CD4⁺ FoxP3⁺ regulatory T-cells in human systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a loss of immune tolerance to self antigens and by the persistent production of pathogenic autoantibodies. Recent studies have suggested a dysregulation of regulatory T-cells (Tregs), particularly CD4⁺CD25 high FoxP3⁺ (forkhead box P3) Tregs, as one of the major factors conferring the risk for expression of human autoimmune diseases, including SLE. However, detailed studies of CD4⁺FoxP3⁺ T-cells in patients with SLE remain limited. We attempt here to integrate the current experimental evidence to delineate the role of CD4⁺CD25 high and other subsets of CD4⁺FoxP3⁺ T-cells in human SLE.

Introduction

Immune tolerance to self-antigens is a tightly regulated process. Deletion of self-reactive T-cells in the thymus is an important mechanism for self-tolerance. However, some autoreactive cells still can escape negative selection into the periphery.

Peripheral tolerance is maintained a number of ways, including CD4⁺CD25⁻ regulatory T-cells (Tregs) that actively suppress autoimmunity and control immune homeostasis. Tregs represent 5–10% of CD4⁺ T-cells in both humans and mice.1,2 They are characterized by the constitutive expression of CD25 [interleukin-2 (IL-2) receptor alpha chain], cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), and transcriptional repressor forkhead box P3 (FoxP3), and a low level or nonexpression of CD127 (IL-7 receptor).3 CD25 used to be seen as the only reliable marker for Tregs; however, activated CD4⁺ T-cells also express CD25. In contrast, FoxP3 has been shown to be specifically expressed by Tregs and able to program the development and function of Tregs.4,5 Further, CD127 expression inversely correlates with FoxP3 irrespective of CD25 expression in both...
humans and mice.\textsuperscript{3,6} Thus, a combination of CD127, CD3, and CD4 expression can be used to isolate viable FoxP3\textsuperscript{+} T-cells (CD3\textsuperscript{+}CD4\textsuperscript{+}CD127\textsuperscript{+}) for functional studies of Tregs.

In addition, to further characterize human CD4\textsuperscript{+}FoxP3\textsuperscript{+} T-cells, they can be separated into three subsets by the expression of CD45RA, FoxP3, and CD25.\textsuperscript{7} These subsets are (1) CD45RA\textsuperscript{+}FoxP3low(CD25low) resting Tregs, (2) CD45RA\textsuperscript{+}FoxP3high(CD25high) activated Tregs, and (3) CD45RA\textsuperscript{+}FoxP3low(CD25low) non-Tregs. Resting Tregs, which come from the thymus, can be differentiated into activated Tregs \emph{in vitro} and \emph{in vivo}. Both resting Tregs and activated Tregs have suppressive activity \emph{in vitro}. In contrast, CD45RA\textsuperscript{+}FoxP3low T-cells can secrete IL-2 and interferon-gamma (IFN-\gamma) and exhibit little suppressive activity. Thus, the identification of FoxP3 and CD45RA expression as a marker for Tregs is important in order to further analyze their role in disease states. Thus, FoxP3 combined with CD127 and CD45RA is currently the best marker for the identification of different subsets of human FoxP3\textsuperscript{+} Tregs.

**The role of FoxP3 in Tregs**

Based on the studies of scurfy mutant mice, which have autoimmune lymphoproliferative disease, the Foxp3 gene has been identified, and it has been shown that FoxP3 is responsible for the scurfy phenotype.\textsuperscript{8} Several lines of evidence demonstrate that FoxP3 is necessary and sufficient for the development and function of Tregs in mice. First, using FoxP3 reporter mice that were knockin with an allele of the Foxp3-green fluorescent protein gene,\textsuperscript{9} it was shown that the predominant type of FoxP3\textsuperscript{+} cells were \(\gamma\delta\) TCR CD4\textsuperscript{+} T-cells irrespective of CD25 expression. In addition, both CD25\textsuperscript{+}FoxP3\textsuperscript{+} and CD25\textsuperscript{+}FoxP3\textsuperscript{+} T-cells can act as suppressors \emph{in vitro}.\textsuperscript{4} Second, ectopic expression of FoxP3 in conventional CD4\textsuperscript{+}CD25\textsuperscript{+} T-cells converted those cells to a regulatory phenotype. These converted FoxP3\textsuperscript{+} T-cells can function as Tregs both \emph{in vitro} and \emph{in vivo}.\textsuperscript{4} Third, when mice lack functional FoxP3 proteins, via either the scurfy mutation or a targeted mutation, they do not display Treg activity and develop severe systemic autoimmune diseases.\textsuperscript{5,9,10} In humans, the FoxP3 mutation also leads to immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) disease with autoimmune manifestations and defective Treg function.\textsuperscript{11,12}

