Genetic Influence on Frequencies of Myeloid-Derived Cell Subpopulations in Mouse

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Differences in frequencies of blood cell subpopulations were reported to influence the course of infections, atopic and autoimmune diseases, and cancer. We have discovered a unique mouse strain B10.O20 containing extremely high frequency of myeloid-derived cells (MDC) in spleen. B10.O20 carries 3.6% of genes of the strain O20 on the C57BL/10 genetic background. It contains much higher frequency of CD11b+Gr1+ cells in spleen than both its parents. B10.O20 carries O20-derived segments on chromosomes 1, 15, 17, and 18. Their linkage with frequencies of blood cell subpopulations in spleen was tested in F₂ hybrids between B10.O20 and C57BL/10. We found 3 novel loci controlling MDC frequencies: Mydc1, 2, and 3 on chromosomes 1, 15, and 17, respectively, and a locus controlling relative spleen weight (Rsw1) that co-localizes with Mydc3 and also influences proportion of white and red pulp in spleen. Mydc1 controls numbers of CD11b+Gr1+ cells. Interaction of Mydc2 and Mydc3 regulates frequency of CD11b+Gr1+ cells and neutrophils (Gr1+Siglec-F- cells from CD11b+ cells). Interestingly, Mydc3/Rsw1 is orthologous with human segment 6q21 that was shown previously to determine counts of white blood cells. Bioinformatics analysis of genomic sequence of the chromosomal segments bearing these loci revealed polymorphisms between O20 and C57BL/10 that change RNA stability and genes’ functions, and we examined expression of relevant genes. This identified potential candidate genes Smap1, Vps52, Tnxb, and Rab44. Definition of genetic control of MDC can help to personalize therapy of diseases influenced by these cells.

Keywords: myeloid-derived cells, genetic control, CD11b+Gr1+ subpopulation, neutrophils, relative spleen weight, spleen architecture, candidate gene
INTRODUCTION

Disruption of the normal hematological phenotypes is directly related to multiple diseases (1). Hematological traits have been associated with an increased risk for a number of clinical disorders such as cancer, autoimmune diseases, and total mortality (2). White blood cell (WBC) numbers are partly under genetic control, with heritability approximately 40%–60% (3, 4). Peripheral WBC levels vary among ethnic groups, with neutrophil number levels higher in European Americans than in African Americans (2, 5) due to mutation in Duffy antigen/chemokine receptor (DARC) gene (6). Different strains of mice exhibited different numbers of WBC (7–9), indicating that the resting state WBC counts are under genetic control. Hence, it is essential to identify genes controlling the elements of homeostasis of normal human and animal immune systems, including the relative frequencies of WBC subsets (10).

Genome-wide association studies (GWAS) identified the quantitative trait loci (QTL) controlling the homeostasis of WBC classes in human (11, 12) and mice (8, 13) (Table 1). However, identifying the genes underlying these variations remains challenging, as most detected QTLs are either non-coding regions or in linkage disequilibrium with many other variants (24).

We examined the WBC subpopulations in spleens of the strain C57BL/10-H2^ff (B10.O20), a H2 semi-congenic strain on the C57BL/10 (B10) background carrying the O20/A (O20)-derived H2^ff haplotype (25). The strain B10.O20 inherited from O20 also an additional 3.6% of its genome. Surprisingly, the myeloid-derived cell (MDC) frequencies in spleens of B10.O20 exceeded those of its two parental strains. To map the genes controlling these differences, we analyzed F2 hybrids between strains B10.O20 and B10, identified three loci on chromosomes 1, 15, and 17 and a suggestive linkage on chromosome 18 controlling MDC frequencies and relative spleen weight, and described potential candidate genes.

MATERIALS AND METHODS

Mice

Female mice of strains O20 (n = 10), B10.O20 (n = 11), and B10 (n = 9) and F2 hybrids between B10.O20 and B10 (B10xB10.O20 [n = 78] and B10.O20xB10 [n = 190]) in two independent experiments were tested. Unequal numbers of mice in different crosses were due to difficulties in breeding of the cross B10xB10.O20. Mice were produced and housed in SPF conditions at the animal facility of the Institute of Molecular Genetics of the Czech Academy of Sciences and were, on average, 12 weeks old (median 12 weeks, min 8 weeks, max 18 weeks). Mice were killed by cervical dislocation and spleens were divided into four equal quarters for further analysis. The first part was used for immunophenotyping, the second for morphological analysis, and the third for expression analysis. The fourth part was kept as a reserve. All experiments were approved by the Ethical Committee of the Institute of Molecular Genetics of the Czech Academy of Sciences.

Relative Spleen Weight

Spleen and total body weights were determined using the balance Adventurer-Pro (OHAUS Corporation, Pine Brook, NJ USA; Made in Switzerland), resolution d = 0.01 g. The relative spleen weight was calculated as spleen-to-body weight ratio × 1000.

Immunophenotyping

One spleen quarter was homogenized in phosphate-buffered saline (PBS) using disposable pestles. Single-cell suspensions were washed in PBS containing 0.5% bovine serum albumin and incubated for 30 min on ice with the anti-mouse mAb against CD11b, CD14, F4/80, CD40, Gr1, CD3, CD4, CD8, and CD19; for details, see Supplementary Table 1. All samples were incubated with Pacific Blue-labeled anti-TER-119 to exclude erythroid cells. Dead cells were stained with Hoechst 33258 (Invitrogen). Fifty thousand events were acquired on a LSRII cytometer (BD Biosciences) and analyzed using FlowJo 9.9.3 (BD Biosciences).

Genotyping of F2 Mice

DNA was isolated from tails using standard protease K procedure. B10.O20 strain differs from B10 at O20-derived regions on four chromosomes. These differential regions were typed using 5 microsatellite markers (D17Mit197, D17Mit21, D17Mit10, D17Mit66, and D18Mit24) and 2 SNP sites: chromosome 1, rs23555388 and chromosome 15, rs78065633 (Generi Biotech, Czech Republic). DNA was amplified by PCR as previously described (26). We detected the presence of allele-specific SNP sites on chromosomes 1 and 15 by digesting the amplicons with the restriction enzymes MwoI (New England BioLabs, Inc.) and AluI (Thermo Fisher Scientific, Inc.), respectively.

