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Dysregulation of Innate and Adaptive Serum Mediators Precedes Systemic Lupus Erythematosus Classification and Improves Prognostic Accuracy of Autoantibodies

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Abstract

Systemic lupus erythematosus (SLE) is a complex autoimmune disease with a poorly understood preclinical stage of immune dysregulation and symptom accrual. Accumulation of antinuclear autoantibody (ANA) specificities is a hallmark of impending clinical disease. Yet, many ANA-positive individuals remain healthy, suggesting that additional immune dysregulation underlies SLE pathogenesis. Indeed, we have recently demonstrated that interferon (IFN) pathways are dysregulated in preclinical SLE. To determine if other forms of immune dysregulation contribute to preclinical SLE pathogenesis, we measured SLE-associated autoantibodies and soluble mediators in samples from 84 individuals collected prior to SLE classification (average timespan = 5.98 years), compared to unaffected, healthy control samples matched by race, gender, age (± 5 years), and time of sample procurement. We found that multiple soluble mediators, including interleukin (IL)-5, IL-6, and IFN-γ, were significantly elevated in cases compared to controls more than 3.5 years pre-classification, prior to or concurrent with autoantibody positivity. Additional mediators, including innate cytokines, IFN-associated chemokines, and soluble tumor necrosis factor (TNF) superfamily mediators increased longitudinally in cases approaching SLE classification, but not in controls. In particular, levels of B lymphocyte stimulator (BLyS) and a

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proliferation-inducing ligand (APRIL) were comparable in cases and controls until less than 10 months pre-classification. Over the entire pre-classification period, random forest models incorporating ANA and anti-Ro/SSA positivity with levels of IL-5, IL-6, and the IFN-γ-induced chemokine, MIG, distinguished future SLE patients with 92% (± 1.8%) accuracy, compared to 78% accuracy utilizing ANA positivity alone. These data suggest that immune dysregulation involving multiple pathways contributes to SLE pathogenesis. Importantly, distinct immunological profiles are predictive for individuals who will develop clinical SLE and may be useful for delineating early pathogenesis, discovering therapeutic targets, and designing prevention trials.

Keywords

Systemic Lupus Erythematosus; Disease Progression; Autoantibodies; Cytokines; Biomarkers; Forecasting

1. Introduction

Systemic lupus erythematosus (SLE) is a clinically and serologically heterogeneous systemic autoimmune disease which causes significant morbidity and early mortality, especially in young women and minorities (1). Immune dysregulation in the form of pathogenic autoantibodies and chronic inflammation contributes to a wide range of clinical manifestations, including skin rashes, arthritis, and life-threatening renal and/or central nervous system damage [1]. A number of antinuclear autoantibody (ANA) specificities have been shown to accumulate prior to SLE classification [2-4]; preclinical use of hydroxychloroquine may abrogate autoantibody accumulation and delay clinical disease onset [4]. Early intervention is an attractive approach to SLE treatment. However, our understanding of pathogenic mechanisms in preclinical SLE is inadequate. Closing this knowledge gap would improve our ability to identify individuals with preclinical SLE, define windows of opportunity for early intervention, and facilitate the development of pathway-targeted treatments.

Current biomarkers in preclinical SLE have limited utility for forecasting the transition to classified disease [2, 3, 5]. Although SLE-associated autoantibody specificities such as anti-dsDNA, anti-spliceosome and anti-Ro/SSA, accumulate in SLE patients years before classification [3], their presence is not sufficient to predict SLE. ANAs are also found in sera from patients with other systemic rheumatic diseases [6], and from healthy individuals who do not go on to develop SLE, including some unaffected family members of SLE patients [7], and up to 14% of the general population [8]. Because individuals may remain healthy despite being ANA-positive, ANA positivity alone is likely not the sole pathogenic driver of SLE [2, 9, 10]. In addition to ANA positivity, the dysregulation of various immune pathways driven by soluble mediators may contribute to the development of clinical disease. High expression of type I interferon (IFN)-related genes has been associated with SLE, yet an elevated IFN signature is not present in all patients [5]. Evidence stemming from lupus-like animal models and SLE patients suggests that breaks in tolerance leading to the activation and persistence of autoreactive B cells arise from amplified crosstalk between innate and adaptive immunity [11, 12]. Key mediators of such crosstalk, including Th-type cytokines

IFN-γ (Th_1), interleukin (IL)-4 and IL-5 (Th_2), and IL-17 and IL-21 (Th_{17}) facilitate lymphocyte recruitment to germinal centers [13-15] and pathogenic autoantibody production [16, 17] with the help of T-follicular helper (T_{fh}) cells [17]. We have recently demonstrated that type II IFN (IFN-γ) becomes elevated prior to and concurrent with the development of lupus-associated autoantibodies [18]. The tumor necrosis factor (TNF) superfamily member B lymphocyte stimulator (BLyS), secreted in response to type I and type II IFNs [19, 20], further supports and propagates autoantibody production as a survival factor for self-reactive B-lymphocytes [21]. In addition to driving the production of pathogenic autoantibodies, these mediators also contribute to inflammation associated with SLE disease flare [22] and organ damage [23]. Although these mediators contribute to SLE disease activity, their role in preclinical autoimmunity and transition to clinical disease are not well understood.

No single factor or mechanism is likely sufficient to explain the complexity and heterogeneity of SLE pathogenesis; thus a multivariate, longitudinal approach is warranted to delineate mechanisms of early disease pathogenesis and discern unique parameters that forecast SLE classification. In the current study, we leveraged longitudinal serum samples from the Department of Defense Serum Repository (DoDSR) to compare levels and determine temporal relationships between autoantibodies and immune mediators from multiple immune pathways in individuals who subsequently developed SLE compared to matched, healthy controls. Our findings shed light on potential mechanisms of early preclinical SLE immunopathogenesis, whereby dysregulation of immune mediators occurs prior to and concurrent with autoantibody accumulation, and is amplified leading up to SLE classification. Further, this study informs the design of reliable and sensitive tools to predict SLE onset. Such tools can be used to identify high risk patients in need of rheumatology referral and enrollment in prospective, preclinical intervention studies, as well as inform the development of novel treatment strategies to avert or delay tissue damage that often accompanies transition to classified disease [24-27].

2. Materials and Methods

2.1 Study population and serum samples

Experiments were performed in accordance with the Helsinki Declaration and approved by the Institutional Review Boards of the Oklahoma Medical Research Foundation and the Walter Reed National Military Medical Center. Samples were obtained from the DoDSR. Demographic and clinical information, including medication history and American College of Rheumatology (ACR) criteria for SLE classification, were extracted from medical records by study personnel. All patients with available serum samples covering periods before and at/after SLE classification (n=84) were selected from a cohort comprised of 130 previously identified individuals [2, 28] and 75 newly identified individuals with classified SLE (≥ 4 ACR criteria for SLE [29, 30]). Cases were compared to healthy controls matched by race, sex, age (± 5 years), and time of sample procurement relative to SLE disease classification, as well as sample availability (n=86; **Supplemental Table 1**). Individuals selected as matched healthy controls had no signs or symptoms of autoimmune disease in their medical record during the time span assessed. In total, 416 samples were analyzed (246 from cases and 170 from controls). Cases had an average of 2.96 available samples (range, 2-3), and

controls had an average of 2 available samples (range, 1-3). For sequential longitudinal analysis, samples from SLE cases and their matched controls were divided into four time periods relative to SLE classification, such that each time period included approximately 60 case samples (range, 61 - 63) (**Supplemental Fig. 1**).

