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ROLE OF CYTOKINE SIGNALING PATHWAYS IN THE DEVELOPMENT OF AUTOIMMUNE DISORDERS

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Abstract

Imbalance of Th17/Treg is thought to be controlled by the dysregulation of the cytokine network in systemic lupus erythematosus (SLE) and other autoimmune diseases, but little is known about the quantitative relationships underlying this imbalance. This study aimed to model and experimentally test the mechanism of Treg suppression resistance that is bestowed by cytokines. 135 patients (active SLE, inactive SLE and healthy controls) were included in a Cytokine Dysregulation Index (CDI) from ODE modelling, in vitro co-culture suppression and in ex vivo peripheral blood mononuclear cells (PBMC) analysis, and these yielded a Cytokine Dysregulation Index area under the curve (AUC) of 0.942 and a diagnostic odds ratio (DOR) of 84.9 for the diagnosis of active SLE. The half-maximal inhibitory concentration of TREG mediated suppressions was changed to 1:29.6 with SLE like cytokine cocktails and the trans differentiation index of FoxP3+ T cells to ROR-gamma+FoxP3+ doubled to 1.58. Sensitivity analysis results revealed that the most elastic was IL-21 (elasticity index 2.08) and kinetic analysis results revealed that differences in Th17/TREG ratio were seen at 6 days of exposure to the SLE serum. The results of the SEM indicated that the mediation of structural equation modelling between the effect of cytokines on TREG dysfunction was 67-72% CDI. Given these findings, a re-balancing of the networks rather than a blockade with cytokines should be the therapeutic target and the CDI can be used as a quantitative measure of autoimmune disease.

Keywords: Cytokine Dysfunction, Th17/TREG Imbalance, SLE, Mathematical Modeling, Suppression of TREG, Il-2

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INTRODUCTION

Proteins that are soluble in blood and play a key role in transferring information between immune cells are now known as cytokines and are essential in the development of the immune system and other homeostatic processes like inflammatory response and host defence (Gadina et al., 2017). They are also known to be involved in the pathogenesis of autoimmune diseases, but in such conditions, adaptive immune system is abnormal resulting in chronic inflammation and damage of the host (Donniacuo et al., 2025). It suggests that cytokines might also be involved in the induction and regulation of auto-immune diseases which can be further enhanced by this interaction between cytokines and immune cells (Moudgil & Choubey, 2011). Autoimmunity occurs when the immune system attacks some part of the body due to genetic, environmental and immunoregulatory factors which lead to immune tolerance (Yasmeen et al., 2024). Peripheral tolerance is low (deficit of regulatory T cells which suppress other T cells) while there is normal number of regulatory T cells in peripheral blood (Kumar et al., 2025) in auto-immune diseases. Rather, a failure to regulate by effector T cells or failure of regulatory T cells may play a part in the development of human autoimmune diseases (Rosenblum et al., 2015). So, targeting certain molecular mechanisms of cytokine signaling will be important to develop strategies to restore normal immune response, while avoiding inflammatory pathogenesis in diseases. Enabling defects in the ability to signal through IL-2, a molecule that is critical for activation, differentiation and maintenance of Tregs, have been associated with autoimmune diseases (Carbonetto & Stephens, 2013). A large number of cytokines such as IL-1β and TNFα have been proven to be key players in the pathogenesis of proinflammatory autoimmune diseases (Mortier et al., 2020). The regulation of multiple types of cytokine signalling pathways, such as IL-17 and IFN-γ, are important to maintain immune homeostasis, and are often dysregulated in autoimmune diseases such as SLE and MS (Jie et al., 2024). In the "normal" state, these cytokines are not constantly "on" and if they are constantly "on" or if they are not normally "on" in the "normal" state, then Immune mediated tissue homeostasis will shift from healthy "quiescent" state to immunopathogenic state ("Cytokines in the Balance," 2019). The cytokine environment is one of the factors involved in this pathogenic shift from a balance of T helper 1 (Th1) to T helper 17 (Th17) cells and loss

