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Authors

Denson, Lee A
Jurickova, Ingrid
Karns, Rebekah
et al.

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Genetic and Transcriptomic Variation Linked to Neutrophil Granulocyte–Macrophage Colony-Stimulating Factor Signaling in Pediatric Crohn's Disease

Lee A. Denson, MD,[*] Ingrid Jurickova, MD,[*] Rebekah Karns, PhD,[*] Kelly A. Shaw, PhD,[†] David J. Cutler, PhD,[†] David Okou, PhD,[‡] C. Alexander Valencia, PhD,[§] Anne Dodd,[‡] Kajari Mondal, PhD,[‡] Bruce J. Aronow, PhD,[¶] Yael Haberman, MD, PhD,[*] Aaron Linn,[*] Adam Price,[*] Ramona Bezold,[*] Kathleen Lake, MSW,[*] Kimberly Jackson,[*] Thomas D. Walters, MD,[‖] Anne Griffiths, MD,[‖] Robert N. Baldassano, MD,[**] Joshua D. Noe, MD,[††] Jeffrey S. Hyams, MD,[§§] Wallace V. Crandall, MD,[¶¶] Barbara S. Kirschner, MD,[‖‖] Melvin B. Heyman, MD,[***] Scott Snapper, MD,[†††] Stephen L. Guthery, MD,[‡‡‡] Marla C. Dubinsky, MD,[§§§] Neal S. Leleiko, MD, PhD,[¶¶¶] Anthony R. Otley, MD,[‖‖‖] Ramnik J. Xavier, MD,[****] Christine Stevens, PhD,[****] Mark J. Daly, PhD,[****] Michael E. Zwick, PhD,[†] and Subra Kugathasan, MD[‡]

Background: Granulocyte–macrophage colony-stimulating factor auto-antibodies (GMAbs) suppress neutrophil-extrinsic GM-CSF signaling and increase risk for stricturing behavior in Crohn's disease (CD). We aimed to define clinical, genomic, and functional associations with neutrophil-intrinsic GM-CSF signaling.

Methods: Missense mutations in *CSF2RA*, *CSF2RB*, *JAK2*, *STAT5A*, and *STAT5B* were identified using whole-exome sequencing in 543 pediatric inflammatory bowel disease (IBD) patients. Neutrophil-intrinsic GM-CSF signaling was defined using the GM-CSF-induced STAT5 stimulation index (GMSI) in 180 pediatric IBD patients and 26 non-IBD controls. Reduced GM-CSF signaling (GMSI-Lo) was defined as the 20th percentile within the control group. Variation in neutrophil phospho-protein abundance, bacterial killing, and the global pattern of gene expression with the GMSI was determined.

Results: We validated 18 potentially damaging missense mutations in *CSF2RA* and *CSF2RB*. *CSF2RA* A17G carriage increased from 10% in those with intact neutrophil GMSI to 32% in those with low GMSI ($P = 0.02$). The frequency of reduced *Staphylococcus aureus* killing increased

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From the *Division of Pediatric Gastroenterology, Hepatology, and Nutrition, Department of Pediatrics, University of Cincinnati College of Medicine and the Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; †Department of Human Genetics and ‡Department of Pediatrics, Emory University, Atlanta, Georgia; §Program and Division of Human Genetics, Molecular Genetics Laboratory, and ¶Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio; ‖Division of Pediatric Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, The Hospital for Sick Children, University of Toronto, Toronto, Canada; **Department of Pediatrics, University of Pennsylvania, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania; ††Department of Pediatric Gastroenterology, Hepatology, and Nutrition, Medical College of Wisconsin, Milwaukee, Wisconsin; §§Division of Digestive Diseases, Hepatology, and Nutrition, Connecticut Children's Medical Center, Hartford, Connecticut; ¶¶Department of Pediatric Gastroenterology, Nationwide Children's Hospital, The Ohio State University College of Medicine, Columbus, Ohio; ‖‖Department of Pediatrics, The University of Chicago, Chicago, Illinois; ***Department of Pediatrics, University of California at San Francisco, San Francisco, California; †††Department of Gastroenterology and Nutrition, Boston Children's Hospital, Boston, Massachusetts; ‡‡‡Department of Pediatrics, University of Utah, Salt Lake City, Utah; §§§Department of Pediatrics, Mount Sinai Hospital, New York, New York; ¶¶¶Department of Pediatrics, Hasbro Children's Hospital, Providence, Rhode Island; ‖‖‖Department of Pediatrics, Dalhousie University, Halifax, Nova Scotia, Canada; ****The Broad Institute of MIT and Harvard, Cambridge, Massachusetts

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Address correspondence to: Lee A. Denson, MD, MLC 2010, 3333 Burnet Avenue, Cincinnati, OH (lee.denson@cchmc.org).

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from 17% in those with intact neutrophil GMSI to 35% in GMSI-Lo neutrophils ($P = 0.043$). Crohn's disease neutrophils with low GMSI exhibited specific alterations in phospho-protein networks and genes regulating cytokine production, wound healing, and cell survival and proliferation. Stricturing behavior increased from 7% in patients with both low GMAb and intact GMSI to 64% in patients with both elevated GMAb and low GMSI ($P < 0.0001$).

Conclusions: Low/normal neutrophil-intrinsic GM-CSF signaling is associated with *CSF2RA* missense mutations, alterations in gene expression networks, and higher rates of disease complications in pediatric CD.

Key Words: GM-CSF, neutrophil, pediatric inflammatory bowel disease, RNA sequencing, STAT5, whole-exome sequencing

INTRODUCTION

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is required for priming of myeloid cell antimicrobial function, and genomic studies have suggested a central role for host:microbe interactions in Crohn's disease (CD) pathogenesis.[1, 2] We discovered that pediatric and adult CD patients exhibit an acquired basis for neutrophil dysfunction, GM-CSF auto-antibodies (GMAb), which increase in titer with older age and suppress GM-CSF:STAT5 signaling in a cell-extrinsic manner.[3, 4] GMAb carriage is associated with reduced neutrophil STAT5 activation, phagocytic capacity, and bacterial killing, and with high rates of antimicrobial serologies (AMS) and stricturing behavior.[5, 6] Crohn's disease patients were found to exhibit increased levels of GMAb in the months preceding clinical relapses, suggesting a role in initiation of mucosal inflammation.[7] Our studies in animal models have shown that GM-CSF promotes intestinal barrier function and effective responses to mucosal injury, suggesting a direct role for genetic or acquired variation in GM-CSF bioactivity in more aggressive CD disease behavior.[4, 8] However, a role for neutrophil-intrinsic variation in GM-CSF signaling in cell function and disease complications in pediatric CD has not previously been explored.

Recently, 2 groups have independently defined a critical role of the GM-CSF receptor for the development and maintenance of immune homeostasis in the human gut.[9, 10] The authors identified increased carriage of the same heterozygous frameshift mutation in the GM-CSF receptor beta common subunit gene, *CSF2RB*, in 2 independent cohorts of Ashkenazi Jewish (AJ) CD patients. Studies using primary monocytes from CD patients with and without heterozygous risk variant carriage confirmed that the *CSF2RB* frameshift mutation resulted in reduced GM-CSF-induced tyrosine phosphorylation of the STAT5 transcription factor in a dominant-negative manner. Cytometry by Time-Of-Flight (CyTOF) analysis defined the predominant myeloid cellular targets for GM-CSF activation of STAT5 in primary human ileal lamina propria leukocytes. Whether genetic variation in *CSF2RA* or *CSF2RB* was also associated with cell-intrinsic reduction in neutrophil GM-CSF signaling in pediatric CD was not known.

We hypothesized that rare genetic variants regulating GM-CSF signaling cause neutrophil dysfunction and thereby contribute to disease pathogenesis in pediatric CD. In the current study, we have focused on the alpha and beta chain genes that comprise the GM-CSF receptor and have tested for associations between neutrophil GM-CSF:STAT5 signaling, rare missense mutations in these genes, and clinical phenotypes. Whole-exome sequencing identified potentially damaging missense mutations in the *CSF2RA* alpha chain and *CSF2RB* beta chain genes. Peripheral blood neutrophil phospho-protein networks, global patterns of gene expression, and antimicrobial functions were defined in cells with intact or reduced GM-CSF responsiveness. We identified a subset of pediatric CD patients characterized by low-neutrophil GM-CSF:STAT5 signaling, which was in turn associated with *CSF2RA* gene missense mutations, alterations in wound healing and cell survival gene programs, and high rates of disease complications.