2.2 Soluble mediator and autoantibody assays

Serum levels of BLyS (R&D Systems, Minneapolis, MN) and a proliferation-inducing ligand (APRIL) (eBioscience/ Affymetrix, San Diego, CA) were assessed using enzyme-linked immunosorbent assay (ELISA) per manufacturer's protocol. Normalized fluorescence intensity values for an additional 30 immune mediators, including cytokines, chemokines, and soluble TNFR superfamily members (**Supplemental Table 2**), were determined by xMAP multiplex assays (eBioscience/Affymetrix) [31]. After performing quality control as described previously [32], four mediators (IFN-α, TNF-α, IL-10, and IL-15) were excluded from further analysis due ≥ 50% of cytokine measurements falling below the lowest level of detection [33]. The average inter-assay coefficient of variance (CV) of the assays performed in this experiment was 10.5%, comparable to the previously reported CV values (10% - 14%) for multiplexed bead-based cytokine assays [34, 35]. Intra-assay precision was high, with an average CV of <10% for duplicate wells in each 30-plex assay. The BioPlex 2200® system (Bio-Rad Technologies) was used to simultaneously detect levels of multiple autoantibody specificities within a single serum sample: dsDNA, chromatin, Ro/SSA, La/SSB, Sm, SmRNP, and RNP [7, 36]. Semi-quantitative values for anti-dsDNA were reported as IU/mL (positive ≥ 10 IU/mL). All other autoantibody specificities were reported in autoantibody index (AI) units based on the fluorescence intensity (range 0-8) using the manufacturer-specified positive cutoff (positive ≥ 1 AI). Factor XIIIb levels were evaluated as a quality control measure, serving as both a serum confirmation and an indicator of sample integrity.

2.3 Statistical analysis

Samples from SLE cases and their matched controls were divided into quartiles based on time of sample procurement relative to SLE classification (**Supplemental Fig. 1**). Z-scores reflecting the number of standard deviations (SD) away from the mean of values for case vs. control samples were calculated and displayed as a heatmap using R (version 2.15.0). Non-parametric rank-based analysis was performed using GraphPad Prism 6.0 (La Jolla, CA) for variables with asymmetric distribution. P-values were adjusted for multiple comparison by false discovery rate (FDR) using the fdrtools package (version 1.2.12) in R (version 2.15.0). Categorical factors were compared by odds ratios with 95% confidence intervals and chi-square tests. Mixed linear regression models were fitted on normalized FI values of each soluble mediator over time using the lme4 package in R (version 2.15.0). Using mixed models, intercepts were modeled as random effects to account for the initial soluble mediator level of each study participant. Disease status was applied as a fixed effect (or population effect) on change of soluble mediator over time. Optimal positive/negative cut-off values for each soluble mediator that best distinguished cases from controls were determined by maximizing the sum of sensitivity and specificity among all possible soluble mediator levels (Youden index/J statistic) from receiver operating curves (ROC) [37]. The timing of soluble mediator dysregulation or autoantibody positivity was visualized by

Kaplan-Meier survival curve analysis, using autoantibody positivity or soluble mediator elevation as the event of interest. Across the entire pre-classification period, the likelihood of soluble mediator dysregulation compared to autoantibody positivity was determined by hazard ratios calculated using a cox proportional hazard model. Statistical significance was determined by robust log-rank test.

A random forest (RF) classification algorithm [38] was implemented using the randomForest R packages (version 4.6-7) to identify factors differentiating individuals who would transition to classified SLE (**Supplemental Fig. 2**). Default settings were used (mtry = $\sqrt{number \quad of \quad variables}$, importance = TRUE, and proximity = TRUE) except that ntree was set to 2,000. For each forest, a randomly selected training set (2/3 of total samples) was used to generate an ensemble of decision trees. The performance of each RF was evaluated using accuracy (1 – out of bag (OOB) error; **Supplemental Fig. 2A**). Variables were selected using the stepwise-like algorithm of Genuer and Tuleau-Malot [38] to predict cases in each quartile time bin relative to time of sample procurement relative to SLE classification (using R package “fifer” [39]): (a) ANA positivity alone (categorical variable), (b) soluble mediator levels alone (continuous variables), and (c) ANA positivity (categorical variable), SLE-associated autoantibodies to dsDNA, chromatin, Ro/SSA, La/SSB, Sm, and RNP (categorical variables), and soluble mediator levels (continuous variables; **Supplemental Fig. 2B**). Final RF models identified the set of predictors that independently contributed to the differentiation of future SLE patients. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated based on the averaged misclassification (2×2 chi-square like) matrix of 50 forests generated using the best model. To generate a predictive model for future SLE onset, a single pre-SLE classification sample from each individual (84 cases and 86 controls) was randomly selected to construct a set of independent pre-SLE samples. To ensure the precision of prediction modeling, ten such datasets were generated with replacement, and the best RF model was selected from each dataset. The final overall pre-SLE model consisted of predictors appearing in at least five of the best RF models. The reliability of the final model was confirmed by calculating the average prediction accuracy using the ten independent pre-SLE datasets (**Supplemental Fig. 2C**). Multidimensional scaling plots of resulting RF proximity matrices were subsequently created using the randomForest R packages (version 4.6-7). Three dimensional scatter plots of cases and controls identified via Random Forest were created using Spotfire [40] and cases contained within clusters compared for differences in age and number of ACR criteria at SLE classification (unpaired t-test), as well as race, medication history, and the presence of individual ACR classification criteria by Fisher's Exact test or Chi-square test, as appropriate.

3. Results

3.1 Innate and adaptive soluble immune mediators are dysregulated more than 3.5 years before SLE classification

Altered levels of multiple adaptive-type soluble mediators, including inflammatory Th_1-, Th_2-, and Th_{17}-type cytokines, as well as innate and regulatory mediators, have been observed in established SLE [31, 41, 42]. To elucidate the possible involvement of soluble

mediators in various stages of preclinical SLE pathogenesis, longitudinal changes in serum cytokine levels were compared in samples spanning pre- and post-classification time periods in cases and controls matched by demographics and time of sample procurement (**Supplemental Table 1**). Samples were grouped into four time periods (<−3.5, −3.5 to −0.9, −0.9 to 0.1, and >0.1 years relative to disease classification), such that each time period included approximately 60 case samples (**Supplemental Fig. 1**).

Cases who later developed SLE exhibited increased inflammatory mediators from multiple immune pathways more than 3.5 years pre-classification (**Fig. 1** and **Table 1**). Innate mediators that influence adaptive immune responses were altered in case vs. controls at this earliest time period, including the T-helper (Th) $Th_2/Th_{17}/T_{fh}$-associated mediator IL-6 (1.69 [1.40-2.15] vs. 1.14 [0.88-1.44], q=6.83×10^{-6}) and Th_1-associated mediator IL-12p70 (1.5 [1.33-1.83] vs. 1.24 [1.00-1.60], *q=0.013*). Additional Th-type mediators elevated in case samples included Th_1-type mediator IFN-γ (2.56 [2.2-2.98] vs. 2.20 [1.74-2.75], *q=0.035*), as well as Th_2-type mediators IL-4 (1.60 [1.31-2.00] vs. 1.25 [1.00-1.61], *q=0.01*) and IL-5 (1.38 [1.24 – 1.62] vs. 0.86 [0.64-1.26], $q=3.6\times10^{-6}$). In addition, the IFN-associated chemokine IFN-γ-inducible protein 10 (IP-10; 2.83 [1.89-4.67] vs. 2.08 [1.45-3.2], *q=0.023*) was elevated in case samples. Concurrently, case samples had significantly lower levels of the regulatory mediator TGF-β (1.84 [1.47 - 2.54] vs. 2.69 [1.58 - 4.93], *q=0.023*). These results suggest that early preclinical SLE pathogenesis is marked by an accumulation of dysregulated innate and adaptive mediators, superimposed on a background of deficient regulatory mechanisms.

