



Meta-analysis of rare and common exome chip variants identifies S1PR4 and other loci influencing blood cell traits

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Meta-analysis of rare and common exome chip variants identifies *S1PR4* and other loci influencing blood cell traits

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Abstract

Hematologic measures such as hematocrit and white blood cell (WBC) count are heritable and clinically relevant. Erythrocyte and WBC phenotypes were analyzed with Illumina HumanExome BeadChip genotypes in 52,531 individuals (37,775 of European ancestry; 11,589 African Americans; 3,167 Hispanic Americans) from 16 population-based cohorts. We then performed replication analyses of novel discoveries in 18,018 European American women and 5,261 Han Chinese. We identified and replicated four novel erythrocyte trait-locus associations (*CEP89*, *SHROOM3*, *FADS2*, and *APOE*) and six novel WBC loci for neutrophil count (*S1PR4*), monocyte count (*BTBD8*, *NLRP12*, and *IL17RA*), eosinophil count (*IRF1*), and total WBC (*MYB*). The novel association of a rare missense variant in *S1PR4* supports the role of sphingosine-1-phosphate signaling in leukocyte trafficking and circulating neutrophil counts. Loss-of-function experiments of *S1pr4* in mouse and zebrafish demonstrated phenotypes consistent with the association observed in humans and altered kinetics of neutrophil recruitment and resolution in response to tissue injury.

Introduction

Erythrocyte and leukocyte blood counts are heritable traits (estimated heritability 0.40–0.90^{1–3} and 0.14–0.40, respectively⁴) that reflect core physiologic functions of oxygen-

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Accession codes for data access

Summary data for all analyses has been deposited into the database of Genotypes and Phenotypes (dbGaP) under the “CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology) Consortium Summary Results from Genomic Studies”. The dbGaP Study Accession is “phs000930” and can be found at: http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000930

Author Contributions

NP, YZ, YZ, EPB, IJD, OHF, MEG, VG, TH, TBH, AH, LJJ, AL, OP, JMS, AD, YH, CJO, APR, and SKG designed the study. YZ, IIB, EPB, MC, IJD, LD, MFF, MEG, VG, TBH, AH, RDJ, JJ, MK, TL, AL, MEN, BMP, OTR, SSR, JMS, BHT, RPT, JW, and CJO recruited and assessed participants. PLA, JB, NG, LL, YZ, FWA, EB, IIB, EPB, PIWdB, MFF, MLG, TL, DCL, YL, SSR, FR, JIR, KDT, and AGU generated genotyping data. YZ, MLA, VC, EJH, BH, KH, XZ, VMN, AMRDS, RLP, and LIZ performed functional experiments. NP, UMS, TSA, MLA, PLA, JB, NG, BH, YL, MAN, RP, AVS, YZ, JSF, NF, MLG, RJFL, BMP, AD, ALW, JGW, RLP, LIZ, CJO, APR, and SKG analyzed and interpreted data. NP, UMS, WZ, TSA, JB, JAB, MHC, JDE, NG, ADJ, ML, YL, LL, AM, REM, MAN, RP, AVS, FvR, MY, JW, and APR performed statistical analysis. NP, UMS, YZ, APR, and SKG wrote the manuscript. All authors were given the opportunity to comment and provide revisions to the manuscript text.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests

carrying capacity and anti-microbial activity. Peripheral blood cell counts are commonly measured in the clinical setting to diagnose and monitor therapy of many acute and chronic conditions, such as infection or anemia. Abnormalities in these clinical measures often reflect primary hematologic disease, blood loss or inflammation. Inter-individual differences in erythrocyte traits, total WBC, and neutrophil counts have been associated with risk of cardiovascular diseases and all-cause mortality.⁵⁻⁷

Previous genome-wide association studies (GWAS) have defined over 100 loci influencing erythrocyte traits⁸⁻¹² and leukocyte counts.^{8,13,14} However, few studies have systematically evaluated the contribution of coding variation, particularly variants at low frequency in the general population.^{15,16} Recently completed exome sequencing in diverse populations has led to international collaboration and creation of a genome-wide catalog of low frequency coding variants. We undertook a large-scale study of erythrocyte and leukocyte traits in up to 52,531 individuals of European, African and Hispanic ancestry to evaluate the impact of both low-frequency and common variants assayed by the Illumina HumanExome BeadChip, also referred to as the exome chip.

Results

Study Samples

In the discovery stage, we analyzed erythrocyte traits (hemoglobin (Hb), hematocrit (Hct), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), red cell distribution width (RDW), and red blood cell count (RBC)) and leukocyte traits (total WBC count and absolute neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts) (Supplementary Table 1) in 52,531 individuals, including 37,775 with European ancestry (EA), 11,589 African Americans (AA), and 3,167 white Hispanics (HA) sampled from 16 population-based cohorts. Sample sizes per trait analyzed in each cohort are provided in Supplementary Table 2. The traits followed expected distributions, and characteristics of the study participants, including age, sex and trait summaries, are presented in Supplementary Table 3. Descriptions of each cohort are provided in the Supplementary Note.

Single variant and gene-based meta-analyses

In single variant analyses, we did not observe significant inflation of the meta-analysis p-values (Supplementary Table 4). A total of 104 unique locus-trait associations exceeded the Bonferroni-corrected significance threshold ($p < 4 \times 10^{-7}$; Supplementary Table 5). These included 49 independent loci associated with erythrocyte traits and 22 loci associated with leukocyte traits (Supplementary Table 6). Many of these were single nucleotide polymorphisms (SNPs) well-established to be associated with hematologic traits (see Supplementary Note), thus confirming the validity of the exome chip. Novel findings reaching study-wide significance ($p < 4 \times 10^{-7}$; $n=9$ for erythrocytes; $n=10$ for leukocytes) are listed in Table 1 and were carried forward to replication in an independent sample. Of these, 4 novel trait-locus associations for erythrocyte traits (*SHROOM*, *CEP89*, and *APOE* were study-wide significant, $p < 0.003$; *FADS2* was only nominally significant, $p=0.02$) and 6 novel trait-locus associations for WBC traits (*BTBD8*, *MYB/HBS1L*, *S1PR4*, and *IL17RA*

were study-wide significant, $p < 0.003$; *IRF1* and *NLRP12* were only nominally significant, $p < 0.05$ were replicated in an independent set of European American samples from WHI (Supplementary Table 7).

Novel, replicated associations with erythrocyte traits

All 4 novel, replicated erythrocyte associations are common SNPs present on the exome chip because of prior associations with non-hematologic phenotypes as listed in the NHGRI GWAS catalog. Two common intronic SNPs previously associated with renal function (*SHROOM3*/rs13146355 and *CEP89*/rs4805834) were associated with both Hb and Hct. The minor allele of *SHROOM3*/rs13146355-A (previously associated with both lower estimated glomerular filtration rate (eGFR)¹⁷ and higher serum magnesium¹⁸) was associated with significantly higher Hb and Hct and nominally higher RBC count in our discovery and replication cohorts. The minor allele of *CEP89*/rs4805834-T was associated with lower Hb and Hct and higher eGFR.¹⁹ The observed directions of effect on Hb and Hct for both *CEP89*/rs4805834 and *SHROOM3*/rs13146355 are opposite of that expected based on the known relationship between lower eGFR and anemia. Conditional analyses performed in a subset of our cohorts demonstrated that the effect of either *CEP89*/rs4805834 or *SHROOM3*/rs13146355 on Hb and Hct was independent of eGFR (see Supplementary Note for more detail).

An intronic SNP of the fatty acid desaturase gene *FADS2* (rs1535) previously associated with transferrin levels²⁰ and polyunsaturated fatty acid (PUFA) levels²¹ was associated with RBC count. Finally, we identified an association between increased RDW and the SNP encoding the canonical *APOE-ε2* variant rs7412, which is known to be associated with cholesterol^{22–25} and inversely associated with dementia.^{26,27} Additional adjustment for LDL-cholesterol, HDL-cholesterol, and triglyceride levels did not attenuate the *APOE-ε2*/RDW association in the ARIC study. A tag for the *APOE-ε4* allele was present on the exome chip, but had no association with either LDL-cholesterol or RDW independent of *APOE-ε2* (Supplementary Note).

