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Multicenter Systems Analysis of Human Blood Reveals Immature Neutrophils in Males and During Pregnancy

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Despite clear differences in immune system responses and in the prevalence of autoimmune diseases between males and females, there is little understanding of the processes involved. In this study, we identified a gene signature of immature-like neutrophils, characterized by the overexpression of genes encoding for several granule-containing proteins, which was found at higher levels (up to 3-fold) in young (20–30 y old) but not older (60 to >89 y old) males compared with females. Functional and phenotypic characterization of peripheral blood neutrophils revealed more mature and responsive neutrophils in young females, which also exhibited an elevated capacity in neutrophil extracellular trap formation at baseline and upon microbial or sterile autoimmune stimuli. The expression levels of the immature-like neutrophil signature increased linearly with pregnancy, an immune state of increased susceptibility to certain infections. Using mass cytometry, we also find increased frequencies of immature forms of neutrophils in the blood of women during late pregnancy. Thus, our findings show novel sex differences in innate immunity and identify a common neutrophil signature in males and in pregnant women. *The Journal of Immunology*, **2017, 198: 2479–2488.**

A number of differences in the immune responses of males and females have been clearly documented. Generally, females mount higher innate and adaptive immune responses than males, and this can result in faster clearance of pathogens and better response to vaccination; however, it may also contribute to an increased susceptibility to inflammatory and autoimmune diseases (reviewed in Refs. 1–3). For instance, after vaccination against influenza, yellow fever, rubella, measles, mumps, hepatitis A and B, herpes simplex 2, rabies, smallpox, and dengue viruses, protective Ab responses can be twice as high in females than in males (4, 5).

Consistent with these more robust immune reactions seen in females, there are also differences in the prevalence and severity of adverse reactions to vaccines, including fever, pain, and inflammation (5, 6). Similarly, autoinflammatory and autoimmune diseases caused by excessive inflammatory reactions are also more frequent in females than in males. For example, it is estimated that >80% of all patients with autoimmune diseases are women (7), with the female bias being highly pronounced for Sjögren's syndrome (9:1 bias), Hashimoto's thyroiditis (8:1), systemic lupus erythematosus (SLE) (8:1), rheumatoid arthritis (RA) (7:1), and multiple sclerosis (MS) (6:1) (7).

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Gene expression data were generated at the Human Immune Monitoring Core (Stanford University) or at the Baylor Institute for Immunology Research. B.G. and G.K.F. contributed mass cytometry control data; J.B., S.G., Y.L., S.H., and M.J.K. designed or performed neutrophil extracellular trap–formation experiments; B.G., E.A.G., D.S., M.S.A., and N.A. designed or conducted mass cytometry experiments on the Stanford pregnancy study; D.F., J.B., C.R.B., and R.S.-D. analyzed data; N.B. and E.A. designed and implemented the NanoString Assay; D.C. and M.C.A. coordinated, designed, and analyzed gene expression data on the Baylor longitudinal pregnancy study; M.J.K., M.M.D., and D.F. provided support, contributed to the planning of the immunological studies, and contributed to study design; and J.B. and D.F. wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CEACAM, carcinoembryonic Ag-related cell adhesion molecule; CSM, cell-staining media; CyTOF, cytometry by time-of-flight; DC, dendritic cell; FDR, false discovery rate; GEO, Gene Expression Omnibus; GR-MDSC, granulocytic myeloid-derived suppressor cell; ILNS, immature-like neutrophil signature; LDG, low-density granulocyte; MS, multiple sclerosis; NDG, normal-density granulocyte; NET, neutrophil extracellular trap; Pd, isothiocyanobenzyl-EDTA/Pd; QuSAGE, quantitative set analysis of gene expression; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

However, the mechanisms underlying these marked sex differences in immunity are not completely understood. The prevailing hypothesis for the immunological differences between sexes is that sex chromosomes and sex hormones influence immune cell functioning. For example, sex hormones can bind to specific receptors on various immune cells and influence the production of cytokines and chemokines. Androgens generally increase the synthesis of anti-inflammatory cytokines, such as IL-10, and suppress the activity of immune cells (8), whereas estrogens can have divergent effects on inflammatory responses, with low doses enhancing proinflammatory cytokine production (e.g., IL-1, IL-6, and TNF-α) and Th1 responses and high or sustained concentrations reducing the production of proinflammatory cytokines and augmenting Th2 responses and humoral immunity (9, 10).

An important role for estrogens in inflammation and in the modulation of the innate immune system is their ability to trigger production of GM-CSF (11), a cytokine with multiple immunological functions that also ensures a normal physiology of pregnancy. Consistent with this, GM-CSF was shown to be elevated in young females compared with young males (20–30 y old); these differences are lost in old age (60 to >89 y old) (12). Because GM-CSF is a crucial cytokine for the maturation of innate immune cells, such as monocyte/macrophages, granulocytes, and dendritic cells (DCs), in this study we aimed to characterize the differences in gene signatures between mature and immature phenotypes of these cell subsets. Despite the fact that multiple studies suggested sex differences in the maturation of monocytes, macrophages, and DCs, with sex hormones having the ability to modulate the expression of key molecules associated with differentiation and function of these cells (reviewed in Ref. 3), no clear sex differences in the maturation status of these cells have been found. In contrast, morphological differences in circulating neutrophils showing increased frequency of segmented cells (a late maturation stage) in healthy females compared with males were described >60 y ago (13); however, very little has been added to those observations, and, to our knowledge, no functional or mechanistic correlates of such differences have been shown. This is not surprising because it was only recently that we learned a great deal about how men and women respond differently to infections, vaccination, and medications (14).

Therefore, evidence of increased immature myeloid cells in circulation is restricted to cases of bacterial infections, certain cancers, and inflammatory states, and this generally reflects early or premature release of myeloid cells from the bone marrow, the site where these cells are generated.

Using data sets from the public domain, as well as those generated prospectively in independent studies conducted in multiple centers in the United States, we find an immature-like neutrophil signature (ILNS), including genes encoding for granule-containing proteins, to be upregulated 3-fold in healthy males compared with females. Males' circulating neutrophils also showed lower levels of cell surface maturation markers, poorer signaling responses to GM-CSF, and lower neutrophil extracellular trap (NET) formation at baseline and upon bacterial and autoimmune stimuli compared with females' neutrophils. Longitudinal analysis of neutrophils' phenotype and blood transcriptome in studies of pregnancy, generally associated with increased susceptibility to and severity of certain infections (15), showed a significant increase in the ILNS during pregnancy. Furthermore, systems-wide phenotypic characterization of cells during pregnancy showed increases in CD15 and decreases in CD10, a profile that is characteristic of less mature neutrophils.

