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in a Heterogenous Disease

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14. ABSTRACT The aim of this project was to compare impact of methotrexate to that of other common lupus immunomodulatory medications (azathioprine, mycophenolate and hydroxychloroquine) on interferon-inducible genes, and as warranted, other patient immunophenotypic clusters. 564 paxgene and 543 plasma tubes have been selected at relevant timepoints from 154 patients to provide samples from active/inactive disease on no immune suppressants, methotrexate, and other common treatments as controls. We completed the RNA expression array of 360 genes. Major findings during this project include confirmation of methotrexate impact on patients with a high interferon signature, and dampening effects on multiple pathways and on a panel of IRGs that we detected. Interference with observable methotrexate effects were found with use of concomitant hydroxychloroquine or corticosteroids. Taken together these observations confirm the feasibility of using immunologic markers to improve selection of treatments and dosing optimization for individuals with a heterogenous disease such as SLE					
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TABLE OF CONTENTS

Cover Page.....	1
Table of Contents.....	3
Abstract.....	4
Introduction.....	5
Keywords.....	5
Accomplishments.....	5
From the SOW Aim 1 (funded Aim)	5
Progress from Year 1 (as already reported)	6
Progress from Year 2 (as already reported)	11
New progress from previous reports (new).....	20
Evaluation.....	28
Assessment and Plan.....	29
Opportunities for training and professional development.....	30
Dissemination of Results.....	30
Plans for Next Reporting Period.....	30
Impact.....	30
Principal Disciplines.....	30
Other Disciplines.....	31
Technology Transfer.....	31
Society Beyond Science and Technology.....	31
Changes/Problems.....	31
Changes in Approach.....	32
Delays and Solutions.....	32
Changes with impact on expenditures.....	32
Human subjects, vertebrate animals, biohazards, select agents.....	32
Products.....	32
Publications, conference papers, and presentations.....	32
Website(s) or other Internet site(s).....	32
Technologies or techniques.....	32
Inventions, patent applications, and/or licenses.....	33
Other Products.....	33
Participants and Other Collaborating Organizations.....	33
Participants.....	33
Change in Other Support.....	34
What other organizations were involved as partners?.....	35
QUAD CHARTS.....	35
Regulatory Protocol and Activity Status.....	35
APPENDICES (includes publications/presentations)	36

Abstract

Purpose: Given the complexity of systemic lupus and the formidable barriers to the development of safe and effective treatments, the main purpose for this project is to determine whether there is a unique impact of methotrexate, as compared to other common, standard lupus treatments, on known and predictable components of lupus pathology, such as expression of cytokines and genes associated with the Type I interferon pathway.

Methods: The approach that we have taken taking in this project combines a comprehensive expression array that includes 360 genes with a multiplex cytokine assay. This supports the assessment of both pre-specified hypotheses, based on our preliminary data, about the potential impact of methotrexate on specific interferon-inducible genes/proteins, but also an alternative way to cluster patients for methotrexate evaluation using definable immune phenotypes based on gene co-expression pathways that we previously observed and more recently reproduced in two non-overlapping lupus populations. Expression of 360 genes is also supporting ongoing, exploratory analysis, since data in support of our primary hypothesis is not as strong as we predicted.

Progress: We completed the selection of 564 high quality RNA, 543 of which are matched to plasma samples from multiple visits of 154 SLE patients. This provided both cross sectional and time-separated studies of patients on or off methotrexate, azathioprine, mycophenolate mofetil or hydroxychloroquine, with active vs inactive disease for all of these treatment variables. Each sample underwent quality testing procedures in our laboratories and each linked clinical visit was subjected to medical review and query-completion as we refined the database for this project. RNA expression arrays were performed in late August 2019, and we performed a preliminary analysis of Methotrexate impact on patients with high vs low interferon-inducible gene signals which was included in previous progress reports. This report contains additional analysis of

Plans: Our original goal was to use the data gained here to generate rational hypotheses suitable for the future design of clinical trials. Unfortunately, we were unable to continue a trial that had enrolled 8 patients during the COVID 19 pandemic that was directly studying methotrexate which would have benefitted from insights from this work. However, some of the results of the current studies will be used to inform biomarker work that will be performed on samples from a multi-center study funded by the NIAMS Autoimmunity Center of Excellence consortium that has just been approved to start. This will be a multi-center trial of mycophenolate with or without voclosporin and our group at OMRF will be leading the biomarker analysis. (PI James, Project Leader, Merrill). Publication of our work is pending, but should be helpful in the design of biomarker analysis for other clinical trials.

Introduction (*this section is not amended from prior progress reports*)

Systemic Lupus (SLE or lupus) is a heterogenous autoimmune disorder with unpredictable manifestations and long-term prognostic uncertainty. Even with optimal current care, patients are frequently under-treated, resulting in a high incidence of flares, or over-treated, resulting

in significant toxicity from medications. More than 30 pivotal trials of promising, targeted biologics have failed to distinguish treatment from placebo. These trials were ubiquitous in adding investigational treatments to aggressive background polypharmacy. Exploratory data from older trials and pre-specified endpoints of recent studies demonstrate that treatment effects are increased using stringent endpoints and design strategies that limit or taper background medications. Future trial designs that taper medications can be considered but are not feasible for patients with severe illness or unfavorable prognosis. These patients have the greatest need for novel treatments, but to protect their safety in trials, they will continue to require background immune modulators.

It is unfortunate, then, that treatments commonly used for this purpose are themselves heterogeneous, empirically prescribed or combined, and poorly understood. Precision medicine is the brass ring of treatment development but remains elusive for complex diseases like SLE. One fundamental problem is our reliance on the use of treatments and treatment combinations for which differential effects on heterogeneous SLE sub-populations are unknown. The current project addressed this issue by utilizing both hypothesis driven and exploratory evaluation of patient samples on and off treatment using two methods to sort out the heterogeneity of the underlying immune pathology and focusing on methotrexate, since our preliminary data suggested differential effects on BLYS and other interferon-inducible gene and protein expression depending on whether the patient has high interferon signals.

Keywords: Systemic lupus, Standard of Care, Immune Modulators, Immune Phenotype, Gene Expression

Accomplishments:

What were the major goals of the project?

1. To determine whether there is a unique impact of methotrexate (compared to other common, standard of care lupus treatments) on expression of cytokines and genes associated with the Type I interferon pathway.
2. To characterize subpopulations (clusters) of lupus patients, based on more comprehensive patterns of upregulation or downregulation of NIAID immune pathway modules, for whom methotrexate has discrete effects, and to distinguish these effects from those of hydroxychloroquine or other immune modulators.
3. To use the data gained here, using existing stored samples from completed clinical research, to generate rational hypotheses suitable for the future design of clinical trials

Accomplishments during this project

PREVIOUSLY REPORTED PROGRESS

What was accomplished under the goals listed on the prior page?

PROGRESS FROM YEAR ONE:

From the SOW: Aim 1 (the funded Aim of this project) was to determine impacts of common lupus immunomodulatory medications (methotrexate, azathioprine, mycophenolate and hydroxychloroquine) on interferon-inducible genes, including OAS2, OASL, LGALS3BP, LY6E and TNFSF13B (BLyS).

The hypothesis was that methotrexate would distinguish itself by a distinct impact on interferon-inducible genes/proteins.

Parts 1 and 2 of AIM 1 from the SOW: 1. Collect already available cohort and stored clinical trial Pax gene and serum samples, blind and code them, transfer to Core Facility. 2. Organize clinical data from the above sources in readiness for analysis of gene expression and protein level results. *AIM 1, parts 1 and 2 were completed in Year 1 of the Study as previously reported and included here.*

Adequate, high quality clinical data and blood samples were obtained and organized. In the SOW it was indicated that these tasks would be finished within two months of funding, but it took many more months to match well-characterized clinical data to high quality RNA and plasma in enough patients meeting specifications to optimally support this project. In the original application we underestimated the number of samples we would need. As listed at the time of submission, we had stored samples (often multiple samples) identified from a total of 78 different subjects who had completed their participation in two investigator-initiated clinical trials at OMRF. This had seemed suitable to begin to examine cross-sectional differences in impact of immune modulators on gene expression and cytokines, as well as provided a pilot basis to examine multiple samples from a number of patients on a given treatment with both low or active disease as well as on no immunosuppressant with low or active disease.

TABLE 1a: Resources in place at the time of grant submission

Study:	ABC	BLAST	Total	To be analyzed for
Total Subjects Screened	72	17	89	Model a Cross sectional validation of preliminary observations
Individual subjects (subtracting those in both studies)	72	5	78	Model b Analysis without overlap of patients.
Subjects with Screening and pre-dose randomization visits after amelioration of disease by steroids	63	14	77	Model c Samples compared at entry with active disease on various background medications vs later after steroids.
Subjects with screening, and 3-month visits who received placebo	29	0	29	Model d Entry samples compared to placebo samples on no background medication
Subjects with an earlier matching sample when on the same treatment as screening but quiescent disease	28	6	34	Model e Screening samples with active disease flares compared to prior samples on the same treatment but quiescent

We were unsure how many samples we could find on and off each individual treatment, however, especially methotrexate. Therefore, we indicated in the SOW that we did plan to supplement with additional samples from cohort visits in order to attain better power for these analyses. Since then, both the ABC and BLAST Studies were completed. Both of these had obtained informed consent to transfer biomarker samples to the Oklahoma Lupus Cohort Repository. This increased the numbers of useful samples, supplemented by matching samples from our cohort as specified in the SOW: Table 1b, below, summarizes our final sample resource for this project. We think the extra time devoted to amassing this substantial resource was well spent.

TABLE 1b: Combined resources from combined sources: Stored samples from clinical trials and the Oklahoma Lupus Cohort

Study:	Total	To be analyzed for
Total Pax gene/plasma samples	564/543	Model a Cross sectional validation of preliminary observations
Total Individual subjects	154	Model b Cross sectional analysis without overlap of patients
Subjects with Screening <u>and</u> pre-dose randomization visits after amelioration of disease by steroids	90	Model c Samples compared at entry with active disease on various background medications vs a short time after steroids given within the conduct of a monitored, prospective trial
Subjects with screening, and visits 3-6 months later who received placebo	33	Model d Entry samples compared to placebo samples on no background medication after 3 months when entry steroids wore off within conduct of a monitored, prospective trial
Subjects on methotrexate with active disease (a)	39	Model e Samples with active disease flares compared to samples taken from same patient at a different time on the same treatment but quiescent An overall evaluation of samples can be performed broken down into immune phenotypes, but we worked on sample selection such that all subjects did provide multiple matched samples for one or more of these analyses. Most patients were able to provide at least one sample with active disease (designated as “a” samples here) and at least one quiescent sample (designated as “q” here either while taking the same treatment or when taking one of the treatments vs no immune suppressant. Note that analysis of samples on immunosuppressants will be controlled for use of hydroxychloroquine and steroids (albeit samples selected were restricted to only low dose steroids and preferably none). The total numbers given for hydroxychloroquine include only patients on no other treatment besides hydroxychloroquine and > 50 have matched samples on no treatment at all.
Subjects on methotrexate with quiescent disease (q)	30	
Subjects on azathioprine with active disease (a)	30	
Subjects on azathioprine with quiescent disease (q)	16	
Subjects on mycophenolate with active disease (a)	23	
Subjects on mycophenolate with quiescent disease (q)	17	
Subjects on no immune suppressant with active disease (a)	101	
Subjects on no immune suppressant with quiescent disease (a)	116	
Subjects on hydroxychloroquine alone with active disease (a)	71	
Subjects on hydroxychloroquine alone with quiescent disease (q)	79	

Quality assurance and preparation of samples and clinical data for evaluation: We completed identification, collection and quality checks on all Pax gene samples, plasma and clinical data from all patients all visits. Clinical datasets were queried to the original clinical investigator who performed the scoring whenever scoring was inconsistent or appeared erroneous (e.g., not matching the clinical note). Data included following clinical disease instruments: *BILAG 2004*, *SLEDAI*, *SLEDAI Flare Index*, *CLASI* and *PGA*. All sample collection was prespecified in the application and previously consented by patients after IRB-approval at OMRF. Use of the cohort repository was by standard operating procedures, including advisory board approval of the project. No patient enrollment was involved since all samples were previously collected.

Gene Expression Profiling and soluble mediator assays: We had previously characterized 7 clusters in adult lupus patients representing patterns of gene expression in 29 calculated modular scores, that each represent relevant immune pathways (see our original preliminary data). **We completed quantifying the expression of genes in all samples described in Table 1b above, generating a raw dataset of > 20 TB of data.** After extensive bioinformatics computation it was possible to reduce the data into meaningful bites for our statisticians to evaluate the expression of interferon-inducible genes and the assortment of patients into immune-module based SLE clusters. **Preliminary findings from Year 1 and Year 2 are shown separately below.** We will continue to interrogate the data using the models described in Table 1a and 1b. **This will enable us to complete the process described in Part 3 of AIM 1 in the SOW.**

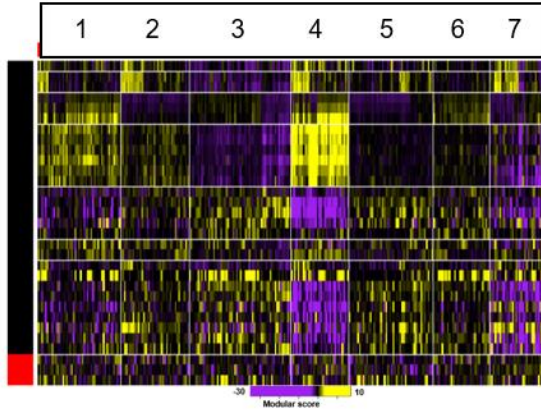
Briefly the ***methods*** that were used were for each stored PAX gene RNA sample for each patient/visit was to extract total RNA with 96 Blood RNA kit (Qiagen, Valencia, California, USA). Expression levels of gene transcripts (n=360) covering the common m1-m5 modules were quantitated by real-time qPCR (Biomark HD) at the OMRF Phenotyping Core. Module scores were calculated compared to healthy controls from the Oklahoma Immune Cohort. Plasma has not yet been assayed but there are stored samples awaiting processing. Plasma soluble mediators (n=50 including IFN- α , IFN- γ , IP10, MCP-1, MIG, MIP-1 α , MIP-1 β , TRAIL, TWEAK, sCD-40 ligand, TNFR I, TNFR II, IL-10, IL-17A, IL-2, IL-2RA, IL-12p70, IL-21, IL-1 α , ICAM-1, SCF and others) will be tested by multiplex, bead-based assay and ELISAs (BLyS, APRIL) as is now standard in our laboratories. We have 543 plasma samples matching most of the RNA samples.

Progress on Part 3 of Aim 1 Part 2 of Aim 1 had progress as of end of Year 1 and is shown again here.

We confirmed the establishment of 7 clusters of adult lupus immunophenotypes with the same relevant immune clusters that had been shown in our preliminary data. Figures 2a and 2b, below allow a visual inspection of the heat maps generated from these module expression profiles. Clearly the original seven clusters of lupus patients have been reduplicated to a significant extent, suggesting a stability of active immune pathways in lupus that may be of an interpretable magnitude. We have observed that some patients may change clusters over time, but several clusters seem to be related in type of increased cluster expression, despite differing degrees (Clusters 1 and 4 or 3 and 6).

Figure 2 Clustering of SLE Patients by Random Forest Modeling of Gene Co-Expression Pathways

2a. Previous data (shown in [application](#))
200 pts from OLC project



2b. Confirmation of cluster patterns
from current selected Rx cohorts

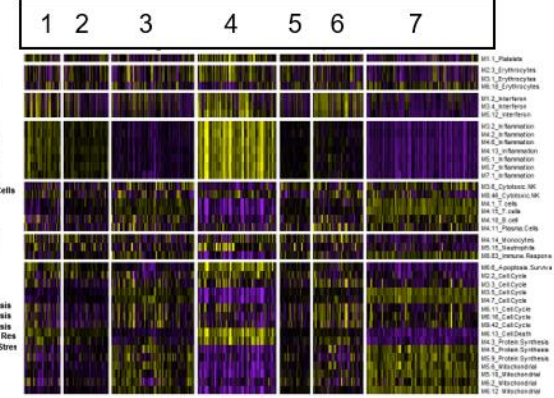


Table 3: Cross Sectional Analysis of Methotrexate on module scores in patients with or without the interferon gene signature. In the table below, module score difference refers to the difference in gene expression in each module between patients receiving methotrexate vs those not receiving methotrexate controlled for disease activity (SLEDAI score) and concomitant hydroxychloroquine use by linear regression analysis.

Module	Module Score Difference (p value)	
	IFN low n=62)	IFN high (n=59)
M4.1_T cells	-0.0000189 (0.975)	-0.00277** (0.002)
M4.15_T cells	-0.000535 (0.269)	-0.00251*** (0.001)
M8.83_Immune Responses	-0.0000879 (0.818)	-0.00177** (0.007)
M5.1_Inflammation	0.000273 (0.412)	0.00111** (0.010)
M6.6_Apoptosis	0.000434 (0.264)	0.00134** (0.009)
M3.5_Cell Cycle	-0.000394 (0.249)	-0.00135** (0.006)
M4.7_Cell Cycle	-0.000438 (0.222)	-0.00137** (0.007)
M9.42_Cell Cycle	-0.000852** (0.009)	-0.000250 (0.621)
M6.12_Mitochondrial	-0.000424 (0.224)	-0.00130** (0.006)

Blue= patients on MTX have lower expression than those not on MTX
Red= patients on MTX have higher expression than those not on MTX

Evaluation: We previously observed decrease in expression of several interferon-inducible genes in patients with the interferon signature on methotrexate. When examining overall impact on immune pathway modules in earlier parts of this study, however, this trend was not found in the summed expression of genes contained in these pathways. Although increase in expression of inflammation and apoptosis pathway modules was associated with methotrexate use in patients with elevated Type I interferon signals along with lower gene expression in T Cell, immune response, mitochondrial and cell cycle pathways. Table 4 (below) depicts differences in module scores in 59 patients with elevated interferon-inducible gene expression taking Methotrexate (reference group) and control patients. In this example all patients had moderately to severely active disease. **Please note that we were concerned about the variables that seemed to impact on discrepant results and during the no cost extension year we re-approached the question of methotrexate effects on interferon pathway expression and patients with or without high interferon expression. See the final part of this progress for data not previously reported to DoD. We do retain the original reported data here for completeness however in the table just below.**

Module	High disease no Rx N=12	High disease MTX+HCQ N=6	High disease HCQ only N=9	* <0.05 **<0.01 ***<0.0001 Blue= control patients in the given column have lower expression than those on MTX Red= control patients in the given column have higher expression than those on MTX
M4.1_T cells	0.00324** (0.005)	0.00350** (0.010)	0.00489*** (0.000)	
M4.15_T cells	0.00317** (0.001)	0.00320** (0.005)	0.00402*** (0.000)	
M8.83_Immune Response	0.00141 (0.111)	0.00164 (0.113)	0.00274** (0.004)	
M3.2_Inflammation	-0.00285* (0.024)	-0.00342* (0.021)	-0.00369** (0.006)	
M4.2_Inflammation	-0.00332* (0.046)	-0.00429* (0.028)	-0.00481** (0.007)	
M4.6_Inflammation	-0.00203* (0.015)	-0.00239* (0.014)	-0.00248** (0.005)	
M5.1_Inflammation	-0.00145* (0.012)	-0.00172* (0.011)	-0.00192** (0.002)	
M5.7_Inflammation	-0.00185* (0.014)	-0.00196* (0.024)	-0.00209** (0.009)	
M5.12_Interferon	-0.00126 (0.139)	-0.00181 (0.071)	-0.00198* (0.030)	
M6.6_Apoptosis / Survival	-0.00164* (0.019)	-0.00171* (0.035)	-0.00212** (0.005)	
M3.3_Cell Cycle	0.00330** (0.005)	0.00156 (0.243)	-0.000162 (0.893)	

M3.5_Cell Cycle	0.00195** (0.003)	0.00201** (0.008)	0.00236*** (0.001)
M4.7_Cell Cycle	0.00193** (0.005)	0.00198* (0.013)	0.00255*** (0.001)
M6.11_Cell Cycle	0.00328** (0.005)	0.000785 (0.549)	-0.000506 (0.669)
M6.16_Cell Cycle	0.00303** (0.001)	0.00188 (0.073)	0.00129 (0.172)
M9.42_Cell Cycle	0.00219** (0.002)	0.00170* (0.038)	0.000798 (0.273)

Preliminary Assessment and Plan as of the End of Year 1

Our preliminary assessment was that methotrexate might have a greater impact on patients with an interferon-inducible gene signature than in those without this underlying immune pattern, with a dampening effect in this subset of patients, on expression of T Cell and Cell Cycle co-expression pathways. However in this first set of data when compared to controls (particularly those on hydroxychloroquine) methotrexate seemed to increase inflammation pathway and perhaps some interferon pathway expression. We thought that this analysis might be complicated by the fact that hydroxychloroquine may interfere with Toll-like receptors and directly suppress interferon pathways, potentially confounding any impact of methotrexate alone. ***Since we did not find a dampening effect of methotrexate on interferon modules as our preliminary data had suggested, we determined to reproduce (or disqualify) our discrepant observation and test several variables that could be causing these inconsistencies, including the possibility that module assignments had gotten reversed somewhere in the analysis, either in the preliminary data or in the later findings. Please see the section on New Progress Since Previous Reports below which will show the results of a more recent analysis.***

Progress from Year 2

After evaluating our data from Year 1 we planned to focus on the following questions:

1. **At the end of Year 1 we planned to acquire more samples of patients taking methotrexate without hydroxychloroquine in order to better determine its impact in the absence of the potentially confounding input from hydroxychloroquine.** We were unable to find sufficient samples since patients taking only methotrexate without hydroxychloroquine are rare. An expanded analysis is planned in a future project facilitated by our collaborations on biomarkers from several industry trials. We adjusted the analysis for this project to examine differential impacts of methotrexate on patients with high vs low disease by controlling for concomitant hydroxychloroquine being taken by many of these patients. **See Table 1, below.** This is an exploratory analysis of differences between methotrexate impact on immune pathway expression modules in patients with high disease (SLEDAI score ≥ 6) vs low disease.

Table 1: MTX (adjusted for hydroxychloroquine) impact on Gene Expression in Patients with High vs Low Disease Activity Blue=lowered Red=elevated

Red arrows: MTX/controls *Left: All pts Middle: pts w/ low disease Right: pts w/ high disease*

* <0.05 **<0.01 ***<0.0001		All (N=121)	MTX	None	SLEDAI	Low SLEDAI (N=67)	MTX	None	High SLEDAI (N=54)	MTX	None
Module score		No vs Yes	No vs Yes	No vs Yes	High vs Low	No vs Yes	No vs Yes	No vs Yes	No vs Yes	No vs Yes	No vs Yes
Module score difference	M4.10_B cell	0.00224	-0.00132	0.00157	-0.00159	0.00272	-0.00123	0.00261	0.000679	-0.00142	-0.000774
p-value		(0.229)	(0.204)	(0.464)	(0.068)	(0.280)	(0.479)	(0.382)	(0.831)	(0.264)	(0.827)
Module score difference	M4.11_Plasma Cells	0.00117	-0.00188*	0.00314	-0.000623	0.000970	-0.00107	0.00387	0.00245	-0.00257*	0.00331
p-value		(0.419)	(0.021)	(0.062)	(0.357)	(0.600)	(0.406)	(0.081)	(0.353)	(0.016)	(0.261)
Module score difference	M4.1_T cells	-0.000643	-0.00110*	-0.00172	-0.000842	0.000567	-0.000654	-0.000735	-0.00311	-0.00136	-0.00385
p-value		(0.492)	(0.037)	(0.114)	(0.055)	(0.568)	(0.343)	(0.532)	(0.117)	(0.084)	(0.081)
Module score difference	M4.15_T cells	-0.000601	-0.00130**	-0.00155	-0.000698	0.000139	-0.000679	-0.000748	-0.00167	-0.00174*	-0.00264
p-value		(0.430)	(0.003)	(0.079)	(0.051)	(0.850)	(0.185)	(0.391)	(0.324)	(0.011)	(0.162)
Module score difference	M3.6_Cytotoxic/NK	-0.000928	-0.00133*	-0.00162	0.0000875	-0.00159	-0.000819	-0.00151	0.00124	-0.00180*	-0.000376
p-value		(0.365)	(0.021)	(0.173)	(0.854)	(0.194)	(0.332)	(0.296)	(0.528)	(0.023)	(0.863)
Module score difference	M8.6_Cytotoxic/NK	-0.000322	0.000186	0.0000798	-0.000670	0.000665	0.0000162	0.000172	-0.00274	0.000427	-0.00118
p-value		(0.728)	(0.719)	(0.940)	(0.122)	(0.573)	(0.984)	(0.902)	(0.096)	(0.508)	(0.513)
Module score difference	M4.14_Monocytes	-0.000238	0.000435	0.0000736	0.000403	-0.000520	0.000375	-0.000332	0.000526	0.000482	0.00101
p-value		(0.706)	(0.216)	(0.919)	(0.171)	(0.472)	(0.455)	(0.698)	(0.681)	(0.343)	(0.477)
Module score difference	M5.15_Neutrophils	0.000777	0.00191	0.00352	0.000182	0.000380	0.00261	0.00397	0.00246	0.00131	0.00419
p-value		(0.669)	(0.061)	(0.095)	(0.829)	(0.871)	(0.112)	(0.157)	(0.454)	(0.317)	(0.253)
Module score difference	M8.83_Immune Responses	-0.000110	-0.000770*	-0.000861	-0.000325	0.000222	-0.000425	-0.000361	-0.000574	-0.00103*	-0.00151
p-value		(0.862)	(0.032)	(0.243)	(0.275)	(0.791)	(0.463)	(0.715)	(0.614)	(0.026)	(0.234)
Module score difference	M3.2_Inflammation	0.000473	0.00130*	0.00127	0.000670	0.000154	0.000544	0.000766	0.000241	0.00185*	0.00117
p-value		(0.624)	(0.017)	(0.257)	(0.138)	(0.896)	(0.506)	(0.584)	(0.896)	(0.014)	(0.570)
Module score difference	M4.2_Inflammation	0.00100	0.00168*	0.00221	0.000490	0.000415	0.000666	0.00143	0.00109	0.00242*	0.00242
p-value		(0.426)	(0.018)	(0.130)	(0.404)	(0.780)	(0.520)	(0.420)	(0.659)	(0.016)	(0.381)
Module score difference	M4.6_Inflammation	0.000202	0.000862*	0.000855	0.000537	-0.0000639	0.000401	0.000442	0.000281	0.00120*	0.00107
p-value		(0.757)	(0.020)	(0.259)	(0.080)	(0.936)	(0.469)	(0.641)	(0.823)	(0.019)	(0.445)
Module score difference	M4.13_Inflammation	0.000233	0.00114*	0.000749	0.000785	0.0000263	0.000458	0.000253	-0.000126	0.00166*	0.000677
p-value		(0.785)	(0.018)	(0.449)	(0.051)	(0.980)	(0.519)	(0.835)	(0.939)	(0.014)	(0.714)
Module score difference	M5.1_Inflammation	0.000277	0.000618*	0.000786	0.000430*	0.0000624	0.000281	0.000432	0.000412	0.000869*	0.00106
p-value		(0.548)	(0.018)	(0.141)	(0.047)	(0.908)	(0.456)	(0.503)	(0.652)	(0.019)	(0.297)
Module score difference	M5.7_Inflammation	0.000236	0.000808*	0.000661	0.000580*	0.0000390	0.000308	0.000189	0.000144	0.00119*	0.000869
p-value		(0.685)	(0.014)	(0.327)	(0.035)	(0.954)	(0.515)	(0.815)	(0.900)	(0.011)	(0.499)
Module score difference	M7.1_Inflammation	0.0000970	0.000614*	0.000487	0.000431	-0.0000479	0.000368	0.000165	0.000212	0.000804*	0.000811
p-value		(0.840)	(0.023)	(0.380)	(0.056)	(0.933)	(0.355)	(0.807)	(0.822)	(0.035)	(0.441)
Module score difference	M1.2_Interferon	-0.00266	-0.0000580	0.000727	-0.000379	-0.00310	0.00116	0.00199	-0.000566	-0.00112	0.000714
p-value		(0.345)	(0.970)	(0.823)	(0.772)	(0.384)	(0.638)	(0.638)	(0.913)	(0.587)	(0.902)
Module score difference	M3.4_Interferon	-0.00116	0.000195	0.000632	-0.000154	-0.00185	0.000388	0.000954	0.000419	-0.0000664	0.000867
p-value		(0.395)	(0.796)	(0.687)	(0.808)	(0.284)	(0.745)	(0.641)	(0.865)	(0.946)	(0.752)
Module score difference	M5.12_Interferon	-0.000439	0.000491	0.000714	0.000156	-0.000991	0.000359	0.000572	0.000598	0.000529	0.00119
p-value		(0.598)	(0.291)	(0.458)	(0.687)	(0.355)	(0.627)	(0.651)	(0.693)	(0.379)	(0.479)
Module score difference	M6.6_Apoptosis / Survival	0.000312	0.000788*	0.000850	0.000385	0.0000230	0.000443	0.000489	0.000595	0.00103*	0.00118
p-value		(0.570)	(0.011)	(0.182)	(0.135)	(0.972)	(0.331)	(0.530)	(0.582)	(0.018)	(0.325)
Module score difference	M2.2_Cell Cycle	0.0000618	0.0000883	0.0000423	0.0000909	0.0000650	2.70e-08	-0.0000565	-0.0000241	0.000162	0.0000811
p-value		(0.677)	(0.286)	(0.805)	(0.190)	(0.686)	(1.000)	(0.767)	(0.938)	(0.189)	(0.814)
Module score difference	M3.3_Cell Cycle	0.00125	-0.000304	0.000332**	-0.000296	0.00103	0.000332	0.000395**	0.00233	-0.000854	0.00335
p-value		(0.166)	(0.545)	(0.002)	(0.480)	(0.381)	(0.684)	(0.006)	(0.138)	(0.169)	(0.056)
Module score difference	M3.5_Cell Cycle	0.000186	-0.000789**	-0.000247	-0.000575*	0.000547	-0.000488	0.000129	-0.000330	-0.000997*	-0.000751
p-value		(0.712)	(0.006)	(0.141)	(0.016)	(0.342)	(0.222)	(0.849)	(0.748)	(0.017)	(0.510)
Module score difference	M4.7_Cell Cycle	-0.000229	-0.000799**	-0.000703	-0.000548*	0.000344	-0.000457	-0.000108	-0.00127	-0.00103*	-0.00174
p-value		(0.664)	(0.007)	(0.250)	(0.028)	(0.568)	(0.274)	(0.879)	(0.236)	(0.017)	(0.145)
Module score difference	M6.11_Cell Cycle	0.00139	-0.000560	0.00317**	-0.000356	0.00104	-0.0000392	0.000389**	0.00250	-0.00105	0.00293
p-value		(0.105)	(0.240)	(0.002)	(0.370)	(0.351)	(0.959)	(0.004)	(0.088)	(0.071)	(0.072)
Module score difference	M6.16_Cell Cycle	0.000169	-0.000708*	0.00109	-0.000632*	0.000179	-0.0000866	0.000170	0.000759	-0.00122**	0.000961
p-value		(0.782)	(0.039)	(0.124)	(0.028)	(0.816)	(0.871)	(0.067)	(0.487)	(0.007)	(0.429)
Module score difference	M9.42_Cell Cycle	0.000799	-0.000580*	0.00129*	-0.000320	0.000857	-0.000278	0.00177*	0.000851	-0.000839*	0.000803
p-value		(0.128)	(0.048)	(0.034)	(0.190)	(0.182)	(0.530)	(0.022)	(0.391)	(0.036)	(0.466)
Module score difference	M6.13_Cell Death	0.000100	0.000972*	0.000833	0.000600	-0.000217	0.000525	0.000418	0.000311	0.00129*	0.00112
p-value		(0.893)	(0.021)	(0.335)	(0.087)	(0.812)	(0.407)	(0.699)	(0.829)	(0.026)	(0.486)
Module score difference	M5.6_Mitochondrial	0.000423	-0.000531*	0.000235	-0.000382*	0.000484	-0.000428	0.000350	0.000389	-0.000610*	0.000140
p-value		(0.256)	(0.011)	(0.584)	(0.029)	(0.297)	(0.185)	(0.524)	(0.582)	(0.033)	(0.858)
Module score difference	M5.10_Mitochondrial	0.000267	-0.000425*	0.0000785	-0.000257	0.000411	-0.000225	0.000204	0.000208	-0.000563*	0.0000733
p-value		(0.371)	(0.012)	(0.820)	(0.066)	(0.252)	(0.365)	(0.631)	(0.720)	(0.017)	(0.909)
Module score difference	M6.2_Mitochondrial	0.000301	-0.000136	0.000165	-0.0000480	0.000260	-0.000129	0.0000502	0.000474	-0.000136	0.000446
p-value		(0.076)	(0.149)	(0.396)	(0.541)	(0.219)	(0.377)	(0.840)	(0.137)	(0.281)	(0.207)
Module score difference	M6.12_Mitochondrial	-0.0000359	-0.000784**	-0.000562	-0.000530*	0.000261	-0.000560	-0.000180	-0.000547	-0.000945*	-0.00117
p-value		(0.942)	(0.005)	(0.325)	(0.023)	(0.646)	(0.158)	(0.790)	(0.584)	(0.020)	(0.294)
Module score difference	M4.3_Protein Synthesis	-0.000882	0.000699	0.0000938	0.000677*	-0.000923	0.000727	-0.0000231	-0.000672	0.000685	0.000416
p-value		(0.216)	(0.079)	(0.909)	(0.043)	(0.285)	(0.225)	(0.982)	(0.628)	(0.216)	(0.787)
Module score difference	M4.5_Protein Synthesis	0.000288	-0.000375*	0.000154	-0.000275*	0.000384	-0.000299	0.000191	0.000188	-0.000422*	0.000145
p-value		(0.315)	(0.020)	(0.641)	(0.041)	(0.292)	(0.237)	(0.657)	(0.724)	(0.049)	(0.807)
Module score difference	M5.9_Protein Synthesis	0.000430	-0.000223	0.000187	-0.0000428	0.000591	-0.0000360	0.000192	0.000396	-0.000339	0.000394
p-value		(0.157)	(0.188)	(0.592)	(0.761)	(0.082)	(0.877)	(0.629)	(0.522)	(0.171)	(0.567)
Module score difference	M1.1_Platelets	-0.000864	-0.0000869	-0.000444	0.00117	-0.000681	0.000667	-0.000379	-0.0000557	-0.000617	0.000654
p-value		(0.536)	(0.911)	(0.783)	(0.075)	(0.717)	(0.608)	(0.865)	(0.981)	(0.516)	(0.806)
Module score difference	M2.3_Erythrocytes	-0.000794	-0.000191	0.000429	0.00127	0.000456	0.00130	0.00128	-0.00154	-0.00119	0.000426
p-value		(0.624)	(0.832)	(0.819)	(0.094)	(0.829)	(0.377)	(0.610)	(0.587)	(0.291)	(0.892)
Module score difference	M3.1_Erythrocytes	-0.000392	0.0000292	0.0000406	0.00119*	0.000355	0.000935	0.000421	-0.000730	-0.000568	0.000331
p-value		(0.753)	(0.966)	(0.978)	(0.043)	(0.829)	(0.414)	(0.829)	(0.737)	(0.510)	(0.891)
Module score difference	M6.18_Erythrocytes	0.00100	0.000362	0.0000826	-0.000714	-0.000130	-0.000585	-0.000836	0.00244	0.000990	0.00112
p-value		(0.414)	(0.596)	(0.953)	(0.212)	(0.934)	(0.593)	(0.655)	(0.270)	(0.258)	(0.647)

Exploratory statistical analysis is not adjusted for multiple comparisons.