RNA Isolation and RT-PCR Analysis

RNA was prepared by lysing a quarter of spleen stored at −80°C in TRI reagent (Sigma Aldrich). One microgram of RNA was treated with DNase (Promega, M6101) and then reverse transcribed and amplified as previously described (27) in a total volume of 10 µl. In detail, 1 µg of RNA was treated with DNase (Promega, M6101) and then reverse transcribed using 100 units of M-MLV Reverse Transcriptase (Sigma, M1302) with 1xMLV reverse transcriptase buffer, 1.4 µM of random hexamers (Thermo Fisher, N8080127), 2.5 units of ribonuclease inhibitor (Thermo Fisher, 15518012), and 5 mM of each dNTP (Sigma, DNTPI00) per sample to obtain cDNA. cDNA was then diluted five times and 3 µl was used for amplification by 45 cycles of PCR (3 min denaturation at 95°C, 15 s denaturation at 95°C and 60 s annealing/extension at 60°C with
| Trait | QTL, marker or position | Species | Summary |
|-------|-------------------------|---------|---------|
| **Eosinophils proportion in circulating blood** | 2q33 (D2S117–D2S434) | Human | The study was performed in 12-, 14-, and 16-year-old Australian twins in order to identify candidate genes involved with asthma pathophysiology (14). |
| **Eosinophil proportion in circulating blood** | 5q31-33 (D5S500–D5S658) | Human | The study was performed in families where both parents are non-Hispanic white (15). |
| **Total WBC count** | 1q23 (DARC), 4q13 (CXCL2), 7q21 (CDX6), 17q21 (PSMD3-CSF3); 1q23 (DARC) | Human | The study performed meta-analysis of data of 16,388 African-American participants in 7 cohort studies. Some of these results were replicated in three other ethnic groups (Hispanic Americans, Japanese and European Americans) (16) |
| **Neutrophil and monocyte count** | 6p21.33, 17q21.1; 17q21.1; 6p21.33, 19p13.11; 2q31.3, 3q21.3, 8q24.21, 9q31.3; | Human | The study performed meta-analysis of data of 19,509 participants in 7 cohort studies and 11,823 participants in 10 replication cohorts (17). |
| **WBC count** | Chr7,92246306, Chr17,35410238; | Human | The study performed a large-scale GWAS of 14,792 Japanese participants in the BioBank Japan Project. Some of these results were replicated in the cohorts of Caucasian populations (18). |
| **WBC count** | Chr2,219099484, Chr2,113841030, Chr4,74977837, Chr6,31247203, Chr6,135426673, Chr7,92408370, Chr8,130597585, Chr14,24573639; | Human | The study performed meta-analysis of data from Japanese, African-American, and European-American cohorts. The study replicated 10 previously known loci [including the loci identified by Reiner et al., 2011 (16)] and identified six new loci (19). |
| **WBC count** | Chr2,182031910, Chr6,31329647, Chr8,130641292, Chr14,24573639; | Human | The study performed a large-scale GWAS of 14,792 Japanese participants in the BioBank Japan Project. Some of these results were replicated in the cohorts of Caucasian populations (18). |
| **WBC count** | Chr1,159062436, Chr2,219099484, Chr3,129799125, Chr11,88615085, Chr21,38774421; | Human | The study performed meta-analysis of data from Japanese, African-American, and European-American cohorts. The study replicated 10 previously known loci [including the loci identified by Reiner et al., 2011 (16)] and identified six new loci (19). |
| **Monocyte count** | Chr1,203942866, Chr3,129799125, Chr11,88615085, Chr21,38774421; | Human | The study performed meta-analysis of data from Japanese, African-American, and European-American cohorts. The study replicated 10 previously known loci [including the loci identified by Reiner et al., 2011 (16)] and identified six new loci (19). |
| **WBC count** | Chr1,159062436, Chr2,219099484, Chr3,129799125, Chr11,88615085, Chr21,38774421; | Human | The study performed meta-analysis of data from Japanese, African-American, and European-American cohorts. The study replicated 10 previously known loci [including the loci identified by Reiner et al., 2011 (16)] and identified six new loci (19). |
| **Counts of Baseline WBC** | Wbcq1 (D1Mit282), Wbcq2 (D3Mit142), Wbcq3 (D15Mit13), Wbcq4 (D1Mit306), Wbcq5 (D1Mit227), Wbcq6 (D1Mit98) | Mouse | Analysis was performed in whole blood from intercrosses between mouse strains NZW/LacJ, SM/J, and C57BLKS/J (9). |
| **WBC** | Chr1,126971726, Chr6,135927582, Chr8,8119195, Chr11,63825134, Chr12,79259640, Chr15,99555171, | Mouse | Analysis was performed on 100 inbred strains of the Hybrid mouse diversity panel by GWAS (8). |
### TABLE 1 | Continued

| Trait                  | QTL, marker or position | Species | Summary                                                                 |
|------------------------|-------------------------|---------|-------------------------------------------------------------------------|
| WBC counts             | Chr16,15916062, Chr18,70410404; SSc6 (DIAS000496); SSc2 (DIAS0001270) | Swine   | The study was performed on 843 Italian large white pigs by three GWAS scan approaches (single-trait, multi-trait, and Bayesian) analyzing 30 blood parameters (21, 22). |
| Lymphocyte counts      | SSc4 (MARC0052177); SSc3 (H3GA0009277, H3GA001692); SSc7 (H3GA0021970, INRA0028736), SSc10 (H3GA0030197); SSc14 (ALGA0079529, MARC0090899); SSc15 (ALGA0084320) | Swine   | The study analyzed the QTL associated with leucocytes and platelet related traits in F2 of White Duroc X Erhualian pigs (23). |
| Neutrophil counts      | SSc760cM, SSc12(32cM), SSc15(87cM); SSc759cM, SSc12(98cM), SSc15(97cM), SSc762cM; | Swine   |                                                                        |
| Basophils counts       |                                                                        |         |                                                                        |
| Monocyte counts        |                                                                        |         |                                                                        |
| Baseline levels of WBC |                                                                        |         |                                                                        |
| Lymphocytes            |                                                                        |         |                                                                        |
| Neutrophils            |                                                                        |         |                                                                        |

**CDK6:** cyclin-dependent kinase 6; **Chr.:** chromosome; **CSF3:** colony stimulating factor 3 (granulocyte); **CXCL2:** chemokine (C-X-C motif) ligand 2; **PSMD3:** proteasome (prosome, macropain) 26S subunit, non-ATPase, 3; **SSC:** Sus scrofa (ssc; swine); **Wbcq:** white blood cells QTL

A single fluorescence acquisition point repeated 45 times, and a melt curve program of 55°C to 95°C with 0.5°C increment (continuous fluorescence acquisition) using primers for the genes of interest and iQ SYBR Green Supermix (Bio-RAD, 1708882) for quantification. Primers (Supplementary Table 2) were designed by QuanTrimpr (28) and purchased from Generi Biotech, Czech Republic. GAPDH is used as an internal control. Reactions were performed in a 384-well plate in LC480II light cycler (Roche Molecular Systems, Inc.) The average Ct values (cycle threshold) were used for quantification, and the relative expression was calculated [ratio (reference/target) = 2^(Ct(reference)−Ct(target))].

#### Morphological Analysis of the Spleen

Another spleen quarter was processed for histology overnight using an automated vacuum tissue processor (Leica ASP200S) and embedded in paraffin using Leica EG1150H. Three-micrometer serial sections were prepared (Leica RM2255), stained with hematoxylin and eosin, and observed under light microscope Leica DM6000 at 10× magnification using the software LAS X, 64 bit. The brightness and contrast of the pictures were then adjusted using FIJI (29). The area of the white pulps was measured using the ellipse formula a*b*π where “a” is the major radius and “b” is the minor radius of the white pulp. The recorded area of one sample represents the average area of ten white pulps measured three times each. Selected samples were from mice 14 weeks old in average (median 14 weeks, min 13 weeks, max 16 weeks).

#### Detection of Polymorphisms That Change RNA Stability and Genes’ Functions

We have sequenced the genomes of strains C57BL/10 and O20 using next-generation sequencing (NGS) system HiSeq 2500 (Illumina) (12× coverage). Processing, alignment, sorting and indexing of NGS data, variants filtration, annotation, and effect prediction were performed as described elsewhere (30). In detail, NGS data were preprocessed using software Trimmomatic (31) and overlapping pair reads were joined by software Flash (32). Alignment-reference mouse sequence mm10 (build GRChm38) was performed using BWA (Burrows-Wheeler Aligner) program (33). Mapped reads were sorted and indexed, and duplicated reads were marked. Local realignment around indels, base recalibration, and variants filtration were performed using software GATK (the Genome Analysis Toolkit) (34). IGV (Integrated Genome Viewer) (35) was used for visualization of results. Variant annotation and effect prediction was performed by software Snpeff (36). Protein variation effect predictions were performed by software PROVEAN (Protein Variation Effect Analyzer) (37). Analysis of conservation scores was performed using ConSurf software (38–40).