3.2 Increasing dysregulation of innate and adaptive immune pathways culminates in elevation of TNF superfamily mediators near SLE classification

Soluble mediators that were altered in cases > 3.5 years prior to SLE classification remained so throughout the preclinical period, with additional immune dysregulation noted as patients approached disease classification (**Fig. 1** and **Table 1**). We therefore assessed the temporal progression of cytokine dysregulation during SLE development. Consistent with the model that SLE pathogenesis entails a deficient regulatory setting [43, 44], the regulatory cytokine TGF-β was significantly decreased in cases compared to controls at all time periods, with no significant longitudinal changes in either group (**Fig. 1** and **Table 1**). However, cases moving toward SLE classification gained an average of 0.5 dysregulated mediators per year, compared to only 0.06 in controls (*p<0.001*; **Fig. 2A and Table 2)**. Cases exhibited a mean of 12.7 elevated mediators at the time of SLE classification (increased from 8.8 mediators > 3.5 years prior to classification), compared to 6.3 in controls (increased from 5.7 mediators) during the comparable time period. Similarly, cases moving toward SLE classification gained an average of 0.3 SLE-associated autoantibody specificities per year, compared to no gain in autoantibody positivity in controls (*p<0.001*; **Supplemental Fig. 3 and Table 2**). Cases exhibited positivity for an average of 3.0 autoantibody specificities at the time of SLE classification (increased from a mean of 1.0 autoantibody specificities > 3.5 years prior to classification), compared to controls, who were consistently positive for an average of 0.1 autoantibody specificities over the matched evaluation period. Cases showed evidence of expanding IFN activity, including increasing levels of the IFN-associated mediators IP-10 (*p<0.001*; **Fig. 2B**) and monocyte induced by IFN-γ (MIG, *p<0.001*; **Fig. 2C**). Growing

dysregulation of innate and adaptive immune pathways throughout the pre-classification period was evidenced by increasing levels of innate and Th-type mediators, including Th1-type IL-2 (*p=0.008*; **Fig. 2D**), Th2-type IL-5 (*p=0.001*; **Fig. 2E**), and Th17-type IL-21 (*p=0.007*; **Fig. 2F**), compared to low and stable levels of these mediators in healthy controls (**Table 2**).

Of note, multiple TNF superfamily members, including TNFRI, TNFRII, BLyS, and APRIL, were dysregulated only as patients approached SLE classification (**Fig. 1 and Table 1**). Mixed linear regression models confirmed that cases had significant longitudinal increases in the levels of these mediators (**Table 2**), including BLyS (*p=0.008*, **Fig. 2G**) and APRIL (*p=0.013*, **Fig. 2H**), compared to minimal changes in controls during the same period. Together, these results support a model in which innate and adaptive immune pathways initiate pathogenic inflammation during early preclinical SLE pathogenesis, followed by expanded immune dysregulation encompassing altered TNF superfamily members as patients approach SLE classification.

3.3 Dysregulated adaptive immune mediators precede autoantibody accumulation in preclinical SLE

To better understand the temporal relationship between soluble mediator dysregulation and autoantibody production, we next compared the timing of autoantibody specificity detection and soluble mediator dysregulation as patients moved toward SLE classification. The proportion of cases with elevated levels of IL-4 and IL-5 (Th_2-type), as well as IL-6 (Th_2 and Th_{17}-type), increased rapidly throughout the pre-classification period (**Fig. 3**). Consistent with our previous findings, IFN-γ (Th_1-type) levels also increased rapidly during the pre-classification period, as did the IFN-γ induced chemokine, MIG ([18] and **Fig. 3**). Each of these mediators was elevated in more than 50% of cases by 2 years pre-classification, and in 85-95% of cases by two years after SLE classification (**Fig. 3**). In addition, cases continued to accumulate autoantibody specificities as they approached SLE classification [18, 45], with anti-Ro/SSA being among the first lupus-associated autoantibody specificities to be detected, followed by autoantibodies reactive to RNP, chromatin, Sm, dsDNA, and La/SSB as patients approached SLE classification (**Fig. 3**). Of interest, the detection of most lupus-associated autoantibody specificities occurred significantly later than the onset of IL-4, IL-5, IL-6, IFN-γ, or MIG dysregulation (**Fig. 3** and **Table 3**). These results suggest that early dysregulation of innate and adaptive immune pathways may contribute to autoantibody development during SLE pathogenesis.

3.4 Autoantibody positivity and dysregulated soluble mediators together reliably distinguish progression to classified SLE

The data presented above suggest that altered soluble mediators are detected years before patients reach SLE classification and may improve the prognostic accuracy of ANA positivity for identifying individuals at high risk of developing SLE. We used random forest (RF) modeling to determine which biomarkers could reliably demarcate patients as they progress from preclinical SLE to classified disease. RF models were generated based on ANA positivity alone, dysregulated soluble mediator levels alone, or the combination of ANA positivity and soluble mediator levels (**Table 4**). Although the ability to differentiate

cases from controls using ANA status alone improved as patients approached SLE classification, the models incorporating soluble mediators consistently exhibited better specificity than ANA-only models (**Table 4**). In the early preclinical period (>3.5 years pre-classification), cases were best distinguished from controls by elevated Th_1- and Th_2-type mediators (IFN-γ, IL-5, IL-6) partnered with ANA and anti-Ro/SSA positivity, with 84% (± 0.12%) accuracy, compared to 58% accuracy using ANA positivity alone and 79% (± 0.6%) accuracy utilizing levels of the soluble mediators IL-5 and IL-6 in the RF models.

As patients moved closer to SLE classification (0.9-3.5 years pre-classification), cases were best distinguished from controls with 92% (± 0.52%) accuracy by elevated serum levels of the IFN-γ-induced chemokine, MIG, and the Th_1-associated mediator IL-12, as well as ANA positivity. When SLE classification was imminent (<0.9 years pre-classification), levels of IL-5, IL-6 and TGF-β independently and optimally predicted SLE classification, and distinguished cases from controls with 90% (± 0.98%) accuracy, highlighting the importance of soluble mediators in the transition to SLE. Finally, in an RF model spanning the entire preclinical period, a combination of ANA positivity, as well as elevated levels of IL-5, IL-6 and MIG, optimally identified individuals who subsequently developed clinical SLE with 92% (± 1.78%) accuracy, positive predictive value (PPV) of 0.96, and negative predictive value (NPV) of 0.84 (**Table 4**).

Confirming the above finding that Th-type mediators are dysregulated prior to the appearance of most lupus-associated autoantibody specificities (**Fig. 3**), a large random forest cluster of cases > 3.5 years prior to SLE classification were ANA negative, but had high levels of IL-5 and IFN-γ (SLE cluster 1, **Fig. 4**). Compared to cases who were ANA positive > 3.5 years prior to SLE classification, cases who were ANA negative with high levels of IL-5 and IFN-γ demonstrated no difference in sex (*p=0.433* by Fisher's exact test), race (p*=0.346* by χ_2), age at SLE classification (*p=0.389* by unpaired t-test), nor medication history as patients approached SLE classification, including hydroxychloroquine (p*=0.115*), azathioprine (*p=0.434*), methotrexate (*p=0.298*), or the use of steroids (*p=1.000*). However, ANA negative, IL-5 and IFN-γ high cases (SLE cluster 1) were more likely to develop nephritis (*p=0.008*), while cases who were ANA positive were more likely to develop arthritis (*p=0.028*) as they transitioned to classified SLE. These results underscore the dual contributions of ANA positivity and progressive, multi-pathway immune dysregulation to preclinical SLE pathogenesis and prognosis.

4. Discussion

Deciphering immune dysregulation that contributes to early lupus pathogenesis is essential for efforts to thwart the development of tissue and organ damage and ensuing morbidity and early mortality associated with progression to clinical SLE. The goal of the current study was to expand and clarify our understanding of SLE pathogenesis prior to and concurrent with the development of clinical disease by determining the nature and temporal relationship of immune pathway dysregulation and the development and accumulation of SLE-associated autoantibody specificities that lead to clinical disease and SLE classification. To this end, we used a unique resource of well-characterized, longitudinal serum samples collected prior to and at/after SLE classification to determine, for the first time in human patients, the extent

and temporal relationship of immune dysregulation relative to the accumulation of autoantibody specificities and SLE classification.