2. **We also planned on the basis of Year 1 findings, to compare phenotypic footprints of other immune modulators to those of methotrexate.** One observation is that use of immune modulators is not evenly distributed among the Clusters. Table X below illustrates the point that patients on methotrexate are more likely to be in Cluster 4, the most active interferon pathway subset, whereas those on azathioprine or mycophenolate are more likely to be in Cluster 1 which has similar patterns but less intense than Cluster 4. This is consistent with our new data confirming that methotrexate use is associated with higher expression of inflammation modules (which is prominent in Cluster 4). Could this just be a patient selection bias? Table 2 below was published in 2020 (not supported by DoD) and was part of the preliminary data for this grant.

Table 2: Data from paper published by Guthridge in 2020

includes preliminary data from original application but not data generated since DoD project began.

Participant demographics and medication usage by cluster.

Cluster Number	1	2	3	4	5	6	7
Total N*	47	39	58	33	48	32	33
Female, n (% of cluster)	41 (87.2)	35 (89.7)	54 (93.1)	29 (87.9)	46 (95.8)	28 (87.5)	30 (90.9)
Age in years, mean	38.6	43.1	42.5	44.3	42.9	41.3	44.7
Race, n (% of cluster)							
European American	14 (29.8)	24 (61.5)	28 (48.3)	15 (45.5)	32 (66.7)	9 (28.1)	9 (27.3)
African American	4 (8.5)	9 (23.1)	19 (32.8)	6 (18.2)	7 (14.6)	7 (21.9)	17 (51.5)
American Indian	13 (27.7)	3 (7.7)	3 (5.2)	3 (9.1)	5 (10.4)	5 (15.6)	3 (9.1)
Asian	6 (12.8)	0 (0)	5 (8.6)	4 (12.1)	1 (2.1)	3 (9.4)	0 (0)
Hispanic	8 (17)	3 (7.7)	3 (5.2)	5 (15.2)	3 (6.3)	8 (25)	4 (12.1)
Mixed Race	2 (4.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Medication use, n (% of cluster)							
Hydroxychloroquine	30 (63.8)	31 (79.5)	40 (69)	19 (57.6)	37 (77.1)	22 (68.8)	21 (63.6)
Mycophenolate Mofetil	18 (38.3)	5 (12.8)	3 (5.2)	4 (12.1)	4 (8.3)	3 (9.4)	4 (12.1)
Azathioprine	9 (19.1)	7 (17.9)	10 (17.2)	4 (12.1)	4 (8.3)	7 (21.9)	5 (15.2)
Methotrexate	5 (10.6)	9 (23.1)	8 (13.8)	7 (21.2)	12 (25)	1 (3.1)	4 (12.1)
Cyclophosphamide	1 (2.1)	0 (0)	0 (0)	4 (12.1)	0 (0)	0 (0)	0 (0)
Rituximab	1 (2.1)	0 (0)	0 (0)	1 (3)	2 (4.2)	0 (0)	0 (0)
Steroids	27 (57.4)	10 (25.6)	22 (37.9)	27 (81.8)	16 (33.3)	14 (43.8)	15 (45.5)
Prednisone dose (mg), median (interquartile range)	0 (0, 10)	0 (0, 0)	0 (0, 0)	10 (5, 25)	0 (0, 0)	0 (0, 5)	0 (0, 10)

* Number of samples; total of 290 samples from 194 individuals.

Table 3 re-examines this question in samples from the current DoD project

Again, methotrexate treatment is associated with use in greater proportions of patients in Cluster 4 compared to azathioprine, but when disease activity is examined, Cluster 4 may be enriched for those doing well on both azathioprine and methotrexate, but not mycophenolate where proportion in Cluster 4 are also inconsistent. These exploratory data are underpowered and need to be confirmed.

Drug	Total #	Cluster 1	Cluster 4	Other Clusters	% Cluster 4	p value % Cluster 4
aza	26	2	4	20	15.4	comparator
mmf	24	1	7	16	29.1	ns
mtx	43	2	13	28	30.2	ns
Drug/Disease Activity						p value high vs low
aza high	14	1	1	12	7.1	comparator
aza low	12	1	3	8	25	ns
mmf high	9	0	3	6	33.3	comparator
mmf low	15	1	4	10	26.6	ns
mtx high	19	2	4	13	21	comparator
mtx low	24	0	9	15	37.5	ns

We recently applied a similar analysis of SLE immunophenotypic clusters to samples from a clinical trial of obixelimab, an agonist of Fc gamma RIIB. This was presented as an abstract at the EULAR meeting (virtual conference) in 2020. Although that is not a study covered by current DoD funding, it is relevant to the “future plans” we described in our application and provided ideas for analyzing the samples from this current project (**Table 4**). In the complementary project we

TABLE 4

CLUSTERS	1 or 4	3 or 6	2, 5 or 7
Total in Clusters	42	39	100
Low disease	24	18	58
high disease	17	21	41
Activity/Treatment			
low Aza	4	2	6
low MMF	5	4	6
low MTX	9	4	11
low HCLq	6	7	28
low no meds	0	1	7
Total Low Dis	24	18	58
high Aza	2	6	6
high MMF	3	2	4
high MTX	6	4	9
high HCLq	3	5	12
high no meds	3	4	10
Total High Dis	17	21	41

observed that combining two Clusters and examining their overlapping expression patterns identified a possible response phenotype. This may be useful when applying insights from our current data to a future project with a larger sample size.

We previously identified that Clusters 1 and 4 share elevated Interferon signals and activated inflammation pathways, although Cluster 1 has a much lower expression of these than Cluster 4. Clusters 3 and 6 share activated B Cell and Plasma Cell pathways and singularly low expression of inflammation modules. Clusters

2,5, and 7 appear to be relatively inactive on Heat Maps compared to the other Clusters, suggesting that more subtle signals may need to be sought. Higher clinical disease activity is observed in 40% of those in Clusters 1 or 4, 54% of those in Clusters 3 or 6 and 41% of those in 2, 5 or 7, suggesting that Clusters 3 and 6 may be most reflective of currently active disease. We plan to compare individual gene expression differences within clusters next, comparing higher and lower clinical

disease scores. We remain underpowered to dice each of the phenotype subcategories yet when restricting samples to patients on methotrexate, but we hope to expand this analysis in a future study.

3. **Cluster Transitions.** As we have previously observed, patients may change from cluster to cluster with repeated measures over time. Below in **Table 5** the transitions of 80 patients were recorded, comparing a visit with high disease to one with low disease.

Table 5: Changes in Immune Phenotype Clusters in Patients with SLE
With Transitions from High to Low Disease (based on SLEDAI score ≥ 6)

Cluster at High Disease	n	Cluster Assignment at Low Disease		
		Cluster 1,4	Cluster 3,6	Cluster 2,5,7
Cluster 1,4	29	18	5	6
Cluster 3,5	22	3	8	11
Cluster 2,5,7	29	4	12	13

When patients assigned to Cluster 1 or 4 at the time of high disease are evaluated in low disease, most do not change to a new cluster. This suggests that the elevated interferon and inflammation signals may be relatively resistant to dramatic modulation by conventional medications. Conversely, it seems less common for the other Clusters to move to that interferon phenotype when their disease improves.

Interferon signals may be dampened by treatments, so it seems possible that the designation of Clusters 1 and 4 define relatively static phenotypes requiring more subtle markers to gauge immunologic indicators for clinical change.

However, Clusters 3,5 and Clusters 2,5,7 seem more likely to change in a somewhat more fluid manner from visit to visit. This suggests that both represent more transitional phenotypes, Clusters 3,5 probably representing, to some extent, higher disease activity than 2,5, or 7. Although we have some indicators suggesting that Clusters 2, 5, 7 may be less immunologically active, these patients may develop multiple clinical features of lupus.

What remains to be determined is the extent to which gene expression levels may precede or be delayed compared to certain clinical changes or, perhaps, be irrelevant to the pathological process at work. Any of these possibilities could explain the imperfection of these current observations.

4. **An approach to understanding methotrexate impact on gene expression when it is not possible to avoid polypharmacy.**

Because we were limited in the numbers of samples with patients on methotrexate alone, much of our analysis has had to control for other medications, especially hydroxychloroquine.

To obtain a more granular view of differences between methotrexate alone and methotrexate with hydroxychloroquine in low and high disease, **Figure 6** examines different combinations of treatments and different degrees of disease activity against a reference of patients on both methotrexate and hydroxychloroquine with well controlled disease. Patients with high disease and no treatment and those with high disease on methotrexate and hydroxychloroquine or on hydroxychloroquine alone have higher expression of T Cell and Cytotoxic T Cell modules as do patients on hydroxychloroquine alone with well controlled disease. This suggests the possibility that methotrexate controls T Cell activation when it is clinically effective.

5. **Cytokine Analysis:** The work on cytokines so far has contributed (in part) to one abstract that was submitted to the American College of Rheumatology meeting in 2020 (virtual national symposium) and a full manuscript. Both are included in the Appendix (abstract: Munroe and paper: Slight Webb).

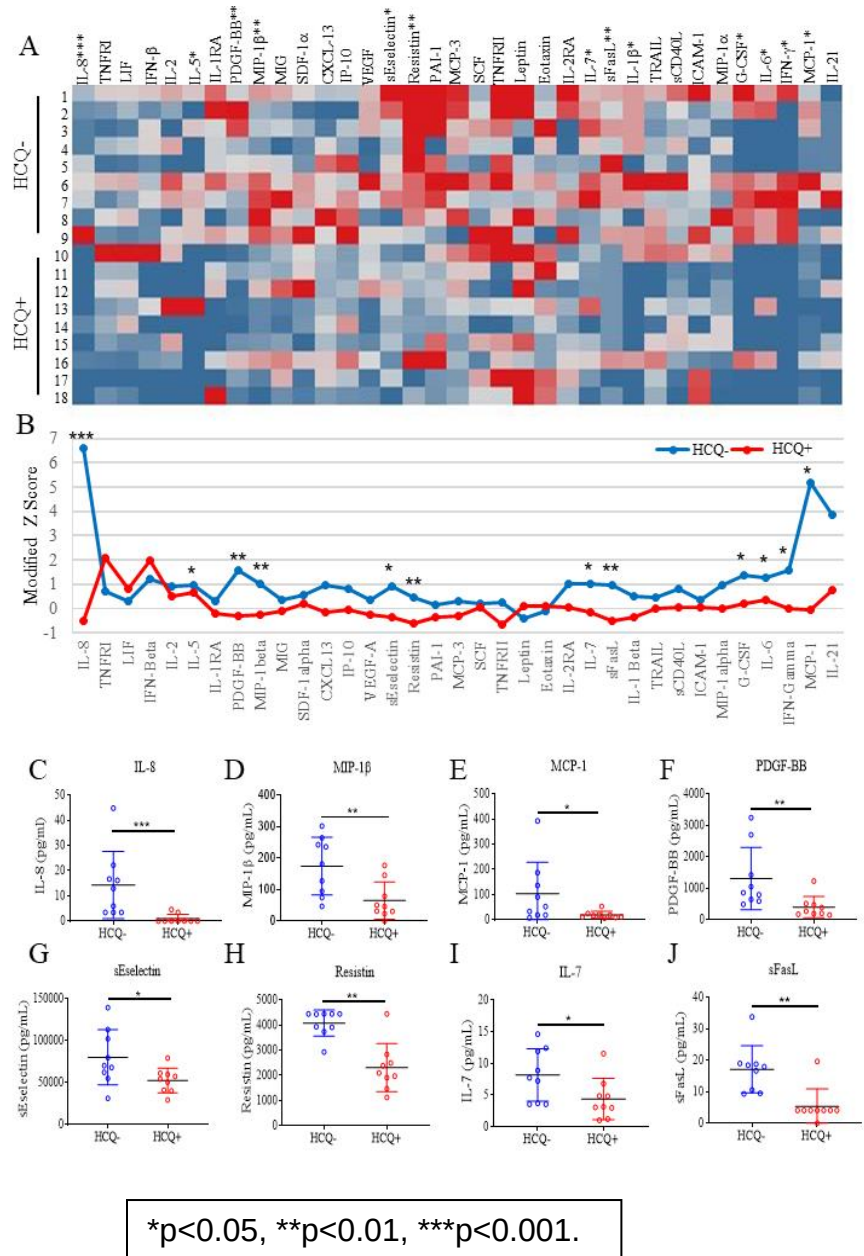
Although we lack samples for definitive studies on patients taking mtx alone, we did complete an experiment identical to one we plan for methotrexate-by comparing cytokine expression in patients who are or are not taking hydroxychloroquine. This is shown in **Figure 7** on next page and also in the appendix as **Figure 5 in a manuscript by our group in the publication submission process. That paper incorporates work funded by several organizations and does cite the DoD for partial support.** Figure 7 shows that monocyte and platelet derived soluble mediators are downregulated in SLE patients who are being treated with hydroxychloroquine.

Fig 6

blue: lower gene expression compared to reference		* <0.05 ** <0.01 *** <0.0001											
red: higher gene expression compared to reference				All (N=121)		Disease/Treatment							
		Module score		Low MTX+HCQ	High/None	High/MTX+HCQ	High/HCQ only	High/MTX only	Low/None	Low/HCQ only	Low/MTX only		
				N=21	N=17	N=13	N=25	N=6	N=11	N=26	N=2		
p-value					(0.000)	(0.400)	(0.048)	(0.946)	(0.013)	(0.018)	(0.363)		
Module score difference				reference group	0.000987	0.00163*	0.00229**	0.00107	0.000614	0.00136*	0.00311		
p-value				(0.198)	(0.198)	(0.050)	(0.001)	(0.325)	(0.481)	(0.050)	(0.075)		
Module score difference				reference group	0.00127*	0.00148*	0.00216***	0.00134	0.000768	0.00174**	0.00167		
p-value				(0.043)	(0.043)	(0.030)	(0.000)	(0.131)	(0.282)	(0.002)	(0.240)		
Module score difference				reference group	0.000932	0.0000367	0.000856	0.00163	0.000189	0.00180*	-0.00124		
p-value				(0.264)	(0.967)	(0.259)	(0.170)	(0.842)	(0.017)	(0.512)			
Module score difference				reference group	0.000533	0.00104	0.00103	0.000377	0.00113	-0.000427	0.00274		
p-value				(0.476)	(0.198)	(0.132)	(0.722)	(0.188)	(0.525)	(0.108)			
Module score difference				reference group	-0.000647	-0.000460	-0.000835	0.0000598	0.00000485	-0.000482	-0.000526		
p-value				(0.215)	(0.414)	(0.079)	(0.935)	(0.993)	(0.304)	(0.656)			

Fig 7: Expression of Soluble Mediators in SLE Patients Taking Hydroxychloroquine

35 pro-inflammatory mediators were measured by multiplex Luminex™ bead-based assays or ELISA as described in our application. Part (A) is a heatmap of plasma mediator levels for each patient. Blue represents protein levels below the mean, white at the mean and red above the mean using a column z-score. In (B) mediator levels are depicted by a line graph using Z-scores. Blue represents patients not taking hydroxychloroquine and red those that are. (C-J) are dot plots of different mediators showing median $\pm$ interquartile ranges. We plan to do the same experiment to compare patients taking methotrexate alone vs methotrexate plus hydroxychloroquine when we amass the required numbers of samples for patients on methotrexate alone.



In additional work for a related project aimed at determining an optimal panel of markers that can distinguish SLE patients with active disease from those with inactive disease, our technology and insights were leveraged and did help to improve our cytokine analysis in this project.

Table 8, below, shows the result when log-transformed, standardized immune mediators, were weighted by the Spearman r correlation coefficient of immune mediator levels vs. either disease activity scores or the number of SLE-associated autoantibody specificities, which correlated with clinical disease activity. After applying a Bonferroni correction $p < 0.0021$, IFN-α, IL-2Rα, TNF-α, TNFR11, IP-10, MIG, and IL-10 remained

significantly correlated with disease activity (Spearman $r=0.221-0.388$), and 22 mediators significantly correlated with accumulated SLE-associated autoantibodies, including the top 12 informative mediators listed in the **Table**. These data confirm previous findings by our group about informative cytokines which seem to associate with disease activity and flare in SLE. This provides a focus for determining the most relevant markers that might change with methotrexate treatment vs different immune-modulators.

Evaluation at the end of Year 2

1. The exploratory data in table 1 indicates that patients taking methotrexate vs controls have suppressed gene expression in plasma cell, T cell and cytotoxic/NK, cell cycle and mitochondrial pathways, seen in all patients and in those with high disease activity while taking methotrexate. This is not observed in those who have more moderated disease while taking methotrexate. This suggests the possibility that methotrexate may cause excessive decrease in these pathways in some patients with a potential feedback that explains the increased inflammatory signals seen in the same group, but when it is working effectively, neither activation or decrease of these pathways will be seen, and these well-modulated patients are not different from controls. Whether this represents a beneficial modulation of what had been previously increased modules in these subjects remains to be determined in prospective studies. Impact of non-compliance with methotrexate is also not known, and it is planned to measure methotrexate levels in future prospective studies.
2. **Methotrexate is associated with increased inflammation module signals in patients who are not responding to it but not in those who are doing well on it.** As mentioned above this is also observed from the data in Table 1. Patients with elevated expression of inflammation modules

Table 8

Most Informative Soluble Mediators Associated With SLE Clinical Disease Activity																		
Rank	Analyte ^a	SLE Patients				Ctl Mean	SEM	Active vs Ctl	Low vs Ctl	Active vs Low	Soluble Mediators vs. # AutoAb Specificities (Cases and Cts)				Soluble Mediators vs. hSLEDAI Score (Cases)			
		Active ^b Mean	SEM	Low ^b Mean	SEM						p-value ^c	p-value ^c	p-value ^c	Spearman r	95% CI	p value ^d	Spearman r	95% CI
1	TNFR1I (p75)	4916	328	4592	720	2071	84	<0.0001	<0.0001	0.0007	0.477	0.371 to 0.571	<0.0001	0.283	0.149 to 0.409	<0.0001		
2	IL-15	9.4	2.2	5.3	0.7	2.1	0.2	<0.0001	<0.0001	0.1887	0.477	0.371 to 0.571	<0.0001	0.167	0.025 to 0.303	0.0181		
	Osteopontin	169844	50532	114957	12278	45203	3485	<0.0001	<0.0001	0.0929	0.346	0.228 to 0.455	<0.0001	0.154	0.011 to 0.290	0.0285		
	SCF	1565	83	1462	88	875	31	<0.0001	<0.0001	0.1515	0.293	0.171 to 0.407	<0.0001	0.096	-0.047 to 0.236	0.1742		
	ILyS	1383	94	1208	80	623	21	<0.0001	<0.0001	0.0425	0.487	0.382 to 0.579	<0.0001	0.182	0.041 to 0.317	0.0098		
	MCP-3/CCL7	19.4	10.9	10.7	4.9	2.9	0.8	<0.0001	<0.0001	0.0178	0.225	0.099 to 0.343	0.0004	0.107	-0.036 to 0.246	0.1303		
	Fas	15080	517	13676	511	9583	380	<0.0001	<0.0001	0.0185	0.345	0.226 to 0.454	<0.0001	0.185	0.043 to 0.319	0.0088		
	MCP-1/CCL2	513	65	388	28	195	11	<0.0001	<0.0001	0.0350	0.430	0.318 to 0.418	<0.0001	0.155	0.012 to 0.291	0.0285		
	IL-10	6.0	0.5	4.5	0.4	1.9	0.1	<0.0001	<0.0001	0.0007	0.494	0.380 to 0.586	<0.0001	0.265	0.127 to 0.393	0.0001		
	TNFR1 (p55)	2124	187	1946	276	961	41	<0.0001	<0.0001	0.0067	0.299	0.177 to 0.412	<0.0001	0.197	0.056 to 0.330	0.0052		
	L-2Rα	2127	110	1775	84	1062	54	<0.0001	<0.0001	0.0013	0.441	0.331 to 0.539	<0.0001	0.238	0.098 to 0.368	0.0007		
	L-8/CXCL8	22.4	5.5	16.8	5.7	31.6	8.6	0.0010	0.0102	0.0344	0.199	0.072 to 0.319	0.0017	0.185	0.043 to 0.319	0.0087		

mediator concentration in pg/ml (Mean and SEM) in 200 SLE Cases vs. 50 race/sex/age-matched Healthy Controls (Ctl)

SLEDAI < 4 (n=100); Active = hSLEDAI ≥ 4 (n=100)

ance determined by Mann-Whitney test; Bonferroni corrected significance **p=0.0042**

Spearman correlation, Bonferroni corrected significance **p=0.0042**

^a mediator concentration in pg/ml (Mean and SEM) in 200 SLE Cases vs. 50 race/sex/age-matched Healthy Controls (Ctl)

^b SLEDAI < 4 (n=100); Active = hSLEDAI $\geq$ 4 (n=100)

^c Spearman correlation, Bonferroni corrected significance $p=0.0042$

^d Spearman correlation, Bonferroni corrected significance $p=0.0042$

are characterized by those in Cluster 4 (and to a lesser extent Cluster 1) who are also the subsets who tend to have the most elevated Type I interferon activity. The following interpretations are possible: 1. Methotrexate is not always an optimal treatment for those with elevated inflammation or propensity to respond by an inflammation exacerbation 2. Methotrexate does control inflammation in subsets of potential clinical responders but not in other subsets. These data underscore, however, the importance of a future prospective analysis of methotrexate impact and confirmation of methotrexate blood levels in the population under study as well as differences in expression of other genes in patients with inflammatory exacerbation.

3. **All patients, with or without methotrexate treatment, demonstrate dampened cell cycle and mitochondrial pathways when patients doing well are compared to those with higher disease.** These findings are shown in the fourth column from the left in Table I. Since this set of immune modules seem to be impacted by disease activity regardless of treatment, the methotrexate effect seen for these modules could be confounded with disease activity effects. This does not seem to be the case for the plasma cell, T cell, cytotoxic T cell, or inflammation pathways so they may remain the focus of more interest in a prospective “before and after” study of methotrexate.
4. **Patients on methotrexate may be clinically well while exhibiting strong inflammation signals and elevated interferon pathway gene expression.** Our data (consistent) with previous reports suggest that this may be a relatively stable phenotype and may not provide a sensitive reflection of clinical disease activity, despite the fact that the patients who exhibit this phenotype seem to be relatively ill overall. Whether gene expression profiling is being caught out of synchronization with clinical manifestations, whether expression of certain genes is more diagnostic than prognostic, or whether there are subtle signals, more sensitive to change within these phenotypes remained to be explored.

We were also concerned about lack of methotrexate impact on interferon markers in this study which did not conform to past findings. See below the Section on New Progress Since Previous Reports for new analyses that we performed as part of our no cost extension which may help explain this.

5. **T Cell and Cytotoxic Pathways May be Dampened by Methotrexate plus hydroxychloroquine in Subsets of Patients with well-controlled disease** as compared to hydroxychloroquine alone, or hydroxychloroquine plus methotrexate when disease is not well controlled. This provides indirect evidence that genes in these pathways might be explored as directly or indirectly relevant to methotrexate activity.

New Progress Since Previous Reports

We wanted a no-cost extension to explore the apparent discrepancy between our preliminary data and the earlier findings from the current project. Our preliminary data indicated that methotrexate use was associated with a decrease in interferon signals (TLDA performed to quantify expression of a panel of IRGs). However our initial gene expression findings in this project utilizing different methods and different definitions of IFN pathway activation suggested that, although methotrexate had more impact in patients with elevated I interferon pathway expression, no decrease in the expression of Type I interferon modules was observed and potential increase occurred in isolated circumstances. To further evaluate this, several hypotheses were tested:

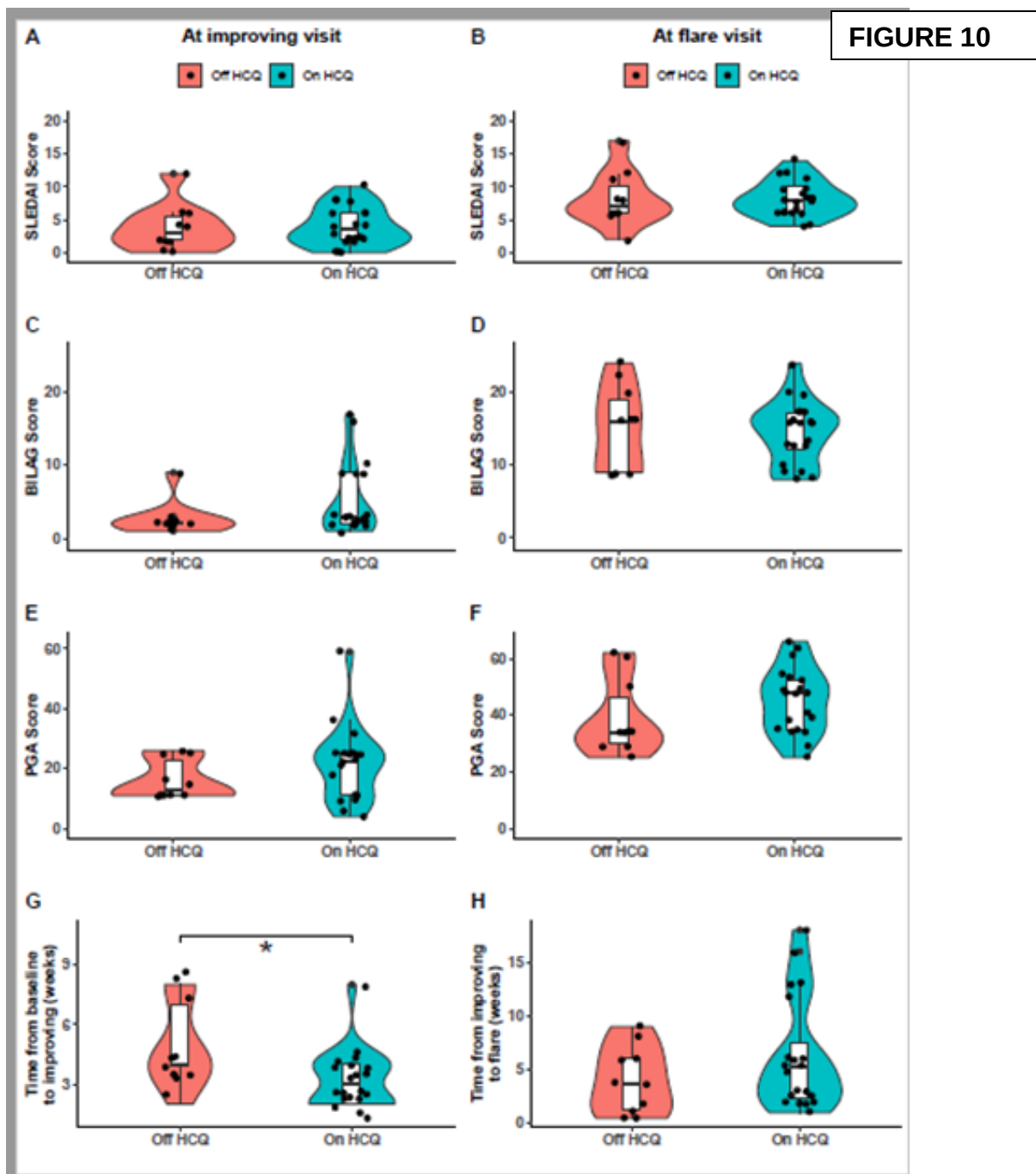
Hypotheses 1 and 2: Differences between findings in this study vs from our preliminary data might have been affected by differences in the handling of background treatments or differences in the defining interferon signals used to stratify the patients. To test these possibilities an analysis of patients defined by expression levels of several interferon gene/pathway measurements was conducted while adjusting for both steroids and hydroxychloroquine. In the Table below, difference of gene expression scores in patients who were using vs not using methotrexate were stratified by defined interferon signals in a multivariate linear regression controlling for hydroxychloroquine and steroid dosing. Little impact was detected.

