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

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Samantha Slight-Webb, PhD,a Miles Smith, PhD,a Aleksandra Bylinska, MS,a Susan Macwana, MS,a Carla Guthridge, PhD,a Rufe Lu, MD, PhD, a,c; Joan T. Merrill, MD,a Eliza Chakravarty, MD,a Cristina Arriens, MD,a,b,c Melissa E. Munroe, MD, PhD,a; Holden T. Macecker, PhD,d Paul J. Utz, MD,a Joel M. Guthridge, PhD,a,c; and Judith A. James, MD, PhD,a,b,c,* Oklahoma City, Okla, and Stanford, Calif

GRAPHICAL ABSTRACT

A unique immune endotype is present in ANA+ healthy individuals with lower lupus risk

Comprehensively define immune profiles using single-cell mass cytometry, nextgen sequencing, phospho-signaling analyses, multiplex cytokine profiling, and serology assessment of viral environmental exposures.

Deep Phenotyping

CMV
EBV EA
EBV VCA
EBNA-1
HSV1/2

Deep Serology

Cytokines

Viral IgG

Hypothesis

AA ANA+ Healthy

Higher Risk

IL-6

CD69

*Relative to ANA- Healthy Control

Abbreviations: ANA (antinuclear antibodies), EA (European American), AA (African American), SLE (systemic lupus erythematosus), CMV (Cytomegalovirus), EBV (Epstein Barr Virus), HSV (Herpes Simplex Virus), IFN (Interferon)

EA ANA+ Healthy

Lower Risk

From a the Department of Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, and the Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City; b the Department of Immunology, Transplantation, and Infection and the Division of Immunology and Rheumatology, Stanford University School of Medicine.

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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

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;1,4 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<sub>eff</sub> 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.7 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<sup>+</sup> 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.

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
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 (IF) and QUANTA Lite ELISA using HEP-2 cells (Inova Diagnostics, Inc, San Diego, Calif) following the manufacturer’s recommended protocols and cutoffs.21,16,17 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, ribonuclease (RNP), Sm RNP, centromere B, ribosomal P, Scl-70, and Jo-1. 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, 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.21 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, ectoX, G-CSF, growth regulated oncogene (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 1B, 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 (Innogenetics, Thermo Fisher 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 (dodecanetetraacetic acid–maleimide [Macrocycles, 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 Fisher 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 ironid isotopes from the intercalator, then on singlets by iridium Ir 191 versus cell length, then on live cells (indiurn-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 neighborhood embedding (tSNE) and manually, as previously published.21

Phosphoepitope flow cytometry
These assays were performed at the Human Immune Monitoring Center at Stanford University. Briefly, PBMCs were suspended at 0.1×10⁷ 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 H₂O₂) 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), CD4 (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‘CD16’77 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 heat map 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 (EuroImmunn, 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 a P value <0.05 considered significant). 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 3.3.3) 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 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, RCColorBrewer, 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, Wallingford, 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,3 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 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).
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). 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^-CD15^-), 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 both ANA^- and 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 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-N, Tables E13 and E14). Specifically, both CD4^+ and CD8^-T cells in memory, naive, and effector T-cell subsets such as TH1-type (C-X-C motif receptor 3^+) and TH17-type (CCR6^-CD161^-) were elevated in patients with SLE compared with ANA^- healthy individuals (Fig 3, E-N, F).

**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), TH1 (purple), TH2 (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.
Furthermore, NK cell subsets were also increased in patients with SLE compared with ANA negative 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 negative versus both ANA positive healthy controls and patients with SLE (Fig 3). The calculated cell numbers indicate elevated T cells in patients with SLE and suppressed T cells in EA ANA negative 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+CD69+CD4+CD8-), (F) CD8+ T cells (CD3+CD69+CD4+CD8+), (G) CD4 memory T cells (CD3+CD66+CD4+CD45RA-), (H) CD8 memory T cells (CD3+CD66+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+CD69+CD4+CD45RA+), (N) CD8+ naive/effector T cells (CD3+CD69+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.
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 receptor 3, 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, pPLCγ2, and pSTAT5 in PBMCs by flow cytometry following treatment with TCR (anti-CD3/CD28) or

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 (cDCa), (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.
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 chemotactic 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 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,2,23 HSV2. 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$n$17-type T cells and SLEDAI instrument score (see Fig E10 in this article’s Online Repository at www.jacionline.org), suggesting these cell subset increases may be influenced by higher SLEDAI instrument score in AA patients with SLE. Furthermore, AA patients with SLE also had HLA-DR$^+$ 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. 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, 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, no correlations were found with herpesvirus seroconversion or reactivation. 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.

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.

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, 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$n$ follicular and regulatory T cell frequencies.

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-28Rα, and (M) SCF. *P < .05, **P < .01. Kruskal-Wallis test with 2-tailed Mann-Whitney for multiple comparisons. Mean ± 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 ≥1:160) in the prior study.\textsuperscript{6} 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.\textsuperscript{31} 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.\textsuperscript{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 IFN− false positives or subjects captured early in the disease process. To address this, Pérez et al\textsuperscript{35} 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.\textsuperscript{31} 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 derived from their resemblance to patients with SLE or their lack thereof. 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 IFN− false positives or subjects captured early in the disease process. To address this, Pérez et al\textsuperscript{35} 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.\textsuperscript{31} 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.

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.

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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\textsuperscript{+} T-cell numbers and STAT4 T-cell gene expression in EAs, and (C) STAT1 and (D) IFNGR2 T-cell gene expression and CD8\textsuperscript{+} T-cell numbers of AAs. All other significant correlations are recorded in the table (E). Corr Coeff, correlation coefficient.
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