SLE-associated autoantibody specificities can be detected years before SLE classification [2], but these autoantibodies are also present in other autoimmune diseases and in healthy populations [26, 31, 46, 47]. Supporting the paradigm that pathogenic autoantibodies are not the sole drivers of SLE pathogenesis, two independent, randomized clinical trials of B cell depletion therapies demonstrated decreased circulating anti-dsDNA autoantibodies, yet produced only modest clinical improvement over standard of care [48, 49]. We demonstrate in the current study that a model combining IL-5,IL-6, and IFN-γ levels reliably distinguishes individuals in the early preclinical stages of SLE from healthy controls. Indeed, we could identify 79% of future SLE cases by evaluating this combination of factors alone more than 3.5 years prior to classification, compared to only 58% of future SLE cases identified using only ANA status. Furthermore, combining immune factors with ANA status resulted in identifying future SLE patients with 84% accuracy > 3.5 years before they reach SLE classification.

These findings suggest that screening for immune pathway dysregulation in conjunction with ANA positivity may improve our ability to identify individuals at high risk for SLE. Although it is possible for up to 14% of the general population [8] without clinical signs or symptoms of SLE to have other facets of immune dysregulation, we have recently demonstrated that autoantibody-positive healthy individuals do not usually display enhanced dysregulation of those mediators compared to SLE patients, including IL-5, IL-6, and IFN-γ (**Table 4**), that are dysregulated in patients at the highest risk of developing SLE [50, 51]. In addition, it is possible that immune pathways found to be dysregulated in asymptomatic individuals who develop SLE may also be present in other rheumatologic autoimmune diseases [52], to date, evaluation of serological samples from the DODSR and other community cohorts in asymptomatic patients who develop other diseases such as rheumatoid arthritis have revealed a combination of dysregulated immune pathways and autoantibody specificities distinct from that of preclinical SLE [53-55]. Future prospective, longitudinal studies of individuals with autoantibody positivity ± immune dysregulation, prior to onset of clinical signs and symptoms, will be necessary to determine which autoimmune disease(s) are associated with particular dysregulated immune pathways that are present before/concurrent with particular autoantibody specificities or clinical rheumatic disease.

Aberrant elevation in Th_1-, Th_2-, and Th_{17}-type cytokines has been reported in multiple SLE cohorts during established disease [22, 47, 56-60]. Our current findings suggest that dysregulation of these cytokines, particularly IL-5 (Th_2-type) and IL-6 ($Th_{2/17}$-type), may be an essential early step in SLE pathogenesis. Indeed, these two mediators were elevated in about 20% of future SLE patients at least 6 years prior to disease transition and in approximately 90% of SLE patients by two years after classification. Further, IL-5 and IL-6 were independent classifiers in most of the multivariate random forest models, revealing that they contribute to all stages of SLE pathogenesis. IL-5 and IL-6 are secreted by both innate and adaptive leukocytes and support T cell survival and antibody production, suggesting that their role in SLE pathogenesis may be to promote autoantibody production. Consistent with this possibility, IL-5 and IL-6 were elevated prior to the development of SLE-associated

autoantibodies, and ANA positivity gradually replaced IL-5 as an independent predictor of future SLE classification. Disruption of regulatory mechanisms may also contribute to autoantibody accumulation, as indicated by the observed early decrease in TGF-β and the current literature showing disrupted Th_{17}/Treg homeostasis during established SLE [61-63].

Previous studies have shown that IFN-γ becomes elevated prior to or concurrent with the appearance of autoantibodies [18], and that elevated levels of IFN-γ are associated with the transition from undifferentiated to defined connective tissue disease [64]. Our current study confirms and expands this finding > 3.5 years prior to SLE classification, during the asymptomatic period of pre-clinical disease pathogenesis. In addition to facilitating autoantibody production by perpetuating Th_1-type responses and modulating Toll-like receptor regulation, IFN-γ drives the production of IFN-α [65]. In turn, IFN-γ and IFN-α stimulate the production of B cell proliferation and activation factors such as BLyS and APRIL [19, 20, 66, 67], which further reinforce inflammation and B cell activation. Interestingly, ANA positivity did not exclude IFN-γ from multivariate random forest models, suggesting that type II IFN dysregulation and ANA production play distinct roles in SLE pathogenesis. Thus, the early elevation of IFN-γ, followed by significant increases in BLyS and APRIL when within one year of disease classification when SLE is imminent, supports the model that simultaneous dysregulation of T helper, regulatory, IFN-related, and TNF-related pathways may unleash an inflammatory cycle that erodes immune tolerance to a point where clinical disease is inevitable [18]. Such alterations are likely due to abnormalities in receptor-mediated proximal and distal signaling pathways [68], many of which are current targets for novel therapeutic approaches to dampen inflammation and target organ damage in SLE [69]. Additional, future studies will be required to determine if dysregulation of signaling pathways that leads to aberrant cellular activation and secretion of inflammatory mediators is due to genetic [23, 70], epigenetic [71], and/or environmental triggers, such as vitamin D deficiency [51] and/or immune dysregulation caused by latent Epstein-Barr viral infection [72, 73].

Early intervention in SLE may be most effective before the immune system enters a feed-forward, self-sustaining cycle of broken tolerance. Immune homeostasis could potentially be maintained by targeting immune pathways that become dysregulated during early pathogenesis. Although the cellular sources of dysregulated soluble mediators in preclinical SLE remain unknown and are the subject of future study, our data suggest that restoring homeostasis within the IL-5, IL-6, and IFN pathways might be effective interventions prior to SLE classification. Of interest, hydroxychloroquine has been shown to activation of TLR7 pathways [74], pathogenic in SLE [74], as well as decrease production of IL-6 and IFN-γ in several small patient cohorts and *in vitro* studies [75-78]. A mainstay of treatment in SLE [79], hydroxychloroquine has already been shown to delay SLE onset and slow the accrual of autoantibodies in patients approaching SLE classification [4]. By identifying high-risk patients via the presence of immune dysregulation coupled with one or more lupus-associated autoantibody specificities during the pre-clinical, asymptomatic period, lower doses of hydroxychloroquine may successfully stave off disease and reduce the risk of ocular toxicity [80].

Alternatively, it may be possible, alone or in conjunction with low-dose hydroxychloroquine, to stave off the accumulation of autoantibody specificities and the development of clinical SLE utilizing pathway-specific, biologic, immune modifiers. Given the predictive nature of IL-5, IL-6, and IFN-γ for future disease development > 3.5 years prior to SLE classification, these would be logical pathways to pursue in early intervention trials. Although no studies to date have explored blockade of Th_2-type cytokines in SLE patients, a number of studies have been performed in patients with asthma and such therapies have been shown to be well-tolerated and provide some clinical benefit [81]. Blockade of the IL-6 receptor in SLE patients has been shown to decrease both B- and T-lymphocyte activation [82] and there is some evidence of clinical improvement in patient-reported outcomes [83]. Given the role of IL-6 in both Th_2 and Th_{17}-type responses, early intervention in patients who exhibit dysregulated levels (89% in the current study) may help delay or prevent both the development of autoantibody specificities and subsequent clinical sequelae, including more serious consequences such as lupus nephritis [83]. For those patients with elevated IFN-γ levels (89% in the current study), treating SLE patients with the anti-IFN-γ monoclonal antibody AMG 811 has been shown to normalized interferon-regulated gene expression and reduce downstream levels of IP-10 [84], which has also become a therapeutic target for rheumatic disease [85]. This may be particularly beneficial as 97% of the future cases in the current study exhibited elevated levels of IFN-γ and/or IP-10 prior to SLE classification. Finally, those patients who may have incomplete lupus, exhibiting signs and symptoms of SLE with concurrent presence of autoantibody specificities and immune dysregulation, may additionally benefit from anti-BLyS therapy, which is elevated proximal to SLE classification and the blockade of which has shown promise clinically, particularly in SLE patients with musculoskeletal and mucocutaneous organ system involvement [86].