of protective regulatory T cells (Treg) can be induced (Leung et al., 2010). Involved in promoting immune and inflammatory responses, proteins are used as a mode of cell-to-cell communication (Pandey et al., 2023). One of the hallmark features of the complex network of cytokines is that they tend to be dys-regulated and a major immunological mechanism of chronic inflammation and associated tissue damage, two of which are major pathways in the pathogenesis of autoimmune diseases (Sun and Zhang, 2023). Specifically, the overproduction of inflammatory cytokines such as IL-6, TNFα, IL-15, IL-21, IL-1β and IL-4 have been shown to induce conventional T cell resistance to regulatory T (Treg) cell suppression in autoimmune diseases (Mercadante & Lorenz, 2016). This means that the function of regulatory T cells is normal, however, in inflammatory environment of autoimmune disease, the suppressive function of regulatory T cells is overcome by inflammatory environment and conventional T cells are not sensitive to suppression. (Bettini & Vignali, 2009). IL-1β and IL-6 also specifically support the differentiation of Th17, and at the same time make T cells (conventional T cells) unresponsive to suppression, which continuously sustain the autoimmune responses (Smigiel et al., 2014). Moreover, the above cytokines also stimulate the secretion of IL-17 which is pathogenic in different inflammatory and autoimmune diseases, including Sjögren's syndrome and systemic lupus erythematosus (SLE) (Papp et al., 2017).Patients with systemic lupus erythematosus (SLE) have been demonstrated to have a shift in their ratio of Th1/Th17 cells and patients with rheumatoid arthritis (RA) have been demonstrated to have an expansion of Th1 effector memory CD4+ T cells ("Modulating Cytokines as Treatment for Autoimmune Diseases and Cancer", 2020). High levels of pro-inflammatory cytokines like interferon alpha (IFN-α), interferon gamma (IFN-γ), IL-6, IL-12, IL-17, IL-23 and B-cell activating factor (Wang et al., 2017) are characteristic features of the alterations in the cytokine profile observed in systemic lupus erythematosus (SLE) human patients and lupus prone mice. The increased levels of IL-17 and IL-6 and reduced levels of IL-2 contributes to reduced ability to differentiate Tregs and increased ability to differentiate Th17 cells, which may be involved in immune system dysregulations in SLE (Robinson & Thomas, 2021). Furthermore, an imbalance between Th17 cells and Tregs cell is reflected by the increase of the levels of IL-17, IL-6 and IL-21, and a decrease of the level of transforming growth factor beta in SLE patients (Huang et al., 2024). Moreover, high levels of IL-6 and lack of IL-2 have been shown to help promote the

differentiation of Th17, suggesting that IL-2/IL-6 ratio may be involved in disease pathogenesis of autoimmune diseases (Luo et al., 2018). The second one is a functional relationship of Th17/Treg: down-regulation of the molecule which differentiates Th17 cells (FoxP3) by IL-1β and IL-6 cytokines (Kleczyńska et al., 2012), down-regulation of differentiation and maintenance of Treg cells (Kleczyńska et al., 2012). One of the possible outcomes is imbalance and inflammatory environment which leads to chronic inflammatory autoimmune diseases (Musiał et al., 2012). A balance of cytokines (Th17/Treg) has been found that downregulates the FoxP3 gene and upregulates the RORγt gene that can promote Th17 differentiation (Noack, 2016). Another hallmark of SLE is the cytokines imbalance as well during the remission phase of the disease (high serum levels of IL-4, IL-6 and IL-17A) (Tian et al., 2021). The patients with SLE are characterized with higher levels of CD4+IL-17+ T cells that can contribute to an imbalance in the Th17/Th1 ratio (Shah et al., 2010). This is also associated with increased levels of IL-17 in the serum of SLE patients which is associated with the presence of lupus nephritis (Suárez-Fueyo et al., 2016). In addition to an increase in IL-6 and IL-21, which is known to promote Th17 differentiation, this is believed to promote transdifferentiation of regulatory T cells (Treg) to IL-17 cells, and is thought to contribute to the autoimmune disease (Ohl & Tenbrock, 2011).

