Sex differences in neutrophil biology modulate response to type I interferons and immunometabolism

Sarthak Gupta[a,1], Shuichiro Nakabo[a], Luz P. Blanco[a], Liam J. O'Neil[a], Gustaf Wigerblad[a], Rishi R. Goel[a], Pragnesh Mistry[a], Kan Jiang[b], Carmelo Carmona-Rivera[a], Diana W. Chan[a], Xinghao Wang[a], Hege L. Pedersen[a], Manasi Gadkari[a], Katherine N. Howe[c], Faiza Naz[d], Stefania Dell'Orso[d], Sarfaraz A. Hasni[a], Caeden Dempsey[e], Ashley Buscetta[f], Pamela A. Frischmeyer-Guerrerio[e], Paul Kruszka[f], Maximilian Muenke[f], Luis M. Franco[a], Hong-Wei Sun[b], and Mariana J. Kaplan[a,1]

[a]Systemic Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), NIH, Bethesda, MD 20892; [b]Biodata Mining and Discovery Section, NIAMS, NIH, Bethesda, MD 20892; [c]Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, MD 20892; [d]Laboratory of Muscle Stem Cells and Gene Regulation, NIAMS, NIH, Bethesda, MD 20892; [e]Food Allergy Research Unit, NIAID, NIH, Bethesda, MD 20892; and [f]Medical Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, MD 20892

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Differences between female and male immunity may contribute to variations in response to infections and predisposition to autoimmunity. We previously reported that neutrophils from reproductive-age males are more immature and less activated than their female counterparts. To further characterize the mechanisms that drive differential neutrophil phenotypes, we performed RNA sequencing on circulating neutrophils from healthy adult females and males. Female neutrophils displayed significant up-regulation of type I IFN (IFN)-stimulated genes (ISGs). Single-cell RNA-sequencing analysis indicated that these differences are neutrophil specific, driven by a distinct neutrophil subset and related to maturation status. Neutrophil hyperresponsiveness to type I IFNs promoted enhanced responses to Toll-like receptor agonists. Neutrophils from young adult males had significantly increased mitochondrial metabolism compared to those from females and this was modulated by estradiol. Assessment of ISGs and neutrophil maturation genes in Klinefelter syndrome (47, XXY) males and in prepubescent children supported that differences in neutrophil phenotype between adult male and female neutrophils are hormonally driven and not explained by X chromosome gene dosage. Our results indicate that there are distinct sex differences in neutrophil biology related to responses to type I IFNs, immunometabolism, and maturation status that may have prominent functional and pathogenic implications.

neutrophils | sex differences | interferons | innate immunity

Females mount more robust immune responses than males and this may contribute to enhanced responses to vaccination (1–3), better outcomes from certain infections, including the recent coronavirus disease (COVID-19) pandemic, and sepsis (4–7) and decreased risk for certain cancers (5). Conversely, enhanced immune responses in females contribute to increased susceptibility to inflammatory and autoimmune diseases (8). Importantly, the mechanisms that drive these stark contrasts between male and female immunity remain insufficiently characterized.

In humans, neutrophils are the most abundant circulating immune cells, represent a critical part of innate immune responses, and the first line of defense against microorganisms. Neutrophils also play key roles in the pathophysiology of various systemic autoimmune diseases, respiratory disorders like acute respiratory distress syndrome (ARDS), and different types of cancer (9–11). Despite their important roles in homeostasis and disease, little is understood at the molecular level about how sex differences modulate neutrophil responses.

We previously reported that young (20 to 30 y old), but not older (60 to 89 y old), healthy adult males, displayed significant enhancement in an immature neutrophil gene signature compared to age-matched females (12). This translated into a less activated male neutrophil phenotype based on cell surface markers, hampered response to cytokine stimulation, and decreased ability to form neutrophil extracellular traps (NETs) following various stimuli (12).

To explore these differences further, we assessed the transcriptome of male and female neutrophils and identified up-regulation of the type I IFN (IFN) pathway in the latter. Single-cell RNA sequencing (RNA-seq) demonstrated that IFN pathway up-regulation is neutrophil specific, driven by a distinct neutrophil subset, and related to neutrophil maturation status. These differences are likely due to hyperresponsiveness of the female neutrophils to IFNs and contribute to enhanced proinflammatory responses to danger signals. Moreover, these differences in neutrophil maturation status promote different bioenergetic profiles in male and female neutrophils, in a hormone-dependent manner.

Significance

Despite clear differences in female and male immunity that may contribute to variations in response to infections and predisposition to cancer and autoimmunity, the exact mechanisms that drive this stark contrast remain insufficiently characterized. Neutrophils play essential roles in homeostasis and disease, but little is known about how sex differences modulate their phenotype and function. Using transcriptomic and functional approaches, we report that healthy young adult females have an activated/mature neutrophil profile characterized by enhanced type I IFN pathway activity, enhanced proinflammatory responses, and distinct bioenergetics. We further show that these differences are cell specific and likely driven by sex hormones. Modulation of these pathways in neutrophils may provide more individualized, sex-specific therapeutic options in a variety of disease states.

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Data deposition: Data from cohort 1 and single-cell data have been uploaded to the Gene Expression Omnibus (GEO) with series no. GSE145231 and data from cohort 2 have been uploaded to the GEO with series no. GSE145033.

[1]To whom correspondence may be addressed. Email: sarthak.gupta@nih.gov or mariana.kaplan@nih.gov.

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Studies in neutrophils from subjects with Klinefelter syndrome (47, XXY) and prepubescent children indicated that sex differences in neutrophil biology are not explained by X chromosome gene dosage and appear to be driven by sex hormones.

Results

Neutrophils from Healthy Young Adult Males and Females Differ in Their Transcriptional Profile. To determine the transcriptional profile of neutrophils from healthy young adult males and females (20 to 31 y old), we performed mRNA-sequencing analysis on density-gradient isolated neutrophils (cohort 1, $n = 7$ per group). Analysis revealed significant differences in gene expression between female and male neutrophils. In female neutrophils, 106 genes were up-regulated and 128 genes were down-regulated, compared to male neutrophils. Neutrophils in earlier stages of development are more transcriptionally active (13). Consistent with our previous report that had used whole blood analysis in other healthy volunteer cohorts (12), primary granule genes, which are expressed at higher levels during earlier stages of neutrophil development, were higher in male than in female neutrophils, suggesting a more immature phenotype (Fig. 1*A* and *SI Appendix*, Table S1). These results indicated differences in the transcriptional program of male and female neutrophils, with confirmation of male neutrophils being more immature compared to female neutrophils.

Genes in the Type I IFN Pathway Are Up-Regulated in Female Neutrophils. Pathway analysis and gene set enrichment analysis (GSEA) showed enrichment of pathways related to type I IFN-signaling/IFN-stimulated genes (ISGs) and neutrophil activation genes in females compared to males (Fig. 1 *B* and *C*). Analysis using a previously validated gene signature comprised of 21 specific type I ISGs (14) showed that their expression values distinctly clustered neutrophils from male and female subjects (Fig. 1*D*). A composite IFN score derived from these 21 ISGs revealed a fourfold higher value of this signature in female compared to male neutrophils (Fig. 1*E*). These results indicated that female neutrophils up-regulate ISGs.

We confirmed the reproducibility of these findings in an independent cohort of healthy adult volunteers (cohort 2), where total RNA sequencing was performed on isolated neutrophils from 8 males and 10 females. All controls in this cohort were between 21 and 50 y of age except 1 female (61 y old) and 1 male (62 y old). Neutrophils were isolated by immunomagnetic negative selection followed by total RNA sequencing. Notwithstanding these differences, transcriptome analysis confirmed that ISGs were up-regulated in female compared to male neutrophils and were sufficient to differentially cluster males and females (Fig. 1*F* and *SI Appendix*, Table S2). The 21-ISG signature score showed a fourfold higher value in female compared to male neutrophils (Fig. 1*G*). We confirmed higher expression of genes characteristic of immature neutrophils in male neutrophils, including *DEFA*, *AZU1*, and *MPO* (*SI Appendix*, Table S2).