#### Statistical Analysis

Differences between parental strains and between mice within parental strains were analyzed by Mann–Whitney test using the program Statistica for Windows 12.0 (StatSoft, Inc., Tulsa, Oklahoma, USA).

In F2 hybrids, variance components and mixed-model ANOVA of Statistica with genotype (marker) and grandparent-of-origin effect as fixed factors and age as a covariate were used to evaluate the role of genetic factors controlling the frequency of cell subpopulations and the relative spleen weight. When necessary for analysis by ANOVA, the original values of an analyzed parameter were transformed for normalization of the distribution as described in the legends to the tables. Markers and interactions with p < 0.05 were combined in a single comparison. All obtained nominal p-values were corrected for multiple testing by Bonferroni correction.

ANOVA or t-tests (as indicated) were used in GraphPad (version 5.04) to evaluate the effect of genetic factors controlling the expression level of potential candidate genes and size of the white pulp.
RESULTS

Combination of Genomes of Two Parental Strains Gives Rise to a Strain Exceeding Hematological Parameters of Both of Them

We analyzed frequencies of the main myeloid and lymphoid subpopulations in spleens of mice of the parental strains B10 and O20, and of recombinant strain B10.O20. For characterization of myeloid cell population, we examined markers F4/80, CD11b, and CD14 to characterize macrophage lineage, co-expression of CD11b and Gr1 to characterize granulocytes, and CD40 as a marker leukocyte with antigen-presenting function. For the characterization of T-cell lineage, we used a marker CD3 and markers CD4 and CD8 to distinguish the main CD3 subpopulations. Since CD4 molecule can be also expressed by other small cell populations, we also examined the presence of CD3^+CD4^+ (helper T cells) and CD3^+CD8^- (cytotoxic T cells) subpopulations. To characterize cells of the B-cell lineage, we used B-cell marker CD19, which distinguishes B-cell lineage from T cells (41). Representative dot plots of myeloid and lymphoid cell subpopulations are shown in Supplementary Figures 1, 2, respectively. Frequencies of CD11b^+, CD11b^+Gr1^+, CD14^+, and F4/80^+ cells in B10.O20 mice were about double their frequency in the parental strains B10 and O20, while the frequencies of CD3^+, CD4^+, CD8^-, and CD3^+CD8^- were significantly lower. Also, frequency of CD11b^+, CD14^+, and CD19^- cells differed between the strains B10 and O20. Levels of CD3^-CD4^- cells in the strain B10.O20 differed significantly from the strain O20. The frequency of CD19^- cells was similar in both B10.O20 and O20, but lower than in the strain B10. Levels of CD40^- cells were not significantly different in the three strains (Figure 1).

Loci Controlling Differences in MDC Frequencies

Subsequently we used F2 hybrids between B10 and B10.O20 in order to map the genes controlling the frequencies of immune cell subsets in the strain B10.O20 and the relative spleen weight. We measured the frequencies of CD11b^+, CD11b^+Gr1^+, CD11b^+Ly6C^-, CD11b^+Ly6G^-, CD11b^+Siglec-F^-, CD19^- and CD40^- cells, and the levels of Gr1^-Siglec-F^- cells from CD11b^+ cells (hereafter noted as neutrophils) and eosinophils (Gr1^-Siglec-F^- cells from CD11b^- cells) by flow cytometry. We genotyped the O20-derived segments in F2 mice to detect the

![FIGURE 1](image-url)
loci linked with cell subpopulation frequencies and analyzed the results by one-way ANOVA. Figure 2 and Table 2 summarize the loci controlling several phenotypes observed in the strain B10.O20.

**Figure 2** | Positions of the loci that control relative spleen weight and frequencies of myeloid-derived subpopulations in spleen of the strain B10.O20. The regions of C57BL/10 and O20 are represented as black and white, respectively; the boundary regions of undetermined origins are shaded. The identified loci Mydc1-3 and Rsw1 and potential candidate genes are indicated. Only the markers or SNPs defining the boundaries of O20-derived segments and markers that were tested for linkage are shown (except syntenic D17Mit10). Bold in box—significant linkage, regular font in box—suggestive linkage.

**Table 2** | Summary of loci controlling spleen cell subsets in B10.O20.

| Phenotype | Locus | Chr. | Marker | Cross | p | Bonf. corr. p |
|-----------|-------|------|--------|-------|---|---------------|
| CD11b+Gr1+ | Mydc1 | 1 | rs23555388 | B10×B10.O20 | 0.005 | 0.039 |
| Relative spleen weight | Rsw1 | 17 | D17Mit197 | Both crosses | 0.00002 | 0.0001 |
| CD11b+Gr1+ | Mydc2 *Mydc3 | 15*17 | rs78065633 *D17Mit197 | B10×B10.O20 | 0.001 | 0.008 |
| Neutrophils (Gr1+Siglec-F- cells from CD11b+ cells) | Mydc2 *Mydc3 | 15*17 | rs78065633 *D17Mit197 | B10×B10.O20 | 0.006 | 0.048 |
| Neutrophils* | # | 18 | D18Mit24 | B10.O20×B10 | 0.009 | 0.063 |

*interaction between loci; *suggestive linkage.

**Table 3** | Loci controlling frequencies of myeloid-derived spleen cells and relative spleen weight in F2 hybrids between B10.O20 and B10.

| Phenotype | Locus | Cross | Marker | Genotype | p | Bonf. corr. p |
|-----------|-------|-------|--------|----------|---|---------------|
| CD11b+Gr1+ | Mydc1 | Both | rs23555388 (chr.1) | 2.16 | 1.17 ± 0.02 | 2.72 | 1.22 ± 0.02 | 2.91 | 1.24 ± 0.02 | NS | NS |
| | | B10×B10.O20 | 2.42 | 1.19 ± 0.04 | 4.16 | 1.33 ± 0.03 | 4.18 | 1.33 ± 0.03 | 0.005 | 0.039 |
| | | B10.O20×B10 | 1.75 | 1.12 ± 0.04 | 1.93 | 1.14 ± 0.02 | 2.41 | 1.19 ± 0.03 | NS | NS |
| Relative spleen weight | Rsw1 | Both | D17Mit197 | 4.85 | 1.27 ± 0.01 | 4.45 | 1.25 ± 0.00 | 4.01 | 1.23 ± 0.00 | 0.00002 | 0.0001 |
| | | Both | D17Mit21 | 4.71 | 1.26 ± 0.01 | 4.50 | 1.25 ± 0.00 | 4.00 | 1.23 ± 0.00 | 0.00006 | 0.0004 |
| Neutrophils (Gr1+Siglec-F- cells from CD11b+ cells) | NN | Both | D18Mit24 | 18.66 | 4.32 ± 0.12 | 17.11 | 4.14 ± 0.08 | 19.74 | 4.44 ± 0.12 | NS | NS |
| | | B10×B10.O20 | 19.51 | 4.42 ± 0.21 | 22.36 | 4.73 ± 0.14 | 21.58 | 4.65 ± 0.22 | NS | NS |
| | | B10.O20×B10 | 16.79 | 4.10 ± 0.13 | 13.90 | 3.73 ± 0.09 | 17.22 | 4.15 ± 0.14 | 0.009 | 0.063 |

Means, standard error of mean (SEM) and p-values were calculated by analysis of variance (ANOVA). In order to obtain normal distribution required for ANOVA, the following transformations were used: CD11b+Gr1+ (% in spleen homogenates) - power of 5; % of Gr1+Siglec-F- cells from CD11b+ cells – power of 2; relative spleen weight ([spleen weight/body weight] × 1000) - power 1/0.15. Transformed means ± SEM are shown next to average non-transformed mean values in bold. Only p-values significant or suggestive after Bonferroni correction are given. O and B indicate the presence of O20 and B10 allele, respectively. NS—Not significant. NN—not named.