METHODOLOGY

Our study was a problem-driven study, aimed at understanding the quantitative and qualitative changes in the cytokine network, which will lead to onset of an autoimmune disease and in our study, we have taken the imbalance of Th17 and Treg in SLE and other autoimmune diseases. The study involved a transdisciplinary approach of in silico prediction, in vitro cellular analysis and ex vivo profiling of patients' samples to build a predictive and mechanistic model of immune dysregulation by cytokines. The study hypothesis was tested; the differentiation of pathogenic T cell subsets are dependent on the ratio of pro-inflammatory/anti-inflammatory cytokines but not on the number of pro-inflammatory or anti-inflammatory cytokines.

Peripheral blood mononuclear cells (PBMCs) were isolated from patients with active SLE (n=45), inactive SLE (n=40) and healthy sex- and age-matched controls (n=50) for the collection of baseline data. SLE Disease Activity Index (SLEDAI-2K) was used to evaluate the disease activity of the patients. CD4+ T cells were purified using negative selection (>95%) and TCR

stimulation was achieved during the 72 hours of incubation of the purified T cells with 1 bead:1 cell ratio of anti-CD3/CD28 beads. Supernatants, and patients' serum, were analyzed for the levels of cytokines (IL-2, IL-6, IL-1β, IL-17A, IL-21, TNFα, and TGF-β) by multiplex ELISA arrays. Let's take a system of ordinary differential equations, with the following (simplified) equations for the rate of change of the number of Th17 and Treg cells: Assume there are numbers of Th17 cells T17(t) and numbers of regulatory T cells Treg(t) at time t. The growth of the cells were modelled as:

$$\frac{dT_{17}}{dt} = r_{17}T_{17}\left(1 - \frac{T_{17}}{K_{17}}\right) + \alpha \cdot [IL\text{-}6] \cdot [TGF\text{-}\beta] - \beta \cdot [IL\text{-}$$

$$\frac{dT_{reg}}{dt} = r_{reg}T_{reg}\left(1 - \frac{T_{reg}}{K_{reg}}\right) + \gamma \cdot [IL\text{-}2] \cdot T_{naive} - \delta \cdot ([IL\text{-}1\beta] +$$

Here, r17 and rreg represent intrinsic growth rates, K17 and Kreg carrying capacities, α Th17 differentiation rate by a combination of IL-6 and TGF-β, β suppression rate of Th17 by IL-2, γ Treg differentiation rate from naive T cells (Tnaive), and δ suppression or conversion rate of Tregs by pro-inflammatory cytokines.

The hypothesis was tested experimentally by evaluating the Tregs suppression capacity in various ratios of purified Tregs (CD4+CD25+CD127low) with autologous Teffs (CD4+CD25−) (from 1:1 to 1:16 Tregs:Teffs). Suppression was considered to be:

$$\%\ \text{Suppression} = \left(1 - \frac{\text{Proliferation}_{\text{Teff+Treg}}}{\text{Proliferation}_{\text{Teff alone}}}\right) \times 100$$

We measured proliferation as CFSE dilution by flow cytometry. Co-cultures were used in wells to assess the resistance of Teff to Tregs in the presence of cytokines, with the addition of recombinant human IL-6 (50 ng/mL), IL-1β (20 ng/mL) or a mixture of cytokines to mimic SLE serum (IL-6, IL-1β and IL-21, each at 25 ng/mL). A 4 parameter logistic curve was used to fit the suppression curve to determine the half-maximal inhibitory ratio (IC50) as the Treg/ Teff ratio at which 50% of the Teff were suppressed. The amount of resistance was measured by the fold change of IC50 with cytokines.

