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

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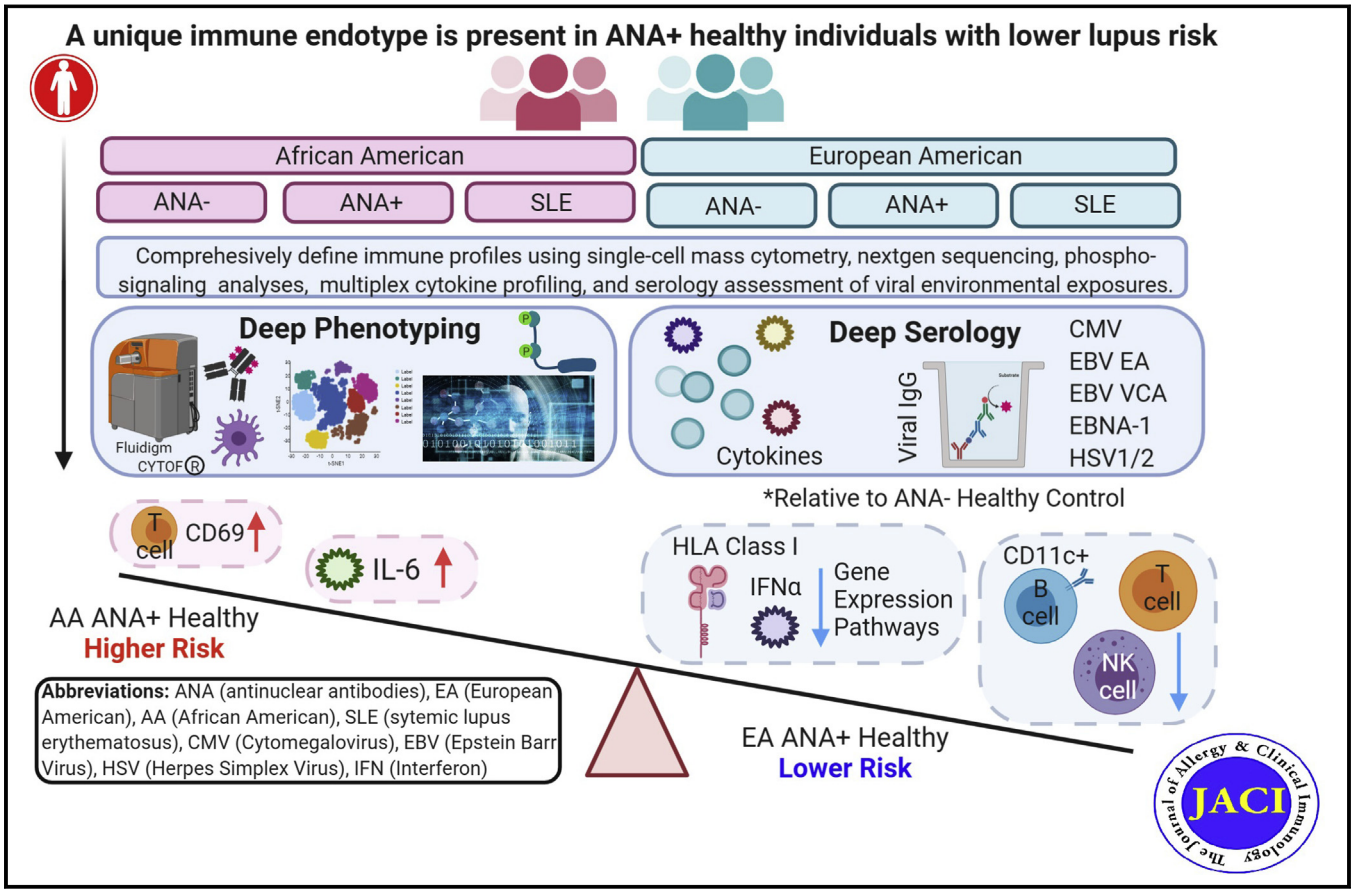
GRAPHICAL ABSTRACT



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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;■■■:■■■-■■■.)

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

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

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 nonwhites;[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 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 autospecificities, 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 SELENA-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-1α, IL-1β, 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 1α, macrophage inflammatory protein 1β, nerve growth factor β, plasminogen activator inhibitor 1, platelet-derived growth factor with 2 B subunits, RANTES, resistin, SCF, stromal cell-derived factor 1a, 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 ≥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 μg/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× 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]

Phosphoepitope 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, pPLCγ2, 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 $CD66b^-CD3^-CD19^-CD56^-HLA\text{-}DR^+CD14^+CD16^{+/-}$ 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 bivariant 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 isotype-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).

