

# CD4+CD25+REGULATORY T CELLS AND THEIR ROLE IN SYSTEMIC LUPUS ERYTHEMATOSUS

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## INTRODUCTION

Systemic lupus erythematosus, or SLE, is an autoimmune disease that currently has no known cause or cure (Postal et al. 2012; Okamoto et al. 2011). According to the Lupus Foundation of America, 1.5 million Americans are thought to be suffering from SLE. It is found in females ages 15-44 but may also be seen in men, teens, and children (Lupus Foundation of America 2012). It is characterized by the loss of the immune system's ability to discern between "self" and foreign antigens. This leads to autoantibody production, abundant production of proinflammatory cytokines, stimulation of complement, and "immune complex depositions" (Okamoto et al. 2011). Multiple organs and tissues are affected due to the dysfunction of the immune system. Patients with SLE show an increased mortality rate compared to the population at large (Okamoto et al. 2011), although the five year survival rate is currently at 90% (Postal et al. 2012). The disease displays periods of advancement, remission achieved through drug therapy, and relapses. Genetic, hormonal, and environmental factors are thought to interact in the initial development of the disease, which can manifest itself in a variety of ways. Current treatment options include corticosteroids and immunosuppressants, which have resulted in some toxic effects in clinical use. To find a cure for SLE and improve patient prognoses, it is necessary to better understand the pathology behind the disease in order to formulate new drug therapies (Okamoto et al. 2011; Postal et al. 2012).

During the formation of T cells, autoreactive T cells, or T cells that target the body's own tissues as opposed to foreign antigens, are generally prevented from maturing and entering into circulating T cell defenses. However, at times, autoreactive T cells developing in the thymus may slip past the body's checks and enter into the circulation. Recent research has found that it is the job of regulatory T cells, or Tregs, to prevent these cells from attacking body tissues, a function known as peripheral tolerance (Okamoto et al. 2011; Venigalla et al. 2008). In effect, the Tregs are aptly called the "mediators of peripheral tolerance and potent suppressors of excessive immune responses" (Okamoto et al. 2011). More knowledge is needed to find out about these Tregs, how they function to prevent autoreactive attacks by responder T cells, and how they mediate cytokine production. A specific group of Tregs, namely the CD4+ CD25+ Tregs, has been avidly studied for their role in peripheral tolerance and autoimmune diseases like SLE. Understanding how these Tregs function, or sometimes fail to function properly, will allow for breakthroughs in SLE treatments and patient prognoses and is the impetus for this literature review (Okamoto et al. 2011).

The experiments discussed here include studies done on amounts and percentages of Tregs in SLE patients' peripheral blood compared to healthy controls (Baráth et al. 2007), how the number of Tregs affects the activity of the disease (Baráth et al. 2007), the lack of suppression by Tregs over responder T cells and responder T cells' cytokine production, including TNF- $\alpha$  and IFN- $\gamma$ , in the blood and skin lesions of SLE patients (Carneiro et al. 2008; Valencia et al. 2007; Venigalla et al. 2008), and finally, the effects of rituximab treatment on the ratio of Th1/Th2 CD4+ T cells (Tamimoto et al. 2008). These experiments give a broad understanding of how the amounts of Treg cells differ between healthy controls and SLE patients and how the Tregs display altered suppressive functioning in SLE patients leading to an

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improved understanding of the course of the disease and possible routes for new treatment options.

## DISCUSSION

### Amounts and percentages of Tregs in SLE patients' peripheral blood

Multiple studies have been conducted to determine the amounts of Tregs cells in SLE patients with differing results (Crispin et al. 2003, Liu et al. 2003, Fathy et al. 2005, Alvarado-Sanchez et al. 2006, Miyara et al. 2005 cited in Venigalla et al. 2008). Baráth et al. did a study using flow cytometry that matched results from prior studies (Crispin et al. 2003, Liu et al. 2003 cited in Baráth et al. 2007) showing that the percentage of CD4+CD25+FoxP3+ Tregs was decreased in SLE patients compared to controls (in percentage per liter of peripheral blood; controls: 4.55%, n=32; SLE patients: 3.21%, n=44) as well as the numbers of Treg cells (controls: 0.038±0.017 G/L; SLE patients: 0.012±0.006 G/L).