It has been demonstrated in both mice and humans that FoxP3-expressing CD4\textsuperscript{+}CD25\textsuperscript{+} T-cells can act as suppressors. However, further characterization of FoxP3 expression shows two major differences between humans and mice. First, human CD4\textsuperscript{+}CD25\textsuperscript{+} T-cells, but not murine cells, can express two isoforms of FoxP3.\textsuperscript{13} One isoform represents the homolog to murine FoxP3, whereas the other isoform is encoded from an mRNA lacking exon 2.\textsuperscript{14} The sequences encoded by exon 2 fall within the repressor domain of the FoxP3 protein.\textsuperscript{14} To date, the role of the \(\Delta e\) exon 2 isoform in the function and development of Tregs, and whether or not the same T-cells express both isoforms simultaneously, remains unclear. Second, the regulation of FoxP3 expression is different between humans and mice. Activation-induced expression of FoxP3 has been reported for \emph{in vitro}-stimulated human CD4\textsuperscript{+} T-cells\textsuperscript{14,15} but not mouse cells.\textsuperscript{4} A recent study has demonstrated that human CD4\textsuperscript{+}FoxP3\textsuperscript{+} T-cells contain cytokine-secreting, non-suppressive effector T-cells that are CD45RA\textsuperscript{+}FoxP3low.\textsuperscript{7} These non-suppressive CD45RA\textsuperscript{+}FoxP3lowCD4\textsuperscript{+} T-cells may correspond to \emph{in vitro} activation-induced FoxP3-expressing cells.\textsuperscript{14,15} In addition, not only the CD45RA\textsuperscript{+}CD25\textsuperscript{high} subset,\textsuperscript{2} but also the CD45RA\textsuperscript{+}CD25\textsuperscript{low} subset in human FoxP3\textsuperscript{+} CD4\textsuperscript{+} T-cells displays suppressive activity \emph{in vitro} and \emph{in vivo}.\textsuperscript{7} Thus, human FoxP3\textsuperscript{+} T-cells are heterogeneous in function and consist of not only suppressive Tregs, but also non-suppressive T-cells.

**Natural Tregs and adaptive Tregs**

There are at least two populations of CD4\textsuperscript{+} Tregs in humans and mice, defined by their origin. The first population of CD4\textsuperscript{+} Tregs is natural Tregs, which are generated during normal T-cell maturation in the thymus. These typically express CD25, as well as CTLA-4 and GITR.\textsuperscript{16} This subset is self-reactive and is involved in protection from autoimmune responses. Recently, two subsets of FoxP3\textsuperscript{+} natural Tregs according to differential inducible costimulator (ICOS) expression have been found in the human thymus and periphery.\textsuperscript{17} ICOS\textsuperscript{+}FoxP3\textsuperscript{+} Tregs secrete IL-10 to suppress dendritic cell (DC) function, and transforming growth factor beta (TGF-\(\beta\)) to suppress T-cell function, whereas ICOS\textsuperscript{+} FoxP3\textsuperscript{+} Tregs mainly secrete TGF-\(\beta\) to mediate the suppressive function.