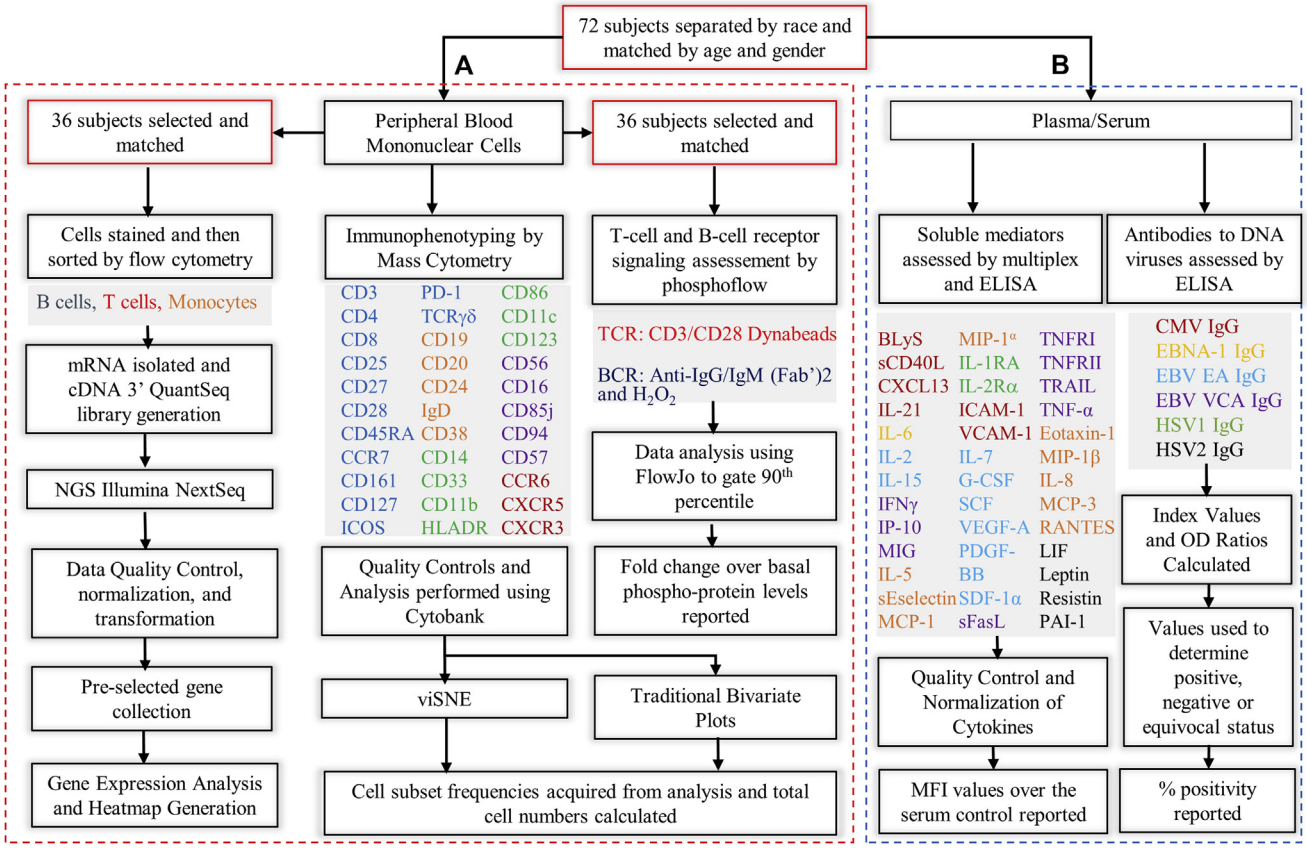


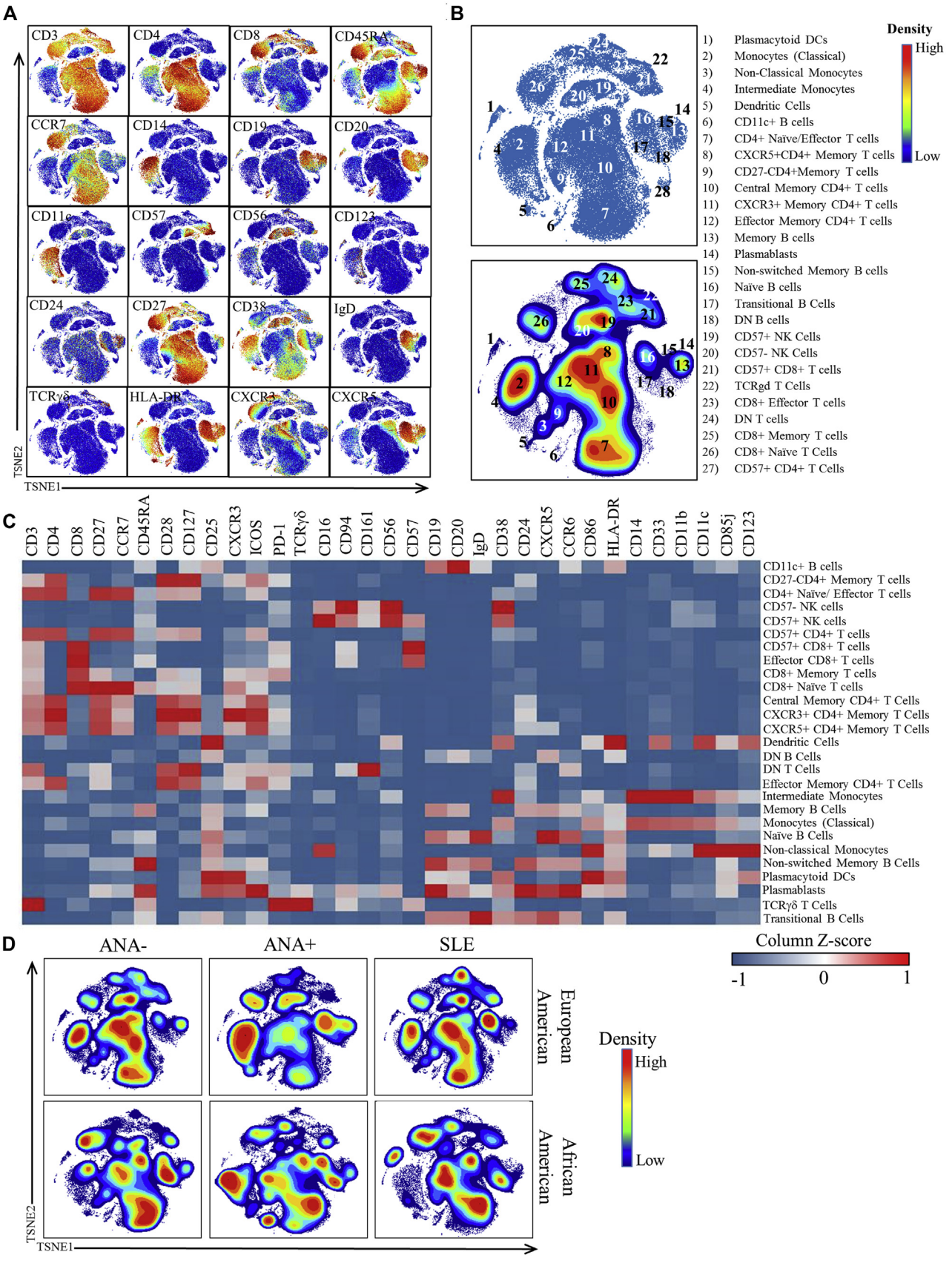
FIG 1. Schematic workflow for ANA+ healthy individual biologic analysis. The workflow is broadly divided into 2 steps. First, 72 samples consisting of EA and AA ANA− healthy individuals, ANA+ healthy individuals, and patients with SLE were matched by age, race, and sex. **A,** PBMCs were collected and 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 in 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,*