### Correlation between disease activity and amount of Tregs after plasmapheresis treatment

Five patients suffering from advanced forms of SLE that were not responding to conventional treatments were subjected to repetitive plasmapheresis treatments to determine the treatments' effects on counts of Treg cells (CD4+ CD25+ FoxP3+ T cells). An inverse correlation was seen between the SLEDAI, or disease activity, and the number of Treg cells, with the disease regressing and the number of Treg cells increasing ( $r=-0.96$ ,  $r^2=0.92$ ,  $P=0.008$ ) (Baráth et al. 2007).

### Treg functioning in the suppression of responder T cells and cytokine production

In one study, responder T cells (Tresp) of active<sup>1</sup> SLE patients were found resistant to the regulatory effects of CD4+ CD25<sup>high</sup> CD127<sup>-/low</sup> regulatory T cells (Tregs) that suppress their proliferation (Venigalla et al. 2008). The researchers built their study off of past research (Crispin et al. 2003; Liu et al. 2003; Fathy et al. 2005; Alvarado-Sanchez et al. 2006; Miyara et al. 2005) that differed as to whether SLE patients displayed differences in the percentage or overall amount of Treg cells. The researchers attributed the differences in the results to the lack of proper purification of the CD4+ CD25<sup>high</sup> Treg cells. SLE patients are known to have an abundance of an effector T cell called CD4+ CD25<sup>++</sup> that is similar to the CD4+ CD25<sup>high</sup> Treg cells. These similar CD4+ CD25<sup>++</sup> T cells cause contamination of counts that are meant to quantify CD4+ CD25<sup>high</sup> Treg cells. A culture of CD4+ CD25<sup>high</sup> Treg cells that shows proliferation is thought to be contaminated with the CD4+ CD25<sup>++</sup> effector T cells since CD4+ CD25<sup>high</sup> Treg cells do not proliferate (Venigalla et al. 2008).

### Procedure for procuring pure Treg samples

To alleviate the contamination problem, the researchers used a simultaneous staining procedure that targeted two T cell antigens: the CD25 and CD127 antigens. The researchers based their procedure on findings that indicated that the antigen CD127 allows for effective sorting between effector and regulatory T-cells; effector T cells express this antigen, or are known as CD127+, while regulatory T cells show little to no expression of this antigen, CD127<sup>-/low</sup> (Liu et al. 2006, Seddiki et al. 2006, Hartigan-O'Connor et al. 2007 cited in Venigalla et al. 2008).

The authors proved that past research that aggregated 'Treg cells' by selecting for CD4+ CD25<sup>high</sup> T cells via flow cytometry used contaminated cultures that contained more than just Treg cells. By testing the proliferation of cells characterized as CD4+ CD25<sup>high</sup> versus cells with CD4+ CD25<sup>high</sup> and the additional CD127<sup>-/low</sup> parameter, results showed significantly fewer of

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<sup>1</sup> Active refers to the disease in a state of advancement while inactive refers to the disease being in a remission state (Valencia et al. 2007; Venigalla et al. 2008).

the latter type of cells. Solidifying the results further, the percentage drop in proliferation seen between the CD4+ CD25<sup>high</sup> 'Treg cells' and the highly purified CD4+ CD25<sup>high</sup> CD127<sup>-low</sup> Tregs cells of SLE patients (P =0.0003) approached the percentage drop seen between these two types of cells in healthy controls (P=0.004) (Venigalla et al. 2008).