To allow sex-stratified comparative visualization of the neutrophil transcriptome data generated in both cohorts, we developed a tool, named NeutGx, that is publicly accessible through the NIAID Bioinformatics Portal (https://neutgx.niaid.nih.gov) (*SI Appendix*, Fig. S1).

Overall, in healthy adult subjects, female neutrophils consistently displayed transcriptomic profiles indicative of a more mature and activated phenotype with an enhanced type I IFN signature.

The Enhanced Type I IFN Signature in Female Leukocytes Is Neutrophil Specific. To assess if the enhanced ISG signature in females is observed in other leukocytes, we performed single-cell RNA sequencing on whole blood from seven young adult healthy volunteers (three males, four females). This sequencing captured the major peripheral blood immune cell types, including neutrophils, with no differences in cell numbers in the major cell clusters between males and females, and no significant transcriptional differences between male and female B cells, $CD4^+$ and $CD8^+$ T cells (Fig. 2 *A* and *B*). In contrast, female neutrophils displayed higher expression of multiple ISGs compared to males, confirmed by pathway analysis (Fig. 2 *C–E* and *SI Appendix*, Table S3). This was further validated with 21-ISG signature, as there were no significant differences between female and male T cell subsets, B cells, monocytes, or natural killer (NK) cells, while female neutrophils displayed higher ISG expression (Fig. 2*E*). Similar to the bulk RNA-sequencing data, higher expression of genes associated with neutrophil immaturity was detected in male neutrophils (*SI Appendix*, Table S3).

To validate further the neutrophil specificity of these findings, we accessed a publicly available RNA-seq gene expression dataset of purified monocytes and T cells from healthy post-pubertal young females and males. No differences were detected in 21-ISG scores in monocytes or T cells between healthy males and females (Fig. 2*F*). These results indicated that ISG up-regulation in young adult females was specific to neutrophils and not observed in other leukocytes.

The IFN Signature in Neutrophils Is Driven by a Distinct Subset. The characterization of neutrophil subsets in peripheral blood and their contribution to innate immunity and inflammation has recently expanded with the use of single-cell RNA-seq approaches (15, 16). To assess if the elevated type I IFN gene signature in female neutrophils is driven by a distinct subset, we examined further the neutrophil cluster from the whole blood single-cell RNA-seq dataset detailed above. Healthy young adult male and female neutrophils clustered into four distinct subsets based on their gene expression profiles (Fig. 3 *A* and *B*), with no obvious differences in number of subsets or cells/subset between male and female neutrophils (*SI Appendix*, Table S4). Analyses of genes enriched in these neutrophil clusters revealed a distinct neutrophil subset (subset 2) displaying enrichment in ISGs (Fig. 3 *B* and *C*). Similar to findings by Xie et al. (15) that were performed in mice and in one human male, we found that ISG expression in other human neutrophil clusters (subsets 0, 1, and 3) was minimal, supporting the notion that a distinct neutrophil subset (subset 2) in human peripheral blood displays elevated ISG expression. We compared the 21-ISG signature between the four neutrophil clusters in males and females and confirmed an elevated IFN signature in the female cluster 2 (F2) compared to male cluster 2 (M2) (Fig. 3*D*). These findings identified a distinct subset of neutrophils that drive the elevated peripheral blood neutrophil type I IFN signature in healthy young adult females compared to males.

Female Neutrophils Are Hyperresponsive to Type I IFNs. We assessed if the increased ISG signature observed in healthy young adult female neutrophils was a consequence of exposure to higher levels of circulating type I IFNs in women. Measuring type I IFN protein in human blood has been challenging and many commercially available kits lack sensitivity and/or specificity (17). Thus, to assess circulating type I IFN levels, we used a previously validated bioassay (18). No significant differences were observed in the ability of female and male sera in inducing ISGs in HeLa cells (Fig. 4*A*). Immunofluorescence microscopy studies (Fig. 4*B*) demonstrated that transcription factors involved in type I IFN signaling, such as IFN regulatory factor 9 (IRF9), phosphorylated IRF3 and p65 subunit of nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) showed preferential nuclear localization in healthy young adult female neutrophils; in contrast, these transcription factors displayed a predominantly cytosolic distribution in male neutrophils, indicative of inactive forms. These

