Hematotoxicity is a life-threatening side effect of many chemotherapy regimens. Although clinical factors influence patient responses, genetic factors may also play an important role. We sought to identify genomic loci that influence chemotherapy-induced hematotoxicity by dosing Diversity Outbred mice with one of three chemotherapy drugs; doxorubicin, cyclophosphamide or docetaxel. We observed that each drug had a distinct effect on both the changes in blood cell subpopulations and the underlying genetic architecture of hematotoxicity. For doxorubicin, we mapped the change in cell counts before and after dosing and found that alleles of ATP-binding cassette B1B (Abcb1b) on chromosome 5 influence all cell populations. For cyclophosphamide and docetaxel, we found that each cell population was influenced by distinct loci, none of which overlapped between drugs. These results suggest that susceptibility to chemotherapy-induced hematotoxicity is influenced by different genes for different chemotherapy drugs.

INTRODUCTION

Forty percent of people in the United States will develop cancer during their lifetimes. The majority of these patients will be treated with repeated intravenous doses of systemic chemotherapy drugs. Many of these drugs cause adverse side effects that can negatively impact treatment. For example, regimens that include doxorubicin (DOX), cyclophosphamide (CYP) and docetaxel (TAX) cause febrile neutropenia (that is, neutropenia with fever) in approximately 30% of patients. Severe neutropenia can lead to treatment delays, increased infection rates, hospitalizations and mortality.

The occurrence and severity of neutropenia is influenced by non-genetic factors, such as age, disease comorbidities, cancer stage and type, as well as by genetic factors. Genetic polymorphisms and the underlying genes that influence chemotherapy-induced toxicity are often interrogated via genome-wide association studies (GWAS), in which many patients receiving the same chemotherapy regimen are genotyped and an association is sought between genotype and the severity of toxicity. A GWAS of chemotherapy-induced neutropenia and leukopenia in a Japanese cohort implicated single-nucleotide polymorphisms (SNPs) on different chromosomes for different drugs, implying that susceptibility to hematotoxicity may be due to distinct genes for different drugs. Results in model organisms support these findings as well. A GWAS of anthracycline-induced cytotoxicity in mouse splenic cells also identified different loci for DOX versus idarubicin toxicity. Another study of chemotherapy-induced toxicity in Drosophila melanogaster also found that the toxicity of each drug was modulated by different loci for each drug. This implies that the toxicity of different drugs may be influenced by different genes and that each drug, or drug class, may have distinct pharmacokinetic and pharmacodynamic mechanisms.

In this study, we sought to identify genomic loci that influence chemotherapy-induced hematotoxicity by mapping genes in Diversity Outbred (DO) mice. DO mice are an outbred stock derived from eight inbred founder strains (A/J, C57BL6/J, 129S1/SvlMj, NOD/ShiLtJ, NZO/HlLtJ, CAST/EiJ, PWK/PhJ and WSB/EiJ) that accumulate recombination events with each outbreeding generation, resulting in fine mapping resolution. DO mice segregate at over 40 million genetic polymorphisms and have balanced allele frequencies, resulting in good power to detect the effects of genetic variants. These properties make the DO an excellent mapping population for the discovery of genes that influence xenobiotic-induced toxicities. Although we did not expect that the genetic polymorphisms in mice would be identical in humans, we expected to identify genes that provide mechanistic insight into the etiology of chemotherapy-induced hematotoxicity and may thereby provide translational relevance.

MATERIALS AND METHODS

Mice housing and feeding

We obtained male and female DO mice, 6–10 weeks of age, from outbreeding generations 3 through 5 (The Jackson Laboratory, Bar Harbor, ME, USA) and allowed them to acclimate for at least one week. The mice were housed in specific pathogen-free conditions in polycarbonate cages with Bed-o-Cob corn cob bedding (The Andersons, Maumee, OH, USA). The females were housed five mice per cage and the males were singly housed. The mice were kept on a 12 h light/dark cycle in rooms maintained at approximately 22 °C ± 4 °C and 50% humidity (±15%). The mice were fed LabDiet 5LL4 (St. Louis, MO, USA) ad libitum and were provided with water acidified to a pH between 2.8 and 3.1. The mice were weighed on the first, third and last day of treatment. All animal procedures were reviewed and approved by The Jackson Laboratory’s Institutional Animal Care and Use Committee (Protocols JW10001 and 13005).
Blood collection
We collected approximately 100 μl of blood from the mice via the retro-orbital vein before dosing. We euthanized the mice by CO₂ asphyxiation and collected post-dose blood via cardiac puncture. We stored the blood in K₂EDTA tubes and analyzed it within 24 h on a Hemavet 950 FS hematology analyzer (Drew Scientific Group, Dallas, TX, USA).