A
CD3
CD4
CD8
CD45RA
CCR7
CD14
CD19
CD20
CD11c
CD57
CD56
CD123
CD24
CD27
CD38
IgD
TCRγδ
HLA-DR
CXCR3
CXCR5
TSNE2
TSNE1
B
1) Plasmacytoid DCs
2) Monocytes (Classical)
3) Non-Classical Monocytes
4) Intermediate Monocytes
5) Dendritic Cells
6) CD11c+ B cells
7) CD4+ Naïve/Effector T cells
8) CXCR5+CD4+ Memory T cells
9) CD27-CD4+Memory T cells
10) Central Memory CD4+ T cells
11) CXCR3+ Memory CD4+ T cells
12) Effector Memory CD4+ T cells
13) Memory B cells
14) Plasmablasts
15) Non-switched Memory B cells
16) Naïve B cells
17) Transitional B Cells
18) DN B cells
19) CD57+ NK Cells
20) CD57- NK Cells
21) CD57+ CD8+ T cells
22) TCRgd T Cells
23) CD8+ Effector T cells
24) DN T cells
25) CD8+ Memory T cells
26) CD8+ Naïve T Cells
27) CD57+ CD4+ T Cells
Density
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Low
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CD11c+ B cells
CD27-CD4+ Memory T cells
CD4+ Naïve/ Effector T cells
CD57- NK cells
CD57+ NK cells
CD57+ CD4+ T cells
CD57+ CD8+ T cells
Effector CD8+ T cells
CD8+ Memory T cells
CD8+ Naïve T cells
Central Memory CD4+ T Cells
CXCR3+ CD4+ Memory T Cells
CXCR5+ CD4+ Memory T Cells
Dendritic Cells
DN B Cells
DN T Cells
Effector Memory CD4+ T Cells
Intermediate Monocytes
Memory B Cells
Monocytes (Classical)
Naïve B Cells
Non-classical Monocytes
Non-switched Memory B Cells
Plasmacytoid DCs
Plasmablasts
TCRγδ T Cells
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TSNE1

