive selection process after the linkage of their TCR with self-antigens with intermediate affinity [26, 27]. These antigens are presented through MHC II molecules from the thymic cortical cells [28]. Co-stimulation via the CD28 molecule induces the FOXP3 promoter either directly or through other genes that increase its activation [29]. It seems that the selection of these CD25+ cells occurs according to a predefined ratio to the respective CD25− cells, which have been generated earlier. They are long-lived cells capable of producing anti-apoptotic molecules that protect them from the process of negative selection [26, 27].

Upon migration to the periphery, nTregs maintain their regulatory phenotype and suppressive capacity, which are mediated through cell-to-cell contact. This mechanism involves certain surface molecules, and it is independent of secreted cytokines [13, 26]. Survival in the periphery is facilitated by CD28 and its respective ligands (CD80, CD86), transforming growth factor β (TGF-β) and IL-2 [12].

Several surface molecules have been considered to allow the laboratory isolation from other cellular subpopulations and are crucial for their function. The most important such molecules are the CD4, CD25^{high}, CD127^{low}, CD45RO and CD45RB^{low}, providing a phenotype of activated memory cells [30].

Moreover, nTregs express other activation markers, such as CD28, CTLA-4, GITR and chemokine receptors, which are implicated in their migration and trafficking [30, 31]. They also express several Toll-like receptors (TLRs), TGF-β, neuropilin-1, perforin and granzymes, L-selectin (CD62L), LAG-3 (lymphocyte activation gene-3, CD223) and the folate receptor FR4 [32–35]. Multiple adhesion molecules are also abundantly expressed on their surface, such as CD11a, CD44, CD54, and CD103 [36]. All the aforementioned markers have been described in other cell types, which are not exclusively expressed in nTregs and cannot be used as differentiation markers.

Other markers that are thought to be highly specific for nTregs were discovered from the Ikaros gene family; the respective transcription factor is called Helios and is preferentially expressed in nTregs as compared to other CD4+ T cells [37].

Recently, it has been demonstrated that certain epigenetic mechanisms are implicated in the regulation and maintenance of FOXP3 expression [38]. In this regard, the methylation state of the Treg-specific demethylated region (TSDR, a conserved non-coding sequence in the

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CNS2 region of the FOXP3 gene) plays a crucial role. Current isolation techniques require this method since only CD4+CD25+FOXP3+ T cells with demethylated TSDR were capable of strongly and permanently expressing FOXP3 and suppressing effector cells [39]. TSDR is incompletely hypomethylated in Tregs that are induced in the periphery and completely methylated in all other CD4+CD25+ T cells [38]. Helios+FOXP3+ Tregs have increased suppressive potential and are fully demethylated at the TSDR region [37].

1.3. Inducible or adaptive Tregs (iTregs)

These Tregs subgroups are not derived from the thymus but they are induced from naive CD4+ T cells in the periphery in response to the occasional micro-environmental conditions, **Figure 1** [40]. Inducible Tregs regulate the immune response against self and non-self antigens and are implicated in the pathophysiology of infections, neoplasias and organ transplantation. Their mechanism of action is usually dependent on the secreted cytokines and not on direct cellular contact. Their characterization is based on the aforementioned surface markers (CD25, CD127, CTLA-4, GITR, etc.), the intensity of intracellular FOXP3 expression as well as their suppressive capacity [13]. As mentioned above, their TSDR is incompletely hypomethylated; thus, FOXP3 expression is transient and unstable. The most important subgroups include the Tr1 and Th3 lymphocytes, the CD4+CD25+ Tregs that are induced from the CD4+CD25− activated T cells, CD103+ Tregs, CD8+ Tregs and the double negative Tregs (CD4−CD8−DN).

Tr1 cells are antigen-specific CD4+ T regulatory lymphocytes that are induced in the presence of IL-10 [41]. They derive from CD4+CD25− naive T lymphocytes after antigenic stimulation with certain costimulatory molecules, such as CD3 and CD46 [42, 43]. Apart from the epigenetic differences, they are phenotypically indistinguishable from natural Tregs, but they secrete large amounts of IL-10. The intensity of the surface expression of CD25 and intracellular FOXP3 is lower than that of nTregs; however, their suppressive capacity is as intense [44]. Their regulatory function is mediated mainly through IL-10 and, secondarily, through TGF-β. They play a major role in the pathophysiology of certain infections and the regulation of allergic reactions [45].

Th3 lymphocytes are CD4+ Tregs that were called helper T cells (T helper 3), although their function is primarily suppressive [46]. Their cardinal characteristic is the secretion of large amounts of TGF-β [47]. The *ex vivo* expansion of the Th3 cells was one of the first reports of clonal expansion of Tregs using an orally administered antigen in mice [48]. Th3 cells are generated and activated through an antigen-specific process but their suppressive function is not specific and mediated through TGF-β. Even in the absence of inflammation, TGF-β secretion induces the expression of FOXP3 in the activated T cells and maintains Tregs' survival in the periphery [49].