TABLE 9

Module Level x 1,000	M1.2 Type 1IFN specific module				M3.2 Type I/2 IFN module				M5.12 Type 1/2 IFN module				M3.4 + M5.12 combined modules				>= 3 high IFN genes elevated			
	High (N=34)		Low (N=36)		High (N=34)		Low (N=36)		High (N=34)		Low (N=36)		High (N=34)		Low (N=36)		High (N=39)		Low (N=31)	
	Diff	p	Diff	p	Diff	p	Diff	p	Diff	p	Diff	p	Diff	p	Diff	p	Diff	p	Diff	p
M4.10_B cell	2.7	0.183	0.3	0.845	2.5	0.188	0.6	0.696	1.6	0.372	1.0	0.484	2.9	0.130	0.1	0.966	2.2	0.169	0.6	0.697
M4.11_Plasma cells	1.9	0.393	-1.6	0.133	0.9	0.682	-1.0	0.349	-0.8	0.685	-0.5	0.693	0.9	0.678	-1.2	0.295	-	0.970	-	0.215
M4.1_T cells	0.5	0.662	-0.4	0.575	0.6	0.531	-0.5	0.492	0.3	0.686	-0.3	0.595	0.7	0.460	-0.7	0.371	0.5	0.544	1.4	0.458
M4.15_T cells	-0.4	0.615	-0.2	0.734	-	0.785	-0.3	0.548	-0.3	0.665	-0.1	0.715	-0.1	0.879	-0.4	0.405	-	0.823	-	0.489
M3.6_Cytotoxic/NK	-1.3	0.367	0.2	0.819	0.2	0.414	0.0	0.999	-1.3	0.315	0.4	0.641	-1.1	0.418	0.1	0.953	0.2	0.515	0.4	0.763
M8.46Cytotoxic/NK	0.0	0.988	-0.4	0.636	1.1	0.671	-0.9	0.331	0.9	0.420	-1.5	0.189	0.5	0.706	-0.9	0.337	0.8	0.988	0.3	0.743
M4.14_Monocytes	1.9	0.016	0.2	0.696	1.2	0.123	0.6	0.352	1.2	0.064	0.4	0.521	1.1	0.118	0.7	0.293	0.0	0.988	0.4	0.848
M5.15_Neutrophils	-0.3	0.913	0.1	0.939	1.4	0.551	-1.5	0.339	1.6	0.446	-2.2	0.180	1.4	0.545	-1.4	0.372	0.1	0.949	0.1	0.692

To confirm the importance of adjustments for the ubiquitous (but not universal) use of hydroxychloroquine in patients taking methotrexate, we identified some of the clinical variables most likely to be impacted by hydroxychloroquine.

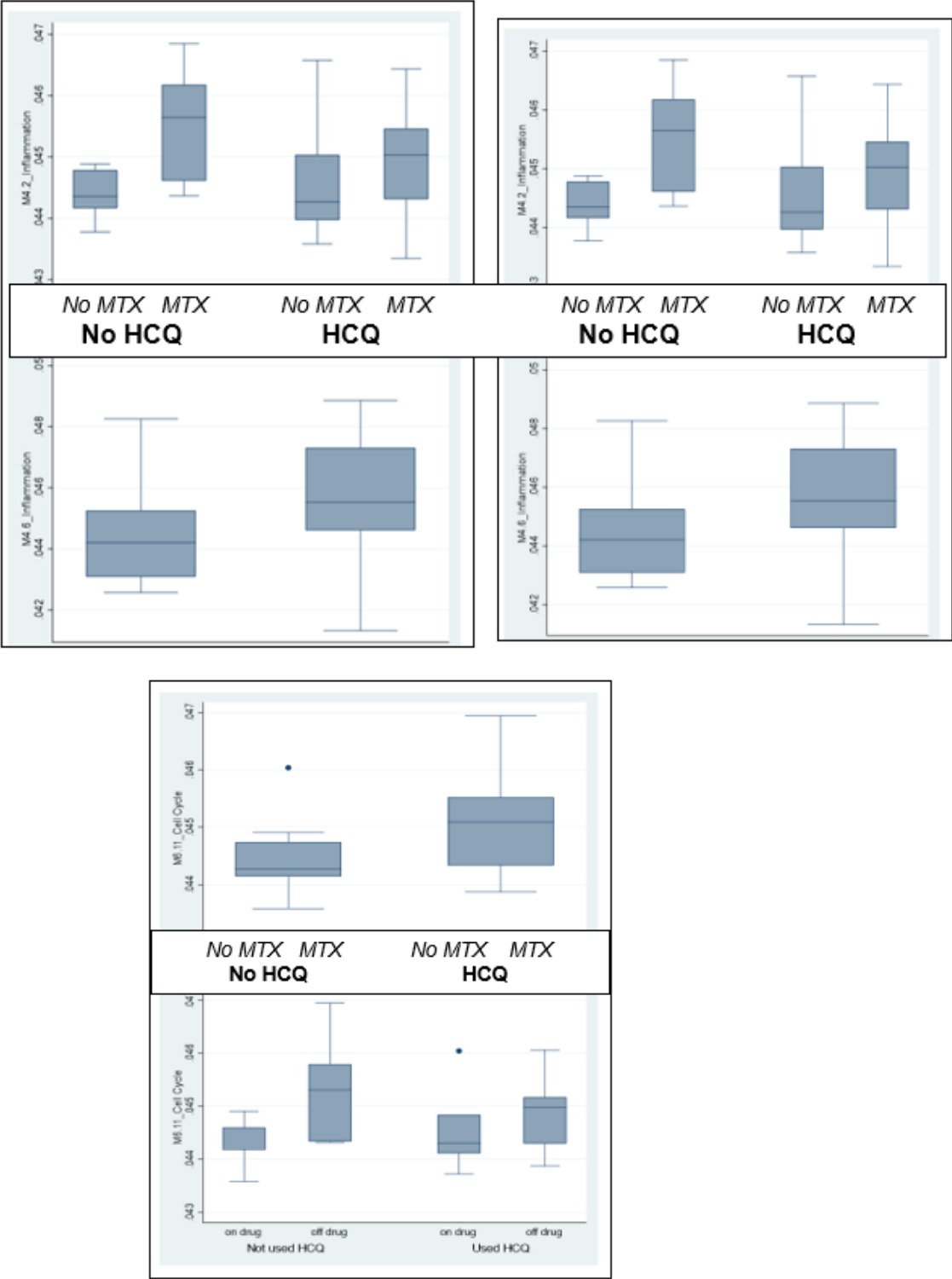
In Figure 10, below, stored samples from our cohort included 14 patients who had participated in projects where background medications were withdrawn, steroid shots were given and time to flare calculated. Thus each donated blood when either taking hydroxychloroquine or not after the steroid treatment (improving visit), then again within the next six months when disease began to reactivate (flare visit). No more than 10 mg prednisone was taken in the interim period and no other SLE treatments or active study treatment were taken by any of these patients at either visit or during the interim period.



it is evident that even when no hydroxychloroquine-associated difference is seen in disease activity (as scored by the SLEDAI, BILAG or the physician's global assessment), those on hydroxychloroquine improve in less time after receiving steroid and exhibited a trend towards a longer time to flare. This impact on clinical endpoints may affect any and all biomarker work that is linked to a clinical results, and seemed likely to be biologically-driven. In future studies, even a limitation of background medications to hydroxychloroquine will not ensure a lack of background drug interference and will need to be accounted for.

Figure 11

Confirmation of Dampening Effect of Hydroxychloroquine on Detection of Methotrexate Effects on Several Key SLE-related Immune Pathway Modules



In summary, since it was clear that the Interferon Modules were not discriminating Methotrexate Effects, additional IRG-defined definitions of Interferon Activity were also examined cross sectionally and little impact was observed. We confirm the importance of accounting for the presence of even low grade background immune modulators such as hydroxychloroquine when attempting to distinguish impact of methotrexate on immune signals.

Hypothesis 3: Discrepancies in impact of interferon inducible gene expression could be due to different genes being measured cross sectionally in studies where different patients were compared to each other. To test this hypothesis we examined various expression patterns in the same SLE patients when taking methotrexate and within six months of stopping methotrexate with limited background immune-modulating medications (defined as no more than 10 mg prednisone and optional hydroxychloroquine). Data were stratified by high or low expression of a set of IRGs we previously studied, or elevated expression of single IRGs. Results with Wilcoxon rank test are in the table below. SLE was moderately to severely active at both visits for all patients to minimize the previously observed impact of disease activity changes on results. Red color indicates increased median gene expression of patients when on methotrexate vs not. Blue indicates lower median gene expression on methotrexate.

TABLE 12

Variables	Total			GBP5			HERC5/ISG15/ISG20/LY6E/OAS2/OAS3/RSAD2						IRF7			MX1		
	on	off	p	on	High off	p	on	High off	p	on	Low off	p	on	High off	p	on	High off	p
Cytokines			N=4			N=3			N=2			N=2			N=4			N=3
IL6	2.69	3.48	0.068	1.46	2.38	0.068	4.88	2.38	0.180	2.06	4.58	0.180	2.69	2.38	0.068	1.46	1.62	0.109
Blys	1176	1546	0.465	1548	1480	1.000	1718	1613	0.655	716	880	0.180	1176	1613	0.465	1548	1546	1.000
Module score			N=6			N=5			N=3			N=3			N=5			N=4
M4.10_B cell	4.35	4.51		4.30	4.47		4.30	4.56		4.76	4.47		4.30	4.56	0.043	4.26	4.51	0.068
M4.11_Plasma Cell	-4.47	-4.41		-4.46	-4.45		-4.46	-4.28		-4.48	-4.45		-4.48	-4.37	0.043	-4.49	-4.36	0.068
M4.1_T cell	4.45	4.41		4.46	4.49		4.46	4.34		4.45	4.49		4.46	4.34	0.043	4.52	4.44	0.068
M4.15_T cell	4.46	4.39		4.47	4.44		4.47	4.35		4.44	4.46		4.47	4.35	0.043	4.49	4.39	0.068
M3.6_Cytotoxicity/NK	4.48	4.32	0.028	4.44	4.25	0.043	4.28	4.22	0.109	4.52	4.40	0.109	4.52	4.25	0.043	4.44	4.23	0.068
M4.14_Monocytes	4.46	4.57		4.40	4.56		4.40	4.59		4.55	4.50		4.40	4.59	0.043	4.39	4.57	0.068
M5.15_Neutrophil	4.44	4.53		4.43	4.52		4.34	4.52		4.55	4.54		4.43	4.54	0.043	4.38	4.53	0.068
LDG1.1	4.25	4.78	0.046	4.13	4.92	0.080	4.13	4.92	0.109	4.36	4.64	0.285	4.13	4.64	0.043	4.07	4.55	0.068
LDG2.1	-4.31	-4.75	0.046	-4.19	-4.74	0.080	-4.19	-4.74	0.109	-4.43	-4.76	0.285	-4.19	-4.74	0.043	-4.16	-4.55	0.068
M3.2_Inflammation	4.39	4.53		4.32	4.52		4.27	4.55		4.67	4.52		4.32	4.55	0.043	4.30	4.53	0.068
M4.2_Inflammation	4.42	4.50	0.046	4.42	4.50	0.080	4.40	4.51	0.109	4.50	4.48	0.285	4.42	4.51	0.043	4.41	4.50	0.068
M4.6_Inflammation	4.43	4.57		4.37	4.56		4.60	4.57		4.34	4.56		4.37	4.57	0.043	4.36	4.57	0.068
M5.1_Inflammation	4.43	4.52	0.046	4.40	4.49	0.080	4.40	4.54	0.109	4.51	4.49	0.285	4.40	4.54	0.043	4.40	4.52	0.068
M7.1_Inflammation	4.47	4.57		4.43	4.54		4.43	4.60		4.51	4.54		4.43	4.60	0.043	4.43	4.57	0.068
M1.2_Interferon	4.69	4.50	0.917	5.25	4.75	0.893	5.29	5.06	0.593	4.09	4.19	0.285	5.25	4.75	0.686	5.27	4.91	0.715
M3.4_Interferon	4.62	4.65	0.249	4.83	4.80	0.345	4.85	4.80	1.000	4.32	4.36	0.109	4.83	4.80	0.500	4.84	4.80	0.715
M5.12_Interferon	4.43	4.41	0.463	4.43	4.41	0.500	4.43	4.40	0.593	4.43	4.41	0.593	4.43	4.41	0.225	4.46	4.40	0.273
M3.5_Cell Cycle	4.50	4.44		4.51	4.44		4.51	4.44		4.43	4.47		4.51	4.44	0.043	4.51	4.44	0.068
M6.11_Cell Cycle	4.42	4.50	0.046	4.42	4.50	0.080	4.41	4.51	0.109	4.46	4.48	0.285	4.42	4.51	0.043	4.41	4.50	0.068
M9.42_Cell Cycle	4.45	4.47		4.46	4.46		4.46	4.48		4.44	4.46		4.44	4.48	0.043	4.44	4.47	0.068
M5.10_Mitochondrial	4.41	4.50		4.39	4.48		4.36	4.52		4.48	4.48		4.39	4.52	0.043	4.38	4.49	0.068
M6.2_Mitochondrial	4.50	4.44		4.54	4.46		4.57	4.42		4.46	4.49		4.54	4.42	0.043	4.55	4.44	0.068
M6.12_Mitochondrial	4.46	4.51	0.046	4.47	4.50	0.080	4.47	4.51	0.109	4.46	4.50	0.285	4.47	4.51	0.043	4.47	4.51	0.068
M4.3_Protein Synthesis	4.76	4.33		4.67	4.35		4.85	4.35		4.40	4.31		4.85	4.31	0.043	4.76	4.33	0.068
M5.9_Protein Synthesis	4.36	4.80	0.046	4.30	5.06	0.080	4.30	5.06	0.109	4.43	4.55	0.285	4.47	4.51	0.043	4.24	4.70	0.068

Summary: Methotrexate induced increases in B Cell, Plasma Cell, monocyte and neutrophil pathways as well as inflammation, cell cycle, mitochondrial and protein synthesis modules. These findings were not apparent examining patients with elevated expression of some single and complexed interferon genes but were found in those who had elevated Interferon regulatory factor 7 (IRF7). This transcription factor is a central regulator of type I interferons (IFNs) in response to pathogens, and is activated by the Epstein Barr Virus protein known as latent membrane protein-1 (LMP1). Both EBV activation and elevated type I interferon inducible proteins are strongly implicated in the pathogenesis of SLE. It is known that both ubiquitination and phosphorylation of IRF7 are involved in its activation (*S Ning Genes Immun. 2011 Sep; 12: 399–414*).

During the course of this no cost extension we have become interested in several other interferon pathway regulators that have links to lupus pathogenesis and host defense and which seem responsive to methotrexate. Data on methotrexate effects are shown in Figures 13-15.

These additional markers of current interest include:

IFI27 codes for interferon alpha inducible protein 27 which has multiple functions including support of binding activities for RNA polymerase II-specific DNA-binding transcription factor, identical protein and lamin, involving it as a key regulator of cellular metabolism and apoptosis. It also regulates RNA transcription nuclear protein export. It is upregulated by TLR7 in plasmacytoid dendritic cells, and may discriminate viral from bacterial infections. It is one of the major ISGs upregulated in SLE, associated with both disease occurrence and progression.

IFI44 (interferon induced protein 4) *aggregates to form microtubules and inactivates signaling such as p44/42 MAPK pathway*. Hypomethylation and overexpression of IFI44 in SLE may discriminate LN patients from healthy controls, and distinguish between active vs inactive LN.

IFI44L (interferon induced protein 4-like) codes for a cytoplasmic protein that may enable GTP binding activity, and is hypomethylated in SLE patients with renal involvement. Methylation levels have been observed to increase in remission.

MX1 is an Interferon-induced GTP-binding protein. Its expression is elevated in SLE and may be a marker for active lupus nephritis

OAS3 codes for the interferon-inducible enzyme 2'-5'-Oligoadenylate Synthetase 3 which catalyzes adenosine to activate RNASE 1. Upregulation leads to activation of T cells, CD19+ B cells, and CD33+ myeloid cells in patients with SLE

RSAD2 which is also elevated in SLE belongs to the S-adenosyl-L-methionine (SAM) superfamily of enzymes and is a key regulator of innate immune signaling.

TRIM25 is a member of the tripartite motif-containing (TRIM) protein superfamily, **upregulated by estrogens**, with ubiquitin ligase activity and associations to innate immunity and lupus.

TSC22D3 is a transcriptional regulator with a leucine zipper structure. ***Expression of this gene is stimulated by glucocorticoids*** which may cause confusion in biomarker studies.

Below, expression of these genes were examined for methotrexate impact, stratified by immunophenotypic cluster (**Figure 13**), Interferon signature status (**Figure 14**) and prednisone dose (**Figure 15**).

FIGURE 13

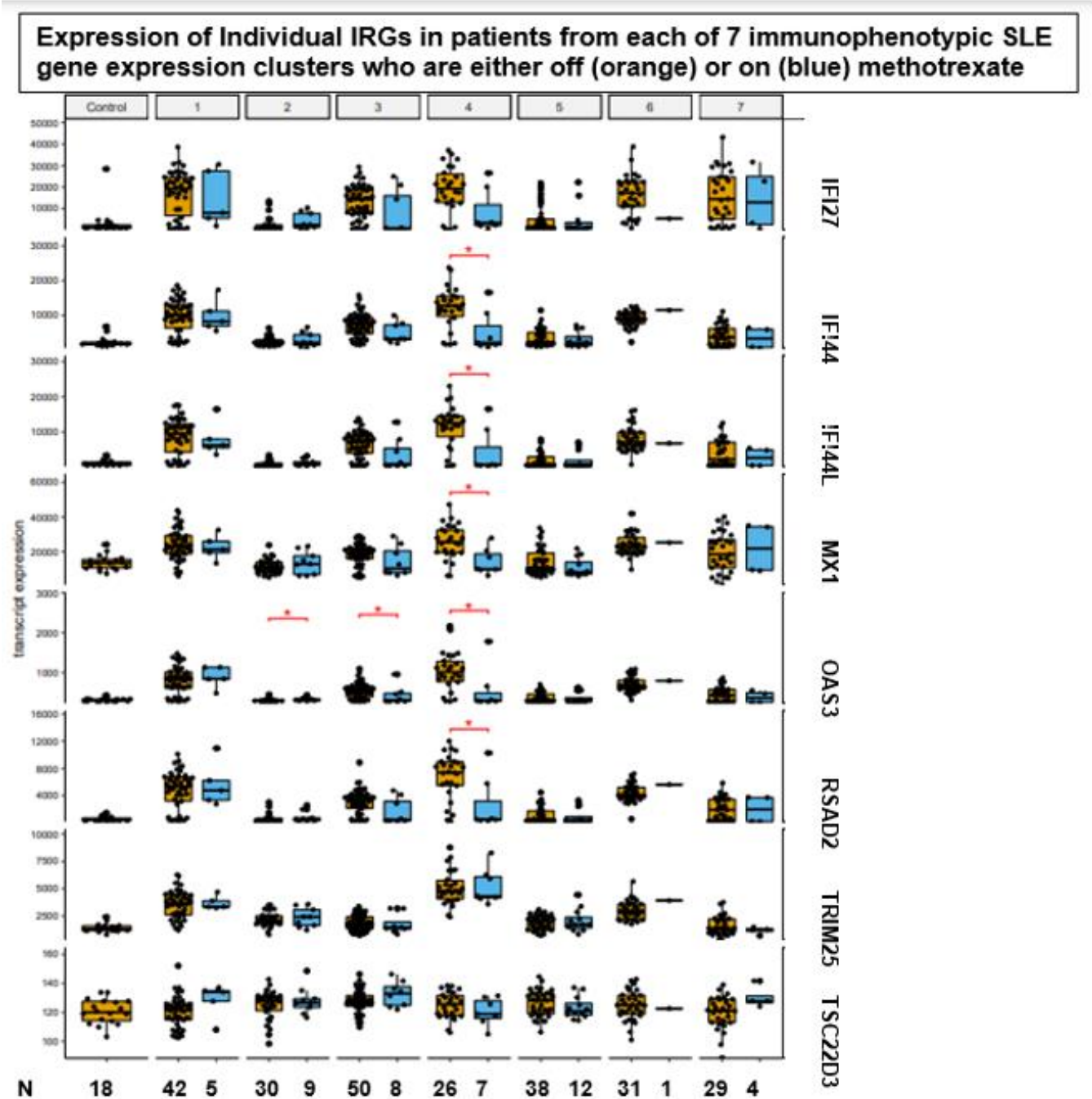


FIGURE 14

Impact of Methotrexate on Expression of Individual IRGs in SLE Patients with High vs low Interferon Inducible Gene Expression

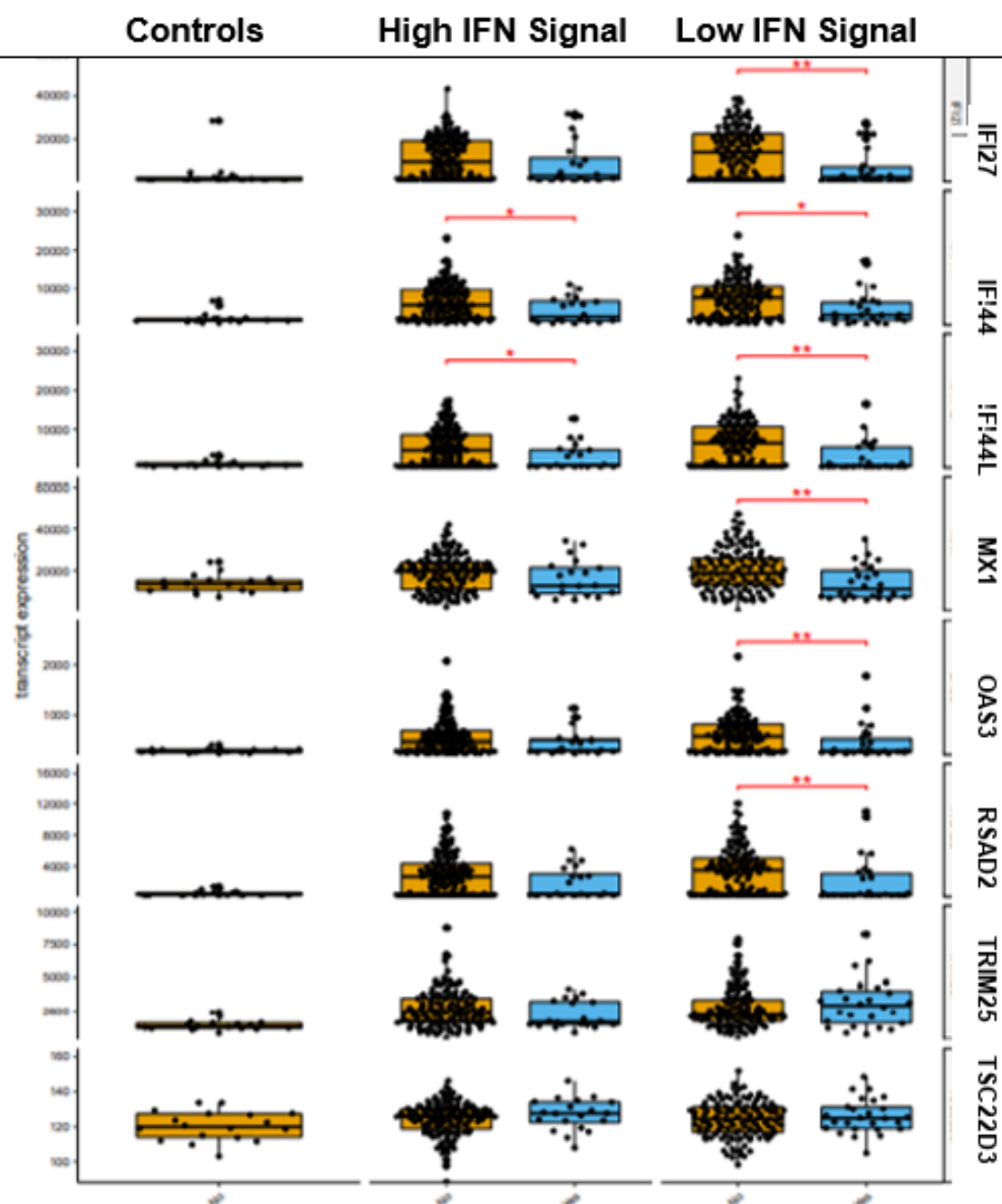
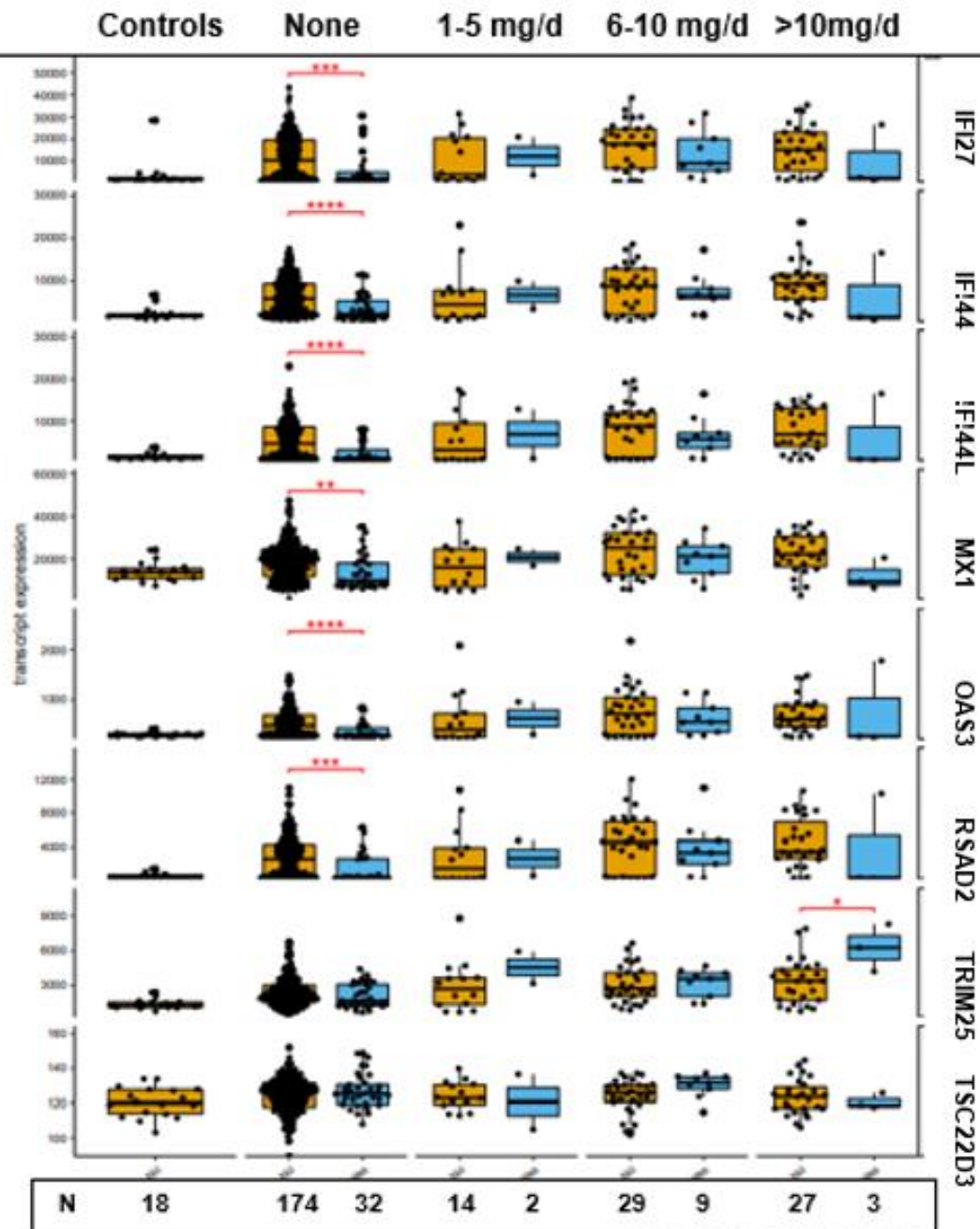


FIGURE 15

Expression of Individual IRGs in SLE patients on different doses of prednisone who are either off (orange) or on (blue) methotrexate



Evaluation:

Although the study of methotrexate effects on the interferon pathway led to confusing data when performing cross sectional studies on disparate patients taking disparate medications using larger pathway-derived gene panels, we have identified a more subtle panel of interferon pathway regulatory genes that may be more relevant. This exploratory panel is by no means assumed to be complete, optimized, or validated at this point, but does provide a proof of concept for the next stages of this work.

The expression of most of these IFN pathway genes was suppressed by methotrexate in phenotype gene expression profile cluster #4 (**Figure 13**) the immunologic patterns for which we have previously characterized. These patients have a consistent pattern of the highest interferon and inflammation signals in our adult lupus population, a finding that has been confirmed in several sets of non-overlapping cohorts, including patients participating in a national clinical trial. The elevated interferon signature may not fully explain this result since non identical results (although overlapping) were obtained when patients were stratified only by interferon signals, including patients from differing expression pattern clusters which may have contributed to the less robust findings in the group specifically designated as high interferon (**Figure 14**), the same finding or designated as having an interferon signature regardless of cluster.

Steroids (as we had found earlier with hydroxychloroquine) seem to mask these effects (**Figure 15**). This observation could potentially help explain discrepancies observed between our preliminary data (and original hypothesis) vs earlier analyses from this project that we presented last year. In fact our original hypotheses appears to be confirmed. Methotrexate is associated with downregulated expression of key interferon pathway regulators and these, in turn, have been previously associated with processes of innate immunity and the diagnosis and activity of lupus.

This suggests the potential value of a panel of interferon pathway regulators as biomarkers to help determine a choice of methotrexate for individual patients and to guide optimal dosing.

Our data also suggest that both steroids and hydroxychloroquine have redundant (or at least partially redundant) effects to methotrexate, resulting in less treatment effect observed with concomitant administration of these drugs. There may be some patients where testing would indicate that methotrexate is not the best choice for add-on therapy when there is ongoing clinical disease while on hydroxychloroquine. Conversely to the extent that methotrexate may have some redundant effects to corticosteroids it might be a good choice as steroid-sparing therapy for those patients.

TRIM25 and TSC22D3 are not downregulated by methotrexate and may even be enhanced in the setting of medium or larger doses of prednisone. Whether this is related to their induction by estrogens and corticosteroids respectively is not known but probably worth examining at a future date.

Assessment and Plan:

The main findings of this project are:

1. Methotrexate use is associated with suppressed gene expression in plasma cell, T cell and cytotoxic/NK, cell cycle and mitochondrial pathways, Methotrexate effects appear to be more apparent in patients with higher disease activity and those that belong to an immunologic cluster in which elevated expression of interferon and inflammatory pathways are characteristic.
2. Methotrexate seems to be associated with greater expression in inflammatory pathways than in those not taking immune suppressants. Whether this represents an adverse feedback loop of excessive downmodulation in some patients or simply designates a discrete population with differing underlying immune patterns and response to methotrexate remains to be determined.
3. Some of the same findings are reproduced in subsets of patients who have high expression of certain (but not all) interferon-inducible gene panels. A panel of IRGs was identified in this project where expression was inhibited by methotrexate. These IRGs include IRF7, IFI27, IFI44, IFI44L, MX1, OAS3, and RSAD2, all of which are regulators of innate immune functions and all associated with SLE. Some subsets with both low and high baseline interferon expression had dampened expression of these genes with methotrexate.
4. Methotrexate had little effect on gene expression of TRIM25 or TSC22D3 where expression may be enhanced by steroids. Our data also demonstrates the likelihood that corticosteroids and hydroxychloroquine, which are commonly prescribed with methotrexate may have redundant effects to methotrexate on gene expression. This has led to an apparent masking of the methotrexate effects that are observed in their absence, which might explain discrepancies observed in earlier analysis of data for this project and may help to decide which treatments to combine (or when) in the future.

Therefore, our data supports the feasibility of developing more rational testing to guide improved treatment selection and dosing strategies for individual members of the clinically and immunologically diverse population with systemic lupus. We have identified some potentially useful biomarkers as well as some important confounding variables that need to be accounted for in developing this process.

We will be pursuing future projects to prospectively refine and/or validate these findings. Continuation of this work is warranted by a significant unmet need in the treatment of SLE. Today, treatment remains largely empiric and unsatisfactory results may last months to years with a standard of care that has a poor evidence base and a primitive rationale compared to what could be possible with the application of current scientific tools.