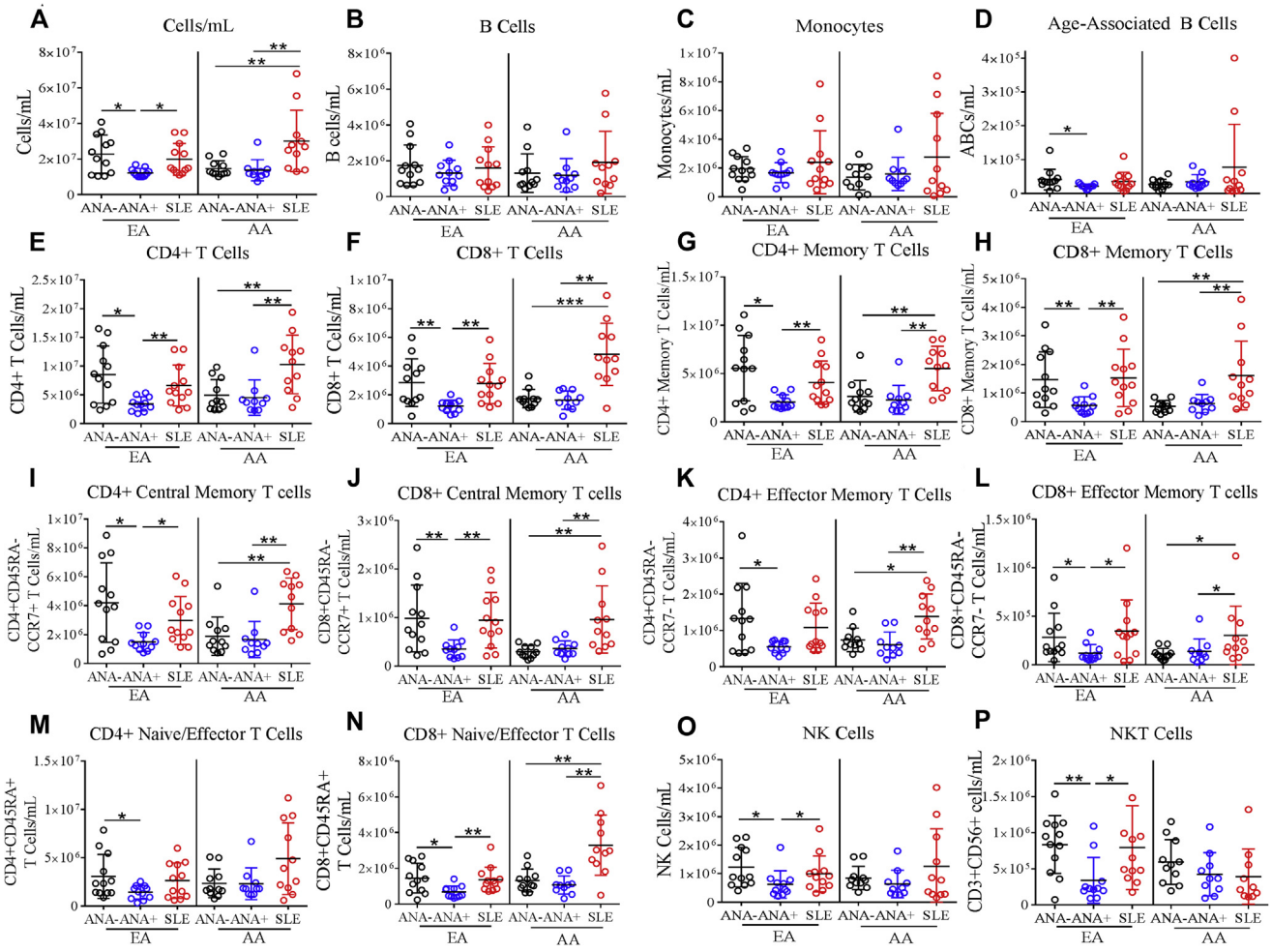


FIG 3. Calculated cell numbers indicate elevated T cells in patients with SLE and suppressed T cells in EA ANA+ healthy individuals. Cells numbers were calculated from cell subsets using frequencies and total cell counts. Cell numbers are shown for **(A)** total cells/mL, **(B)** B cells ($CD3^-CD19^+$), **(C)** monocytes ($HLA\text{-}DR^+CD11c^+CD14^+CD16^{+/-}$), **(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 ± 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.

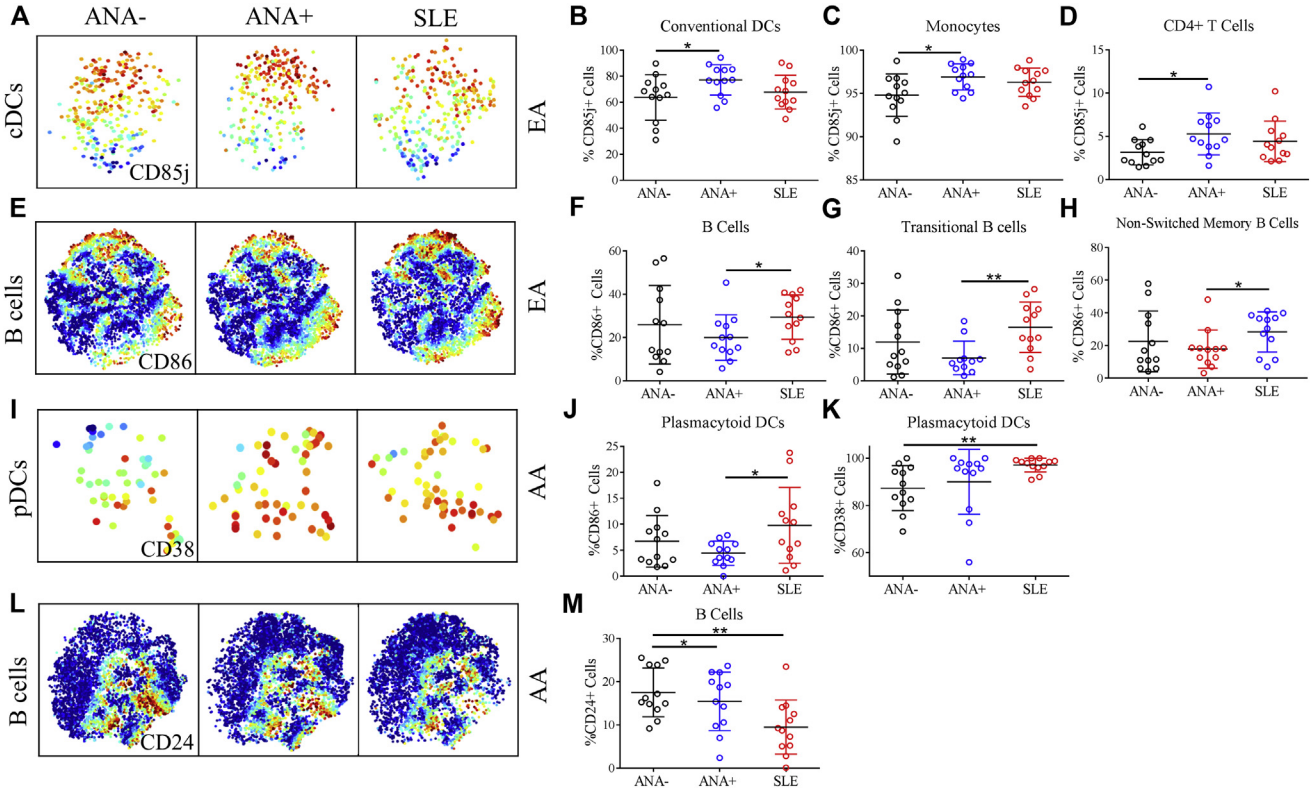


FIG 4. Cell subset marker expression identify activated cell subsets in patients with SLE and elevated regulatory marker expression in ANA+ healthy individuals. All 55 cell subsets were manually gated and assessed for frequencies of activation and regulatory surface markers. tSNE plots were used to illustrate 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 ± 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 nonswitched 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, pPLCγ2, 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 TNFRII, 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, *TGFBR1* 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.