Other types of Tregs include the CD4+CD25+ Tregs deriving from CD4+CD25− T lymphocytes under specific conditions, the CD103+ Tregs (expressing integrin alpha-E beta-7), the CD8+CD28− Tregs and the CD4−CD8− double negative Tregs [13]. All these subpopulations express FOXP3 upon activation and are able to suppress immune responses in a non-antigen specific fashion.

Further research using certain surface markers revealed the existence of novel subpopulations of iTregs, including the CD4+CD25−FOXP3+ T cells, the CD4+CD45RA+FOXP3+ Tregs,

the CD4+CD161+ Tregs, and the CD4+CXCR5+FOXP3+ Tregs [50–52]. Although the CD25−FOXP3+ T cells could suppress effector cells *in vitro*, it is still uncertain if they represent dysfunctional Tregs or, simply, activated T cells. The CD161+ Tregs represent an excellent paradigm of T cell plasticity, as they are capable of producing pro-inflammatory cytokines such as IL-2, IFN-γ, and IL-17, behaving like Th1 or Th17 cells under proper cytokine micro-environment [53]. In spite of their cytokine-producing properties, these cells also retain their regulatory functions and have the already mentioned demethylated TSDR in the FOXP3 locus, like the nTregs. Finally, the CXCR5+ Tregs are follicular T cells, which are able to gain access into the germinal centres (through the CXCR5) and directly suppress the B cells that undergo hypermutation and isotype switch at those sites. These cells are decreased in active and new onset SLE, seemingly allowing for the activation of B cells [54].

2. Mechanisms of action

The mechanisms of action of Tregs have been studied mostly in *in vitro* systems. Thus, it is not clear how accurately these studies may reproduce Treg activity *in vivo.* Tregs delete auto-reactive T cells and induce tolerance and dampen inflammation, while their cellular targets include CD4+CD25− T cells, CD8+ T cells, B cells, monocytes, DCs, and NK cells [55]. These cells appear to inhibit the target cells via IL-2 deprivation, cell-to-cell contact and cytolysis, secretion of inhibitory cytokines, metabolic disruption and modulation of DC maturation and function [56–60], **Figure 2**.

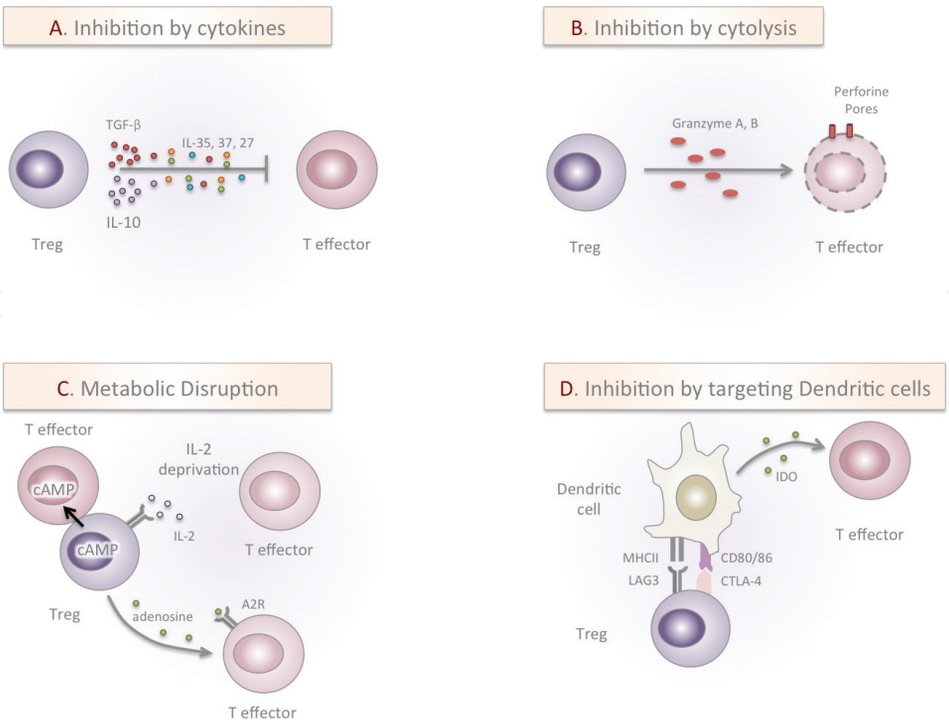

Figure 2. Treg mechanisms of action.

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IL-2 is not required for the thymic development of nTregs; however, in the periphery, it acts as a growth factor, essential for their survival and functional integrity. Tregs have more requirements in IL-2 than conventional T cells. IL-2 drives the production of IL-10, CTLA-4, TGF-β, and the activation of FOXP3 [61]. Simultaneously, CD25 expression is induced, further amplifying the affinity of Tregs for IL-2. In co-cultures of Tregs and T effector cells, addition of exogenous IL-2 led to active proliferation and activation of Tregs [62]. In addition, Tregs inhibit the function of other T helper cells or cytotoxic cells by deprivation of other cytokines that share the common γ chain, such as IL-4 and IL-7; this leads to apoptosis of the effector cells [63]. Moreover, the prioritized usage of IL-2 may modify the function of Tregs by the increased IL-10 production [64].