Thus, our study describes novel differences in the innate immune system between the sexes and during pregnancy in healthy subjects and adds to a large body of evidence showing more potent immunity in females than in males.

Materials and Methods

Study subjects and sample collection

This study is composed of several independent cohorts from different centers.

Stanford-Ellison longitudinal cohort. This cohort was composed of 91 subjects (ages 20 to >89) who were enrolled in an influenza vaccine study at the Stanford–Lucile Packard Children's Hospital Vaccine Program (Stanford, CA) during the years 2008–2012 (12, 16, 17) (ClinicalTrials.gov registration NCT#01827462). Because baseline samples were obtained from all individuals prior to vaccination with the influenza vaccine, no randomization or blinding was done for this study. The protocol for this study was approved by the Institutional Review Board of Stanford University School of Medicine. Informed consent was obtained from all subjects. All individuals were ambulatory and generally healthy, as determined by clinical assessment. At the time of initial enrollment, volunteers had no acute systemic or serious concurrent illness, no history of immunodeficiency, or any known or suspected impairment of immunologic function, including clinically observed liver disease, diabetes mellitus treated with insulin, moderate to severe renal disease, blood pressure >150/95 at screening, chronic hepatitis B or C, and recent or current use of immunosuppressive medication. In addition, on each annual vaccination day, none of the volunteers had been recipients or donors of blood or blood products within the past 6 mo and 6 wk, respectively, and none showed any signs of febrile illness on the day of baseline blood draw. Peripheral blood samples were obtained from venipuncture, and whole blood was used for gene expression analysis.

Stanford pregnancy cohort. The study was conducted at Lucile Packard Children's Hospital (Stanford, CA). The study was approved by the Institutional Review Board of Stanford University School of Medicine, and all participants gave informed consent. Five healthy pregnant women were prospectively enrolled in the study. Maternal demographic characteristics included age (31 ± 3.1 y, range: 29–36 y), gravidity (range 0–3), parity 1, and maternal body mass index (19.7 ± 5.3 kg/m^2, range: 18.6–31.3 kg/m^2). Four peripheral blood samples were collected by venipuncture into heparinized tubes (sodium heparin; BD Biosciences, San Jose, CA) during the first, second, and third trimester of pregnancy and at 6 wk postpartum. Within 30 min of sample collection, whole-blood samples were fixed with a proteomic stabilization buffer (Smart Tube, San Carlos, CA) for 10 min at room temperature and frozen at −80°C for storage until further processing.

National Institutes of Health cohort. This study cohort included 22 healthy individuals (11 males and 11 females) (ages 22–30 y) and 5 SLE patients (all females) who were recruited at the Clinical Center, National Institutes of Health. All individuals signed informed consent on Institutional Review Board–approved protocol NIH 94-AR-0066: the Studies of the Pathogenesis and Natural History of SLE that evaluates subjects with SLE, their relatives, and healthy individuals to understand the disease and mechanisms leading to organ damage. Peripheral blood was obtained from venipuncture, and cells were isolated as described below.

Baylor cohort. A total of 12 pregnant women and 10 healthy controls was recruited at the Baylor Institute for Immunology Research (Dallas, TX) for a study of immunological signatures of pregnancy (IRB# 009-257). All participants signed informed consent. The protocol for this study was approved by the Institutional Review Board of the Research Compliance Office at the Baylor Institute for Immunology Research. Peripheral blood samples were obtained at 2-wk intervals, using a noninvasive finger prick method, in microcapillary tubes for further RNA extraction and transcriptomic analysis.

Gene expression analysis

Stanford cohort. Two microarray platforms were used to generate expression data from whole-blood samples obtained from a total of 91 individuals recruited as part of the Stanford-Ellison cohort (12, 16, 17), the Human HT12v3 Expression Bead Chip (Illumina, San Diego, CA) for the years 2008 and 2009, and the GeneChip PrimeView Human Gene Expression Array (Affymetrix, Santa Clara, CA) for the years 2010, 2011, and 2012. For the Illumina platform, biotinylated, amplified antisense cRNA targets were prepared from 200–250 ng of the total RNA using the Illumina RNA amplification kit (Applied Biosystems/Ambion). A total of 750 ng of labeled cRNA was hybridized overnight to Illumina HumanHT-12 v3 BeadChip arrays (Illumina), which contained >48,000 probes. The arrays were washed, blocked, stained, and scanned on an Illumina BeadStation 500, following the manufacturer's protocols.

BeadStudio/GenomeStudio software (Illumina) was used to generate signal intensity values from the scans. For normalization, the software was used to subtract background and scale average signal intensity for each sample to the global average signal intensity for all samples. A gene expression analysis software program, GeneSpring GX version 7.3.1 (Agilent Technologies), was used to perform further normalization. For the Affymetrix platform, standard Affymetrix 3′IVT Express protocol was used to generate biotinylated cRNA from 50–500 ng of total RNA. DNA polymerase was used for the production of double-stranded cDNA. T7 RNA polymerase, in the presence of biotinylated nucleotides, was used for in vitro transcription of biotinylated cRNA. The fragmented and labeled targets were hybridized to the PrimeView Human Gene Expression Array cartridge, which measures gene expression of >36,000 transcripts and variants per sample using multiple independent measurements for each transcript (11 probes per set for well-annotated sequences, 9 probes per set for the remainder). The standard Affymetrix hybridization protocol includes a 16-h (overnight) hybridization at 45°C at 60 rpm in an Affymetrix GeneChip Hybridization Oven 645. The arrays were then washed and stained in an Affymetrix GeneChip Fluidics Station 450. The arrays were scanned using the Affymetrix GeneChip Scanner 3000 7G, and Affymetrix GeneChip Command Console Software was used for gene expression data processing and extraction. The raw data for years 2008 through 2012 were submitted to the Immunology Database and Analysis Portal (https://immport.niaid.nih.gov) under accession numbers SDY314, SDY312, SDY311, SDY112, and SDY315, respectively.