Dosing
We aimed to model the first-cycle response to chemotherapy by dosing mice and measuring blood cell counts at an acute time point near the nadir of neutrophil counts. We performed preliminary dose–response studies in the founder strains and determined the dose and the euthanasia time point for each drug (data not shown). The mice were randomly assigned to a single dosing group in batches of approximately 50 animals (25 females and 25 males). DOX, CYC and TAX were administered via the tail vein (intravenous). We measured body weights daily and monitored mice for signs of clinical distress. Mice that experienced a weight loss of 20% or more were euthanized and were not included in this analysis.

Docorubicin
A total of 396 DO mice (198 females and 198 males) were dosed intravenously with 20 mg kg⁻¹ of a 2 mg ml⁻¹ solution of DOX (Pfizer, New York, NY, USA). We collected post-dose blood five days after dosing. Three mice died during dosing and 14 were removed from mapping analysis due to failed genotyping. We analyzed data for 379 mice (191 females and 188 males) and had power to detect peaks that explain 9.5% of the phenotypic variance.

Doxorubicin and CSF3
A total of 195 DO mice (100 females and 95 males) were dosed intravenously with 20 mg kg⁻¹ of a 2 mg ml⁻¹ solution of DOX. On days 3 through 6 after dosing, the mice were injected subcutaneously twice per day with 150 μg kg⁻¹ of a 30 μg ml⁻¹ solution of Neupogen (Amgen, Thousand Oaks, CA, USA). Post-dose blood was collected 6 days after dosing. We removed 16 mice from the mapping analysis that failed genotyping. We analyzed data for 179 mice (84 females and 95 males) and had power to detect peaks that explain 19% of the phenotypic variance.

Cyclophosphamide
A total of 200 DO mice (102 females and 98 males) were dosed intravenously with 200 mg kg⁻¹ of a 20 mg ml⁻¹ solution of CYC (Pfizer, Bridgewater, NJ, USA). Post-dose blood was collected 5 days after dosing. Three mice died during dosing and six were removed from mapping analysis due to failed genotyping. We analyzed data for 191 mice (97 females and 94 males) and had power to detect peaks that explain 18% of the phenotypic variance.

Docetaxel
A total of 181 DO mice (107 females and 74 males) were dosed intravenously with a total of 30 mg kg⁻¹ by administering a 10 mg ml⁻¹ solution of TAX (Sanofi Aventis, Bridgewater, NJ, USA) for three consecutive days. Post-dose blood was collected 7 days after the first dose. Four mice died during dosing and we removed 23 mice from mapping analysis that failed genotyping. We analyzed data for 154 DO mice (85 females and 69 males) and had power to detect peaks that explain 20% of the phenotypic variance.

Statistical analyses
For pre-dose hematology values, we log-transformed all the values and fit a mixed-effects model with sex and drug as fixed effects and dosing date as a random effect. We calculated the geometric mean and standard error on a log scale and transformed these values back to the natural scale. We tested for differences in pre-dose parameters between drugs using a mixed-effects model with sex and drug as fixed effects and experimental batch as a random effect. We tested for an interaction between the pre- and post-dose parameter values by fitting a mixed-effects model with sex and treatment as fixed effects and experimental batch as a random effect. We performed a likelihood ratio test between the null model (that is, excluding treatment) and the full model. P-values were adjusted using a Bonferroni correction. The investigator was not blinded regarding the status of the mice.

Genotyping
We collected tail tips after euthanasia from each animal and isolated DNA. GeneseeK (Lincoln, NE, USA) genotyped each mouse at 7,854 markers using the Mouse Universal Genotyping Array (MUGA). Samples with allele call rates below 90% were removed due to low quality.

