Research Article

The Expansion of CD25highIL-10highFoxP3high B Regulatory Cells Is in Association with SLE Disease Activity

Zahava Vadasz, Regina Peri, Nasren Eiza, Gleb Slobodin, Alexandra Balbir-Gurman, and Elias Toubi

1 Division of Allergy & Clinical Immunology, Bnai Zion Medical Center, Faculty of Medicine, Technion, 4940 Haifa, Israel
2 Rheumatology Unit, Bnai Zion Medical Center, Faculty of Medicine, Technion, 4940 Haifa, Israel
3 B. Shine Rheumatology Unit, Rambam Health Care Campus, Faculty of Medicine, Technion, 4940 Haifa, Israel

Correspondence should be addressed to Elias Toubi; elias.toubi@b-zion.org.il

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B regulatory cells (Bregs) belong to a subgroup of activated B cells tasked with maintaining self-tolerance and preventing autoimmunity. While sharing similar regulatory mechanisms such as IL-10 dependency, they also defer in exhibiting their suppressive effects by expressing Fas-Ligand, TGF-beta, and PDL-1. In this study we show, for the first time, the expansion of CD25highFoxP3high Bregs in systemic lupus erythematosus (SLE) patients compared to healthy individuals (18.5 ± 3.052% versus 11.0 ± 1.654%, 𝑝 < 0.001, resp.). This expansion was also shown to correlate with SLE disease activity (𝑟 = 0.75). In addition, CD25highFoxP3high Bregs were also IL-10high expressing and further expanded when stimulated with semaphorin 3A. In sum we show that CD25highFoxP3high are an additional subtype of Bregs, involved in regulating SLE disease activity. Being IL-10 expressing, we may assume that they are one of the sources of increased serum IL-10 in SLE patients. Further studies are required in order to assess the relation between high serum IL-10 and CD25highFoxP3high Breg cells.

1. Introduction

Among the many immune mediated responses involved in systemic lupus erythematosus (SLE) is the imbalance between T-helper cells (Th) subsets, namely, Th1/Th2/Th17, and both T and B regulatory (reg) cells [1]. Th1 proinflammatory cytokine levels such as IL-12, IL-6, and IFNs are usually increased in association with SLE disease activity index (SLEDAI). Th17 related cytokines such as IL-17 and IL-21 are also reported to be enhanced and contribute to inflammatory processes in SLE and other rheumatic diseases such as rheumatoid arthritis (RA) and psoriasis. Th2 related cytokines, that is, IL-4 and IL-10, are known for their ability in driving humoral immune responses, B cell overactivation, and the production of many specific autoantibodies [2–5]. Many studies during the last decade have reported on the failure of Treg cells to maintain self-tolerance, allowing the development of many autoimmune diseases. The failure in suppressing effector Th cell proliferation is mainly considered to be IL-10 dependent (lower expression and/or production of IL-10) due to the altered expression of FoxP3 and/or inhibitory molecules such as CTLA-4 in Treg cells [6]. Breg cells are involved in regulating/suppressing immune mediated inflammation but act earlier than Treg cells. They use similar suppressive modalities, that is, IL-10, TGF-beta, and the expression of proapoptotic membrane molecules which vary across different Breg subtypes [7]. Among these different subtypes, CD19+CD24highCD38high and CD19+CD25highCD86highCD1dhigh were both described as being involved in suppressing autoimmune processes, both in an IL-10 dependent way and with an altered function in SLE [8, 9]. Breg cells have also been characterized as CD5high, FoxP3high, and Fas-Ligand expressing cells. CD19+CD25highFoxP3high Breg cells were reported to be involved in non-IgE-mediated food allergies, namely, in maintaining tolerance to milk allergies [10]. In addition to this subtype, Breg cells were defined as being...
2. Patients and Methods