METHODS

Whole-Exome Sequencing

Whole-exome sequencing (WES) was performed in pediatric IBD patients enrolled in the Crohn's and Colitis Foundation (CCF)–sponsored RISK cohort study and the National Institutes of Health (NIH)–sponsored Emory African American gene discovery study.[11, 12] Clinical and demographic features are summarized in Supplementary Table 1. Genomic DNA was extracted from whole blood from 567 early-onset (age 0–18 years) IBD samples; 543 (95.8%) of these samples passed DNA quality control. Libraries were prepared according to the manufacturer's instructions, and whole-exome capture was performed with the SureSelect Human All Exon 50-Mb Kit (Agilent Technologies). Libraries were validated with the KAPA Library Quantification Kit (KAPA Biosystems) and were sequenced on the Illumina HiSeq platform according to standard protocols at Broad Institute's Genomics Platform (Cambridge, MA, USA).

Exome Sequencing Data Analysis

Raw sequence reads were mapped relative to the human genome reference sequence (hg38), and variants were called using PEmapper/PECaller.[13] Unique variants were functionally annotated using SeqAnt, version 2.0 (Beta 3; https://seqant.genetics.emory.edu/), which reports the variant's functional classification (nonsense, replacement, silent, 5' or 3' UTR, intronic, intergenic), presence in databases including dbSNP, and Combined Annotation-Dependent Depletion (CADD) scores.[14, 15] We used the Exome Aggregation Consortium (ExAC) reference data set for estimation of allele frequency (http://exac.broadinstitute.org/).[14, 15]

Filtering and Validation of Variants

We filtered all variants by minor allele frequency (MAF) <5% and nonsynonymous variants by CADD score >10 based on SeqAnt annotation to identify likely damaging variants. A scaled C-score above 10 indicated that the variant was within the top 10% of deleterious substitutions.[16] Variants were validated by Sanger sequencing to confirm bioinformatics analysis. Primers used for validation were designed using EmPrime or Primer3. M13 forward and reverse tags (Forward tag:TGTAAAACGACGGCCAGT; Reverse tag:CAGGAAACAGCTATGACC) were added to the primer sequence to facilitate Sanger downstream analysis. The polymerase chain reaction amplicons were sent to Genewiz (South Plainfield, NJ, USA) for sequencing. Sanger sequencing results were analyzed using 4Peaks DNA sequence trace viewer software. Primers used for the validation are provided in Supplemental Table 2. Not all variants required Sanger validation as they were validated by either frequency, cluster, or 1000 genomes.

Enzyme-Linked Immunosorbent Assays

GM-CSF was measured using a high-sensitivity enzyme-linked immunosorbent assay (ELISA), as per the manufacturer's protocol (BioLegend, San Diego, CA, USA). GM-CSF auto-antibodies were measured by ELISA, as previously reported.[6]

Neutrophil Function Studies

Neutrophil function assays were performed using peripheral blood samples obtained from 180 pediatric CD and UC patients and 26 healthy controls, as summarized in Table 1. Washed neutrophils were employed to remove circulating GMAb. Peripheral blood neutrophil CD64 index, CD116 (GM-CSF receptor alpha chain encoded by *CSF2RA*) cell surface abundance, GM-CSF-induced STAT5 tyrosine phosphorylation, N-formyl peptide (FMLP)–induced reactive oxygen species (ROS) production, and *Staphylococcus aureus* phagocytosis and killing were determined by flow cytometry or light microscopy using our published methods.[5] The GM-CSF:STAT5 stimulation index (GMSI) was defined as: ((granulocyte pSTAT5 mean fluorescence intensity [MFI] following GM-CSF stimulation – basal granulocyte pSTAT5 MFI)/ basal granulocyte pSTAT5 MFI)*100. A low GMSI was defined as ≤25%, the 20th percentile within controls.

Circulating Neutrophil Purification and RNA and Protein Preparation

Peripheral blood neutrophils were purified using the MACxpress Neutrophil Isolation Kit according to the manufacturer's protocol (Miltenyi Biotec). For RNA preparation, after purification, the neutrophils were centrifuged and resuspended in RNAlater (Qiagen) and stored at –80°C until further processing. Neutrophil RNA was extracted using TRIzol LS reagent (Life Technologies) according to the manufacturer's protocol. For protein preparation, cell pellets were flash-frozen and stored at –80°C until further processing. MILLIPLEX MAP Lysis Buffer with freshly added protease inhibitors was added to cell pellets and filtrated over a filter (EMD Millipore Catalog #UFC 0DV 25). Cell lysates were stored at –80°C and later processed for phospho-protein immunoassay according to the manufacturer's protocol.

Neutrophil Phospho-Protein Quantification

Neutrophil cell lysates were processed for the Milliplex phospho-protein immunoassay, including CREB (pS133), ERK (pT185/pY187), NFκB (pS536), JNK (pT183/pY185), p38 (pT180/pY182), p70 S6K (pT412), STAT3 (pS727), STAT5A/B (pY694/699), Akt (pS47; 48-680MAG, EMD Millipore), and STAT1 (Tyr701; 46-655MAG), determined using Luminex technology on the Milliplex Analyzer (MilliporeSigma, Darmstadt, Germany). The presence of GAPDH (46-667MAG) and total β-Tubulin (46-713MAG) in cell lysates was assessed. Concentrations were calculated from standard curves using recombinant proteins and were expressed in pg/mL.

RNA Sequencing

Total RNA was amplified using the Ovation RNA-Seq System, version 2 (NuGEN), according to the manufacturer's protocol. The libraries were prepared with the Nextera XT DNA Sample Preparation kit (Illumina Technologies). Libraries were

TABLE 1. Clinical and Demographic Characteristics of the Participants With Neutrophil Function Testing

	Age at Diagnosis, Mean (Range), y	Age at Testing, Mean (Range), y	Male, No. (%)	White, No. (%)	AA, No. (%)	L1, No. (%)	L2, No. (%)	aL3, No. (%)	E1E2, No. (%)	E3E4, No. (%)
Control (n = 26)	NA	14 (6–19)	13 (50)	21 (81)	4 (15)	NA	NA	NA	NA	NA
UC (n = 54)	10 (1–18)	15 (4–22)	24 (41)	48 (89)	5 (9)	NA	NA	NA	8 (14)	50 (86)
CD (n = 126)	11 (1–20)	15 (4–23)	79 (61)	115 (89)	11 (9)	13 (10)	18 (14)	98 (76)	NA	NA

One control, 1 UC subject, & 3 CD subjects were Asian.
Abbreviations: AA, African American; E1E2, left-sided colitis; E3E4, pan-colitis; L1, ileal locaton; L2, colon-only location, L3, ileo-colonic location; NA, not applicable.

sequenced on the Illumina HiSeq2500 following the manufacturer's protocol with paired-end, 75-bp reads (Illumina, San Diego, CA, USA). Following removal of barcodes and primers, raw sequences were aligned to the Hg19 human genome, with reference annotations provided by University of California Santa Cruz. Alignment was performed under the following parameters: (1) minimum identity of 90%; (2) maximum gaps of 5%; (3) minimum aligned read length of 25; (4) 1 match to output per read; and (5) reads with >5 matches were ignored. Reads were aligned using a proprietary Burrow-Wheeler transform-based method, COBWeb. Using the expectation–maximization algorithm, reads per transcript per million (RPKM) were computed from aligned reads. RPKM were thresholded at 1, normalized using the DESeq algorithm, and baselined to the median of all samples (n = 40,448 transcripts). Transcripts with reasonable expression, >3 reads in 100% of samples in at least 1 of the 3 conditions (control, normal-GMSI Crohn's, low-GMSI Crohn's), were included in differential analyses (n = 13,339 transcripts).

Neutrophil Function and Phospho-Protein Statistical Analysis

Neutrophil function and phospho-protein data were tested for differences between CD cases with high and low GMSI and relative to non-IBD healthy controls. We also tested for variation in the GMSI with gene variant carriage in the prespecified GM-CSF:STAT5 pathway genes (Table 2). Cases for this analysis were patients who carried the candidate variant of interest and no other potentially damaging variants in the pathway genes that could affect the GMSI (Table 2). Although potentially damaging missense mutations in the downstream pathway genes *JAK2*, *STAT5A*, and *STAT5B* were identified in the overall cohort of 543 subjects; these were not detected in the patients in this study who had neutrophil function studies performed. Therefore, this analysis was limited to *CSF2RA* and *CSF2RB* variants. Controls for this analysis were defined as not carrying any potentially damaging variants in the GM-CSF:STAT5 pathway genes (Table 2). We recorded age, sex, disease activity, and concurrent medications at the time of the neutrophil function studies and accounted for these. Statistical analyses were performed using GraphPad PRISM, version 7. Continuous variables were analyzed using the unpaired *t* test, 2-sample *t* test, Mann-Whitney test, 1-way analysis of variance with Bonferroni multiple comparison test, Kruskal-Wallis with Dunn's test for multiple comparisons, or linear test for trend. Discrete variables were analyzed using the Fisher exact test or chi-square test. A *P* value <0.05 was considered significant.