Quantitative trait locus mapping
We reconstructed the genome of each DO mouse in terms of founder haplotypes using DOQTL, a software package that performs haplotype reconstruction and genetic mapping. We transformed cell counts into normal scores and performed linkage mapping by fitting an additive haplotype model with a kinship adjustment. We regressed the post-dose cell counts on the pre-dose cell counts, sex, dosing batch and the genotype at each MUGA marker. We imputed all single high quality (that is, FILTER = PASS) SNPs from the Sanger Mouse Genome Project onto DO genomes and fit an additive genotype model at each SNP using the same covariates as the additive haplotype model, but with the imputed Sanger genotypes rather than founder haplotypes. We calculated genome-wide P-values (p_{HAP}) via permutation of the phenotype values and selection of the top 5% of the P-values in each permutation. We estimated heritabilities by fitting a mixed-effects model that estimated the additive genetic variance and report the ratio of the additive genetic variance over the total phenotypic variance.

Knockout mice
We obtained three knockout mouse strains, each targeting a different gene or pair of genes: Abcb1a/FVB.129P2-Abcb1a11R7, N7, Taconic, Hudson, NY), Abcb1a/b (FVB.129P2-Abcb1ad108bAbcb1b1311, N12, Taconic, Hudson, NY) and Abcb4 (FVB.129P2-Abcb4s2835, The Jackson Laboratory). Male homozygous knockout mice were mated to two FVB/NJ female mice (FVB, The Jackson Laboratory) to produce heterozygotes knockouts. We mated a pair of heterozygous knockouts to produce mice with wild-type, heterozygous and homozygous knockout alleles. We dosed each of the cohorts with 20 mg kg⁻¹ of DOX as described above and measured neutrophil counts before dosing and 5 days after dosing.

Repeat of Abcb4 knockout
We repeated the Abcb4 knockout test using a different experimental design at the Saarland University Medical Center in Homberg, Germany. We obtained FVB.129P2-Abcb4s1123 and bred them to obtain 72 progeny (36 females and 36 males). We housed mice in individually ventilated cages (three per cage) under standard conditions (12 h light/dark cycle) and mice received water and a standard rodent diet (V1534, ssniff, Germany) ad libitum. The mice were divided by genotype into three groups of 24 mice each; wild-type, heterozygous and homozygous knockout for Abcb4. We dosed 12 of the mice (6 males, 6 females) in each group with 0.9% saline solution and dosed the other 12 mice with 20 mg kg⁻¹ of DOX; both were administered via tail vein injection. We collected blood from all mice 5 days after dosing and measured neutrophil counts on a Sysmex XE-5000 automated hematology analyzer. All animal experiments were approved by the respective government agency (Landesamt für Verbraucherschutz, Saarbrücken; TV43/2012).

Comparison with human GWAS
We obtained data from the Biobank Japan human GWAS of chemotherapy-induced neutropenia and leukopenia, which contained results for DOX, CYC and TAX. This study contained 13 122 patients treated with one or more drugs. There were 234, 758 and 523 patients dosed with DOX, CYC and TAX, respectively. We compared our candidate gene list for each drug by intersecting their gene symbols with the genes in our QTL support intervals.

Genome, gene and variant annotation
We used mouse genome build GRCh38 to report genome coordinates. We obtained gene locations from Mouse Genome Database, (version MGl.20160711.gff3.gz) and mouse genome variants from version 5 (REL-1505) of the Sanger Mouse Genomes Project.
RESULTS

We dosed DO mice with one of three chemotherapy drugs that induce neutropenia in humans. Each drug has a different mode of action against tumor cells; DOX is a topoisomerase II inhibitor, CYC is a DNA alkylating agent and TAX is a microtubule stabilizer. DOX is used to treat many tumor types, including breast, childhood solid tumors and lymphomas. CYC is used to treat leukemias, lymphomas and solid tumors, and as a myeloablative agent. TAX is used to treat breast cancer in both the adjuvant and metastatic setting. These drugs are rarely administered alone and are commonly administered in combination with other chemotherapeutic agents. For example, the AC-T regimen in which DOX (A, Adriamycin) and CYC (C) are followed by TAX (T, Taxotere). Our goal in this study was not to mimic the exact details of clinical regimens. Rather, we aimed to determine whether genetic variants affect variation in hematotoxicity, whether genetic variants affect all hematopoietic cell subpopulations identically and to identify genomic loci that influence variation in hematotoxicity. We chose to model first-cycle responses because the first-cycle response in patients is often used to guide future dose modifications. In addition to dosing three cohorts with either DOX, CYC or TAX, we dosed a cohort of mice with DOX followed by granulocyte colony-stimulating factor (CSF3), which is commonly co-administered with chemotherapeutic agents to attenuate myelosuppression. For each of these four dosing groups, we identified genomic loci associated with pre- and post-treatment blood cell counts.