2.1. Patients Population. This study examined 21 SLE patients (20 females and 1 male; age range 16–59 years; mean 30.5 ± 9.2). All patients are routinely followed up by well-trained rheumatologists and all fulfill the ACR criteria for the classification of SLE [16]. Clinical and serological data (skin involvement; arthritis; renal involvement; full cell blood count; serum complement levels; anti-dsDNA and other extractable nuclear autoantibodies) were all available, enabling the determination of SLEDAI. The serological workup was performed at the Bnai Zion Medical Center by a single experienced technician to ensure uniformity of all analyses, utilizing identical kits. Patients in whom SLEDAI was between 4 and 6 points were treated with hydroxychloroquine and in some patients prednisolone (2.5 mg/daily) was added. When SLEDAI was above 7 points, azathioprine was added, but only after analyzing specific serology and purifying B cells. When SLEDAI was above 12 points the addition of cyclophosphamide or MMF was considered again, only after performing SLE serology and purifying B cells. Twenty healthy controls, sex and age matched, were assessed and analyzed for all above-mentioned parameters. This study was approved by both the local Helsinki Committee of the Bnai Zion Medical Center and the Rambam Health Care Campus, Haifa, Israel.

2.2. B Cell Purification. B cells were purified from peripheral blood of healthy controls and SLE patients. To do so, peripheral blood mononuclear cells (PBMCs) were isolated on Lymphoprep (Axis-Shield, Oslo, Norway), and B lymphocytes were then twice purified by positive selection using CD22 microbeads (20 μL/10⁶ cells; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions, achieving this >99% purity.

2.3. FoxP3 and IL-10 Expression in CD19⁺CD25⁺ B Cells. The expression of FoxP3 and IL-10 in CD19⁺CD25⁺ B cells (considered as Breg cells) from healthy controls and SLE patients was initially assessed by staining purified B cells after 48 hours of activation with ODN-CpG and CD40L. The staining was performed by using monoclonal antibodies, human anti-CD19-PE/Cy7 (BD Horizon, Becton Dickinson, NJ, USA) and human anti-CD25 BV595 (BD Horizon, Becton Dickinson, NJ, USA) as outer membrane antibodies, and FoxP3 PE/Cy5 and IL-10 APC (BD Horizon, Becton Dickinson, NJ, USA) as intracellular staining, using a “Fix and Perm” kit (Invitrogen, NY, USA) according to the manufacturer’s instructions. The staining was evaluated using flow cytometry software (FC500 and CXP software, Beckman Coulter, Brea, CA, USA, and Becton Dickinson, NJ, USA). CD3 positive cells in the purified cell culture were determined by using monoclonal CD3 PerCP-Cy5.5 antibody (BD Pharmingen, Becton Dickinson, NJ, USA) and analyzed by Becton Dickinson FACS-Fortessa. The results are shown as % of CD19⁺CD25⁺ Breg cells expressing FoxP3 or IL-10, taking into consideration that the absolute number of Breg cells in all groups was found to be comparable. Standard deviation (STDEV) was used to quantify the amount of variation of a set of data values (e.g., percentage of Breg cells expressing FoxP3 among the patients in each indicated group of disease or normal control).

2.4. Semaphorin 3A Enhances FoxP3 Expression. Aiming to evaluate the effect of sema3A on FoxP3 expression, conditioned media from HEK293- cells, which were infected by NSPI-CMV-FLAG lentivirus with or without human sema3A cDNA, a kind gift from Professor Gera Neufeld and Dr. Ofra Kessler, Ruth and Bruce Rappaport Faculty of Medicine, Technion, Israel, as previously described [17], were added to the above-mentioned purified B cells activated by ODN-CpG and CD40L and incubated for 48 hours. After incubation, CD19⁺CD25⁺ cells were analyzed for the possible change in FoxP3 expression using the above-mentioned specific monoclonal antibodies and evaluated using an FC500 flow
cytometer and Becton Dickinson FACS Fortessa. The results are shown as % of Breg cells expressing FoxP3, taking into consideration that the absolute number of Breg cells in all groups was found to be comparable.

2.5. Clinical Correlation and Statistical Analysis. Comparison of FoxP3 expression in B cells from SLE patients and healthy controls was done using the unpaired Student t-test. The correlation coefficient (r) of clinical correlation between SLEDAI score and % of Breg cells expressing FoxP3 was determined using the Pearson correlation test. A two-tailed p value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. CD19+ CD25high Activated B Cells Are FoxP3high. First, we examined whether CD19+ CD25high B regulatory cells are also FoxP3 expressing cells. Purified resting B cells (immediately following purification) were FoxP3dim (weakly detectable) (data not shown). However, following their stimulation with CpG-ODN and CD40L for 48h, CD19+ CD25high B cells turned to become FoxP3high (Figure 1(a)). As also seen, there are less than 0.5% gated CD3 T cells and therefore B cell contamination with CD3 is unlikely and FoxP3 expression in CD25high B cells is very prominent (Figure 1(b)).