The suppressive function of nTregs is mediated through direct cell-to-cell contact and is not dependent on the presence of inhibitory cytokines like IL-10 and TGF-β [13, 56, 57]. Surface molecules that are involved in this process include CTLA-4 [65, 66], membrane-bound TGF-β [67, 68], LAG-3 (lymphocyte activation gene-3, CD223) [69], GITR (glucocorticoid-induced TNFR-a family-related protein) [70, 71], PD-1 (programmed death-1, CD279) [72] and perforin and granzymes, which lead to cytolysis of the target cell [73].

Granzyme B, in particular, has been implicated as an effector mechanism in Treg-mediated suppression, since its up-regulation was associated with the killing of target cells in a granzyme B-dependent, perforin-independent manner [73]. Granzyme B-deficient Tregs display reduced suppressive activity [74]. Other studies proved that the activated Tregs could lyse CD4+, CD8+ T cells and B cells through granzyme A and perforin [56–58].

The intracellular signal transduction pathways have not been elucidated yet; however, it has been demonstrated that CTLA-4 induces the expression of ICER (inducible cAMP early repressor) and, subsequently, inhibition of IL-2 signals to target cells [75]. Membrane-bound TGF-β activates the Smad proteins and inhibits genes that are required for the functional differentiation of the effector cells [68].

Suppression by inhibitory cytokines is an important mode of action utilized by iTregs. The most important cytokines with regulatory/suppressive capacity are TGF-β [76, 77], primarily produced by the Th3 cells and IL-10 [78, 79] by the Tr1 cells. IL-10 activates the JAK/STAT intracellular pathway and the MAP kinases [78]. The net result is the inhibition of genes that control the synthesis and secretion of pro-inflammatory cytokines. Another regulatory cytokine that is produced from Tregs is the IL-35 [80]. This cytokine is assembled by two chains, IL-12α and EBI3, and is required for the suppressive capacity of Tregs *in vivo*, since inability to express these chains results in uncontrolled expansion of T effector cells in systemic autoimmune diseases [81]. Other anti-inflammatory cytokines that have been implicated in the mechanisms of action of Tregs include IL-27 and IL-37 [82]; more recently, it has been shown that fibrinogen-like protein 2 is also secreted by Tregs and mediates immune suppression [38].

Metabolic disruption of the target cells is another mechanism utilized by Tregs to regulate immune responses. nTregs possess large amounts of cAMP (cyclic adenosine monophosphate), which exerts potent inhibitory action against the proliferation and differentiation of

the effector T cells and the expression of genes that control the synthesis of IL-2 and IFN-γ [83]. Gene expression is inhibited through the suppression of the protein kinase A of NF-κB or through activation of ICER. Tregs induce intracellular cAMP within the effector T cells with cell-to-cell cAMP transfer through the gap junctions. Neutralization of cAMP or blockage of the gap junctions led to significant weakening of Tregs' suppressive function [83]. In addition, co-expression of CD39 and CD73 on the surface of Tregs induces the secretion of large amounts of adenosine that suppresses T effectors [84]. The linkage of adenosine to its receptor A2A on Tregs induces TGF-β secretion and inhibits IL-6, generating appropriate circumstances for new Treg development [85, 86].

Tregs seem capable of limiting the capacity of DCs to stimulate effector cells. In this context, their interaction with the dendritic cells and the inhibition of their maturation is of particular importance [87, 88]. Tregs induce the production of regulatory molecules from DC, such as indoleamine-2,3-dioxygenase (IDO), IL-10 and TGF-β, through interactions between CTLA-4 and CD80/CD86 [89, 90]. The same investigators showed that Tregs reduce the expression of the costimulatory molecules CD80 and CD86 on DCs. Moreover, the catabolism of tryptophan and arginine through IDO leads to Tregs activation and induction of regulatory phenotype in naive T cells and T effector cell apoptosis [91].