For further verification that the cells collected from the purification procedure were actual Tregs, the researchers compared the amounts of CD4+ CD25<sup>high</sup> CD127<sup>-low</sup> Treg cells to counts done of verified regulatory T cells, or CD4+ CD25<sup>high</sup> FoxP3+ Treg cells. FoxP3, or forkhead box P3, is a transcription factor that is critical for normal Treg cell development (Fontenot et al. 2003 cited in Venigalla et al. 2008). The researchers could not use the FoxP3 transcription factor as a limiter for isolating true Treg cells with flow cytometry (instead of antigen CD127) due to the fact that FoxP3 is an intracellular marker. However, counts of Treg cells with FoxP3 (or FoxP3+) were done as a check to compare to amounts of CD127<sup>-low</sup> Treg cells collected. If the values between the two types of cells showed a significant positive correlation, it could be concluded that the CD4+ CD25<sup>high</sup> CD127<sup>-low</sup> T cells were the true Treg cells. When comparing the purification results of the CD4+ CD25<sup>high</sup> CD127<sup>-low</sup> Treg cells to the CD4+ CD25<sup>high</sup> FoxP3+ Treg cells, a high positive correlation was seen between the total amount of each type of cell both for the SLE patients ( $r^2 = 0.77$ , P=0.0009, n = 13) and for the normal controls ( $r^2 = 0.70$ , P<0.0001, n = 17), proving the likelihood that the CD127<sup>-low</sup> cells were indeed classic Treg cells (Venigalla et al. 2008).

Use of CD4+ CD25+ FoxP3+ Treg cells from control and SLE patients, both active and inactive cases, also highlighted that active SLE patients had an increase in the average percentage of CD4+ CD25<sup>low</sup> FoxP3+ T cells and CD4+ CD25<sup>high</sup> FoxP3+ T cells compared to healthy controls and patients with inactive SLE. Both types of cells, however, did not show significantly increased amounts when measured in comparison to a set volume of peripheral blood mononuclear cells (Venigalla et al. 2008).

### **Effects of Tregs on Tresp cells in SLE patients**

After procuring pure samples of the Treg cells (CD4+ CD25<sup>high</sup> CD127<sup>-low</sup>), the researchers determined the effects of CD4+ CD25<sup>high</sup> CD127<sup>-low</sup> Treg cells on T cell responders (Tresp). The researchers cultured measured amounts of the Treg cells with the Tresp cells. Different ratios of cells were created (Treg:Tresp), spanning from 1:1 to 0.03125:1 ratios. Results of the experiment from the 1:1 ratio of control Treg cells to control Tresp cells showed an 80±2% (n=9) inhibition of proliferation of the Tresp cells. With the decreasing amount of control Treg cells compared to the set value of control Tresp cells, the percentage of inhibition gradually decreased. On the other hand, the experimental group consisting of participants with active SLE showed decreased Treg-related suppressive functioning, with baseline inhibition of Tresp proliferation at a 1:1 ratio of only 53±6% (n=9). This statistically significant decrease in inhibition of proliferation ranged from the 1:1 ratio down until the 0.125:1 ratio (P=0.0006). Similar findings to a lesser extent were found when controls were compared to patients with inactive SLE (88±1% (n=8) versus 77±2% (n=8), ratios from 1:1 down until 0.25:1, P=0.0006) (Venigalla et al. 2008).

Responder T cells of SLE patients were not sensitive to the regulation of the Treg cells; two opposing hypotheses were conjectured to understand where the decreased sensitivity to the Treg cells stemmed from. The first hypothesis ascertained that the Treg cells in SLE patients were dysfunctional and lacked the ability to suppress the Tresp cells. Conversely, the second hypothesis implicated that the Tresp cells became resistant to the signals sent by the Treg cells and proliferated of their own accord. To test these two opposing theories, Tresp cells of active