Proteomic analyses were performed using SAS, version 9.3. Background levels were subtracted from all phospho-protein values, and the distribution of each protein shifted up so that each protein's minimum value was 0. Data were corrected for total protein submitted, using a total protein normalization (test protein/total protein*100). Samples with total protein levels <500 were excluded from analyses. Wilcoxon rank-sum tests, which do not rely on normally distributed data, were used to compare background- and total protein-corrected phospho-protein levels between controls, CD patients with high GMSI, and CD patients with low GMSI. A false discovery rate–corrected *P* value <0.05 was considered significant.

RNA Sequencing Analysis

Expression values were compared between low-GMSI CD neutrophils, normal-GMSI CD neutrophils, and normal-GMSI control neutrophils. The analysis was carried out using 2 pipelines (Supplementary Fig. 1). The first pipeline determined genes differentially regulated between low-GMSI CD neutrophils and controls (n = 1450 transcripts) and between normal-GMSI CD neutrophils and controls (n = 1039 transcripts) using unpaired Welch's *t* tests (assuming unequal variance) with significance at *P* <0.05 and fold-change >1.5. Gene lists were overlapped using a Venn diagram (bioinfogp.cnb.csic.es/tools/venny/index.html) to identify gene sets specific to and shared between low- and normal-GMSI neutrophils compared with controls (Supplementary Fig. 2). The second pipeline directly compared low- and normal-GMSI neutrophils within CD, applying the same tests and cutoffs used in the first pipeline (550 differentially regulated transcripts).

Ontological Analysis

To determine the processes and pathways contributing to increased disease severity associated with low-GMSI CD neutrophils, overlapping and specific transcripts were assessed at the mechanism level. Genes up- and downregulated from each analysis were submitted to ToppGene and ToppCluster for assessment of ontological enrichments (toppgene.cchmc.org, toppcluster.cchmc.org), focusing on molecular functions, biological processes, and pathways. In ontological analyses of genes derived from comparing low- and normal-GMSI CD neutrophils with controls, up/down genes included those shared between the low/normal-GMSI CD vs control comparisons and those that were specific to low/normal-GMSI CD (see the Venn diagram in Supplementary Fig. 2). Ontological maps were generated through Toppcluster and Cytoscape. The full analytic pipeline is described in Supplementary Figure 1.

Sample Size

The primary outcome variable for the neutrophil function studies was the GM-CSF-induced STAT5 stimulation index in patients with genetic mutations predicted to influence the GM-CSF:STAT5 pathway, as summarized in Table 2. A significant effect on the GM-CSF-induced STAT5 stimulation index was defined as a reduction in the mean (SD) from 100 (40) in disease controls lacking a specific genetic variant to 60 (20) in cases carrying a specific genetic variant. Ten subjects carrying the variant of interest, compared with 10 subjects not carrying the variant, were sufficient to test for this difference with >80% power and an α of 0.05.

TABLE 2. Validated GM-CSF Receptor Alpha and Beta Chain Mutations

Gene	Chrom	Hg38 **P**osition	Transcript **C**hange	AA change	CADD	Allele Frequency
CSF2RA	X	1288843	c.428C>T	p.Thr143Met	24.3	0.0001789
CSF2RA	X	1288840	c.425C>T	p.Pro142Leu	22	0.004308
CSF2RA	X	1309462	c.1288G>A	p.Val430Met	23.8	0.0001228
CSF2RA	X	1282753	c.50C>G	p.Ala17Gly	14.9	0.08972
CSF2RB	22	36938236	c.2428C>G	p.Pro810Ala	23.4	1.80E-05
CSF2RB	22	36937781	c.1973C>T	p.Pro658Leu	13	7.24E-05
CSF2RB	22	36937748	c.1940G>T	p.Gly647Val	16.9	0.0003314
CSF2RB	22	36930695	c.877C>A	p.Leu293Met	23.2	4.07E-06
CSF2RB	22	36936558	c.1474G>A	p.Ala492Thr	12	3.26E-05
CSF2RB	22	36937615	c.1807C>A	p.Pro603Thr	24.8	0.03198
CSF2RB	22	36937894	c.2086C>T	p.Pro696Ser	4.46	0.02814
CSF2RB	22	36937858	c.2050G>C	p.Gly684Arg	16.5	0.0002284
CSF2RB	22	36938203	c.2395C>T	p.Arg799Cys	23.2	0.0001259
CSF2RB	22	36937435	c.1627A>G	p.Lys543Glu	9.58	4.06E-06
CSF2RB	22	36930752	c.934G>A	p.Asp312Asn	9.9	0.01259
CSF2RB	22	36937855	c.2047G>A	p.Val683Met	11.43	8.17E-06
CSF2RB	22	36937658	c.1850G>A	p.Ser617Asn	2.83	4.90E-05
CSF2RB	22	36937528	c.1720C>T	p.Pro574Ser	1.28	4.09E-06
JAK2	9	5064922	c.1096G>A	p.Val366Met	28.7	2.26E-05
JAK2	9	5022180	c.193G>C	p.Glu65Gln	26	4.06E-06
JAK2	9	5089798	c.2696T>C	p.Ile899Thr	27.3	0.000148
JAK2	9	5072541	c.1691G>T	p.Arg564Leu	25.9	6.17E-05
JAK2	9	5050742	c.525G>C	p.Gln175His	25.5	1.63E-05
JAK2	9	5077514	c.1926A>T	p.Lys642Asn	23.6	0.00001222
JAK2	9	5022129	c.142G>C	p.Gly48Arg	17.4	4.06E-06
JAK2	9	5126343	c.3188G>A	p.Arg1063His	26.4	0.004721
JAK2	9	5089679	c.2577T>C	p.Asn859Asn	19.1	0.0002943
JAK2	9	5069134	c.1439G>T	p.Cys480Phe	23.4	0.0006252
STAT5A	17	42310593	c.2309G>A	p.Arg770Gln	32	0.0001828
STAT5A	17	42304380	c.1208T>C	p.Met403Thr	26.5	4.06E-06
STAT5A	17	42309091	c.2107G>A	p.Val703Ile	22.7	5.05E-05
STAT5A	17	42292027	c.341A>G	p.Tyr114Cys	23.7	0.0003102
STAT5A	17	42301451	c.1166G>A	p.Arg389His	23.1	0.001076
STAT5B	17	42223427	c.505C>G	p.Gln169Glu	24.1	4.06E-06
STAT5B	17	42224826	c.328C>T	p.Arg110Cys	27.3	3.25E-05
STAT5B	17	42218849	c.863A>T	p.Gln288Leu	23.8	8.55E-05
STAT5B	17	42223477	c.455G>A	p.Arg152Gln	24.1	5.28E-05

Minor allele frequency for the pediatric IBD whole-exome sequencing cohort, summarized in Supplementary Table 1, is shown.
Abbreviations: AA, amino acid; CADD, Combined Annotation-Dependent Depletion score; Chrom, chromosome.

Study Approval

This study was approved by the institutional review boards at each of the RISK and Emory African American gene discovery study sites, including Emory and Cincinnati Children's Hospital Medical Center, and consent was obtained from parents and adult subjects and assent from pediatric subjects age 11 years and above.

RESULTS

Variation in Neutrophil-Intrinsic GM-CSF:STAT5 Signaling in Pediatric IBD

The clinical and demographic characteristics of the subjects with neutrophil function testing are summarized in Table 1. We first asked whether neutrophil GM-CSF-induced

STAT5 tyrosine phosphorylation would vary between CD or UC patients and healthy controls. Peripheral blood leukocytes were stimulated with GM-CSF, and STAT5 tyrosine phosphorylation was measured by flow cytometry. Representative fluorescence-activated cell sorting (FACS) plots for unstimulated and stimulated control and CD patient samples are shown in Figure 1A. The median (interquartile range [IQR]) GM-CSF-induced neutrophil STAT5 stimulation index (GMSI) did not differ between healthy controls, UC patients, or CD patients (Fig. 1B). We therefore subsequently defined a low GM-CSF response as a GMSI ≤25% (GMSI-Lo), the 20th percentile within controls, and tested for differences in cellular phospho-protein networks and antimicrobial functions compared with neutrophils with a GMSI >25% (GMSI-Hi).