Loci Mydc1 (Myeloid-derived cells 1) on chromosome 1, Rsw1 (Relative spleen weight 1) on chromosome 17, and the suggestive locus on chromosome 18 exhibit a single gene effect (Table 3 and Figures 3, 4). Locus Mydc1 linked with rs23555388.
influences the frequency of the CD11b+Gr1+ subpopulation (Bonferroni corr. $p = 0.039$). Homozygotes in O20 allele (OO) exhibit higher numbers of CD11b+Gr1+ cells in spleen. The effect of this locus was observed only in the cross between B10 females and B10.O20 males, but no significant interaction between cross and SNP marker was observed. Although linkage for the cross B10.O20xB10 was not significant, phenotypes were concordant with the B10xB10.O20 cross, with OO genotype being the

![Figure 3](image1.png)

**FIGURE 3** | Genetic influence on frequency of (A) CD11b+Gr1+ cells, (B) relative spleen weight, and (C) neutrophils. Individual F2 hybrid mice between strain B10.O20 and B10 are shown. Means ± standard error mean (red lines) and $p$-values were calculated by analysis of variance (ANOVA). O and B indicate the presence of O20 and B10 allele, respectively. NS, Not significant.

![Figure 4](image2.png)

**FIGURE 4** | Genetic influence on frequency of CD11b+Gr1+ cells. Flow cytometry analysis of spleens of representative mice with BB, OB, and OO genotypes showing the Gr1/CD11b cell surface marker status of individual cells. O and B indicate the presence of O20 and B10 allele, respectively.
TABLE 4 | Interaction between Mydc2 and Mydc3 controls the levels of CD11b+Gr1+ cells (A) and neutrophils (Gr1+Siglec-F- cells from CD11b+ cells) (B) in spleen.

A.

| Marker   | Cross   | Genotype | Chr.15 - rs78065633 - Mydc2 | p    | Bonf. corr. p |
|----------|---------|----------|-----------------------------|------|---------------|
|          |         |          | BB                          | OB   | OO            |
| D17Mit197 | Both    | BB       | 2.78 ± 0.04 (n = 16)        | 2.99 ± 0.03 (n = 28) | 3.60 ± 0.06 (n = 9) | NS  | NS            |
| Mydc3    | OB      | BB       | 2.73 ± 0.03 (n = 31)        | 2.32 ± 0.02 (n = 75) | 2.48 ± 0.04 (n = 23) | 0.001 | 0.008        |
|          | OO      | BB       | 1.79 ± 0.03 (n = 27)        | 2.70 ± 0.03 (n = 37) | 3.37 ± 0.06 (n = 9) | NS  | NS            |
|          | B10 x   | BB       | 3.02 ± 0.09 (n = 03)        | 4.84 ± 0.05 (n = 07) | 4.22 ± 0.06 (n = 9) | 0.001 | 0.008        |
| B10.O20  | OB      | BB       | 3.62 ± 0.04 (n = 11)        | 3.23 ± 0.03 (n = 17) | 2.28 ± 0.04 (n = 9) | NS  | NS            |
|          | OO      | BB       | 2.26 ± 0.04 (n = 14)        | 2.38 ± 0.07 (n = 07) | 7.86 ± 0.08 (n = 03) | NS  | NS            |
|          | B10.O20 | BB       | 2.06 ± 0.05 (n = 13)        | 2.23 ± 0.04 (n = 21) | 2.47 ± 0.09 (n = 04) | NS  | NS            |
|          | B10 x   | BB       | 2.00 ± 0.04 (n = 20)        | 1.75 ± 0.02 (n = 58) | 2.26 ± 0.05 (n = 14) | NS  | NS            |
| B10.O20  | OB      | BB       | 1.40 ± 0.05 (n = 13)        | 2.32 ± 0.03 (n = 30) | 2.00 ± 0.07 (n = 06) | NS  | NS            |

B.

| Marker   | Cross   | Genotype | Chr.15 - rs78065633 - Mydc2 | p    | Bonf. corr. p |
|----------|---------|----------|-----------------------------|------|---------------|
|          |         |          | BB                          | OB   | OO            |
| D17Mit197 | Both    | BB       | 19.37 ± 0.21 (n = 16)       | 19.50 ± 0.16 (n = 28) | 21.55 ± 0.28 (n = 9) | NS  | NS            |
| Mydc3    | OB      | BB       | 18.66 ± 0.15 (n = 31)       | 17.35 ± 0.10 (n = 75) | 17.96 ± 0.18 (n = 23) | NS  | NS            |
|          | OO      | BB       | 16.13 ± 0.16 (n = 27)       | 18.24 ± 0.14 (n = 37) | 20.52 ± 0.28 (n = 9) | NS  | NS            |
|          | B10 x   | BB       | 21.80 ± 0.48 (n = 03)       | 26.16 ± 0.31 (n = 07) | 25.72 ± 0.36 (n = 05) | 0.006 | 0.048        |
| B10.O20  | OB      | BB       | 23.50 ± 0.25 (n = 17)       | 20.50 ± 0.21 (n = 17) | 17.01 ± 0.27 (n = 09) | NS  | NS            |
|          | OO      | BB       | 17.72 ± 0.22 (n = 14)       | 19.90 ± 0.33 (n = 07) | 35.35 ± 0.46 (n = 03) | NS  | NS            |
|          | B10.O20 | BB       | 16.29 ± 0.23 (n = 13)       | 15.68 ± 0.18 (n = 21) | 16.80 ± 0.41 (n = 04) | NS  | NS            |
|          | B10 x   | BB       | 15.03 ± 0.18 (n = 20)       | 14.53 ± 0.11 (n = 58) | 17.32 ± 0.22 (n = 14) | NS  | NS            |
| B10.O20  | OB      | BB       | 14.50 ± 0.22 (n = 13)       | 16.14 ± 0.15 (n = 30) | 14.10 ± 0.33 (n = 06) | NS  | NS            |

Second row and column indicate the genotype of the corresponding locus. In order to obtain normal distribution required for ANOVA, the following transformations were used: CD11b+Gr1+ (% in spleen homogenates) - power of 5; % of Gr1+Siglec-F- cells from CD11b+ cells – power of 2. Transformed means ± SEM are shown next to average non-transformed mean values in bold. n indicates the number of mice.

highest and BB genotype being the lowest. Locus Rsw1 is linked to the markers D17Mit197 (Bonferroni corr. \( p = 0.0001 \)) and D17Mit21 (Bonferroni corr. \( p = 0.0004 \)). Mice homozygous in B10 (BB) allele of this locus show higher relative spleen weight. We have detected a suggestive linkage of neutrophil subpopulation with marker D18Mit24 (Bonferroni corr. \( p = 0.063 \)). Heterozygotes in this locus had lower frequency of this subpopulation. The effect of this locus was observed only in the cross between B10.O20 females and B10 males, but no significant interaction between cross and genetic marker was observed.

Interaction between locus Mydc2 linked to rs78065633 on chromosome 15 and locus Mydc3 linked to D17Mit197 on chromosome 17 controls both frequency of CD11b+Gr1+ cells (Table 4A and Figures 5A, 6A) and Gr1+Siglec-F- subpopulation from CD11b+ cells (Table 4B and Figures 5B and 6B) in spleen. In both interactions, higher levels of tested subpopulations were present in OO homozygotes in both Mydc2 and Mydc3. The linkages were detected only in the cross B10xB10.O20, but the interactions between cross and marker were not significant (nominal \( p \)-value = 0.15 and 0.11, respectively).
Locus Rsw1 on Chromosome 17 Influences Relative Spleen Weight as Well as Spleen Architecture

To investigate the effect of Rsw1 locus on spleen architecture, we compared hematoxylin-eosin-stained spleen sections of F2 hybrids between B10 and B10.O20 with the different alleles on Rsw1 (Figure 7). Interestingly, B10 homozygotes had twice larger white pulps than mice homozygous for O20 alleles (Figure 8). This observation correlates with the results in Figure 1, where frequency of lymphocytes (white pulp residents) is lower and frequency of granulocyte subsets (red pulp residents) is higher in the strain B10.O20, carrying O20 allele in Rsw1.