## CD4+CD25+REGULATORY T CELLS

SLE patients were grown with control Treg cells and vice versa. Tresp cells of active SLE patients cultured with control Tregs allowed for the following conclusions; if the suppression of the propagation of Tresp occurred by the control Treg cells at the elevated rate (around 80%), the problem could be isolated to the malfunctioning of the SLE patients' Treg cells. If, however, the suppression remained at the same level as that seen normally in active SLE patients, the SLE patients' Tresp cells were thought to have become immune to the mediation of SLE Treg cells. When testing the reverse (culturing Tresp cells of controls with Treg cells of active SLE patients), if the inhibition of the proliferative effects of the Tresp was very high as in the control patients, then the Tresp cells of the SLE patients' were thought to be resistant to SLE Treg cells. Just the opposite, if the reduction in Tresp proliferation was lower as in the active SLE patients, then the Tregs of the SLE patients were thought to have lost their mediating capacity. In the first case (control Treg cells + active SLE Tresp), the control Treg cells were unable to suppress the proliferation of the Tresp cells of the SLE patients, implicating a problem with the Tresp ( $74\pm 5\%$ ,  $n=15$  for control Treg cell/control Tresp cells vs.  $48\pm 6\%$ ,  $n=15$  for control Treg cells/active SLE Tresp cells;  $P=0.001$ ). The results were further confirmed to be related to the development of resistance by the active SLE patients' Tresp cells in the second experiment (active SLE Treg cells + control Tresp cells) where the active SLE Treg cells could suppress the control Tresp cells ( $48\pm 6\%$ ,  $n=15$  active SLE Treg cells/active SLE Tresp cells vs.  $63\pm 6\%$ ,  $n=15$  active SLE Treg cells/control Tresp cells;  $P=0.03$ ) (Venigalla et al. 2008).

From the testing, the Tresp cells were found to be resistant to the mediation of the Treg cells in active SLE patients. Other testing was conducted that further solidified the view that the Tresp were indeed not responsive to the Treg inhibition in active SLE patients. Statistically, the degree of inhibition of Tresp cell proliferation was inversely proportional to the level of activity seen in the disease, or more diseased states showed a decrease in the ability of the Treg cells to control the Tresp proliferation ( $r^2 = 0.37$ ,  $P < 0.0001$ ,  $n=28$ ). Finally, the researchers used ELISA during the assay on the control and active SLE T cells that showed the production of interferon-gamma ( $IFN-\gamma$ ), a Th1 class of cytokines produced by Tresp cells and regulated by Treg cells, with the following results:

Treg cells of:	$IFN-\gamma$ production by:	Able to suppress $IFN-\gamma$ production?
Control	Control Tresp cell	Yes
Control	Active SLE Tresp cell	No (not as well suppressed)
Active SLE	Active SLE Tresp cell	No (unable to suppress)
Active SLE	Control Tresp cell	Yes

These results further prove the lack of Tresp cells of active SLE patients to respond to Treg cell mediation, whether it is the cell directly (i.e. via proliferation) or the products that the cell makes (i.e.  $IFN-\gamma$  production). An interesting supposition that the authors mention is the possibility that cytokines, like  $IFN-\gamma$ , may initiate the active SLE patients' Tresp cells to 'retaliate' and build resistance to the regulation of the Treg cells. To determine the voracity of this hypothesis, the role of the cytokines must be determined (Venigalla et al. 2008).

Contradictory research proving Tregs are malfunctioning.  $CD4+ CD25^{high}$  Treg cells help in the maintenance of "immunologic homeostasis" and prevention of autoimmunity (Valencia et

al. 2007). Deficient Tregs, like those seen in SLE, attack target cells, increase cytokine production, increase cell-to-cell contact, and lead to apoptosis of target cells. In another study on Tregs (Valencia et al. 2007), the researchers hypothesized that the characteristic breakdown of “self” tolerance in SLE patients was due to altered Treg functioning. Findings from their research were in line with their hypothesis. Active SLE Tregs showed a decrease in FoxP3 mRNA and protein expression compared to controls and inactive SLE participants (controls: mean  $85\pm 5\%$ ,  $n=40$ ; inactive SLE: mean  $64.4\pm 15\%$ ,  $n=8$ ; active SLE: mean  $45\pm 10\%$ ,  $n=10$  [ $P=0.003$  comparison between controls and active SLE;  $P=0.2$  comparison between inactive SLE and controls]). Decreases in this gene expression were not mentioned in Venigalla and colleagues’ experiment (2008). Differing from results seen with Venigalla et al. (2008), Tregs were implicated as malfunctioning, doing a poor job of inhibiting the proliferation of CD4+ CD25- T responder (Tresp) cells and their cytokine secretions. The significant decrease that was evident in the suppressive functioning of the CD4+ CD25<sup>high</sup> Treg cells from the active SLE patients extended to both Tresp from controls and “self” (active SLE) Tresp cells. Further supporting the premise that the Tregs were defective, the problem could not be contributed to resistance conferred on the Tresp cells (like the findings of Vanigella et al. in 2008), as Tregs from the controls were able to suppress active SLE Tresp cell proliferation. With respect to inactive SLE patients, their Tregs were markedly different from those of the active SLE patients, displaying similar suppressive capabilities as control Tregs (Valencia et al. 2007).