5. Conclusions

In conclusion, data from our study delineate a complex and cumulative pathogenic process in preclinical SLE, involving a number risk factors and gradual dysregulation of innate and T-helper, adaptive immune pathways (**Fig. 5**). Abnormalities in multiple Th-type cytokines arise in early preclinical SLE pathogenesis and could be leveraged to identify individuals at highest risk of future SLE clinical onset with >90% accuracy. This study also describes multifactorial models that improve the prediction of SLE classification during early disease development, and thus provides tools to select at-risk individuals for prospective mechanistic studies and clinical prevention trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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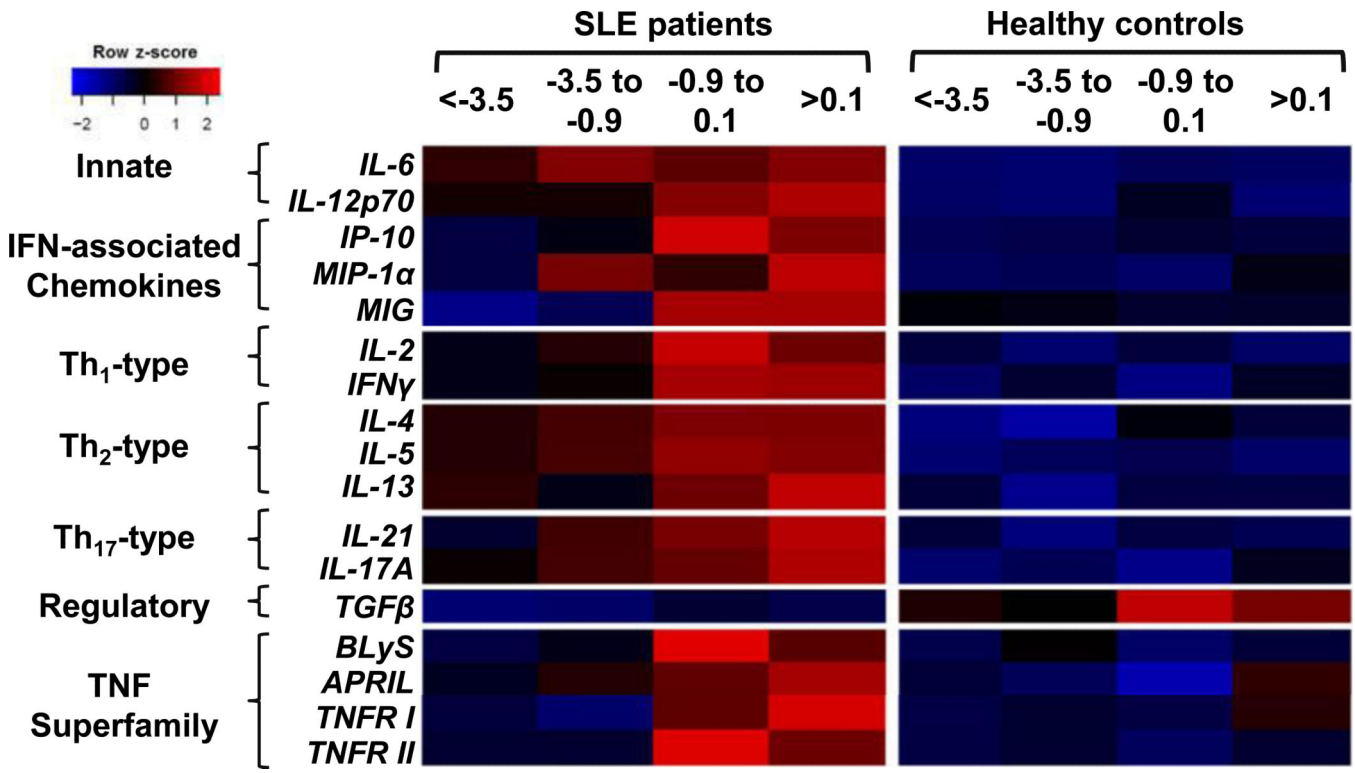

Figure. 1. Individuals moving toward SLE classification have distinct preclinical soluble mediator profiles compared to healthy controls

Heat map color type and intensity were determined by median normalized fluorescence intensity values in cases vs. race, gender, age (± 5 years), and time of sample procurement-matched healthy controls at four different quartile periods relative to SLE classification. Blue is lower expression and red is higher expression.

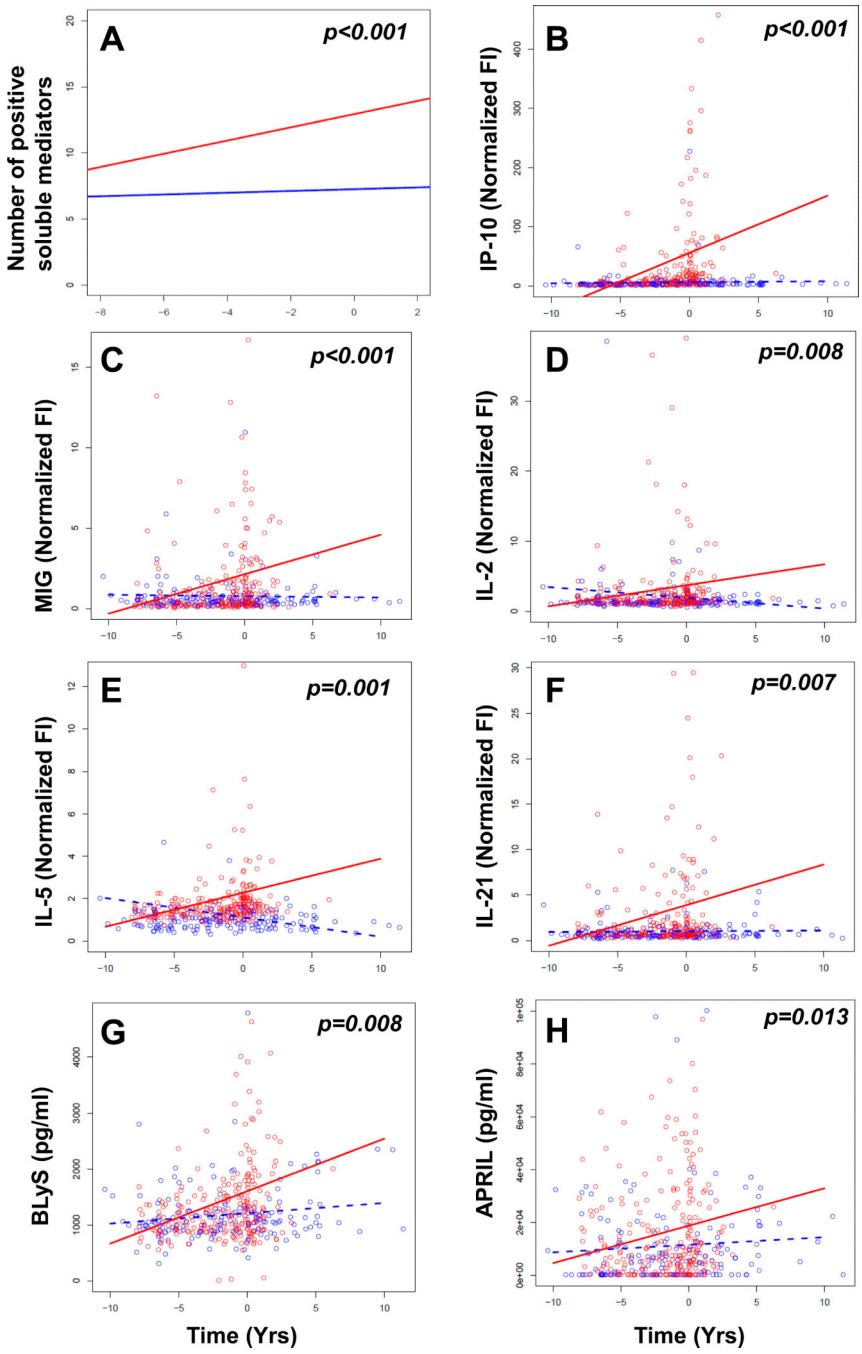

Figure 2. Select soluble mediators increase in cases as they approach SLE classification, but not in healthy controls

(**A**) Number of positive soluble mediators over time in patients prior to SLE classification (red), vs. race, gender, age (± 5 years) and time of sample procurement-matched healthy controls (blue). P-values for the fixed effect of disease status are shown. Normalized FI of IP-10 (**B**), MIG (**C**), IL-2 (**D**), IL-5 (**E**), and IL-21 (**F**), with pg/ml concentration of BLyS (**G**), and APRIL (**H**) are compared in cases (red) vs. controls (blue) over time relative to SLE classification by mixed linear regression models. Slope of line for cases (red) vs. matched healthy controls (blue) is presented in Table 2.