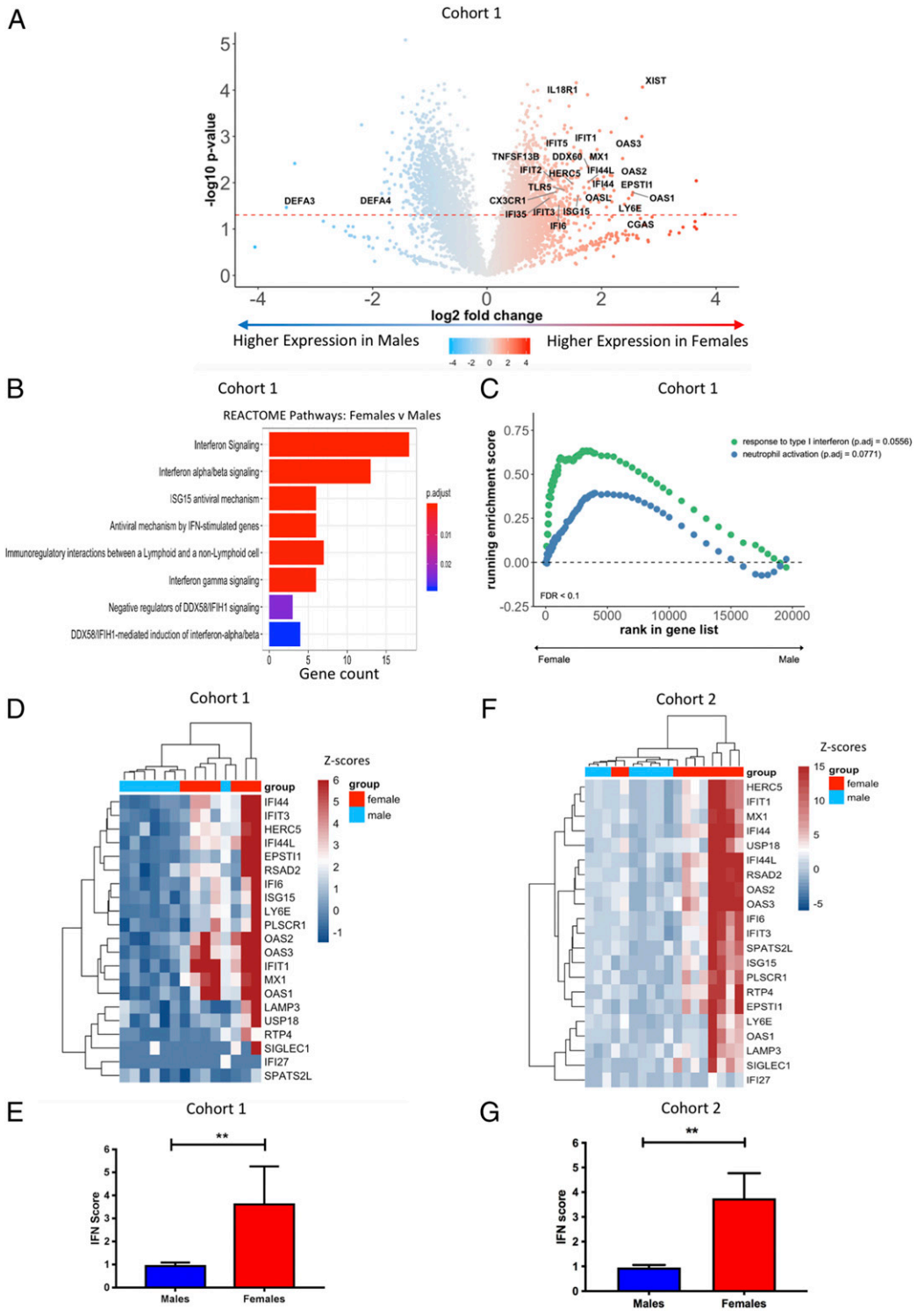

Fig. 1. Adult female neutrophils up-regulate ISGs. (*A*) Volcano plot of differentially expressed genes comparing healthy young adult female and male neutrophils (cohort 1; $n = 7$/group). The dataset was filtered to include the 10,000 genes with highest average expression. Genes in red have higher expression in female neutrophils and genes in blue have higher expression in male neutrophils. (*B*) Pathway enrichment analysis of differentially expressed genes between female and male neutrophils (cohort 1; $n = 7$/group). Statistical analysis was performed with the clusterProfiler package in RStudio. (*C*) GSEA plots of ISGs and neutrophil activation genes in female vs. male neutrophils (cohort 1; $n = 7$/group). Differentially expressed genes were ordered using a calculated rank metric = −log10(*P* value) * log2(fold change). Adjusted *P* values for each gene set are shown. (*D*) Heatmap and hierarchal clustering of ISG expression in female and male neutrophils (cohort 1; $n = 7$/group). Data are represented as z scores as compared to average of the male samples. (*E*) Sex differences in type I IFN score between female and male neutrophils (cohort 1; $n = 7$/group). IFN score was calculated for each individual using 21-gene ISGs from RNA-sequencing data. *P* values were calculated with Mann–Whitney *U* test; $^{**}P \leq 0.005$. (*F*) Heatmap and hierarchal clustering of ISG expression in female ($n = 10$) and male ($n = 8$) neutrophils in cohort 2. Data are represented as z scores as compared to average of the male samples. (*G*) Sex differences in IFN score between female ($n = 10$) and male ($n = 8$) neutrophils in cohort 2. IFN score was calculated for each individual using 21-gene ISGs from RNA-sequencing data. *P* values were calculated with Mann–Whitney *U* test; $^{**}P \leq 0.005$.

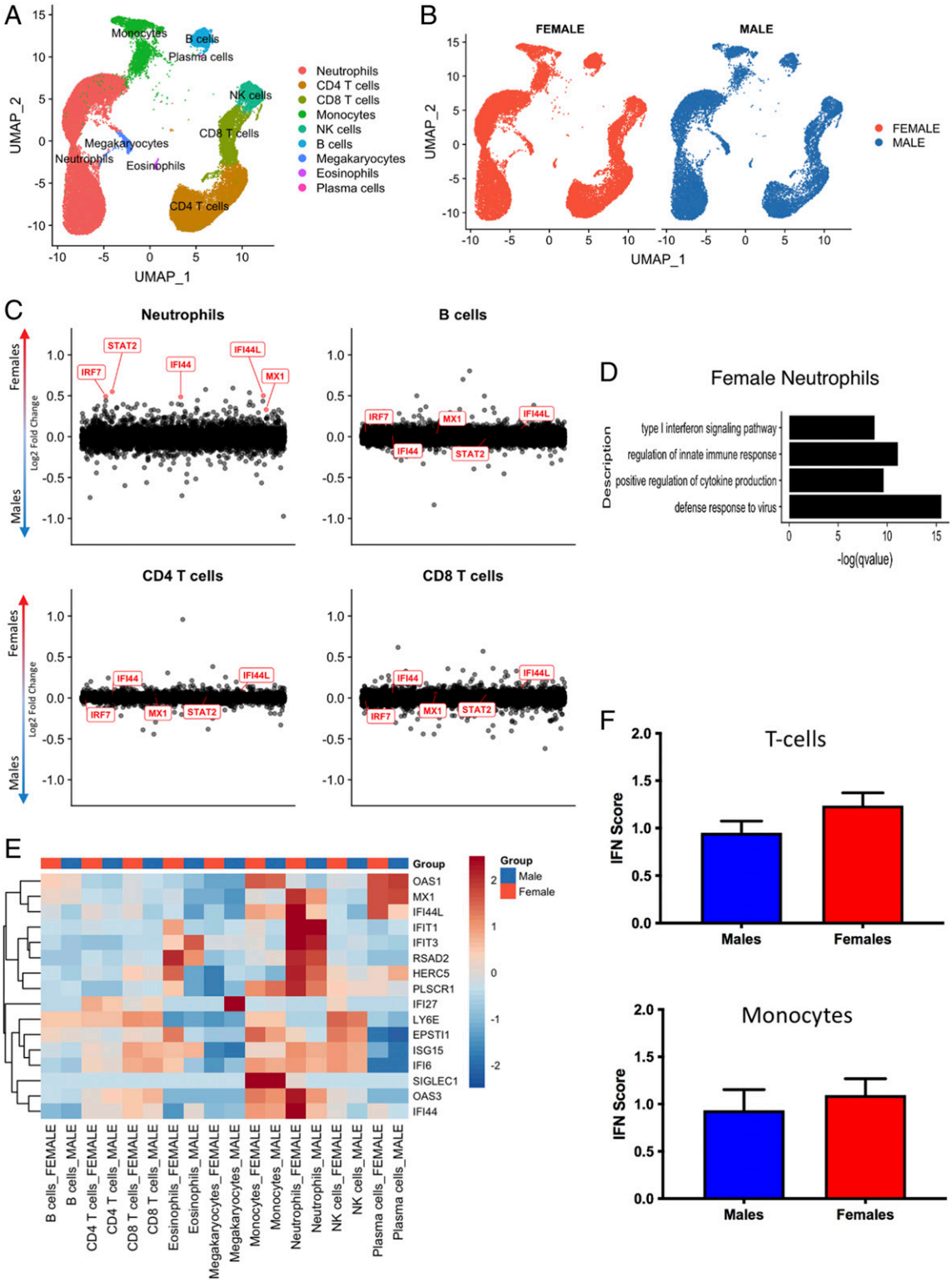

Fig. 2. Enhanced type I IFN response in female whole blood leukocytes is restricted to neutrophils. (*A*) Uniform manifold approximation and projection (UMAP) clustering of whole blood human leukocytes from seven healthy human controls (n = 40,724 cells, females = 4, males = 3). (*B*) UMAP representation of female (red, n = 24,083 cells; 4 donors) and male (blue, n = 16,641 cells; 3 donors) whole blood leukocytes. (*C*) Log2 fold change difference between main male and female white blood cell clusters with differentially expressed ISGs highlighted in red (female = 4, male = 3). (*D*) Up-regulated GO pathways in female neutrophils relative to male neutrophils (female = 4, male = 3). (*E*) Heatmap representation of the 21 ISGs in each cell cluster split by sex (female = 4, male = 3). (*F*) IFN score in T cells and monocytes from healthy postpubertal young males and females from a publicly available database (GSE103147). IFN score was calculated for each individual using 21 ISGs from RNA-sequencing data publicly available performed on sorted T cells (female = 40, male = 17) and monocytes (female = 25, male = 17). Mann–Whitney *U* test was used to determine differences in the score between males and females.