In gene-based tests, several loci were significantly associated with erythrocyte traits in the discovery sample (Table 2; Supplementary Table 8). The *EPO* gene-based association was driven by a single low-frequency missense variant (p.Asp70Asn/rs62483572), confirming the recent association of this variant with lower Hb.¹⁶ Similarly, a single novel variant drove the *ITFG3* association (p.Asp534Asn/rs144091859). The *HFE* and *G6PD* associations were driven by population-specific common variants identified in prior GWAS that were included in the gene-based test because they are common in one population but absent in another and therefore averaged out to below the minor allele frequency (MAF) < 0.05 threshold for inclusion in the trans-ethnic analysis. Significant associations in *ANK1*, *NLRC3*, and *HBS1L* were supported by multiple rare variants (Supplementary Table 9a; Supplementary Note).

Novel, replicated leukocyte associations

We discovered and replicated 6 novel WBC trait-locus associations. In the single-variant analysis, we identified a single missense variant in the type 4 sphingosine-1-phosphate

receptor (*SIPR4*), p.Arg365Leu/rs3746072, that was associated with lower total WBC ($p=1.5\times 10^{-7}$) and lower neutrophil counts ($p=3.4\times 10^{-7}$) (Supplementary Figure 1). The association was consistent across cohorts (Figure 1) and validated in both replication samples (WHI EA women $p=0.001$; PUUMA Han Chinese $p=0.003$; $p\text{-meta}_{\text{discovery+replication}}=5\times 10^{-12}$) (Supplementary Table 7). The variant is rare ($\text{MAF}_{\text{meta}}=0.006$) and not in linkage disequilibrium with variants in the region (Supplementary Figure 1). In both discovery and replication analyses, p.Arg365Leu was the only variant contributing to the significant gene-based association. Neutrophil counts were approximately 10% lower in the p.Arg365Leu minor allele carriers (Figure 2). The *SIPR4* p.Arg365Leu amino acid substitution is located in the intracellular cytoplasmic tail of *SIPR4*, is at a conserved site (GERP: 3.94), and is predicted to be “possibly damaging” by PolyPhen-2.²⁸

Two missense variants were associated with lower monocyte count: a low-frequency p.Val60Ile variant in *BTBD8* (rs34856868; $\text{MAF}_{\text{EA}}=0.03$) and a common p.Gly39Val variant in *NLRP12* (rs34436714; $\text{MAF}_{\text{EA}}=0.217$). Three common, intergenic variants included on the exome chip as GWAS index SNPs originally associated with non-leukocyte phenotypes were newly associated with WBC traits in our analysis. The common intergenic regulatory variant of *HBSIL-MYB* (previously associated with erythrocyte and platelet traits) was associated with total WBC count. Common non-coding SNPs in the regions of *IL33* and *IRF1* previously associated with asthma²⁹ and other allergic/autoimmune disorders^{30–33} were associated with eosinophil count.

Gene-based analyses identified an association between low frequency variation in the *IL17RA* locus and monocyte count ($p=6.4\times 10^{-7}$). We confirmed the recently reported multi-variant association between *CXCR2* and lower neutrophil count; 6 of the 9 rare *CXCR2* missense variants in our analysis had a p-value less than 0.05, with the strongest associations from p.Arg153His (rs55799208; $p=2.4\times 10^{-5}$) and p.Arg248Gln (rs61733609; $p=6.1\times 10^{-5}$). Several additional single-variant and gene-based associations with WBC traits were observed within the AA or HA discovery samples, but not in the larger EA discovery sample. Three of these associations were driven by low frequency (MAF 0.01–0.05) variants in AAs (*IQCJ* and *SEC24D*) or HAs (*SEC14L1*) (Table 1b and Supplementary Tables 8 and 9b). Further assessment in independent AA or HA samples will be needed to validate these ethnicity-specific associations.

Characterization of variants in previously known GWAS loci

To evaluate whether variants identified in our analysis overlap previously known GWAS results or whether we identified independent associations, we conducted conditional analyses in ARIC, adjusting for previously known variants associated with erythrocyte and leukocyte traits in several regions overlapping the findings in this study (Supplementary Table 10). Specifically, we interrogated any variant that was rare ($\text{MAF}<5\%$) and meeting study-wide significance (Supplementary Note).

We identified a novel association between a low-frequency variant in *ANK1* (p.Ala1462Val/rs34664882; $\text{MAF}_{\text{EA}}=0.029$; $\text{MAF}_{\text{AA}}=0.015$; $\text{MAF}_{\text{HA}}=0.013$) and MCHC that is independent of the original GWAS result (rs4737009; 1000G CEU $\text{MAF}=0.27$; ARIC

MAF_{EA}=0.24). We also identified several low-frequency missense variants in the *HBA1-HBA2* region on chromosome 16. The most prominent was an AA-specific variant in *ITFG3* (p.Asp534Asn) that was associated with several erythrocyte parameters (MCH, MCHC, MCV, RBC count) and is independent of the common GWAS association (see Supplementary Note). Significant associations with the same traits were also seen for rare variants in *MRPL28*, *NARFL*, *RGS11*, *TMEM8A*, and *TPSD1* (see Supplementary Note).

Expression quantitative trait loci (eQTL) analysis

We used eQTL analysis³⁴ to determine if newly identified non-coding variants are associated with expression of nearby genes across a range of tissue types (Supplementary Table 11). The most notable eQTL findings were in the *FADS2* locus, which was associated with RBC count in our discovery analysis and met a nominal significance level in the replication analysis (p=0.02). In this region, *FADS1*, *FADS2*, and *FADS3* all showed evidence of strong cis eQTL association to either the index SNP (rs1535) in multiple tissues, including *FADS1* (minimum p= 8.0×10^{-31} in CD19+ B cells) and *FADS2* (minimum p= 3.0×10^{-57} in blood lymphocytes). The *SIPR4* p.Arg365Leu variant does not demonstrate an association with expression levels of *SIPR4* or any nearby transcript (Supplementary Table 12).

Among the novel and independently replicated loci, rs4895441 at the *HBS1L-MYB* locus showed the expected eQTL association with *HBS1L* expression in multiple tissues (minimum p= 3.1×10^{-34} in aortic endothelial cells). In the *SHROOM3* locus, rs131463 exhibited a weak eQTL association (p= 7.3×10^{-6}) with *SHROOM3* transcript expression in subcutaneous adipose tissue. In the *CEP89* locus, rs4805834 was associated with expression of *SLC7A9* in multiple tissue (p= 1.9×10^{-24} in whole blood). The *IRF1* SNP, rs12521868, was associated with expression of *IRF1* in multiple tissues (p= 1.4×10^{-125} in whole blood).

Pleiotropy in the associated loci

In addition to pleiotropy between our novel findings and the known associations with kidney function (*CEP89* and *SHROOM3*) and with dementia and dyslipidemia (*APOE*), we also identified variants with pleiotropy across multiple blood cell lineages, most notably for the *HBS1L-MYB* and *SH2B3* loci as well as other subthreshold associations (see Supplementary Note; Supplementary Table 13).

Confirmation of *SIPR4* as a causal gene in model systems

The primary hypothesis of our exome chip study was that focused evaluation of coding variation would yield novel genetic associations of rare variants with hematologic traits, and that these variants would be more likely to be functionally relevant owing to the selection of variants for the exome chip. Our study yielded many novel associations, in part due to coverage of noncoding variation included in the exome chip as follow-up of previous GWAS, as is the case for the three novel RBC loci we report here. The association of a rare missense variant in *SIPR4* associated with total WBC and neutrophil count was consistent with our *a priori* hypothesis, and we therefore undertook further follow-up studies of this gene's functional impact on neutrophil traits in model systems.