3.2. Activated CD19+ CD25high FoxP3high Are Also IL-10high. Gating on activated CD25high FoxP3high one can see that most of these cells (>85% of these cells) are IL-10high (Figure 2) in contrast to B cells that are FoxP3dim being also IL-10dim.

3.3. CD19+ CD25high FoxP3high in SLE. The percentage of Breg cells (CD19+ CD25high cells) in peripheral blood (highly
3.4. Semaphorin 3A Increases FoxP3 Expression in Breg Cells. We then sought to determine if sema3A increases the expression of FoxP3 in these Breg cells. As is demonstrated in Figure 3, sema3A increases the percentage of Breg cells (CD19⁺CD25⁺Breg cells) in peripheral blood expressing FoxP3, in normal controls and to a higher extent in SLE patients (in normal controls up to 13.6 ± 1.806% from baseline, \( p < 0.002 \), and in SLE patients up to 28.5 ± 3.506%, \( p < 0.0001 \)) (Figure 3).

3.5. FoxP3 Expression in B Cells Is Correlated with SLEDAI. Figure 4 demonstrates the correlation between the percentage of CD19⁺CD25⁺FoxP3⁺ Breg cells of SLE patients and the SLEDAI score of these patients. As can be seen, there is a positive correlation with an “r” Pearson coefficient of 0.75. This result is in line with the known correlation between IL-10 level in SLE patients and their SLEDAI.

4. Discussion

In most autoimmune diseases, immune mediated inflammatory damage is always the result of a net balance between the overactivity of self-reactive cells (T and B effector cells) and immune regulatory mechanisms (T and B regulatory cells). Most B regulatory cells are defined as being IL-10 expressing/producing cells; however, they have different subtypes, are heterogeneous, and have different mechanisms in diseases in which they are involved. Their homology to Treg subtypes, namely, Br1 cells (expressing IL-10), Br3 expressing FoxP3) was significantly higher in SLE patients when compared to that of healthy individuals (18.5% ± 3.052 versus 11.0 ± 1.654%, resp., \( p < 0.005 \)) (Figure 3).

![Figure 3: The percentage of CD19⁺CD25⁺FoxP3⁺ Breg cells in normal controls (\( n = 20 \)) and in patients suffering from SLE (\( n = 21 \)). One can see that this subtype of B cells is significantly increased in SLE patients. In addition, the addition of sema3A to these cells increased significantly the percentage of these cells.](image)

![Figure 4: Clinical correlation between the percentage of CD19⁺CD25⁺FoxP3⁺ Breg cells of SLE patients and the SLEDAI score of these patients. The correlation was done using the Pearson correlation test.](image)

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expansion of B regulatory cells was attributed to the compensatory attempt of these cells to maintain immune regulatory processes [21]. In contrast to this study, rheumatoid arthritis patients had significantly lower proportions of peripheral blood CD19<sup>+</sup> FoxP3<sup>+</sup> B cells as compared to healthy controls, particularly in patients with interstitial lung disease. This finding suggests that Breg phenotypes may have different functions in the pathogenesis of different rheumatic diseases [22]. The fact that serum IL-10 is increased in SLE and in association with SLE disease activity has been established in many previous studies. In one, increased IL-10 was shown to exhibit a modulatory effect by suppressing the differentiation and function of monocyte-derived dendritic cells [23]. In a recent study, increased IL-10 in the sera of SLE patients was capable of inducing Fas and FasL expression on CD4<sup>+</sup>T cell surfaces, promoting apoptosis of this cell subset, thus contributing to many other mechanisms of self-tolerance [24]. However, we still need to explain the mechanisms by which serum IL-10 is increased in SLE. In this regard, the expansion of IL-10 producing B cells was shown to be in part the result of increased B cell activating factor (BAFF). Enhanced serum BAFF in SLE was described in many studies as being associated with increased expression of TLR-9 and other markers of B cell activation [25, 26]. This may explain our finding of increased IL-10<sup>+</sup>FoxP3<sup>+</sup>Bregs as well as increased serum IL-10 in SLE. Another significance of FoxP3<sup>+</sup>B cells being increased in SLE is the possibility that by multiplying they also increase their IL-10 production improving by this their regulatory function. When B cells were cocultured with sema3A they responded by increasing their FoxP3 expression. This raises the possibility that if provided with the proper stimulation Bregs may develop higher regulatory properties and that by increasing their IL-10 production they may induce a better regulatory mechanism in SLE.