What opportunities for training and professional development has the project provided?

None. This project was not intended to provide formal training, although we are all learning from it.

How were the results disseminated to communities of interest?

See Appendix. The paper recently published (Slight Webb) cites the DoD funding as partial support, since Figure 5 is directly relevant to the current project. The abstract by Munroe made a direct contribution to the current project as we expanded our analysis of methotrexate effects on cytokine expression. Other abstracts and papers from 2020 that are in the Appendix are not directly related to the DoD funding but have either published preliminary data that had been used in the application (paper by Guthridge) or (in the case of the other abstracts) provided concepts and/or technology that will inform our work over the next year on this project. The major publication from this project awaits completion of a new study which will enable us to increase the sample size of paired samples from the same individuals before and after prospectively withdrawing methotrexate.

What do you plan to do to accomplish the goals?

Our goal is to seek funding and initiate a prospective study of methotrexate initiation and withdrawal in patients with SLE, applying or adjusting for the variables identified in this project that seem to impact on the biometric changes associated with methotrexate. The aim will be to establish immunologic guidance testing to optimize dosing towards clinical efficacy in individuals.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have been making progress on the consistency and reproducibility of our gene expression array and computational approaches. Under outside funding, we have already reproduced the 7 characteristic lupus patient clusters (based on gene-co-expression immune modules) in an SLE population that has no overlap with the original 200 patients we had reported in our preliminary data. We did reproduce the same clusters again with the samples we ran for the current project (which does overlap somewhat with our original test population but extends the ranges of dates, time, disease activity and medication variables).

This have now addressed several questions that we discussed in last year's report that may improve our understanding of the significance of these patient clusters. Some but not all patients seem to remain in one cluster over time, and this may be more likely in those who have high interferon and high inflammation signals. We have already (not in

this project) begun to distinguish subsets of patients at baseline who are most likely to respond to treatment with a given targeted agent. How the effects of different background medications affect this kind of analysis is not yet known. It is hoped that the impact of the current project will be to set the stage for addressing that problem, since most trials in SLE include polypharmacy. The data from this project will be important to know in determining more optimal use of methotrexate, while contributing to a larger body of evidence illuminating some fundamental questions about the pathologic process in SLE.

What was the impact on other disciplines?

There are many similarities between lupus flare and innate immune responses especially in patients (defined by us as Clusters 1 and 4) with elevated interferon and inflammation signals. We believe that this may have significant implications for the severe autoimmune-like thrombotic diathesis of COVID-19 (which is often accompanied by antiphospholipid antibodies and bearing a strong relationship to catastrophic antiphospholipid syndrome and other complement-mediated TMA syndromes). We published a theoretical commentary on this earlier in the year **Merrill JT, Erkan D, Winakur J and James, JA *Nature Reviews Rheumatology* 2020 <https://doi.org/10.1038/s41584-020-0474-5> PMID: 32733003**. Our group has an established track record over decades in connecting the pathology of SLE to viral infection, mechanisms of thrombosis, and vaccination responses. We believe that insights from the current DoD-funded project will help us advance the technology needed to better understand responses of SLE patients to vaccination and viral infection. In line with this we are intrigued by the observation that high expression the EBV-inducible interferon regulatory factor 7 may be a marker for patients who are more responsive to biomarker changes associated with methotrexate. Some of our staff, who have a particular interest in EBV, Type I interferons and SLE are planning to pursue this connection at the level of signal-induced transcription, post-translational and epigenetic modifications. In turn, this work may apply well beyond the discipline of rheumatology to the study of adverse sequelae of new and emerging pathogens.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

There have been no changes in the approaches other than the analysis of patients taking concomitant hydroxychloroquine and prednisone as described above, due to a paucity of samples of patients on pure methotrexate..

Actual or anticipated problems or delays and actions or plans to resolve them

Discussed in previous reports. The project is now completed.

Changes that had a significant impact on expenditures

No changes were expected to have a significant impact on expenditures over the funding period of this project. We completed our work during the no-cost extension period with no additional funding.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

This project utilized stored samples from our post-trial and cohort repositories that had been IRB-approved and consented in advance for this use. There was no involvement of vertebrate animals, biohazards and/or select agents in this project.

PRODUCTS: none

Publications, conference papers, and presentations

Please see the Appendix. We have published one paper that reports the preliminary data used in the application for this project (Guthridge et al). Another paper is appended that was published at the end of 2020 (Slight-Webb et al). That paper utilized data directly related to this project, so DoD is credited with partial support of that work. Other abstracts not directly arising from this project are also in the Appendix as we cite them for providing technology or ideas that was applied to this study and will be applied to our further work arising from this project. The main findings reported here did not necessarily disqualify our hypotheses, but are subject to several variables which we have identified. Prior to publishing what became primarily an exploratory, hypothesis-generating analysis, we would like to validate these observations in a better controlled, prospective study of methotrexate initiation and withdrawal for which we are currently seeking funding.

Website(s) or other Internet site(s)

None

Technologies or techniques

None

Inventions, patent applications, and/or licenses

None

Other Products

■ data or databases; We incorporated codes specific to this project in our database and foresee the possibility of a searchable dataset that could be made available to others.

■ biospecimen collections; Not attributable to this project. We are utilizing biospecimens that were created through other funding for other projects, then entered into our repositories.

■ *audio or video products*; None

■ *software*; None

■ *models*; None

■ *educational aids or curricula*; None

■ *instruments or equipment*; None

■ *research material (e.g., Germplasm; cell lines, DNA probes, animal models)*; None

■ clinical interventions; We completed organizing the clinical information and integrated some of it in our analysis at the end of Year 1 and in Year 2. We foresee the possibility of a potential clinical tool to determine whether immune phenotyping can improve selection and dosing of treatments. To the extent that this current project affects our ability to characterize the pharmacodynamics of methotrexate in our future repository (from an ongoing clinical trial with no overlap to this project) than this project could leverage the development of such a tool.

■ *new business creation*; None

■ *Other*; None

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Joan T Merrill, M.D.

Project Role: PI

Research Identifier:

Nearest person month worked: 2.4 (no salary)

Contribution to Project: Dr. Merrill performed the selection and quality assurance of all of the clinical data and continues to supervise the integration of the biomarker, medication and clinical assessments. She collaborates closely with the laboratory team in decisions that affect the objectives of the project and will lead the data analysis in an ongoing manner. For this report she performed the preliminary statistical analysis.

Funding Support: Dr. Merrill's contribution to this project has been leveraged by Departmental Funds.

Name: Melissa Munroe, Ph.D.

Project Role: Sub investigator

Research Identifier:

Nearest person month worked: 3.45

Contribution to Project: Dr. Munroe has significant experience with gene expression profiling and immunophenotyping. She has been a key driver of formulating the initial algorithms for defining transition events in SLE, including mechanisms of disease flare. She has been working with Dr. Merrill to design and oversee lab and analysis algorithms and provided daily scientific direction of the technical personnel during the arduous quality assurance, set up and running of this extensive phenotyping project. She has extensive experience with a variety of platforms for cytokine detection, including serum/plasma detection with an Affymetrix platform and Mesoscale discovery and will be completing that part of the project during the no cost extension period. She will continue to participate in meetings for this study. and will be integral to the process while we analyze data, prepare reports, and write/edit manuscripts.

Funding Support: Not applicable

Name: Nic Dominguez

Project Role: Research Technician

Research Identifier:

Nearest person month worked: 3.1

Contribution to Project: Mr. Dominguez is an experienced member of the OMRF Phenotyping Core and CAP-Certified OMRF Biorepository. He has helped to locate and pull samples, update inventory, and handle all samples for the study, execute the RNA isolation, library generation and BiomarkHD experiments. He has assisted and will continue to help Dr. Munroe perform soluble mediator analyses, quality control and data analyses.

Funding Support: not applicable

Has there been a change in the active other support of the PD/PI(s) or senior/key? personnel since the Year 1 reporting period?

Research Grant – PI Merrill Joan

12/18/2020 – 06/30/2022

Funding Agency: Astra Zeneca

Total cost:

Project Title: Nature of Anifrolumab Impact on Vaccine-Emergent Immunity in patients with moderately to severely active Systemic Lupus Erythematosus: A Multi-Centre Open Label Parallel Group Trial: The NAÏVE study

Short Explanation: This project will compare immunization responses (including changes in cytokine and gene expression and production of neutralizing antibodies following quadrivalent influenza vaccination in ten lupus patients taking anifrolumab vs ten taking standard of care. Anifrolumab is an antagonist of signaling through the main type I interferon receptor (IFNAR).

Funding Agency: Lupus Research Alliance partnered with Celgene/BMS

Project Title: Dissecting Molecular Heterogeneity of SLE Patient Treatment Response

Short Explanation: This project will examine cell-type specific epigenetic profiles associated with abatacept-responsive patient immunophenotype clusters. assess the full cell-type specific transcriptional profiles, including alternative transcription start sites, alternative splicing and non-coding RNAs, and validate a predictive model for abatacept response based on multiomic data and clinical data.

What other organizations were involved as partners?

- Nothing to Report.

QUAD CHARTS:

- Submitted under separate cover as power point slide

APPENDICES:

- See below p 35

Describe the Regulatory Protocol and Activity Status (if applicable)

- Not applicable

APPENDIX

The Following Presentations and Publications are Appended in the Following Order

Type	Venue/Journal	Relationship to This Project	Relevance
1. Abstract <i>Munroe et al</i>	American College of Rheumatology 2020	Indirect	Is providing technical information useful for this project
2. Abstract <i>Ding et al</i>	American College of Rheumatology 2020	Indirect	Relates to our future plans that will utilize insights from this project and in return, findings from this abstract may inform our analysis in this project in the next year
3. Abstract <i>Merrill et al</i>	European League Against Rheumatism 2020	Indirect	Relates to our future plans that will utilize insights from this project and in return, findings from this abstract may inform our analysis in this project in the next year
4. Paper published <i>Slight Webb et al</i>	J Allergy Clin Immunol 2020 146(6):1419-1433	Some of it has direct relevance	Some of the data are relevant (see Figure 5) and DoD was given credit for partial support
5. Paper Published <i>Guthridge et al</i>	E-Clinical Medicine Volume 20 2020 eclinm.2020.100291	Direct Relevance but preceded the current project	This is the publication of the Preliminary Data used in the application for this project

The major publication resulting from this work awaits completion of a new project to expand the sample size and obtain ability to focus on patients taking methotrexate alone. However, several off shoot projects are envisioned by members of our team which will utilize some of our findings as key preliminary data.

ABSTRACT NUMBER: 1803

Ability of Innate, Adaptive, and TNF-Superfamily Immune Pathways to Characterize Disease Activity and Inform a Refined Lupus Disease Activity Immune in a Confirmatory Cohort of SLE Patients

Melissa Munroe¹, Wade DeJager², Susan Macwana², Ly Tran², Joel Guthridge², Eldon Jupe³, Daniele DeFreese³, Ryan Newhardt³, Mohan Purushothaman³, Sanjiv Sharma³, Nancy Redinger², Teresa Aberle², Stan Kamp², Cristina Arriens², Eliza Chakravarty², Joan Merrill⁴ and Judith James⁵, ¹Oklahoma Medical Research Foundation/Progentec Diagnostics, Inc., Oklahoma City, OK, ²Oklahoma Medical Research Foundation, Oklahoma City, OK, ³Progentec Diagnostics, Inc., Oklahoma City, OK, ⁴Oklahoma Medical Research Foundation, Oklahoma City, ⁵Arthritis and Clinical Immunology Research Program, Oklahoma Medical Research Foundation;Department of Pathology, University of Oklahoma Health Sciences Center;Department of Medicine, University of Oklahoma Health Sciences Center, Edmond, OK

Meeting: ACR Convergence 2020

Keywords: Autoantibody(ies), cytokines, Disease Activity, prognostic factors, Systemic lupus erythematosus (SLE)

SESSION INFORMATION

Date: Monday, November 9, 2020

Session Type: Poster Session D

Session Title: SLE – Diagnosis, Manifestations, & Outcomes Poster III: Bench to Bedside

Session Time: 9:00AM-11:00AM

Background/Purpose: Systemic lupus erythematosus (SLE) is a chronic autoimmune disease driven by complex immune dysregulation, involving altered immune mediators and accumulation of autoantibody (AutoAb) specificities. We have previously identified patterns of disordered immunity associated with development of SLE (Munroe et al. Ann Rheum Dis. 2016; Lu et al. J. Autoimmun.



2016). This study seeks to determine an optimal panel of markers that can distinguish those SLE patients with active disease and help to refine a Lupus Disease Activity Immune Index (LDAII).

Methods: We procured 200 samples from 150 patients classified with SLE on dates of low disease scores measured by the hybrid SLEDAI (hSLEDAI) (< 4 , range 0-3, $n=100$) or active disease (≥ 4 , range 4-30, $n=100$). Race/sex/age-matched healthy control (Ctl) samples ($n=50$) were also evaluated. SLE-linked plasma immune mediators ($n = 35$) were evaluated by EllaTM microfluidic immunoassay and serum SLE-associated AutoAb specificities, including dsDNA, chromatin, Ro/SSA, La/SSB, Sm, SmRNP, RNP, and ribosomal P, were assessed by Bioplex® 2200 xMAP assay. A subset of 24 log-transformed immune mediators were further evaluated using random forest applied machine learning modeling to determine an optimal subset of mediators to inform the LDAII. The LDAII is the sum of log-transformed, standardized immune mediators, weighted by the Spearman r correlation coefficient of immune mediator levels vs. either hSLEDAI scores or number of SLE-associated AutoAb specificities, which correlates with clinical disease activity (Spearman $r = 0.289$, $p < 0.0001$).

Results: No difference in age, ethnicity, or sex was noted between low or active clinical disease. After adjusting for multiple comparisons (Bonferroni corrected $p < 0.0021$), IFN- α , IL-2R α , TNF- α , TNFRII, IP-10, MIG, and IL-10 remained significantly correlated with hSLEDAI scores (Spearman $r=0.221-0.388$), and 22/35 soluble mediators significantly correlated with accrued SLE-associated autoantibodies, including the top 12 LDAII informative mediators listed in the **Table**. Forward selection of the 24 mediators ranked by variable importance (**Figure [A]**) drew 12 mediators that best distinguished between controls, low disease and active disease (**Table**). These top 12 immune mediators best informed and maximized the performance of a newly refined LDAII (**Figure [B]**). The LDAII, weighted either by hSLEDAI score ($p < 0.0001$) or number of AutoAb specificities ($p=0.0060$), significantly correlated with disease activity in SLE patients and identified patients with renal involvement ($p < 0.01$), where SCF and TNFRII ($p < 0.0001$) were significantly altered. In addition, the LDAII distinguished patients with dsDNA binding and low complement as well as clinically and serologically active vs. quiescent disease states ($p < 0.0001$), differentiated by TNF- α , IL-2R α , IP-10, and IL-10 levels ($p \leq 0.001$).

Conclusion: We have refined the LDAII in order to characterize SLE patients with active clinical disease. Treatment to target using a sensitive and objective biologic surrogate for clinical disease activity could help improve disease management and prevent organ damage in SLE.



Most Informative Soluble Mediators Associated With SLE Clinical Disease Activity

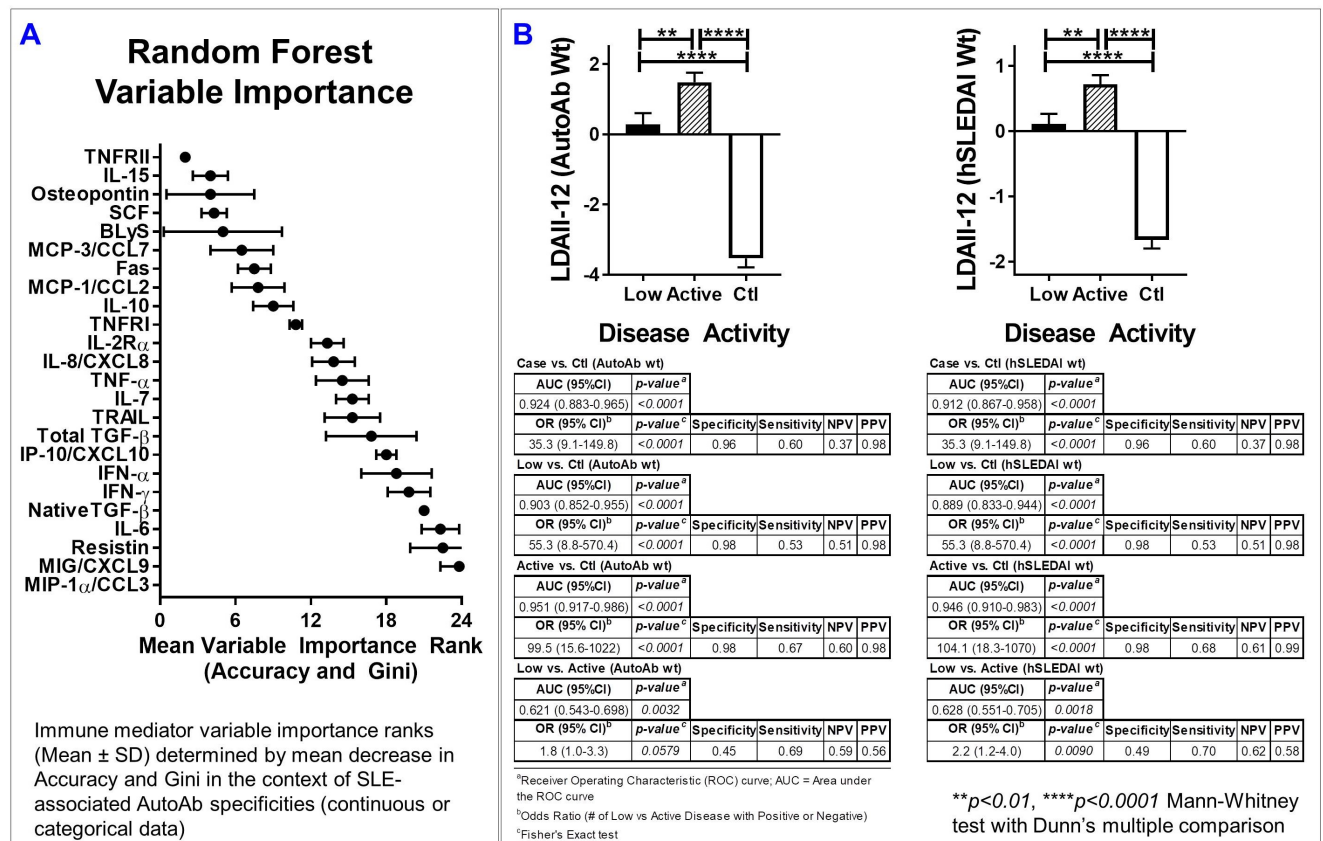
		SLE Patients				Active vs Ctl			Low vs Ctl			Active vs Low			Soluble Mediators vs. # AutoAb Specificities (Cases and Ctls)			Soluble Mediators vs. hSLEDAI Score (Cases)		
Rank	Analyte ^a	Active ^b Mean	SEM	Low ^b Mean	SEM	Ctl Mean	SEM	p-value ^c	p-value ^c	p-value ^c	Spearman r	95% CI	p value ^d	Spearman r	95% CI	p value ^d				
1	TNFR1 (p75)	4916	328	4592	720	2071	84	<0.0001	<0.0001	0.0007	0.477	0.371 to 0.571	<0.0001	0.283	0.149 to 0.409	<0.0001				
2	IL-15	9.4	2.2	5.3	0.7	2.1	0.2	<0.0001	<0.0001	0.1881	0.477	0.371 to 0.571	<0.0001	0.167	0.025 to 0.303	0.0181				
3	Osteopontin	169844	50532	114957	12278	45203	3485	<0.0001	<0.0001	0.0929	0.346	0.228 to 0.455	<0.0001	0.154	0.011 to 0.290	0.0285				
4	SCF	1565	83	1462	88	875	31	<0.0001	<0.0001	0.1515	0.293	0.171 to 0.407	<0.0001	0.096	-0.047 to 0.236	0.1742				
5	BLyS	1383	94	1208	80	623	21	<0.0001	<0.0001	0.0425	0.487	0.382 to 0.579	<0.0001	0.182	0.041 to 0.317	0.0098				
6	MCP-3/CCL7	19.4	10.9	10.7	4.9	2.9	0.8	<0.0001	<0.0001	0.0178	0.225	0.099 to 0.343	0.0004	0.107	-0.036 to 0.246	0.1303				
7	Fas	15080	517	13676	511	9583	380	<0.0001	<0.0001	0.0185	0.345	0.226 to 0.454	<0.0001	0.185	0.043 to 0.319	0.0088				
8	MCP-1/CCL2	513	65	388	28	195	11	<0.0001	<0.0001	0.0350	0.430	0.318 to 0.418	<0.0001	0.155	0.012 to 0.291	0.0285				
9	IL-10	6.0	0.5	4.5	0.4	1.9	0.1	<0.0001	<0.0001	0.0007	0.494	0.390 to 0.586	<0.0001	0.265	0.127 to 0.393	0.0001				
10	TNFR1 (p55)	2124	187	1946	276	961	41	<0.0001	<0.0001	0.0067	0.299	0.177 to 0.412	<0.0001	0.197	0.056 to 0.330	0.0052				
11	IL-2R α	2127	110	1775	84	1062	54	<0.0001	<0.0001	0.0013	0.441	0.331 to 0.539	<0.0001	0.238	0.098 to 0.368	0.0007				
12	IL-8/CXCL8	22.4	5.5	16.8	5.7	31.6	8.6	0.0010	0.0102	0.0344	0.199	0.072 to 0.319	0.0017	0.185	0.043 to 0.319	0.0087				

^aSoluble mediator concentration in pg/ml (Mean and SEM) in 200 SLE Cases vs. 50 race/sex/age-matched Healthy Controls (Ctl)

^bLow = hSLEDAI < 4 (n=100); Active = hSLEDAI $\geq$ 4 (n=100)

^cSignificance determined by Mann-Whitney test; Bonferroni corrected significance $p=0.0042$

^dSpearman correlation, Bonferroni corrected significance $p=0.0042$



Diagnostics, Inc., 3; **R. Newhardt**, Progentec Diagnostics, Inc., 3; **M. Purushothaman**, Progentec Diagnostics, Inc., 3; **S. Sharma**, Progentec Diagnostics, Inc., 3; **N. Redinger**, None; **T. Aberle**, None; **S. Kamp**, None; **C. Arriens**, BMS, 1, 2, GSK, 1; **E. Chakravarty**, None; **J. Merrill**, Bristol Myers Squibb, 2, 5, GlaxoSmithKline, 2, 5, AstraZeneca, 5, AbbVie, 5, Amgen, 5, Aurinia, 5, EMD Serono, 5, Remegen, 5, Janssen, 5, Provention, 5, UCB, 5; **J. James**, Progentec Diagnostics, Inc., 9.

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<https://acrabstracts.org/abstract/ability-of-innate-adaptive-and-tnf-superfamily-immune-pathways-to-characterize-disease-activity-and-inform-a-refined-lupus-disease-activity-immune-in-a-confirmatory-cohort-of-sle-patients/>. Accessed May 5, 2022.

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ABSTRACT NUMBER: 1840

A Novel Biomarker Identifies Systemic Lupus Erythematosus (SLE) Patients Who Benefit from Obexelimab (XmAb®5871) Treatment

Ying Ding¹, Debra Zack¹, Bart Burington¹, Allen Yang¹, Joan Merrill², Judith James³, John Desjarlais¹, Raphael Clynes¹ and **Joel Guthridge**⁴, ¹Xencor Inc, Monrovia, CA, ²New York University, New York, ³Oklahoma Medical Research Foundation, Oklahoma City, ⁴Oklahoma Medical Research Foundation, Oklahoma City, OK

Meeting: ACR Convergence 2020

Keywords: Biologicals, Biomarkers, Systemic lupus erythematosus (SLE)

SESSION INFORMATION

Date: Monday, November 9, 2020

Session Type: Poster Session D

Session Title: SLE – Treatment Poster II

Session Time: 9:00AM-11:00AM

Background/Purpose: We previously reported Phase 2 SLE trial results for obexelimab, a CD19-targeted FcγRIIb engager that suppresses B-cell activation [1]. The primary endpoint, which measured loss of baseline response to steroids, was not met, but a strong trend in favor of obexelimab and statistically significant efficacy in secondary endpoints supported further analysis. Here, we describe a novel whole blood gene expression biomarker that characterized patients with improved outcomes on obexelimab therapy.

Methods: Expression of individual genes and gene pathway scores were evaluated for 68 patients who either completed the study or terminated early for loss of response using a five-fold cross validation framework. The relevance of each gene to a potential biomarker predictive model was measured by the degree to which high or low expression identified a patient subgroup (cDx+) with greater reduction in risk of flare by obexelimab than in cDX- patients.



Results: Obexelimab treatment was associated with reduction of B-cell genes and gene-sets reflective of activated B-cells and plasma cells/plasmablasts (Figure 1), as previously reported [2].

Importantly, our cross-validated analyses determined that the baseline expression levels of 2 predictive genes, CD27 and APP, identified a cDx+ group (50% of all patients) with greatly reduced risk of flare on obexelimab (cross-validated HR=0.262, full data set HR=0.166) (Figure 2). Conversely, in the placebo group, the CDx+ subjects had higher flare risk, thus cDx+ also identifies poor-prognosis patients who might particularly benefit from obexelimab. The cDx+ group demonstrated increased obexelimab effects compared to cDx- patients over a range of clinical endpoints (SRI-4: 56% cDx+ vs 14% cDx-, SRI-6: 33% vs 6.2%, LLDAS: 50% vs 6.2%, BICLA: 33% vs 12.5%) (Figure 3). CD27, the top predictive biomarker, is highly expressed by T-naïve and memory cells. Consistent with the notion that CD27 expression is important within the T-cell lineage, increased expression of other T-cell genes was also associated with reduced flare risk, including CD28 ($p=0.04$), TCF7($p=0.03$) and FOXP3 ($p=0.05$). Taken together with the reduction of B cell signatures seen on treatment, this new identification of CD27 as a potential T-cell associated predictive biomarker suggests an important therapeutic role of obexelimab in suppression of B-cell and T-cell interactions.

Conclusion: A novel two-gene classifier developed from whole blood transcriptomic data by RNA-sequencing identifies a biomarker positive group of patients with a high baseline resting and stem-like T-cell (CD27+, CD28+ and TCF7+) signature. These cDx+ patients had superior response rates compared to placebo across multiple clinical endpoints, suggesting that adaptive immune responses such as B-cell antigen presentation and/or T-cell costimulation may be key components of obexelimab effects. This exploratory biomarker analysis supports further assessment of the two gene signature as a patient stratification/companion diagnostic strategy in obexelimab clinical development for SLE and other autoimmune conditions.

[1] Merrill JT, et al Arth Rheum 2018;70 Abs S10.

[2] Merrill JT, et al EULAR 2020; Abs SAT0187



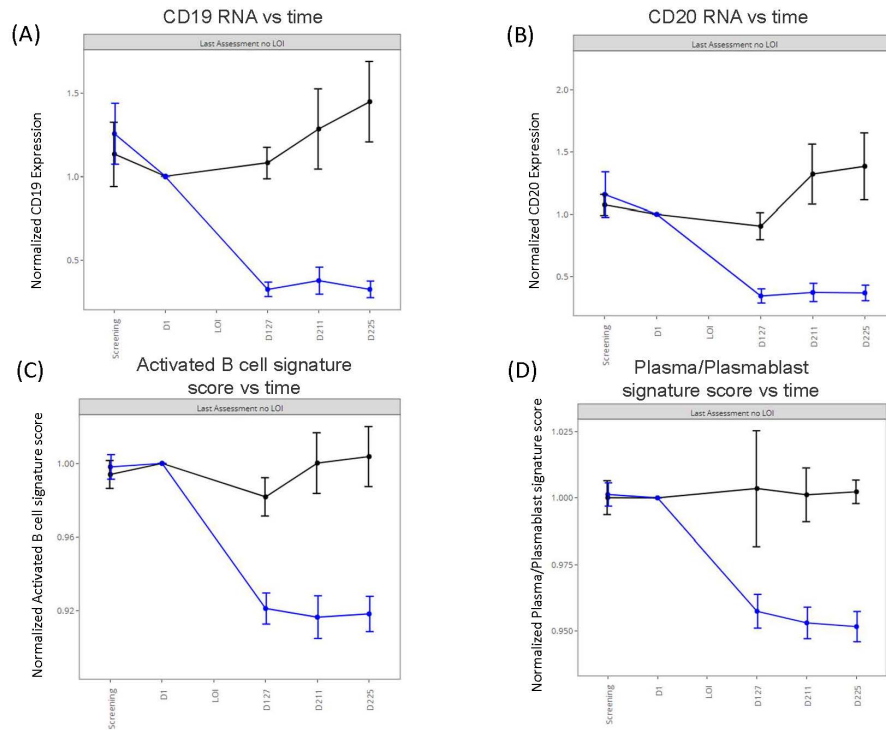


Figure 1. Pharmacodynamic effects of obexelimab (blue curve) compared with placebo (black curve) in patients with systemic lupus erythematosus (SLE) who had evaluable baseline whole blood transcriptomic (RNA-seq) data and either completed the study without a flare or experienced a flare on study. The RNA expression and signature scores are relative to baseline. Signature scores are based on immune cell signatures described in [3] and generated by the Singscore method[4] (A) B-cell marker gene CD19 gene expression over time (B) B-cell marker gene CD20 (transcript MS4A1) gene expression over time. (C) Activated B-cell signature score over time (D) Plasma/plasmablast signature score over time. [3] Arazi, A. et al. Nat. Immunol. 2019. 20;902-914. [4] Foroutan M, et al. . BMC Bioinformatics, 2018 19; 404.



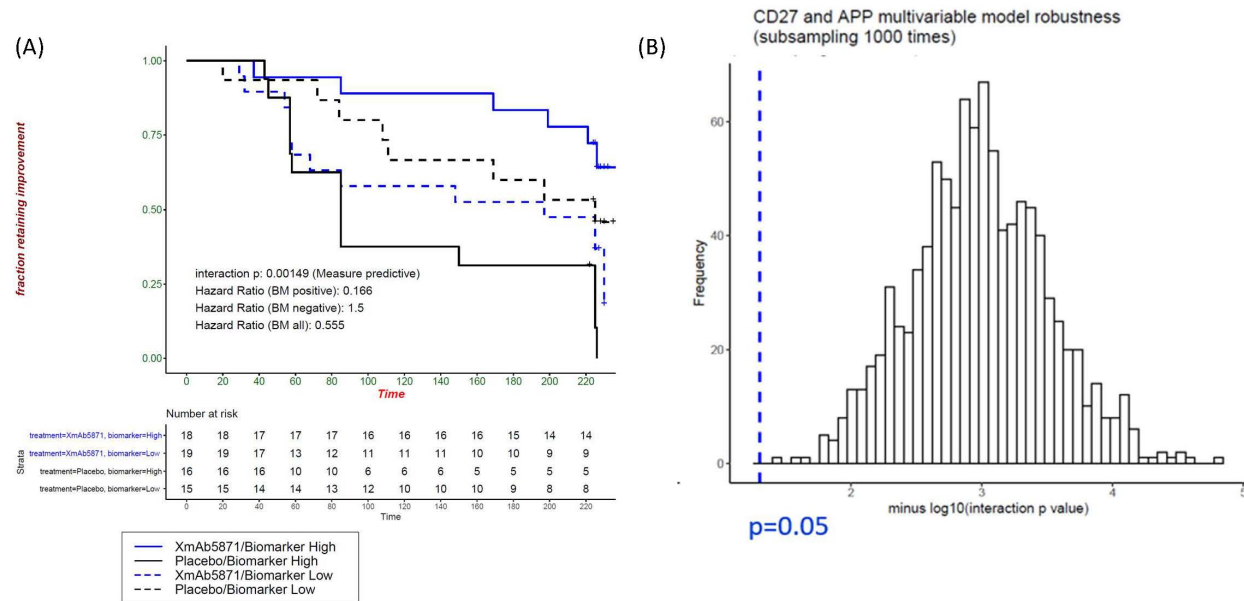


Figure 2. Two-gene predictive model (CD27 and APP). A five-fold cross validation framework was developed to ensure the generalizability of the results and to determine the number of genes needed and what fraction of patients to assign as cDx+. Classifications are based on a simple threshold for the sum of gene expression levels that have been normalized by the training sample mean and standard deviation. (A) Normalized expression levels of two genes classify patients as cDx+ or cDx-, with an estimated 50% of patients assigned to each group. The benefit from obexelimab over placebo in the cDx+ group (solid blue and solid black curves) was significantly greater than the benefit in the cDx- group (dashed blue and dashed black curves) ($p=0.000149$, HR cDx+ = 0.166 vs HR cDx- = 1.5). (B) Robustness of the two-gene predictive model as shown by the distribution of the interaction p-value based on subsampling 80% of the datasets 1000 times.