Analysis of pre-dose parameters

We measured complete blood counts and body weight in each mouse before and after dosing with one of four dosing protocols: DOX, DOX with granulocyte colony-stimulating factor (DOX +CSF3), CYC or TAX. We quantified the differences in mean pre-dose cell counts to determine whether there were any baseline differences between drug groups (Supplementary Table 1, Figure 1). We found no differences between pre-dose dosing groups for absolute neutrophil counts (NEUT, PBonf = 1.0), lymphocyte counts (LYMPH, PBonf = 0.44), red blood cell counts (RBC, PBonf = 0.22) and body weight (BW, PBonf = 1.0). We found that pre-dose values differed between drug groups for monocyte counts and platelet counts (PLT), but not for lymphocytes, red blood cells or body weight.

Figure 1. Changes in cell counts and body weight before and after dosing. The plots are organized with drugs in rows (a) doxorubicin, (b) doxorubicin with CSF3, (c) cyclophosphamide and (d) docetaxel) and phenotypes in columns. Each panel plots the pre-dose (x axis) value versus post-dose value (y axis) for each mouse. The females are red and the males are blue. Dashed lines plot the pre- and post-dose means for each sex. The black solid line along the diagonal shows where pre- and post-dose values would be equal. Points below the diagonal indicate a decrease in cell counts after dosing. CSF, colony-stimulating factor.

Data availability

The phenotype and genotype data are available at http://churchill-lab.jax.org/website/gattietal2017a.

Code availability

The DOQTL software is freely available as an R package from the Bioconductor project at http://bioconductor.org/packages/release/bioc/html/DOQTL.html.
counts (MONO, \(P_{\text{bonf}} = 0.041\)) and platelets (PLT, \(P_{\text{bonf}} = 2.71 \times 10^{-4}\)). We categorized mice as neutropenic if NEUT fell below 120 cells \(\mu l^{-1}\), which was the first percentile of the pre-dose NEUT distribution (that is, 99% of pre-dose mice had NEUT > 120 cells \(\mu l^{-1}\)). This is similar to the neutropenia threshold of 110 cells \(\mu l^{-1}\) reported in ICR outbred mice.\(^{33}\) We defined anemia as RBC < 7.88 \(\times 10^6\) cells \(\mu l^{-1}\) using the same 1% threshold in DO mice before dosing.

Effect of chemotherapy drugs on blood cell counts

DOX had a general suppressive effect on the hematopoietic system, reducing mean LYMPH, NEUT, RBC and PLT (Figure 1a, Supplementary Table 1). This effect is consistent with the neutropenic effects of DOX in the clinic.\(^{36}\) The variance of post-dose BW was unchanged \((P_F > 0.05)\), but DOX increased the variance of all post-dose cell populations \((P_F < 10^{-4})\). There was no difference in neutropenia between sexes. The Pearson correlation between pre- and post-dose NEUT was 0.241, indicating that pre-dose values are not predictive of DOX response. The heritability of the change in NEUT was 0.516 (Table 1). DOX reduced mean BW by 11.7% in both the sexes.

The addition of CSF3 to the DOX regimen increased mean NEUT by 54.6% (Figure 1b, Supplementary Table 1), consistent with clinical experience.\(^{37}\) The variance of NEUT increased after dosing with CSF3 \((P_F = 1.80 \times 10^{-15})\), indicating that genetic variation affects the response to CSF3 as well. The variance of BW and RBC was unchanged \((P_F > 0.05)\), but DOX increased the variance of post-dose LYMPH, MONO, NEUT and PLT \((P_F < 10^{-4})\). The Pearson correlation between pre- and post-dose NEUT was −0.039, again indicating that there was little predictive value in pre-dose counts. Mean LYMPH, MONO, PLT and RBC also decreased. The addition of CSF3 to the DOX regimen lowered the heritability of the change in NEUT to 0.395 (Table 1). Mean BW decreased by 11.9%.