5. Conclusion

CD25<sup>+</sup>FoxP3<sup>+</sup>Bregs (highly expressing IL-10) are significantly increased in SLE, in correlation with SLEDAI. Semaphorin 3A increases FoxP3 expression in Breg cells improving by this their regulatory properties. We assume that the expansion of these cells is the attempt of our regulatory immune responses to maintain self-tolerance and to suppress as much as possible SLE disease activity. Further studies are required in order to better understand the role of this subset of B cells in autoimmunity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] R. M. Talaat, S. F. Mohamed, I. H. Bassyouni, and A. A. Raouf, “Th1/Th2/Th17/Treg cytokine imbalance in systemic lupus erythematosus (SLE) patients: correlation with disease activity,” Cytokine, vol. 72, no. 2, pp. 146–153, 2015 (Chinese).

[2] K. Shah, W.-W. Lee, S.-H. Lee et al., “Dysregulated balance of Th17 and Th1 cells in systemic lupus erythematosus,” Arthritis Research & Therapy, vol. 12, no. 2, article R53, 2010.

[3] D. Li, B. Guo, H. Wu, L. Tan, C. Chang, and Q. Lu, “Interleukin-17 in systemic lupus erythematosus: a comprehensive review,” Autoimmunity, vol. 48, no. 6, pp. 353–361, 2015.

[4] B. Terrier, N. Costedoat-Chalumeau, M. Garrido et al., “Interleukin 21 correlates with T cell and B cell subset alterations in systemic lupus erythematosus,” The Journal of Rheumatology, vol. 39, no. 9, pp. 1819–1828, 2012.

[5] S. Futatsugi-Yumikura, K. Matushita, A. Fukuoka et al., “Pathogenic Th2-type follicular helper T cells contribute to the development of lupus in Fas-deficient mice,” International Immunology, vol. 26, no. 4, pp. 221–231, 2014.

[6] B. Yan, S. Ye, G. Chen, N. Shen, and S. Chen, “Dysfunctional CD4+CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-α-producing antigen-presenting cells,” Arthritis and Rheumatism, vol. 58, no. 3, pp. 801–812, 2008.

[7] A. Ray, L. Wang, and B. N. Dittel, “IL-10-independent regulatory B-cell subsets and mechanisms of action,” International Immunology, 2015.

[8] P. A. Blair, L. Y. Noreña, F. Flores-Borja et al., “CD19<sup>+</sup>CD24<sup>+</sup>CD38hi B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus,” Immunity, vol. 32, no. 1, pp. 129–140, 2010.

[9] A. Kessel, T. Haj, R. Peri et al., “Human CD19<sup>+</sup>CD25<sup>+</sup> B regulatory cells suppress proliferation of CD4<sup>+</sup>T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells,” Autoimmunity Reviews, vol. 11, no. 9, pp. 670–677, 2012.

[10] J. Noh and G. Noh, “Allergen specific responses of CD19<sup>+</sup> and CD19<sup>+</sup>B cells in non-IgE mediated food allergy of late eczematous reactions in atopic dermatitis: presence of IL-17 and IL-32 producing regulatory B cells (Br17 & Br32),” Inflammation & Allergy-Drug Targets, vol. 11, no. 4, pp. 320–329, 2012.

[11] S. K. Lundy, “Killer B lymphocytes: the evidence and the potential,” Inflammation Research, vol. 58, no. 7, pp. 345–357, 2009.