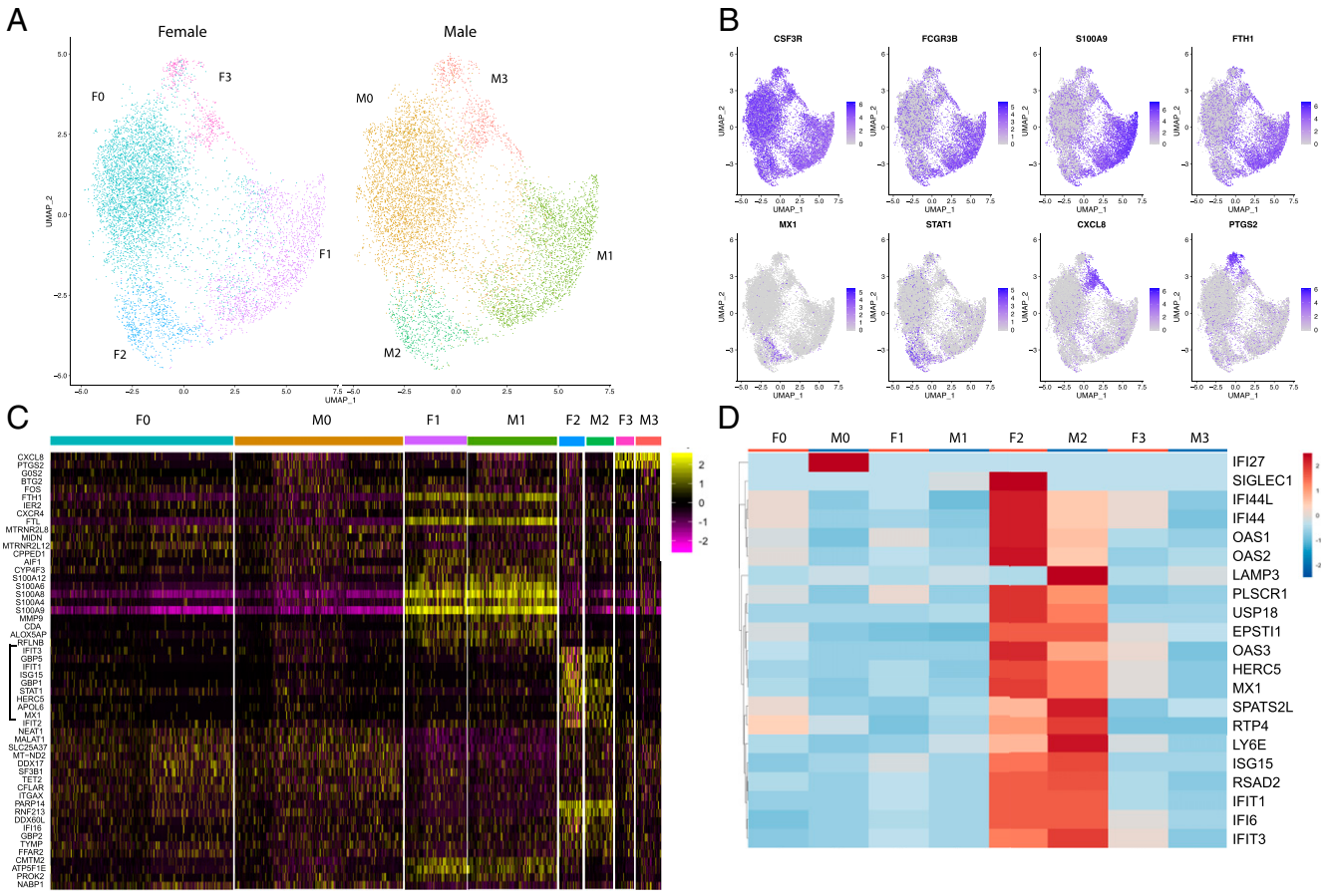

Fig. 3. The IFN signature in neutrophils is driven by a distinct subset and ISGs in this subset are elevated in females. (*A*) UMAP clustering of neutrophils from human whole blood leukocytes from females ($n = 4$) and males ($n = 3$). (*B*) UMAP feature map showing expression of various neutrophil genes in different neutrophil subsets from all healthy controls ($n = 7$, female = 4, male = 3). Colony-stimulating factor 3 receptor (*CSF3R*), Fc fragment of IgG receptor IIIb (*FCGR3B*), S100 calcium-binding protein A9 (*S100A9*), ferritin heavy chain 1 (*FTH1*), MX dynamin-like GTPase 1 (*MX1*), signal transducer and activator of transcription 1 (*STAT1*), C-X-C motif chemokine ligand 8 (*CXCL8*, *IL8*), and prostaglandin-endoperoxide synthase 2 (*PTGS2*, *COX2*). (*C*) The top defining genes of the four neutrophil clusters from females (F, $n = 4$) and males (M, $n = 3$) based on gene expression, with cluster 2 showing high expression of ISGs. (*D*) Expression of 21 ISGs in different neutrophil subsets display increased expression of ISGs in subset 2 in females ($n = 4$) and males ($n = 3$), and higher ISG expression in females compared to males.

observations were supported by transcriptomic data from cohort 1 that showed increased expression of *STAT1*, a key transcription factor that modulates multiple ISGs, in female neutrophils (Fig. 4*C*) (19). These results suggested that, even in the absence of in vitro stimulation, the type I IFN pathway was constitutively more activated in young adult female than male neutrophils, which may explain the enhanced IFN gene signature. By flow cytometry, we found no differences in the cell surface expression levels of type I IFN receptor (IFNAR1) between male and female neutrophils (Fig. 4*D*).

Overall, these results indicated that the differences in ISG levels between young adult female and male neutrophils were driven by enhanced activity of the type I IFN signaling pathway rather than by exposure to enhanced circulating levels of these cytokines or enhanced expression of IFNAR1 in females.

Type I IFNs Prime Neutrophils to Respond to Toll-Like Receptor Stimulation. Type I IFNs play important roles in neutrophil biology (20). IFNα can enhance inflammatory cytokine synthesis by human neutrophils, following stimulation with Toll-like receptor 8 (TLR8) agonist (21). To assess if priming with type I IFNs, mimicking the enhanced response to these cytokines observed in female neutrophils, would lead to enhanced proinflammatory responses following TLR stimulation, we preincubated healthy young adult male neutrophils with IFNα (10 ng/mL) for 15 min, followed by stimulation with the TLR7/8 agonist R848 for 3 h. IFNα-primed male neutrophils showed significant up-regulation of *TNF* mRNA expression (but not *IL6*), in response to R848, when compared to cells treated with TLR agonist alone (Fig. 5*A*). These results suggested that enhanced responses to type I IFNs observed in young adult female neutrophils may endow them with an increased ability to respond to danger signals and acquire a more proinflammatory phenotype with augmented levels of TNF synthesis.