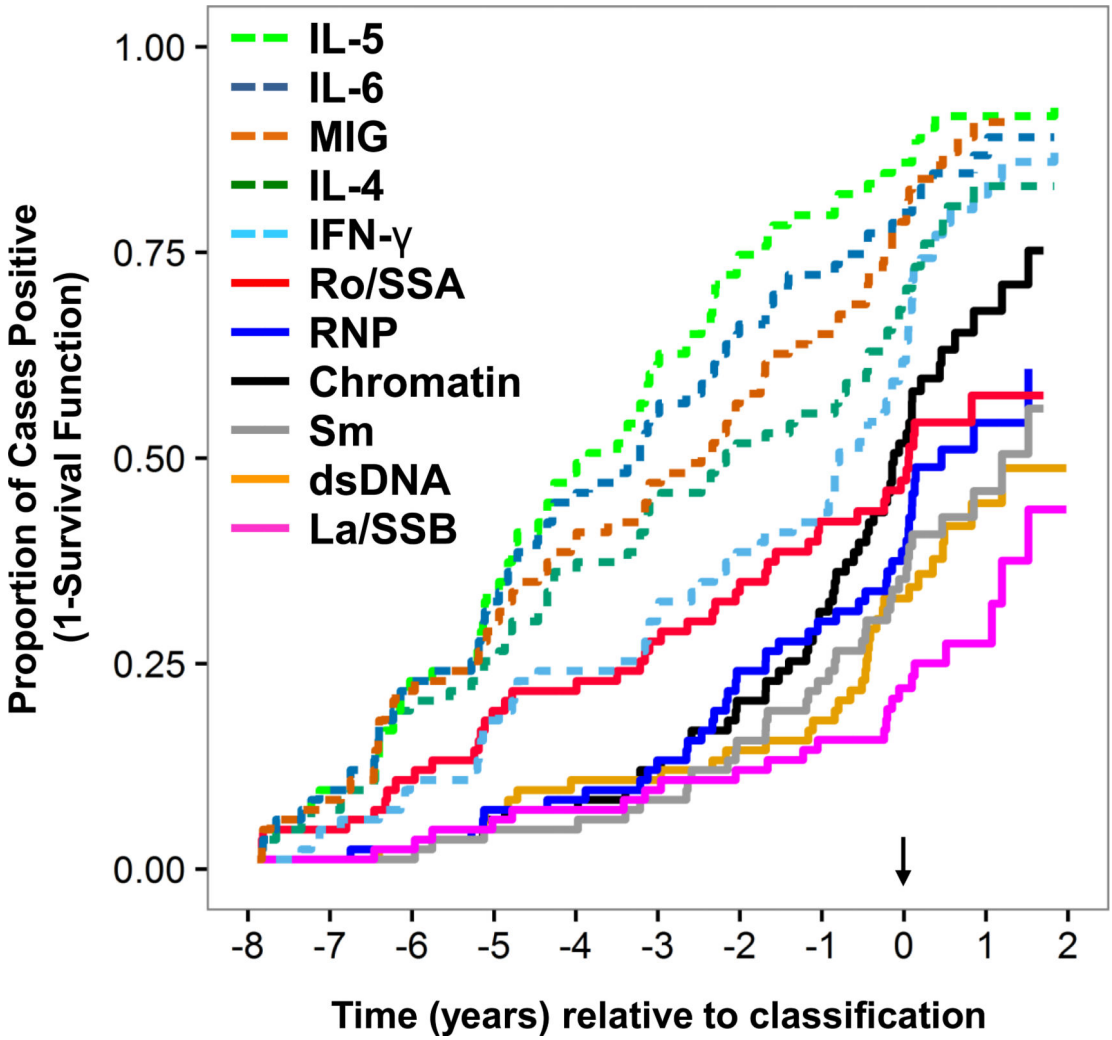

Figure 3. Dysregulation of Innate and Th-type mediators occurs prior to or concurrent with lupus-associated autoantibodies during early SLE pathogenesis

Kaplan-Meier plots demonstrating proportion of cases positive for serum cytokines IL-5 (green dotted line), IL-6 (blue dotted line), MIG (orange dotted line), IL-4 (green dotted line), and IFN-γ (teal dotted line) vs. SLE-associated autoantibody specificities against Ro/SSA (red solid line), RNP (blue solid line), chromatin (black solid line), Sm (grey solid line), dsDNA (orange solid line), and La/SSB (pink solid line) relative to time of SLE classification (arrow) are shown. Hazard ratios are presented in Table 3.

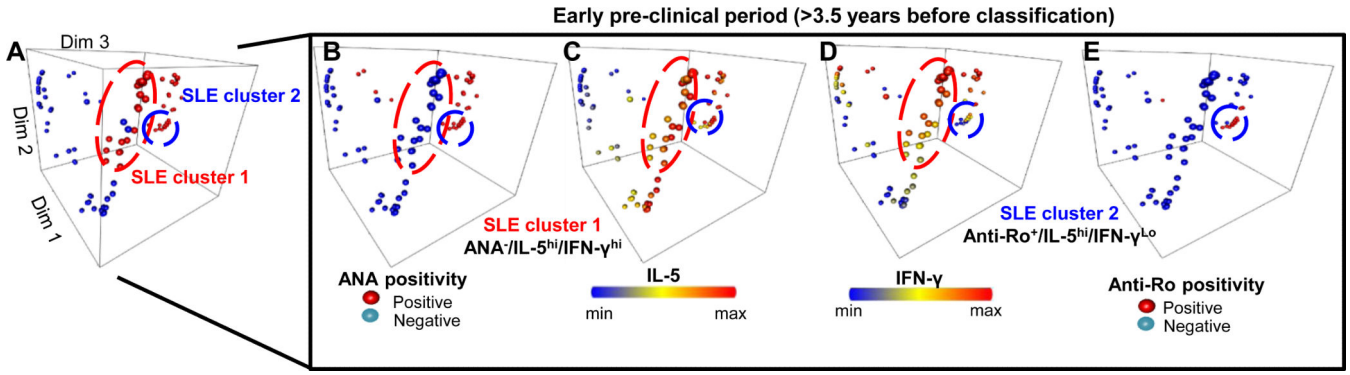

Figure 4. ANA Negative and ANA/Anti-Ro/SSA Positive, pre-clinical SLE patients show elevated IL-5 and IFN-γ >3.5 years before disease classification

(**A**) Scatter plots, showing individual cases (red dots) and matched healthy controls (blue dots) as separate points, were generated using multidimensional reduction analysis of the random forest proximity matrix. ANA positivity (**B**), IL-5 levels (**C**), IFN-γ levels (**D**), and anti-Ro/SSA positivity (**E**) are shown. SLE cluster 1 (red circle), made up of ANA^{-}/$IL\text{-}5^{Hi}$/$IFN\text{-}\gamma^{Hi}$ cases, and SLE cluster 2 (blue circle), made up of $Anti\text{-}Ro^{+}$/$IL\text{-}5^{Hi}$/$IFN\text{-}\gamma^{Lo}$ cases, are highlighted.