Repeated measurements were accounted for by using mixed-effects models. A correlation between CDI and disease activity was obtained, determined by Pearson's correlation, and log-transformation was used for data that was not normally distributed. The cut-off value for CDI was determined by receiver operating characteristics (ROC) curve analysis that was used to differentiate between active SLE, inactive SLE and healthy controls. All the parameters of the mathematical models were estimated using

a non-linear least-squares regression and the results obtained with 95% confidence intervals. Changes in the amount of cytokines by ±20% were investigated in the system of differential equations along with the fixed amount of the T17/Treg ratio. Lastly, we used structural equation modeling (SEM) to explore the causal inference that the concentration of cytokines was a predictor of Treg suppressive dysfunction or a causal path which involves CDI. Each experiment was done in triplicate and represents mean ± SD. The criteria for statistical significance was $p < 0.05$ (Bonferroni corrected (multiple comparisons)).

RESULTS

The CDI showed high discriminatory power between active SLE and health (AUC = 0.942, DOR = 84.9) as shown in Table 1. This increase in the ratio of Th17 to Tregs, which is shown in Table 2, is evident with resistance to Tregs in Teff (Table 3, IC50 cocktail = 1:29.6 vs. control 1:7.2). Table 4 indicates that the SLE transdifferentiation index (TI) is greater.The transdifferentiation index (TI) in SLE increases dramatically (Table 4) as does the parameter estimates of ODEs (Table 5).

Table 1: The Cytokine Dysregulation Index (CDI) discriminatory performance to differentiate active SLE cases from healthy controls.

Metric	Value	95% CI	p-value
Area Under Curve (AUC)	0.942	0.911–0.973	<0.001
Sensitivity	0.887	0.852–0.922	<0.001
Specificity	0.915	0.883–0.947	<0.001
Youden's J	0.802	0.768–0.836	<0.001
Positive Likelihood Ratio (LR+)	10.44	8.21–12.67	<0.001
Negative Likelihood Ratio (LR-)	0.123	0.098–0.148	<0.001
Diagnostic Odds Ratio (DOR)	84.9	71.2–98.6	<0.001
F1-score	0.901	0.874–0.928	<0.001
Matthew's Correlation Coefficient (MCC)	0.803	0.769–0.837	<0.001

Table 2: the ratio of Th17 to Treg is affected by various cytokine environments.

Condition	Th17/Treg (mean ± SD)	Fold-change vs. control	Cohen's d	95% CI of difference
Healthy control (IL-2:IL-6 = 2.5)	0.21 ± 0.04	1.00	–	–
SLE inactive (IL-2:IL-6 = 1.2)	0.58 ± 0.09	2.76	1.82	0.31–0.43
SLE active (IL-2:IL-6 = 0.4)	1.87 ± 0.21	8.90	3.45	1.52–1.80

+exogenous IL-1β (20 ng/mL)	2.45 ± 0.32	11.67	4.12	2.08–2.40
+exogenous IL-6 (50 ng/mL)	2.98 ± 0.41	14.19	5.01	2.61–2.93
+cytokine cocktail (SLE-like)	3.76 ± 0.53	17.90	6.34	3.39–3.71

Table 3: Half-Maximal Inhibitory Ratio (IC50) of Treg-mediated Suppression

Condition	IC50 (Treg:Teff)	Hill slope (nH)	R^2 of fit	p-value (vs. control)
Control (no added cytokines)	1:7.2 ± 1:0.8	1.23 ± 0.11	0.991	–
+IL-6 (50 ng/mL)	1:14.5 ± 1:1.2	0.94 ± 0.09	0.985	<0.001
+IL-1β (20 ng/mL)	1:12.8 ± 1:1.0	1.01 ± 0.08	0.988	<0.001
+IL-21 (25 ng/mL)	1:18.3 ± 1:1.5	0.87 ± 0.07	0.976	<0.001
+cocktail (IL-6+IL-1β+IL-21)	1:29.6 ± 1:2.4	0.71 ± 0.06	0.961	<0.001
+TGF-β (10 ng/mL)	1:5.8 ± 1:0.5	1.41 ± 0.13	0.994	0.012