Young Adult Male and Female Neutrophils Differ in Bioenergetics, which Is Modulated by Sex Hormones. Recent reports indicate that immature bone marrow-derived neutrophils have enhanced basal and maximal mitochondrial respiration when compared with mature neutrophils (22, 23). As male neutrophils had an enhanced immature neutrophil signature, we performed metabolic assays in healthy young adult female and male neutrophils using a Seahorse analyzer ($n = 6$ each; Fig. 5 *B*–*D*). We quantified oxygen consumption rate (OCR, to assess mitochondrial respiration) and extracellular acidification rate (ECAR, to measure glycolysis). Throughout the mitochondrial stress assay, OCR was significantly higher in male than female neutrophils, while there

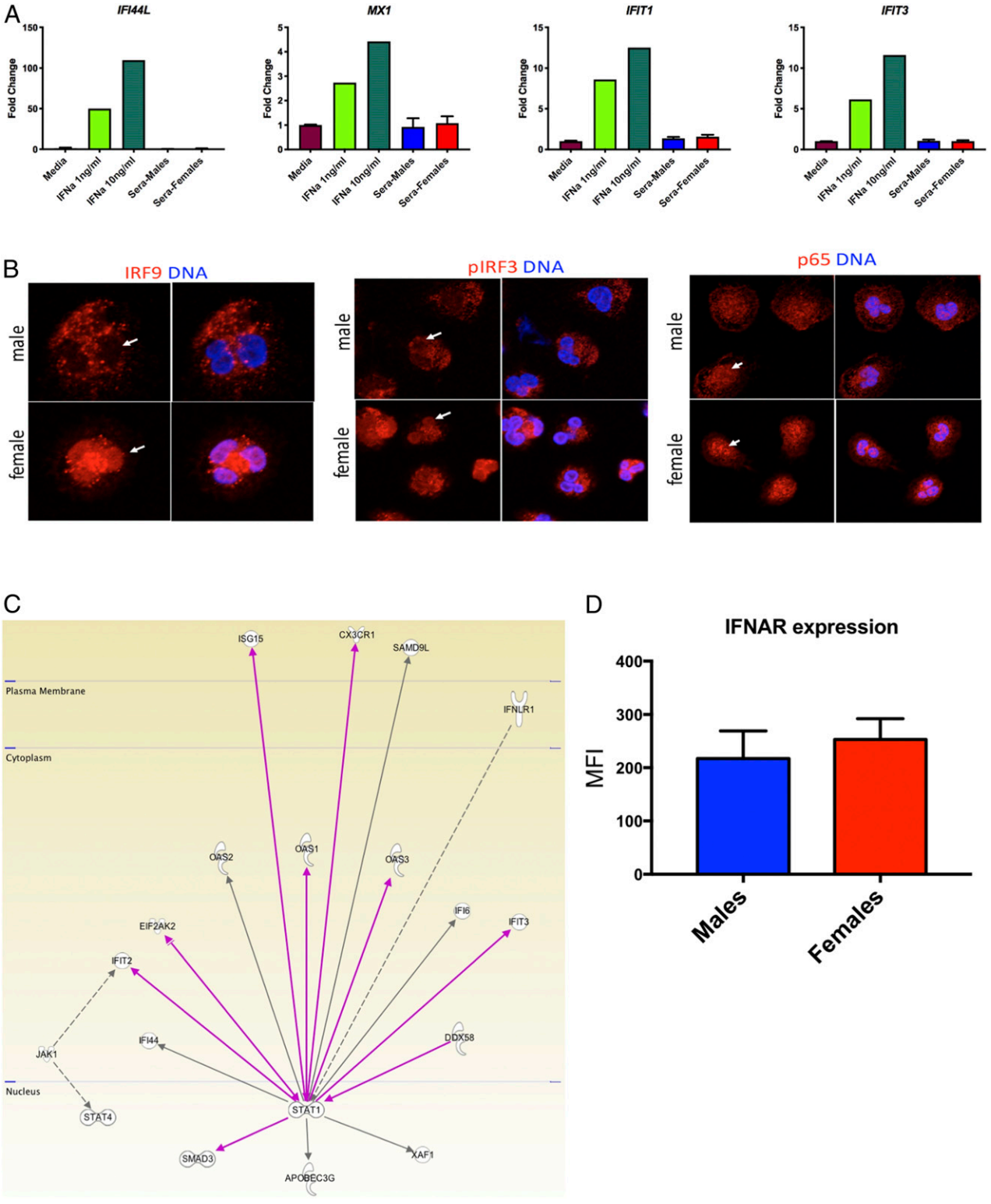

Fig. 4. Increased type I IFN gene signature in female neutrophils is driven by enhanced response to these cytokines. (*A*) Serum type I IFN activity in healthy young adult females and males. ISGs were evaluated by RT-PCR in HeLa cells after culturing them with male or female sera (n = 5/group) or recombinant IFNα (1 ng/mL or 10 ng/mL) for 6 h. Each subpanel represents an individual ISG. Results represent mean + SEM. (*B*) Immunofluorescence analysis of intracellular localization of IFN-related transcription factors (IRF9 in red, highlighted by white arrows; p65 in red, highlighted by white arrows; and pIRF3 in red, highlighted by white arrows) was performed on permeabilized neutrophils isolated from male and female subjects. Nuclei were counterstained with Hoechst (blue). Original magnification, 400×. Images shown are from one representative dataset of three independent experiments. (*C*) Transcription factor analysis of RNA-sequencing data from cohort 1 showed increased expression of *STAT1* and its regulated genes in female compared to male neutrophils (n = 7/group). (*D*) Cell membrane expression of IFNAR was quantified by flow cytometry in neutrophils from healthy males and females (n = 4/group). Mann–Whitney *U* test was used to calculate significance. *P* value was nonsignificant.