Using previously generated *S1pr4* null mice,³⁵ we evaluated peripheral circulating blood neutrophil and monocyte counts, bone marrow neutrophil counts, and spleen neutrophil counts in *S1pr4*^{-/-} mice and *S1pr4*^{+/+} littermates. We analyzed 12 mice in each genotype group (total n=24 mice), with equal numbers of males and females in each group, and found the mean percentage of total cells analyzed by fluorescence-activated cell sorting (FACS) that were Gr1⁺ CD11b⁺, marking neutrophils, was 31% lower in *S1pr4*^{-/-} mice compared to wild-type mice. We repeated the experiment again in an additional 24 mice, again with 12 mice in each genotype group and equal numbers of males and females in each group and saw a similar decrease. Across the 48 mice, both the percentage of white blood cells that were neutrophils (28.0% decrease; p=0.11) and the absolute neutrophil count (54.3% decrease; p=0.03) were lower in *S1pr4*^{-/-} mice compared to wild-type mice (Figure 3; Supplementary Figure 2; Supplementary Table 14). To evaluate the effects on circulating monocyte counts, FACS analysis was conducted in the same samples, and the mean percentage of total leukocytes analyzed by FACS that were Gr1⁻ CD11b⁺ was equivalent in the two mouse groups (6.36% in *S1pr4*^{+/+} mice and 6.20% in *S1pr4*^{-/-} mice, p=0.80, Supplementary Figures 3–4). Since abnormalities of leukocyte bone marrow egress have been described in the setting of *S1pr1* deficiency,³⁶ we evaluated the proportion of neutrophils in the bone marrow and spleen, to evaluate whether cells may be abnormally retained in these tissues, and the expression of specific adhesion molecules involved in leukocyte trafficking. No significant differences in neutrophil proportions or absolute counts were observed in the bone marrow or spleen (Supplementary Table 14, Supplementary Figures 3–6). *Cd49b* and *Cxcr4* expression on bone marrow neutrophils were not differentially expressed (p>0.05), whereas *Cd62l*, or L-selectin, expression measured on circulating neutrophils was reduced approximately 2-fold in the *S1pr4*^{-/-} mice (p=0.003) across both groups of mice studied. Since lower L-selectin expression may reflect shedding upon activation and cell extravasation in tissues, we evaluated tissue neutrophil numbers in the liver and lung of *S1pr4*^{-/-} and wild-type mice. Neutrophil numbers were lower in both tissues in *S1pr4*^{-/-} mice, with a 29.2% reduction in liver (p=0.12) and 40.2% reduction in lung (p=0.02) Supplementary Figure 7).

To further assess the impact of disrupted *s1pr4* expression *in vivo*, we conducted parallel experiments in zebrafish in which gene expression may be manipulated readily using morpholino (MO) antisense technology to specifically knock down the expression of target genes.³⁷ In the comparison of embryos injected with ATG-MO's designed against two independent sequences (Supplementary Note) within the single exon of *s1pr4* (n=14 and 19) to non-specific MO (n=22), we confirmed a 36.6% and 34.3% decrease in neutrophil count in the two batches of whole embryos at two days post fertilization (dpf) (p=3.8×10⁻⁶ and p=4.4×10⁻⁷, respectively) (Figure 4; Supplementary Table 15).

Finally, to assess neutrophil behavior in response to injury, a cutaneous wound was made on the ventral side of the tail fin of the embryos at 2 dpf after treatment with the *s1pr4* MO versus uninjected controls, and the numbers of neutrophils around the wound area at intervals up to five hours post injury were counted to quantify neutrophil recruitment and resolution in response to the injury. The overall number of neutrophils recruited to the wound was higher and took place faster in embryos treated with *s1pr4* MO; however, after initial recruitment a trend for higher reverse migration rate and fewer cells retained at the

site of injury in the *s1pr4* morphants (Supplementary Table 16) suggests that the time course of neutrophil response to injury and/or resolution of inflammation may be altered in the setting of decreased *s1pr4* expression (Figure 5).

Discussion

Using a custom genotyping array with focused coverage of missense and loss-of-function variants in exonic regions, we conducted an analysis of erythrocyte and leukocyte traits in as many as 52,531 individuals of European, African and Hispanic ancestry. We identified and replicated 9 novel genetic loci associated with inter-individual differences in blood cell traits and have extended the role of several common variants previously associated with non-hematologic traits to erythrocyte or WBC phenotypes. Of these new findings, we identified a novel association between a rare missense variant in *SIPR4* and WBC and neutrophil counts, and confirmed a role for this gene in two model organisms. Our findings highlight the importance of genes involved in erythrocyte membrane composition and leukocyte trafficking in the regulation of peripheral erythrocyte and WBC phenotypes.

The rare missense variant in *SIPR4* (p.Arg365Leu/rs3746072; MAF_{meta} 0.006) was robustly associated with total WBC count and neutrophil count. *SIPR4* belongs to a family of G-coupled protein receptors for spingosine-1-phosphate (S1P), a lysophospholipid which functions as an extracellular signaling molecule with diverse biologic functions, including leukocyte trafficking.³⁸ Another S1P receptor subtype, *SIPR1*, plays an important role in regulating immune cell function and lymphocyte trafficking by regulating egress of lymphocytes from bone marrow and lymphoid tissues;^{39–41} however, much less is known about the function of *SIPR4*. *SIPR4* is expressed on hematopoietic and lymphoid cells and has been implicated in terminal megakaryocyte differentiation to platelets,⁴² and the regulation of dendritic cell function and T(H)17-cell⁴³ and plasmacytoid dendritic cell⁴⁴ differentiation. *SIPR4* is highly expressed in neutrophils and lymphocytes. In the setting of combined s1p lyase and *S1pr4* deletion in mice, neutrophilia and inflammation are decreased compared to S1P lyase deficiency alone. This suggests that *SIPR4* may mediate the higher neutrophil count that accompanies highly elevated S1P levels in mice with S1P lyase deficiency.^{35,45}

Here, we confirm in two *in vivo* vertebrate model systems (mouse and zebrafish) that loss of *S1pr4* function leads to lower basal numbers (and proportion) of circulating neutrophils, consistent with the association observed in human p.Arg365Leu carriers. The mild reduction in neutrophil count suggests a hypothesis of abnormal neutrophil trafficking, rather than a critical role in neutrophil development. Bone marrow egress of leukocytes is known to be impaired in the setting of *S1pr1* deficiency.^{39–41} We therefore examined the expression of previously defined key adhesion molecules for leukocyte migration in response to S1P signaling in the bone marrow neutrophils of *S1pr4* null mice, including CD49b, which is abnormally expressed in *S1pr1* and S1P lyase deficient states,³⁶ and CXCR4, which interacts with the cytokine peptide SDF-1 required for cellular bone marrow egress.^{46,47} We did not find any alterations of these adhesion molecules, and we did not observe accumulation of neutrophils in the bone marrow or spleen, which corroborates a lack of effect on egress of neutrophils. Because neutrophil recruitment to injured or infected tissue

is a key process, we evaluated neutrophil CD62L surface expression, which mediates interactions between the neutrophil and endothelium and is required for leukocyte trafficking across the endothelial border.⁴⁸ CD62L neutrophil expression was reduced approximately two-fold in the *S1pr4* null mouse. Since CD62L is shed from the surface of neutrophils upon activation and mediates leukocyte extravasation into tissues, we examined whether tissue neutrophil counts were elevated in the *S1pr4* null mouse, accounting for lower circulating neutrophil counts. However, tissue neutrophil numbers were not increased in the *S1pr4* null mouse compared to the wild-type; rather they were similarly decreased as in the blood.

In the zebrafish *s1pr4* morphants, neutrophil accumulation and resolution at the site of a cutaneous wound occurred earlier than in controls, suggesting impaired cellular inflammation in response to tissue injury. Further experiments to delineate neutrophil-endothelial cell interactions will be needed to delineate further the precise mechanisms by which *S1PR4* impacts circulating neutrophil counts. Together, our observations support the role of *S1PR4* in the regulation of neutrophil counts and potentially clinically relevant impairment in response to injury or infection.