Baylor cohort. A NanoString Assay was executed at the Baylor Institute for Immunology Research Genomics Core. A total of 100 ng of total RNA was hybridized overnight (18 h) to target genes contained in custom gene expression nCounter Plex^2 for GEx NanoString Assay, following the manufacturer's Gene Expression Assay protocol. Enrichment of hybridized reporter/capture complexes and RNA target was carried out using SamplePrep Station, whereas signal detection was carried out in an nCounter Digital Analyzer set for high-resolution scanning. NanoString data analysis guidelines were followed to carry out normalization to assay positive controls and to subtract background noise. Normalization to housekeeping genes (included in custom gene panel) was carried out using housekeeping-gene global geometric mean approach. Resulting normalized values were reported for downstream statistical analysis.

Differential expression of genes expressed during cell differentiation or maturation

To identify gene modules enriched for genes expressed during monocyte/macrophage, DC, and neutrophil maturation, we used gene modules that were previously derived from our studies of aging (16). We searched for the presence of modules that were annotated (enriched) for maturation or differentiation of these cell types across a total of 109 gene modules. A gene module corresponds to a set of coexpressed genes sharing regulatory programs (18, 19). Briefly, a total of 6234 highly variant genes were normalized by centering and scaling the expression. Data were log transformed to approximate to normal distribution. We used hierarchical agglomerative clustering with average linkage, Euclidean distance, and a height cutoff value of 1.5 to derive 109 modules. For each gene module, a set of regulatory genes (regulatory program) was assigned, based on regression analysis of genes in the modules onto expression of 188 known transcription factors (20). The genes in modules 1 through 109 and their regulatory programs can be found at http://cs.unc.edu/~vjojic/fluy2-upd/.

We used gene set enrichment analysis (21) and found three gene modules whose regulatory program was annotated for genes expressed during DC differentiation (GM14, GM29, GM64) and one gene module enriched for genes expressed during neutrophil maturation (GM77). For these gene modules, a Student *t* test was computed on the mean module expression (first year of study, 2008) between males and females for both the young and older cohorts. The p values < 0.05 were considered statistically significant.

To determine the robustness of the sex differences for GM77, we used the QuSAGE gene set analysis method (22), which creates a probability distribution representing the mean and SD of a set of genes and enables comparisons of gene sets across different groups. For this analysis, samples from the individuals' first appearance in the study were used to analyze the sex associations for module expression (22 males and 22 females). To further validate these differences, we used publicly available gene expression data from 23 males and 33 females (Gene Expression Omnibus [GEO] accession numbers GSE29615 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29615] and GSE29617 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29617]) (23).

Longitudinal immune cell phenotyping of pregnancy by cytometry by time-of-flight

Sample processing and RBC lysis. Fixed samples were thawed for 10 min at 4°C and then for 10 min in a water bath kept at room temperature. Samples were then filtered using a 100-μm membrane into a hypotonic lysis solution (Smart Tube) and incubated for 5 min at room temperature. Samples were centrifuged and resuspended in lysis solution for 5 min and washed twice with cell-staining media (CSM; PBS with 0.5% BSA, 0.02% NaN_3).

Mass-tag cellular barcoding. To minimize experimental variability, samples were barcoded as previously described (24, 25). Briefly, isothiocyanobenzyl-EDTA/Pd (Pd)-based reagents were prepared for mass-tag barcoding. Twenty-well barcode plates were prepared with each well containing a unique combination of three Pd isotopes (102, 104, 105, 106, 108, or 110) at a concentration of 200 nM in DMSO. After sample thawing and RBC lysis, cells were washed with CSM, with PBS, and finally with 0.02% saponin in PBS. Barcoding plates were thawed and resuspended in 1 ml of 0.02% saponin in PBS. Barcoding reagent was added to each sample, and each sample was incubated with shaking at room temperature for 15 min. Samples were subsequently washed twice with CSM and pooled into a single tube for Ab staining. All 20 samples were analyzed using the same barcoded plate.

Abs. Abs for mass cytometry analysis were obtained preconjugated from the manufacturer (Fluidigm, South San Francisco, CA) or were conjugated in-house with the appropriate metal isotopes. Purified unconjugated Abs in protein-free PBS carrier were labeled using the Maxpar Ab conjugation kit (Fluidigm), according to the manufacturer's instructions. Conjugated Abs were stored at a concentration of 0.2 mg/ml (based on percentage yield calculated from measured absorbance at 280 nm) in Candor PBS Ab Stabilization Solution (Candor Biosciences, Wangen, Germany) at 4°C.

Ab staining. Pooled barcoded samples were incubated with a mixture of 26 Abs against surface Ag (Supplemental Table III) for 30 min with shaking at room temperature and then washed with CSM. Samples were incubated with an iridium intercalator (Fluidigm) with 1.6% PFA in PBS overnight at 4°C.

Mass cytometry. Intercalated samples were washed once with CSM and twice with double-distilled water and then resuspended in a solution of normalization beads (Fluidigm). Samples were filtered prior to mass cytometry analysis through a 35-μm membrane and analyzed at a flow rate of ~500 cells per second on a CyTOF 2.0 mass cytometry instrument (Fluidigm). Samples were normalized and debarcoded using software, as described previously (25, 26). The gating strategy is shown is Supplemental Fig. 2. Data were analyzed using viSNE analysis (27).

Isolation of neutrophils and analysis of NETs

To isolate neutrophils, heparinized blood was layered on a Ficoll density gradient, as described previously (28). RBCs were lysed with hypertonic solution, and neutrophils were resuspended in neutrophil medium (RPMI 1640 without phenol red; Life Technologies, Waltham, MA). Isolated neutrophils (1×10^6 cells per milliliter) were incubated on poly-L-lysine–coated coverslips with LPS (1 μg/ml), with 15% sera isolated from SLE patients that fulfilled the revised American College of Rheumatology criteria for the disease (29, 30), or without any stimulus for 3 h at 37°C.

Visualization and quantification of NETs by fluorescence microscopy

Neutrophils, seeded on coverslips and stimulated as above, were fixed with 4% paraformaldehyde. NETs were detected by washing the fixed cells with PBS and incubating with rabbit anti-human myeloperoxidase Ab (1:1000; Dako) for 60 min at 37°C, followed by incubation with secondary fluorochrome-conjugated Ab (1:200, A31572, Alexa Fluor 555 donkey anti-rabbit Abs; Life Technologies) for 30 min at 37°C. Nuclear DNA was detected by incubating cells with Hoechst 33342 (1:1000; Life Technologies) for 10 min at room temperature. After mounting (Prolong; Life Technologies), cells were visualized using fluorescence microscopy (Leica DMI4000B; Leica Microsystems).