The second population is adaptive Tregs, which originate from the thymus but are developed throughout the course of the immune response \emph{in vivo}. This population includes TGF-\(\beta\)-expressing type 3 T-helper cells, IL-10-producing type 1 regulatory T-cells, and peripherally converted FoxP3\textsuperscript{+} Tregs.\textsuperscript{18} Research has shown that naive CD4\textsuperscript{+} T-cells can be converted, \emph{de novo}, into CD25\textsuperscript{+}FoxP3\textsuperscript{+} and CD25 FoxP3\textsuperscript{+} suppressor T-cells on subimmunogenic stimulation.\textsuperscript{19} This \emph{de novo} Treg conversion may be mediated either by DCs with tolerogenic properties\textsuperscript{20} or by intestinal DCs from lamina propria.\textsuperscript{21}

CD4\textsuperscript{+}FoxP3\textsuperscript{+} T-cells in systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is characterized by dysregulated immunity with hyperactive T-cells and B-cells. Lupus-prone mice with CD4\textsuperscript{+}CD25\textsuperscript{+} Treg depletion from thymectomy have an enhanced expansion of autoreactive T-cells and accelerated autoantibody production.\textsuperscript{22} In contrast, treatment with CD4\textsuperscript{+}CD25\textsuperscript{+} Tregs from syngeneic normal mice can effectively abrogate the progress of autoimmune disease,\textsuperscript{23} as well as supplement with \emph{in vitro}-expanded Tregs\textsuperscript{24} and TGF-\(\beta\)-generated Tregs.\textsuperscript{25} Thus, the dysregulated immunity in lupus may be correlated with the altered homeostasis or defective function of Tregs.

A few studies have been performed to quantify the frequency of CD4\textsuperscript{+}CD25\textsuperscript{+/high} T-cells in patients with SLE. These studies have shown that the frequency of CD4\textsuperscript{+}CD25\textsuperscript{+/high} T-cells is decreased in patients with SLE.\textsuperscript{26,27} However, no correlation has been established between the level of CD4\textsuperscript{+}CD25\textsuperscript{+/high} T-cells and disease
activity. Lee et al have shown an inverse correlation between percentages of CD4+CD25high T-cells and disease activity, including SLE Disease Activity Index (SLEDAI) score and serum anti-double-stranded DNA levels in pediatric patients.28 Interestingly, this finding showed higher FoxP3 mRNA levels in CD4+ T-cells in active SLE compared with normal controls and those with inactive SLE.

A recent study by Miyara et al29 also observed that CD4+CD25high T-cell depletion in lupus patients was associated with the clinical severity of the flare. These Tregs with normal suppressive function did not redistribute to lymph nodes or tissues and were sensitive to Fas-induced apoptosis.29 This study suggests that the inappropriate induction of Treg apoptosis is, at least in part, associated with the depletion of CD4+CD25high T-cells in lupus patients and thus relevant to SLE pathogenesis. However, Valencia et al showed that CD4+CD25high T-cells from patients with active SLE expressed reduced levels of FoxP3 (corresponding to activated Tregs7; the R1 region in Fig. 1) and displayed a poor inhibitory activity on the proliferative response of responding T-cells.32 Alvarado-Sanchez et al showed that about one-third of lupus patients exhibited a diminished suppressive function of CD4+CD25high T-cells, but had a normal frequency of Tregs in their peripheral blood.31

These conflicting results may be due to an imprecise phenotypic definition of Tregs. Due to the unique expression pattern of human CD25 on activated CD4+ T-cells and Tregs, it is difficult to draw the line between CD25high and CD25low T-cells in blood.31 Therefore, if only CD25 serves as a Treg marker, sorted CD25high Tregs could display poor suppressive ability in vitro if they were contaminated by CD25low effector T-cells. In contrast, strict gating for high CD25 expression on CD4+ T-cells may result in falsely low Treg numbers/frequency in human peripheral blood mononuclear cells.

Five studies have examined FoxP3-expressing CD4+ T-cells in patients with SLE (Table 1). All the studies showed that SLE patients had higher CD4+CD25+FoxP3+ and/or CD4+CD25+FoxP3+ T-cell frequencies than normal controls.7,32–35 According to these studies, and based on the findings of heterogeneous subsets in the function of human FoxP3+ cells,7 SLE patients may have a defect in the homeostatic control of different subsets of FoxP3+ cells. These human FoxP3+ subsets are described separately as follows.