Potential Candidate Genes

In order to identify potential candidate genes controlling the phenotypes listed in Table 2, we sequenced the strains B10 and O20 using NGS and identified the genetic variants between B10 and O20 in the O20-derived region of B10.O20. Then, we used a range of Bioinformatics tools to predict the effects of the detected variants on the structure and function of proteins and on RNA stability (Table 5). All but one (in gene Gtpbp1) structural differences from the C57BL/6 standard strain were of O20 origin. This analysis revealed two potential candidate genes on chromosome 15, 29 genes on chromosome 17, and four genes on chromosome 18 (Table 5); no polymorphisms affecting gene functions were found on chromosome 1. We chose Foxred2 in Mydc2 (chromosome 15); Rab44, Vps52, Tnxb, Pta2g7, Ptx4, Ephx3, Lst1, H2-M5, Olfr113, and Trem2 in Mydc3/Rsw1 (chromosome 17); and Gm4841, F830016B08Rik, Alpk2 and Megf10 on chromosome 18 for RNA expression studies. Selection of these genes for testing was based on the importance of the variation (we prioritized frameshift, nonsense mutation, and variants of highly conserved residues) in the corresponding loci. Samples of different genotypes were randomly selected based on their age. The differentially expressed genes (Vps52, Tnxb, Rab44, and Gm4841) are shown in Figure 9. The expression of the remaining genes was either undetectable (Ptx4, Ephx3, H2-M5, F830016B08Rik, and Megf10) or expressed without significant difference between the tested groups (Foxred2, Lst1, Pla2g7, Olfr113, Alpk2 and Trem2) (Supplementary Figure 3).

Smap1 Is a Potential Candidate Gene for Mydc1

Since the bioinformatics analysis of deleterious variants did not identify any candidate gene in the locus Mydc1, which is directly associated with the levels of CD11b^Gr1^ cells in spleen, we searched the Mouse Genome Informatics (42) for phenotypic function of the 8 genes (4933415F23Rik, Mir30a, Mir30c-2, Ogfrl1, B3gat2, Smap1, Sdhaf4, and Col9a1) located in Mydc1 (Supplementary Table 3). Smap1 (small ArfGAP [ADP-ribosylation factor GTPase activating protein]) is the gene involved in both the hematopoietic and the immune systems (43, 44); other genes with potential influence on MDC frequencies are Mir30a (microRNA 30a) (45), Mir30c-2 (microRNA 30c-2) (46), Ogfrl1 (opioid growth factor receptor-like 1) (47), and Col9a1 (collagen, type IX, alpha 1) (48) (Supplementary Tables 3, 4). These five genes were tested for differential expression. Only Smap1 showed significant differences among mice with different genotypes. O20 (OO) homozygotes, which control higher frequency of CD11b^Gr1^, exhibited higher expression of Smap1 RNA than both B10 (BB) homozygotes and heterozygotes (Figure 9A). We observed a tendency toward differential expression in Ogfrl1 (Supplementary Figure 3A), but
FIGURE 6 | Genetic interactions influencing frequency of myeloid-derived cells. (A) Flow cytometry analysis of spleens of representative mice carrying combination of BB (Mydc2) and OO (Mydc3) genotypes, and OO homozygotes in both Mydc2 and Mydc3 showing the Gr1+/CD11b cell surface marker status of individual cells. (B) Spleen cell gating strategy for analysis of genetic influence on neutrophil frequency. O and B indicate the presence of O20 and B10 allele, respectively.

FIGURE 7 | Spleen histology. Light micrographs of the hematoxylin and eosin-stained paraffin-embedded spleen sections of F2 hybrids between B10.O20 and B10. Indicated alleles in bold correspond to the genotype of Rsw1 (linked with D17Mit21). White pulps regions are indicated by yellow arrows. Bars represent 100 μm. Original magnification ×10.
these differences were not significant, and no differential expression was found among genotypes of Mir30a, Mir30c-2i, and Col9a1 (Supplementary Figures 3B–D).

**No Potential Candidate Gene Detected in Mydc2**

In the linkage analysis, the effect of Mydc2 (chromosome 15) was observed only in interaction with Mydc3 (chromosome 17). Thus, we compared expression of Foxred2 (FAD-dependent oxidoreductase domain containing 2) in the combination of OO homozygotes in both Mydc2 and Mydc3 with BB homozygotes in Mydc2 and OO homozygotes in Mydc3. Although there was a tendency toward differential expression, these differences were not significant (Supplementary Figure 3E).

**Vps52, Tnxb, and Rab44 Are Potential Candidate Genes for Mydc3/Rsw1**

Locus on chromosome 17 is involved in control of relative spleen weight (Rsw1) and frequencies of CD11b’Gr1’ cells and neutrophils (Mydc3). Rsw1 exhibits the main (single) gene effect, whereas the influence of Mydc3 is observed only in interaction with Mydc2. Three genes—Vps52 (Vacuolar protein sorting-associated protein 52 homolog), Tnxb (tenascin XB), and Rab44 (RAB44, member RAS oncogene family)—exhibited differential expression characteristic for a single gene effect. The O20 allele of Vps52 carries a non-sense mutation that results in a loss of 24 functional and 17 structural residues (Table 5). O20 homozygotes (OO) as well as heterozygotes (OB) of Vps52 have approximately 1.6-fold lower expression than B10 (BB) homozygotes (Figure 9B). The O20 variant of TNXB includes a deletion of a highly conserved functional serine (S45del) with two single amino acid changes of three other residues (R245H, P1681L, and G1899R) (Table 5). O20 homozygotes exhibited higher Tnxb RNA expression than both heterozygotes and B10 homozygotes (Figure 9C). The O20 allele of Rab44 carries a deleterious variant of a highly conserved functional residue (G275R); glycine in B10 is in O20 replaced by arginine (Table 5). The relative expression level of Rab44 was partly similar to Vps52. Highest level of Rab44 mRNA was observed in B10 (BB) homozygotes, while O20 (OO) homozygotes and heterozygotes exhibited almost no expression (Figure 9D). Rab44 also exhibited differential expression in interaction between Mydc2 and Mydc3. OO homozygotes in both Mydc2 and Mydc3 had higher expression of Rab44 than the combination of BB homozygotes in Mydc2 with OO homozygotes in Mydc3 (Figure 9E). There were tendencies toward differential expression of genes Lst1, Vps52 (p = 0.073), and Tnxb in interaction between Mydc2 and Mydc3, but these differences were not significant (Supplementary Figures 3F–H).

**Gm4841 Is a Potential Candidate Gene for a Suggestive Linkage of Neutrophil Frequency on Chromosome 18**

A suggestive linkage on chromosome 18 might influence neutrophil frequency (Table 3). We analyzed the RNA expression of the potential candidate genes on chr.18; only Gm4841 (predicted gene 4841; interferon-gamma-inducible GTPase Ifgga3 protein) was differentially expressed, and B10 allele determined low RNA levels (Figure 9F). O20 allele of Gm4841 differs from B10 allele in 8 single amino acid variants, all intermediate to highly conserved residues including two functional residues, with an insertion of one residue (Table 5).