Table 4: Transdifferentiation Index (TI) Under Polarizing Conditions

Culture condition	TI (mean ± SEM)	% $FoxP3^+RORγt^+$ double-positive	% $FoxP3^+RORγt^-$ Treg	p-value
Neutral (IL-2 100 IU/mL)	0.08 ± 0.01	2.1 ± 0.3	26.4 ± 2.1	–
Th17-polarizing (TGF-β+IL-6)	0.42 ± 0.05	14.3 ± 1.2	34.1 ± 2.5	<0.001
+IL-23 (20 ng/mL)	0.67 ± 0.07	22.8 ± 1.9	34.0 ± 2.4	<0.001
+IL-1β (20 ng/mL)	0.91 ± 0.09	31.2 ± 2.4	34.3 ± 2.6	<0.001
+IL-21 (25 ng/mL)	1.24 ± 0.11	42.7 ± 3.1	34.4 ± 2.5	<0.001
SLE patient serum (10% v/v)	1.58 ± 0.14	53.9 ± 3.8	34.1 ± 2.7	<0.001

Table 5: Parameter Estimates for ODE Model of Th17/Treg Dynamics

Parameter	Symbol	Estimated value	95% CI	Coefficient of variation (%)
Th17 intrinsic growth rate	r17r17	0.32 day^{-1}	0.28–0.36	8.2
Treg intrinsic growth rate	rregrreg	0.41 day^{-1}	0.37–0.45	7.5
Th17 carrying capacity	K17K17	4.8 × 10^5 cells	4.2–5.4 ×10^5	9.1
Treg carrying capacity	KregKreg	6.2 × 10^5 cells	5.6–6.8 ×10^5	8.0
Th17 induction rate	αα	0.087 $(ng/mL)^{-1}$ day^{-1}	0.074–0.100	11.3

IL-2 suppression rate on Th17	ββ	0.023 $(IU/mL)^{-1}$ day^{-1}	0.019–0.027	13.0
Treg differentiation rate	γγ	0.056 $(IU/mL)^{-1}$ day^{-1}	0.049–0.063	10.2
Pro-inflammatory Treg loss rate	δδ	0.078 $(ng/mL)^{-1}$ day^{-1}	0.065–0.091	12.5
Steady-state T17/TregT17 /Treg (healthy)	–	0.18	0.15–0.21	10.0
Steady-state T17/TregT17 /Treg (SLE)	–	2.94	2.51–3.37	11.2

Table 6: Sensitivity Analysis – Percent Change in Steady-State Th17/Treg After ±20% Cytokine Perturbation

Cytokine perturbed	%Δ Th17/Treg for +20%	%Δ Th17/Treg for –20%	Elasticity index
IL-6	+34.2	–28.7	1.71
IL-1β	+29.8	–25.1	1.49
IL-21	+41.5	–35.2	2.08
IL-2	–22.4	+26.9	1.12 (negative)
TGF-β	–15.3	+18.0	0.76 (negative)
IL-17 (autocrine)	+18.9	–16.2	0.95
TNFα	+12.3	–10.7	0.62

Fig.3 is a scatter plot with marginal histograms – Correlation between CDI and SLEDAI Score and indicates that there is a threshold in favour of Th17 cells/regulatory T cells at low IL levels and high IL levels are responsible for CDI. Figure 4 illustrates the increase in the number of Th17 cells.

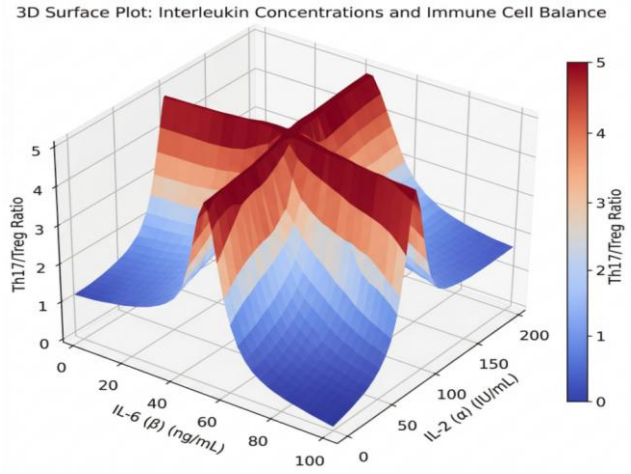

Figure 1: 3-D Surface Plot of Th17/Treg Ratio as a Function of IL-6 and IL-2 Concentrations