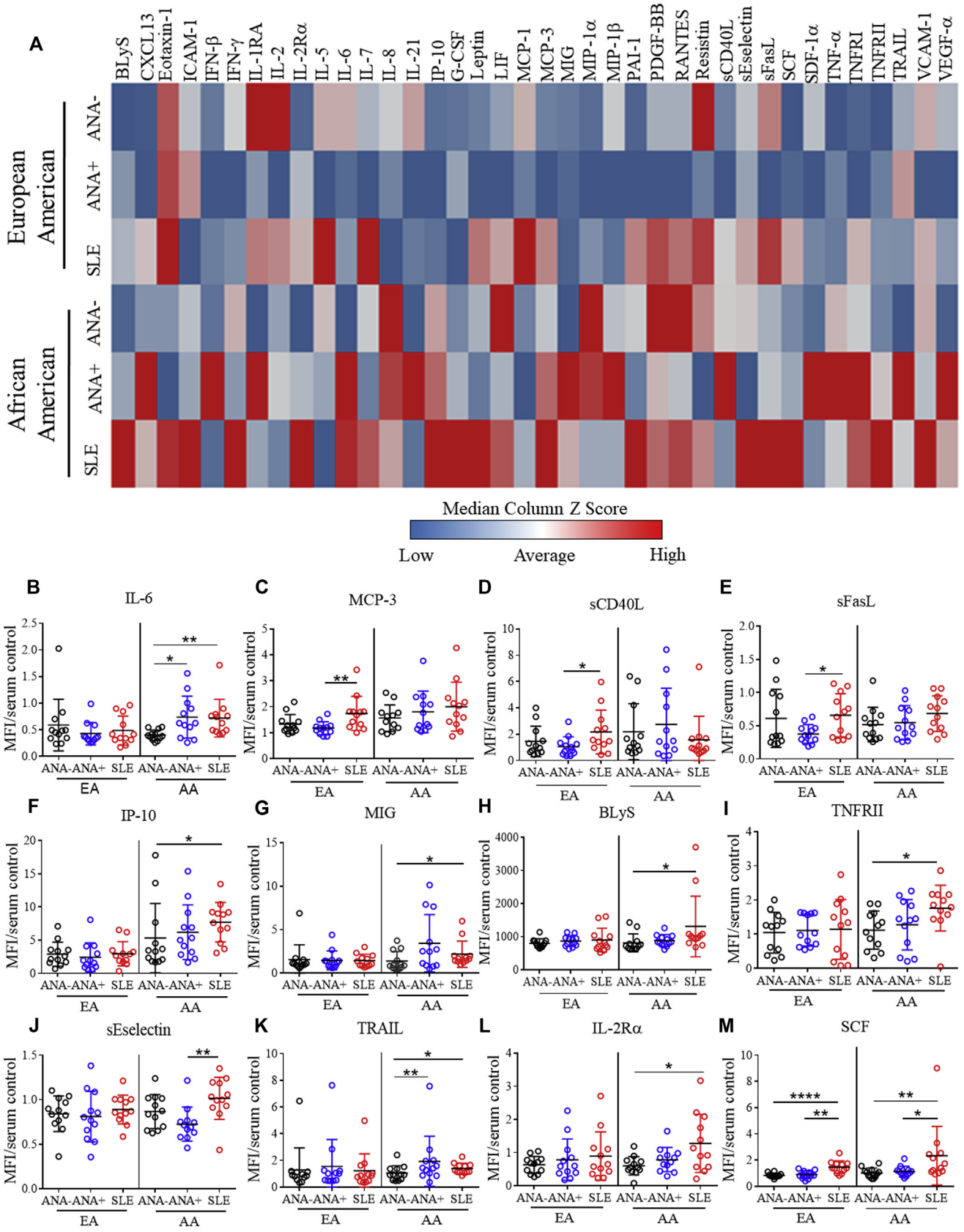


FIG 5. SCF distinguishes patients with SLE from ANA+ healthy individuals in both EAs and AAs. Proinflammatory soluble mediators were measured by multiplex or ELISA. **A,** A heatmap summary of the plasma levels for each individual are shown. Soluble mediator levels are displayed on a color scale ranging

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 $HLA\text{-}DR^{Hi}$ 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.

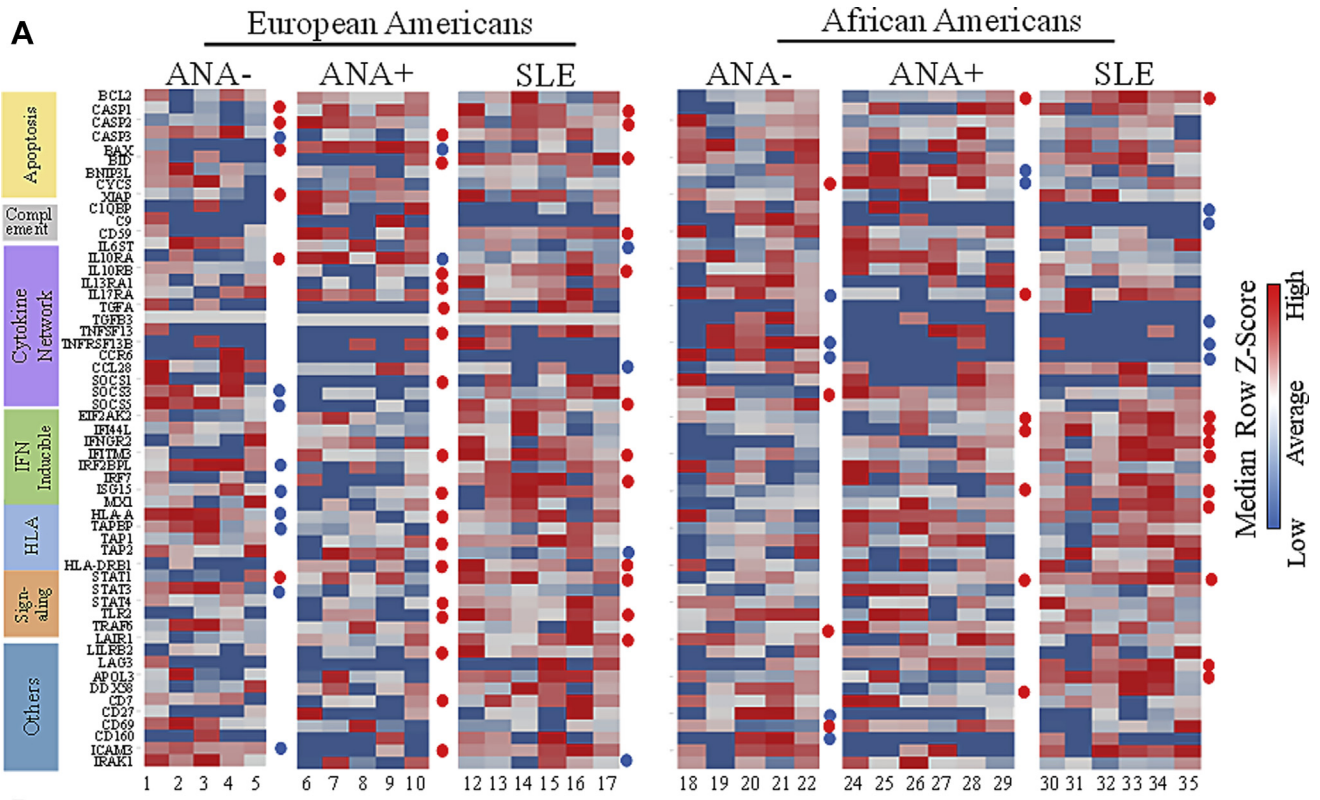
Individuals with AA ancestry are at a higher risk for developing autoimmune diseases, such as SLE, than are those with EA ancestry.[9] We discovered a unique and potentially protective immune profile associated with asymptomatic autoimmunity in EA individuals. Unlike EA ANA− healthy controls, EA ANA+ 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.