Blood monocyte counts are altered in the setting of chronic inflammatory disease and various infections, both viral and non-viral. *NLRP12* is a member of a sub-group of a non-inflammasome forming NLR family that attenuates inflammation by suppressing NF- κ B signaling in activated monocytes.⁴⁹ *NLRP12* loss-of-function mutations have been identified in families with hereditary periodic fever syndromes⁵⁰. The missense variant reported here, p.Gly39Val, was not reported in these families and is not present in the ClinVar database.⁵¹ In humans and mice, *NLRP12* is highly expressed in bone marrow, and macrophages from *Nlrp12*-deficient mice exhibit decreased chemotaxis in response to chemokines *in vitro* suggesting that *NLRP12* is important for leukocyte cell trafficking.⁵² *IL17RA* is a proinflammatory cytokine with a role in hematopoietic cell maturation, and vascular IL-17RA supports monocyte adherence.^{53,54} Mutations in *IL17RA* are associated with familial candidiasis.⁵⁵

Eosinophil counts are altered in parasitic infection, allergic and autoimmune diseases such as asthma and inflammatory bowel disease. The *IL33* variant rs1342326 has previously been associated with asthma,²⁹ and IL33 activates eosinophils.⁵⁶ The *IRF1* variant (rs12521868), which we show to be associated with *IRF1* expression, has been previously associated with Crohn's disease.⁵⁷ *IRF1* is also near *IL5*, a known regulator of eosinophil production previously associated with eosinophil count.⁵⁸

We observed novel erythrocyte trait associations for common non-coding SNPs representing two genomic regions previously associated with kidney function, near *SHROOM3* and near *CEP89*.^{19,59} For both loci, the allele associated with lower Hb and Hct was associated with higher eGFR,¹⁷ suggesting that these erythrocyte trait associations are not mediated through an effect of renal dysfunction and related decreased erythropoietin production. The *SHROOM3* locus has additionally been associated with serum magnesium levels.¹⁸ The effects of these two loci on erythrocyte, renal, and electrolyte traits may occur instead through cytoskeleton-dependent solute/ion channels shared between kidney epithelia and erythrocyte membranes, as has been demonstrated in other examples such as *PIEZO1*,

another GWAS locus for erythrocyte traits. *PIEZO1* is a RBC membrane mechano-sensitive cation channel that appears to require actin cytoskeleton reorganization^{60,61} and senses mechanical forces associated with fluid flow and/or circumferential stretch in epithelial cells at the basolateral side of renal proximal convoluted tubules.^{62–64} Dominant missense mutations of *PIEZO1* have been reported in patients with hereditary xerocytosis,⁶⁵ a congenital hemolytic anemia characterized by dehydrated, shrunken erythrocytes and the presence of stomatocytes due to increased potassium permeability. By analogy, *SHROOM3* is an actin-binding protein involved in epithelial shape regulation, modulating ion channel activity through myosin II-dependent cytoskeletal re-organization in the kidney.⁶⁶ Its role in erythrocyte function has yet to be tested experimentally. *CEP89* is a ubiquitously expressed and highly conserved gene for which biologic function is not well known. *CEP89* is flanked by *SLC7A9*, a kidney solute transporter. Mutations in *SLC7A9* result in congenital cystinuria.⁶⁷ Our eQTL analyses showed a significant association of the index SNP associated with Hb and Hct and *SLC7A9* transcript levels in multiple tissue types and most strongly in whole blood, supporting a possible hematologic function.

FADS1 and *FADS2* encode the two rate-limiting desaturases in the conversion of dietary essential medium-chain PUFAs (e.g., α -linoleic acid [ALA]), to long-chain PUFAs (arachadonic acid, eicosapentaenoic acid [EPA], docosahexaenoic acid [DHA]). The minor allele of the *FADS2* intronic variant rs1535-G is associated with higher levels of ALA and lower levels of EPA and DHA. This suggests less efficient conversion due to decreased FADS activity,²¹ as well as cholesterol levels and pro-inflammatory eicosanoids.⁶⁸ Here we report that the same *FADS2* allele is associated with higher RBC count, Hb and Hct. rs1535 is in strong LD with other common SNPs in the *FADS1-FADS2* region on chromosome 11q12.2, including several eQTL SNPs for *FADS1*. Our eQTL analysis of this region showed strong associations of these SNPs with *FADS1*, *FADS2* and *FADS3* expression levels. Long-chain PUFAs are incorporated into erythrocyte membrane glycerolipids, affecting erythrocyte membrane fluidity, permeability, and sensitivity to oxidative damage and subsequent hemolysis.⁶⁹ Nonetheless, the association of rs1535 with higher RBC count suggests additional mechanisms. In this regard, rs1535 is also located ~100 kb from *FTH1*, which encodes the heavy subunit of ferritin, the major intracellular iron storage protein which is expressed in both mature erythrocytes and early erythroid precursors.

Pleiotropy (i.e., more than one trait associated with the same locus) was observed for erythrocyte associations at the *CEP89*, *FADS1* and *HFE* loci, and we extended the association of the well-characterized common *HBSL1-MYB* regulatory variant, previously associated with erythrocyte and platelet traits, to WBC count. *MYB* encodes c-Myb, a transcription factor and proto-oncogene expressed in immature hematopoietic cells and leukemic cells that plays an essential role in the regulation of normal hematopoiesis and leukemogenesis.⁷⁰ In addition, we confirmed the previously reported association of the chromosome 12q24 *SH2B3* region with erythrocyte and WBC traits; this locus has been associated with multiple cardiovascular and inflammatory traits and diseases.^{9,71–73}

Our results add to recent observations that rare coding variants contribute to phenotypic differences in complex blood cell traits among community-dwelling individuals unselected for hematologic disorders. Experimental testing of *SIPR4* loss of function *in vivo*,

performed to follow up a *SIPRA* rare missense variant association in our study, showed novel biologic effects on neutrophil count and function. Common variants originally associated with a single blood cell trait through GWAS, such as *SH2B3*, have subsequently been associated with traits related to all 3 blood cell lineages,^{10,74} as well as non-hematologic traits,^{73,75,76} and these pleiotropic effects will be useful to discern patterns suggesting specific biologic hypotheses for further mechanistic hypothesis testing.

Methods (online)

Study Samples

Our discovery sample consisted of exome chip data from 52,531 individuals, including 37,775 European Americans (EA), 11,589 African Americans (AA), and 3,167 Hispanic Americans (HA) sampled from 16 population-based cohorts participating in the CHARGE Consortium⁷⁷: Age, Gene/Environment Susceptibility study (AGES), Atherosclerosis Risk in Communities (ARIC) Study, Cardiovascular Health Study (CHS), Family Heart Study (FamHS), Framingham Heart Study (FHS), Health ABC (HABC), Health2006/2008, the Mount Sinai Institute for Personalized Medicine BioMe Biobank Project (BioMe), Jackson Heart Study (JHS), the Lothian Birth Cohorts 1921/1936 (LBC), Multi-Ethnic Study of Atherosclerosis (MESA), the Rotterdam Study (RS), the Women's Health Initiative (WHI; AAs only), and the Cardiovascular Risk in Young Finns Study (YFS). The replication sample consisted of 17,500 samples from the Women's Health Initiative (WHI; EAs only) and 5,261 Han Chinese individuals from the Peking University – University of Michigan Study of Atherosclerosis (PUUMA). Descriptions of each of the cohorts and the techniques used to measure the hematologic traits are provided in previous publications (Supplementary Note) and summarized in Supplementary Table 1. All participants provided written informed consent as approved by local human-subjects committees.

Erythrocyte and Leukocyte Phenotypes

The hematology traits we studied included hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell (RBC) count, red cell distribution width (RDW), total white blood cell (WBC) count, and counts of the WBC subtypes neutrophils, monocytes, lymphocytes, basophils, and eosinophils, using the transformations defined in Supplementary Table 1. Traits were harmonized across cohorts for the same units of measurement, and within each cohort, traits were transformed according to standard convention (Supplementary Table 1). We Winsorized values greater than three standard deviations of the population mean for each trait in each cohort in order to reduce false positives caused by extreme outliers while still maintaining power to identify a potential signal with strong effect.

Genotyping and quality control

Genotypes were assayed using the Illumina HumanExome Beadchip (Illumina, Inc., San Diego, CA) in accordance with the manufacturer's instructions. Genotype calls were assigned using GenomeStudio v2010.3. Samples were excluded if any of the following applied to them: a call-rate less than 95%, ethnic outlier in a principal components analysis,

evidence of contamination, sex mismatch, or unexpected cryptic relatedness. SNPs were excluded with call-rates less than 95% or if they deviated from Hardy-Weinberg at $p < 5 \times 10^{-6}$. For the SNPs identified by the association analyses, the cluster plots were visually inspected.