**CD4+CD25highFoxP3+ Tregs**

Patients with active SLE have a significantly decreased percentage and number of CD4+CD25highFoxP3+ Tregs (corresponding to activated Tregs7; the R1 region in Fig. 1) with normal suppressive activity in their peripheral blood. This decrease is also correlated with disease activity.29,32,34 The global depletion of CD4+CD25highFoxP3+ Tregs may be associated with their hypersensitivity to Fas-induced apoptosis (Fig. 2).29 However, the mechanisms responsible for the exacerbated susceptibility to apoptosis of human lupus CD4+CD25highFoxP3+ Tregs still need to be clarified.
differentiation dynamics of CD4+CD25lowFoxP3+ T-cells in lupus still need to be explored.

**CD4+CD25−FoxP3+ T-cells**

Another important subset of FoxP3+ T-cells that has been found to show a significant increase in frequency in SLE patients is CD4+FoxP3+ T-cells without CD25 expression (the R3 region in Fig. 1).32 The CD4+CD25− T-cells, irrespective of their CD45RA expression, express little FoxP3 in normal individuals.7 Thus, this subset seems relatively unique to SLE. However, the origin and function of this subset are at present largely unknown.

There are several issues worth discussing here. The first one is the function and origin of this unique subset in lupus. Their FoxP3 protein may be transiently induced by activation, and this subset may display non-Treg activity. Second, systemic inflammation can induce the differentiation of naïve CD4+ T-cells into CD25+FoxP3+ and CD25lowFoxP3+ Tregs, or so-called adaptive Tregs.18,19,32,37 This subset may represent the adaptive Tregs in patients with SLE. Finally, there are several issues worth discussing here. The first one is the function and origin of this unique subset in lupus.

<table>
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<th>Reference</th>
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<td>Anti-dsDNA IgG</td>
<td>CD4+CD25high normal suppressive activity</td>
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**Table 1** Analysis of CD4+FoxP3+ T-cell subpopulations in human systemic lupus erythematosus.

dsDNA = double-stranded DNA; NA = not analyzed; Ig = immunoglobulin; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; Teff = effector T-cell (represented by CD4+CD25+FoxP3− T-cells); Treg = regulatory T-cell.

Figure 2 Altered homeostasis of CD4+FoxP3+ T-cell subsets in patients with active systemic lupus erythematosus. Patients with active lupus have a significantly decreased frequency of activated regulatory T-cells (Tregs; CD25highCD45RA−FoxP3high), increased CD25lowFoxP3low, CD25lowFoxP3low, and CD25lowFoxP3− T-cell subsets. The depletion of activated Tregs may be associated with their hypersensitivity to Fas-induced apoptosis. Activated Tregs can suppress the proliferation of resting Tregs via negative feedback. CD25lowFoxP3+ T-cells may contain resting Tregs and FoxP3+ non-Tregs; however, the CD45RA expression level of this subset has not yet been clarified. Another unique subset, CD25 FoxP3low T-cells, also has been observed in patients; however, their origin and function are largely unknown. This unique subset may represent adaptive Tregs that are differentiated from naïve T-cells or FoxP3-expressing non-Tregs. In addition, a significantly increased number of activated T-cells (CD25lowCD45RA−FoxP3+ Tregs, or so-called adaptive Tregs, 18,19,32,37 This subset may represent the adaptive Tregs in patients with SLE. Finally,
the increased frequency of CD25<sup>+</sup> FoxP3<sup>+</sup> T-cells in patients may compensate for the loss of CD25<sup>high</sup>FoxP3<sup>+</sup> Tregs in active SLE. However, this compensation may not be enough to regulate the autoimmune response, as SLE patients have altered relative ratios of CD25<sup>high</sup>FoxP3<sup>+</sup> Tregs and CD25<sup>low</sup>FoxP3<sup>+</sup> T-cells versus effector T-cells (Fig. 2).<sup>32</sup>

In contrast to those with SLE, Rheumatoid arthritis (RA) patients have similar frequencies of CD4<sup>+</sup> FoxP3<sup>+</sup> T-cells irrespective of their CD25 expression compared with normal controls.<sup>32,33</sup> In addition, Franz et al showed that patients with cutaneous lupus erythematosus had normal levels of CD4<sup>+</sup> FoxP3<sup>+</sup> T-cells in their peripheral blood, but decreased levels in local skin lesions.<sup>38</sup> This suggests that the global dysregulation of decreased CD25<sup>high</sup>FoxP3<sup>+</sup> Tregs and increased CD25<sup>low</sup>/FoxP3<sup>+</sup> CD4<sup>+</sup> T-cells seems to be relatively unique to SLE. The mechanism clearly needs to be investigated further.