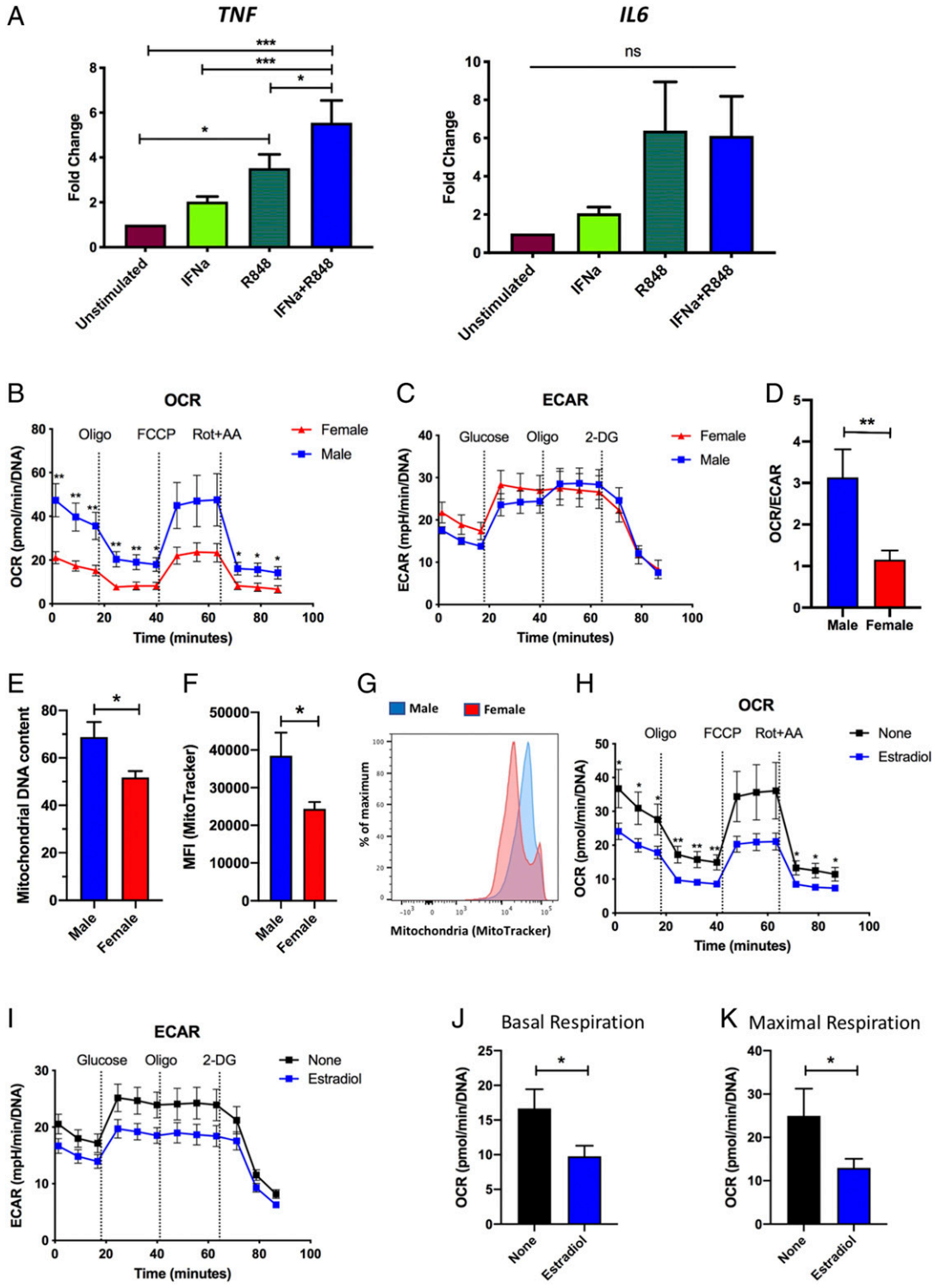

Fig. 5. (*A*) Priming of neutrophils with type I IFNs enhances responses to TLR agonists: *TNF* and *IL6* mRNA were quantified after exposing male neutrophils ($n = 8$) to IFNα (10 ng/mL) or media for 15 min, followed by incubation in the presence or absence of R484 for 3 h. Data were analyzed using Mann–Whitney *U* test; $^*P < 0.05$, ns, not significant. Results represent mean + SEM. (*B–D*) Female and male neutrophils differ in their bioenergetic capacity. Mitochondrial and glycolysis stress tests analyses of peripheral blood neutrophils. Graphs display (*B*) OCR, to assess mitochondrial respiration, and (*C*) ECAR to measure glycolysis (females = 6; males = 6). (*D*) Bar graph shows ratio of basal OCR vs. basal ECAR. (*E–G*) Male neutrophils have increased mitochondrial content. (*E*) Quantification of mitochondrial DNA by qPCR in neutrophils from males ($n = 6$) and females ($n = 6$). (*F*) Quantification of MitoTracker by flow cytometry in neutrophils from males ($n = 6$) and females ($n = 6$). (*G*) Representative example of a male and female neutrophil stained with MitoTracker green dye. (*H–K*) Estradiol stimulation decreases mitochondrial metabolism in male neutrophils. Effect of estradiol (200 pg/mL) stimulation on male neutrophils ($n = 6$) on (*H*) OCR and (*I*) ECAR. Also shown, male neutrophils' (*J*) basal respiration and (*K*) maximal respiration after incubation with estradiol. Oligo, oligomycin (inhibitor of ATP-synthase); FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (mitochondrial uncoupler); Rot + AA, rotenone + antimycin (complex I and complex III inhibitors, respectively). Data (*D*, *E*, *F*, *J*, and *K*) were analyzed using Mann–Whitney *U* test. Data (*B* and *H*) were analyzed using multiple *t* tests; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Results represent mean + SEM.

were no differences in glycolytic capacity (Fig. 5 *B* and *C*). The OCR/ECAR ratio was significantly higher in male compared to female neutrophils (Fig. 5*D*).

We asked if increases in mitochondrial metabolism in healthy young adult male neutrophils were associated with higher number of mitochondria. Using qPCR and flow cytometry, we found significantly higher mitochondrial DNA content and mitochondrial mass, respectively, in male neutrophils compared to females ($n = 6$ each; Fig. 5 *E*–*G*). This is consistent with the immature phenotype observed in male neutrophils, as mitochondria numbers and activity decrease during neutrophil differentiation (24). These results suggested that neutrophils from healthy young adult males and females significantly differ in their bioenergetic potential, with the former displaying enhanced mitochondrial metabolic activity and increased mitochondrial mass suggestive of a more immature phenotype.

We examined if the female hormone estradiol could modify the bioenergetics of male neutrophils to make them more similar to female neutrophils. Estradiol treatment (200 pg/mL) of male neutrophils ($n = 6$) had significant effects on OCR (Fig. 5*H*), minimal effects on ECAR (Fig. 5*I*), and promoted a decrease in both basal respiration (Fig. 5*J*) and maximal respiration (Fig. 5*K*). The mitochondrial metabolism of estradiol-treated male neutrophils was very similar to the pattern observed in unstimulated female neutrophils. These results indicated that the female sex hormone estradiol modulates neutrophil metabolism and contributes to the differences in bioenergetics observed between male and female neutrophils.

X Chromosome Gene Dosage Does Not Contribute to the Enhanced Female Neutrophil Type I IFN Signature. Subjects with Klinefelter syndrome are phenotypically male but have an extra copy of the X chromosome (47, XXY). Characteristically, males with Klinefelter syndrome have hypogonadism and low testosterone. To further define if differences in type I IFN signaling and maturation status detected between male and female neutrophils are driven by X chromosome gene dosage and/or sex hormone influences, we compared neutrophil ISG expression between young adult healthy control males ($n = 10$) and females ($n = 15$), and in males with Klinefelter syndrome ($n = 17$, 19 to 56 y old; *SI Appendix*, Table S8). The vast majority of subjects with Klinefelter syndrome we studied were receiving testosterone supplementation and were therefore not hypogonadic. There were no statistically significant differences in neutrophil ISG levels between adult males with or without Klinefelter syndrome, while ISGs in young adult female neutrophils were significantly higher compared to males in either group (Fig. 6*A*).

We purified neutrophils from healthy prepubescent pediatric males ($n = 8$, 4 to 12 y old) and females ($n = 4$, 7 to 12 y old) and found that, in contrast to healthy young adults, there were no significant differences in ISG levels between boys and girls (Fig. 6*B*). As mature neutrophils are more responsive to type I IFNs than immature neutrophils (25), we assessed if prepubescent male and female neutrophils differed in their maturation status. In contrast to young adults, there were no significant differences in expression of primary granule genes when comparing prepubescent male and female neutrophils (Fig. 6*C*). These results indicated that the differences in maturation status and response to type I IFNs between young adult male and female neutrophils are not explained by X chromosome gene dosage and may be driven by sex hormone status.

Discussion

Despite clear differences in female and male immunity that may contribute to variations in response to infections (6, 7) and predisposition to autoimmunity, the exact mechanisms that drive this stark contrast remain insufficiently characterized. Neutrophils play important roles in homeostasis and disease, but little is known about how sex differences modulate their phenotype and function. Using transcriptomic and functional approaches, we report significant sex differences in neutrophil biology related to responses to type I IFNs, maturation status, and bioenergetics that may have prominent functional and pathogenic implications.

Transcriptome profiling revealed that peripheral blood female neutrophils displayed ex vivo up-regulation of ISGs and enhanced maturation status, compared to male neutrophils. Strikingly, this differential female ISG expression was not observed in other innate or adaptive immune cell types and was not driven by variations in levels of circulating type I IFNs or neutrophil type I IFN receptor. Instead, differences in maturation and activation of the female neutrophil may drive this hyperresponsiveness, with clear differences in cellular localization of IFN-regulatory factors between female and male neutrophils. We previously reported an immature-like neutrophil gene signature and phenotype in circulating neutrophils from healthy young males compared with females (12). Previous reports also indicated that mature neutrophils show enhanced response to type I IFNs in vitro when compared to more immature forms, suggesting that the cellular signaling pathways leading to ISG transcription are more operational in terminally differentiated stages of neutrophil maturation (25). As IFN priming on mature neutrophils enhances the response to NET-inducing stimuli, this enhanced hyperresponsiveness to these cytokines by female neutrophils may be implicated in their enhanced ability to form NETs and generate autoantigens (12), adding an additional putative pathway that enhances autoimmunity risk in women (9). Plasmacytoid dendritic cells (pDCs) are major producers of type I IFNs following TLR7/9 activation (26). Sex differences in TLR-mediated pDC responses have been described and attributed to both X chromosome genes and sex hormones (27, 28). However, as we found no differences in circulating IFNs between healthy young adult males or females and no differential ISG expression in other cell types, our findings provide further evidence that these differences are related to neutrophil-intrinsic responses to these cytokines. It is important to notice that the changes seen may represent sex differences in the epigenetic status of neutrophils and this should be addressed in future studies.