3. Homeostasis of Tregs

3.1. Functional differentiation of Tregs in the periphery

Certain transcriptional factors are implicated in the process of maturation and functional specialization of the CD4+ T cells. The most important factors are T-bet for Th1 cells, GATA-3 for Th2 cells, RORγt for Th17 cells, Bcl6 for follicular T helper cells (Tfh), and FOXP3 for Tregs [92]. For Tregs, in particular, their functional integrity depends on the dynamic interaction of different transcriptional regulators, which is shaped by the occasional micro-environmental circumstances. These regulators include members of the nuclear factor of activated T (NFAT) cell family, the NF-κB, the activator protein-1 (AP-1) and STAT5 [93]. Furthermore, functional specialization of Tregs has been documented; for instance, these cells are using Th-related transcription factors during Th1, Th2 or Th17 immune responses. In this context, T-bet+ Tregs migrate into the inflamed tissue in cases of Th-1-mediated inflammation and suppress the Th1 effectors [94]. Accordingly, the expression of IRF4 in Tregs is required for the suppression of Th2 responses, while the deletion of STAT3 is linked to uncontrolled Th17 responses [95]. The precise mechanisms by which these transcription factors control Tregs differentiation are unknown. However, the experimental inhibition of these factors was associated with impaired expression of certain surface chemokine receptors, such as CXCR3 (for Th1), CCR8 (for Th2) and CCR6 (for Th17 immune response) [96]. Moreover, deletion of the respective genes of these chemokine receptors led to decreased Tregs activity and renders Tregs incapable of migrating into the site of Th-1-mediated inflammation [97]. Based on these data, it seems possible that phenotypically and functionally distinct Tregs may be active against different effector arms of the immune response.

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3.2. Clonal expansion of Tregs

The question if nTregs numbers remain stable through life or if their pool is constantly enriched with new cells was based on the findings of stable numbers of CD4+CD25+ T cells in mice from the age of 2 weeks up to 1 year. In thymectomized mice with no T cells, adoptive transfer of Tregs was followed by an expansion of these cells to the extent of the nonthymectomized animals of similar age [98].

In humans, it has demonstrated that nTregs, after they leave thymus, are constantly proliferating after cytokine (TGF-β, IL-2, IL-10) stimulation and in the presence of tissue antigens. DCs can also induce Tregs in the presence of IL-2 [99].

3.3. Tregs recruitment at the site of inflammation

Natural Tregs are generated in the thymus and migrate into the periphery where their population will be enriched with inducible Tregs. The precise site of their clonal expansion (peripheral lymphoid organs or the site of inflammation) is not known. Apart from the thymus, Tregs have been found in the bone marrow, lymph nodes, intestine, liver, synovial fluid, skin, vessel wall, etc.

More than 25% of the total CD4+ T cells residing in the bone marrow have regulatory phenotype and properties [100]. In this regard, the bone marrow acts as a reservoir for Tregs that is able to release them upon inflammation. Bone marrow Tregs express CXCR4 (the CXCL12 receptor), which is crucial for their migration and their return to the bone marrow after suppression [100]. Integrins are also implicated in their migration; intense expression of CD62L and CCR7 along with poor expression of CD103 (integrin αEβ7) allows for penetration into the lymph nodes. On the contrary, strong expression of CD103 is required for migrating into the inflamed tissues [94].

Integrins are crucial for the homeostasis of iTregs; integrin α4β7 tissue expression (usually in the mucosal vessels) attracts Tr1 cells, whereas the α4β1 (on the endothelium of inflamed tissues) engages the Th3 cells [101]. Furthermore, it has been showed that when Tregs migrate to the T-zone of lymph nodes, they utilize the CCL19/CCR7 ligation, while when they migrate to the B-zone, they utilize the CXCL13/CXCR9 interaction [102].

3.4. How effector cells escape Treg-mediated suppression

Tregs are also regulated by the immune system in a fashion that allows the control of their action either through negative feedback or through the development of escape mechanisms for the effector cells [103, 104]. The negative feedback is maintained through various mechanisms, such as TLR activation on DCs [104] and the direct regulation by cytokines like IL-21, IL-7, IL-15, TNF-α and IL-6. In particular, IL-21 increases the resistance of the effector T cells against Tregs in experimental diabetes [105]. DC-derived IL-6 renders CD4+ T cells resistant to Tregs suppression [106]. Additional mechanisms include the amplification of co-stimulatory molecule expression on the surface of T effectors and DCs, such as the CD80 and CD86 molecules, CD28, NFATc1, c2, c3 and TRAF6, which protect the integrity of intracellular signal transduction [10].

4. Tregs in systemic lupus erythematosus

4.1. A matter of numbers and function?

SLE is characterized by the breakdown of immune tolerance against self-antigens. The net result is the induction and proliferation of auto-reactive lymphocytes, the production pro-inflammatory soluble mediators, the formation of pathogenic autoantibodies and immuno-complexes that cause tissue damage [1]. Tregs are thought to play a critical role before and during this pathophysiological process. Most studies in lupus-prone mice and humans demonstrated quantitative and/or qualitative defects of these cells [14, 107–113]. Other reports present insignificant variations in Tregs numbers between lupus patients and healthy controls [50, 114, 115] or even higher numbers [116], probably as a result of significant differences in protocol designs. With regard to the functional capacity of these cells, studies are again controversial with reportedly defective [110, 111] or normal [14, 114, 115] function. In the latter case, T effectors showed decreased sensitivity to the suppressive function of Tregs [117].