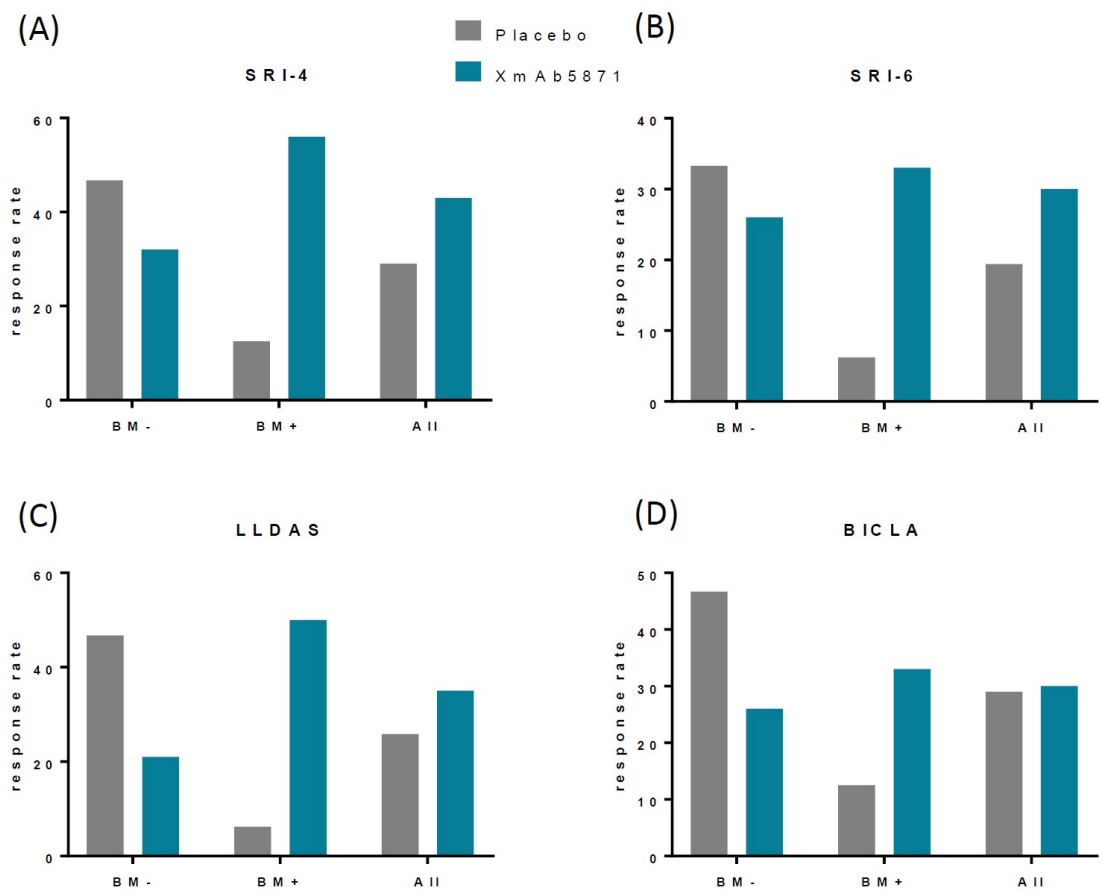


Figure 3. Response rates are enriched among cDX+ (50%) patients' four landmark endpoints at 32 weeks. (A) SRI-4 (B) SRI-6 (C) LLDAS (D) BICLA.

Disclosure: Y. Ding, Xencor Inc, 1, 3; D. Zack, Xencor Inc, 1, 5, Exagen, 1, 3, 6; B. Burington, Xencor Inc, 1, 3; A. Yang, Xencor Inc, 1, 3, 6; J. Merrill, None; J. James, None; J. Desjarlais, Xencor Inc, 1, 3, 6; R. Clynes, Xencor Inc, 1, 3; J. Guthridge, None.

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<https://acrabstracts.org/abstract/a-novel-biomarker-identifies-systemic-lupus-erythematosus-sle-patients-who-benefit-from-obexelimab-xmab5871-treatment/>. Accessed May 5, 2022.

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SAT0187 (2020)

DISCRIMINATION OF SYSTEMIC LUPUS (SLE) PATIENTS WITH CLINICAL RESPONSE TO OBEXELIMAB (XMAB®5871) BASED ON A PATTERN OF IMMUNOLOGIC MARKERS

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Background: We recently reported Phase 2 SLE trial results of obexelimab, an FcγRIIb agonist (suppressor of B cell activation). Obexelimab did not meet the primary endpoint (% of patients without flare at Day 225) ($p=0.183$) but other endpoints such as time to flare ($p=0.025$) were met.

Objectives: 1. To assign SLE patients to phenotypic subsets based on patterns of gene expression in immune-related pathways. ¹ 2. To explore the association of immune patterns and clinical response to obexelimab.

Methods: This analysis included 71 of the 104 participants in the obexelimab study, those who either completed the protocol or terminated for disease flare, if there were adequate blood samples (biomarker subset). At screening, patients were assigned to clusters based on 41 SLE co-expression signature modules from the Human Immune Phenotyping Consortium via unsupervised random forest and K-means clustering. ² Other markers of SLE disease were also examined. The *BOLD* ³ study design mandated withdrawal of background immunosuppressants, supporting less ambiguous pharmacodynamic analysis as the trial progressed.

Results: Immune pathway expression patterns of 7 patient clusters (Fig 1a) confirmed our prior characterization of 200 non-overlapping SLE patients. ² The biomarker subset retained a trend of longer time to first flare in patients receiving obexelimab ($n=38$) vs placebo ($n=33$) (Fig1b, HR 0.61, $p=0.11$). A smaller set of only Clusters 3 and 6 demonstrated marked increased time to flare in the obexelimab group ($n=13$) compared to placebo ($n=14$) (Fig 1c , HR 0.22, $p=0.025$). Obexelimab had no effect on other clusters (Fig 1d). The responder clusters shared low expression of inflammation modules ($p < 0.001$) compared to other clusters and high expression of T Cell, immune response, cell cycle, mitochondrial modules (all $p < 0.001$) and B Cell modules ($p=0.006$). We therefore sorted patients by these specific features regardless of cluster assignment. Figure 2 shows significant impact of obexelimab on time to flare in 64 patients with B Cell pathway activation (HR 0.5, $p=0.038$), although less robust by itself than found in Clusters 3 and 6. In a group with high B or plasma cell modules but low inflammation ($n=46$), treatment effect increased (HR 0.35, $p=0.019$). Between Screening and Baseline, as brief steroids were given and background treatments withdrawn, expression of B Cell and Plasma Cell pathways increased. Both then decreased after treatment with obexelimab but not placebo ($p < 0.0001$ and $p < 0.001$ respectively), an effect not seen with other immune pathway modules.

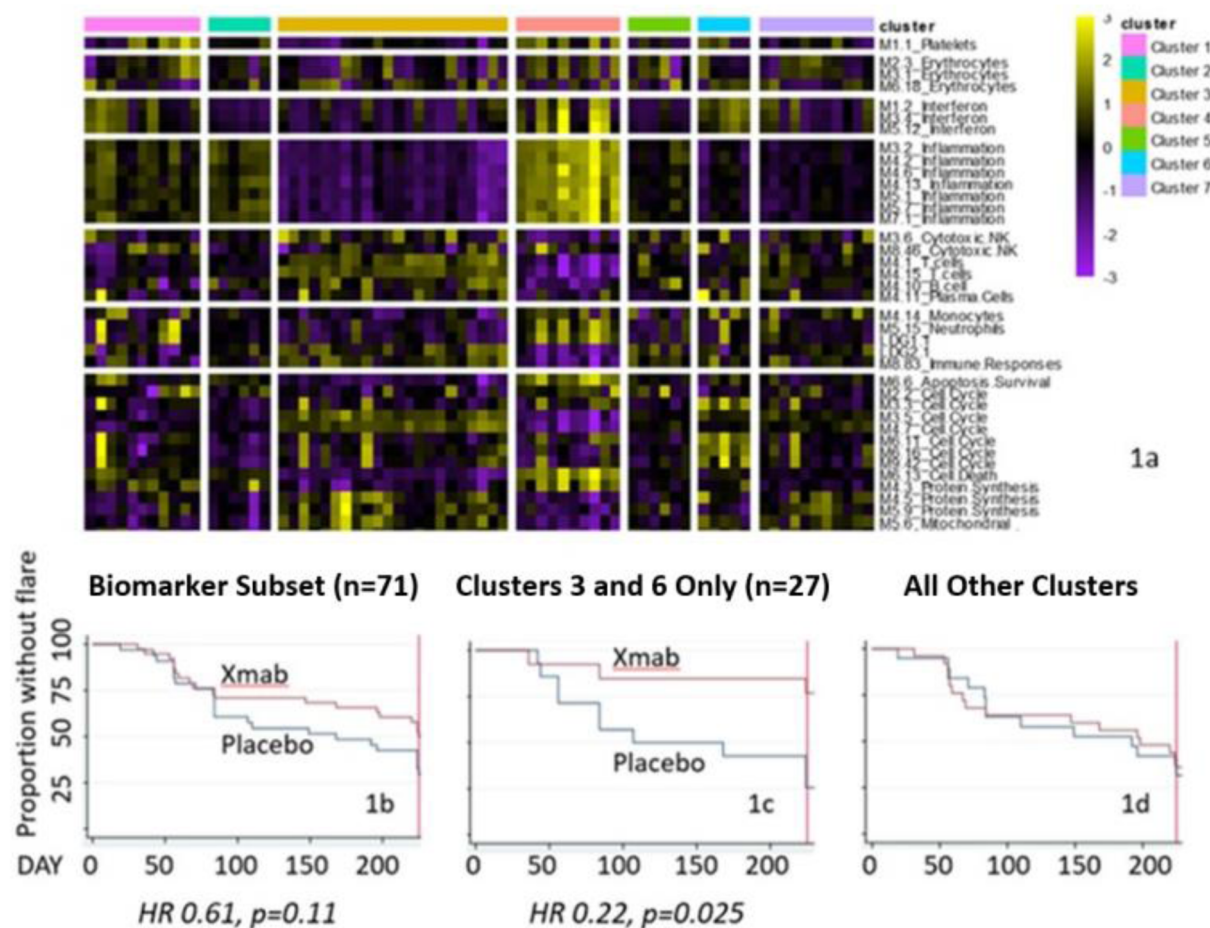
: Precision medicine for SLE has been hampered by heterogeneous immune signals with variable clustering of patients by gene co-expression pathways enabled an efficient, hierarchical array that (https://www.eular.eu) cohort, suggesting these are not random phenotypes. Of these 7 reproducible clusters, the combination of clusters 3 and 6 distinguished an obexelimab responder population of 27 out of 38%) with high expression of B and T Cell modules and cell activation pathways. Focus on the

defining features shared by these clusters revealed specific factors associated with response, enabling inclusion of some patients from other clusters in an optimized responder population of 46/71 (65% of subjects). B Cell and Plasma Cell pathways demonstrated mechanism-related pharmacodynamic effects of obexelimab. Lack of responders with high expression of inflammation modules could implicate inhibitory factors to obexelimab within inflammatory pathways, potentially targetable by complementary treatments.

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- [2]Lu ACR Abstract #2977 2017
- [3]Merrill Arthritis Rheumatol 69: 1257 2017

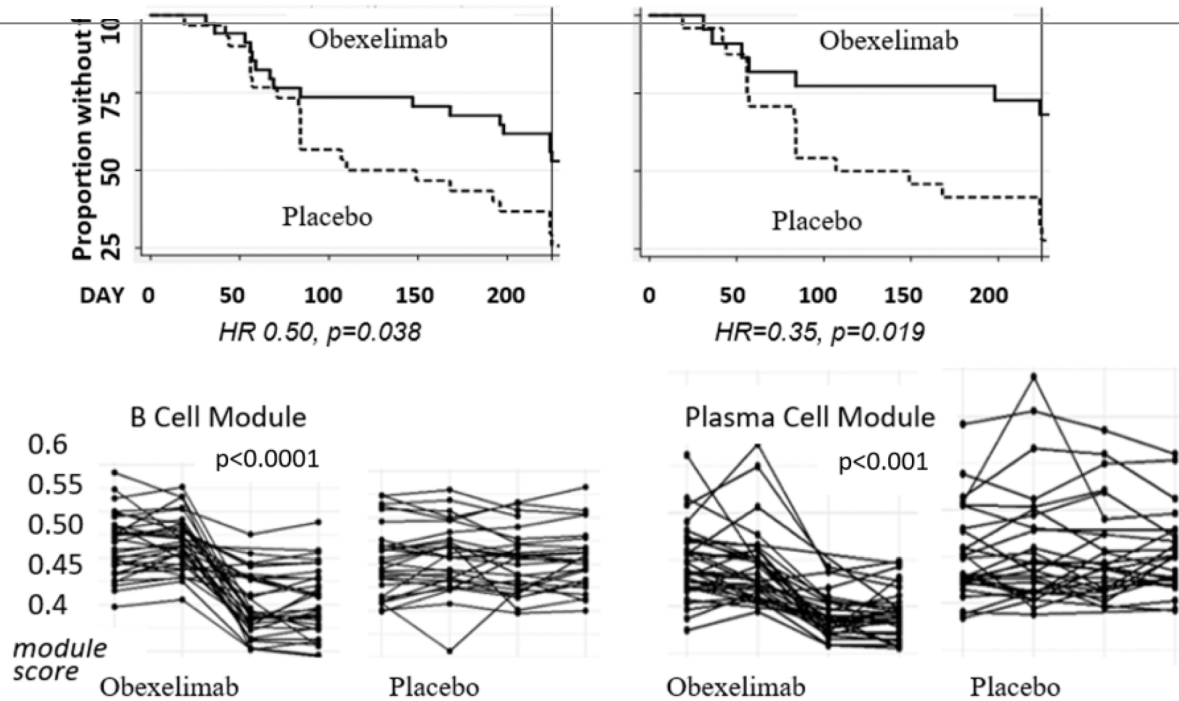
Fig 1: Effects of Obexelimab Treatment on Time to Flare in SLE Patient Subsets Defined by Patterns of Gene Co-Expression Pathways



(<http://www.eular.org/>)

Increased B Cell Module Expression

High B/Plasma Cell, Low Inflammation



Pathway Module Expression Over Time: Screening-Baseline-Next to Last-Last Visit

Disclosure of Interests : Joan T Merrill Grant/research support from: Xencor, Bristol Myers Squibb, Glaxo Smith Kline, Consultant of: Xencor, Abbvie, UCB, Glaxo Smith Kline, EMD Serono, Astellas, Remegen, Celgene/Bristol Myers Squibb, Exagen, Astra Zeneca, Amgen, Janssen, Servier, ILTOO, Daichi Sankyo, Lilly, Paid instructor for: Abbvie, Bristol Myers Squibb, Joel Guthridge Grant/research support from: Xencor, Bristol Myers Squibb, DXterity, Debra Zack Shareholder of: Xencor, Employee of: Xencor, Paul Foster Shareholder of: Xencor Inc, Employee of: Xencor Inc, Bart Burington Shareholder of: Xencor Inc, Employee of: Xencor Inc, Ly Tran: None declared, Miles Smith: None declared, Judith A. James Grant/research support from: Progentec Diagnostics, Inc, Consultant of: Abbvie, Novartis, Janssen

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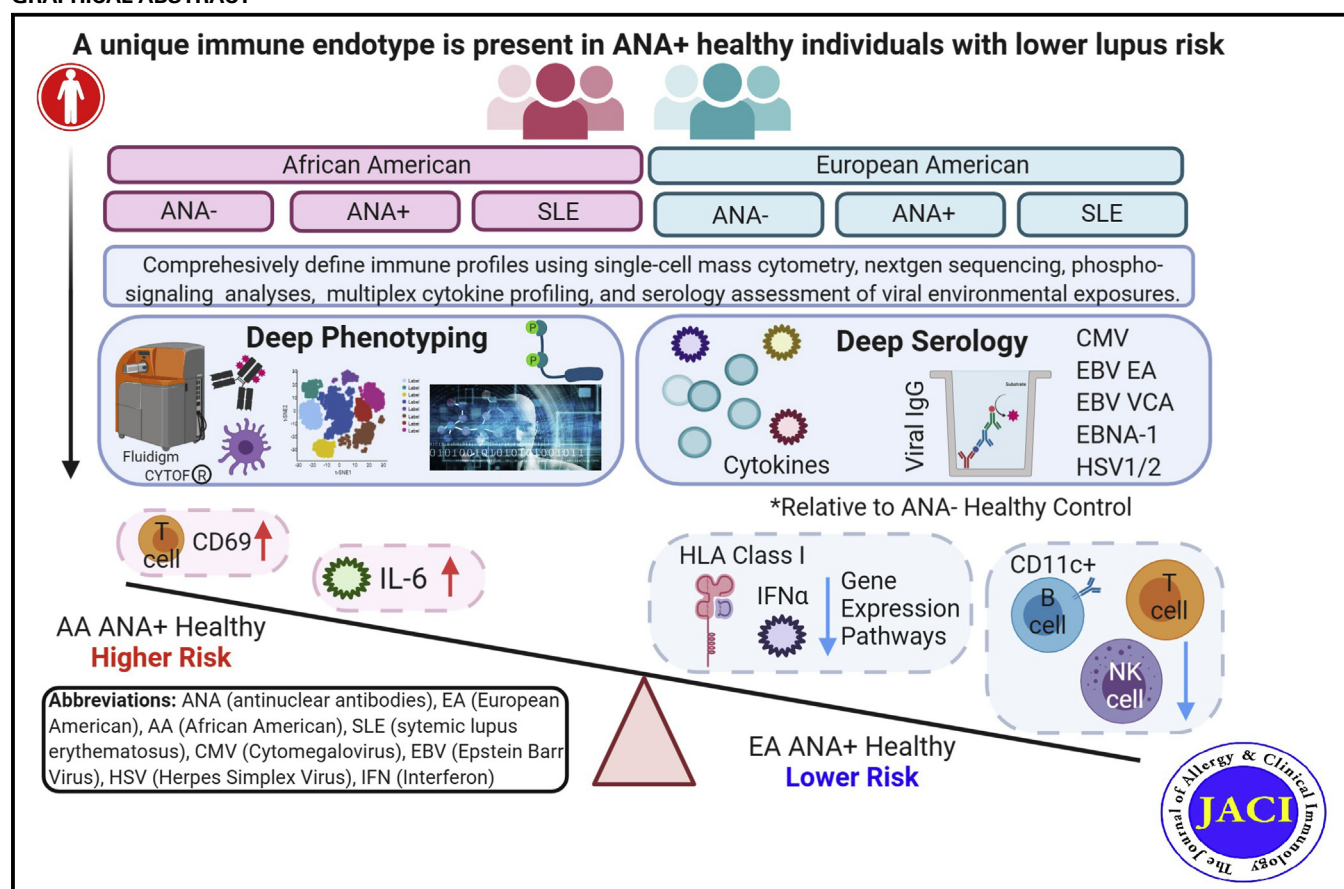
Session: SLE, Sjön's and APS - treatment (Poster Presentations)

Autoantibody-positive healthy individuals with lower lupus risk display a unique immune endotype



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GRAPHICAL ABSTRACT



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*These authors contributed equally to this work.

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Fellowship Support to R.L. and the Oklahoma Medical Research Foundation Lou C. Kerr Chair in Biomedical Research to J.A.J.

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Background: Autoimmune diseases comprise a spectrum of illnesses and are on the rise worldwide. Although antinuclear antibodies (ANAs) are detected in many autoimmune diseases, up to 20% of healthy women are ANA-positive (ANA+) and most will never develop clinical symptoms. Furthermore, disease transition is higher among ANA+ African Americans compared with ANA+ European Americans.

Objective: We sought to determine the immune features that might define and prevent transition to clinical autoimmunity in ANA+ healthy individuals.

Methods: We comprehensively phenotyped immune profiles of African Americans and European Americans who are ANA-negative (ANA-) healthy, ANA+ healthy, or have SLE using single cell mass cytometry, next-generation RNA-sequencing, multiplex cytokine profiling, and phospho-signaling analyses.

Results: We found that, compared with both ANA- and ANA+ healthy individuals, patients with SLE of both races displayed T-cell expansion and elevated expression of type I and II interferon pathways. We discovered a unique immune signature that suggests a suppressive immune phenotype and reduced CD11C⁺ autoimmunity-associated B cells in healthy ANA+ European Americans that is absent in their SLE or even healthy ANA- counterparts, or among African American cohorts. In contrast, ANA+ healthy African Americans exhibited elevated expression of T-cell activation markers and higher plasma levels of IL-6 than did healthy ANA+ European Americans.

Conclusions: We propose that this novel immune signature identified in ANA+ healthy European Americans may protect them from T-cell expansion, heightened activation of interferon pathways, and disease transition. (*J Allergy Clin Immunol* 2020;146:1419-33.)

Key words: ANA+ healthy, SLE, Autoantibodies, immune suppression, T cells, race, cytokines

Autoimmune diseases, such as SLE, are driven by both environmental and genetic factors. Approximately 8% of the population has a classified autoimmune disease, often associated with the presence of antinuclear antibodies (ANAs).^{1,2} ANAs and other autoantibodies can be detected up to 10 years before autoimmune disease onset. Indeed, ANAs are detected in about 20% of healthy people, particularly females, the elderly, and non-whites;^{1,3} however, the presence of autoantibodies alone does not predict the development of clinical symptoms.⁴⁻⁶ Whether a subset of ANA-positive (ANA+) individuals possess protective cellular factors and mechanisms that prevent disease transition is unknown.

Significant effort has focused on understanding the mechanisms that drive autoimmune disease. Compared with patients with SLE, ANA+ healthy individuals have lower levels of stem cell factor (SCF), B lymphocyte stimulator (BLyS), and type I interferons (IFN- α and IFN- β), as well as higher levels of the regulatory cytokine, IL-1 receptor antagonist.⁷ However, we recently found that immune pathways are already dysregulated in ANA+ versus ANA-negative (ANA-) healthy individuals; ANA+ healthy subjects show a modest elevation of proinflammatory cytokines in serum.⁷ The frequencies of monocytes, B cells, and T_H follicular cells were also elevated in ANA+ versus ANA- healthy individuals.^{7,8} In addition, we discovered that some soluble mediators are elevated in serum approximately 3.5 years before SLE classification and even prior

Abbreviations used

AA:	African American
ANA:	Antinuclear antibody
ANA-:	ANA-negative
ANA+:	ANA-positive
BCR:	B-cell receptor
BLyS:	B lymphocytes stimulator
CMV:	Cytomegalovirus
CytoF:	Cytometry by time-of-flight
DC:	Dendritic cell
ds:	Double-stranded
EA:	European American
HSV:	Herpes simplex virus
NK:	Natural killer
pDC:	Plasmacytoid DC
RNP:	Ribonucleoprotein
SCF:	Stem cell factor
TCR:	T-cell receptor
tSNE:	t-Distributed stochastic neighboring embedding

to ANA-positivity, including IL-5, IL-6, and IFN- γ .⁶ Other innate cytokines, interferon-associated chemokines (such as monokine induced by IFN- γ /C-X-C motif ligand 9), and BLyS increase ~10 months before SLE classification.⁶ Nevertheless, we lack a comprehensive evaluation of immunophenotypes and immune function for healthy ANA+ individuals versus patients with SLE and for ANA+ versus ANA- subjects. As a result, it is not fully understood why only a subset of ANA+ healthy individuals will transition to disease.

In addition to ANA-positivity and elevated soluble mediators, race and ethnicity contribute to the risk for developing autoimmune disease. For instance, systemic autoimmune rheumatic diseases often show a later onset and milder clinical presentation in those with European American (EA) versus African American (AA) ancestry.⁹ Genetic, environmental, and socioeconomic factors likely influence the diverse biological mechanisms that contribute to autoimmunity. Autoantibody-dependent interferon-activation pathways, BLyS serum cytokine levels, and DNA methylation in naive CD4⁺ T cells differ between AA and EA patients with SLE.¹⁰⁻¹⁵ Although ancestral backgrounds display distinct genetic factors and gene signatures,¹⁵ no studies have examined ANA+ healthy individuals by race. This analysis is needed to identify potentially clinically relevant, but unknown, mechanisms that regulate the transition from an ANA+ healthy status to SLE.

Here, we used an in-depth immune screening platform to identify regulatory and inflammatory immune features that are critical for the development of clinical autoimmunity. We examined populations that are at higher risk (AA) and lower risk (EA) for transitioning to SLE, and compared ANA+ healthy individuals with ANA- controls and patients with SLE to discover putative regulatory mechanisms. Unexpectedly, EA ANA+ healthy subjects exhibited a unique immune suppression signature in T cells that was not present in EA ANA- controls or patients with SLE or the AA cohorts. Our results identify the first protective immune profile displayed during development of clinical autoimmune disease and thus point to potential therapeutic avenues to activate a pathway that could delay or prevent disease transition.

METHODS

Study population and autoantibody screening

All experiments were performed in accordance with the Helsinki Declaration and approved by the Institutional Review Board of the Oklahoma Medical Research Foundation. Healthy individuals were recruited through 15 health fairs, and screened initially for 11 serum autoantibody specificities using the BioPlex 2200 system (Bio-Rad Technologies, Hercules, Calif). Positive individuals were also assessed by NOVA Lite indirect immunofluorescence (IIF) and QUANTA Lite ELISA using HEP-2 cells (Inova Diagnostics, Inc, San Diego, Calif) following the manufacturer's recommended protocols and cutoffs.^{7,16,17} IIF testing was performed by College of American Pathologists—Clinical Laboratory Improvement Amendments—certified Morris Reichlin, MD, Clinical Immunology Laboratory. Briefly, BioPlex 2200 ANA tested autoantibody specificities include double-stranded (ds)DNA, chromatin, Ro/Sjögren's syndrome-related antigen A, La/Sjögren's syndrome-related antigen B, Sm, ribonucleoprotein (RNP), SmRNP, centromere B, ribosomal P, Scl-70, and Jo-1. All autoantibodies, except anti-dsDNA, were reported in antibody index units based on a fluorescent intensity range of 0 to 8. The manufacturer-specified cutoff was used to determine positivity (positive ≥ 1 antibody index unit) for all autoantibodies, except for anti-dsDNA where semiquantitative values were reported as IU/mL with positive being ≥ 10 IU/mL. Confirmation assays included INNO-LIA ANA Update EIA testing (Innogenetics NV, Zwijnaarde, Belgium). ANA- and ANA+ healthy individuals had no probable autoimmune rheumatic disease determined by a connective tissue disease screening questionnaire,¹⁸ as well as review of medical and medication history. ANA+ healthy individuals were identified as having 1 or more of the 11 tested autoantibodies by BioPlex and no probable autoimmune disease.

ANA+ healthy individuals ($n = 24$) were matched by sex, age ± 5 years, and race to ANA- healthy controls ($n = 24$) and patients with SLE ($n = 24$) (see Table E1 in this article's Online Repository at www.jacionline.org). Patients with SLE met at least 4 American College of Radiology classification criteria and were assessed for disease activity by SELANA-SLEDAI (Safety of Estrogens in Systemic Lupus Erythematosus National Assessment—Systemic Lupus Erythematosus Disease Activity Index) instrument score.¹⁹ ANA- healthy controls, ANA+ healthy individuals, and patients with SLE were divided and analyzed by race, which was self-reported as African American or European American and verified using genetic ancestry informative markers.²⁰ PBMCs were isolated using Lymphocyte Separation Medium (Mediatech, Inc, Manassas, Va) and stored in freezing media (20% human serum and 10% dimethyl sulfoxide in RPMI) in liquid nitrogen until use. Plasma was also collected and stored at -80°C until testing.

Soluble mediator measurement

Plasma levels of BLyS were assessed using ELISA per the manufacturer's protocol and reported in pg/mL (R&D Systems, Minneapolis, Minn). All other soluble mediators (soluble CD40 ligand, C-X-C motif ligand 13, eotaxin, G-CSF, growth regulated oncogen (GRO)- α , intercellular adhesion molecule 1, IFN- α , IFN- β , IFN- γ , IL-1 α , IL-1 β , IL-1 receptor antagonist, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-2, IL-21, IL-23, IL-2 receptor α , IL-4, IL-5, IL-6, IL-7, IL-8, induced protein 10, leptin, leukemia inhibitory factor, monocyte chemoattractant protein 1, monocyte chemoattractant protein 3, monokine induced by IFN- γ , macrophage inflammatory protein 1 α , macrophage inflammatory protein 1 β , nerve growth factor β , plasminogen activator inhibitor 1, platelet-derived growth factor with 2 B subunits, RANTES, resistin, SCF, stromal cell-derived factor 1 α , soluble E-selectin, soluble Fas ligand, TGF- β , TNF- α , TNF receptor 1, TNF receptor 2, TNF-related apoptosis-inducing ligand, vascular cell adhesion molecule 1, and vascular endothelial growth factor) were assessed using xMAP (ProcartaPlex) multiplex assays (Invitrogen, Thermo Fischer Scientific, Waltham, Mass) and run on Bioplex 200 suspension array reader (Bio-Rad). All data were normalized across plates using a serum control (Cellgro human AB serum, Mediatech) and reported as both MFI over serum control and concentration (pg/mL). As previously described,²¹ soluble mediators were excluded from analysis if $\geq 50\%$ of measurements reported were below the lowest level of detection resulting in 38 soluble mediators that passed quality control.

CyTOF immunophenotyping

Assays were performed in the Human Immune Monitoring Center at Stanford University. Antibody clones, staining protocols, and gating strategies were previously described.²² Briefly, PBMCs were thawed, washed, and resuspended in cell sorting buffer (PBS supplemented with 2% BSA, 2 mmol/L EDTA, and 0.1% sodium azide), and viable cells were counted by Vi-CELL (Beckman Coulter Life Sciences, Indianapolis, Ind). Viable cells (1.5 million cells/well) were stained with antibody-polymer conjugate cocktail (see Table E2 in this article's Online Repository at www.jacionline.org). All antibodies were unconjugated and carrier-free and then conjugated using polymer and metal isotopes from Fluidigm (San Francisco, Calif). Cells were washed and resuspended in cell sorting buffer. The cells were resuspended in 2 $\mu\text{g/mL}$ Live/Dead (dodecane tetraacetic acid-maleimide [MacroCyclics, Inc, Plano, Tex], containing natural-abundance indium). The cells were washed and resuspended in 2% paraformaldehyde in PBS and placed at 4°C overnight. The next day, cells were washed and placed in eBiosciences (Thermo Fischer Scientific) permeabilization buffer ($1\times$ in PBS) and incubated on ice. Cells were then washed twice in PBS and acquired using cytometry by time-of-flight ([CyTOF]; Fluidigm). Data analysis was performed using Cytobank by gating on intact cells based on the iridium isotopes from the intercalator, then on singlets by iridium Ir 191 versus cell length, then on live cells (indium-Live/Dead minus population) (see Fig E1 in this article's Online Repository at www.jacionline.org), followed by cell subset-specific gating as shown in Fig 2 by t -distributed stochastic neighboring embedding (tSNE) and manually, as previously published.²²

Phosphopeptide flow cytometry

These assays were performed at the Human Immune Monitoring Center at Stanford University. Briefly, PBMCs were suspended at 0.1×10^6 viable cells and stimulated with T-cell receptor (TCR) stimuli (CD3/CD28 Dynabeads, Thermo Fisher Scientific) or B-cell receptor (BCR) stimuli (anti-human IgG, anti-human IgM, and H_2O_2) and incubated at 37°C for either 30 minutes (TCR) or 4 minutes (BCR). The PBMCs were then fixed with paraformaldehyde and permeabilized with methanol. Cells were bar-coded using a combination of Pacific Orange and Alexa-750 dyes (Invitrogen) and then stained with the following antibodies (all from BD Biosciences, San Jose, Calif): CD3, CD4, CD20, CD33, CD45RA, p38, pPLC γ 2, pSTAT-5, and pERK1/2. Cells were collected (100,000/sample) using DIVA 6.0 software on an LSRII flow cytometer (BD Biosciences). Data analysis was performed using FlowJo (version 9.3, BD) by gating on live cells based on forward versus side scatter profiles, then on singlets using forward scatter area versus height, followed by cell subset-specific gating of the 90th percentile. Fold change was calculated over basal phospho-protein levels to assess differences following BCR and TCR stimulation.