Several pieces of evidence suggest that lupus DCs may be responsible for this dysregulation.<sup>39,40</sup> It has been shown that IFN-α-producing antigen-presenting cells, such as plasmacytoid DCs, play a vital role in the pathogenesis of SLE.<sup>40</sup> These IFN-α-producing antigen-presenting cells may block Treg cell-mediated suppression in SLE patients.<sup>34</sup> In addition, the overproduction of IL-6 by DCs in lupus-prone mice may mediate the impaired Treg function.<sup>41</sup>

Another major issue is the antigen specificity of Tregs in SLE patients. Hahn et al demonstrated that functional human Tregs can be induced by exposure to anti-DNA immunoglobulin-based peptides.<sup>42</sup> In addition, the FoxP3 expression level in lupus CD4<sup>+</sup> CD25<sup>high</sup> T-cells also clearly increased when peripheral blood mononuclear cells from patients stimulated with these self-peptides in vitro. Two possibilities may explain the increase in number or FoxP3 level of CD4<sup>+</sup> CD25<sup>high</sup> T-cells with in vitro culture. First, self peptides may directly promote the expansion of lupus CD4<sup>+</sup> CD25<sup>high</sup> T-cells in vitro. Second, CD4<sup>+</sup> CD25<sup>high</sup> T-cells may be derived from FoxP3-expressing CD25<sup>low</sup> or CD25<sup>+</sup> T-cells after in vitro stimulation, which leads to the change in phenotype from CD25<sup>low</sup>/ to CD25<sup>high</sup> without losing FoxP3 expression. Thus, these studies suggest that dysregulated Treg homeostasis may play an important role in the pathogenesis of SLE.

**Potential implications of Treg immunotherapy in SLE patients**

In humans, small-scale trials have demonstrated that in vitro-expanded Tregs under good-manufacturing practice conditions can provide a beneficial effect in the management of bone marrow transplantation.<sup>43–45</sup> This suggests that Treg therapy is another therapeutic strategy to control systemic autoimmunity. Two approaches can be considered regarding Treg therapy in patients with SLE. First, we could isolate patients’ resting Tregs (CD25<sup>low</sup>/CD45RA<sup>−</sup>CD127<sup>−</sup>FoxP3<sup>+</sup>) and expand them in vitro to perform Treg therapy. Second, it has been found that administration of a histone deacetylase inhibitor in vivo significantly increases FoxP3 gene expression and the suppressive function of Tregs through epigenetic modification of FoxP3.<sup>46</sup> Thus, it is worth examining whether histone deacetylase inhibitor treatment can increase the FoxP3 expression level in FoxP3-expressing lupus subsets (as shown in Fig. 2) and whether the increased FoxP3 expression enables those cells to display the suppressive activity in vivo. If so, the autoimmune responses in patients may be downregulated by these high percentages of lupus FoxP3-expressing subsets.<sup>32</sup>

**Summary**

SLE patients have altered homeostasis in their CD4<sup>+</sup> FoxP3<sup>+</sup> T-cell subsets, that is, a decreased frequency of CD4<sup>+</sup> CD25<sup>high</sup>FoxP3<sup>+</sup> Tregs and an increased number of CD4<sup>+</sup> FoxP3<sup>+</sup> T-cells with CD25<sup>low</sup> or CD25<sup>+</sup> expression. One should remember that FoxP3 is also transiently expressed in human nonregulatory T-cells.<sup>12,13</sup> In contrast, murine FoxP3 expression is sufficient for the suppressive function of Tregs.<sup>4</sup> Thus, functional characterization is required for each subset of lupus FoxP3<sup>+</sup> T-cells in humans. Furthermore, analyzing the function and origin of each subset will help us to better understand the immune responses in cases of lupus and to design antigen-specific immunotherapy for SLE.

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