In a seminal paper, Miyara et al. described the characteristics of Tregs kinetics and the strongly inverse the correlation with SLE disease activity [14]. They found that Tregs (CD4+CD25bright) were globally depleted from the periphery of active lupus patients. They provided evidence that these cells do not accumulate in involved organs (by kidney biopsies) or lymphoid tissue (by lymph node biopsies). In fact, Tregs were found to be more sensitive to Fas-mediated apoptosis although they were still functionally intact. Moreover, they were the first to show that Tregs are increased after the successful treatment of disease flare. FOXP3 expression was found in 85.6% of the CD4+CD25^{high} compartment.

The issue of functional integrity of Tregs within the lupus inflammatory microenvironment was questioned later by the findings of Valencia et al. [110] and Lyssouk et al. [111]. These groups reported that CD4+CD25^{high} Tregs were defective in terms of both proliferation and suppression against CD4+ and CD8+ T effector cells. They also showed that FOXP3 expression was decreased in Tregs from active lupus patients, generating doubt about the appropriate immunophenotype that should be used for cell isolation and study. These findings were confirmed later by using the mean fluorescence intensity (MFI) in newly diagnosed, untreated lupus patients [118].

At the same time, Barath et al. were the first to utilize the CD4+CD25^{high}FOXP3+ immunophenotype for Tregs characterization [112]. They found these cells in significantly lower levels as compared to healthy controls, whereas the inducible CD4+IL-10+ Tregs did not display any significant quantitative differences. At the tissue level, CD4+CD25+FOXP3+ Tregs were found in decreased numbers in the skin lesions of active cutaneous lupus as compared to other inflammatory skin diseases, such as psoriasis, atopic dermatitis, and lichen planus [119].

In 2008, a new subpopulation was described, namely the CD4+CD25−FOXP3+ T cells [50]. These cells were found in increased numbers in newly diagnosed SLE patients and were associated with other indices of disease activity, such as low complement C3 and C4 [51]. Their function was primarily regulatory as they were able to suppress the effector T cell proliferation but not IFN-γ production [51]. Other investigators opposed these findings by performing

the measurements in untreated lupus patients, reaching the conclusion that not all FOXP3 expressing T cells are Tregs [120].

The association of Tregs with SLE disease activity was tested in several studies with a small number of patients. Most of them reported a strongly inverse correlation [14, 113], whereas others found insignificant correlations [109, 112].

In the first large-scale (n = 100 patients), longitudinal (mean follow up of 5 years) study of CD4+CD25^{high}FOXP3+ Tregs as a biomarker of disease activity, we found that these cells are gradually decreased from healthy controls to patients with inactive, mild, or severe disease [113]. Moreover, we observed inverse alterations in cases of changing disease activity; these cells were reduced during disease flare and increased upon remission, while numbers remained stable during stable disease activity. Their sensitivity and specificity to assess a clinically significant change in global disease activity was 88 and 74%, respectively. Their positive and negative predictive values were 85 and 79%, respectively. In the same study, we reported decreased Tregs numbers in active lupus nephritis and active neuropsychiatric involvement, whereas no differences were observed in patients with active antiphospholipid syndrome (APS). The ability of CD4+CD25^{high}FOXP3+ Tregs to predict disease flares was low (positive predictive value 17%).

Concerning the influence of certain therapeutic approaches, we prospectively showed that Tregs' numerical restoration after treatment is independent of the occasional medication administered [121]. In that study, patients achieved remission after administration of various immunomodulatory agents, including glucocorticoids (oral and intravenous), cyclophosphamide, intravenous immuno globulins, azathioprine and hydroxy chloroquxine. In all cases, a significant increase of CD4+CD25^{high}FOXP3+ Tregs was observed. That restoration was faster with the intravenous regimens as compared to oral therapies. Cyclophosphamide pulse therapy, in particular, led to a significant increase of Tregs after the fourth pulse in patients with active lupus nephritis and/or neuropsychiatric involvement [122]. Of note, an even faster response (shortly after the first infusion) was documented after treatment with intravenous tocilizumab in patients with rheumatoid arthritis [123].

4.2. Novel theories for Tregs in the pro-inflammatory environment of SLE

As mentioned above, most studies on Tregs have been conducted *in vitro*; thus, their reliability and accuracy pertaining to the actual function of these cells *in vivo* are questionable. After the discovery that a fully demethylated TSDR is required for intense and sustained expression of FOXP3, the hallmark of regulatory function, many beliefs have been revised [39]. In this context, Helios+FOXP3+ T cells, with a fully demethylated TSDR, were found in increased numbers in active lupus patients; their function was intact [124]. It is not yet known if Helios represents a unique marker for Tregs; however, the epigenetic change is believed to differentiate between natural and inducible Tregs. Nevertheless, there is disequilibrium between Tregs and effector cells that is more prominent in the pro-inflammatory environment of SLE.