Association analysis of single variants and implementation of gene-based tests

Variants were annotated using dbNSFP v2.0.^{78,79} Phenotypes were first transformed (either natural log transform, square root, or none at all, as delineated in Supplementary Table 1 and then Winsorized at 3 standard deviations (mean and standard deviation was computed separately for each cohort and the threshold was computed as $\text{mean} \pm 3$ standard deviations; any individual with a value exceeding this threshold was replaced with this threshold). Age, sex, study (if needed), and principal components were included as covariates in the analyses. The R *skatMeta* (v1.4.2) package was used for all cohort-level analyses. Each study used either the *skatCohort* or the *skatFamCohort* function to create an R object that was then uploaded to a central server.

After performing quality control of the genotypes as described previously,⁸⁰ we analyzed 247,870 SNPs meeting quality control, using single variant association tests and gene-based tests of aggregate variants. For single variant association tests, a minor allele count filter of at least 40 was used for each trait. As a secondary analysis, we lowered this filter to a minor allele count of 10 or greater, to evaluate for any lower frequency alleles with strong effects (Supplementary Note; Supplementary Figure 8). For gene-based testing, only coding variants putatively affecting protein structure (missense, stop-gain, stop-loss, and splice variants) that also had a frequency $< 5\%$ in a given population (~200,000 SNPs) were included.

In parallel with the single-variant association tests, we conducted aggregate variant testing using two methods: the T5 test⁸¹ (MAF < 0.05) and the SKAT test⁸² (MAF < 0.05 , Wu weights). The T5 test identifies those genes where multiple samples have private or rare mutations leading to a strong effect in a single direction. The SKAT test allows for different variants to have effects in different directions. In both tests, only those variants with a possible effect on amino acid sequence (missense, stop-gain, stop-loss, and splice variants) were included in the analysis.

Meta-analysis of single variant and gene-based tests

Single variant and gene-based association statistics were combined in a fixed-effects, inverse-variance weighted meta-analysis and performed in parallel at two different sites using the same *skatMeta* package. Analyses were stratified first by ancestry and then combined in a trans-ethnic analysis using the same methodology. Results for single variant analyses were reported only when 40 or more minor allele counts were observed, and a Bonferroni correction for the number of tests was employed to determine significance. For gene-based tests, two different methods were employed. The first was the Combined Multivariate and Collapsing (CMC) approach,⁸¹ where the number of qualifying variants in each gene were added together for each individual separately and then used as the predictor in a linear regression model. To be included, a variant had to have an average allele

frequency less than 5% across all cohorts and also change the amino acid sequence of an mRNA, either as a missense, stop-gain, stop-loss, frameshift or splice site variant. The second method was the SKAT method⁸² and used the same set of variants as the CMC/T5 approach. Only those genes with a minor allele count greater than 40 were analyzed, and a Bonferroni correction for the number of genes tested was employed to determine significance. The number of individuals with each of the hematologic traits under study differed, and consequently the number of markers reaching our minor allele count threshold of 40 varied by trait. We therefore applied trait-specific p-value thresholds, according to the number of variants available for the individuals with each trait (Supplementary Table 5).

Independent replication analysis

We conducted follow-up replication analysis in 18,018 independent EA samples from the Women's Health Initiative (WHI) and 5,261 Han Chinese individuals from the Shijingshan district of Beijing that participated in the Peking University – University of Michigan Study of Atherosclerosis (PUUMA) (Supplementary Note). Both studies were genotyped using an Illumina HumanExome BeadChip genotyping array and had erythrocyte and WBC traits available.¹⁶ All novel, significant ($p < \text{trait-specific Bonferroni threshold}$) variant associations from the discovery results were tested in the replication analysis. Gene-based test results that were significant in the discovery analyses were tested in the replication samples, with the exception of *HFE* and *PIGM/DARC* since these loci have previously well-defined, known signals and were also seen in the single variant analyses. In the case where an association was identified in the discovery analysis with an erythrocyte trait other than Hb or Hct, we analyzed the association with Hb and Hct in the replication analysis. Similarly, in the case where a leukocyte subtype association was found in the discovery analyses, we analyzed those variants' association with total WBC in the replication analysis. We applied a Bonferroni correction to the number of replication tests we conducted for the single variant analyses ($p\text{-value} = 0.05 / 19 = 0.003$) and for the gene-based tests ($p\text{-value} = 0.05 / 10 = 0.005$).

Expression quantitative trait loci (eQTL) analysis—We identified proxy SNPs in high linkage disequilibrium (LD; $r^2 > 0.8$) with associated index SNPs in 3 HapMap builds and 1000 Genomes with SNAP⁸³. SNP rsIDs were searched for primary SNPs and LD proxies against a collected database of expression SNP (eSNP) results (Supplementary Note). The collected eSNP results met criteria for statistical thresholds for association with gene transcript levels as described in the original papers.

Mouse experiments—*S1pr4*^{+/-} mice on a C57Bl/6 background (stock number 005799) were obtained from The Jackson Laboratory, Bar Harbor, ME.³⁵ Mice were housed in a clean conventional facility that excluded specific mouse pathogens. All animal procedures were approved by the National Institute of Diabetes and Digestive and Kidney Diseases and were performed in accordance with the National Institutes of Health guidelines. Because neutrophil counts are known to exhibit a high degree of variability within the same mouse and between mice, and by sex,^{84,85} we studied a total of 48 mice. The first 24 mice (6 *S1pr4*^{-/-} females, 6 *S1pr4*^{-/-} males, 6 *S1pr4*^{+/+} females, and 6 *S1pr4*^{+/+} males) were all littermates (“Experiment 1” in Supplementary Table 14). In a second set of confirmatory

experiments, 12 *S1pr4*^{-/-} mice were compared to 12 C57BL6 controls (Jackson Labs), again with equal proportions of males and females in each genotype group (“Experiment 2” in Supplementary Table 14). Mice were genotyped by multiplex PCR from tail snips using the set of primers and conditions as previously described.³⁵ Mice were analyzed between 2 and 4 months after birth.

Total bone marrow cells were isolated from mice by flushing the femur and tibia from both legs two times with 1 ml of PBS. To obtain total leukocytes, spleen was dissected and mechanically disaggregated. Single-cell suspensions were obtained using a 40- μ m cell strainer. Blood samples were obtained by cardiac puncture. Erythrocytes were removed by ammonium chloride lysis. Absolute blood cell counts were determined by flow cytometry using CALTAG counting beads (Life Technology, Grand Island, NY), and % neutrophils of the total leukocyte pool were calculated and analyzed to account for any possible pipetting error. Neutrophils were analyzed by flow cytometry as previously described.³⁵ All antibodies were purchased from BD Bioscience, San Jose, CA and were used in 1/50 dilutions. Briefly, cells were diluted in 1% BSA-PBS and incubated with anti-Fc γ R antibody (catalog # 553141 clone 2.4G2) followed by the antibodies anti-mouse Gr-1 (allophycocyanin [APC]-conjugated) (catalog # 553129 clone RB6-8C5) and anti-mouse CD11b (phycoerythrin [PE]-conjugated) (catalog # 553311 clone m1/70). Cells were also incubated with anti-mouse CD62L (catalog # 553150 clone MEL-14), CD49d (catalog # 553156 clone R1-2) and CXCR4 (catalog # 551967 clone 2B11/CXCR4) (all three antibodies were fluorescein-conjugated). After cells were labeled for 30 minutes on ice, and fixed in 1% paraformaldehyde in PBS, then subjected to flow cytometry on a FACScalibur (BD Bioscience). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR). Neutrophils were identified as Gr-1+ CD11b+ cells, and monocytes were identified as Gr-1- CD11b+ cells.