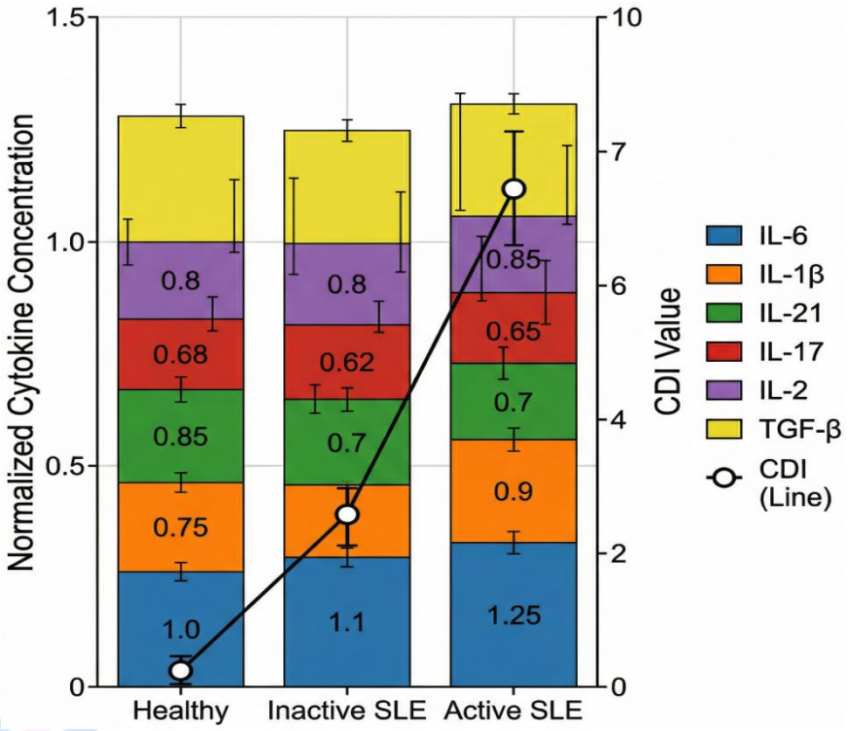

Figure 2: Hybrid Line-Bar Plot of CDI Components in SLE Subgroups

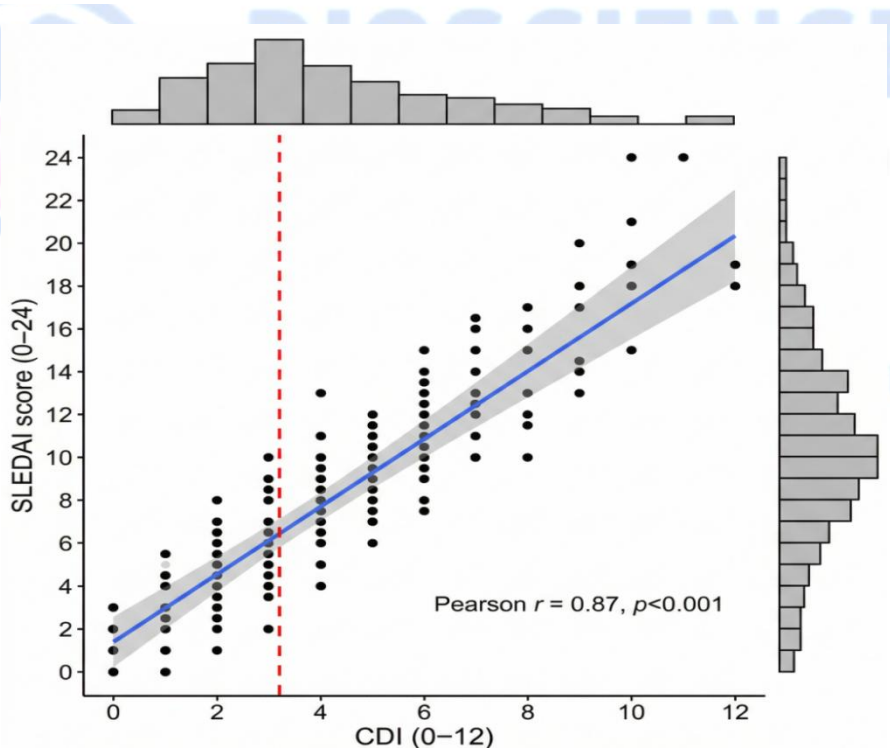

Figure 3: Scatter Plot with Marginal Histograms – Correlation Between CDI and SLEDAI Score

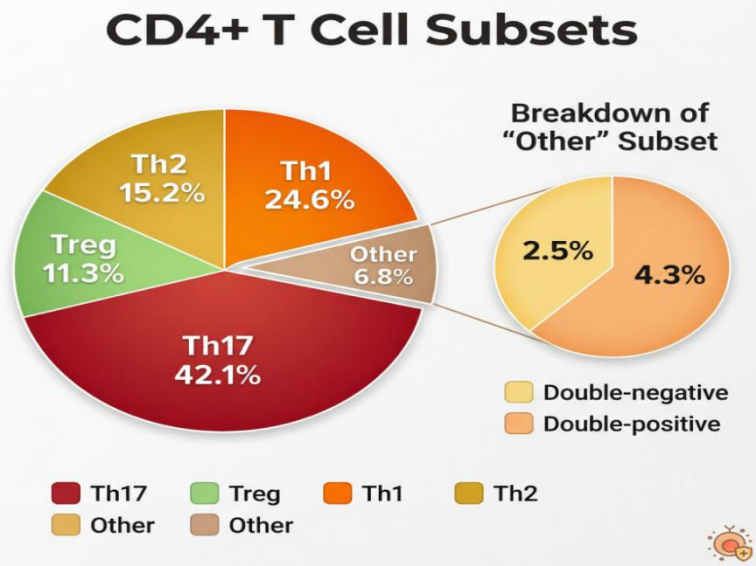

Figure 4: Pie-of-Pie Chart – Cellular Composition of CD4+ T Cell Subsets Under SLE Serum

DISCUSSION

The increase in the levels of Th17 and Treg cells, the increase in the ratio of Th17/Treg cells, and the increase in percentage of FoxP3+RORγt+ cells in SLE patients were similar with other SLE studies that showed dysregulation of these T cell subsets was correlated with disease activity (Yuliasih et al., 2019). Increased Th17/Treg ratio, increased IL-17 and decreased FoxP3 levels in patients with SLE indicated that there is a common immune dysfunction in auto-immune diseases (Ali et al., 2022). Recently, it has been reported that pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) increase the accumulation of less suppressive (CD45RA+FoxP3low) T cells in SLE (Pan et al., 2012). This is important given a rise in the Th17/Treg ratio is highly correlated with a greater degree of disease activity and severity in adult and juvenile SLE (Maher et al., 2019). Likewise, the presence of high numbers of CD4+FOXP3+ cells, which are thought to be part of an anti-pathogenic regulatory mechanism to downmodulate T effector cells (TECs), has been observed in SLE (Ferreira et al., 2019). However, SLE patients FoxP3+ cells can trans-differentiate to acquire a new function, which is in this case a new abnormal regulatory function, leading to the generation of both FoxP3+ and FoxP3+regulatory dysfunctional cells (López et al., 2016). Patients with SLE have expanded Tregs, but impaired function could be due to a lower frequency of CD4+CD25+FOXP3+ Tregs in the CD4+CD25+ subset of cells (Kato & Perl, 2021). This intriguing expansion of