CYC had a broad suppressive effect on the hematopoietic system (Figure 1c, Supplementary Table 1), consistent with CYC’s role as a myeloablative agent.\(^{38}\) Mean counts for all blood cell subpopulations decreased. The variance of BW and RBC was unchanged \((P_F > 0.05)\), but CYC increased the variance of post-dose LYMPH, MONO, NEUT and PLT \((P_F < 10^{-7})\). The Pearson correlation between pre- and post-dose NEUT was 0.125, again indicating little predictive value in pre-dose NEUT. The heritability of the change in NEUT was 0.272 (Table 1). Mean BW decreased by 3.9%.

TAX produced a lesser myelosuppressive effect in DO mice when compared with the other drugs (Figure 1d, Supplementary Table 1). There was a modest decrease mean NEUT and 2.8% of the mice were neutropenic. TAX suppressed mean RBC, causing 49.7% of the mice to become anemic. In contrast to the other drugs in this study, mean PLT increased in treated mice, which is consistent with limited clinical observations\(^{39}\) and may be significant due to the relationship between PLT and metastasis.\(^{39}\) The variance of BW and RBC was unchanged \((P_F > 0.05)\), but TAX increased the variance of post-dose LYMPH, MONO, NEUT and PLT \((P_F < 10^{-5})\). The heritability of the change in NEUT was 0.352 (Table 1). TAX produced a 9.2% decrease in mean BW.

Genetic architecture of chemotherapy-induced hematotoxicity

We performed genome-wide association (GWA) mapping on the change in cell counts to identify genomic loci that influence susceptibility to chemotherapy-induced hematotoxicity. We found that the hematotoxicity of each drug is influenced by a distinct set...
of genomic loci that have varying effects on the hematopoietic system. DOX toxicity is influenced by a pleiotropic locus on proximal chromosome 5 that affects cell counts for all of the blood cell populations as well as BW (Figure 2a). In the DOX+CSF3 cohort, the same locus on chromosome 5 had a major effect on white blood cell populations and BW, but not on PLT or RBC (Figure 2b). For CYC, we found no pleiotropic loci; NEUT associated with a locus on chromosome 11, and PLT associated with two loci on the distal arm of chromosomes 5 and 19 (Figure 2c). Mice dosed with TAX showed peaks with less significant loci overall, which may be due to the modest change in mean NEUT at the dose we selected. There were loci that affect LYMPH on chromosome 3, a locus on chromosome 11 that affects RBC and loci on chromosomes 4 and 12 that affect NEUT (Figure 2d).

We found a total of 21 loci met genome-wide significance (Table 2). We focused on genomic loci that affect NEUT because neutropenia is the most common dose-limiting toxicity of these drugs. NEUT in mice dosed with DOX or DOX+CSF3 showed a peak on proximal chromosome 5 (Figures 3a and b). We mapped the change in NEUT for mice dosed with CYC to a locus on chromosome 11 (Figure 3c) and for mice dosed with TAX to loci on chromosomes 4 and 12 (Figure 3d). We mapped NEUT using the pre-dose values from all 950 mice and did not find any peaks that overlapped with these peaks, indicating that the peaks we found for each drug are related to treatment and not to constitutive NEUT (Figure 3e). The large peak on chromosome 1 contains chemokine receptor 4 (Ccr4), which contains a missense SNP (rs8256191) and is involved in neutrophil trafficking from the bone marrow.5

Genetic influences of doxorubicin on neutrophil counts and body weight

We dosed 379 DO mice with DOX and mapped the change in NEUT before and after dosing. The additive heritability was 0.52 ($P=1.94 \times 10^{-4}$), indicating that genetic background has a strong influence on the response to DOX. We mapped pre-dose NEUT and found that the highest $-\log_{10}(P\text{-value})$ in the interval from 3 to 30 Mb on chromosome 5 was 1.95, indicating that the chromosome 5 peak is not related to constitutive neutrophil levels (Figure 3e). We performed GWA mapping of the change in NEUT and found a peak on chromosome 5 at an uncorrected $P\text{-value}$ of $1.51 \times 10^{-33}$ that explains 36.3% of the phenotypic variance (Figure 3a). This locus had pleiotropic effects on the hematopoietic system, influencing white blood cell, NEUT, neutrophil, PLT, platelet, RBC, red blood cell. For each peak, the drug regimen, cell type, chromosome, proximal and distal peak location, nominal $P$-value and adjusted $P$-values are listed.