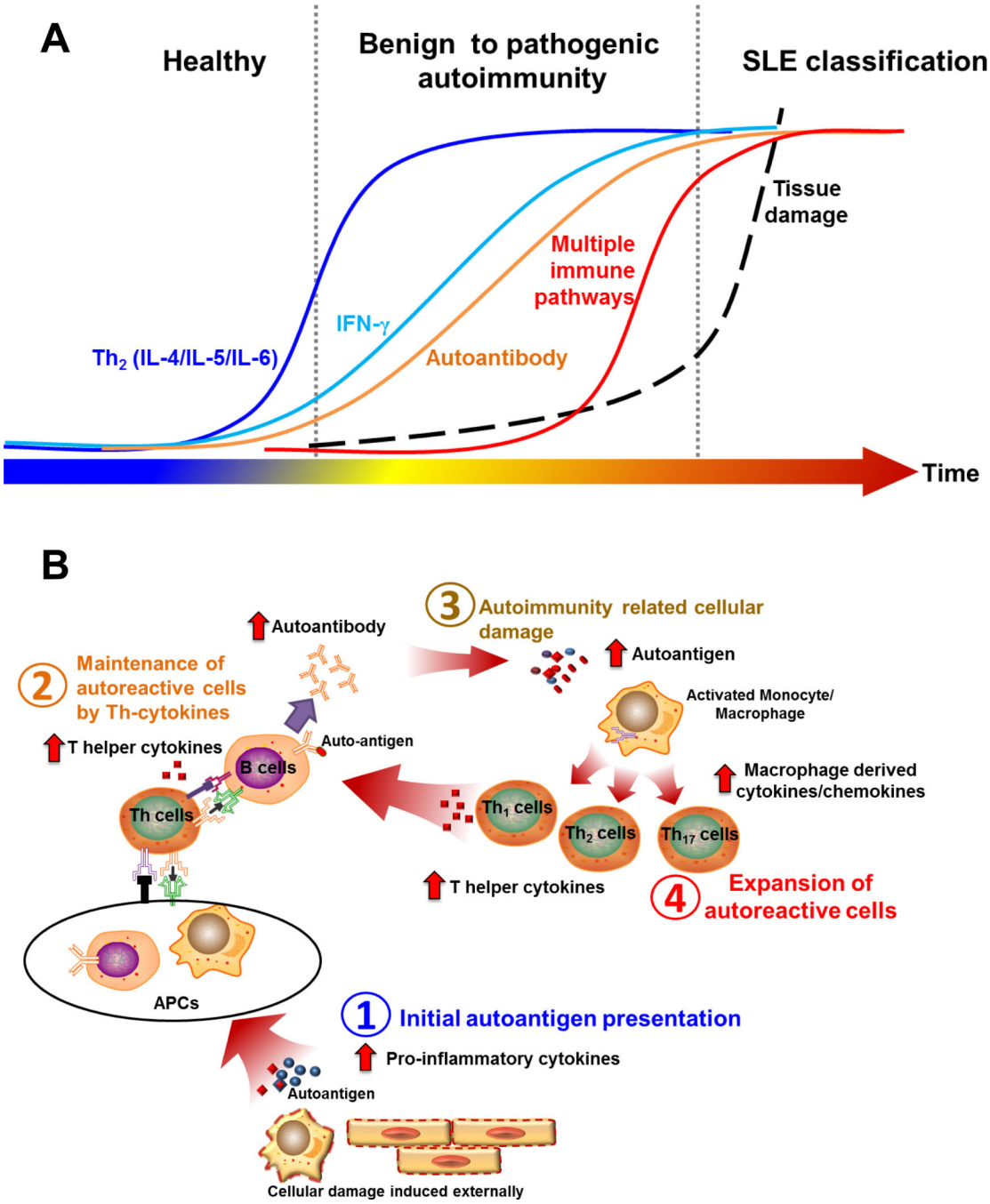

Figure 5. Proposed model of immune dysregulation leading to pathogenic autoimmunity and SLE classification

(**A**). Temporal relationship among dysregulated Th-type immune mediators associated with pre-clinical SLE is shown. (**B**) Hypothesis model of pre-clinical SLE pathogenesis: Genetic predisposition affecting apoptotic clearance, antigen-presentation, and lymphocyte responses may contribute to the appearance and maintenance of autoreactive cells (**B1**), leading to aberrant elevation of T helper (Th)-type cytokines, providing further co-stimulatory signals for the expansion of auto-reactive cells and potentiating the accrual of lupus-associated

autoantibodies (**B2**). Immune dysregulation results in tissue damage and further exposure to intracellular auto-antigens, which may result in hyperactivation of innate immune cells (**B3**), leading to further dysregulation of soluble mediators that contribute to enhanced apoptosis and intracellular auto-antigen exposure, perpetuating the cycle of autoimmunity (**B4**).

Table 1

Altered Preclinical Soluble Mediators in Individuals Who Develop SLE

		>3.5 years before classification			0.9 years before to 0.1 years after classification		
	Soluble Mediator (Normalized FI)	**Case (n=61) Median (IQR)**	**Control (n=56) Median (IQR)**	***q-value***	**Case (n=63) Median (IQR)**	**Control (n=23) Median (IQR)**	***q-value***
Innate	IL-6	1.69 (1.4 - 2.15)	1.14 (0.88 - 1.44)	***8.26E-06***	1.86 (1.5 - 3.29)	1.21 (0.81 - 1.43)	***1.72E-05***
	IL-12p70	1.5 (1.33 - 1.83)	1.24 (1 - 1.6)	***1.54E-02***	1.75 (1.5 - 2.17)	1.39 (1.07 - 1.77)	***1.86E-02***
IFN-Associated Chemokines	IP-10	2.83 (1.89 - 4.67)	2.08 (1.45 - 3.2)	***2.45E-02***	18.75 (6.09 - 51.5)	4.08 (2.63 - 5.79)	***8.79E-05***
	MIP1α	2.41 (1.51 - 4.81)	2.13 (1.49 - 3.95)	*9.27E-01*	3.34 (2.16 - 8.52)	2.06 (1.38 - 4.51)	*1.91E-01*
	MIG	0.33 (0.16 - 0.6)	0.54 (0.34 - 0.77)	***1.54E-02***	0.82 (0.26 - 1.91)	0.48 (0.38 - 1)	*4.85E-01*
Th1-type	IL-2	1.4 (1.14 - 2)	1.33 (1.17 - 1.81)	*9.27E-01*	1.8 (1.2 - 2.5)	1.33 (1.12 - 2.62)	*4.75E-01*
	IFN-γ	2.56 (2.2 - 2.98)	2.2 (1.74 - 2.75)	***3.72E-02***	3.43 (2.75 - 4.38)	2.06 (1.81 - 2.87)	***2.01E-03***
Th2-type	IL-4	1.6 (1.31 - 2)	1.25 (1 - 1.61)	***1.25E-02***	1.8 (1.43 - 2.4)	1.5 (1.17 - 1.9)	*7.05E-02*
	IL-5	1.38 (1.24 - 1.62)	0.86 (0.64 - 1.26)	***4.35E-06***	1.75 (1.31 - 2.08)	1 (0.61 - 1.14)	***7.43E-07***
	IL-13	1.32 (1.02 - 1.86)	1.21 (0.91 - 1.63)	*1.16E-01*	1.4 (1.11 - 2.33)	1.2 (0.95 - 1.6)	***3.28E-02***
Th17-type	IL-21	0.69 (0.51 - 1.08)	0.67 (0.43 - 1.01)	*6.72E-01*	0.99 (0.55 - 2.3)	0.65 (0.4 - 1.34)	*7.05E-02*
	IL-17A	1.87 (1.32 - 2.29)	1.51 (1.25 - 1.9)	*1.08E-01*	2.15 (1.75 - 2.95)	1.42 (1.25 - 2.02)	***8.29E-03***
Regulatory	TGF-β	1.84 (1.47 - 2.54)	2.69 (1.58 - 4.93)	***2.45E-02***	2.21 (1.69 - 3)	3.67 (2.47 - 6.54)	***2.01E-03***
TNF Superfamily	BLyS*	1049.55 (883.31 - 1319.46)	1042.96 (905.99 - 1241.23)	*9.27E-01*	1374.12 (1018.56 - 1788.81)	1014.64 (792.86 - 1225.4)	***3.02E-03***
	APRIL*	5524 (0 - 18877.33)	4869.57 (0 - 14735.83)	*6.72E-01*	9010.05 (3232.99 - 22773.56)	1615.03 (0 - 7540.67)	***1.94E-02***
	TNFRI	1.05 (0.94 - 1.14)	1.03 (0.91 - 1.17)	*9.27E-01*	1.22 (1.02 - 1.5)	1.04 (0.91 - 1.14)	***2.28E-02***
	TNFRII	1.01 (0.92 - 1.21)	1 (0.9 - 1.11)	*5.94E-01*	1.24 (1.09 - 1.42)	0.97 (0.85 - 1.08)	***2.41E-04***