Several studies have reported on altered ratios between Tregs and T effectors in lupus patients [125]. The most striking feature, among other findings, was that there is plasticity between

Tregs and Th17 populations, and the latter may derive from the former under certain circumstances [126]. In this regard, the presence of TGF-β alone will drive naive CD4+ T cells towards Tregs differentiation, while the simultaneous presence of IL-6 will lead to Th17 proliferation [127]. Other transitions have also been described between Th1 and Th17 cells, based on the presence of IL-12 receptors on the surface of Th17 cells; upon activation with IL-12, these cells are capable of producing IFN-γ [125, 127]. Of note, it has been documented that some Tregs down-regulate FOXP3 expression and act as effectors, promoting inflammation through the secretion of IL-17 and IFN-α [39]. These cells are called ex Tregs and believed to derive from the Tregs lineage prior to natural Tregs commitment. They acquire pro-inflammatory characteristics in the periphery, probably in the context of a generalized immune response.

The concept of Tregs/Th17 imbalance, in particular, seems of paramount importance in SLE. It has been demonstrated that disease relapses may occur as a consequence of an impaired Tregs/Th17 ratio, in favour of the latter, in animal models [128]. Those findings were later confirmed in lupus patients, in whom the altered Tregs/Th17 ratio was documented even in clinically quiescent disease; this may represent a hallmark of SLE [129, 130]. In this context, it is believed that the sole targeting of the Th17 arm of the immune response will not render meaningful results; approaches aiming at the restoration of the Tregs/Th17 balance will be more likely to exert beneficial effects [125, 131].

The disturbed balance between T effectors (Th1, Th2, Th17) and Tregs is driven by a relative IL-2 deficiency in SLE [132]. Treatment with low doses of IL-2 re-established the equilibrium between Tregs and T effectors in animal models of the disease; accordingly, IL-2 neutralization or CD25 depletion accelerated disease onset [39]. Studies in lupus patients showed that FOXP3+Helios+ Tregs were capable of proliferating despite the reduced IL-2 levels; however, the integrity of their suppressive function has not been confirmed [124]. Apart from IL-2, other cytokines, such as IL-6, IL-21 and IFN-α may inhibit the Tregs function and/or render T effectors resistant to regulation. All these cytokines are found in abundance in SLE and are positively correlated to disease activity. On the other hand, the main regulatory cytokine, TGF-β, is lower in active disease, generating hypotheses that the cytokine disequilibrium drives the imbalance of Tregs and T effectors. The exact mechanisms by which these cytokines increase the T effectors' resistance to Tregs suppression have not been elucidated yet [39].

4.3. Epigenetics and Tregs

Latest studies revealed that certain epigenetic mechanisms, such as methylation, histone modification and miRNAs, play a significant role in Tregs biology [38]. In this regard, the methylation status of the TSDR is of paramount importance for the sustained expression of FOXP3 and, hence, the intensity of Treg suppressive function. Histone modification is another mechanism involved in Tregs functional differentiation. The acetylation of histones H3 and H4 has been shown to reliably differentiate Tregs than FOXP3+ effectors [133]. Modification of the FOXP3 promoter by other genes influenced dramatically the rate of differentiation of iTregs in the periphery [38]. Finally, miRNA-155 is associated with less Tregs, though functionally intact, in mice, while miRNA-126 up-regulates Tregs and enhances their function [134].

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Epigenetic regulation of the FOXP3 gene has been reported in lupus patients. In this context, decreased peripheral Tregs were associated with hypermethylation of the promoter of the FOXP3 gene [135]. Genome-wide studies have shown that virtually all immune cells in SLE, including Tregs, had severe hypomethylation in interferon-type I-related genes [38]. Treatment with a histone modification inhibitor the enhanced Tregs number and function in lupus-prone mice [136]; the same results were reached with an inhibitor of the protein kinase IV [137]. It is believed that these approaches will soon be tested in lupus patients [38].

5. Tregs-based therapeutic approaches in SLE

5.1. Adoptive transfer of *ex vivo* expanded Tregs

Based on the aforementioned data, Tregs may represent a promising target in SLE therapeutics. Several groups have tried to manipulate this cellular subpopulation in order to restore the defective immune tolerance that is a crucial component of disease pathophysiology [138]. Early experiments in mice models showed that adoptive transfer of *ex vivo* expanded Tregs was capable of ameliorating the disease [139, 140]. In the first experiment, T cells treated with IL-2 and TGF-β lost their ability to induce a graft-versus-host disease and prevented other effector T cells from activating B cells [139]. In addition, when transferred to animals with high titers of anti-dsDNA antibodies, they led to a significant reduction of their titers and doubled survival. In New Zealand black/New Zealand white mice, a well-studied lupus model, transfer of thymic Tregs (CD4+CD25+CD62L^{high}) decreased the rate of glomerulonephritis, the severity of proteinuria and improved survival [140]. The precise mechanism by which these Tregs suppress the autoimmune response has not been elucidated; however, it was demonstrated that the induction of tolerogenic DCs plays a critical role [141]. These DCs were also able to expand the recipient's CD4+FOXP3+ Tregs (infectious tolerance).