### Table 2. List of significant QTL peaks

| Drug | Phenotype | Chromosome | Proximal position | Distal position | $P$-value | Adjusted $P$-value |
|------|-----------|------------|-------------------|----------------|----------|------------------|
| Doxorubicin+GCSF | NEUT | 2 | 148 150 899 | 148 150 944 | 6.00E−07 | 0.049 |
| Doxorubicin+GCSF | MONO | 2 | 14150 899 | 141 50 944 | 8.70E−06 | 0.469 |
| Docetaxel | LYMHP | 3 | 88 377 902 | 88 587 928 | 3.49E−08 | 0.001 |
| Docetaxel | MONO | 3 | 90 106 020 | 90 106 020 | 4.27E−06 | 0.219 |
| Docetaxel | NEUT | 3 | 90 106 020 | 90 106 020 | 2.10E−05 | 0.598 |
| Docetaxel | NEUT | 4 | 142 873 065 | 142 988 627 | 7.27E−08 | 0.004 |
| Doxorubicin+GCSF | EOS | 5 | 5 809 501 | 6 132 452 | 4.53E−12 | 0.000 |
| Doxorubicin | NEUT | 5 | 6 289 554 | 6 509 240 | 1.51E−33 | 0.000 |
| Doxorubicin+GCSF | LYMHP | 5 | 6 513 094 | 7 276 358 | 5.06E−15 | 0.000 |
| Doxorubicin+GCSF | MONO | 5 | 6 513 094 | 7 276 358 | 2.80E−13 | 0.000 |
| Doxorubicin+GCSF | NEUT | 5 | 6 513 094 | 7 276 358 | 2.78E−17 | 0.000 |
| Doxorubicin | LYMHP | 5 | 8 122 454 | 8798106 | 1.73E−05 | 0.555 |
| Doxorubicin | PLT | 5 | 8 125 162 | 8 797 053 | 6.15E−10 | 0.000 |
| Doxorubicin+GCSF | BW | 5 | 8 796 831 | 9 265 645 | 4.88E−07 | 0.040 |
| Doxorubicin | BW | 5 | 9 187 736 | 9 234 163 | 7.17E−06 | 0.294 |
| Doxorubicin | MONO | 5 | 9 285 547 | 9 302 450 | 2.17E−18 | 0.000 |
| Doxorubicin | RBC | 5 | 10 685 282 | 10 685 282 | 2.25E−06 | 0.123 |
| Cyclophosphamide | PLT | 5 | 127 467 289 | 127 475 691 | 3.51E−07 | 0.029 |
| Doxorubicin | RBC | 6 | 27 582 992 | 27 583 007 | 6.98E−09 | 0.000 |
| Doxorubicin+GCSF | MONO | 6 | 92 622 580 | 92 858 576 | 2.19E−07 | 0.021 |
| Doxorubicin+GCSF | EOS | 6 | 92 622 580 | 92 858 576 | 4.09E−06 | 0.291 |
| Doxorubicin+GCSF | MONO | 7 | 143 262 081 | 143 262 081 | 1.16E−06 | 0.093 |
| Docetaxel | RBC | 11 | 44 576 416 | 44 845 071 | 1.19E−17 | 0.005 |
| Cyclophosphamide | NEUT | 11 | 69 579 943 | 69 686 580 | 2.83E−10 | 0.000 |
| Docetaxel | NEUT | 12 | 75 408 009 | 75 837 799 | 1.01E−06 | 0.062 |
| Docetaxel | LYMHP | 12 | 80 024 381 | 80 024 381 | 8.40E−07 | 0.054 |
| Doxorubicin | NEUT | 13 | 24 678 372 | 24 872 531 | 6.19E−07 | 0.041 |
| Doxorubicin+GCSF | BW | 13 | 62 961 059 | 63 347 403 | 5.26E−06 | 0.340 |
| Doxorubicin+GCSF | LYMHP | 13 | 63 942 114 | 63994071 | 5.13E−07 | 0.045 |
| Doxorubicin+GCSF | LYMHP | 16 | 39 615 002 | 39 615 002 | 2.81E−07 | 0.028 |
| Doxorubicin | BW | 17 | 84 505 914 | 84 505 914 | 2.79E−07 | 0.025 |
| Cyclophosphamide | PLT | 19 | 18 875 754 | 19 340 012 | 1.07E−07 | 0.006 |

Abbreviations: BW, body weight; GCSF, granulocyte colony-stimulating factor; LYMHP, lymphocyte; MONO, monocyte; NEUT, neutrophil; PLT, platelet; RBC, red blood cell. For each peak, the drug regimen, cell type, chromosome, proximal and distal peak location, nominal $P$-value and adjusted $P$-values are listed.