#### **Reversible loss of Treg suppressive functioning**

The researchers also found that the loss of suppressive abilities of the active SLE Tregs could be reversed, not studied in the subsequent experiment done by Venigalla et al. in 2008. Incubating the Tregs in vitro with anti-CD3 led to activation of the Tregs. As a result, the Tregs exhibited an increase in FoxP3 mRNA and protein expression and restoration of their suppressive functioning on Tresp cell proliferation and IFN- $\gamma$  secretion (Valencia et al. 2007).

Phenotypic changes in Tregs of SLE patients. Valencia et al. (2007) also studied the phenotype of the Treg cells harvested from controls and SLE patients. The Treg cells from active SLE patients differed phenotypically from Tregs collected from controls and inactive SLE patients. In particular, TNFR2, which has been linked to another autoimmune disease (Valencia et al. 2006 cited in Valencia et al. 2007), was increasingly expressed. TNFR2 is part of a signaling pathway that downregulates CD4+ CD25<sup>high</sup> Tregs’ suppressive function on CD4+ CD25- Tresp cells. Since active SLE Treg cells displayed an inability to regulate Tresp proliferation, the researchers tested whether TNF moderated the suppressive capacity active SLE Tregs. Results from the experiment displayed that adding anti-TNFR2 antibodies or TNF stopped control and anti-CD3 activated active SLE Tregs from being able to inhibit CD4+ CD25- Tresp cells. The cells lost their regulatory capacity, indicating that expression of TNFR2 affects the Tregs ability to contain Tresp multiplication (Valencia et al. 2007).

#### **Correlations between SLE disease activity and Treg phenotype and suppressive capacity**

Two notable inverse correlations were discovered in this study. The first correlation was found between SLE Treg FoxP3 expression and SLEDAI score, the systemic lupus erythematosus activity score<sup>2</sup>. Interpreting the correlations, a patient with a more active form of the disease had decreased FoxP3 expression and vice versa. A second inverse correlation was found between the percentage of suppression that the Tregs exhibited over the Tresp and the SLEDAI score. A patient with a more active form of the disease displayed Tregs with a lessened

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<sup>2</sup> To be classified as an inactive SLE patient, the SLEDAI score needed to be less than 3, while an active SLE patient scored at or above 3 (Valencia et al. 2007).

capacity for suppressing the proliferation of their Tresp cells. Interestingly, no correlation existed between therapeutic drugs (glucocorticoids) and Treg functioning, indicating that malfunctioning of the Tregs was not related to the therapeutic treatment (Valencia et al. 2007).

Treg suppressive functioning and cytokine secretions in SLE patients' skin lesions. It is evident that proper control over Tresp and their cytokine secretions is lacking in SLE patients, whether it is due to the malfunctioning of the Tregs or the resistance of the Tresp to the Tregs' regulation. Cytokine secretions are increased, such as TNF- $\alpha$  and IFN- $\gamma$ , due to the lack of regulation over the Tresp that produce these two cytokines.

Immunologic disorders in several inflammatory diseases have been characterized according to the dominant cytokine pattern of the infiltrating CD4+ T cells. A Th1 pattern is characterized by predominance of interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ), whereas a Th2 pattern is characterized by predominance of IL-4, IL-5, IL-6, IL-10, IL-13 (Carneiro et al. 2011).