Studies in humans also showed that *in vitro* expanded Tregs, both polyclonal [142] and antigen-specific [143] may display enhanced regulatory activity. These encouraging results led to the implementation of this strategy in phase I and II clinical trials in other autoimmune diseases. Seminal studies in type 1 diabetes (T1D) proved the feasibility of generating purified iTregs for therapeutic purposes [144, 145]. Bluestone et al. demonstrated that Tregs could survive for more than 1 year after infusion in 14 patients with T1D; although there were no significant reactions to infusion, from an efficacy standpoint there was no significant improvement in C-peptide levels and HbA1c [144]. In another study of 12 children with T1D, adoptive transfer of Tregs led to significant reduction in exogenous insulin needs and improvement in C-peptide levels. Of note, two children were insulin independent after 12 months [145]. In chronic graft-versus-host disease (GVHD), adoptive transfer of Tregs ameliorated symptoms in two out of five patients, while the remaining patients did not show any deterioration after 21 months of follow-up [146]. In another study, where umbilical cord-derived Tregs were used in 11 patients, there was a significant reduction in the rate of severe acute GVHD, whereas chronic GVHD at 1 year was 0% in Treg-treated patients and 14% in patients who received the conventional therapy with immunosuppressives (sirolimus and mycophenolate mofetil)

[147]. All the aforementioned studies reported a purity of approximately 90%, demonstrating that this approach is feasible; on the other hand, survival of Tregs *in vivo* (after infusion) was limited with a dramatic decline after 14 days from infusion. There are several currently ongoing clinical trials based on adoptive Treg transfer mainly in solid organ transplantation [147]. Such therapeutic approaches have not been published yet in lupus patients; one phase I clinical trial aiming to assess Treg efficacy in cutaneous lupus started in 2015 [148].

5.2. Hematopoietic and mesenchymal stem cell transplantation

Hematopoietic and mesenchymal stem cell transplantation (HSCT and MSCT, respectively) aim at immune reconstitution after intensive chemotherapy and have been implemented in cases with refractory autoimmune diseases.

In the context of SLE, HSCT has been shown to induce long-term remission for approximately 5 years in half patients, whereas relapse was usually mild [149, 150]. On the other hand, MSCT exerts potent immunosuppressive capacity since mesenchymal stem cells do not require MHC (major histocompatibility complex) restriction for their function [151]. The effects of these therapeutic approaches on Tregs numbers and function have a critical role with respect to their efficacy. Zhang et al. showed that CD4+CD25^{high}FOXP3+ Tregs were reconstituted in levels comparable to those of normal individuals after autologous HSCT in 15 SLE patients [152]. In addition, a novel Tregs subset (CD8+LAPhighCD103^{high}) was induced and capable of maintaining remission through TGF-β mediated suppression. On the contrary, Szodoray et al. did not find any significant differences in Tregs numbers (pre- and post-transplant) in 12 patients with various systemic autoimmune diseases; only three lupus patients were enrolled in that study [153].

Concerning MSCT, a report on nine patients with refractory SLE showed good safety profile after 6 years; unfortunately, Tregs were not assessed in this study [154]. Limited case reports demonstrated a significant increase of peripheral Tregs in three lupus patients; however, clinical remission was not achieved [155, 156]. Of note, mesenchymal stem cells were shown to increase Tregs in 30 active lupus patients, in a dose-dependent fashion, even after 1 week after transplantation, and this was sustained for 1 and 3 months after transplant [157]. In the same study, Th17 cells were accordingly reduced after 3 months.

5.3. IL-2-based approaches

Extensive research on IL-2 and IL-2 receptor (IL-2R) biology has shed light on its critical importance for the maintenance of immune tolerance by influencing Tregs number and function [132]. Administration of low doses of IL-2 led to remission and decreased glucocorticoid dose in lupus patients [158], while it was shown that Tregs expansion (CD4+CD25^{high}CD127low) and a decrease in T effectors/Tregs ratio were the primary mechanism [159]. The same results were observed in other diseases, such as GVHD and HCV-related vasculitis [160]. Interestingly, IL-2/anti-IL-2 immunocomplexes were capable of reducing the severity of renal inflammation in NZB/W F1 mice by inducing CD4+CD25+FOXP3+ Tregs. With regard to proteinuria, this approach was superior to the combination of glucocorticoids and mycophenolate mofetil, the current standard of care for LN [161].

5.4. All-trans retinoic acid (atRA)

This approach has been used in various autoimmune diseases with inconsistent and contradictory results, possibly due to the small sample sizes [162]. Limited data in lupus patients showed that Tregs could be induced by atRA [163]; however, these results were not confirmed [164]. In a more recent study, retinoic acid increased Treg numbers (and decreased Th17 cells) in lupus patients with low levels of vitamin A [165].