The recorded images were loaded onto Adobe Photoshop (Adobe Systems) for further analysis, in which NETs were manually quantified. Strands positive for both neutrophil myeloperoxidase and nuclear staining (Hoechst) were considered a NET, and the number of cells undergoing NETosis was digitally recorded to prevent multiple counts. The percentage of NETs was calculated as the average of four fields (×20) normalized to the total number of cells.

Statistical analysis of NETs

The Fisher combined probability test (31) was used to calculate p values when comparing the percentages of NETs issued from two independent experiments.

Quantitative set analysis of gene expression

We used quantitative set analysis of gene expression (QuSAGE) to identify differences in gene sets that quantify gene-set activity using a probability density function. This method is particularly well suited for data sets showing intergene correlations and improves the estimation of the variance inflation factor. The analysis was performed using QuSAGE package in R (22).

Significance analysis of microarrays

Significance analysis of microarrays identifies genes with statistically significant changes in expression by assimilating a set of gene-specific *t* tests. Each gene is assigned a score on the basis of its change in gene expression relative to the SD of repeated measurements for that gene. Genes with scores greater than a threshold are deemed potentially significant. The percentage of such genes identified by chance is the false discovery rate (FDR) (32).

Venn diagram

A Venn diagram was generated using the VennDiagram package in R (33). The enrichment *p* value was calculated using the hypergeometric test.

Results

Upregulation of immature-like neutrophil gene signature in young, but not older, males compared with females

We first investigated whether males and females differed in their expression levels of maturation/differentiation genes in monocyte/macrophages, granulocytes, and DCs. We used transcriptomic data collected in the year 2008 from the Stanford-Ellison longitudinal cohort (1, 12, 16, 17, 34, 35), which consists of a total of 91 young (20–30 y) and older (60 to >89 y) individuals (Supplemental Table I) who are monitored yearly with a comprehensive analysis of immune biomarkers, including whole-blood gene expression, immune cell phenotypes, serum cytokines and chemokines, and signaling responses to a variety of stimuli. Because the sex differences in circulating GM-CSF levels were identified previously in young, but not older, adults (12), we analyzed young (n = 30) and older (n = 61) subjects separately. We used gene modules that were previously derived from gene expression data by cluster analysis and assignment of a set of transcription factors (regulatory program) to each module's profile [(16) http://cs.unc.edu/~vjojic/fluy2-upd/]. We searched for gene modules that were enriched for genes expressed in different stages of cell maturation and identified three gene modules (GM14, GM29 and GM64) whose regulatory programs were enriched for DC maturation genes ($p < 0.05$ for enrichment) and one gene module (GM77) enriched for genes expressed during neutrophil maturation (see below). No gene modules were found to be associated with the maturation of monocytes or macrophages. The complete list of genes in the GM14, GM29, GM64 and GM77 modules, as well as their regulatory programs, can be found in Supplemental Fig. 1 and Supplemental Table II, respectively.

Next, we searched for differences in the expression of GM14, GM29, GM64, and GM77 between males and females in the young group and in the older cohort and found no significant differences for GM14, GM29, or GM64 in either age group. However, the mean expression levels for GM77 were substantially higher in males compared with females in the young group ($p < 0.001$) but not in the older group ($p = 0.83$) (Fig. 1A, 1B). The genes contained in this module overlapped with a gene signature identified in a previous study that compared gene expression from immature neutrophils (obtained from bone marrow samples) with mature ones (obtained from peripheral blood samples) (36), as depicted in a Venn diagram (Fig. 1C), with an enrichment *p* value of 10^{-22} (by the hypergeometric test). Thus, the gene module GM77 contains genes representing an ILNS, including those encoding for granule-containing serine proteases, such as AZU (azurocidin), CTSG (cathepsin G), ELANE (elastase), and MPO (myeloperoxidase), among others. Of note, it is known that mRNA expression of these genes is highest during neutrophil development and decreases in late stages of maturation, during which the antibacterial proteins encoded by these genes are being packed in cytoplasmic granules for further release upon cell activation (37, 38).

To validate our findings, we computed the differences in the expression levels of GM77 in a larger cohort consisting of 22 males and 22 females (age 20–35) who were recruited as part of the Stanford-Ellison longitudinal cohort during the years 2008–2012. We used the first visit for each subject and applied QuSAGE (22), which is a method that summarizes expression information that is used to identify differences in gene sets to quantify gene-set activity using a probability density function. This method is particularly well suited for data sets showing intergene correlations, as is the case for the gene module approach (see *Materials and Methods*). In this new analysis, we confirmed the increased levels of the ILNS observed in males (expressed as a summarized module mean expression fold change in males versus females, Fig. 1D).

Next, we analyzed an independent data set of 23 males and 33 females (age 21–47 y) using data from the public domain that contained metadata, including gender (GEO accession numbers GSE29615 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29615] and GSE29617 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29617]) (23). Using the same approach, we again found a significant increase in the ILNS in males compared with females (Fig. 1E). Taken together, these data demonstrate that males' peripheral blood neutrophils have an immature-like phenotype compared with their female counterparts.

Immature-like neutrophil phenotype and function in young males compared with females

To further characterize the sex differences observed in neutrophils at the mRNA level, we investigated differences in neutrophil phenotype and function. To do so, we first reanalyzed existing data from a study of immune mechanisms of recovery from surgical trauma, consisting of a cohort of 16 males and 9 females (26–74 y old) whose blood was screened by cytometry by time-of-flight (CyTOF) for 34 markers expressed in a variety of cells (39). To identify features that were significantly associated with the variable sex, we conducted multiple regression analysis with permutations (>200 resamplings) to estimate significance (*p* value) for each regression coefficient. Because the age distribution of this cohort was relatively large and because the expected differences were age dependent, age and sex were included in the multiple regression models. At an FDR of 5%, we found that only two cell surface markers from the granulocyte subset of analyzed cells differed between males and females (Fig. 2A). These were CD11c and CD16, both of which were expressed at higher levels in females compared with males (Fig. 2B, 2C). In agreement with these results, the genes encoding for these molecules (ITGAX and FCGR3A, respectively) were found to be downregulated in immature neutrophils compared with mature ones (36), and the expression of both markers increases during neutrophil maturation (40).