In a study by Carneiro et al. (2011), the researchers sampled epidermal keratinocytes of SLE patients extracted from areas of unaffected skin and skin lesions commonly found in SLE patients (both discoid and acute). Using PCR, the researchers quantified the amounts of 4 cytokines (IFN- $\gamma$ , IL-2, IL-5, TNF- $\alpha$ ) found in the skin cells. The results showed overexpression of at least one of the four cytokines in 47% of the 38 skin samples. Examining IFN- $\gamma$  in particular (which is produced by the Tresp cells), 8 out of the 38 biopsies taken showed overexpression of this cytokine (7/8 of the biopsies were from acute/discoid lesions while the 8<sup>th</sup> came from an unaffected area of skin). TNF- $\alpha$  was overexpressed in only 3 samples taken from areas with skin lesions. From the results showing overexpression of IFN- $\gamma$  specifically, the authors indicated that this suggests that Th1 class of CD4+ T cells, related to Treg cells and their functioning, may play some role in SLE's skin-related pathology (Carneiro et al. 2011).

#### **Effects of rituximab on the ratio of Th1/Th2 CD4+ T cells**

One drug on the market for SLE that was created to target B cells called rituximab has shown an effect on the ratio of Th1/Th2 CD4+ T cells (Postal et al. 2012). Rituximab has a tendency to tip the ratio in favor of Th1 CD4+ T cells over Th2 CD4+ T cells and may yield these effects by decreasing serum levels of TNF- $\alpha$ <sup>3</sup> (Tamimoto et al. 2008), mentioned previously with regard to the ability of this cytokine to inhibit Tregs' suppressive capacity over Tresp cells (Valencia et al. 2007). The study of rituximab, showed its safety, but more importantly, the drug generated remission in approximately 89% percent of the patients (n=9). Improvement in SLE patients leading to remission is, therefore, linked to changes in T cells and cytokine secretions (Tamimoto et al. 2008).

#### **CONCLUSION**

Treg cells are an important area of research in helping treat SLE patients. SLE patients suffer from having fewer of these important regulatory T cells, which help prevent autoimmunity. Other concerns include the changes in the phenotype of the remaining Treg cells and the production of TNF affecting the suppressive capacity of Tregs over Tresp (Valencia et al. 2007).

However, it is encouraging that plasmapheresis treatments in patients not responding to conventional SLE treatment showed increases in their Treg counts and decreases in their SLEDAIs, or disease activity (Baráth et al. 2007). Another promising finding is the similarity between inactive SLE patients and healthy controls with regard to appropriate Treg functioning

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<sup>3</sup> The ratio for Th1/Th2 CD4+ T cells was calculated from flow cytometry of the CD4+ T cells. Th1 cells were determined as the percentage of IFN- $\gamma$ + cells while Th2 cells were determined as interleukin-4 positive, or IL-4+ cells (Tamimoto et al. 2008).

(Valencia et al. 2007). This indicates that patients in remission, which could be brought about by use of rituximab (Tamimoto et al. 2008), may generate restored Treg functioning. It is also interesting to hypothesize whether activation of Tregs, ex or in vivo, can be used clinically to restore proper Treg functioning in SLE patients like the in vitro findings seen in Valencia et al.'s experiment in 2007.

Review of this research is important for clarifying how Treg cells in particular relate to the current knowledge of SLE pathology and bring the researchers closer to finding which mechanism can be targeted as a cure for this disease. From the research presented, it is evident that impairment of proper Treg functioning, whether on the part of the Tregs or the Tresp, leads to the detrimental proliferation of Tresp cells and their respective cytokines that is characteristic of the autoimmune disease. It is necessary to conduct more research to determine whether Tregs or Tresp are malfunctioning, as the research differed on this point. Determination of the source of increased Tresp cells and cytokine production will allow for the creation of treatment options that target the deficient cells.

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