Cell sorting and bulk RNA-sequencing

PBMCs from 36 subjects were stained with antibodies for CD3 (UCHT1), CD19 (SJ25-C1), HLA-DR (G46-6), CD14 (61D3), CD16 (3G8), CD56 (NCAM16.2), CD66b (G10F5), and CD66b $^{-}$ CD19 $^{-}$ CD3 $^{+}$ T cells, CD66b $^{-}$ CD3 $^{-}$ CD19 $^{+}$ B cells, and CD66b $^{-}$ CD3 $^{-}$ CD19 $^{-}$ CD56 $^{-}$ HLA-DR $^{+}$ CD14 $^{+}$ CD16 $^{+/-}$ monocytes were sorted using a FACS Aria III (BD Biosciences). RNA was isolated using TRIzol reagent (Invitrogen), purified with Direct-zol RNA MicroPrep Kit (Zymo Research, Irvine, Calif), and quantitated using 2100 Bioanalyzer (Agilent, Santa Clara, Calif). QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria) was used to create cDNA libraries and to amplify and sequence 3 million reads/sample using NextSeq 550 (Illumina). Libraries were evaluated for poor sample quality using FASTQC (version 0.11.8),²³ and adaptors and contaminating nonspecific reads were removed using BBTools (version 38.56).²⁴ Using STAR (version 2.5.3a),²⁵ reads were aligned to the GENCODE release 28 transcriptome.²⁶ The .bam files were then converted to a gene count matrix using StringTie (version 1.3.6).²⁷ Counts were normalized using the DESeq2 package (version 1.24.0)²⁸ in R (R Foundation, Vienna, Austria) and transformed using variance stabilizing transformation for heatmap visualizations.²⁹ All low expressed genes were removed prior to analysis.

A preselected gene list of 500 genes was used for differential expression analysis between disease groups by race. Significant differentially expressed genes were calculated between either ANA[−] and ANA⁺, ANA⁺ and SLE, or ANA[−] and SLE using a Kruskal-Wallis test with *P* value <0.05 considered significant.

Virus IgG detection ELISAs

The cytomegalovirus (CMV) IgG, EBV early antigen IgG, EBV viral capsid antigen IgG, herpes simplex virus 1 (HSV1) IgG, and HSV2 IgG levels were measured according to manufacturer's instructions using 1:21 dilution of sera (Zeus Scientific, Inc, Branchburg, NJ).³⁰ The anti-EBV nuclear antigen 1 IgG ELISA was also performed according to the manufacturer's specifications using a 1:101 dilution of sera (EuroImmun, Eubeck, Germany). Samples determined as equivocal were rerun to determine positive, negative, or equivocal status.

Data analysis and statistics

tSNE analysis were performed using Cytobank.³¹ The .fcs files were uploaded to Cytobank and gated off live intact singlet cells. To generate tSNE plots, 22,000 events were used per sample with data for 33 cell surface markers. Concatenated files of 110,000 cells were used for representative tSNE images and cell subset profiling. Frequencies of cell subsets were exported from tSNE for cell number calculations and analysis. Traditional bivariate gating was performed in Cytobank. Cytokine data were non-normally distributed; therefore, continuous data were analyzed using the Kruskal-Wallis test with Wilcoxon-Mann-Whitney 2-tailed test for 2-group comparisons. The *q* values were calculated using the qvalue R package (version 3.3.3) to correct for multiple comparisons and estimate the false discovery rate to control for the expected proportion of incorrectly rejected null hypotheses. All analyses, heatmaps, and plots were performed and generating using GraphPad Prism (version 6.0 for Windows; GraphPad Software, San Diego, Calif) or TIBCO Spotfire (version 6.0.1; TIBCO Software Inc, Boston, Mass). The 3-dimensional bar graphs were generated in R (version 3.2.2) using the latticeExtra, RColorBrewer, and gridExtra packages.

RESULTS

EA and AA ANA⁺ healthy individuals have distinct autoantibody specificities

We recruited and screened 1035 healthy subjects for autoantibodies, using both indirect immunofluorescence and Luminex bead-based assays (Invitrogen, ThermoFischer Scientific, Waltham, Mass) that measure common lupus, Sjogren's, systemic sclerosis, and myositis autoantibodies, as previously described.^{7,16} Approximately 25.6% of the cohort were ANA⁺, with an ANA titer ≥120 defined by indirect immunofluorescence.

Using BioPlex 2200 ANA testing, 41 EA (7.32% of total EAs) and 12 AA individuals (7.84% of total AAs) were ANA⁺, having at least 1 of 11 autoantibody specifications, yet without a diagnosed autoimmune rheumatic disease. Autoantibody specificity varied between EA and AA ANA⁺ healthy individuals. In EA ANA⁺ healthy individuals, anti-RNP was the primary autoantibody (41.5% in EAs vs 20.0% in AAs), followed by antibodies against centromere B (17.0%), dsDNA (14.6%), Ro (14.6%), and La (12.2%). Anti-dsDNA antibody was the most prevalent autoantibody in AA ANA⁺ healthy individuals, with 50.0% of subjects testing positive, followed by anti-RNP (20.0%), anti-La (20.0%), and anti-Ro (8.3%).

We identified EA (*n* = 12) and AA (*n* = 12) individuals that were ANA⁺ and healthy by BioPlex as defined above and matched them to healthy ANA[−] controls (*n* = 24) and patients with SLE patients (*n* = 24) according to age (±5 years), sex, and

race (Table E1). All ANA⁺ subjects, except for 1 RNP⁺ AA ANA⁺ individual were also positive by IIF and/or ELISA (see Table E3 in this article's Online Repository at www.jacionline.org). All control participants completed a connective tissue disease screening questionnaire to assess whether participants had possible autoimmune rheumatic disease. No probable disease was found in ANA⁺ subjects or ANA[−] controls.¹⁸ Autoantibody specificities of the ANA⁺ healthy individuals were selected to reflect the EA or AA cohort (see Table E4 in this article's Online Repository at www.jacionline.org). To reduce variability associated with sex, and given that there were fewer ANA⁺ healthy males (11.3%), all individuals selected for this study were female.

The selected EA patients with SLE had a higher prevalence of anti-Ro/Sjögren's syndrome-related antigen A antibodies (33.3%), and AA patients with SLE had a higher prevalence of anti-RNP antibodies (50.0%) (see Tables E5 and E6 in this article's Online Repository at www.jacionline.org). As had been previously reported,⁹ AA patients with SLE had a more active clinical disease presentation, evidenced by higher prevalence of renal disease (58.3% vs 16.7%) and a higher average SELENA-SLEDAI instrument score (5.5 vs 2.8 in EA patients). At the time of this study, there were no significant differences in proteinuria (0.0% EA vs 8.3% AA) between groups, and no EA or AA patients with SLE had lymphopenia, increased DNA binding, central nervous system issues, or hematuria.

EA ANA⁺ healthy individuals have reduced numbers of T cells, NK cells, and autoimmunity-associated B cells

To identify biologic mechanisms that regulate autoimmune disease progression, we collected PBMCs and plasma from matched subjects of EA and AA ancestry for ANA[−] controls, ANA⁺ healthy individuals, and patients with SLE (6 groups of 12 individuals are shown in Fig 1). PBMCs were assessed using (1) mass cytometry (CyTOF) to detect differences in immune cell frequencies, (2) phospho-flow cytometry to detect differences in signaling responses, and (3) RNA-sequencing to detect differences in gene expression in T cells, B cells, and monocytes (Fig 1, A). In addition, we assessed the plasma levels of 51 soluble mediators associated with innate immunity, adaptive immunity, regulation, growth, adhesion, and migration (Fig 1, B). Serum was used to assess prior environmental exposure to herpesviruses.

We investigated cell lineages that are essential for the development of ANA-positivity and autoimmune disease transition by race. Briefly, we designed a CyTOF panel of 33 metal isotope-tagged mAbs specific for cell lineage markers that discriminate the major immune cell subsets and subpopulations.²² Additional markers were included to distinguish the activation status and homing properties of specific cell subsets. Cell frequencies were visualized both by a high-dimensionality reduction method (tSNE) and by a standard hand-gating scheme that identified 55 different cell subsets (Fig 2). The tSNE analysis incorporated over 110,000 cells and distinguished 27 phenotypically distinct clusters (Fig 2, A and B). Marker expression of gated phenotype clusters is summarized by median intensity in a heatmap (Fig 2, C). For major immune cell subsets, tSNE and manual gating found similar differences in frequencies between ANA[−] controls, ANA⁺ healthy individuals, and patients with SLE (see Tables E7-E14 in this article's Online Repository at www.jacionline.org).

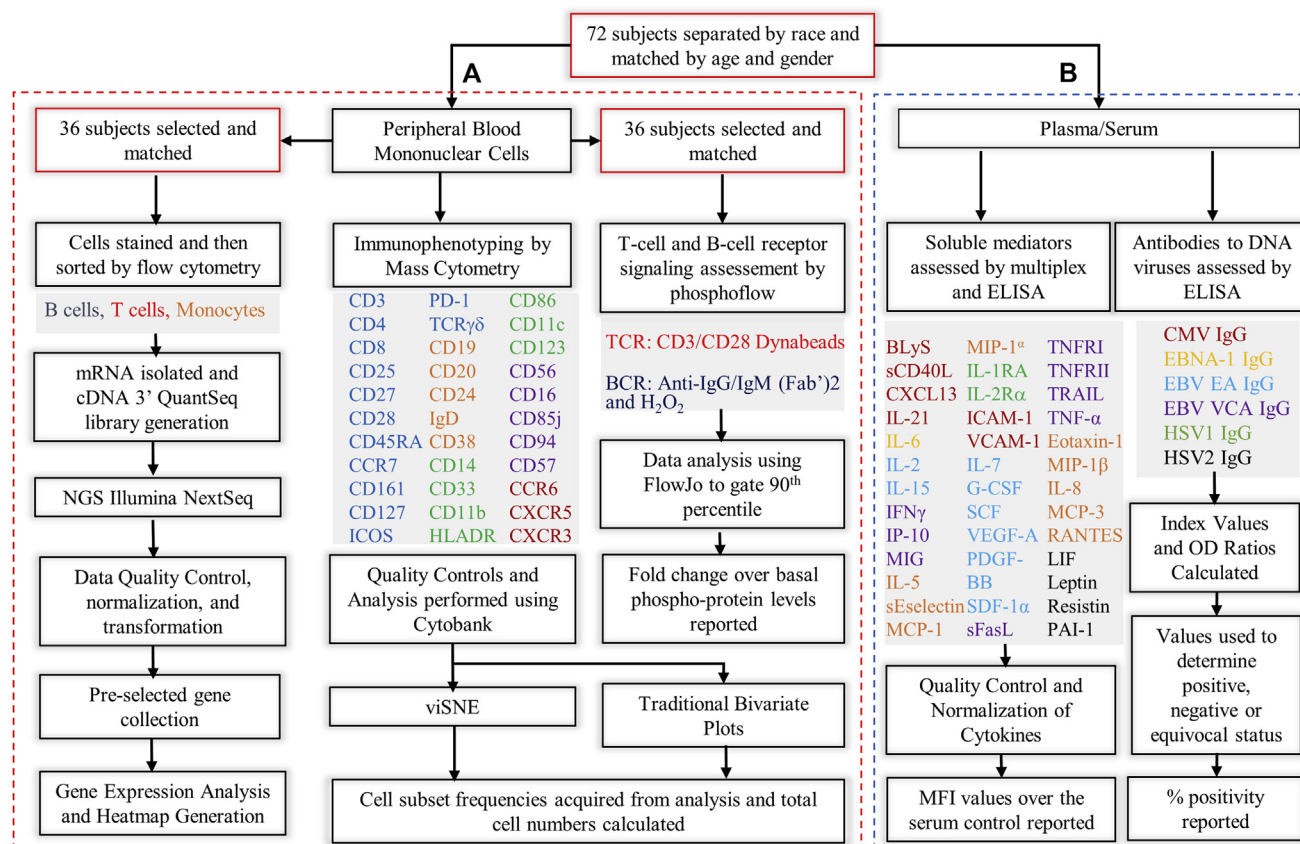


FIG 1. Schematic workflow for ANA+ healthy individual biologic analysis. The workflow is broadly divided into 2 steps. First, 72 samples consisting of EA and AA ANA+ healthy individuals, ANA+ healthy individuals, and patients with SLE were matched by age, race, and sex. **A**, PBMCs were collected and used for immunophenotyping by mass cytometry, TCR and BCR signaling analysis by phospho-flow, and gene expression analysis by 3' QuantSeq. **B**, Plasma was collected for soluble mediator analysis of 51 different metabolites using multiplex bead-based assays and ELISAs, and serum was used for viral IgG ELISAs. Immunophenotyping markers are colored according to cell association/pathway: T cell (blue), B cell (orange), myeloid cell (green), NK cell (purple) and chemokine receptors (red). Soluble mediators are also grouped by cell association/pathway using color: B cells (red), T cell (blue), T_H1 (purple), T_H2 (orange), regulatory (green), adhesion (red), precursor/growth factors (light blue), apoptotic (purple), myeloid/neutrophil (orange), and adipose (black). MFI, Median fluorescent intensity; NGS, next-generation sequencing; OD, optical density; viSNE, t-distributed stochastic neighbor embedding-based visualization.

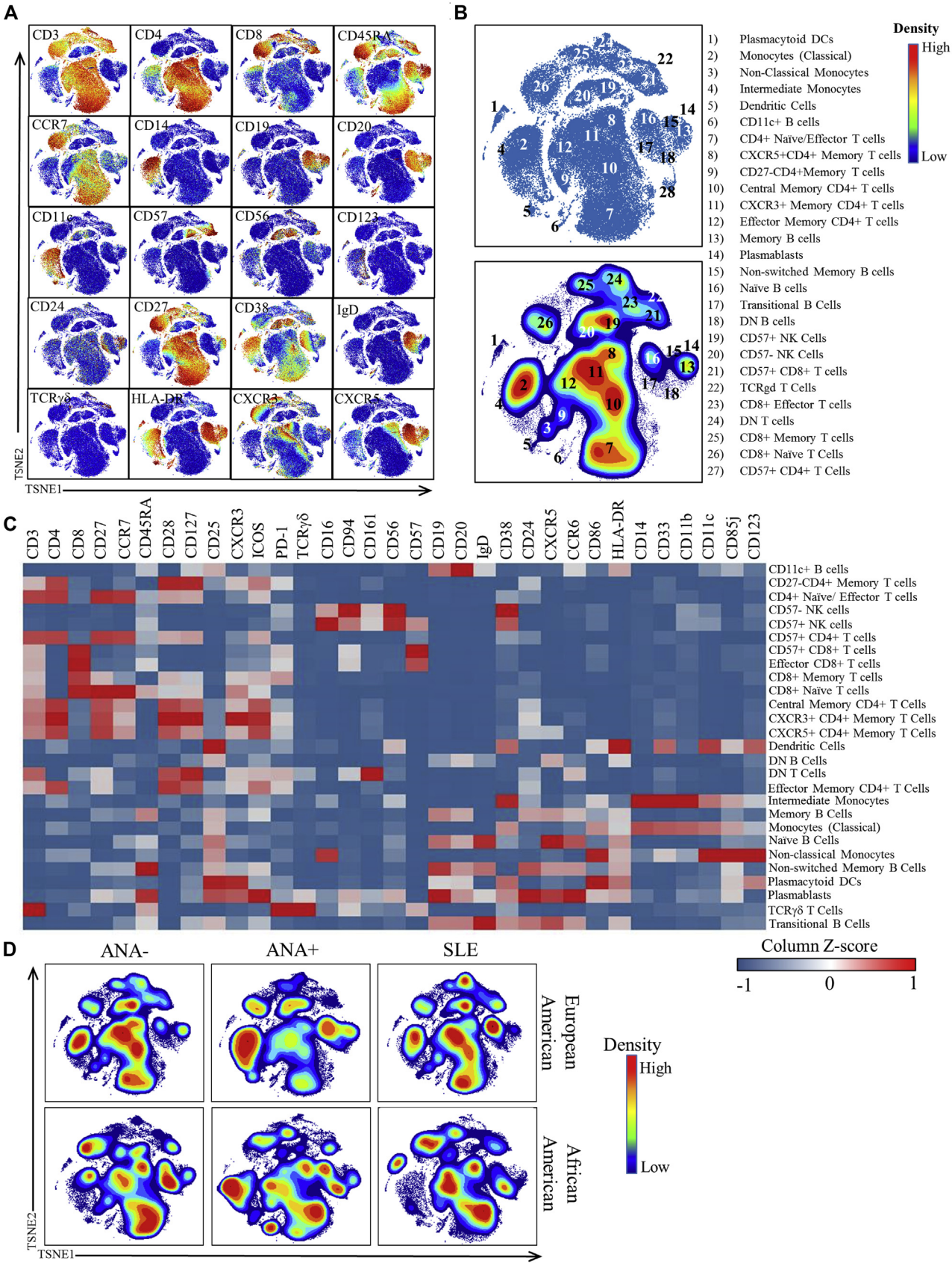
B cell (CD3⁺CD19⁺) and monocyte (CD3⁺CD19⁺HLA-DR⁺CD11c⁺CD14⁺) frequencies were elevated in EA ANA+ healthy individuals compared with ANA+ healthy controls (see Fig E2 in this article's Online Repository at www.jacionline.org, Tables E7-E10).⁷ The frequency of CD8⁺ T cells (CD3⁺CD56⁺CD8⁺) was elevated in patients with SLE compared with ANA+ healthy individuals in both the EA and AA groups (Fig E2). Some cell populations, including dendritic cells (DCs) (CD3⁺CD19⁺HLA-DR⁺CD11c⁺CD14⁺), plasmacytoid DCs (pDCs) (CD3⁺CD19⁺HLA-DR⁺CD11c⁺CD123⁺), and natural killer (NK) cells (CD3⁺CD19⁺CD56⁺), showed decreased frequencies in AA patients with SLE compared to ANA+ or ANA+ healthy controls (Fig E2).

To determine the source of these different frequencies in EA and AA ANA+ healthy individuals, total cell subsets/mL were back-calculated from cell frequencies (Tables E11-E14). Cell numbers (total cells/mL) were reduced in EA ANA+ healthy individuals compared with in both ANA+ healthy controls and patients with SLE (Fig 3, A). Although the cell numbers

were reduced in AA ANA+ healthy individuals versus patients with SLE, they were similar to ANA+ healthy controls (Fig 3, A). ANA titers did not correlate with decreases in cell numbers (see Fig E3 in this article's Online Repository at www.jacionline.org).

Although the frequencies of monocytes and B cells were elevated in EA ANA+ healthy individuals compared with in controls, the number of total monocytes and B cells were similar in the 6 groups (Fig 3, B and C). However, autoimmunity-associated B cells, characterized as CD11c⁺CD27⁺IgD⁺, were reduced in EA ANA+ healthy individuals relative to controls (Fig 3, D, Tables E13 and E14).

T-cell numbers were significantly increased in both AA and EA patients with SLE compared with ANA+ healthy individuals (Fig 3, E and F, Tables E13 and E14). Specifically, both CD4⁺ and CD8⁺ T cells in memory, naive, and effector T-cell subsets such as T_H1-type (C-X-C motif receptor 3⁺) and T_H17-type (CCR6⁺CD161⁺) were elevated in patients with SLE compared with ANA+ healthy individuals (Fig 3, E-N,



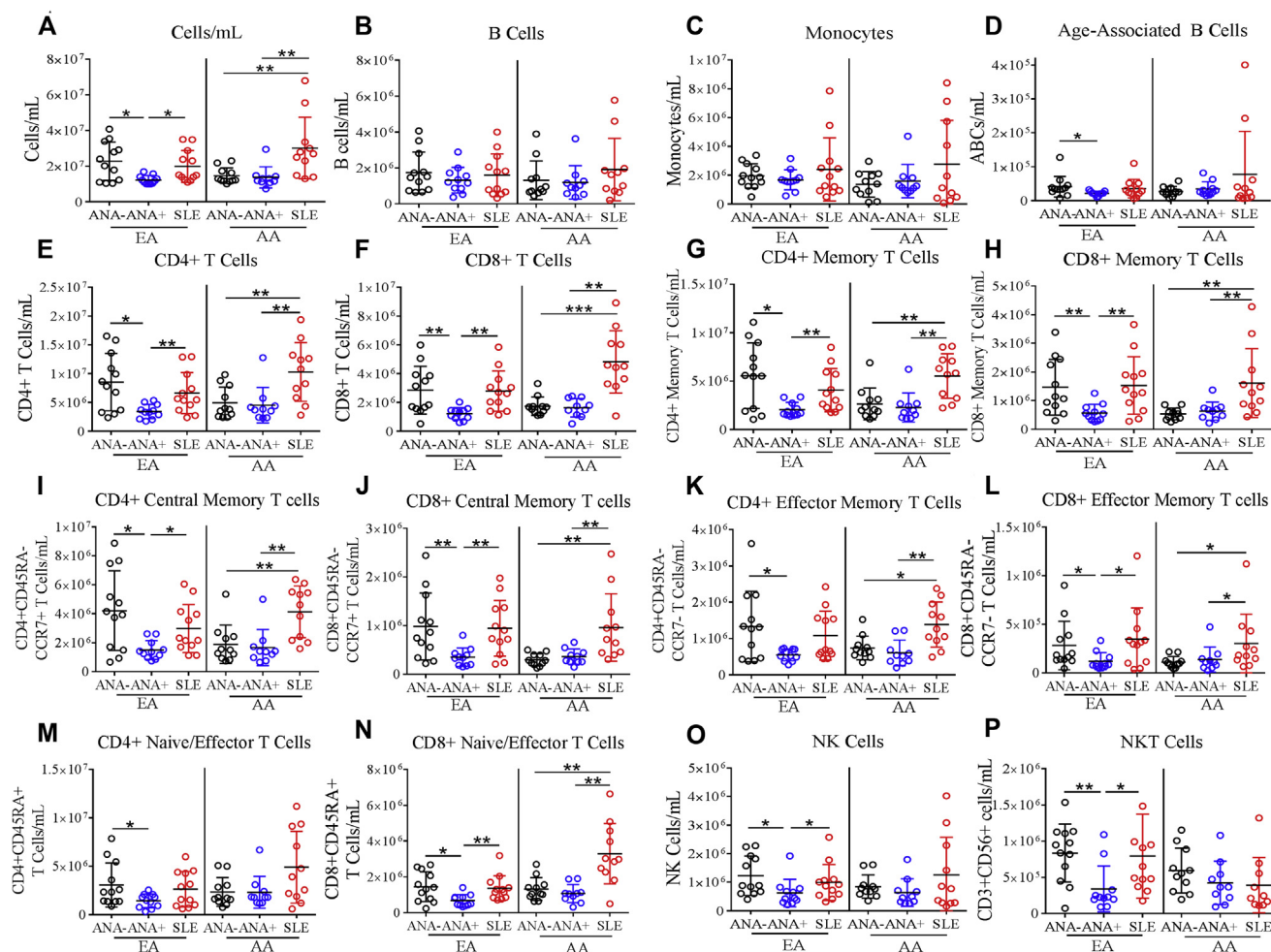


FIG 3. Calculated cell numbers indicate elevated T cells in patients with SLE and suppressed T cells in EA ANA+ healthy individuals. Cells numbers were calculated from cell subsets using frequencies and total cell counts. Cell numbers are shown for (A) total cells/mL, (B) B cells ($CD3^+CD19^+$), (C) monocytes ($HLA-DR^+CD11c^+CD14^+CD16^{+/-}$), (D) autoimmunity-associated B cells ($CD3^-CD19^+IgD^-CD27^-CD11c^+$), (E) $CD4^+$ T cells ($CD3^+CD56^-CD4^+CD8^-$), (F) $CD8^+$ T cells ($CD3^+CD56^-CD4^+CD8^+$), (G) $CD4^+$ memory T cells ($CD3^+CD56^-CD4^+CD45RA^-$), (H) $CD8^+$ memory T cells ($CD3^+CD56^-CD8^+CD45RA^-$), (I) $CD4^+$ central memory T cells ($CD3^+CD4^+CCR7^+CD45RA^-$), (J) $CD8^+$ central memory T cells ($CD3^+CD8^+CCR7^+CD45RA^-$), (K) $CD4^+$ effector memory T cells ($CD3^+CD4^+CCR7^-CD45RA^-$), (L) $CD8^+$ effector memory T cells ($CD3^+CD8^+CCR7^-CD45RA^-$), (M) $CD4^+$ naive/effector T cells ($CD3^+CD56^-CD4^+CD45RA^+$), (N) $CD8^+$ naive/effector T cells ($CD3^+CD56^-CD8^+CD45RA^+$), (O) NK cells ($CD3^-CD19^-CD56^+$), and (P) NK T cells ($CD3^+CD19^-CD56^+$). Black correlates to ANA- healthy controls, blue to ANA+ healthy individuals, and red to patients with SLE. * $P < .05$, ** $P < .01$, *** $P < .001$. Kruskal-Wallis test with 2-tailed Mann-Whitney for multiple comparisons. Mean $\pm$ SD shown.

Tables E13 and E14). Furthermore, NK cell subsets were also increased in patients with SLE compared with ANA+ healthy individuals in both races, whereas NK T cells were only elevated in EA patients with SLE (Fig 3, O and P, Tables E13 and E14).

These data further support the involvement of T and NK cells in SLE pathogenesis.

Intriguingly, NK- and T-cell populations were decreased in ANA+ versus both ANA- healthy controls and patients with

FIG 2. tSNE analysis pipeline identifies 27 phenotypically distinct populations in PBMCs. A, Twenty-cell surface marker expression is shown using dimensionality reduced tSNE plots from PBMC data (110,000 cells) derived from 72 samples. Dot plots are individually colored by channel using ArcSinh-5-transformed expression values. B, A dot plot and density map are shown depicting the density of cells and are numbered according to phenotypic subset. C, A heatmap summary of the expression values of all 33 cell surface markers are used to distinguish identified cell subsets. Marker values are displayed on a color scale ranging from blue (levels below the mean) through white (levels equal to the mean) to red (levels greater than the mean) using a column Z-score. D, Density maps depicting PBMC tSNE plots created using all 33 surface markers are plotted for EA and AA ANA- and ANA+ individuals and patients with SLE. All plots were derived from cumulative data from 12 individuals per group.

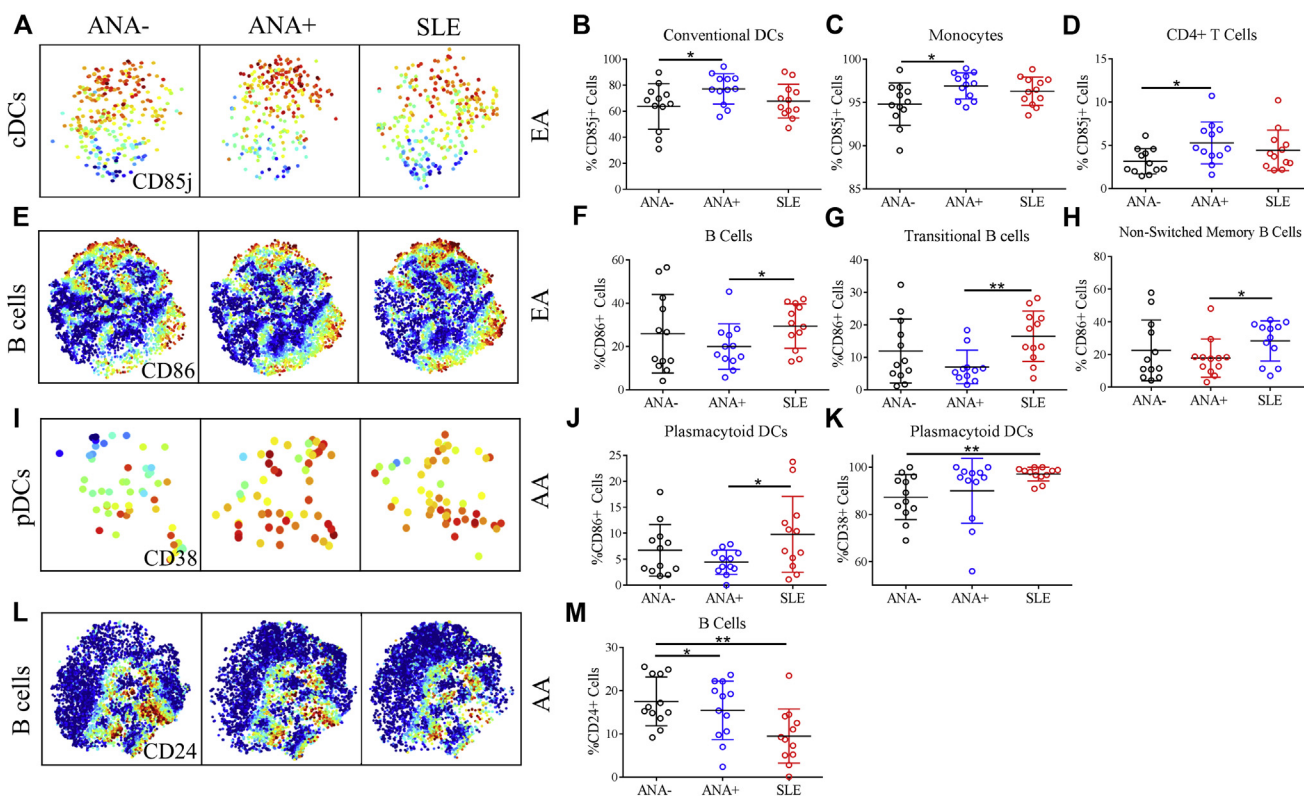


FIG 4. Cell subset marker expression identify activated cell subsets in patients with SLE and elevated regulatory marker expression in ANA+ healthy individuals. All 55 cell subsets were manually gated and assessed for frequencies of activation and regulatory surface markers. tSNE plots were used to illustrate specific expression differences among immune cell populations. CD85j expression on EA ANA– and ANA+ individuals and patients with SLE is shown using (A) tSNE plots with red indicating elevated expression and blue low expression, along with dot plot of significant differences in (B) conventional DCs (cDCs), (C) monocytes, and (D) CD4⁺ T cells. CD86 expression of B cells in EA subjects is shown by (E) tSNE plot, and dot plots in (F) B cells, (G) transitional B cells, and nonswitched memory B cells. Differences in pDCs in AA subjects is shown by (I) CD38 expression in tSNE plot and dot plots depicting (J) CD86 expression in EA subjects and (K) CD38 expression in AA subjects. B-cell CD24 expression of AA subjects is shown via (L) tSNE plot and (M) dot plot. * $P < .05$, ** $P < .01$. Kruskal-Wallis test with 2-tailed Mann-Whitney for multiple comparisons. Mean $\pm$ SD shown.