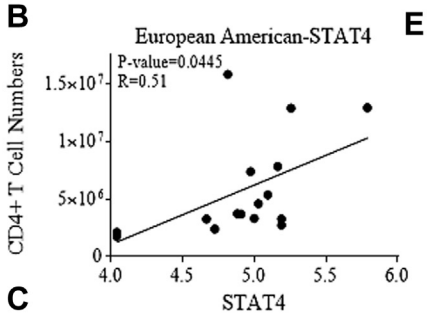
Although the suppressed immune features in EA ANA+ healthy are reminiscent of certain viral infections,[32,33] no correlations were found with herpesvirus seroconversion or seroreactivation. However, an expanded panel to assess comprehensive infection history and chronic low-level viral infection could 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]

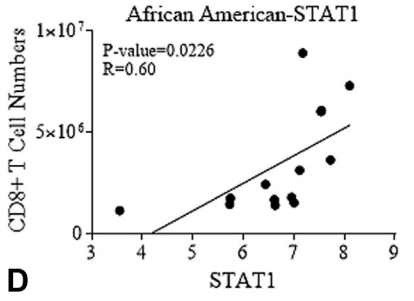
Suppression may be a form of regulation in response to early autoreactivity or a pathogenic result of unseen immune activation in EA ANA+ healthy individuals. However, elevated levels of the inhibitory receptor CD85j, which is expressed on DCs, monocytes, and T cells, and a lack of elevated activation 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.

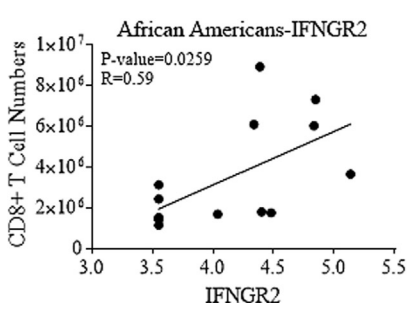
Previous work in ANA+ healthy individuals, compared with ANA− controls, has found elevated CD86 expression on B cells and elevated T_H follicular and regulatory T cell frequencies.[8]

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)** TNFRII, **(J)** sEselectin, **(K)** TNF-related apoptosis-inducing ligand (TRAIL), **(L)** IL-2Rα, and **(M)** SCF. $*P < .05$, $**P < .01$. Kruskal-Wallis test with 2-tailed Mann-Whitney for multiple comparisons. Mean ± SD shown.









E

Gene	P-value	Corr. Coeff. R	Gene	P-value	Corr. Coeff. R
European American			African American		
T cell gene expression correlate with CD4+ T cell numbers					
STAT4	0.0445	0.51	SOCS5	0.0237	-0.60
			BCL2	0.0262	0.59
			CCL28	0.0491	0.53
			STAT1	0.0525	0.53
T cell gene expression correlate with CD8+ T cell numbers					
IL13RA1	0.0268	0.55	IFITM3	0.00402	0.72
ICAM3	0.0284	0.55	LAG3	0.00773	0.68
IL6ST	0.0381	-0.52	STAT1	0.0226	0.60
LAG3	0.0488	0.5	IFNGR2	0.0259	0.59
B cell gene expression correlate with T cell numbers					
IRF2BPL	0.002	0.73	IFITM3	0.00375	0.76
LILRB2	0.00451	0.69	OAS1	0.00522	0.75
CCL5	0.0115	0.63	IRF2	0.0097	0.71
ID2	0.0156	0.61	CD86	0.0126	0.69
TNFSF13B	0.0457	0.52	CXCR4	0.046	-0.68
			APOL6	0.019	0.66
			CD55	0.0215	-0.65
			STAT1	0.0362	0.61
			IFITM2	0.0374	0.60
			LILRB2	0.0504	0.56
Monocyte gene expression correlate with T cell numbers					
C2	0.00841	0.67	TGFBR1	0.00304	-0.80
BNIP3L	0.0218	-0.61	TNIP2	0.016	0.70
TGFB3	0.0339	0.57	APOL3	0.0227	0.67
			BAD	0.0479	-0.61

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 ≥1:160) in the prior study.[8] ANA testing was performed for various reasons including noninflammatory arthritis/arthralgia (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 al[45] 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.