5.5. Tolerogenic peptides

The rationale behind the use of tolerogenic peptides in SLE therapeutics is that a dysregulated immune system can be modified by inducing tolerance against a specific antigen. This is a crucial component of this approach since non-specific tolerance may lead to generalized immune suppression and secondary immunodeficiency. In this context, such different molecules (hCDR1, pCons, P140, etc.) have been administered in lupus prone mice with subsequent expansion of Tregs and suppression of effector cells and pro-inflammatory cytokines [166, 167]. These encouraging results led to the first peptide-based randomized controlled trial in SLE with 149 patients [168]. Although the effect on Tregs was not assessed, approximately 62% of the peptide-treated patients achieved the primary clinical end-point as compared to 38.6% of the placebo arm (all patients received standard of care therapy).

5.6. Effect of other medications on Tregs

Apart from the aforementioned approaches that implicate Tregs in their mechanism of action, medications commonly used in SLE patients have been demonstrated to increase their numbers and/or restore their function. Several studies have demonstrated a significant Tregs expansion after treatment with glucocorticoids [121, 122, 169–171]. Moreover, intravenous methylprednisolone pulses led to a dramatic and sustained increase in $CD4+CD25^{high}FOXP3+$ Tregs numbers, regardless of the initial clinical indication [121]; this was noted even from the first few days after the pulses [172]. The mechanism by which these medications lead to Treg proliferation is yet unknown; however, a steroid-mediated up-regulation of FOXP3 has been described [171].

Immunosuppressive drugs have also been shown to affect Tregs in active SLE. Cyclophosphamide pulse therapy led to a significant increase in Tregs numbers after the 4th month of administration, which reflected clinical remission [121], although the effect of concomitant glucocorticoid treatment may have a role. Similar results were obtained with azathioprine and hydroxychloroquine [121]. Of note, polyclonal intravenous immunoglobulins (IVIGs) also led to Tregs increase, possibly through up-regulation of FOX3 and intracellular IL-10 and TGF-β [173]. Rituximab was demonstrated to enhance the Tregs numbers and function in lupus patients whereas the increased and sustained FOXP3 mRNA expression was associated with favourable outcome [174]. In general, *in vivo* expansion of Tregs after treatment might be the result of a change of Th1/Th17 to Th2 balance, which could lead to disease remission and not a direct drug-specific reaction [121].

Other medications that are increasingly used in lupus patients and may affect Tregs include statins. These drugs display multiple beneficial effects in atherosclerosis through different mechanisms among which immune modulation is critical [175]. Several experiments in animal models showed that statins increase the numbers and suppressive capacity of Tregs as well as their accumulation in the atherosclerotic plaque [176]. Atorvastatin, in particular, exerted similar results in human Tregs [177].

All the pre-mentioned therapeutic interventions are summarized in **Table 1**.

5.7. Barriers in Tregs-based therapeutic approaches

Although the above-mentioned data are encouraging for SLE patients, several challenges still exist. The multiple phenotypes that have been used to characterize Tregs in the different studies have demonstrated that all Tregs are not functionally capable of suppressing autoimmune responses [160]. In the chronic inflammatory environment of SLE, it cannot be predicted which regulatory cells are likely to function more beneficially; furthermore, effector cells are more capable of escaping regulatory mechanisms under these circumstances [106]. Furthermore, tissue distribution of Tregs, after infusion, is unknown, while their survival and maintenance of regulatory capacity have not been precisely defined in the context of SLE. Other considerations include technical aspects, such as the purity and cost-effectiveness of these approaches.

Therapy	Mechanism	Approach	Efficacy	Notes
Adoptive transfer of *ex vivo* expanded Tregs	Increase of Tregs pool	Experimental and clinical trials in other immune-mediated diseases	Moderate	High purification rates, low Tregs survival
HSCT/MSCT	Immune system reconstitution	Limited clinical trials	Moderate	Inconsistent clinical results
IL-2	Enhanced survival and function of Tregs	Limited clinical trials	Moderate	IL-2/anti-IL-2 complexes provided favourable results in LN
Retinoids	Induction of Tregs	Limited clinical trials	Inconsistent	Mainly in patients with low vitamin A
Tolerogenic peptides	Induction of Tregs	Experimental studies and one RCT	Moderate	Ongoing phase III clinical trials
Glucocorticoids	Up-regulation of FOXP3	Limited observational trials	Good	Rapid induction of Tregs
Immunomodulating agents	Induction of Tregs	Limited observational trials	Good	Regardless of the agent used, probably an epiphenomenon to disease remission
Statins	Enhanced numbers and function of Tregs	Experimental and limited observational trials	Good	Accumulation of Tregs in the atherosclerotic plaques

Table 1. Therapeutic approaches targeting Tregs in SLE.

6. Conclusion

Most well-designed studies have concluded that Tregs are significantly depleted from the periphery of active lupus SLE patients and this reduction is in accordance with disease activity. Moreover, Tregs follow alterations in disease activity (with inverse changes) quite reliably; numeric increase is not drug specific but characterizes disease remission. Their value as an activity biomarker has been demonstrated and may be helpful in assessing disease status in controversial circumstances. Their potential to be used for therapeutic purposes, either by direct adoptive transfer or by approaches aiming to increase their numbers, is quite promising in the field of SLE.

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