To gain more insights into the mechanisms of these sex differences and the function of neutrophils between males and females, we analyzed neutrophil signaling responses to GM-CSF stimulation using data from the same study (39). As mentioned above, GM-CSF plays a crucial role in neutrophil differentiation and signals through heterodimeric receptor complexes by activating the JAK/STAT, MAPK, and PI3K pathways (41). GM-CSF also enhances functionality of mature neutrophils and elicits their

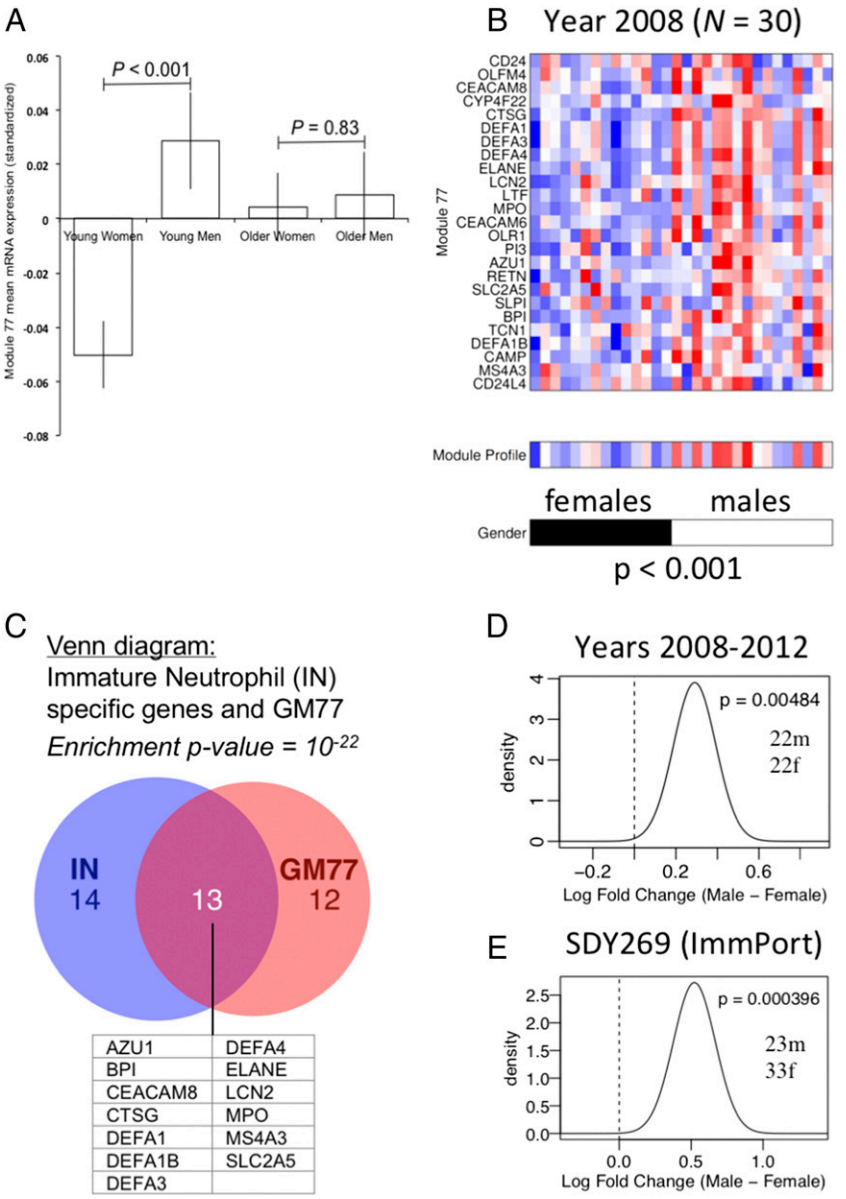

FIGURE 1. ILNS in males compared with females. (**A**) Differences in GM77 gene expression between males and females. Mean expression of module 77 was computed and compared between young women and young men or between older women and older men. Differences were estimated by the Student *t* test. (**B**) Heat map of gene expression of GM77 in young (20–30-y-old) individuals (14 females and 16 males), shown for different genes individually, as well as averaged expression for the whole module (module profile). (**C**) Venn diagram depicting overlaps between GM77 and immature neutrophil (IN)-specific genes. The 13 common genes are listed. Validation of the ILNS in two additional data sets of 22 males and 22 females (**D**) and of 23 males and 33 females from public domain data (GEO accession numbers GSE29615 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29615] and GSE29617 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29617]) (23) (**E**), by QuSAGE, expressed as a summarized module mean expression fold change of males versus females.

chemotaxis through ribosomal S6 phosphorylation (42). Using the same multiple-regression analysis described above, we identified 2 of 244 signaling features that were significantly different at 5% FDR (Fig. 2D). We found that, upon stimulation, phosphorylation of NF-κB and S6 ribosomal protein was higher in neutrophils from females compared with males (Fig. 2E, 2F). These results point to a more robust response to stimulation of neutrophils in females, in concordance with their more mature phenotype.

To further explore the functional differences between males' and female's peripheral blood neutrophils, we prospectively investigated the formation of NETs in vitro at baseline and in the presence of bacterial LPS or sera isolated from female patients with SLE (see *Materials and Methods*). NETs are chromatin structures loaded with antimicrobial molecules, and their release occurs in mature neutrophils as a response to cell activation. Healthy volunteers were recruited at the National Institute of Arthritis and Musculoskeletal and Skin Diseases (National Institutes of Health), and neutrophils from 11 females and 11 males were isolated for the evaluation of NET formation upon stimulation with LPS. Significantly higher levels of NET formation were observed in females' neutrophils in unstimulated and LPS-stimulated conditions (Fig. 2G, 2H). In addition, we measured NET formation after stimulation with sera from five female patients with SLE, an autoimmune disease in which the formation of NETs was described to be enhanced and plays an important pathogenic role (43, 44). Similar to the LPS stimulation, we observed that female neutrophils exposed to sera from patients with SLE developed significantly enhanced NET formation compared with males' neutrophils (Fig. 2H). Altogether, these data support the observation that females' neutrophils are in a more activated state at baseline and are more sensitive to activation by bacterial and sterile proinflammatory stimuli.