[12] F. Mion, S. Topnon, B. Toffoletto, D. Cesselli, C. E. Pucillo, and G. Vitale, “IL-10 production by B cells is differentially regulated by immune-mediated and infectious stimuli and requires p38 activation,” Molecular Immunology, vol. 62, no. 2, pp. 266–276, 2014.

[13] K. Yanaba, J.-D. Bouaziz, T. Matushita, T. Tsubata, and T. F. Tedder, “The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals,” Journal of Immunology, vol. 182, no. 12, pp. 7459–7472, 2009.

[14] Z. Vadasz, T. Haj, A. Balbir et al., “A regulatory role for CD72 expression on B cells in systemic lupus erythematosus,” Seminars in Arthritis & Rheumatism, vol. 43, no. 6, pp. 767–771, 2014.

[15] P. López, E. Alonso-Pérez, J. Rodríguez-Carrio, and A. Suárez, “Influence of Atg5 mutation in SLE dependson functional IL-10 genotype,” PLoS ONE, vol. 8, no. 10, Article ID e78756, 2013.

[16] C. Bombardier, D. D. Gladman, M. B. Urowitz, D. Caron, and C. H. Chang, “Derivation of the SLEDAI. A disease activity index for lupus patients. The committee on prognosis studies in SLE,” Arthritis and Rheumatism, vol. 35, no. 6, pp. 830–840, 1992.
[17] B. Kigel, A. Varshavsky, O. Kessler, and G. Neufeld, “Successful inhibition of tumor development by specific class-3 semaphorins is associated with expression of appropriate semaphorin receptors by tumor cells,” *PLoS ONE*, vol. 3, no. 9, article e3287, 2008.

[18] J.-M. Berthelot, C. Jamin, K. Amrouche, B. Le Goff, Y. Maugars, and P. Youinou, “Regulatory B cells play a key role in immune system balance,” *Joint Bone Spine*, vol. 80, no. 1, pp. 18–22, 2013.

[19] Y. Iwata, T. Matsushita, M. Horikawa et al., “Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells,” *Blood*, vol. 117, no. 2, pp. 530–541, 2011.

[20] D. Bonardelle, K. Benihoud, N. Kiger, and P. Bobé, “B lymphocytes mediate Fas-dependent cytotoxicity in MRL/lpr mice,” *Journal of Leukocyte Biology*, vol. 78, no. 5, pp. 1052–1059, 2005.

[21] C. de Andrés, M. Tejera-Alhambra, B. Alonso et al., “New regulatory CD19+CD25+ B-cell subset in clinically isolated syndrome and multiple sclerosis relapse. Changes after glucocorticoids,” *Journal of Neuroimmunology*, vol. 270, no. 1-2, pp. 37–44, 2014.

[22] Y. Guo, X. Zhang, M. Qin, and X. Wang, “Changes in peripheral CD19+Foxp3+ and CD19+TGF-β+ regulatory B cell populations in rheumatoid arthritis patients with interstitial lung disease,” *Journal of Thoracic Disease*, vol. 7, no. 3, pp. 471–477, 2015.

[23] Z. Sun, R. Zhang, H. Wang et al., “Serum IL-10 from systemic lupus erythematosus patients suppresses the differentiation and function of monocyte-derived dendritic cells,” *Journal of Biomedical Research*, vol. 26, no. 6, pp. 456–466, 2012.

[24] X. Yang, B. Sun, H. Wang, C. Yin, X. Wang, and X. Ji, “Increased serum IL-10 in lupus patients promotes apoptosis of T cell subsets via the caspase 8 pathway initiated by Fas signaling,” *Journal of Biomedical Research*, vol. 29, no. 3, pp. 232–240, 2015.

[25] M. Yang, L. Sun, S. Wang et al., “Cutting edge: novel function of B cell-activating factor in the induction of IL-10-producing regulatory B cells,” *The Journal of Immunology*, vol. 184, no. 7, pp. 3321–3325, 2010.

[26] D. Yehudai, A. Snir, R. Peri et al., “B cell-activating factor enhances interleukin-6 and interleukin-10 production by ODN-activated human B cells,” *Scandinavian Journal of Immunology*, vol. 76, no. 4, pp. 371–377, 2012.