SLE in the EA cohort but not in the AA cohort. These results imply that reduced levels of T, NK, and autoimmunity-associated B cells protect EA ANA+ healthy individuals from transitioning to SLE and might contribute to the reduced risk of EA versus AA populations.

Cell-surface activation markers show increased expression in patients with SLE

To uncover potential functional differences, we assessed all 55 immune cell populations for surface expression of activation markers (CD86, HLA-DR, CD38, inducible costimulator), inhibitory receptors (CD85j, CD94, CD33, programmed cell death 1), and chemokine receptors (CCR6, CCR7, C-X-C receptor3, and C-X-C receptor 5) (see Tables E15 and E16 in this article's Online Repository at www.jacionline.org). CD85j was more highly expressed on conventional DCs, monocytes, and CD4⁺ T cells from EA ANA+ versus ANA– healthy individuals (Fig 4, A-D, Tables E15 and E16). The activation marker CD86 showed higher expression on B cells, primarily transitional B cells and non-switched memory B cells, from patients with SLE versus ANA+ healthy individuals of EA ancestry (Fig 4, E-H). pDCs,

NK, and NK T cells showed higher expression of activation markers in patients with SLE versus ANA+ and ANA– controls of AA ancestry (Fig 4, I-K, Tables E15 and E16). Thus, activation markers were elevated in patients with SLE, but not in ANA+ healthy individuals for both EA and AA cohorts.

Some receptors associated with immune regulation were significantly decreased in AA ANA+ healthy individuals and patients with SLE compared with AA ANA– controls. These include B-cell expression of CD24, which is associated with regulatory B-cell subsets (Fig 4, L and M) as well as T-cell, monocyte, and DC expression of the regulatory receptor CD94, which binds to HLA α chain E (Tables E15 and E16). These data support a potentially important role for inhibitory receptors in attenuating preclinical autoimmunity.

Phospho-signaling dysregulation in T-cell signaling pathways of ANA+ healthy individuals

To assess the relationship between the T- and B-cell phenotypes of these cohorts and TCR and BCR signaling, we evaluated pERK1/2, p38, pPLC γ 2, and pSTAT5 in PBMCs by flow cytometry following treatment with TCR (anti-CD3/CD28) or

BCR (anti-IgM/IgG F(ab')₂) stimuli (Fig 1, A, see Table E17 in this article's Online Repository at www.jacionline.org). Innate immune cells (non-B-/T-cell subsets) had elevated basal levels of pERK1/2 and p38 in EA ANA+ healthy individuals and in patients with SLE, respectively, compared with in ANA– controls (see Figs E4 and E5 in this article's Online Repository at www.jacionline.org, see Tables E18 and E19 in this article's Online Repository at www.jacionline.org). No significant differences were identified in either the EA or AA cohort following BCR stimulation (Fig E5). Following TCR stimulation, memory CD8⁺ T cells from EA ANA+ healthy patients, compared with those from patients with SLE and ANA– controls, had elevated pERK1/2 (Figs E4 and E5, Table E18). In AA patients with SLE, basal levels of pERK1/2 and p38 were increased in CD4⁺ and CD8⁺ T cells (Figs E3 and E4, Table E18). AA patients with SLE memory CD4⁺ and CD8⁺ T cells appear to have elevated basal levels of phosphorylated proteins that were not amplified, but slightly decreased, in response to TCR stimulation. These data suggest that these cells were already activated and maximally utilizing this signaling cascade.

Soluble mediator levels trend lower in EA ANA+ healthy individuals

To examine peripheral levels of cytokines, chemokines, and other soluble mediators, we assessed the levels of 51 soluble mediators in plasma (Fig 1, B). Plasma-soluble mediator levels trended lower in EA ANA+ versus ANA– healthy individuals (Fig 5, A, see Tables E20–E23 in this article's Online Repository at www.jacionline.org). In the AA cohort, ANA+ healthy individuals and patients with SLE, compared with ANA– controls, had elevated IL-6 levels (Fig 5, B). Monocyte chemoattractant protein 3, produced primarily by monocytes, along with the B-cell activating soluble CD40 ligand and apoptosis associated soluble Fas ligand, were increased in EA patients with SLE compared with in ANA+ healthy individuals (Fig 5, C–E). AA patients with SLE also had elevated interferon-associated soluble mediators, including induced protein 10, monokine induced by IFN-γ, BLyS, and TNFR1I, along with selectins (soluble E-selectin), TNF-related apoptosis-inducing ligand, and IL-2 receptor α (Fig 5, F–L). SCF was the only cytokine elevated in both EA and AA patients with SLE compared with in both ANA– and ANA+ healthy controls (Fig 5, M). Thus, SCF production is a critical indicator of SLE autoimmune disease that is independent of race.

T-cell immune suppression signature in EA ANA+ healthy individuals

To determine the mechanisms underlying the reduced numbers of T cells in EA ANA+ healthy individuals, we sorted T cells, monocytes, and B cells and assessed RNA expression of 500 genes involved in cell regulation, HLA inhibition, cytokine regulation, apoptosis, signal transducer and activator of transcription and cytokine pathways, adhesion, and cell activation (Fig 1, A, see full list in Table E24 in this article's Online Repository at www.jacionline.org). We found that interferon-inducible genes and HLA class I genes were markedly downregulated in T cells from ANA+ healthy individuals compared with those from ANA– controls and patients with SLE (Fig 6, A). Furthermore, *STAT1* upregulation and a

pyroptosis signature, characterized by elevated *CASP1*, distinguished T cells from EA ANA+ healthy individuals versus ANA– controls and patients with SLE (Fig 6, A). Compared with the EA cohorts, the AA cohorts showed distinct and fewer changes in T cells from ANA+ versus ANA– healthy individuals, including upregulation of the activation element *CD69* (Fig 6, A). T cells from patients with SLE of both races demonstrated increased expression of interferon-inducible genes, HLA genes, and other proinflammatory cytokine genes (Fig 6, A). Overall, these data suggest that immune suppression occurs in T cells of EA ANA+ healthy individuals, which may help to prevent clinical autoimmune onset.

To determine the genetic pathways associated with T-cell suppression in EA ANA+ individuals, we examined whether T-cell numbers correlated with gene expression patterns in T cells, B cells, and/or monocytes (see Figs E6 and E7 in this article's Online Repository at www.jacionline.org). Expression of *STAT4*, which is driven by type II interferons, was positively associated with CD4⁺ T-cell numbers in EA individuals (Fig 6, B). In AA individuals, gene expression of both types I and II interferon pathways (*STAT1*, *IFNGR2*) was positively correlated with CD4⁺ and CD8⁺ T-cell numbers (Fig 6, C–E). Regulation and activation of B cells and monocytes likely contribute to T-cell expansion, and the expression of interferon-inducible genes, CD85 regulatory molecules (*LILRB2*), *CD86*, and BLyS (*TNFSF13B*) positively correlated with T-cell numbers (Fig 6, E). Furthermore, *TGFBRI* expression negatively correlated with T-cell numbers in AA individuals. These data suggest that activation of types I and II interferon pathways in T and B cells, and reduced TGF-β signaling in monocytes, coincide with T-cell dysregulation and clinical autoimmune disease.

CMV and EBV seroconversion is not associated with immune suppression

Our data so far show that ANA+ EA individuals have reduced T-cell numbers; decreased plasma-soluble mediators; dysregulated T-cell signaling; and altered expression of HLA class I-, type I interferon-, apoptosis-, and *STAT1*-associated genes in T cells. These features are reminiscent of virus immune-evasion strategies and virus-induced immune suppression, often seen with members of the *Herpesviridae* family, specifically human CMV.^{32,33} Furthermore, environmental factors, particularly herpesvirus infection and reactivation, most notably EBV, are associated with the development of SLE.³⁴ To determine whether persistent infection or reactivation of herpesviruses were involved in T-cell suppression observed in EA ANA+ healthy individuals, we examined the IgG responses against human CMV, EBV early antigen, EBV viral capsid antigen, EBV nuclear antigen, HSV1, and HSV2.

Consistent with previous reports, there was a higher frequency of patients with SLE with antibodies directed against EBV early antigen, in conjunction with viral capsid antigen- and EBV nuclear antigen 1-directed antibodies, suggesting current or recent reactivation of EBV (see Fig E8, A, in this article's Online Repository at www.jacionline.org). The frequency of ANA+ healthy individuals with antibodies to EBV early antigen was higher than the frequency in healthy ANA– controls, but reduced when compared with the frequency in patients with SLE.

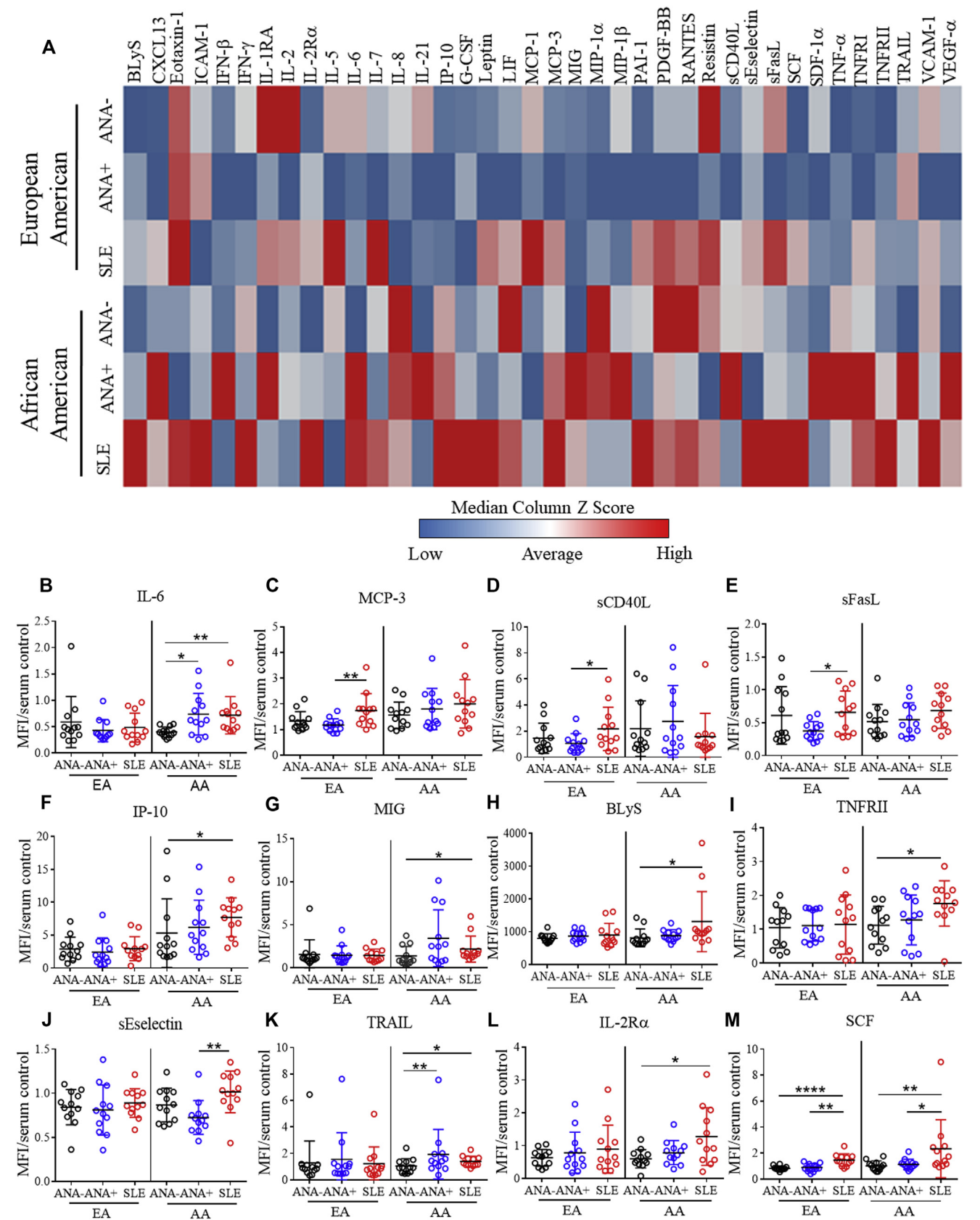


FIG 5. SCF distinguishes patients with SLE from ANA+ healthy individuals in both EAs and AAs. Proinflammatory soluble mediators were measured by multiplex or ELISA. **A**, A heatmap summary of the plasma levels for each individual are shown. Soluble mediator levels are displayed on a color scale ranging

However, EBV, CMV, and HSV infection did not correlate with T-cell numbers in EA individuals (Fig E8, B-D). No correlations were observed between reduced systemic soluble mediators and viral positivity; however, IFN- β levels were higher among CMV+ individuals (Fig E8, E). CD4⁺ and CD8⁺CD57⁺ T cells, a marker of replicative senescence, were also elevated among CMV+ healthy subjects and patients with SLE, which may influence disease symptoms and outcome.

Suppressed immune phenotype does not correlate with ANA specificity

Although we strongly attempted to perfectly match all subjects within this study, some differences remained. Differences were primarily found within the AA patients with SLE who, when compared with the EA patients with SLE and controls, had a trend higher in age, disease activity, and slightly more DNA-specific autoantibodies (Tables E4 and E5, see Fig E9 in this article's Online Repository at www.jacionline.org). In an attempt to control for these differences, linear regression analyses were done with age and SLEDAI instrument score in patients with SLE between all significant differences in the study. When all patients with SLE were assessed, a positive correlation was observed with CD4⁺ memory T-cell numbers and CD4⁺ T_H17-type T cells and SLEDAI instrument score (see Fig E10 in this article's Online Repository at www.jacionline.org), suggesting these cell subset increases may be influenced by higher SLEDAI instrument score in AA patients with SLE. Furthermore, AA patients with SLE also had HLA-DR^{Hi} expression on transitional and naive B cells that positively correlate with SLEDAI instrument score (Fig E10). Soluble E-selectin was the only finding that positively associated with age in AA patients with SLE (see Fig E11 in this article's Online Repository at www.jacionline.org).

Finally, ANA+ healthy subjects or patients with SLE were grouped by ANA specificity, either DNA- or RNA-specific autoantibodies. No significant differences were observed in either the ANA+ healthy individuals or the patients with SLE in the significant findings of this study.

Data availability

All RNA-sequencing data that support the findings of this study have been deposited in National Center for Biotechnology Information Gene Expression Omnibus and are accessible through Gene Expression Omnibus series accession number GSE138400. The authors declare all other data supporting this study are available within the paper and the Online Repository; in addition, all data files are available on reasonable request from the corresponding author.

DISCUSSION

Understanding the early cellular events that occur during preclinical autoimmunity in the absence of confounding

medication use is valuable for delineating important pathways in lupus pathogenesis. Studying healthy individuals with lupus-associated autoantibodies, in particular, is crucial for identifying not only early cellular dysregulation, but also regulatory pathways that may prevent clinical disease development and progression in most individuals. Our study provides the most comprehensive analysis of ANA+ healthy individuals to date.

Individuals with AA ancestry are at a higher risk for developing autoimmune diseases, such as SLE, than are those with EA ancestry.⁹ We discovered a unique and potentially protective immune profile associated with asymptomatic autoimmunity in EA individuals. Unlike EA ANA- healthy controls, EA ANA+ healthy individuals had a suppressed immune profile with decreased T-, NK-, and NK T-cell numbers, a trend lower in cytokine levels, dysfunctional T-cell signaling, and altered T-cell transcriptional profiles. Although lymphopenia is common in patients with SLE and often associated with disease activity,³⁵ no EA ANA+ healthy subjects demonstrated or reported lymphopenia or leukopenia. T-cell numbers also did not correlate with ANA titers.

Although the suppressed immune features in EA ANA+ healthy are reminiscent of certain viral infections,^{32,33} no correlations were found with herpesvirus seroconversion or seroreactivation. However, an expanded panel to assess comprehensive infection history and chronic low-level viral infection could uncover possible viral involvement in immune suppression. Whether these EA ANA+ individuals are more susceptible to infection is unknown, but heightened TCR phospho-signaling suggests a normal or elevated T-cell immune response to stimuli. Reduced T-cell numbers may arise from a number of mechanisms, but we observed altered gene expression of apoptosis pathways and altered cytokine profiles, which could contribute to the decreases in circulating cell populations.³⁶

Suppression may be a form of regulation in response to early autoreactivity or a pathogenic result of unseen immune activation in EA ANA+ healthy individuals. However, elevated levels of the inhibitory receptor CD85j, which is expressed on DCs, monocytes, and T cells, and a lack of elevated activation markers on B cells and pDCs in EA ANA+ healthy individuals suggest that enhanced regulatory pathways contribute to a suppressed immune phenotype. Furthermore, disruption of regulatory pathways, such as programmed cell death 1, is associated with autoimmune disease development, and upregulation of these exhaustion receptors are associated with better disease outcomes.³⁷⁻³⁹ The absence of this suppression signature in AA ANA+ healthy individuals may be attributed to the known stronger inflammatory responses in individuals of African ancestry,⁴⁰ which could contribute to more ANA+ subjects transitioning to SLE, or progressing more rapidly to SLE.

Previous work in ANA+ healthy individuals, compared with ANA- controls, has found elevated CD86 expression on B cells and elevated T_H follicular and regulatory T cell frequencies.⁸

from blue (protein levels below the mean) through white (protein levels equal to the mean) to red (protein levels greater than the mean) using a column Z-score. Significant cytokines included (B) IL-6, (C) monocyte chemoattractant protein 3 (MCP-3), (D) soluble CD40 ligand (sCD40L), (E) soluble Fas ligand (sFasL), (F) induced protein 10 (IP-10), (G) monokine induced by IFN- γ (MIG), (H) BLYS, (I) TNFR11, (J) sEselectin, (K) TNF-related apoptosis-inducing ligand (TRAIL), (L) IL-2R α , and (M) SCF. * $P < .05$, ** $P < .01$. Kruskal-Wallis test with 2-tailed Mann-Whitney for multiple comparisons. Mean $\pm$ SD shown.

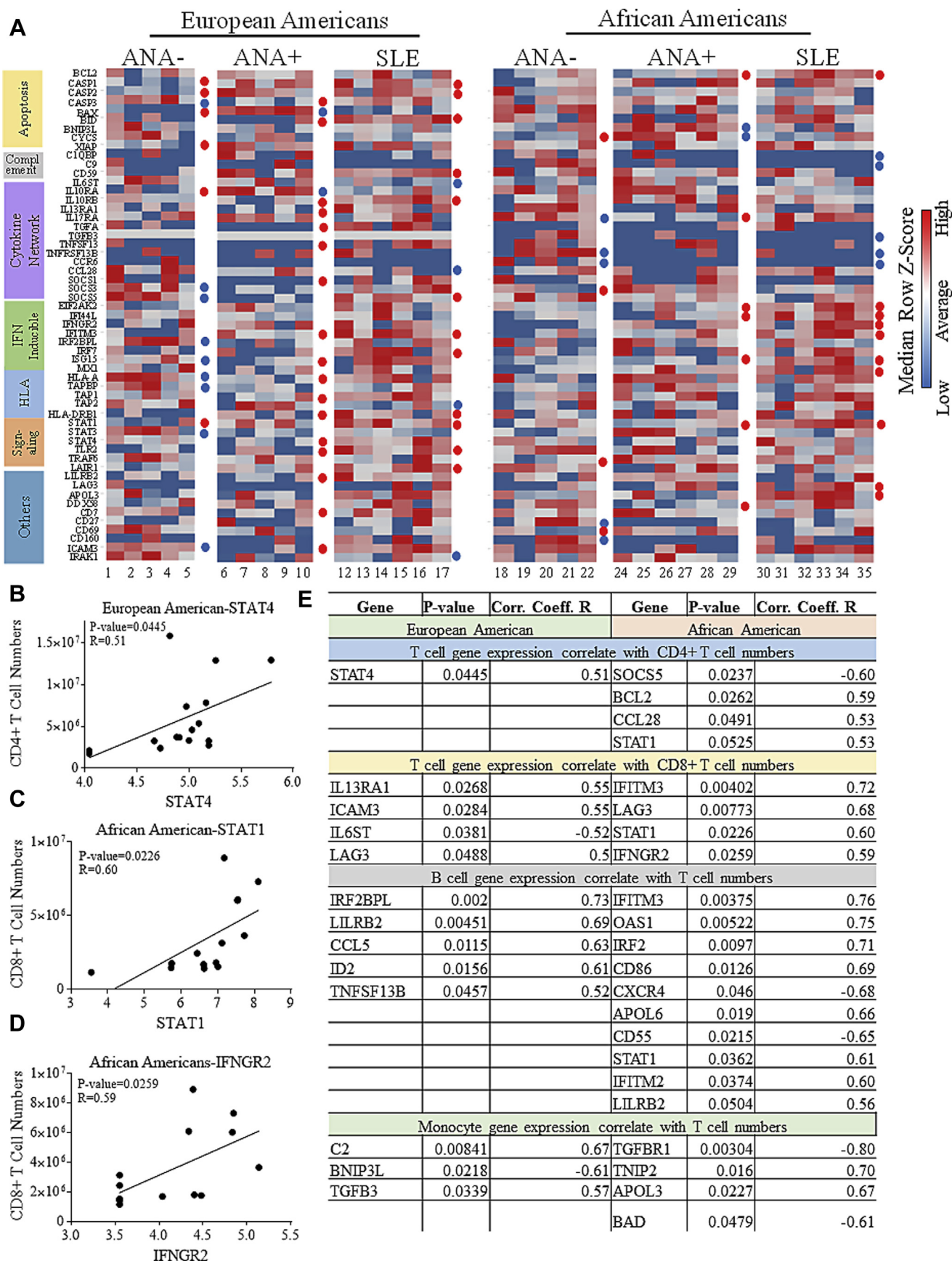


FIG 6. EA ANA+ healthy individuals exhibit a gene expression signature in T cells consistent with virus-induced immune evasion. RNA-sequencing of PBMCs for 36 subjects were used to assess differences

Although this differs from our results, sample recruitment differed between studies, with ANA+ healthy subjects being referred to rheumatology clinics for positive ANA (using IIF with ANA $\geq 1:160$) in the prior study.⁸ ANA testing was performed for various reasons including noninflammatory arthritis/arthritis (41%), family history of autoimmune disease (7%), urticarial/nonspecific rash (7%), and recurrent miscarriages or a child with neonatal lupus (13%), with some subjects already taking hydroxychloroquine (8.2%). In the current study, asymptomatic subjects were recruited through health fairs and ANA-positivity was determined by BioPlex and confirmed by either ELISA or IIF. Furthermore, almost one half of healthy individuals were ethnic minorities in the previous study, which may contribute to a more activated profile.⁴¹ The importance of expanding and assessing ANA+ healthy individuals longitudinally cannot be understated, as determining whether ANA-positivity is maintained and whether EA ANA+ healthy individuals with a suppressed immune profile will ever develop disease, or whether this signature changes to a more activated profile seen in higher risk subjects, is of significant interest.

ANA-positivity can be determined by a number of methods, and more recently testing ANA+ subjects by various ANA assays has identified discordant results.⁴²⁻⁴⁴ To aid in interpretation and future discussion, we assessed our subjects by 3 different methods (BioPlex, ELISA, and IIF) with all subjects ANA+ by BioPlex and 1 other method, with exception of 1 RNP+ AA individual who was only positive by BioPlex (specificity was confirmed by INNO-LIA [Innogenetics NV, Belgium]). The discordance of ANA testing results has led to questions of whether healthy subjects with BioPlex ANA-positivity, which is a specific indicator for RNA- and DNA-specific autoantibodies common to rheumatic diseases, but who are IIF- false positives or subjects captured early in the disease process. To address this, Pérez et al⁴⁵ followed 411 healthy Mediterranean subjects that were positive according to BioPlex and negative according to IIF for 3 years. At follow-up, 76% of subjects were positive by IIF, and 87% had developed a classified autoimmune disease, which suggested BioPlex has a greater sensitivity for autoimmune-specific antibody detection. Thus, although other ANA+ healthy studies have used ELISA or IIF as indicators of positivity, we used BioPlex as a primary indicator of early autoimmune-specific ANA-positivity.^{8,17} Longitudinal studies assessing these results in other ethnic populations will assist in the understanding and importance of changes in ANA-positivity by different methodology prior to disease diagnosis or lack thereof.

Other notable differences in ANA+ healthy individuals are derived from their resemblance to patients with SLE or their lack thereof. We noted SCF, BlyS, IL-12p40, and type I interferon levels were significantly elevated only in patients with SLE when compared with ANA+ healthy individuals.⁷ We found that SCF

was the only cytokine significantly elevated in both EA and AA patients with SLE in this study. SCF is most commonly known as a niche component for hematopoietic stem cell renewal and for driving the development and survival of mast cells.⁴⁶⁻⁴⁸ More recently, c-kit (the receptor for SCF), was found to be expressed by NK cells and DCs in the periphery, suggesting an important role for SCF in these cell types.^{49,50} DCs produced more IL-6 following SCF/c-kit signaling, which drives expansion of T_H2 and T_H17 immune responses, an environment commonly associated with SLE.⁵¹ Furthermore, we found patients with SLE had strong interferon signatures among various cell types, and elevated interferon-associated soluble mediators were more visible in AA patients. IL-6, IFN- γ and T_H2 cytokines were previously found to be the first soluble mediators to increase in healthy subjects who transition to SLE.⁶ We found IL-6 was already elevated in AA ANA+ healthy individuals and increased T-cell numbers correlated with elevated gene expression in types I and II interferon signaling pathways in patients with SLE, suggesting dysregulation of these cytokines contributes to autoimmune pathogenesis.

The current study is not without its limitations. The cross-sectional study design allows us to only capture the immune profile of these subjects at 1 time point. A secondary cohort with a larger sample size to confirm the findings of this study and a longitudinal assessment of ANA+ healthy individuals will be important in verifying the findings and the changes in this immune profile over time and up to the point of transition. Furthermore, the use of established patients with SLE with controlled disease in this study may contribute to differences in the immune profile versus patients with newly onset or active disease.

Collectively, our findings highlight the importance of race on early autoimmune profiles and identify a novel immune endotype with hallmarks of suppression in EA ANA+ healthy individuals. The racial differences in early autoimmune regulation likely influence which individuals may transition to SLE or other classified autoimmune diseases and offer potential pathways to target for disease prevention.

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Clinical implications: A protective immune signature identified in preclinical autoimmunity may be pivotal in finding novel autoimmune disease therapeutic targets and in identifying those at greatest risk for transition to clinical disease.

in inflammatory and regulatory associated gene expression among groups. **A**, A heatmap summary of individual gene expression in T cells is shown. Significant differences between groups are indicated by dots between columns. Genes significantly upregulated are indicated by a red dot and significantly downregulated by a blue dot. Dots between columns 1 and 2 indicate a significant difference between ANA- and ANA+ individuals, between columns 2 and 3 indicate significant differences between ANA+ individuals and patients with SLE, and after column 3 indicate significant differences between ANA- individuals and patients with SLE. Correlations between T-cell numbers and gene expression were conducted between significant T-cell, B-cell, and monocyte genes. Significant correlations were observed between **(B)** CD4⁺ T-cell numbers and *STAT4* T-cell gene expression in EAs, and **(C)** *STAT1* and **(D)** *IFNGR2* T-cell gene expression and CD8⁺ T-cell numbers of AAs. All other significant correlations are recorded in the table **(E)**. *Corr Coeff*, correlation coefficient.

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Research Paper

Adults with systemic lupus exhibit distinct molecular phenotypes in a cross-sectional study

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ABSTRACT

Background: The clinical and pathologic diversity of systemic lupus erythematosus (SLE) hinders diagnosis, management, and treatment development. This study addresses heterogeneity in SLE through comprehensive molecular phenotyping and machine learning clustering.

Methods: Adult SLE patients ($n = 198$) provided plasma, serum, and RNA. Disease activity was scored by modified SELENA-SLEDAI. Twenty-nine co-expression module scores were calculated from microarray gene-expression data. Plasma soluble mediators ($n = 23$) and autoantibodies ($n = 13$) were assessed by multiplex bead-based assays and ELISAs. Patient clusters were identified by machine learning combining K-means clustering and random forest analysis of co-expression module scores and soluble mediators.

Findings: SLEDAI scores correlated with interferon, plasma cell, and select cell cycle modules, and with circulating IFN- α , IP10, and IL-1 α levels. Co-expression modules and soluble mediators differentiated seven clusters of SLE patients with unique molecular phenotypes. Inflammation and interferon modules were elevated in Clusters 1 (moderately) and 4 (strongly), with decreased T cell modules in Cluster 4. Monocyte, neutrophil, plasmablast, B cell, and T cell modules distinguished the remaining clusters. Active clinical features were similar across clusters. Clinical SLEDAI trended highest in Clusters 3 and 4, though Cluster 3 lacked strong interferon and inflammation signatures. Renal activity was more frequent in Cluster 4, and rare in Clusters 2, 5, and 7. Serology findings were lowest in Clusters 2 and 5. Musculoskeletal and mucocutaneous activity were common in all clusters.

Interpretation: Molecular profiles distinguish SLE subsets that are not apparent from clinical information. Prospective longitudinal studies of these profiles may help improve prognostic evaluation, clinical trial design, and precision medicine approaches.

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1. Introduction

Systemic lupus erythematosus (SLE) is characterized by remarkable clinical and pathogenic diversity, which hinders prompt diagnosis, accurate prognosis, and the optimization of therapies. Molecular profiles could be used to better understand the underlying disease processes of SLE, define features that are shared or unique in different

categorical subsets, and advance precision medicine approaches to trial designs and clinical management.

Dozens of candidate molecules with robust scientific rationale have been tested in either a broad range of SLE patients or in clinically defined subsets, such as active nephritis or cutaneous lupus patients [1]. These trials have almost universally failed to meet their pre-specified primary outcomes. Although multiple factors contribute to the failure of lupus trials [2,3], many treatments have shown efficacy in secondary endpoints or exploratory analyses [1]. Moreover, an exploratory analysis revealed differential effects of standard of care medications on the expression of genes that represent the targets for treatments currently under development. These effects varied, not only by the standard of care medication, but also by

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Research in context

Evidence before this study

Clinical and immunologic heterogeneity in systemic lupus erythematosus (SLE) hinders effective clinical trials and treatment optimization. Recent studies have used autoantibody profiles, whole blood gene expression profiles, or immunophenotyping individually to group SLE patients into more homogenous subsets. In a large, longitudinal cohort of pediatric SLE patients, Banchereau and colleagues identified transcriptional modules that change over time with disease activity and identify clinical subsets of pediatric SLE patients. However, these approaches have not yet been tested and confirmed in adult SLE patients, nor have the intricacies of immune dysregulation been distilled into a robust tool that can be readily applied in clinical trials or in clinical care for individual patients.

Added value of this study

This study establishes an approach that identifies seven phenotypic clusters of adult SLE patients by incorporating multiple types of immunologic data through machine learning. This analysis leveraged a large, diverse, and carefully-characterized cohort to support the application of these findings to a broader population of adult patients. Disease activity and clinical manifestations varied between clusters, with certain clusters enriched for more severe disease manifestations. Clinical features that are often used to select patients for clinical trials, such as arthritis or lupus nephritis, were present across multiple clusters with distinct patterns of immune activation.

Implications of all available evidence

Multi-dimensional molecular and immunological profiles distinguish unique subsets of SLE patients that are not apparent based on clinical information, thus laying the foundation for precision medicine in lupus treatment and clinical trials.

learning approaches to cluster adult SLE patients according to their molecular phenotypes, and evaluated demographic and clinical features enriched in each cluster.