We would like to thank Sarah Kleckner, Wade DeJager, Virginia Roberts, Jeremy Levin, and Wendy Klein for technical assistance and Rebecka Bourn, PhD, and Angela Andersen, PhD, for scientific editing of this manuscript. In addition, we would also like to thank the Stanford Human Immune Monitoring Center for executing the immunophenotyping and phospho-flow assays in this manuscript, and biorender.com for the software used to create the graphical abstract.

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.

REFERENCES

1. Wandstrat AE, Carr-Johnson F, Branch V, Gray H, Fairhurst AM, Reimold A, et al. Autoantibody profiling to identify individuals at risk for systemic lupus erythematosus. J Autoimmun 2006;27:153-60.
2. National Institutes of Health. NIH Autoimmune Diseases Coordinating Committee: autoimmune diseases research plan. 2005. Available at: niaid.nih.gov/sites/default/files/adccfinal.pdf. Accessed December 2019.
3. Li X, Liu X, Cui J, Song W, Liang Y, Hu Y, Guo Y. Epidemiological survey of antinuclear antibodies in healthy population and analysis of clinical characteristics of positive population. J Clin Lab Anal 2019;33:e22965.
4. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, Harley JB. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med 2003;349:1526-33.
5. Sokolove J, Bromberg R, Deane KD, Lahey LJ, Derber LA, Chandra PE, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. PLoS One 2012;7:e35296.
6. Lu R, Munroe ME, Guthridge JM, Bean KM, Fife DA, Chen H, et al. Dysregulation of innate and adaptive serum mediators precedes systemic lupus erythematosus classification and improves prognostic accuracy of autoantibodies. J Autoimmun 2016;74:182-93.
7. Slight-Webb S, Lu R, Ritterhouse LL, Munroe ME, Maecker HT, Fathman CG, et al. Autoantibody-positive healthy individuals display unique immune profiles that may regulate autoimmunity. Arthritis Rheumatol 2016;68:2492-502.
8. Baglaenko Y, Chang NH, Johnson SR, Hafiz W, Manion K, Ferri D, et al. The presence of anti-nuclear antibodies alone is associated with changes in B cell activation and T follicular helper cells similar to those in systemic autoimmune rheumatic disease. Arthritis Res Ther 2018;20:264.
9. Lewis MJ, Jawad AS. The effect of ethnicity and genetic ancestry on the epidemiology, clinical features and outcome of systemic lupus erythematosus. Rheumatology (Oxford) 2017;56(suppl 1):i67-77.
10. Weckerle CE, Mangale D, Franek BS, Kelly JA, Kumabe M, James JA, et al. Large-scale analysis of tumor necrosis factor alpha levels in systemic lupus erythematosus. Arthritis Rheum 2012;64:2947-52.
11. Ko K, Koldobskaya Y, Rosenzweig E, Niewold TB. Activation of the interferon pathway is dependent upon autoantibodies in African-American SLE patients, but not in European-American SLE patients. Front Immunol 2013;4:309.
12. Ritterhouse LL, Crowe SR, Niewold TB, Merrill JT, Roberts VC, Dedeke AB, et al. B lymphocyte stimulator levels in systemic lupus erythematosus: higher circulating levels in African American patients and increased production after influenza vaccination in patients with low baseline levels. Arthritis Rheum 2011;63:3931-41.
13. Lyn-Cook BD, Xie C, Oates J, Treadwell E, Word B, Hammons G, Wiley K. Increased expression of Toll-like receptors (TLRs) 7 and 9 and other cytokines in systemic lupus erythematosus (SLE) patients: ethnic differences and potential new targets for therapeutic drugs. Mol Immunol 2014;61:38-43.
14. Coit P, Ognenovski M, Gensterblum E, Maksimowicz-McKinnon K, Wren JD, Sawalha AH. Ethnicity-specific epigenetic variation in naive $CD4^+$ T cells and the susceptibility to autoimmunity. Epigenetics Chromatin 2015;8:49.
15. Sharma S, Jin Z, Rosenzweig E, Rao S, Ko K, Niewold TB. Widely divergent transcriptional patterns between SLE patients of different ancestral backgrounds in sorted immune cell populations. J Autoimmun 2015;60:51-8.
16. Kheir JM, Guthridge CJ, Johnston JR, Adams LJ, Rasmussen A, Gross TF, et al. Unique clinical characteristics, autoantibodies and medication use in Native American patients with systemic lupus erythematosus. Lupus Sci Med 2018;5:e000247.
17. Li QZ, Karp DR, Quan J, Branch VK, Zhou J, Lian Y, et al. Risk factors for ANA positivity in healthy persons. Arthritis Res Ther 2011;13:R38.
18. Karlson EW, Sanchez-Guerrero J, Wright EA, Lew RA, Daltroy LH, Katz JN, Liang MH. A connective tissue disease screening questionnaire for population studies. Ann Epidemiol 1995;5:297-302.
19. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997;40:1725.
20. Price AL, Butler J, Patterson N, Capelli C, Pascali VL, Scarnicci F, et al. Discerning the ancestry of European Americans in genetic association studies. PLoS Genet 2008;4:e236.
21. Rosenberg-Hasson Y, Hansmann L, Liedtke M, Herschmann I, Maecker HT. Effects of serum and plasma matrices on multiplex immunoassays. Immunol Res 2014;58:224-33.
22. Slight-Webb S, Guthridge JM, Chakravarty EF, Chen H, Lu R, Macwana S, et al. Mycophenolate mofetil reduces STAT3 phosphorylation in systemic lupus erythematosus patients. JCI Insight 2019;4:e124575.