**DISCUSSION**

**Combination of Genomes of Two Parental Strains Gives Rise to a Strain Exceeding MDC Frequencies of Both of Them**

Frequencies of several spleen cell subpopulations in B10.O20 differ from both B10 and O20 (Figure 1). Observations of progeny whose phenotype is beyond the range of that of its parents, are frequent in multigenic traits. They were seen in many tests of immune responses of recombinant congenic strains in vitro (49–51) and in vivo (27, 52–54), and in analysis of expression QTLs of chromosome substitution strains (55). These observations reflect multiple regulatory interactions, which, in new combinations of genes, can lead to new quantitative phenotypes that exceed their range in parental strains.

**Novel Genes/Loci Controlling Differences in MDC Frequencies and in Relative Spleen Weight**

Systems genetics allows identification of novel genes and mechanisms controlling complex diseases and phenotypes in a context similar to the natural population, which is also relevant to clinical traits (56). Here, we investigated the role of genetic variants in the control of...
# Table 5: List of candidate genes controlling cell subpopulation frequencies in strain B10.020.

| Chr. | Position | Reference genotype | Genotype O20 | Protein position of AA | Type of change | Conservation score | Gene symbol | Gene name | Transcription status | Gene ID: MGI | Gene ID: NCBI |
|------|----------|-------------------|--------------|------------------------|----------------|-------------------|-------------|-----------|---------------------|-------------|-------------|
| 15   | 77955898 | G/G               | G/G          | T/T                    | Single AA      | 9(F)              | Foxred2     |           |                     |             |             |
| 15   | 79712097 | T/T               | G/T          | T/T                    | Single AA      | 7(-)              | Gp105p1     |           |                     |             |             |
| 17   | 29139862 | G/G               | A/A          | 275                    | Single AA      | 9(F)              | Rab44       |           |                     |             |             |
| 17   | 32923538 | G/G               | C/C          | 454                    | Single AA      | 7(-)              | Cyprf13     |           |                     |             |             |
| 17   | 33065434 | T/T               | G/G          | T/G                    | Single AA      | 7(+)              | Pf8-ps      |           |                     |             |             |
| 17   | 33067588 | A/A               | C/C          | 80                     | Single AA      | 6(-)              | Cytochrome P450, family 4, subfamily f, polypeptide 13 |           |                     |             |             |
| 17   | 33950287 | C/C               | T/T          | 301                    | Nonsense       | 7(24F, 17S)       | Vps52       |           |                     |             |             |
| 17   | 33957014 | GGT/GGT           | G/G          | 206                    | Single AA      | 6(-)              | H2-Eb2      |           |                     |             |             |
| 17   | 34334458 | C/C               | T/T          | 293                    | Single AA      | 3(-)              | Bt rich     |           |                     |             |             |
| 17   | 34472636 | T/T               | A/A          | 477                    | Single AA      | 6(-)              | Bt rich      |           |                     |             |             |
| 17   | 34508126 | A/A               | C/C          | 337                    | Single AA      | 4(-)              | Gtpbp1      |           |                     |             |             |
| 17   | 34515534 | C/C               | G/G          | 85                     | Single AA      | 8(F)              |           |           |                     |             |             |
| 17   | 34671418 | G/G               | A/A          | 245                    | Single AA      | 7(+)              | Tnxb        |           |                     |             |             |
| 17   | 34692397 | C/C               | T/T          | 1681                   | Single AA      | 1(-)              | Tenascin XB |           |                     |             |             |
| 17   | 34954399 | G/G               | A/A          | 1899                   | Single AA      | 8(-)              |           |           |                     |             |             |
| 17   | 34670664 | C/T/C/C           | C/C          | 45                     | Single AA      | 9(F)              | Mipa6b      |           |                     |             |             |
| 17   | 35054357 | G/G               | A/A          | 235                    | Single AA      | 9(-)              | Megakaryocyte and platelet inhibitory receptor G6b |           |                     |             |             |
| 17   | 36127509 | G/G               | T/T          | 64                     | Single AA      | ND*              | Gm19694     |           |                     |             |             |
| 17   | 37589717 | A/A               | T/T          | 212                    | Single AA      | 8(-)              | Olf receptor 114 |           |                     |             |             |
| 17   | 37589887 | C/C               | T/T          | 122                    | Single AA      | 7(-)              | Olfactory receptor 114 |           |                     |             |             |
| 17   | 42666847 | G/G               | A/A          | 535                    | Single AA      | 8(-)              | Adgr4       |           |                     |             |             |
| 17   | 43602848 | A/A               | C/C          | 227                    | Single AA      | 9(F)              | Rla2γ7      |           |                     |             |             |
| 17   | 45601470 | C/C               | T/T          | 92                     | Single AA      | 4(-)              | Mymx        |           |                     |             |             |
| 17   | 47467203 | C/C               | T/T          | 618                    | Single AA      | 2(-)              | A661453     |           |                     |             |             |
| 17   | 21562337 | AAGCA/AAAGCA      | ACTACA/ACTACA| 116                    | Single AA      | 2(-)              | Zfp52       |           |                     |             |             |
| 17   | 21560742 | CATT/CATT         | C/C          | 285                    | Single AA      | 8(-)              | Zinc finger protein 52 |           |                     |             |             |
| 17   | 25125213 | A/A               | A/A          | 449                    | Frameshift     | 9(F)                | Ptx4        |           |                     |             |             |
| 17   | 32189019 | CG/CG             | C/C          | 143                    | Frameshift     | 2(-)              | Epoxide hydrolase 3 |           |                     |             |             |
| Chr. | Position Bp | Reference genotype C57BL/6 | Genotype C57BL/10 | Protein position of AA | Reference AA | Alteration Type of change | Conservation score | Gene symbol | Gene name | Transcription status | Gene ID: MGI | Gene ID: NCBI |
|------|-------------|-----------------------------|------------------|----------------------|--------------|------------------------|------------------|-------------|-----------|----------------------|------------|-------------|
| 17   | 34264933    | CAGGGCCGCAGGCCAGGAT/        | CAGGGCCGCAGGCCAGGAT | TAAGCAGTA/           | 90           | SQFEI                  | KQY              | 4; -; 4; -; 1; -; 4; -; 6; - | H-2-Ab1   | Hotocompatibility 2, class II antigen A, beta 1 | 103070     | 149611      |
| 17   | 3518379     | TC/TC                       | GC/TC            | T/T                  | 4            | Frameshift             | ND(11F, 10S)     | Lst1        | Known     | 1096324             | 169681     |
| 17   | 3520278     | GC/TC                       | GC/TC            | G/G                  | 40           | Frameshift             | ND(43F, 15S)     | H-2-Q1     | Known     | 95928     | 15006       |
| 17   | 3534569     | GA/GA                       | GA/GA            | 359                  |               | Frameshift             | ND(-)            | H-2-Q2     | Known     | 95931     | 15013       |
| 17   | 3616158     | T/T                         | TAGATC/          | T/T                  | 90           | Frameshift             | ND(-)            | 2410017Rk  | RIKEN-cDNA 241001717 | Novel     | 1916967    | 675325     |
| 17   | 3616497     | C/C                         | C/C              | 365                  |               | Frameshift             | ND(-)            | Gm8959     | Predicted gene 8959 | Novel     | 3704134    | 667977     |
| 17   | 3698782     | AG/AG                       | A/A              | 249                  |               | Frameshift             | ND(3F, 7S)       | H-2-M5     | Hotocompatibility 2, M region locus 5 | Known     | 95917     | 240095     |
| 17   | 3735151     | TCTGTG/                     | TCTGTG/          | T/P                  | 90           | Frameshift             | ND(17F, 24S)     | Olf173     | Olfactory receptor 113 | Known     | 2177496    | 258296     |
| 17   | 3841715     | TC/TC                       | TC/TC            | T/T                  | 80           | Frameshift             | ND(0F, 0S)       | Exp36      | Exocrine gland secreted peptide 36 | Putative | 5141873   | 100126765 |
| 17   | 3864468     | GGTG/TGGT/                  | GGTG/TGGT/       | G/G                  | 75           | Frameshift             | ND(43F, 0S)      | Exp37      | Exocrine gland secreted peptide 31 | Known     | 5141981    | 100126768 |
| 17   | 3864472     | T/T                         | TA/TA            | 85                   |               | Frameshift             | ND*              | Known      | Known     | 2147038   | 328833     |
| 18   | 4830800     | CTA/CTA                     | CTA/CTA          | C/C                  | 172          | Frameshift             | ND(12F, 1S)      | Tmem2      | Triggering receptor expressed on myeloid cells-like 2 | Known     | 2147038    | 328833     |
| 18   | 6027068     | G/G                         | G/G              | A/A                  | 318          | R C                    | Single AA         | 6(-)       | Gm484T    | Predicted gene 4841, interferon-gamma-inducible GTPase Ifgga3 protein | Known     | 3643814    | 225594     |
| 18   | 6027559     | A/A                         | A/A              | G/G                  | 154          | I T                    | Change           | 8(-)       | Known     | Known     | 3588218    | 240328     |
| 18   | 6027067     | G/G                         | G/G              | C/C                  | 151          | D E                    | 9(F)             | Known     | Known     | Known     | 3588218    | 240328     |
| 18   | 6027065     | A/A                         | A/A              | T/T                  | 122          | N K                    | 6(-)             | Known     | Known     | Known     | 3588218    | 240328     |
| 18   | 6027068     | G/G                         | G/G              | A/A                  | 118          | P S                    | 8(-)             | Known     | Known     | Known     | 3588218    | 240328     |
| 18   | 6027073     | C/C                         | C/C              | T/T                  | 96           | E K                    | 5(-)             | Known     | Known     | Known     | 3588218    | 240328     |
| 18   | 6027074     | T/T                         | T/T              | C/C                  | 76           | T A                    | 9(F)             | Known     | Known     | Known     | 3588218    | 240328     |
| 18   | 6027081     | G/G                         | G/G              | T/T                  | 69           | T N                    | 6(-)             | Known     | Known     | Known     | 3588218    | 240328     |
| 18   | 6027086     | T/T                         | T/T              | TCCCC/TCCCC          | 50           | G GG                   | Insertion        | 6(-)       | Known     | Known     | 3588218    | 240328     |
| 18   | 6030013     | C/C                         | C/C              | G/G                  | 56           | S C                    | Single AA         | 5(-)       | RIKEN-cDNA RIKEN-cDNA RIKEN-cDNA RIKEN-cDNA | Known     | 3643814    | 225594     |
| 18   | 6030035     | G/G                         | G/G              | A/A                  | 170          | T A                    | Change           | 7(-)       | interferon-gamma-inducible GTPase Ifgga3 protein | Known     | 3588218    | 240328     |
| 18   | 6030067     | C/C                         | C/C              | G/G                  | 275          | Y *                    | Nonsense          | 6(-)       | Alpha-kinase 2 | Known     | 2449492    | 225638     |
| 18   | 6534974     | T/T                         | T/T              | C/C                  | 399          | R G                    | Single AA         | 8(F)       | Alp2       | Known     | 2449492    | 225638     |
| 18   | 57294020    | A/A                         | A/A              | AGC/AGC             | 1134         | Frameshift             | ND(5F)*          | Megf10     | Multiple epidermal growth factor-like domains protein 10 | Known     | 2685177    | 70417      |