Low-Neutrophil GM-CSF:STAT5 Signaling Is Associated With Reduced Bacterial Killing

GM-CSF plays a critical role in priming neutrophil antimicrobial functions.[1] We therefore next asked whether these would vary with the GMSI. Peripheral blood leukocytes were stimulated with FMLP, and neutrophil ROS production was measured by flow cytometry using the dihydrorhodamine 123 (DHR) dye. Representative FACS plots for unstimulated and stimulated samples are shown in Supplementary Figure 1A. FMLP-induced neutrophil ROS production did not vary within IBD patients stratified by the GMSI (Supplementary Fig. 1B). Peripheral blood neutrophils were plated on glass slides, exposed to *S. aureus,* and stained with acridine orange to identify live and dead intracellular bacteria by light microscopy. Representative microscopic images are shown for live (green) and dead (red) intracellular bacteria in a CD sample in Supplementary Figure 1C. *S. aureus* uptake (phagocytosis) did not vary within IBD patients stratified by the GMSI (Supplementary Fig. 1D). However, we did observe a modest reduction in median (IQR) *S. aureus* killing, from 88% (80%–92%) killed in GMSI-Hi neutrophils to 84% (77%–89%) killed in GMSI-Lo neutrophils (P = 0.0325) (Fig. 1C). GM-CSF pretreatment increased *S. aureus* killing in both groups and corrected the difference observed in unprimed neutrophils (Fig. 1C). Consistent with this, the frequency of subjects with low bacterial killing (<80% killed) increased from 17% in GMSI-Hi neutrophils to 35% in GMSI-Lo neutrophils (P = 0.043) (Fig. 1D). This was also largely corrected by GM-CSF pretreatment. Collectively, these data identified an association between low/normal neutrophil GMSI and reduced *S. aureus* killing in IBD patients.

Neutrophil GM-CSF:STAT5 Signaling and GM-CSF Receptor Mutations

Disease duration, frequency of moderate to severe disease activity, and treatment exposures did not vary within the UC or CD patient groups stratified by GMSI at the time of neutrophil function testing (Supplementary Table 3). Circulating GM-CSF, GMAb, neutrophil cell surface GM-CSF receptor alpha chain (CD116) abundance, and the neutrophil activation marker CD64 did not vary between the GMSI-Lo vs GMSI-Hi groups (Supplementary Table 4). Moreover, we did not detect any association between either circulating GMAb levels (Fig. 2A) or cell surface GM-CSF receptor alpha chain (CD116) abundance (Fig. 2B) and the neutrophil GMSI. This suggests that at least a portion of the variation in the GMSI could be related to genetic variants. We therefore next asked whether IBD patients with low-neutrophil GMSI would be more likely to carry genetic variants in *CSF2RA*, the GM-CSF receptor alpha chain gene, or *CSF2RB*, the GM-CSF receptor beta chain gene.

Potentially damaging missense mutations in the protein-coding regions of *CSF2RA* and *CSF2RB* and the downstream signaling genes *JAK2*, *STAT5A*, and *STAT5B* were determined using the WES of 543 pediatric IBD patients (Supplementary Table 1). As summarized in Table 2, we identified 37 potentially damaging heterozygous or homozygous missense mutations in *CSF2RA*, *CSF2RB*, *JAK2*, *STAT5A*, and *STAT5B*. The IBD case MAF for each of these variants did not differ from control MAFs obtained from publicly available databases. The MAFs for the IBD cases are shown in Table 2; the MAFs reported for each of these variants in the ExAC database for healthy individuals of European, African, or East/South Asian ancestry are included in Supplementary Table 5. Forty-eight of the IBD patients with WES also had neutrophil function testing completed. We therefore next asked whether any of the missense mutations summarized in Table 2 would be associated with a reduction in the neutrophil GMSI.

The median (IQR) neutrophil GMSI was equal to 45% (8%–111%) in IBD patients lacking any potentially damaging variants in these genes detected by WES (wild-type, n = 27). These data and those for patients carrying the indicated missense mutations are shown in Figure 2C. Inflammatory bowel disease patients carrying the *CSF2RA* A17G mutation were more likely to exhibit a neutrophil GMSI below the median value for patients lacking mutations (wild-type group), whereas most patients carrying 1 of several *CSF2RB* missense mutations exhibited a neutrophil GMSI above the median value for patients lacking mutations (wild-type group). The median (IQR) neutrophil GMSI increased from 18% (–9% to 36%) in the group carrying the *CSF2RA* A17G mutation (n = 7) to 61% (34%–93%) in the group carrying any of the indicated *CSF2RB* mutations (n = 14, P = 0.025). To confirm the association between the *CSF2RA* A17G mutation and the GMSI in CD, we sequenced additional CD patients with data for the GMSI for this variant (n = 90). The median (IQR) neutrophil GMSI decreased from 63% (38%–102%) in CD patients lacking the *CSF2RA* A17G variant to 29% (–3% to 69%) in CD patients carrying the *CSF2RA* A17G variant (P = 0.02) (Fig. 2D). Consistent with this, *CSF2RA* A17G carriage increased from 10% in those with intact neutrophil GMSI to 32% in those with low GMSI