Zebrafish experiments—Zebrafish ortholog *s1pr4* was identified by sequence homology searches and gene synteny analysis, and MO design also incorporated information about gene structure and translational initiation sites (Gene-Tool Inc., Philomath, OR). Two separate MO's were designed against *s1pr4*, which is a single exon gene, in the ATG region to inhibit its mRNA translation (see Supplementary Table 15) MOs were injected at multiple doses into one-cell stage embryos of the *mpx1-gfp* zebrafish line to find the optimal dose, 2 ng/embryo, and the number of *gfp*-expressing cells was imaged under a spinning-disk confocal microscope and counted at 2 days post fertilization. Experiments were conducted in >10 each of control and morphant embryos. The day 2 cutaneous injury was created 2 days after MO injection by nicking the tail fin, and the number of *gfp*+ cells at the site of the cutaneous wound was counted at 30 minutes, and 1, 2, 3, 4, 5, 6, and 8 hours post injury. Paired, one-tailed t-tests were computed for the comparison groups, and linear regression analysis of neutrophil numbers at the cutaneous wound in the time series was performed. Experiments were done in replicates of at least 10 embryos by a technician and analysis was checked by a postdoctoral fellow blinded to MO injection status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Exome chip analysis identified loci associated with RBC and WBC traits that were replicated in an independent sample
- Systematic assessment of coding variation identified candidate causal genes
- A low frequency *SIPR4* missense variant was robustly associated with neutrophil counts
- Loss-of-function experiments *in vivo* in murine and zebrafish models confirmed *SIPR4* function in maintaining circulating neutrophil counts, consistent with the effect observed in humans
- *SIPR4* appears to play a role in recruitment and resolution of neutrophils in response to tissue injury

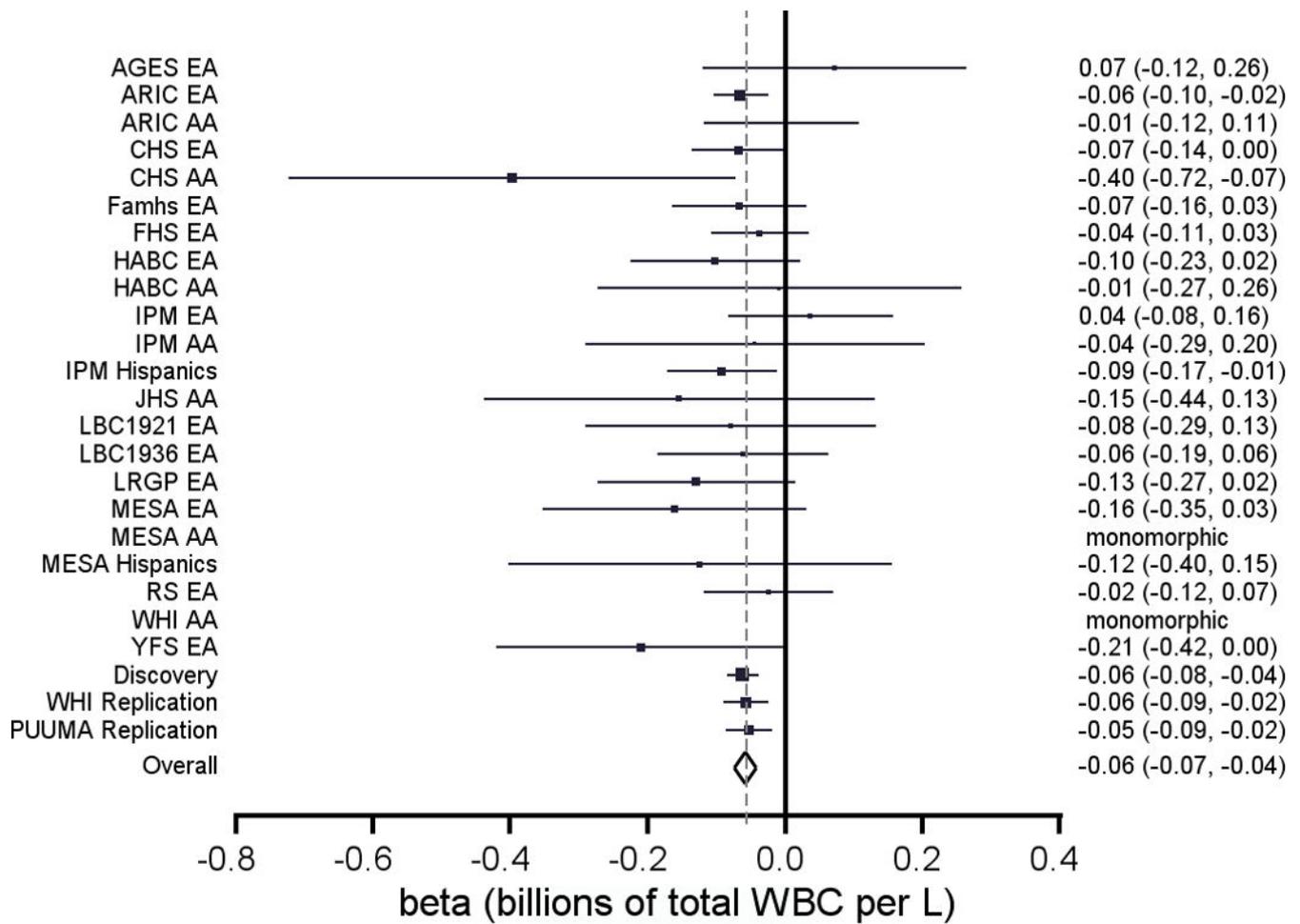


Figure 1. Forest plot of *SIPR4*p.Arg365Leu for neutrophil count and total WBCs. Betas and 95% confidence intervals for each contributing study and for each meta-analysis