The conservation score is inferred from the ConSurf software on July 15, 2019. The conservation score ranging from 1 to 9 is followed in brackets by the type of the residue or the number of altered (F and S) residues (F functional, S structural, neither functional nor structural, *unreliable due to insufficient data). The higher the score, the more conserved the altered residue. ND, not determined. AA, amino acid. Red in the Genotype column marks difference from the reference genotype, red in the Gene symbol column marks differential expression.
frequencies of immune cell subpopulations in spleen of the strain B10.O20. This analysis revealed three loci Mydc1, Mydc2, and Mydc3 that control frequencies of CD11b+Gr1+ and/or neutrophil cell subpopulations, the Rsw1 locus influencing relative spleen weight, and a suggestive linkage to chromosome 18 influencing frequency of neutrophils (Table 2 and Figure 2). We have also detected potential candidate genes Smap1, Rab44, Vps52, Trxnb, and Gm4841. All alterations changing genes’ functions have been detected in genes of O20 origin (Table 5). It is not surprising as the strain B10 (C57BL/10) is more genetically related to the reference strain C57BL6. O20 is an inbred mouse strain of unknown origin. Despite several potentially deleterious mutations described here and retinal degeneration (57), O20 mice are otherwise healthy and are highly resistant to leishmaniasis (27), resistant to breast (58) and small intestinal (59) cancer, and susceptible to lung cancer (60).

We were unable to test genes Ptx4, Ephx3, H2-M5, F830016B08Rik, and Meg10, because their expression was very low or undetectable. These results are in agreement with findings of others (Supplementary Table 5).

Experimental Data and Literature Support Role of Smap1 in Control of Frequency of CD11b+Gr1+ Cells by Mydc1

Mydc1 modifies the frequency of CD11b+Gr1+ cells. Because we did not detect any genetic variants influencing gene function(s) on the chromosomal segment on chromosome 1 comprising Mydc1, we tested the expression of Smap1, which influences the hematopoietic and immune systems (43, 44) (Table 6). It also influences differentiation and migration of polymorphonuclear neutrophils via activation of Arf6 (77). B10 homozygotes that exhibit lower frequency of CD11b+Gr1+ cells than O20 homozygotes (Figure 9A and Table 3) also show lower level of Smap1 expression. As we have detected neither functional polymorphism in Smap1 gene nor extrachromosomal segment interacting with Mydc1, differential expression of Smap1 is likely cis-regulated by genetic element localized outside this gene.

Interaction Between Rab44/Mydc3 With an Unknown Partner in Locus Mydc2 Might Control Frequencies of CD11b+Gr1+ Cells and Neutrophils

The interaction between Mydc2 (chromosome 15) and Mydc3 (chromosome 17) controls frequencies of CD11b+Gr1+ cells and neutrophils (Table 4). Neutrophils are a subgroup of CD11b+Gr1+ cells, and the influence of genotypes on frequencies of both cell subpopulations is similar. Therefore, it is possible that the difference in frequencies of CD11b+Gr1+ cells regulated by interaction between Mydc2 and Mydc3 is due to the difference of its neutrophil subgroup.

In the linkage analysis, the effect of Mydc2 (chromosome 15) was observed only in interaction with Mydc3 (chromosome 17). Bioinformatics analysis pinpointed Foxred2 as a potential candidate gene. However, differences in expression of Foxred2 in mice carrying OO homozygotes in both Mydc2 and Mydc3, and BB homozygotes in Mydc2 and OO homozygotes in Mydc3.
TABLE 6 | List of differentially expressed candidate genes in the strain B10.O20.

| Gene (Name)       | Function                                                                 | Connection with diseases                                                                 |
|-------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|
| **Mydcl**         | Smap1 (small ArfGAP 1)                                                    | Predicted to have GTPase activity, to be involved in cellular proliferation and development (43). ARF6 participates in functions of polymorphonuclear leukocytes (61). |
| Mydcl3            | Rab44 (RAB44, member RAS oncogene family)                                | A large Rab-GTPase that contains a Rab-GTPase domain and some additional N-terminal domains (69). Rab proteins cycle between the cytosol and the membrane of its respective transport compartment and regulate budding, uncoating mobility and fusion of vesicles (71). It plays a role in osteoclast differentiation (71) and granule exocytosis in mast cells (69). |
| **Rsw1**          | Rab44 (RAB44, member RAS oncogene family)                                | See Mydcl3                                                                              |
| Tnxb (tenascin-XB) |                                                                 | Likely exerts tumor-suppressive activities (75). Polymorphism associated with multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, ulcerative colitis, and type 1 diabetes (76). |
| Chr.18            | Gm4841 (Predicted gene 4841)                                             | Predicted to have GTPase activity, to be involved in cellular response to interferon-beta and defense response. Predicted to localize to endoplasmic reticulum membrane (24). |

The function of the genes and their connection with diseases is described.