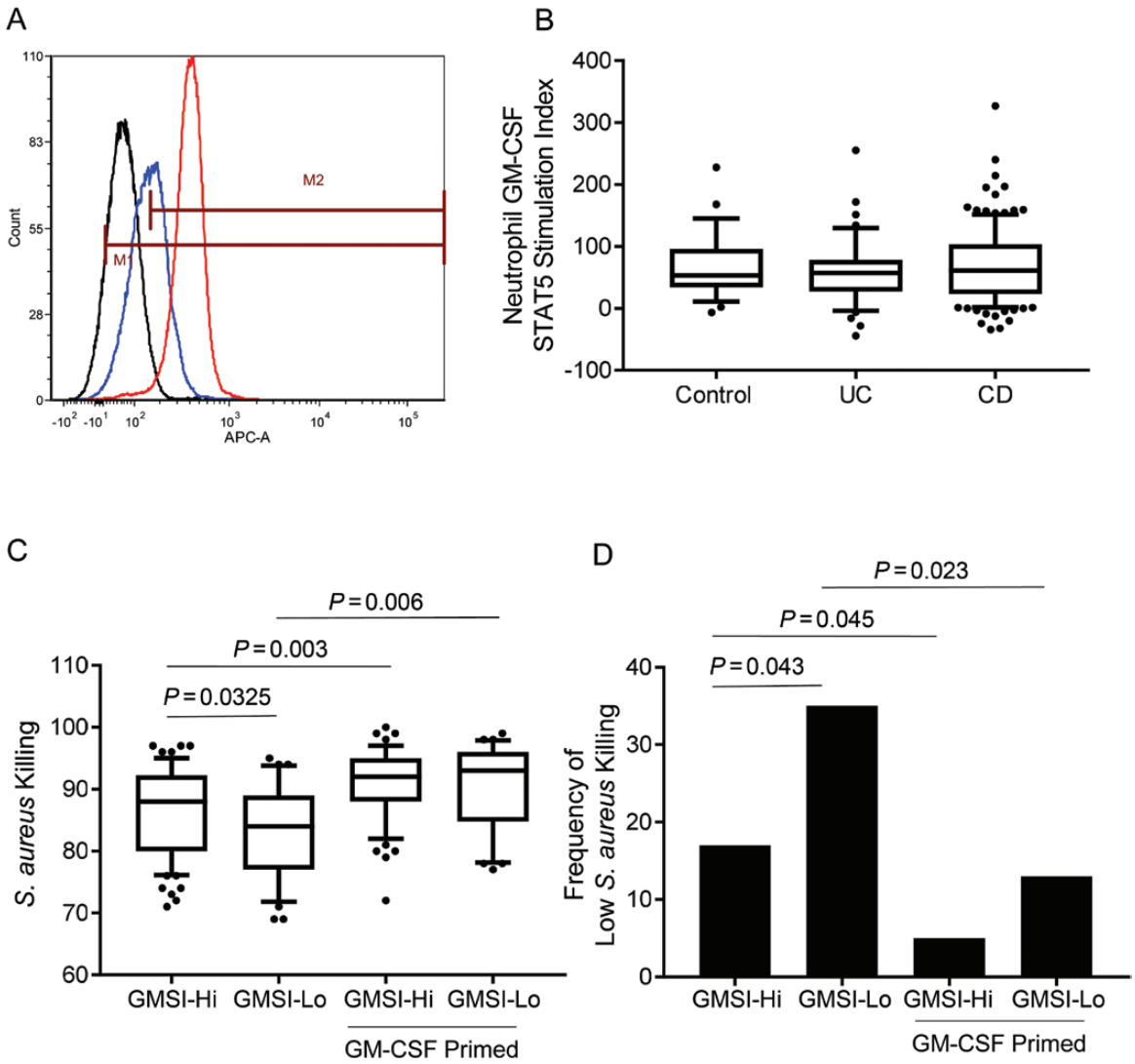

FIGURE 1. Neutrophil granulocyte–macrophage colony-stimulating factor signaling and bacterial killing. A, Peripheral blood leukocytes were stimulated with GM-CSF, and STAT5 tyrosine phosphorylation was measured by flow cytometry. Representative FACS plots for unstimulated and stimulated control and CD patient samples are shown. B, The GM-CSF-induced neutrophil STAT5 stimulation index in healthy controls (n = 26), ulcerative colitis patients (n = 54), and Crohn's disease patients (n = 126) is shown as median (IQR). C, *Staphylococcus aureus* killing is shown as the median (IQR) of the percentage of intracellular bacteria killed with and without GM-CSF priming in IBD patients stratified by the GM-CSF STAT5 stimulation index (n = 31 for GMSI-Lo and n = 70 for GMSI-Hi). D, The frequency of subjects with low bacterial killing, defined by less than 80%, is shown in IBD patients stratified by the GM-CSF STAT5 stimulation index (n = 31 for GMSI-Lo and n = 70 for GMSI-Hi). Abbreviations: GMSI-Lo, GM-CSF stimulation index ≤25%; GMSI-Hi, GM-CSF stimulation index >25%; differences between the groups were tested using the Mann-Whitney or chi-square test.

($P = 0.02$) (Fig. 2E). Collectively, these data demonstrated that a portion of the variance in the neutrophil GMSI index was associated with *CSF2RA* A17G missense mutation carriage.

Clinical and Demographic Characteristics of IBD Patients With Low-Neutrophil GMSI

We previously reported that both pediatric and adult-onset CD patients with reduced GM-CSF bioactivity due to elevated GM-CSF Ab experience higher rates of disease complications requiring surgery.[3,4] We therefore next asked whether these would also vary with the neutrophil GMSI as a measure of cell-intrinsic GM-CSF responsiveness. As shown in Table 3, age of onset, sex, race, and disease location did not vary within the CD or UC groups with the neutrophil GMSI. However, the frequency of both stricturing and penetrating complications was significantly higher within the GMSI-Lo CD group. The frequency of stricturing complications increased from 22% in the CD GMSI-Hi group to 40% in the CD GMSI-Lo group ($P = 0.05$), whereas the frequency of internal penetrating complications increased from 8% in the CD GMSI-Hi group to 23% in the CD GMSI-Lo group ($P = 0.03$). We observed a trend toward an overall increase in surgeries within the CD GMSI-Lo

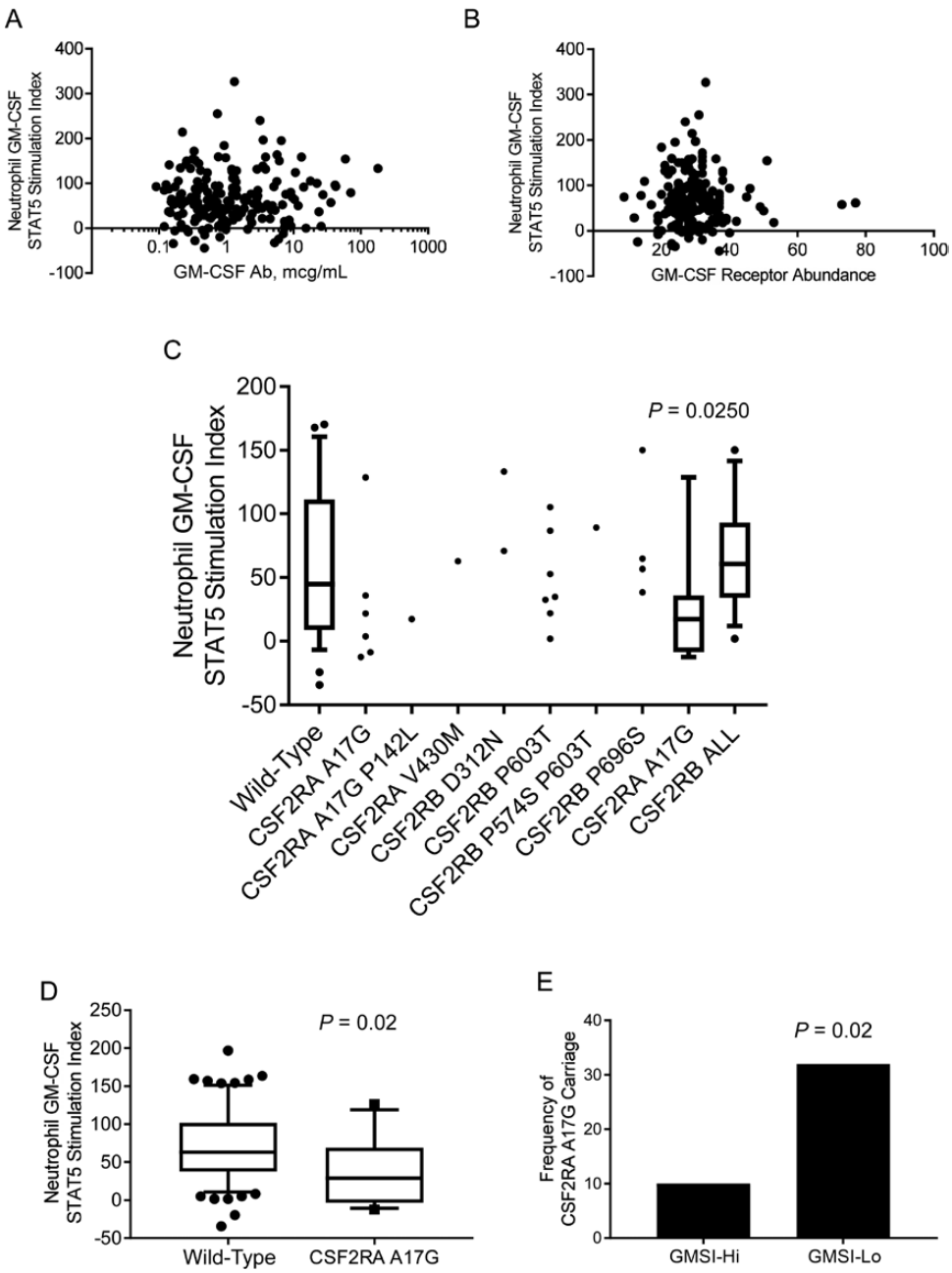

FIGURE 2. Neutrophil granulocyte–macrophage colony-stimulating factor signaling and GM-CSF receptor genetic variation. The relationships between (A) circulating GM-CSF Ab measured by ELISA or (B) neutrophil cell surface GM-CSF receptor alpha chain abundance (CD116) measured by flow cytometry and the neutrophil GM-CSF STAT5 stimulation index determined by flow cytometry are shown. C, Potentially damaging missense mutations in the protein coding regions of CSF2RA and CSF2RB were determined using whole-exome sequencing and were tested for association with neutrophil GM-CSF-induced STAT5 tyrosine phosphorylation in IBD patients. The GM-CSF-induced neutrophil STAT5 stimulation index is shown as the median (IQR) for IBD patients lacking any potentially damaging variants in these genes (wild-type, n = 27) or patients carrying the indicated missense mutations. Differences between the group carrying the CSF2RA A17G mutation (n = 7) and the group carrying any of the indicated CSF2RB mutations (n = 14) were tested by the Mann-Whitney test. D, The GM-CSF-induced neutrophil STAT5 stimulation index is shown as median (IQR) for CD patients with (n = 14) or without (n = 76) CSF2RA A17G carriage. Differences between groups were tested by unpaired *t* test. E, The frequency of CSF2RA A17G carriage is shown for CD patients stratified by a GM-CSF-induced STAT5 stimulation index of 25%, GMSI-Hi vs GMSI-Lo. Differences between groups were tested by chi-square test.

group, which reached significance for the subgroup with surgeries for colonic disease (10% in the GMSI-Hi group vs 30% in the GMSI-Lo group, $P = 0.009$). Importantly, these differences in disease complications were not associated with any differences in treatment exposures during the first 3 years after diagnosis (Supplementary Fig. 5). By comparison, there was no difference in rates of surgeries within the UC group.