Immature-like forms of neutrophils in late pregnancy

The observed differences in the expression levels of the ILNS, as well as in neutrophil phenotype and function between males and females, may explain, in part, the usually poorer immune responses in males compared with females and the higher rates of autoimmune disease in females.

Pregnancy is also associated with increased severity of illness from infectious diseases and increased susceptibility to some infections (45, 46). This may be due to the major immunological

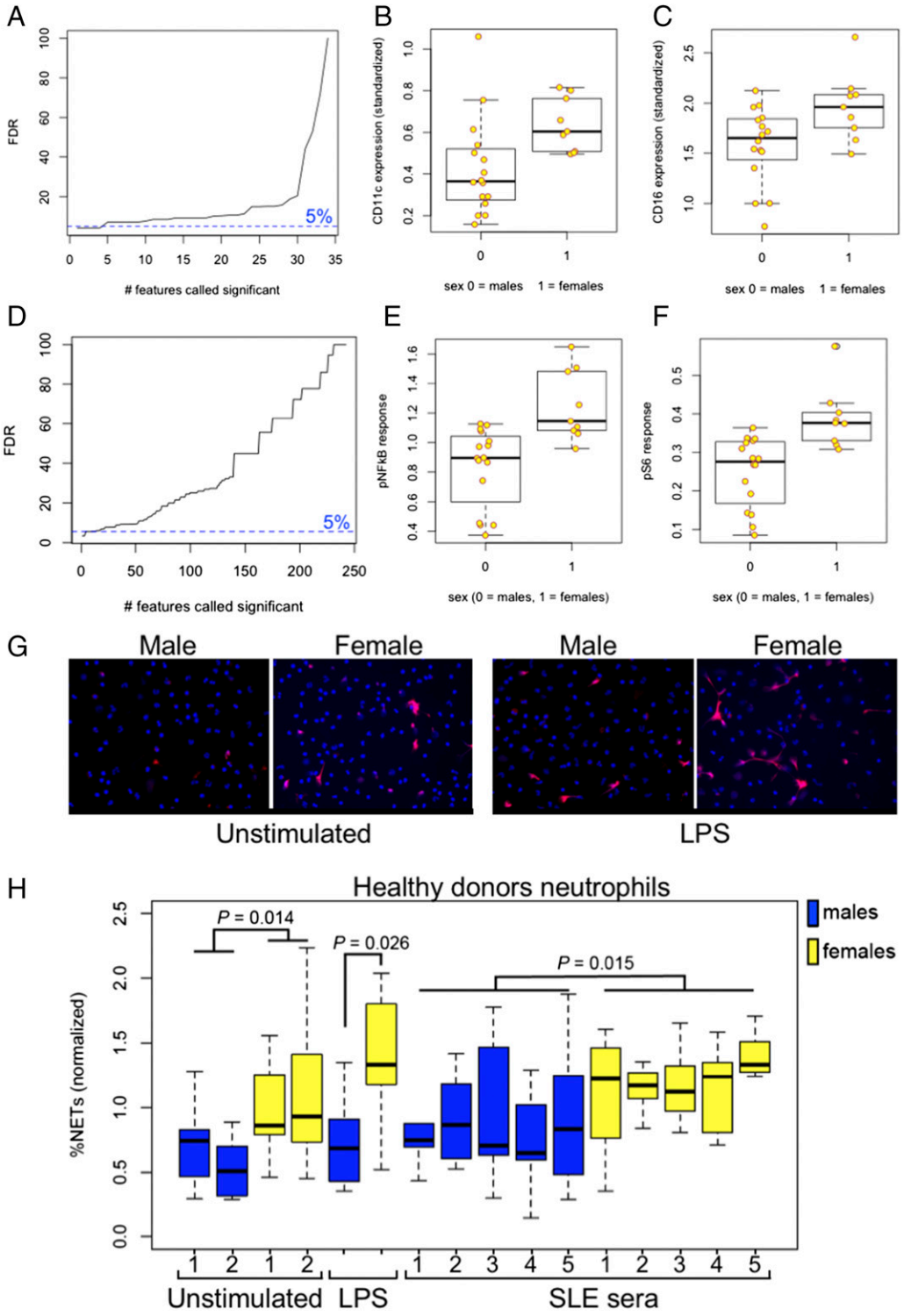

FIGURE 2. Cell surface markers and functional analysis of males' and females' neutrophils. Analysis of cell surface marker expression in males and females was conducted using a CyTOF instrument. (**A**) Multiple regression analysis with permutations for 34 molecules, with cut-off line at 5% FDR. Box plots showing standardized CD11c (**B**) and CD16 (**C**) expression in males and females. (**D**–**F**) Neutrophil responses to GM-CSF stimulation. (D) Multiple regression analysis with permutations for 244 features, with cut-off line at 5% FDR. Box plots showing the expression of standardized phosphorylated NF-κB (E) and phosphorylated S6 ribosomal protein (F) in males and females. (**G** and **H**) NET formation in vitro. (G) Fluorescent microscopy of NETs in neutrophils from healthy young males and females, in unstimulated conditions and after stimulation with bacterial LPS. (H) Box plots showing normalized percentage of NETs in healthy males and females in unstimulated conditions, after stimulation with LPS, and after stimulation with sera from five SLE patients. Significance of difference between males and females was calculated by combined *p* values (31) for two unstimulated conditions and five SLE sera–stimulated conditions.

transformations that ensue in pregnant women, which are likely a consequence of the immunosuppressive effect of elevated estrogen levels in late pregnancy (9) and the increasing levels of androgens, also suppressive, that were reported (47, 48). These changes are thought to be essential for maintaining feto–maternal tolerance and ensuring a successful pregnancy and include an increase in

anti-inflammatory cytokines, a decrease in proinflammatory mediators, a shift from Th1 to Th2 responses (3, 46, 49), an increase in regulatory T cells, and striking elevations in granulocytic myeloid-derived suppressor cells (GR-MDSCs). Thus, it is not surprising that for some autoimmune diseases, such as MS and RA, disease activity decreases throughout pregnancy (50). This is in contrast to SLE, which appears to worsen or remain unchanged during pregnancy.