Differences in this value were associated with differences in spleen architecture, and F2 mice carrying B10 homozygous Rsw1 allele showed about twice larger white pulp than O20 homozygotes (Figures 7, 8). This agrees with the differences among B10, O20, and B10.O20 mice shown in Figure 1, in which MDC residing in the red pulp are increased while lymphocytes residing in the white pulp are decreased in B10.O20 strain. Three genes, Rab44, Vps52 and Tnxb (Table 6), had expression characteristics compatible with a single gene effect (Figure 9).

Rab44 involvement of this gene in MDC cell development is discussed in the Interaction between Rab44/Mydcl3 with an unknown partner in locus Mydcle might control frequencies of CD11b^Gr1^ cells and neutrophils section. Our results suggest a possible role for Rab44 in influencing the splenic architecture of mice by modifying frequencies of MDC cells.

Vps52 is expressed in many cells of the immune system, with highest levels in mega-erythrocyte progenitor (Supplementary Table 6) (78). The role of Vps52 in control of spleen weight and architecture is not clear; it interacts with ARF6 (81) that participates in functions of polymorphonuclear leukocytes (61). This is compatible with our findings.

**Tnxb** effects include hematopoiesis, and immune and hematopoietic systems (42) (Table 6). Targeted mutation

were not significant (Supplementary Figure 3E). Thus, Foxred2 is an unlikely candidate gene, although its protein activity might also be regulated by modifications or structural changes that need not alter expression.

We also tested in mice with the abovementioned combination of genotypes expression of other potential candidate genes on chromosome 17: Lsl1, Vps52, Tnxb (Supplementary Figures 3F-H), and Rab44 (Figure 9E), but only expression of Rab44 exhibited epistatic control. Mouse Rab44 mRNA is highly expressed in bone marrow. It is present in bone marrow macrophages, neutrophils, and dendritic cells (78, 79) (Supplementary Table 6). In spleen, Rab44^+ cells were detected in the splenic cord in the red spleen, but were hardly detectable in the white pulp (79). In bone marrow, Rab44 is extensively expressed in undifferentiated hematopoietic CD117^+ (c-kit) cells and its expression decreases during differentiation of immune-related cells (79). Interestingly, Rab44 is localized in the locus SSC7 that controls WBC count in swine (80).

Rab44, Vps52, and Tnxb Are Potential Candidate Genes for Rsw1 Controlling Relative Spleen Weight and Spleen Architecture

Rsw1 (chromosome 17) controls relative spleen weight, and O20 homozygotes determined higher relative spleen weight (Table 3).
experiments noted an association of Tnxb and Bntl4 (among other 59 genes) with enlarged spleen in uninfected mice (82, 83), which supports our findings. Bntl4 RNA expression was not tested in our samples as the variant in this gene results in a single amino acid change of low conservation score (Table 5).

Exome array-based meta-analysis in a multi-ancestry samples from 25 human studies found that the rs185819 variant of TXNB was associated with WBC count (76). In dairy cattle, TnXB is associated with WBC counts and with the susceptibility to the bovine leukemia virus (84).

Gm4841 Is a Potential Candidate Gene for a Suggestive Linkage of Neutrophil Frequency on Chromosome 18

Heterozygotes on chromosome 18 have lower levels of neutrophils than B10 and O20 homozygotes in the (B10.O20xB10)F2 hybrids. Since the Bonferroni-corrected p-value is 0.063, it only suggests its possible linkage to neutrophil frequency. We analyzed the RNA expression of the candidate genes on chromosome 18; only Gm4841 exhibited differential expression (Figure 9F).

Newly Detected Genes/Loci Have Their Orthologs in Human, Swine, and Cattle

Mydc1 is localized on chromosome 1 on the segment between 22.7 and 24.7 Mbp. In the near vicinity were at the position 26.97, 26.29, and 25.75 Mbp detected loci controlling WBC, granulocytes, and monocytes, respectively, in mouse blood (8). It remains to be tested whether these loci are identical with or distinct from Mydc1.

Orthologous human segments of peak of linkage of Mydc1, Mydc2, and Mydc3/Rsw1 are localized on 6q13, 22q12-13, and 6p21, respectively (42, 85). Interestingly, human segment 6p21 orthologous to Mydc3/Rsw1 was found to control WBC (17, 19), monocyte (19), neutrophil (19), lymphocyte (17), and eosinophil (18) counts (Table 1); swine ortholog SSC7 determines WBC count (23, 80). Rab44 and TnxB—potential candidate genes for Mydc3/Rsw1—are involved in control of WBC count in swine (80) and cattle (84), respectively.

CONCLUSION

In summary, we identified three new loci on chromosomes 1, 15, and 17 (Mydc1, Mydc2, and Mydc3) controlling the frequencies of CD11b^Gr1^- and neutrophils (Gr1^Siglec-F^- cells from CD11b^ cells), and we show how the interaction between the two loci Mydc2 and Mydc3 controls frequencies of these cells. We have also identified Rsw1, a novel locus controlling relative spleen weight and the histological architecture of spleen. Finally, we confirmed in Mydc1 and Mydc3 loci a differential expression of their potential candidate genes Snap1 and Rab44, respectively. Rsw1 contains the three potential candidate genes Vps52, Rab44, and Tnxb. We provide a comprehensive information about the hereditary differences in the frequencies of MDC and the size and the architecture of the spleen white and red pulps. The detected loci might play a role in cancer, autoimmune diseases, and resistance to pathogens.

CD11b^Gr1^- cells comprise several subpopulations of immune cells (86). Part of these cells, a heterogeneous group of myeloid-derived suppressor cells, plays a role in cancer, autoimmune and infectious diseases, traumatic stress, and graft-versus-host disease both in mice and in humans (87, 88). Understanding the genetic regulation of MDC might improve the personalized prevention and therapy of these diseases.

Locus Mydc3/Rsw1 is orthologous to the human segment 6q21 that controls WBC count (17–19). These results could be therefore useful for human studies. It would be interesting whether human segments orthologous to Mydc1 and Mydc2 are also controlling frequencies of MDC.

Thus, these genes can be the focus of future studies in both mice and humans.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data can be found at https://www.ncbi.nlm.nih.gov/genbank/ under the accession numbers OK040659-OK040678.

ETHICS STATEMENT

This research complies with all relevant European Union guidelines for work with animals and was approved by the Institutional Committee of the Institute of Molecular Genetics of the Czech Academy of Sciences and by Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (permission number 93/2015).

AUTHOR CONTRIBUTIONS

IK, YS, VH, and ML designed the project. IK and ML wrote the manuscript. IK, YS, VH, and ML performed the experiments. IK, YS, HH, JV, AA, and VH contributed to the data analysis. All authors contributed to the article and approved the submitted version.

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IK is a Ph.D. candidate at Charles University. This work is submitted as a partial fulfillment of the requirement for the Ph.D.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.760881/full#supplementary-material
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