CD Neutrophil Phospho-Protein Signaling Networks Associated With GM-CSF Responsiveness

Unstimulated Hi- and Lo-GMSI neutrophils failed to exhibit differential phospho-protein levels in the 10-protein immunoassay (Fig. 3A). STAT1, however, exhibited nominal significance ($P = 0.014$) that did not survive correction for multiple testing. NFKB, JNK, and p70S6K all exhibited nominal trends toward significance ($P < 0.1$). Pairwise analysis between Hi-GMSI and controls focusing on the 4 proteins with nominal trends revealed that the initial associations in NFKB ($P = 0.032$) and STAT1 ($P = 0.003$) were driven by elevation in Hi-GMSI and not Lo-GMSI samples. Testing of the same proteins between Lo-GMSI and controls indicated that the association observed in p70S6K was driven by lower levels in the Lo-GMSI samples ($P = 0.022$). The initial trend seen in JNK was driven by elevated levels in Hi-GMSI samples in comparison with low levels in Lo-GMSI samples ($P = 0.027$), independent of levels observed in controls.

CD Neutrophil Gene Expression Patterns Associated With GM-CSF Responsiveness

Differences in the neutrophil GMSI were not associated with differences in overall *CSF2RA*, *CSF2RB*, *JAK2*, or *STAT5A*/*B* expression (Supplementary Table 6). Direct comparison between CD patients with low and high GMSI revealed that the majority of differentially regulated transcripts ($P < 0.05$, fold-change > 1.5) were upregulated in CD-Lo patients (523 of 550 transcripts). Next, we identified 1450 differentially regulated transcripts between CD-Lo subjects and controls (1072 up and 378 down in CD-Lo) and 1039 differentially regulated transcripts between CD-Hi and controls (583 up and 456 down in CD-Hi) (Supplementary Table 7). Using Venn diagrams, we overlapped up- and down-regulated genes to identify central gene sets associated with the disease process and those specific to neutrophil GMSI. We observed a greater number of transcripts specific to the CD-Lo gene set (764 down and 194 up compared with controls), compared with the CD-Hi gene set (272 down and 275 up), confirming the over-representation of upregulated

TABLE 3. Clinical and Demographic Characteristics of CD and UC Patients Stratified by GM-CSF-Induced STAT5 Tyrosine Phosphorylation

	CD GMSI-Lo (n = 30), **No. (%)**	CD GMSI-Hi (n = 96), **No. (%)**	UC GMSI-Lo (n = 14), **No. (%)**	UC GMSI-Hi (n = 40), **No. (%)**
Age < 6 **y**	1 (3)	16 (17)	3 (21)	6 (15)
Age 6–9 **y**	9 (30)	24 (25)	5 (36)	8 (20)
Age 10–16 **y**	20 (67)	56 (58)	6 (43)	26 (65)
Male	19 (63)	58 (60)	7 (50)	17 (43)
White	26 (87)	87 (91)	11 (79)	37 (93)
Ileal location	3 (10)	9 (9)	NA	NA
Colon-only location	3 (10)	15 (16)	NA	NA
Ileo-colonic location	24 (80)	72 (75)	NA	NA
Perianal disease	15 (50)	39 (41)	NA	NA
Left-sided colitis	NA	NA	2 (14)	5 (13)
Pan-colitis	NA	NA	12 (86)	35 (87)
Stricturing **b**ehavior	12 (40)*	21 (22)	NA	NA
Penetrating **b**ehavior	7 (23)*	8 (8)	NA	NA
Surgery	14 (47)	29 (30)	2 (14)	3 (8)
CD **small bowel surgery**	7 (23)	18 (19)	NA	NA
CD **colon surgery**	9 (30)**	10 (10)	NA	NA

GMSI-Lo was defined as <25%.
Abbreviations: GMSI, GM-CSF-induced STAT5 stimulation index; NA, not applicable.
*$P < 0.05$; **$P < 0.01$.

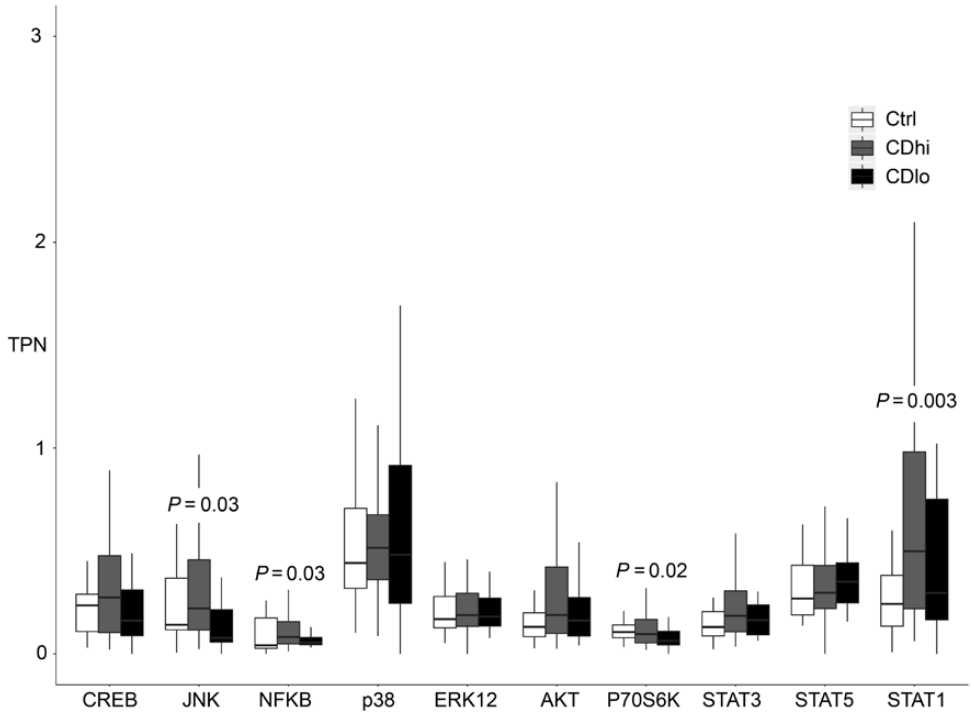

FIGURE 3. Metabolic and immune functions differentially expressed in CD neutrophils with normal and low GM-CSF stimulation index. A, Protein was isolated from unstimulated control (n = 20) and CD (n = 104) neutrophils, and phospho-protein abundance was determined using a multiplex assay. Differences between groups were tested using Wilcoxon rank-sum tests. Candidate gene prioritization through functional enrichment analysis used genes differentially regulated between normal or low-GMSI CD neutrophils and control neutrophils. Differentially expressed genes were submitted to ToppCluster to identify mechanistic enrichments. B, Pathways and mechanisms enriched in genes that were upregulated in low-GMSI neutrophils vs control neutrophils or normal-GMSI neutrophils vs control neutrophils. C, Pathways and mechanisms enriched in genes that were downregulated in low-GMSI CD neutrophils vs control neutrophils or normal-GMSI CD neutrophils vs control neutrophils. Abbreviations: GMSI, neutrophil GM-CSF STAT5 tyrosine phosphorylation stimulation index; GMSI-Lo ≤25%; GMSI-Hi >25%.

transcripts in CD-Lo neutrophils. Further, among the genes shared (184 down and 308 up) between CD-Lo vs controls and CD-Hi vs controls, we see an augmentation of down- or upregulation in the CD-Lo neutrophils (effect size increase of 26.8%, $P < 0.0001$).

Ontological analysis focused on identifying shared and independent pathways and biological processes enriched in up- and downregulated transcripts in Hi- and Lo-GMSI neutrophils in comparison with Controls (Fig. 3; Supplementary Table 8). Among the shared upregulated pathways, we identified enrichments in classical CD-related mechanisms, including complement pathways and innate immune response, wound healing and Wnt signaling, Toll-like cascades and inflammatory response (Fig. 3B). Providing insight into the biological specificity of Hi-GMSI neutrophils, we see enrichment of interferon gamma signaling, which substantiates our observation of elevated STAT1 phospho-proteins in Hi-GMSI neutrophils. Similarly, we identified upregulation of the NFKB pathway in Hi-GMSI neutrophils only, again confirming our phospho-protein findings. Conversely, among mechanisms specific to upregulated genes in Lo-GMSI samples, we identified extracellular matrix and adhesion molecules indicative of a profibrotic transcriptomic signature, which is highly concordant with the observed higher incidence of stricturing and penetration in the Lo-GMSI subgroup.