To investigate changes in neutrophil maturation during pregnancy, we prospectively recruited five pregnant women at Stanford University and followed them longitudinally throughout pregnancy. Peripheral blood was obtained from each subject at each study time point (first, second, and third trimester and postpartum) and analyzed using CyTOF with an Ab panel targeting neutrophils (for the list of molecules see Supplemental Table III, for the gating strategy see Supplemental Fig. 2). We conducted viSNE analysis (27) and compared the levels of all of the cell surface markers using significance analysis of microarrays (32). We found significant differences in the expression of two markers of neutrophil maturation in late pregnancy compared with baseline samples: a decrease in CD10 and an increase in CD15 (Fig. 3A, 3B), a phenotype characteristic of less mature neutrophils (40).

To further validate these findings, we recruited 12 additional pregnant women and 10 age-matched healthy controls at the Baylor Institute for Immunology Research and followed them at 2-wk intervals from week 10 until delivery. Whole-blood RNA was extracted at each time point, and the changes in the expression of three representative ILNS genes, defensin α-4, carcinoembryonic Ag-related cell adhesion molecule (CEACAM)6, and CEACAM8, were analyzed using a NanoString gene expression assay. Nonpregnant women were used as controls. We observed a linear increase in the ILNS during the course of pregnancy compared with the controls ($p < 0.001$) (Fig. 3C). These results validate the less mature neutrophil phenotype indicated by the cell surface marker profiles during late pregnancy.

Taken together, these results demonstrate major changes in the phenotype and mRNA expression of neutrophils throughout pregnancy, with the most immature forms of neutrophils in women at late stages of pregnancy.

Discussion

In this study, we profiled peripheral blood from males and females using different technological platforms and multiple study cohorts and found an ILNS whose expression is elevated considerably in males compared with females. Males' neutrophils also show an immature cell surface marker phenotype, lower responses to cytokine stimulation, lower spontaneous rates of NET formation, and weaker NET production upon microbial and autoimmune stimuli. In independent cohorts of healthy pregnancy, the levels of this ILNS increased linearly, reaching significantly higher levels compared with baseline levels or those found in nonpregnant women. Furthermore, substantial changes in neutrophil phenotype with pregnancy indicate a switch to less mature forms carrying higher levels of CD15 and lower levels of CD10.

This increase in the ILNS in males and in late pregnancy suggests that the mechanisms leading to these relatively suppressed,

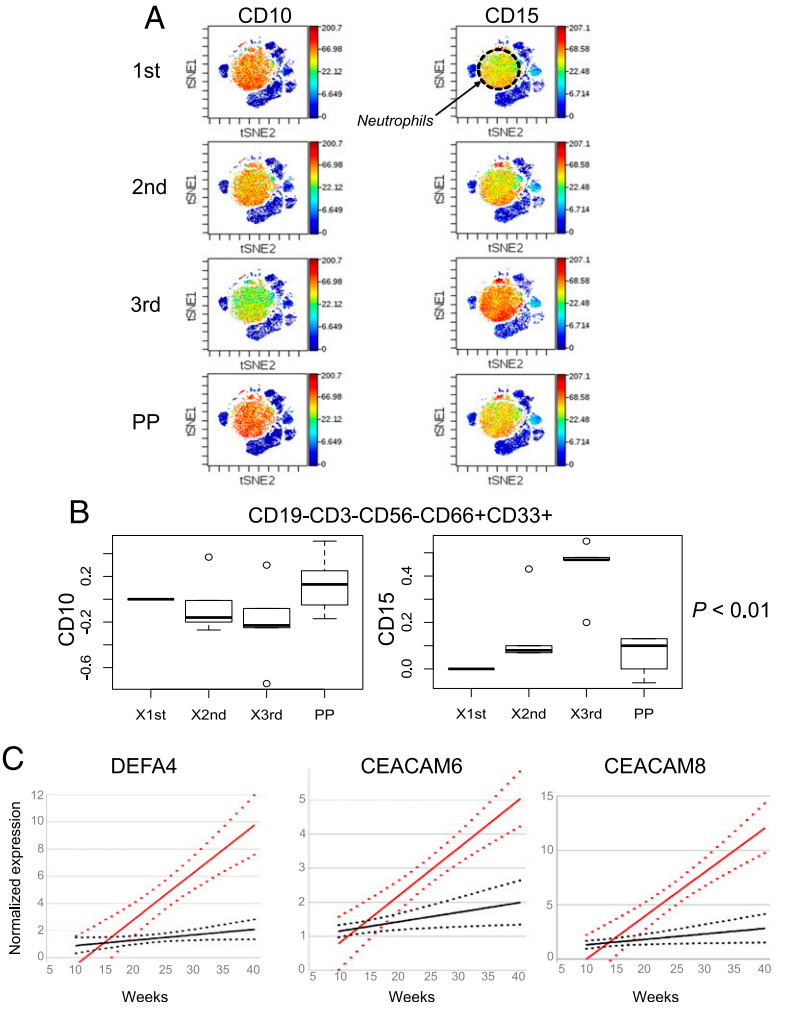

FIGURE 3. Neutrophil phenotype in pregnancy. Cell surface markers CD10 and CD15, measured by CyTOF, differentially expressed in four stages of pregnancy (first trimester, second trimester, third trimester, and postpartum [PP]), expressed as viSNE plots (**A**) and as standardized expression levels (**B**). Neutrophils, highlighted in the top right plot by arrow and dotted circle, were defined by expression of the following markers: $CD19^-$, $CD3^-$, $CD56^-$, $CD66^+$, and $CD33^+$. For gating strategy, see Supplemental Fig. 2. The level of expression is depicted by the color scale, as shown on the right side of each plot. (**C**) Differential RNA expression of three representative ILNS genes (defensin α-4, CEACAM6, and CEACAM8) in whole blood of pregnant women (red lines) and healthy controls (black lines) during pregnancy. Solid lines represent median expression; dotted lines indicate the boundaries in which >95% of the values for the group are contained.

yet normal, immune states may be common to these two conditions. Although some immune responses in pregnant women, such as those to influenza vaccination, are preserved (51), there is clear epidemiological evidence of higher morbidity and mortality due to increased severity of infections, such as influenza, hepatitis E, and herpes simplex, among others (45, 46, 52, 53). Furthermore, compared with nonpregnant women, males and pregnant women are less susceptible to developing inflammation-driven autoimmune diseases (50, 54).