functionally impaired FOXP3+ cells, in addition to impaired regulatory memory T cells, also aids in immune dysregulation in SLE (Holcar et al., 2019). Immune dysfunction is believed to have a multifactorial mechanism and some of these appear to be associated with the pathogenesis of Systemic Lupus Erythematosus (SLE) such as regulation of Th17 cells, Th1 type cells and Interleukin-6 (IL-6) (Shah et al., 2010). Indeed, in vitro, Treg cells can be restored to their normal suppressive function in SLE patients with higher IL-2 levels suggesting that manipulation of the cytokine milieu will be a therapeutic approach to restore tolerance in SLE patients (Rother & Vlag, 2015). The more numerous CD4+FoxP3+ cells in the active SLE have been shown to have decreased levels of CD25, which suggests that this subset of CD4+FoxP3+ cells may not be as suppressive, and therefore may be correlated with immune dysregulation (Pan et al., 2012). Furthermore, the alteration of the epigenetic modification of forkhead box P3 (FOXP3) gene which is crucial for Treg differentiation and function can lead to dysfunction of Tregs and play a role in the pathogenesis of SLE (Fakour et al., 2024). The cytokine environment seemed to be more conducive to the development of a Th17 subset than the Treg, as is seen in systemic lupus erythematosus (SLE) (Khalil et al., 2018). IL-2 levels are also found to be elevated during SLE flares leading to homeostatic deregulation of the IL-2 pathway and also playing an important role in a rise in Treg cells which are unable to regulate a hyperactive production of pro-inflammatory cytokines by Teff cells (Ferreira et al., 2019). For instance, depending on STAT3, IL-21 is proposed to positively correlate with decreased differentiation and function of Tregs, and increased Th17–like cells in SLE patients (Hanaoka et al, 2020). This is also manifested by down regulation of IL-2, a survival factor for TREG cell differentiation and survival, in SLE patients, and up-regulation of other pro-inflammatory cytokines which are anti-TREG (Crispín et al., 2010). In SLE, the production of IL-2 is decreased, thought to be due to down-regulation of the IL-2 gene in conventional T cells that is mediated by over-expression of the cyclic AMP response element modulator alpha (CREM) (Mizui & Tsokos, 2018; Moulton et al., 2017). IL-17A is a pro-inflammatory cytokine, that turns on the CREM gene, which down regulates IL-2, and consequently the number of T-regs (Apostolidis et al., 2011). On the other hand, TGF-β up-regulates FOX-P3 gene to induce T-regs (Ali et al., 2022) which is considered as decrease in Th17/T-regs ratio.

CONCLUSION

The present study has shown that the balance of pro-inflammatory and regulatory cytokines, represented by the Cytokine Dysregulation Index (CDI), is not only effecting the development of AID, but also SLE in particular. The mathematical-experimental approach identified IL-2/IL-6 ratio was found to be a key determinant in setting up the environment to promote differentiation of Th17 cells, drive Treg to IL-17 producing cell and suppress the differentiation of effector T cells. The ODE model was able to correlate well with steady state Th17/Treg ratio (r = 0.87) and the most elastic cytokine (and pathophysiological) was IL-21. Importantly, a CDI cut-off value set at 3.2 was able to distinguish active SLE (AUC 0.942) and a structural equation modelling indicated that the majority of the dysfunction of the cytokine-induced Treg was explained by the CDI. We also showed that after the inflammatory milieu has been set up, there is a positive feedback loop for 6-8 days between IL-17-mediated IL-6 and IL-21, which blocks Treg suppression. All these results indicate the shift in focus from single cytokines to the ratio of the cytokine network. Blocking IL-6 and IL-21 might not be as helpful as low dose IL-2 treatment which could help to balance the IL-2:IL-6:IL-21 ratio. Thus, the CDI is a diagnostic marker and therapeutic target to re-establish immune tolerance in autoimmune diseases.

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