In our study, consistent with previous reports (21), IFN-primed neutrophils up-regulated inflammatory cytokine genes in response to TLR stimulation. This enhanced response to a danger signal may enhance antimicrobial responses but also contribute to deleterious proinflammatory roles in tissues. IFN-primed neutrophils increase reactive oxygen species (ROS) production (29), migration (30), NET formation (25), and adhesion molecule expression (20) and is associated with a more advanced maturation state (31). All these observations indicate that the neutrophil-specific activation of the type I IFN pathway in healthy adult females is physiologically important, given the critical role of neutrophils in homeostatic immune responses (1–3). Conversely, dysregulation in the IFN pathway plays key features in many autoimmune diseases like systemic lupus erythematosus (32, 33) where there is a significant sex bias (8) and an increase in incidence after puberty (34, 35). Furthermore, dysregulation in neutrophil biology has been proposed to play key roles in many autoimmune diseases (9). Thus, elevated type I IFN signaling and enhanced activation status seen in the reproductive-age female neutrophils could contribute to the enhanced risk for autoimmunity and perpetuation of inflammatory responses observed in women.

Circulating neutrophils are more heterogeneous than previously appreciated but delineation of neutrophil subsets and their various functions has been technically challenging (16, 36). Using single-cell RNA-seq, we identified that a subset of circulating neutrophils is defined by the elevated expression of ISGs. A recent study using single-cell RNA-seq, identified a similar subset in peripheral blood of female mice and in a 32-y-old healthy human male (15). We corroborated that the ISG signature in human neutrophils is driven by this subset in a larger set of healthy human

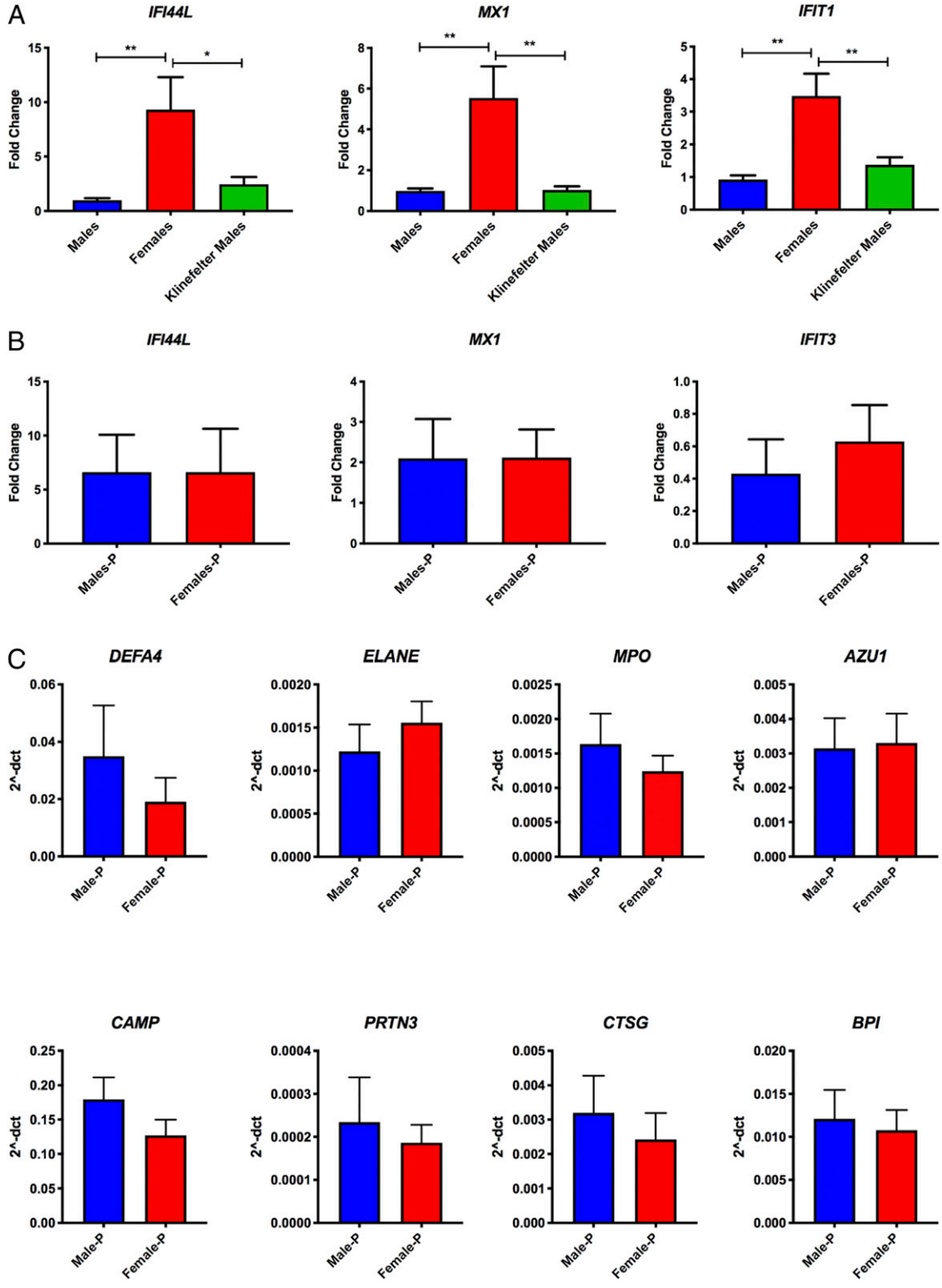

Fig. 6. The enhanced response to type I IFNs and maturation status in female neutrophils is not explained by X chromosome gene dosage. ISGs were quantified by RT-PCR in (*A*) neutrophils from young adult males ($n = 10$), and females ($n = 15$) and males with Klinefelter syndrome (47, XXY) ($n = 17$); (*B*) and in neutrophils from healthy prepubertal males ($n = 8$) and females ($n = 4$). Data were analyzed with Mann–Whitney *U* test; $^{*}P < 0.05$, $^{**}P < 0.01$. Results represent mean + SEM. (*C*) Primary granule genes were quantified by PCR in neutrophils from prepubertal healthy males ($n = 8$) and females ($n = 5$). Results represent mean + SEM.

controls, finding augmented ISG expression within this population in healthy young adult females. Future studies should explore the phenotypic characteristics of this subset and its role in antimicrobial responses and autoimmune phenotype.

The increased IFN-gene signature in healthy adult female neutrophils is likely not explained by differences in X chromosome gene dosage, as neutrophils from Klinefelter syndrome males displayed lower ISG expression than young females. Likewise, as

prepubescent male and female neutrophils did not differ in maturation status or ISG levels, these observations support that the differences between adult female and male neutrophils are likely driven by sex hormones. This is supported by the observation that neutrophil bioenergetics characteristic of immature neutrophils (as observed in male neutrophils) were modified in the presence of estradiol. The dissimilar bioenergetic profiles of male and female neutrophils further suggest a differential maturation status of these cells, potentially driven by sex hormones. Indeed, male neutrophils displayed enhanced mitochondrial respiration and mitochondrial mass, characteristic of earlier neutrophil/myeloid forms (24). Indeed, c-kit-positive neutrophils (considered immature) possess significantly higher reserve and maximal mitochondrial OCR than mature c-kit-negative neutrophils, relying more on mitochondria to meet energy demands (22, 23). How these bioenergetics differences impact neutrophil function in males versus females, in the context of infection and inflammation, should be the focus of future studies.