Hence, it is plausible that the altered immunity observed in males and during late pregnancy involves an expansion of immature-like forms of circulating neutrophils observed in these cases because, in addition to neutrophils' role in innate immunity as controllers of the first line of defense, they can interact with other immune cells and regulate innate and adaptive immune responses. For example, a B cell helper subset of neutrophils, able to potentiate Ig class switching and somatic hypermutation, was recently reported in humans, rhesus macaques, and mice (55). Neutrophils also were shown to induce IFN-γ–polarizing naive T cells (56, 57) and to modulate T cell responses by production of diverse cytokines, such as TNF-α, upon interaction with APCs (56–58). Lastly, NETs formed by neutrophils can activate a type I IFN response, which is an important pathogenic mechanism in autoimmunity, and can prime $CD4^+$ Th cells by reducing their activation threshold (59).

Alternatively, the overall immune response to Ags and the development of autoimmune disease can also be tuned by the levels of different peripheral blood neutrophil subsets. For instance, based on their density, neutrophils can be divided into two groups: normal-density granulocytes (NDGs) and low-density granulocytes (LDGs). NDGs are represented by mature neutrophils, whereas LDGs consist of mature and immature neutrophils (60). Depending on disease context, LDGs may have more proinflammatory effect and display greater NET formation, such as in SLE (28, 61), but they also were described as being immunosuppressive (62–64). Although we did not directly measure the frequencies of LDGs and NDGs in our cohorts, previous studies showed that, during pregnancy, an increase in LDGs (which express higher levels of immature-like molecules CD15, CD33, and CD66b [CEACAM8] compared with NDGs) is characteristic (65); this could explain, at least in part, the skewed immune responses and amelioration of inflammation-driven autoimmune disease outcomes during late pregnancy (54, 66–68). Higher levels of LDGs also were described in HIV infection, for which an association with disease severity was reported (69). In both cases, pregnancy and worsened outcomes of HIV infection, immune suppression was associated with LDG-mediated impaired T cell responses (65, 69). Alternatively, other studies described increases in the proportion of GR-MDSCs in healthy pregnancies, where they seem to be responsible for the observed suppression of some immune reactions, as well as the induction of regulatory T cells (70, 71). The GR-MDSC subset is also characterized by a low-density and immature phenotype with high expression of CD11b, CD15, CD33, and CD66b molecules; thus, it is seemingly difficult to be able distinguish this subset from the neutrophil subset within the LDG fraction (60, 72, 73). Therefore, it is possible that the neutrophil phenotype observed in males and in pregnant women is due to an expansion of immature forms of LDGs, which may affect the overall inflammatory condition and immune responsiveness to Ags.

The mechanisms leading to a change in the proportion of responsive (mature) versus unresponsive (immature-like) forms of neutrophils in males and during late pregnancy observed in our study are not clear. However, our observations suggest that hormonal differences between males and females and hormonal fluctuations during pregnancy account for this immunological switch. In particular, high levels of androgens are observed in adult males and in women during late pregnancy (47, 48). Because the expansion of immature LDGs (which include GR-MDSCs) and the activation of these cells under certain conditions can lead to an upregulation of immune-suppressive factors, such as arginase (encoded by *ARG1*) and inducible NO synthase (also known as NOS2) (74), it is tempting to hypothesize that androgens are implicated in the regulation of these molecules. In support of this hypothesis, experiments in hypogonadic animals showed that NOS2 levels are significantly reduced and can be restored upon treatment with testosterone (75). Furthermore, an analysis of circulating testosterone and gene expression data from our previous studies of sex differences in immunity (12) shows that, in young adults (20–30 y old), the levels of free testosterone from serum correlate with expression of the NOS2 gene ($p = 0.01$) (Supplemental Fig. 3). Lastly, increased arginase activity in activated LDGs was found during pregnancy, and this contributes to the suppression of the maternal immune system to ensure successful pregnancies (65, 76).

Although testosterone seems to be crucial for neutrophil production and differentiation, as shown in androgen receptor–knockout mice (77), its effect on cell maturation has not been determined. However, low levels of testosterone were associated with an increased risk for autoimmune RA in men (78); in addition, testosterone can suppress superoxide production and microbicidal activity in neutrophils and, thus, influence the inflammatory response (79). Furthermore, an increase in testosterone levels suppresses the expression of proinflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) and potentiates the expression of the anti-inflammatory cytokine IL-10 (80, 81), which was shown to inhibit neutrophil phagocytic and bactericidal activity and downregulate some cell surface proteins (e.g., CD11b), pointing toward a less mature phenotype (82, 83). Consistent with our observations, IL-10 levels increase in pregnant women (84, 85) and were associated with diminished symptoms of RA during pregnancy (86). Although, to the best of our knowledge, there is no evidence of higher baseline levels of IL-10 in males compared with females, males produce substantially higher IL-10 levels upon viral infections (87), which could directly impact neutrophil phenotype and function. Thus, more studies addressing the effects of androgens in the development of LDGs and GR-MDSCs are warranted.

This study generates a number of hypotheses on the sex differences in the human immune system, but one limitation includes the lack of data about the mechanisms that lead to the expansion of immature neutrophils in males and pregnant women. Another limitation derives from the fact that, although we observe a significant increase in ILNS in males compared with females in several independent cohorts and a robust expansion of this signature in our longitudinal studies of pregnancy, in some cases, because of the use of public domain data sets, the markers investigated and age ranges in the different cohorts do not fully overlap. This results in discrepancies, for example, in the expressed cell surface markers found in higher proportions in females' compared with males' neutrophils (CD11c and C16) and those changing with pregnancy (CD15 and CD10). However, it is well established that high expression of CD11c, CD16, and CD10 is characteristic of mature neutrophils (36, 40, 88) and that CD15 expression on neutrophils decreases during late stages of maturation (segmented versus band cells) (40). Thus, we conclude that immature neutrophil states are found in males and during late pregnancy.

In summary, using longitudinal monitoring and systems-level phenotyping of blood samples, we find immature-like forms of neutrophils in healthy and ambulatory males, with similarities observed during late stages of pregnancy, where a switch to more immature profiles is accompanied by an increase in the ILNS. Thus, our results add to previous evidence showing marked sex differences in immune cell function. The differences found in our study are also relevant to the prevalence of autoimmunity in females and suggest a common mechanism for the decreased immune responses in males and in pregnant women.

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Disclosures

The authors have no financial conflicts of interest.

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