23. Andrews S, Segonds-Pichon A, Biggins L, Krueger C, Wingett S. FASTQC: A quality controls tool for high throughput sequence data. 2019. Available at: bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed December 2019.
24. Bushnell B. BBTools: a suite of fast, multithreaded bioinformatics tools designed for analysis of DNA and RNA sequence data. 2019. Available at: jgi.doe.gov/data-and-tools/bbtools/. Accessed December 2019.
25. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15-21.
26. Frankish A, Diekhans M, Ferreira AM, Johnson R, Jungreis I, Loveland J, et al. GENCODE reference annotation for the human and mouse genomes. Nucleic Acids Res 2019;47:D766-73.
27. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol 2015;33:290-5.
28. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.
29. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol 2010;11:R106.
30. Vista ES, Weisman MH, Ishimori ML, Chen H, Bourn RL, Bruner BF, et al. Strong viral associations with SLE among Filipinos. Lupus Sci Med 2017;4:e000214.
31. Amir el-AD, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nat Biotechnol 2013;31:545-52.
32. Urban SL, Welsh RM. Out-of-sequence signal 3 as a mechanism for virus-induced immune suppression of CD8 T cell responses. PLoS Pathog 2014;10:e1004357.
33. Bego MG, St Jeor S. Human cytomegalovirus infection of cells of hematopoietic origin: HCMV-induced immunosuppression, immune evasion, and latency. Exp Hematol 2006;34:555-70.
34. Jog NR, Young KA, Munroe ME, Harmon MT, Guthridge JM, Kelly JA, et al. Association of Epstein-Barr virus serological reactivation with transitioning to systemic lupus erythematosus in at-risk individuals. Ann Rheum Dis 2019;78:1235-41.
35. Fayyaz A, Igoe A, Kurien BT, Danda D, James JA, Stafford HA, Scofield RH. Haematological manifestations of lupus. Lupus Sci Med 2015;2:e000078.
36. de Vries RD, de Swart RL. Measles immune suppression: functional impairment or numbers game? PLoS Pathog 2014;10:e1004482.
37. Guo Y, Walsh AM, Canavan M, Wechalekar MD, Cole S, Yin X, et al. Immune checkpoint inhibitor PD-1 pathway is down-regulated in synovium at various stages of rheumatoid arthritis disease progression. PLoS One 2018;13:e0192704.
38. Granados HM, Draghi A 2nd, Tsurutani N, Wright K, Fernandez ML, Sylvester FA, Vella AT. Programmed cell death-1, PD-1, is dysregulated in T cells from children with new onset type 1 diabetes. PLoS One 2017;12:e0183887.
39. McKinney EF, Lee JC, Jayne DR, Lyons PA, Smith KG. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. Nature 2015;523:612-6.
40. Nedelec Y, Sanz J, Baharian G, Szpiech ZA, Pacis A, Dumaine A, et al. Genetic ancestry and natural selection drive population differences in immune responses to pathogens. Cell 2016;167:657-69.e21.
41. Menard LC, Habte S, Gonsiorek W, Lee D, Banas D, Holloway DA, et al. B cells from African American lupus patients exhibit an activated phenotype. JCI Insight 2016;1:e87310.
42. Shovman O, Gilburd B, Barzilai O, Shinar E, Larida B, Zandman-Goddard G, et al. Evaluation of the BioPlex 2200 ANA screen: analysis of 510 healthy subjects: incidence of natural/predictive autoantibodies. Ann N Y Acad Sci 2005;1050:380-8.
43. Au EY, Ip WK, Lau CS, Chan YT. Evaluation of a multiplex flow immunoassay versus conventional assays in detecting autoantibodies in systemic lupus erythematosus. Hong Kong Med J 2018;24:261-9.
44. Bruner BF, Guthridge JM, Lu R, Vidal G, Kelly JA, Robertson JM, et al. Comparison of autoantibody specificities between traditional and bead-based assays in a large, diverse collection of patients with systemic lupus erythematosus and family members. Arthritis Rheum 2012;64:3677-86.
45. Pérez D, Gilburd B, Cabrera-Marante O, Martinez-Flores JA, Serrano M, Naranjo L, et al. Predictive autoimmunity using autoantibodies: screening for anti-nuclear antibodies. Clin Chem Lab Med 2018;56:1771-7.
46. Wang Z, Mascarenhas N, Eckmann L, Miyamoto Y, Sun X, Kawakami T, Di Nardo A. Skin microbiome promotes mast cell maturation by triggering stem cell factor production in keratinocytes. J Allergy Clin Immunol 2017;139:1205-16.e6.
47. Cho KA, Park M, Kim YH, Woo SY. Th17 cell-mediated immune responses promote mast cell proliferation by triggering stem cell factor in keratinocytes. Biochem Biophys Res Commun 2017;487:856-61.

48. Asada N, Kunisaki Y, Pierce H, Wang Z, Fernandez NF, Birbrair A, et al. Differential cytokine contributions of perivascular haematopoietic stem cell niches. Nat Cell Biol 2017;19:214-23.
49. Ray P, Krishnamoorthy N, Ray A. Emerging functions of c-kit and its ligand stem cell factor in dendritic cells: regulators of T cell differentiation. Cell Cycle 2008;7: 2826-32.
50. Pradier A, Tabone-Eglinger S, Huber V, Bosshard C, Rigal E, Wehrle-Haller B, Roosnek E. Peripheral blood CD56(bright) NK cells respond to stem cell factor and adhere to its membrane-bound form after upregulation of c-kit. Eur J Immunol 2014;44:511-20.
51. Krishnamoorthy N, Oriss TB, Paglia M, Fei M, Yarlagadda M, Vanhaesebroeck B, et al. Activation of c-kit in dendritic cells regulates T helper cell differentiation and allergic asthma. Nat Med 2008;14:565-73.