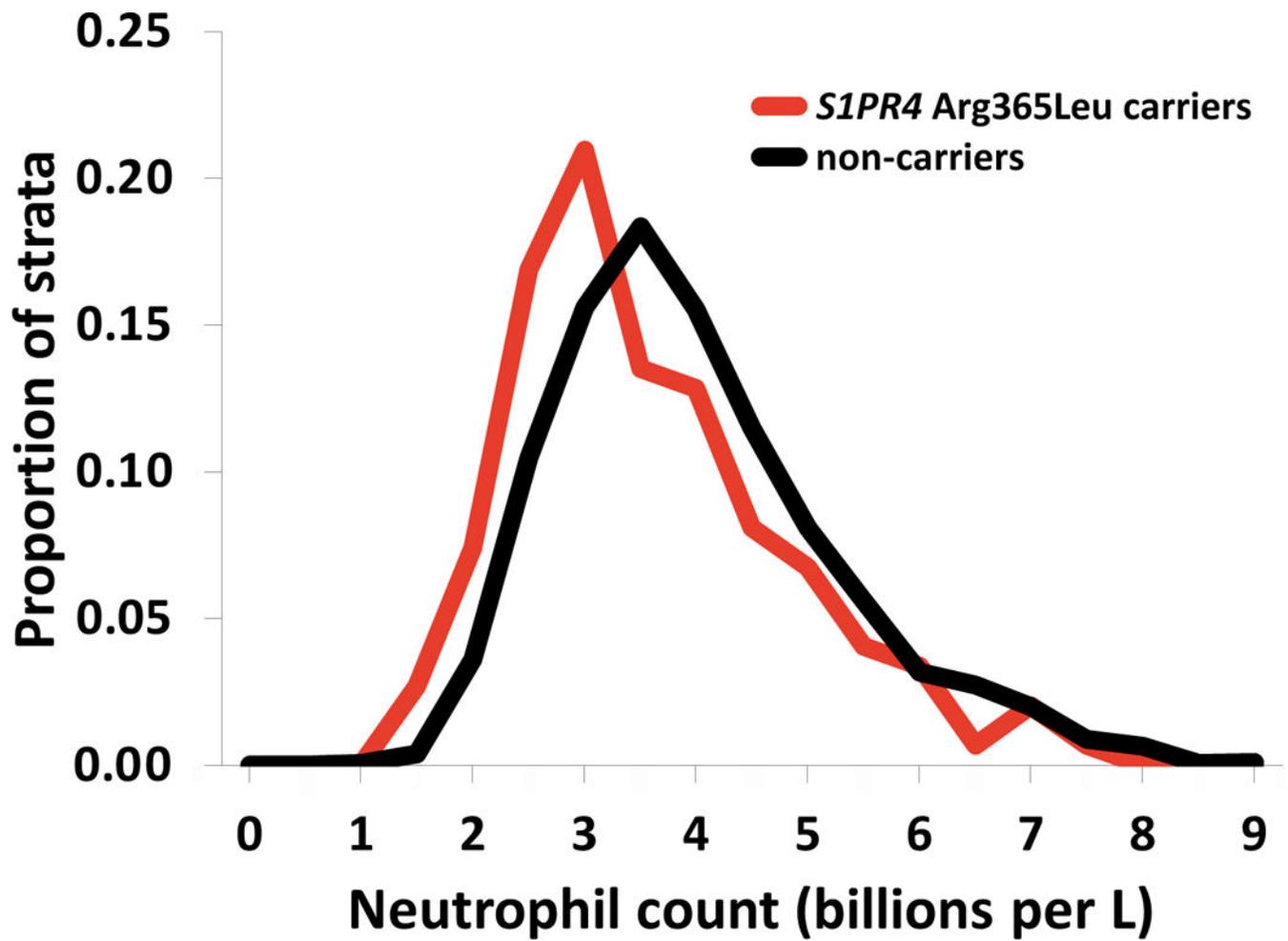


Figure 2. Distributions of neutrophil counts for carriers and non-carriers of *S1PR4* p.Arg365Leu in ARIC.

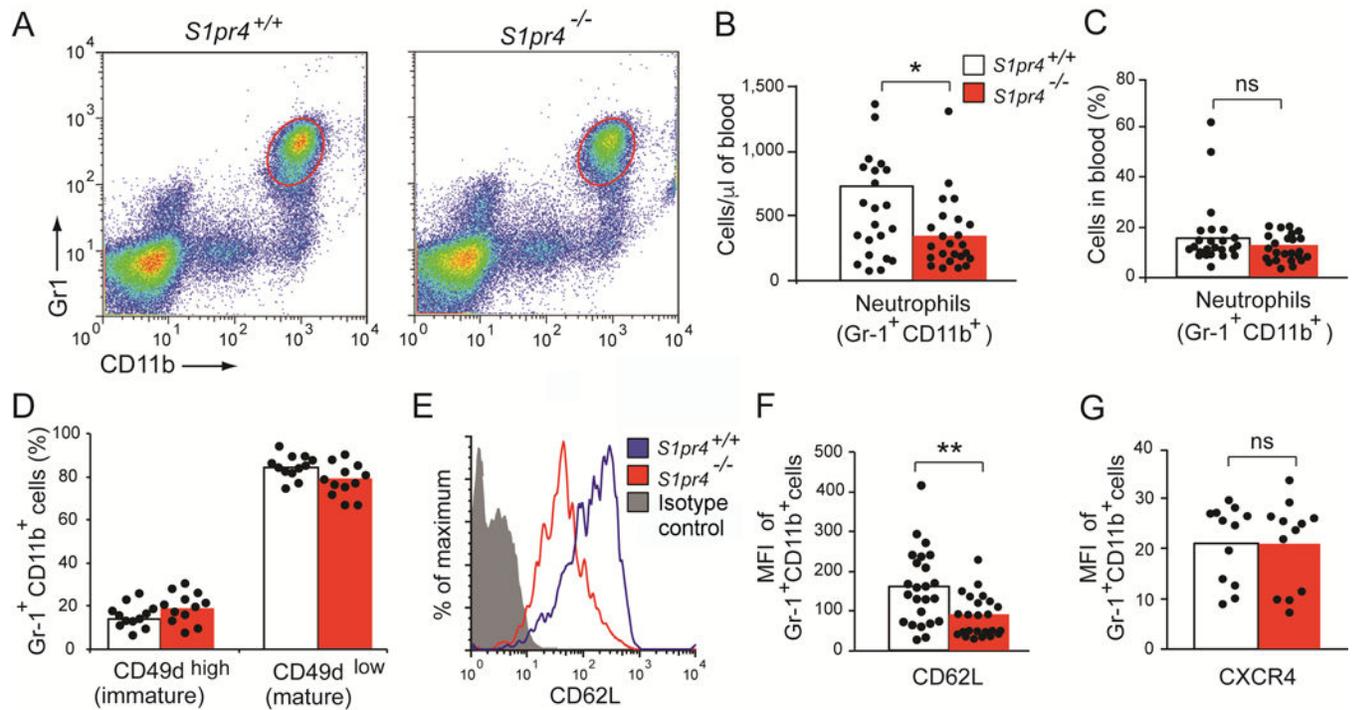


Figure 3. Blood neutrophils in *S1pr4*^{-/-} mice

(A–C) Neutrophil numbers. Blood cells from 2–4 month–old *S1pr4*^{+/+} (n=24) and *S1pr4*^{-/-} (n=24) mice were stained with anti–Gr–1 and anti–CD11b antibodies and analyzed by flow cytometry. Neutrophils were identified as Gr–1^{high} CD11b⁺. Results are shown as density plots (A), as absolute numbers per μ l of blood (B) and as the percentage of cells analyzed (C). (D–G) Adhesion molecule expression on blood neutrophils. Blood neutrophils from *S1pr4*^{+/+} and *S1pr4*^{-/-} mice were analyzed by flow cytometry for the expression of CD49d (D), CD62L (E, F) and CXCR4 (G). Expression of CD49 is shown as percentage of Gr1⁺ CD11b⁺ CD49^{high} (immature neutrophils) and Gr1⁺ CD11b⁺ CD49^{low} (mature neutrophils) (D). Expression of CD62L (F) and CXCR4 (G) on Gr1⁺ CD11b⁺ cells are shown as mean fluorescence intensity (MFI). Representative histogram analysis showing the CD62L expression for *S1pr4*^{+/+} neutrophils (blue line), *S1pr4*^{-/-} neutrophils (red line) and the corresponding isotype control staining (green line) (E). The bars represent mean values, and the closed circles are individual mice. *S1pr4*^{+/+} (open bars) and *S1pr4*^{-/-} (red bars). Student's *t* test **p* < 0.05; ***p* < 0.01; ns, not significant.

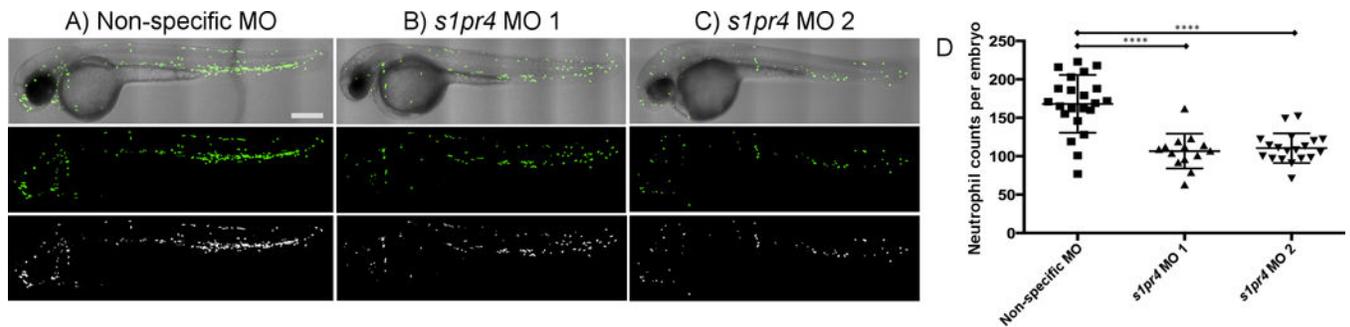


Figure 4. Reduction in neutrophil counts in zebrafish embryos with decreased *s1pr4* expression by morpholino knock-down with two independent morpholino oligonucleotides

Representative images of zebrafish *mpx-gfp* fish are shown, demonstrating decreases in neutrophil number in *s1pr4* morphants at 2 dpf. (A-C) The top set of panels are composite images of differential interference contrast (DIC), the middle panels are images using fluorescence (green channel), and the bottom panels are black and white images of the fluorescent signal of the same embryo injected at 2 dpf with either (A) non-specific MO, (B) 2 ng/embryo morpholino 1, or (C) 2 ng/embryo morpholino 2; D) distribution of average numbers of neutrophils across *s1pr4* MO 1 (n=14), *s1pr4* MO 2 (n=19) and non-specific MO (n=22). ****Student t-test p-value < 0.0001. Scale bar represents 300 μ m and is the same for all panels.