2. Methods

2.1. Patients and samples

Study procedures followed were in accordance with the ethical standards of the OMRF Institutional Review Board and with the revised Helsinki Declaration of 2000. This study was approved by the Institutional Review Board of the Oklahoma Medical Research Foundation, and all participants provided written informed consent prior to study-specific procedures. A subset of the Oklahoma Cohort for Rheumatic Disease (OCRD) at the Oklahoma Medical Research Foundation comprises 198 SLE patients meeting SLE classification by the 1997 update to the 1982 ACR criteria [21], with recruitment beginning in 2001. This subset of SLE patients from the OCRD had an average of 13 visits per subject (total cohort visits 2585, range 2–34 visits). Disease activity was measured at each visit by the modified SELENA-SLE Disease Activity Index (mSELENA-SLEDAI) [22]. SLEDAI scores ≥ 4 were considered high, and SLEDAI scores < 4 were considered low. Efforts were made to minimize bias based upon concurrent medications by the elimination of patients who had recently (within 1 year) taken rituximab, cyclophosphamide, or pulse IV steroids. In addition, samples were tested in a blinded fashion by technical personnel who were performing and initially analyzing the data. The sample size was also large enough to minimize bias. Non-autoimmune rheumatic disease controls from the Oklahoma Immune Cohort were cohort-matched for age (within 5 years), race, gender, and time of sample procurement, such that matched samples were stored at -80°C for the same length of time prior to the assays ($n = 48$) (see Supplemental Table 1 for demographics).

Blood was collected into PAXgene blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) for gene expression profiling. On the day of collection, the College of American Pathologists certified sample processing and biorepository core at OMRF (CAP# #9418302) isolated undiluted plasma from heparin tubes and undiluted serum from serum tubes, and froze aliquots at -80°C . Assays were performed on freshly thawed samples to maximize consistent detection of analytes, even when present in the pg/mL range.

2.2. Gene expression profiling

Total cellular RNA was isolated and purified from PAXgene tubes (PAXgene Blood RNA kit, Qiagen Inc, Valencia, CA). RNA quality and quantity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After depletion of globin mRNA and ribosomal RNAs (Globin-Zero Gold kit, Illumina, San Diego, CA, USA), RNA was amplified, in vitro transcribed, and labeled using the Illumina Bead-Expression Kit for Illumina Human HT-12 v4.0 whole genome expression chip, and cDNA was hybridized to the chips. Chips were scanned using the Illumina iScan system. Quality control of gene expression data was performed with GenomeStudio Version 2011.1 (Illumina) per manufacturer protocol. Background-subtracted expression data were log2 transformed and normalized with the rank-invariant method using the lumiR package [23]. System-based modular co-expression analysis was performed calculating the individual-level modular co-expression network scores (M1-M6) for each patient relative to the aggregate of controls, using second generation modular frameworks as described [18,24–26]. These modular scores were then used as individual variables in subsequent analyses described below.

underlying patient immune phenotype [4]. These observations suggest that a systematic approach is needed to address lupus heterogeneity by subdividing patients into subsets with shared immunologic and genomic characteristics.

Historical approaches to address heterogeneity have employed autoantibody profiles [5–7], clinical patterns [8–10], whole blood gene expression profiles [11–13], or immunophenotyping [14] individually to group SLE patients into more homogenous subsets; however, these approaches individually have not yet been very useful in sorting through the complex array of data acquired in clinical trials [15–17] or in developing optimized clinical care. In a large, longitudinal cohort of pediatric SLE patients, Banchereau and colleagues [18] identified transcriptional modules that change over time with disease activity and identify clinical subsets of pediatric SLE patients. In addition, our group has identified soluble mediators, including cytokines, chemokines, adhesion molecules, and soluble receptors that associate with risk of SLE disease flare [19,20]. Although the specific pathways that distinguish an impending disease flare differ somewhat between European American and African American cohorts and likely vary between individual patients, an assay that simultaneously surveys multiple immune pathways accurately identified SLE patients at higher risk of future enhanced disease activity [19,20].

Together, these findings demonstrate the value of high-dimensional data for understanding the diversity of pathogenic mechanisms in SLE and the relevance of molecular phenotypes to clinical studies and patient care. This cross-sectional study used machine

2.3. Autoantibody detection

Serum antinuclear autoantibodies were measured by indirect immunofluorescence against Hep-2 cells in the College of American Pathologists -certified Morris Reichlin Clinical Immunology Laboratory at OMRF (CAP# 2036101) [27]. ANAs present at $\geq 1:120$ dilution were considered positive. Autoantibodies were also detected using bead-based, multiplex assays on a Bio-Rad BioPlex 2200 platform (Bio-Rad Technologies, Hercules, California, USA) as previously described [28]. Antibodies against chromatin, ribosomal P, Sm, SmRNP, nRNP composite (nRNP A and/or nRNP 68), Ro/SSA composite (52 kDa Ro and/or 60 kDa Ro), La/SSB, centromere B, Scl-70, and Jo-1 were quantified as an antibody index (AI) value based on the fluorescence intensity of each of the autoantibody specificities, with a manufacturer-recommended positive cutoff of $AI \geq 1$ (range 0–>8). Anti-dsDNA was quantified in IU/mL with a manufacturer-recommended positive cutoff of 10 IU/mL.

2.4. Soluble mediator detection

Plasma levels of B lymphocyte stimulator (BlyS) and a proliferation-inducing ligand (APRIL) were measured by enzyme linked immunosorbent assays (ELISAs) per manufacturer protocol (Human BAFF/BlyS/TNFSF13B Quantikine ELISA, R&D Systems, Minneapolis, MN, USA; and Human TNFSF13/APRIL ELISA, eBioscience/Affymetrix, San Diego, CA).

Plasma levels of other soluble mediators were assessed using a custom, multiplex panel (ProcartaPlex, Thermo Fisher Scientific/Invitrogen) on the Bio-Rad Bioplex 200 Luminex xMAP plate reader (Bio-Rad Technologies) as previously described [19]. This panel uses an optimized and validated multiplex design and pre-purchase quality controls to maximize assay specificity and sensitivity, particularly for analytes present in low quantities. A bridge control serum sample was included on each plate (Collect human AB serum, Cat#2931949, Lot#Q8823, MP Biomedicals, Solon, OH, USA) to control for plate-to-plate variation of soluble mediator assays. The mean inter-assay coefficient of variance (CV) of these assays (10.5%) was within that previously shown for bead-based assays [29]. Limit of blank, limit of detection, and limit of quantification were determined and used for quality control as previously described [30]. Analytes with >60% of samples below the limit of detection were excluded from subsequent analyses. Analytes passing quality control included IFN- α , IFN- γ , IP10, MCP-1, MIG, MIP-1 α , MIP-1 β , TRAIL, TWEAK, sCD-40 ligand, TNFR I, TNFR II, IL-10, IL-17A, IL-2, IL-2RA, IL-12p70, IL-21, IL-1 α , ICAM-1, and SCF. Concentrations were interpolated from 5-parameter logistic nonlinear regression standard curves, or assigned a value of 0 if a sample was below the limit of detection.

2.5. Statistical analysis

Variables with <60% missing data were retained for univariate analyses. Quantitative variables were compared by Kruskal Wallis test, and categorical variables by Chi-square or Fisher's exact test, as appropriate, with Bonferroni adjustment for multiple corrections. No imputation was performed. Instead, samples with missing data were excluded from analyses requiring complete data, and retained for other analyses. Additionally, four outlier samples were removed from downstream clustering analyses. After these criteria, 290 samples from 194 patients were used for final analyses of molecular clusters. All variables including modular co-expression scores, autoantibody and cytokine concentrations were used for univariate, Pearson correlation and multivariate analysis in R 3.3-2.

To identify patient clusters using the informative molecular and cytokine variables, clustering and regression were performed using the unsupervised randomForest module (version 4.6–12) in R (<https://cran.r-project.org/>) with mtry = the square root of the

number of variables, ntree = 2000, and dissimilarity matrix defined as $\sqrt{1 - \text{similarity}}$, where the similarity matrix was stabilized by averaging similarities generated by 100 random forest unsupervised clustering models. The dissimilarity matrix was then reduced to three principle components using the t-Distributed Stochastic Neighbor Embedding (t-SNE) R package to minimize information loss compared to conventional dimensional reduction analysis. The first two principle components were used for K-mean clustering with n-max of 20 using pamK R package.

2.6. Data sharing

Microarray data will be available through GEO (accession number GSE138458). Other data generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

3. Results

3.1. Characteristics of the study cohort

From the Oklahoma Cohort for Rheumatic Disease, this study identified 196 unique SLE patients with at least one sample that passed predefined quality control metrics for molecular phenotype data. Of these, 98 patients provided samples at two visits, which mainly had different disease activity scores (Supplemental Table 1). Of the 196 participants in this study, 89.8% were female. The racial composition was 46.9% European American, 23.0% African American, 12.2% American Indian, 11.2% Hispanic, 5.6% Asian, and the rest more than one race (Supplemental Table 1). All patients in this study were ANA positive by medical record review.

3.2. Correlations among co-expression module scores, soluble mediator levels, and SLEDAI scores

Transcriptional co-expression module scores were calculated from Illumina microarray gene expression data for previously defined immune pathway related modules [24]. Module scores showed the expected correlations (p -values <0.05) with other modules and with soluble mediator levels based on previously established immunological pathways. For example, inflammation module scores significantly correlated with each other and with the neutrophil module score. Interferon modular scores strongly correlated with one another and with levels of interferon associated soluble mediators, such as IFN α , IFN γ , IP-10 (CXCL10), MIG, BlyS, and TNFR II. Interferon module scores also significantly correlated with inflammation module scores, as well as with levels of inflammatory mediators, such as MCP-1, MIP-1 α , MIP-1 β , IL-1 α , and other cytokines (e.g. IL-2RA, IL-21, IL-12p70, IL-2 and IL-10 (Supplemental Fig. 1). B cells module scores moderately correlated with TRAIL, sCD-40 ligand, IL-21, and stem cell factor, but negatively correlated with IL-10 and TNFR I (Supplemental Fig. 1).

Total SLEDAI scores positively correlated with interferon ($r > 0.2$) and plasma cell ($r = 0.25$) module score in this adult lupus collection (Supplemental Fig. 1). Levels of IFN α , IP-10, and IL-1 α also correlated with SLEDAI scores (Supplemental Fig. 1; p -value <0.05; r range from 0.11 to 0.27). Together, these correlations demonstrate the internal validity of these datasets, the expected coordinated regulation of the expression of these markers, and correlation with clinical disease activity.

3.3. Expression modules and soluble mediators stratify SLE patients into seven subsets

To enable more precise patient stratification for future clinical studies, clinical trial designs, potential prognosis and improved clinical care, seven patient subsets with distinct molecular phenotypes were identified by k-means clustering with a t-SNE reduced random

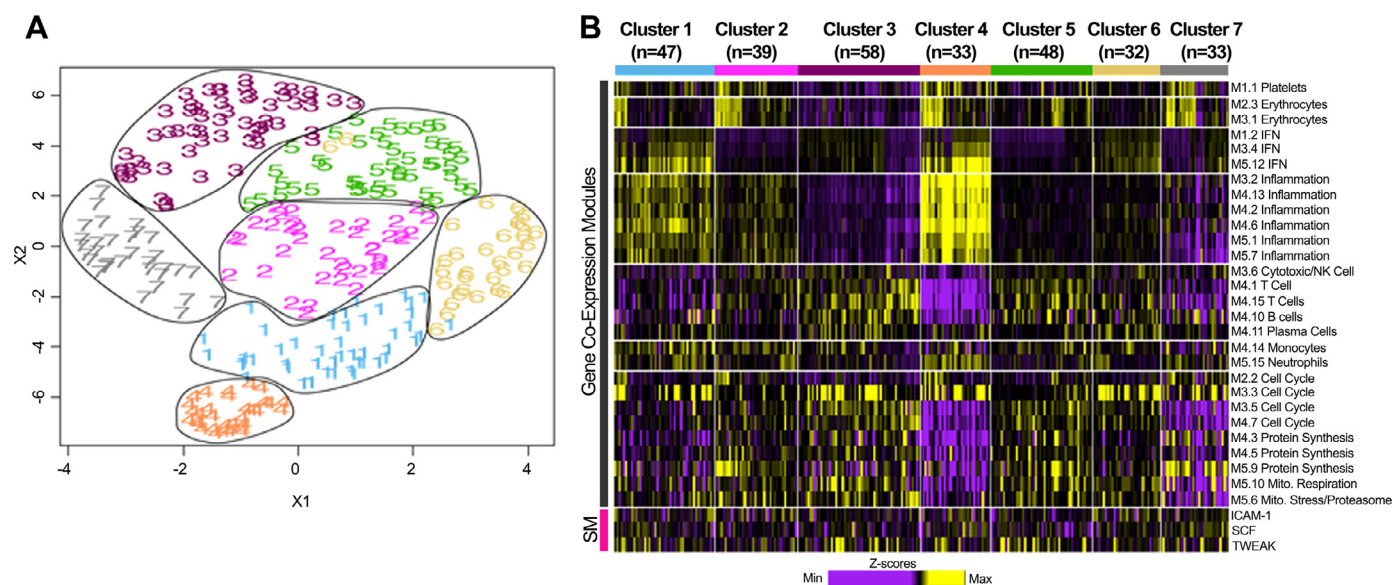


Fig. 1. Gene expression modules and soluble mediators stratify SLE Patients into seven molecular phenotypic subsets. (A) Unique patterns of gene co-expression modules and soluble mediators (SM) distinguished seven phenotypic clusters of SLE patients, indicated by colored numbers in the plot. X1 and X2 indicate the top two principal components defined by tSNE on a random forest dissimilarity matrix. These components were used in k-means clustering to identify the seven clusters. (B) The heat map presents the informative gene expression modules and soluble mediators used in clustering (see methods). Each row is a gene expression module or soluble mediator and each column is a patient. Colors indicate row z-scores, from purple (low) to yellow (high). The seven clusters are color coded as in other figures, and the number of samples in each cluster are indicated.

forest dissimilarity matrix of soluble mediators and previously defined co-expression modules [18,24–26] (Fig. 1(A)). Three clusters (Clusters 1, 4, and 6) demonstrated substantially higher IFN modular scores compared to the other subgroups. Another three clusters (Clusters 2, 3, and 5) demonstrated predominant lymphoid and monocytoid modular scores. One cluster (Cluster 7) showed minimal activation of interferon, lymphoid, neutrophilic and monocytoid related modules. Demographics varied between the clusters (Table 1, Supplemental Fig. 2). A higher percentage of European American patients were in Cluster 5 (24.4%) (Supplemental Fig. 2). African American patients ($n = 69$) were most often in Cluster 7 (27.5%) or Cluster 3 (24.6%), and were rarely in Cluster 1 (5.8%). American Indian patients had the highest frequency of patients in Cluster 1 (37.1%).

Clusters 1 and 4 were characterized by significant elevation of inflammation module scores, along with elevations in the interferon

module scores (Figs. 1(B) and 2(A)). Cluster 4 also had the highest neutrophil module scores (Figs. 1(B) and 2(A)) and the highest levels of soluble IP-10, MIG, APRIL, TNFR1/II, and IL-10 (Fig. 2(B)). Cluster 1 demonstrated only moderate levels of each of those variables, but had the highest levels of IL-21, IL-17A, and MIP-1 β (Fig. 2(B)). Cluster 6 had the highest levels of IL-1 α and IL-2RA. The distinguishing features based on relative expression of the gene co-expression modules and soluble mediators are shown in Fig. 2. Variations in the molecular phenotypes among these clusters suggest that different groups of lupus patients may have different active immune pathways at a given time point, or different levels of activation of particular pathways. These differences may be due to altered transcriptional regulation within cells, or differing frequencies of certain cell populations. These results suggest that distinct directed therapeutics or combination treatments may be needed to target both shared and cluster-specific immune dysregulation.

Table 1
Participant demographics and medication usage by cluster.

Cluster Number	1	2	3	4	5	6	7
Total N*	47	39	58	33	48	32	33
Female, n (% of cluster)	41 (87.2)	35 (89.7)	54 (93.1)	29 (87.9)	46 (95.8)	28 (87.5)	30 (90.9)
Age in years, mean	38.6	43.1	42.5	44.3	42.9	41.3	44.7
Race, n (% of cluster)							
European American	14 (29.8)	24 (61.5)	28 (48.3)	15 (45.5)	32 (66.7)	9 (28.1)	9 (27.3)
African American	4 (8.5)	9 (23.1)	19 (32.8)	6 (18.2)	7 (14.6)	7 (21.9)	17 (51.5)
American Indian	13 (27.7)	3 (7.7)	3 (5.2)	3 (9.1)	5 (10.4)	5 (15.6)	3 (9.1)
Asian	6 (12.8)	0 (0)	5 (8.6)	4 (12.1)	1 (2.1)	3 (9.4)	0 (0)
Hispanic	8 (17)	3 (7.7)	3 (5.2)	5 (15.2)	3 (6.3)	8 (25)	4 (12.1)
Mixed Race	2 (4.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Medication use, n (% of cluster)							
Hydroxychloroquine	30 (63.8)	31 (79.5)	40 (69)	19 (57.6)	37 (77.1)	22 (68.8)	21 (63.6)
Mycophenolate Mofetil	18 (38.3)	5 (12.8)	3 (5.2)	4 (12.1)	4 (8.3)	3 (9.4)	4 (12.1)
Azathioprine	9 (19.1)	7 (17.9)	10 (17.2)	4 (12.1)	4 (8.3)	7 (21.9)	5 (15.2)
Methotrexate	5 (10.6)	9 (23.1)	8 (13.8)	7 (21.2)	12 (25)	1 (3.1)	4 (12.1)
Cyclophosphamide	1 (2.1)	0 (0)	0 (0)	4 (12.1)	0 (0)	0 (0)	0 (0)
Rituximab	1 (2.1)	0 (0)	0 (0)	1 (3)	2 (4.2)	0 (0)	0 (0)
Steroids	27 (57.4)	10 (25.6)	22 (37.9)	27 (81.8)	16 (33.3)	14 (43.8)	15 (45.5)
Prednisone dose (mg), median (interquartile range)	0 (0, 10)	0 (0, 0)	0 (0, 0)	10 (5, 25)	0 (0, 0)	0 (0, 5)	0 (0, 10)

* Number of samples; total of 290 samples from 194 individuals.

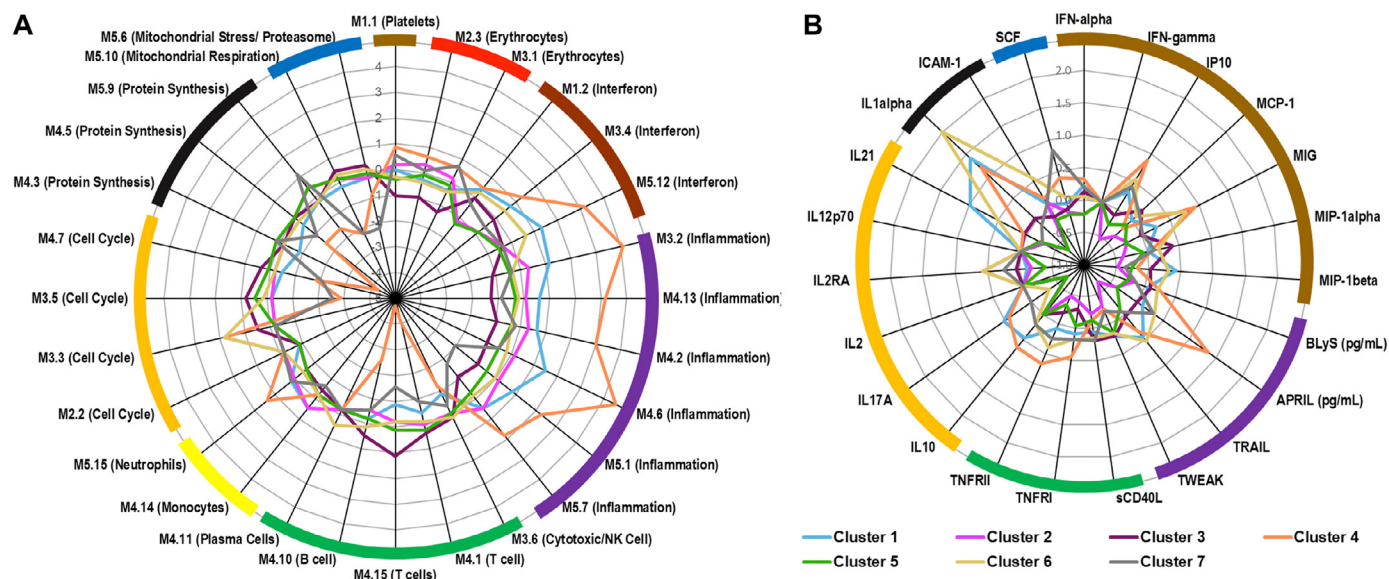


Fig. 2. Molecular profiles of seven SLE patient clusters. Radar plots show modified z-scores of relative gene expression module scores (A) and plasma soluble mediator levels (B) in each of the molecularly-defined patient clusters, indicated by colored lines as shown in the legend at bottom right. Modules and soluble mediators are grouped by function, indicated by colored arcs around each plot and labeled with the co-expression module name (A) or with the soluble mediator tested (B).

3.4. Clinical features of molecularly defined patient clusters

Every cluster included patients with higher disease activity, as well as patients with less active disease. Although there were trends towards increased SLEDAI scores in Clusters 1 and 4, there were no statistically significant differences between clusters (Fig. 3(A)). Clinical SLEDAI scores, calculated without the complement and DNA binding components, were also not statistically significant between clusters, but trended higher in Clusters 3 and 4 (Supplemental Table 2 and Supplemental Fig. 3). Furthermore, patients grouped by anti-dsDNA and complement status show substantial variability in expression modules and soluble mediators (Supplemental Fig. 4). When considering SLEDAI organ systems, each system was active in patients from multiple molecular clusters (Fig. 3(B), Supplemental Fig. 5). For example, patients with renal involvement were evenly divided between the molecularly distinct Cluster 1 ($n = 5$; 25% of renal patients), Cluster 3 ($n = 6$, 30%), and Cluster 4 ($n = 6$, 30%), with additional patients from Clusters 2 and 4 (Fig. 3(B)). Among the SLEDAI components, low complement was significantly more common in Cluster 1 than in Clusters 2 or 5 (Bonferroni-corrected $p < 0.0021$) (Fig. 3(C), Supplemental Table 3). Increased DNA binding was significantly more common in Clusters 1 and 4 than in Cluster 2 (Bonferroni-corrected $p = 0.042$ for both) (Fig. 3(C), Supplemental Table 3). Anti-dsDNA positivity and low complement levels trended higher in Clusters 1, 3, 4, and 6 (Supplemental Table 3). Other active lupus manifestations that commonly drive the selection of patients for clinical trials, such as musculoskeletal and mucocutaneous manifestations, occurred in nearly equal frequencies across all seven clusters (Fig. 3(C); Bonferroni-corrected $p > 0.05$) (Supplemental Table 3).

Cumulative ACR criteria, representing a history of organ activity, were also compared across the clusters (Supplemental Figure 6). Overall, cumulative criteria were quite similar across all of the clusters, with each criterion being represented in each cluster. Immunologic criteria were found in 75% or more of patients across all clusters, whereas renal disorder trended to more frequent in Clusters 1 and 4.

Medication use also differed somewhat between certain clusters (overall $p = 0.0001$). Frequency of steroid use was highest in Cluster 4 (Table 1). Prednisone dose was highest in Cluster 4 (all Bonferroni-corrected $p \leq 0.0092$ vs. each cluster, by Kruskal–Wallis); higher in Cluster 1 than in Clusters 2, 3, and 5 (Bonferroni-corrected $p = 0.0216$, 0.0050, and 0.0289, respectively), and higher in Cluster 7

than Cluster 3 (Bonferroni-corrected $p = 0.0428$) (Table 1, Supplemental Table 4, and Supplemental Table 5). Rates of mycophenolate mofetil use were approximately three times higher in Cluster 1 (38%) than any other cluster (5.2–13%). Hydroxychloroquine use was common across all clusters, but highest in Clusters 2 (79.5%) and 5 (77.1%) and lowest in Cluster 4 (57.6%).

3.5. Autoantibody profiles in molecularly defined SLE patients clusters

Autoantibody profiles were compared by autoantibodies against common lupus RNA- and DNA-binding proteins, such as chromatin, nRNP, Sm and Ro (Supplemental Fig. 7). Each autoantibody was present in patients across multiple clusters. For example, anti-dsDNA and anti-RNA-binding protein autoantibodies were found in clusters 1, 4, 6, and 7, which are molecularly quite different. Further, the levels of anti-dsDNA were nearly equivalent between these clusters. Clusters 2 and 5 had the lowest levels of all autoantibodies compared to the other clusters. Anti-ribosomal P autoantibodies were infrequent, but when present were most common in Cluster 4. Cluster 6 had the highest frequencies of anti-Ro and anti-La specificities (Supplemental Fig. 7).

4. Discussion

Lack of understanding of the molecular heterogeneity of SLE remains a major deterrent to optimized individualized therapy, novel target identification, therapeutic development, and clinical trial success. Indeed, dissection of disease heterogeneity has been considered one of the ten most important contemporary challenges in SLE management [31] and is highlighted as a key obstacle for development of novel treatments [2]. Three decades of failed clinical trials and limited evidence to guide the selection of standard of care treatments hinder optimal care for individual SLE patients [1]. Key roadblocks in the development of new lupus treatments include incomplete understanding of the underlying molecular mechanisms of disease, the heterogeneity of disease across poorly defined clusters of patients, high placebo response rates, the cacophony of background therapies, and other factors [2,3]. This study set forth to address the heterogeneity of lupus by applying machine learning approaches to extensive gene expression, soluble mediator, autoantibody, and clinical information in a large cohort of carefully clinically-characterized patients. These

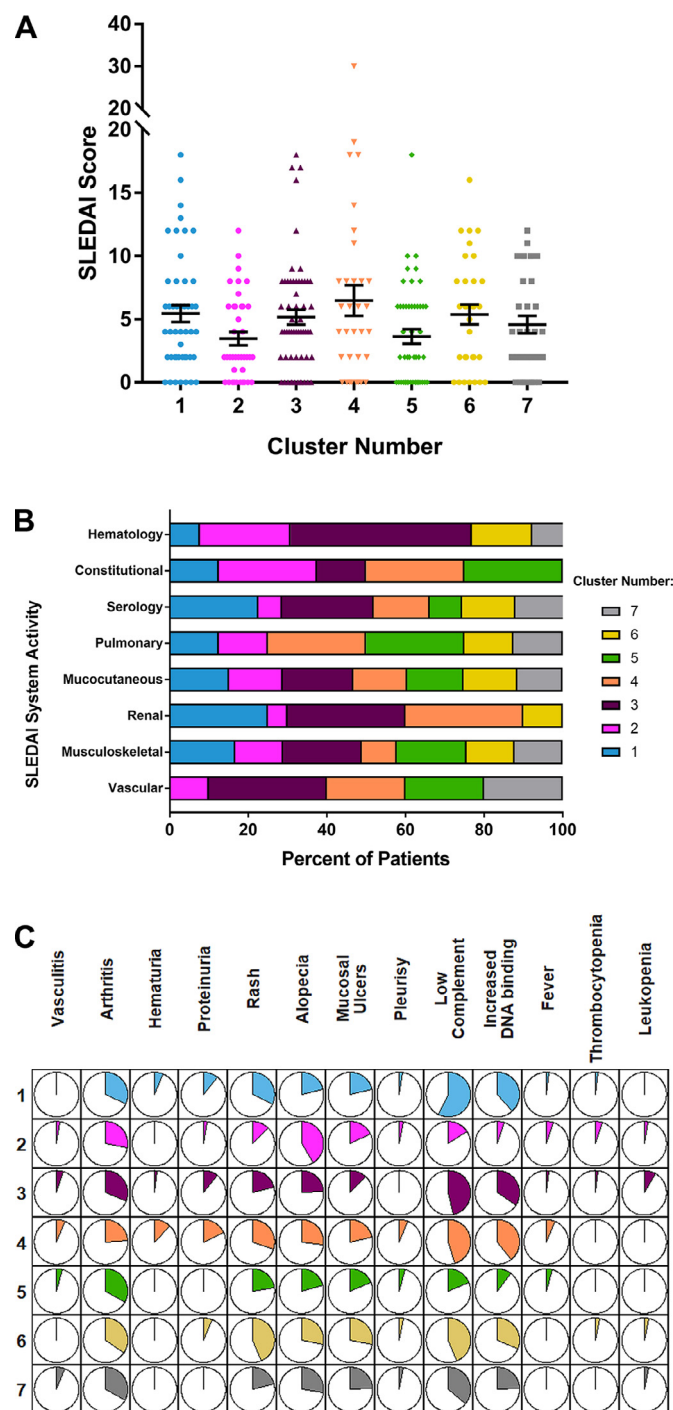


Fig. 3. Clinical phenotypes of molecularly defined SLE patient clusters, based on SLEDAI variables. (A) Mean $\pm$ SEM SLEDAI scores in each cluster. Comparison is not significant ($p > 0.05$) by Kruskal–Wallis. (B) Each bar represents 100% of patients who have active manifestations in the given organ system, with colored segments indicating the percentage of patients from each cluster. Activity in an organ system is defined as activity in at least one of the corresponding individual components (e.g., thrombocytopenia or leukopenia in the hematologic domain; low complement or increased DNA binding in the serologic domain, etc.). (C) Frequency of SLEDAI components in each cluster. SLEDAI components not present in any patients are not shown (seizure, psychosis, organic brain, visual, cranial nerve, lupus headache, CVA, myositis, pericarditis). A pie chart showing a single line indicates 0%.

approaches identified seven phenotypic clusters of adult SLE patients that were not apparent from clinical information.

Subsetting of lupus patients has been attempted both in trials and in prognostic studies based on clinical features (such as nephritis vs.

no nephritis) or based on autoantibodies and complement. All of these approaches have taught us something, but it has long been suspected that these distinctions are somewhat incomplete, and that the next step must be to examine a more comprehensive set of immune variables. Our work and others who have utilized gene co-expression module signatures with or without the integration of soluble mediators [13,30,32,33] confirms that many features that can somewhat define patient subsets, such as autoantibodies or nephritis, are found in immunologically distinct patients who may benefit more or less from given combinations of immune modulating treatments.

Although molecular subsetting alone will not resolve every problem in the design and interpretation of lupus trials, these results may help reduce the confusion of heterogeneity in lupus based on seven characteristic patterns of gene expression and soluble mediator profiles. Important implications for treatment development are suggested, including better identification of promising treatment targets in rationally defined patient subsets, improved study of pharmacodynamic effects by sorting through their impact on molecularly similar patients, and the potential development of diagnostic tests suitable for selecting optimal treatments and monitoring individual responses based on better characterized pathologic mechanisms.

Strengths of this study include the large population of racially diverse, adult lupus patients who have donated samples when disease was both active and inactive. In addition, this project begins to integrate soluble mediators, autoantibodies, gene co-expression modules, demographic, and clinical information. The application of machine learning approaches generated seven robust clusters with strong internal validity. For example anti-dsDNA antibodies, known to be associated with nephritis, are enriched in modules that are associated with other features of nephritis.

Limitations of this study suggest major opportunities for further research. Modules evaluated were associated with a limited set of immunologic pathways. Significant additional gene expression data information is available, and novel modules within this dataset may exist which are important in adult lupus pathogenesis. The modeling being examined now might also be improved by further inclusion of genetics, epigenetics, metabolomics, lipidomics, immunophenotyping, microbiome/other environmental information, or other evolving technologies. Evaluation of larger numbers of prospectively-collected, protocolized samples across time would also be helpful to establish the stability or movement of patients between various clusters with changes in disease activity, clinical manifestations, therapeutic interventions, hormonal changes with estrus or menopause, or variation with aging. Closely related clusters, like Clusters 1 and 4, may represent two unique pathotypes, or the higher frequency of MMF use in Cluster 1 may partially suppress signatures that would otherwise be assigned to Cluster 4. Resolving this question will require studies designed to directly test the impact of therapeutics during treatment and/or disease, especially with respect to downregulation of interferon and inflammatory markers and upregulation of other pertinent immune modulators.

Synthesizing and simplifying these algorithms to a limited number of analytes may allow individual patients to be more precisely diagnosed and treated. Refinements over time will be expected, but should not delay the implementation of a good disease model which could serve clinical trial designs and future studies that will be necessary to fully integrate disparate SLE pathophysiology with prognosis for individual patients.

Declaration of Competing Interest

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Supplementary materials

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