*Units in pg/ml

Table 2

Soluble mediator levels increase prior to SLE classification

Type	Soluble mediator	Slope (Case)	Slope (Control)	*p-value*
Innate	IL-12p70	0.26	−0.26	*0.011*
	IL-23	0.13	−0.20	*0.041*
IFN-associated chemokines	IP-10	9.68	0.17	*<0.001*
	MIG	0.25	−0.01	*<0.001*
Th1-type	IL-2	0.30	−0.15	*0.008*
	IFN-γ	0.22	−0.23	*0.035*
Th2-type	IL-5	0.16	−0.09	*0 001*
Th17-type	IL-21	0.45	0.01	*0.007*
TNF superfamily	BLyS	93.93	18.38	*0.008*
	APRIL	1415	288	*0.013*
	TNFRI	0.05	0.01	*<0.001*
	TNFRII	0.03	0.00	*<0.001*
# Positive Mediators		0.50	0.06	*<0.001*
# DNA/RNA-Binding AutoAbs		0.30	−0.01	*<0.001*

Table 3

Dysregulation of T-helper-type mediators detected prior to autoantibody positivity[a]

	IL-4	IL-5	IL-6	IFN-γ	MIG
anti-dsDNA	3.14 (2.13, 4.63)	5.28 (3.56, 7.82)	4.22 (2.85, 6.25)	3.06 (2.11, 4.44)	4.11 (2.80, 6.06)
	p=9.65E-08	*p=2.92E-13*	*p=1.29E-10*	*p=7.67E-09*	*p=4.61E-13*
anti-chromatin	1.69 (1.22, 2.35)	3.05 (2.17, 4.29)	2.35 (1.70, 3.24)	1.54 (1.12, 2.11)	2.28 (1.68, 3.10)
	p=0.002	*p=3.30E-09*	*p=1.72E-06*	*p=0.008*	*p=8.82E-07*
anti-Ro/SSA	1.86 (1.27, 2.71)	2.96 (2.01, 4.36)	2.43 (1.67, 3.52)	1.74 (1.17, 2.60)	2.38 (1.71, 3.31)
	p=0.001	*p=2.56E-08*	*p=4.32E-06*	*p=0.006*	*p=6.49E-07*
anti-La/SSB	4.44 (2.92, 6.76)	7.13 (4.57, 11.11)	5.72 (3.60, 9.07)	4.31 (2.77, 6.69)	6.01 (3.99, 9.04)
	p=4.58E-10	*p=2.18E-14*	*p=1.94E-11*	*p=2.55E-10*	*p=2.62E-13*
anti-RNP	2.19 (1.50, 3.20)	3.78 (2.65, 5.39)	2.91 (2.06, 4.12)	2.03 (1.45, 2.84)	2.92 (2.01, 4.23)
	p=9.30E-05	*p=1.67E-11*	*p=2.84E-08*	*p=4.51E-05*	*p=4.69E-08*
anti-Sm	2.73 (1.91, 3.90)	4.59 (3.21, 6.55)	3.62 (2.57, 5.10)	2.54 (1.76, 3.66)	3.67 (2.56, 5.24)
	p=4.58E-07	*p=9.20E-13*	*p=3.29E-10*	*p=1.06E-06*	*p=1.61E-10*

[a]Likelihood of soluble mediator dysregulation compared to autoantibody positivity is shown as hazard ratio (95% confidence interval), with p-values determined by robust log-rank test. The hazard ratio is the composite ratio of cases with elevated soluble mediator: cases with positive autoantibody at any given time. A hazard ratio >1 indicates that the soluble mediator is more likely to be positive than the SLE-associated autoantibody.

Table 4

Soluble mediators improve predictive accuracy of ANA prior to SLE classification

Years Pre-SLE Classification	Factors	Independent Predictors of Developing SLE	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy
>3.5	ANA only	ANA	0.86 (0.71, 0.95)	0.65 (0.53, 0.76)	0.58 (0.45, 0.70)	0.89 (0.77, 0.96)	58%
	Soluble Mediators	IL-5 and IL-6	0.79 (0.68, 0.89)	0.79 (0.65, 0.89)	0.82 (0.71, 0.91)	0.76 (0.62, 0.86)	79% ± 0.60%
	Combined	**ANA, IL-5, IL-6, anti-Ro/SSA and IFN-γ**	**0.83 (0.72, 0.91)**	**0.86 (0.73, 0.94)**	**0.89 (0.78, 0.95)**	**0.8 (0.66, 0.89)**	**84% ± 0.12%**
3.5-0.9	ANA only	ANA	0.96 (0.86, 1)	0.73 (0.58, 0.85)	0.8 (0.68, 0.89)	0.94 (0.81, 0.99)	80%
	Soluble Mediators	IL-5	0.8 (0.69, 0.89)	0.76 (0.57, 0.90)	0.89 (0.78, 0.95)	0.63 (0.45, 0.79)	79% ± 0.37%
	Combined	**IL-12, MIG and ANA**	**0.93 (0.83, 0.98)**	**0.91 (0.76, 0.98)**	**0.95 (0.86, 0.99)**	**0.87 (0.71, 0.96)**	**92% ± 0.52%**
<0.9	ANA only	ANA	0.95 (0.85, 0.99)	0.68 (0.45, 0.86)	0.88 (0.77, 0.95)	0.83 (0.59, 0.96)	88%
	Soluble Mediators	IL5, IL6 and TGF-β	0.94 (0.84, 0.98)	0.79 (0.55, 0.94)	0.93 (0.84, 0.98)	0.81 (0.56, 0.95)	90% ± 0.98%
	Combined	**ANA and IL-1RA**	**0.92 (0.82, 0.97)**	**0.78 (0.52, 0.94)**	**0.93 (0.84, 0.98)**	**0.74 (0.49, 0.91)**	**89%**
ALL	ANA only	ANA	0.92 (0.85, 0.97)	0.61 (0.48, 0.72)	0.75 (0.66, 0.83)	0.86 (0.73, 0.94)	78% ± 2.42%
	Soluble Mediators	IP-10, IL-5 and IL-6	0.87 (0.79, 0.92)	0.77 (0.62, 0.89)	0.91 (0.84, 0.96)	0.68 (0.54, 0.81)	84% ± 2.95%
	Combined	**IL-6, anti-Ro/SSA, IL-5, ANA, MIG**	**0.93 (0.87, 0.97)**	**0.89 (0.77, 0.96)**	**0.96 (0.90, 0.99)**	**0.84 (0.71, 0.93)**	**92% ± 1.78%**