Among shared downregulated ontologies, we observed the expected downregulation of mitochondrial function and metabolism, coupled with factors regulating the inflammatory cascade, and a dysregulation of cell proliferation (Fig. 3C). Specific to the Hi-GMSI neutrophils, we observed dysregulation of bioenergetics through cellular carbohydrate metabolism, glucokinase activity, and gluconeogenesis indicative of more metabolically active neutrophils. In the Lo-GMSI neutrophils, we identified indicators of dysregulation of the cell death process, which correlated with our observation of low p70S6k levels in Lo-GMSI samples. This further underscores the profibrotic tendencies of Lo-GMSI patients.

Disease Location and Complications in CD Patients Stratified by Circulating GM-CSF Auto-antibodies and Neutrophil GM-CSF STAT5 Stimulation Index

We previously reported that increased GMAb levels were associated with reduced neutrophil GM-CSF-induced STAT5

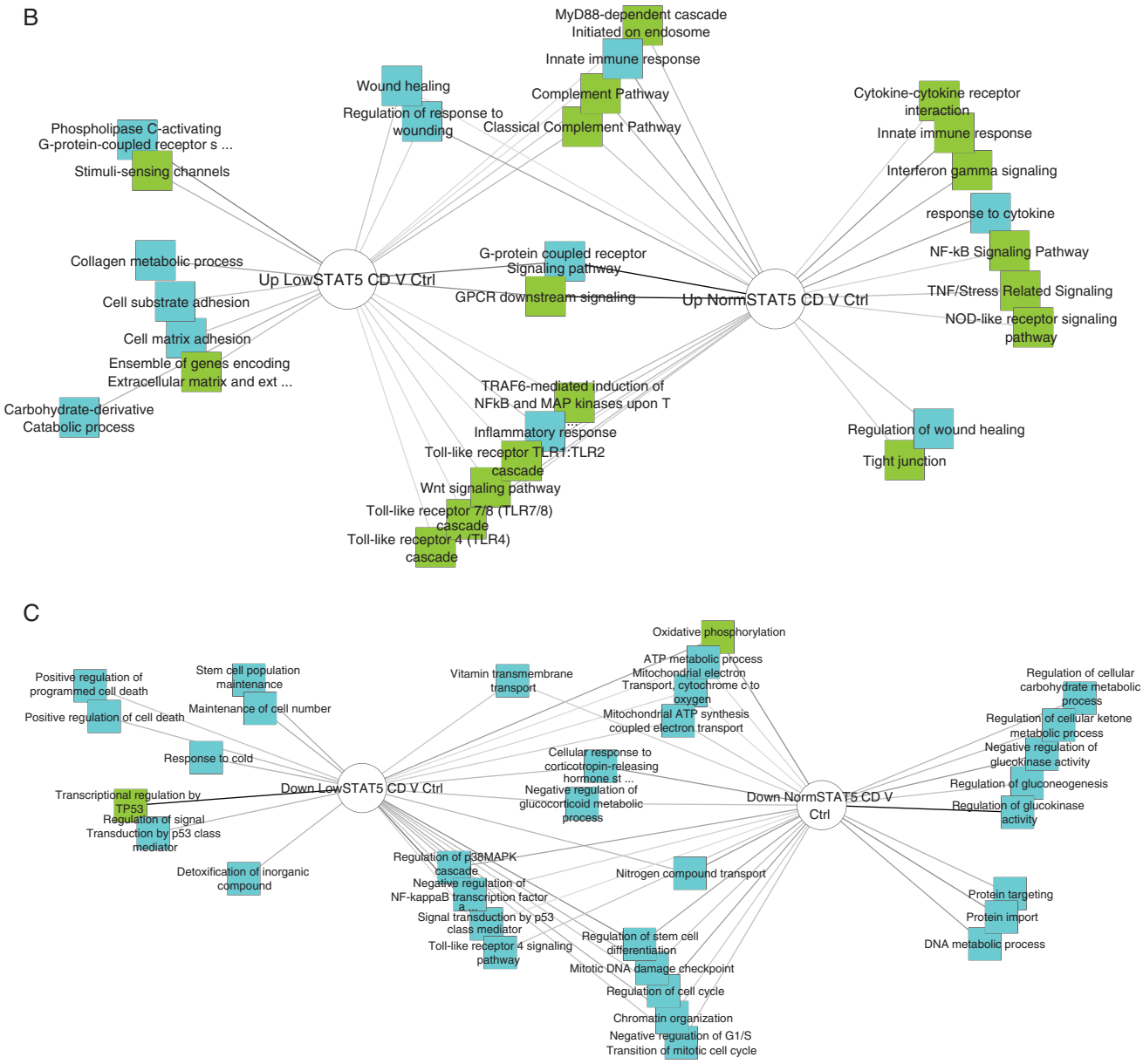

FIGURE 3. Continued

tyrosine phosphorylation in whole-blood samples, which contained these auto-antibodies at physiologic levels.[5] This reduction in GM-CSF bioactivity was in turn associated with higher rates of stricturing complications. We therefore next asked whether disease location or complications would vary within CD patients stratified by both circulating GM-CSF auto-antibodies and neutrophil GM-CSF STAT5 stimulation index (Supplementary Table 9). We identified a progressive decrease in the frequency of colon-only disease location (P = 0.0217) (Fig. 4A) and an increase in the frequency of jejunal and proximal ileal location and perianal involvement (P = 0.0007 and P < 0.001, respectively) (Figs. 4B, C) in patients with both elevated GMAb and reduced GMSI. The frequency of stricturing behavior increased from 7% in CD patients with low GMAb and high GMSI to 64% in patients with both elevated GMAb and reduced GMSI (P < 0.0001) (Fig. 4D). Similarly, the frequency of internal penetrating behavior increased from 0% in CD patients with low GMAb and high GMSI to 29% in patients with both elevated GMAb and reduced GMSI (P = 0.0004) (Fig. 4E). Consistent with this, the frequency of surgery increased from 19% in CD patients with low GMAb and high GMSI to 64% in patients with both elevated GMAb and reduced GMSI (P = 0.0005) (Fig. 4F). Collectively, these data demonstrated a high rate of disease complications and surgeries in patients with reduced