Previous observations have suggested a role for sex hormones in the modulation of neutrophils' phenotype. Through its ability to trigger granulocyte-macrophage colony-stimulating factor (GM-CSF) production, estradiol may affect neutrophil maturation (37). GM-CSF is elevated in adult young females versus males and this difference disappears after menopause (38). Estradiol and progesterone decrease neutrophils' apoptosis, potentially enhancing their half-life (39). Estrogen regulates multiple STATs, important transcription factors for ISGs, by either enhancing their phosphorylation or DNA-binding activity (40–43). The genomic-binding site of the progesterone receptor is enriched and overlaps with the binding sites of STAT1, STAT3, and STAT5, suggesting a role for progesterone in ISG activation (44). In contrast, while androgens can increase neutrophil recruitment to inflamed tissues (45–47) they reduce their bactericidal efficiency and ROS synthesis (48). However, the effects of sex hormones and blocking of their respective receptors on neutrophil maturation and function are difficult to elucidate due to the terminally differentiated nature of these cells. Absence of reliable neutrophil cell lines or efficient protocols to differentiate bone marrow-derived precursor cells into neutrophils, also limits the ability to study the in vitro or ex vivo effect of sex hormones in these cells. Additionally, different levels of exposure to sex hormones between males and females could have chronic effects on the epigenome, which would not necessarily be reflected in differences in the transcriptional response to a short-term exposure to steroid hormones in vitro. Future studies should expand the assessment of sex differences of neutrophils in vivo during their whole life span, to further dissect the exact impact of hormonal modulation.

In conclusion, healthy young adult females have an activated/mature neutrophil profile characterized by enhanced type I IFN pathway activity, enhanced proinflammatory responses, and distinct bioenergetics. These differences between females and males are cell specific and likely driven by in vivo exposure to sex hormones. Modulation of these pathways in neutrophils may provide more individualized, sex-specific therapeutic options in a variety of disease states.

Materials and Methods

Study Subjects and Neutrophil Isolation. Subjects were recruited at the Clinical Center, NIH, Bethesda, MD and provided informed consent prior to participation in the study. The study was approved by the institutional review boards of the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), the National Institute of Allergy and Infectious Diseases (NIAID), and the National Human Genome Research Institute (NHGRI) (NIH 94-AR-0066, NIH 16-I-0126, NIH 15-I-0162, and NIH 12-HG-0181). Neutrophils were isolated as described (49). Additional details are in *SI Appendix, Supplemental Methods*.

Data Availability. Data from cohort 1 and single-cell data have been uploaded to the Gene Expression Omnibus (GEO) with series no. GSE145231. Data from cohort 2 have been uploaded to the GEO with series no. GSE145033. All of the other information is included in the manuscript and supplemental material. The NeutGx platform is available through the NIAID Bioinformatics Portal (https://neutgx.niaid.nih.gov).

RNA Purification, Sequencing, Analysis, Pathway Mapping, IFN Signature Score, and Generation of the Web Tool for Data Visualization. For cohort 1 neutrophils, TruSeq-Stranded mRNA library prep for Neoprep (Illumina, cat. no. 20020594) was used to prepare cDNA libraries. For cohort 2 neutrophils, total RNA was isolated with TRIzol reagent followed by column-based purification with RNA Clean & Concentrator-5 Kit (Zymo Research; cat. no. R1016). RNA-seq libraries were prepared with TruSeq-Stranded Total RNA with Ribo-Zero Gold Kit (Illumina, cat. no. 20020599) and TruSeq RNA CD Index Plate (Illumina, cat. no. 20019792). The cBot system (Illumina, cat. no. SY-301-2002) was used for paired-end cluster generation with a TruSeq PE Cluster Kit v3-cBot-HS (Illumina, cat. no. PE-401-3001). Paired-end sequencing (2 × 101 bp) was performed on an Illumina HiSEq. 2500 (Illumina, cat. no. SY-401-2501) with the TruSeq SBS Kit v3-HS (Illumina, cat. no. FC-401-3001). Additional details, including use of publicly available T cell and monocyte NIH GEO datasets and data access of sequencing data are in *SI Appendix, Supplemental Methods*.

Single-Cell RNA Sequencing. Single cells were sequenced using the 10× Genomics platform. Whole blood leukocytes (four males, four females) were isolated using a red blood cell depletion kit (Erythroclear Red Blood Cell Depletion Reagent Kit; STEMCELL Technologies, cat. no. 01738). Male and female samples were processed and sequenced in pairs to reduce batch effect. One male sample was excluded because of poor capture. Cells were encapsulated using microfluidics technology, barcoded using a unique molecular identifier (UMI), cDNA was prepared using manufacturer's specifications, and sequenced on the Illumina 3000 HiSeq system. Data were demultiplexed using Cell Ranger software (version 2.2.0) that generated FAST-Q files, which were aligned to a human reference genome (GrCh38) using STAR. Cell ranger output files were loaded into R (v3.5.3) for analysis using Seurat v3.0 (50, 51). Additional information is in *SI Appendix, Supplemental Methods*.

qRT-PCR and TLR Stimulation. Total RNA was isolated using Zymo Research Direct-zol RNA Miniprep kit (Zymo Research, cat. no. R2050). Gene expression was measured by qRT-PCR with transcript-specific primers (detailed in *SI Appendix, Supplemental Methods*) using SYBR Green or TaqMan gene expression master mix and specific probes in the Biorad CFX96 Real-Time PCR System. In some experiments, neutrophils were preincubated with/without IFNα (10 ng/mL) in RPMI/10% fetal bovine serum (FBS) for 15 min in a 24-well plate, followed by incubation with/without R848 (25 μg/mL, Invivogen, cat. no. tlrl-r848-5) for 3 h. The plate was centrifuged (200 × *g*/2 min), cell pellets were resuspended in TRI Reagent, and qRT-PCR was performed.

Serum IFN Reporter Assay. IFN reporter assay quantified ability of male and female sera to induce ISGs in HeLa cells, as reported (18).

Fluorescent Microscopy Assessment of Cellular Localization of IFN Transcription Factors. Neutrophils were fixed, permeabilized, and incubated with antibodies to assess transcription factor cellular localization. Additional details are in *SI Appendix, Supplemental Methods*.

Flow Cytometry. Neutrophils were incubated for 15 min with Human TruStain Fc-receptor Blocking Solution (BioLegend, cat. no. 422302), followed by anti-human IFNAR1 phycoerythrin (R&D Systems, cat. no. FAB245P) or isotype control for 15 min in dark, then fixed with 2% paraformaldehyde. In addition, neutrophils were incubated with MitoTracker Green FM (100 nM; Thermo Fisher) and immediately analyzed. Data were acquired on a FACSCanto II (BD Biosciences) and analyzed using FlowJo Software version 9.9.

Metabolism Assessment by Seahorse Technology and Mitochondrial DNA Quantification. Neutrophils were plated on Corning Cell-Tak (Fisher Scientific 354240)-coated Seahorse culture plates (300,000 cells/well) in XF RPMI (pH 7.4). Analysis was performed at 37 °C without CO_2, on an XF-96e analyzer (Agilent). For mitochondrial DNA, a qPCR adapted from Venegas (52) was performed on neutrophil DNA. Details are in *SI Appendix, Supplemental Methods*.

Statistics. Statistical analysis was performed using Fisher's, Wilcoxon's, Mann–Whitney *U* test, or Kruskal–Wallis tests, as indicated. The results were considered significant at $P < 0.05$.

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