Table 1 Novel RBC (a) and WBC (b) discovered associations in the discovery samples, with replication results

(a) Trait	rsID	Chr	Position	Gene	Function	EA+AA+HA beta	EA+AA+HA p-value	EA / AA / HA -log10(p)	EA / AA / HA MAFs	WHI EA Beta	WHI EA p-value
(a) Red Blood Cell traits											
Hct	rs587404	1	39,908,506	<i>MACF1</i>	p.Ala6577Thr	-0.107	2.9×10⁻⁷	4.6 / 2.5 / 0.3	0.298 / 0.462 / 0.349	0.009	0.79
MCV	rs2229032	3	142,178,144	<i>ATP</i>	p.Arg2425Gln	-0.003	1.1×10⁻⁷	6.4 / 0.1 / 1.5	0.160 / 0.077 / 0.110	0.000	0.98
Hct	rs13146355	4	77,412,140	<i>SHROOM3</i>	intronic	0.105	4.1×10⁻⁷	4.2 / 2.0 / 2.6	0.444 / 0.150 / 0.317	0.144	4.7×10⁻⁶
MCV	rs853678	6	28,297,313	<i>ZSCAN31</i>	p.Thr50Ser	-0.003	8.1×10⁻⁸	8.3 / 0.6 / 0.3	0.134 / 0.351 / 0.220	0.001	0.81
MCH	rs4909444	8	139,701,209	<i>COL22A1</i>	p.Ala938Asp	-0.003	2.7×10⁻⁷	5.0 / 2.0 / 0.9	0.328 / 0.265 / 0.310	0.002	0.22
RBC	rs1535	11	61,597,972	<i>FADS2</i>	intronic	0.020	3.1×10⁻⁹	7.3 / 0.8 / 1.3	0.344 / 0.149 / 0.381	0.028	0.02
MCV	rs2748427	17	76,121,864	<i>TMC6</i>	p.Trp125Arg	-0.002	1.6×10⁻⁵	6.9 / 2.0 / 1.4	0.210 / 0.468 / 0.291	-0.001	0.96
Hb	rs4805834	19	33,453,659	<i>CEP89</i>	intronic	-0.059	2.2×10⁻⁸	6.1 / 2.6 / 0.7	0.145 / 0.025 / 0.080	-0.052	4.0×10⁻⁴
RDW	rs7412	19	45,412,079	<i>APOE</i>	p.Arg202Cys	0.009	5.6×10⁻⁸	6.1 / 1.7 / -	0.068 / 0.111 / -	0.012	0.003
(b) White Blood Cell traits											
Monocytes	rs34856868	1	92,554,283	<i>BTBD8</i>	p.Val60Ile	0.079	1.2×10⁻¹²	10.7 / 1.0 / 1.0	0.029 / 0.005 / 0.015	0.118	1.2×10⁻⁵
Total WBC	rs147630966	3	158,970,516	<i>IQCJ</i>	p.Asn25Lys	0.082	1.8×10⁻⁷	3.4 / 5.5 / 0.1	0.0004 / 0.013 / 0.006	0.122	0.35
Total WBC	rs116085696	4	119,745,814	<i>SEC24D</i>	p.Gly70Val	0.095	2.4×10⁻⁷	- / 6.7 / 0.2	- / 0.011 / 0.003	-	-
Eosinophils	rs12521868	5	131,784,393	<i>IRF1</i>	intronic	-0.008	7.5×10⁻⁸	8.3 / 1.0 / 1.2	0.415 / 0.090 / 0.280	-0.009	0.03
Total WBC	rs7776054	6	135,418,916	<i>MYB/HBS1L</i>	intergenic	-0.010	6.1×10⁻⁷	7.1 / 0.1 / 0.2	0.262 / 0.217 / 0.195	-0.011	1.0×10⁻⁴
Eosinophils	rs1342326	9	6,190,076	<i>IL33</i>	intergenic	0.009	1.5×10⁻⁷	8.2 / 0.4 / 0.3	0.164 / 0.349 / 0.206	-0.009	0.10
Lymphocytes	rs3744064	17	75,211,208	<i>SEC14L1</i>	UTR3	0.004	0.49	0.3 / 0.3 / 8.1	0.030 / 0.006 / 0.026	-0.024	0.44
Total WBC	rs3746072	19	3,179,884	<i>S1PR4</i>	p.Arg365Leu	-0.062	1.5×10⁻⁷	5.4 / 0.6 / 1.7	0.006 / 0.003 / 0.010	-0.056	0.001
Monocytes	rs34436714	19	54,327,313	<i>NLRP12</i>	p.Gly39Val	-0.022	1.0×10⁻⁷	5.9 / 0.5 / 1.7	0.217 / 0.370 / 0.263	-0.026	0.02
Monocytes	rs149771513	22	17,588,658	<i>IL17RA</i>	p.Asp363Asn	0.284	3.2×10⁻⁸	6.7 / 1.4 / 0.3	0.001 / 0.0002 / 0.0002	-0.073	0.82

Bold indicates a significant association in either the discovery ($p < 5 \times 10^{-7}$) or replication samples ($p < 0.003$); *Italics* indicates nominal significance ($p < 0.05$)

Table 2

Top results for gene-based tests in the discovery and replication samples

Red Blood Cell Traits		T5Count p-value				SKATwu5 p-value				Replication p-value*					
Trait	Gene	EA+AA+HA	EA	AA	HA	EA+AA+HA	EA	AA	HA	EA+AA+HA	EA	AA	HA	WHI EA Hb	WHI EA Hct
Hb	<i>EPO</i>	0.0024	9.9E-04	0.72	0.90	1.0E-08	2.3E-08	0.17	0.30	0.0001	6.4E-05				
Hb, Hct, MCH, MCHC, MCV	<i>HFE</i>	4.7E-31	0.18	0.08	0.89	2.2E-23	0.06	0.03	0.92	N/A	N/A				N/A
Hb, Hct, MCH, MCV, RBC	<i>G6PD</i>	2.6E-19	0.02	0.32	0.07	1.6E-19	0.08	0.25	0.07	N/A	N/A				N/A
MCH	<i>NLR3</i>	0.0028	0.38	0.44	4E-04	0.06	0.77	0.87	2.5E-07	0.96					0.96
MCH, MCHC, MCV, RBC	<i>ITFG3</i>	1.3E-04	0.95	9.9E-17	0.49	5.2E-26	0.85	1.1E-40	8.0E-04	0.095					0.94
MCHC	<i>ANK1</i>	5.7E-04	2.5E-06	0.39	0.56	1.3E-10	1.1E-09	0.48	0.42	0.70					0.92
MCV	<i>HBS1L</i>	0.01	0.001	0.62	0.68	6.1E-07	1.5E-06	0.44	0.94	0.84					0.71

White Blood Cell Traits		T5Count p-value				SKATwu5 p-value				Replication p-value				
Trait	Gene	EA+AA+HA	EA	AA	HA	EA+AA+HA	EA	AA	HA	EA+AA+HA	EA	AA	HA	WHI EA trait
Monocytes	<i>BTBD8</i>	1.1E-06	8.3E-06	0.26	0.06	6.6E-12	1.5E-10	0.3	0.16					5.0E-6
Monocytes	<i>IL17RA</i>	0.24	0.22	0.81	0.5	6.4E-07	1.4E-07	0.77	0.08	0.004				
Total WBC	<i>IQCI</i>	2.7E-05	5.6E-04	8.0E-05	0.16	1.8E-07	5.1E-04	3.5E-06	0.39	0.67				
Total WBC, Neutrophils	(<i>DARC</i> locus)	0.47	0.14	3.6E-08	0.59	0.36	0.23	1.2E-07	0.86	N/A				
Total WBC, Neutrophils	<i>SIPR4</i>	4.8E-06	4.5E-05	0.33	0.05	1.8E-07	5.1E-06	0.26	0.02	3.9E-05				

* Multiple associations in DARC, HFE and G6PD with Hb and Hct which were previously known and also seen in the single variant analyses were not evaluated.