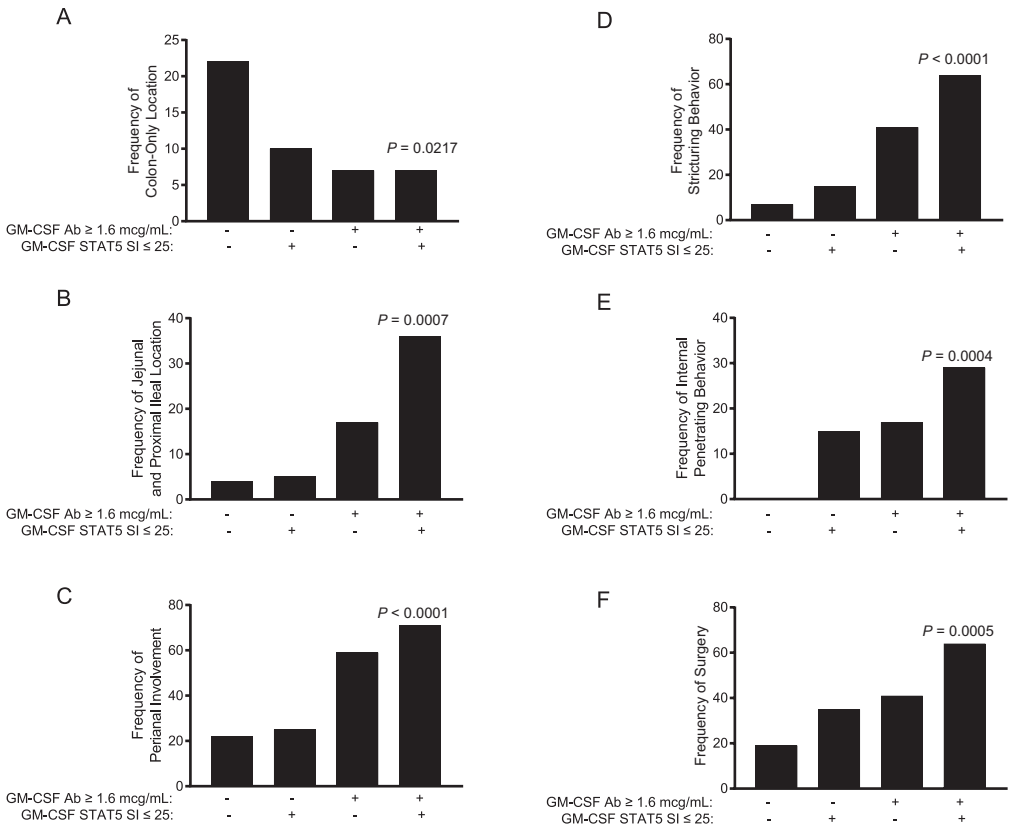

FIGURE 4. Disease location and complications in CD patients stratified by circulating GM-CSF auto-antibodies and neutrophil GM-CSF STAT5 stimulation index. The frequency of (A) colon-only disease location, (B) jejunal and proximal ileal disease location, (C) perianal involvement, (D) stricturing disease behavior, (E) internal penetrating disease behavior, or (F) surgery is shown in CD patients stratified by circulating GM-CSF auto-antibodies and neutrophil GM-CSF STAT5 stimulation index. The number of subjects in each subgroup was 54 for those with GM-CSF Ab <1.6 mcg/mL and GM-CSF STAT5 SI >25%, 20 for those with GM-CSF Ab <1.6 mcg/mL and GM-CSF STAT5 SI ≤25%, 46 for those with GM-CSF Ab ≥1.6 mcg/mL and GM-CSF STAT5 SI >25%, and 14 for those with GM-CSF Ab ≥1.6 mcg/mL and GM-CSF STAT5 SI ≤25%. Differences between groups were tested by chi-square test for trend.

GM-CSF bioactivity due to extrinsic GMAb and cell-intrinsic mechanisms reflected by the GMSI.

DISCUSSION

Crohn's disease patients typically exhibit inflammatory disease behavior at diagnosis, with varying rates of progression to stricturing and internal penetrating complications and surgery over time.[17] Despite increased use of anti–tumor necrosis factor–α therapy, a population-level decline in rates of surgeries for CD strictures has not been observed.[18, 19] This suggests that alternate inflammatory and molecular pathways drive stricturing complications. In this regard, GM-CSF exerts pleotropic epithelial and immune effects in the gut.[1] A rare dominant negative frameshift mutation in the GM-CSF receptor beta chain gene *CSF2RB* reduces GM-CSF activation of STAT5 in monocytes and increases risk for adult-onset CD.[9, 10] We discovered that common neutralizing GMAb, which inhibit GM-CSF signaling in a cell-extrinsic manner, are associated with reduced neutrophil bacterial killing and higher rates of stricturing complications in pediatric and adult-onset CD.[4, 5] In the current study, we have identified a subset of pediatric CD patients characterized by low/normal neutrophil-intrinsic GMSI, which was in turn associated with a *CSF2RA* A17G missense mutation, alterations in wound healing and cell survival gene programs, and high rates of disease complications.

We did not detect any association between circulating GM-CSF or GMAb levels, cell surface GM-CSF receptor alpha chain (CD116) abundance, and the neutrophil-intrinsic GMSI. We therefore asked whether IBD patients with reduced neutrophil GMSI would be more likely to carry genetic variants in *CSF2RA* or *CSF2RB*. We conducted the largest pediatric IBD WES study to date and identified 18 potentially damaging heterozygous or homozygous missense mutations in *CSF2RA* or *CSF2RB*.[20] Only 1 of these mutations, *CSF2RA* A17G, was present at a high enough frequency to test for an association with the neutrophil GMSI in primary patient-derived cells. We confirmed that *CSF2RA* A17G carriage was associated with a 2-fold reduction in the neutrophil GMSI. CD patients

with GMSI-Lo neutrophils were 3-fold more likely to carry the *CSF2RA* A17G missense mutation. Collectively, these data demonstrated that the neutrophil GMSI is regulated in part by *CSF2RA* or *CSF2RB* genetic variation, although other acquired mechanisms involving cell signaling and gene expression are likely to play a critical role.

CSF2RA is located on the X chromosome, and *CSF2RA* A17G carriage is 5-fold more common in individuals of African ancestry compared with those of European ancestry.[14] It is interesting to note that African American race was associated with higher rates of internal penetrating complications in our recent multicenter cohort study, potentially involving *CSF2RA* A17G carriage and reduced cellular GM-CSF responsiveness.[12] However, we were not adequately powered to test for interactions between sex, race, *CSF2RA* A17G carriage, and neutrophil function and disease behavior in the current study. This will be the focus of a future multicenter study guided by the current results.

GM-CSF plays a critical role in priming neutrophil antimicrobial functions. Neither *S. aureus* uptake (phagocytosis) nor FMLP-induced ROS production varied within IBD patients stratified by the GMSI. However, we did observe a reduction in *S. aureus* killing in GMSI-Lo neutrophils. GM-CSF pretreatment before exposure to *S. aureus* killing increased killing in both groups and corrected the difference observed in unprimed neutrophils. Consistent with this, the frequency of subjects with low bacterial killing also increased 2-fold from GMSI-Hi neutrophils to GM-CSF-Lo neutrophils. This was also largely corrected by GM-CSF pretreatment. Collectively, these data identified a subgroup of CD and UC patients characterized by low/normal neutrophil GMSI in association with reduced *S. aureus* killing and suggest the potential for correcting this with ex vivo GM-CSF priming of myeloid cells.[1]

We therefore asked whether differences in neutrophil bacterial killing with GMSI would be specific to the GM-CSF:STAT5 pathway or if they reflect a broader dysregulation of cell signaling networks.[9] We conducted an unbiased survey of 10 key cell signaling phospho-protein targets under steady-state conditions. Although we did not observe significant differences in phospho-protein abundance that survived corrections for multiple comparisons, we did observe nominal increases in STAT1 and NFkB activation in the GMSI-Hi neutrophils, which were supported by the gene expression data.

We have defined for the first time the global pattern of gene expression in purified neutrophils from CD patients and controls and tested for specific variation with cell-intrinsic GM-CSF responsiveness. We were specifically interested to know whether gene programs expressed in circulating neutrophils would reflect those that we recently reported in the gut.[12] The gene expression results with GMSI-Lo neutrophils were remarkably similar to those obtained in pretreatment ileal biopsies for CD patients who went on to develop strictures.[12] This included upregulation of an extracellular matrix production gene signature and suppression of a mitochondrial respiration gene signature in GMSI-Lo neutrophils relative to control neutrophils. These data suggest an important role for GM-CSF in regulating these critical gene programs linked to patient outcomes, which will now be tested in purified ileal epithelial, myeloid, and myofibroblast cells.

We previously reported that increased GMAb levels were associated with reduced neutrophil GM-CSF-induced STAT5 tyrosine phosphorylation in whole-blood samples that contained these Ab at physiologic levels.[5] This reduction in GM-CSF bioactivity was in turn associated with higher rates of stricturing complications.[3, 4] We therefore asked whether disease location or complications would vary within CD patients stratified by both circulating GMAb and the neutrophil-intrinsic GMSI. We identified a progressive decrease in the frequency of colon-only disease location and an increase in the frequency of jejunal and proximal ileal location and perianal involvement in patients with both elevated GMAb and reduced GMSI. Consistent with this, the frequency of both stricturing and internal penetrating disease complications and associated surgeries increased substantially in CD patients with both elevated GMAb and reduced GMSI. These assays, which are now clinically available, may be used to estimate risk for disease complications and in the future to guide therapeutic approaches to boost GM-CSF bioactivity in targeted patient populations.

CONCLUSIONS

In the current study, we found that low/normal neutrophil-intrinsic GM-CSF signaling is associated with *CSF2RA* missense mutations, alterations in gene expression networks, and higher rates of disease complications in pediatric CD. A recent line of research has defined effects of GM-CSF priming upon human and murine monocytes, which may inform a novel cell-based therapy for CD.[1] These data suggested that GM-CSF priming of monocytes ex vivo might provide a future cellular therapy for CD that would avoid the potential limitations of systemic GM-CSF administration. Whether patients with genetic or acquired reductions in GM-CSF bioactivity due to rare *CSF2RA* or *CSF2RB* mutations or common GMAb, respectively, might receive particular benefit is unknown but ideally should be considered in future clinical trials.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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