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We focused on the 15,266 SNPs with the most significant associations having \(-\log_{10}(P\text{-value}) > 24\) (Figure 4c). For all of these SNPs, the NOD and PWK strains contribute a resistant allele that is associated with a 1.3-fold increase in NEUT (Figure 4d), which is consistent with the increase of 1.3 that results from adding a NOD allele in Figure 4b. We added the genotype of the resistant allele in Figures 4b and d, which is consistent with the increase of 1.3 that results from adding a NOD allele in Figure 4b. We added the genotype of the resistant allele that increases the log2-ratio NEUT. Red lines show the \(P_{GW} = 0.05\) significance threshold. Orange triangles plot the locations of genes reported in the literature as being involved in the metabolism or transport of each drug. The peaks on distal chromosome 4 in (d) and (e) are 20 Mb apart and do not overlap. CSF, colony-stimulatin factor; NEUT, neutrophil; SNP, single-nucleotide polymorphism.

There are 178 annotated features in the region covered by the NOD/PWK allele SNPs in Figure 4c; 52 are protein coding genes, 56 are pseudogenes, 48 are noncoding RNAs and 22 are unclassified. We selected SNPs and indels that produce a coding or a splice site change in proteins and found 269 variants that intersect with 42 genes (Supplementary Table S2). We accumulated annotation data about the genes under the NEUT association peak to select the most plausible candidates by combining evidence from missense and splice site variants, expression and protein quantitative trait loci (eQTL, pQTL) from separate liver data in DO mice and genes annotated in the Comparative Toxicogenomics Database. Sorcin (Sri) contains one missense SNP (rs248635887) and is involved in calcium homeostasis. Sri is upregulated in DOX-resistant cell lines and Sri downregulation in vitro induces apoptosis and renders cells less resistant to DOX. The chromosomal region also contains three ATP-binding cassette genes; Abcb1a, Abcb1b and Abcb4. Abcb1a and Abcb1b are multidrug efflux transporters that are orthologs of the human multidrug-resistance gene 1 (MDR1 or P-glycoprotein). They are both upregulated in DOX-resistant tumors and cell lines and are capable of exporting DOX from the cell. Abcb1a and Abcb1b carry either splice site or missense variants (rs31421794, rs8268091, rs6277917, rs16800179) for which NOD and PWK carry the alternate allele. Abcb4 is annotated as a phospholipid transporter in the liver, but it is also highly expressed in the megakaryocyte/erythroid progenitor. It is upregulated along with Abcb1a/b in cell lines exposed to DOX and the zebrafish ortholog has been shown to transport DOX from cells. Abcb4 also contains both splice and missense SNPs (rs8279985, rs225705157) as well as an in-frame deletion that removes a lysine from the protein product (rs255450780).

We obtained mice with one or more of the Abcb1a/b and Abcb4 genes knocked out and investigated whether these genes influence DOX-induced neutropenia. All knockouts were produced on the FVB/N (FVB) background, which is a strain with high fecundity and is genetically similar to the A/J, C57BL/6J, 129S1/SvJev and NZO/HILJ strains on proximal chromosome 5. The peaks on distal chromosome 4 in (d) and (e) are 20 Mb apart and do not overlap. CSF, colony-stimulatin factor; NEUT, neutrophil; SNP, single-nucleotide polymorphism.

| a | b | c | d | e |
|---|---|---|---|---|
| Genome-wide association mapping plots of change in NEUT for (a) doxorubicin, (b) doxorubicin +CSF3, (c) cyclophosphamide, (d) docetaxel and (e) pre-dose NEUT. Each point plots the \(-\log_{10}(P\text{-value})\) for the association between one SNP and the change in NEUT. Red lines show the \(P_{GW} = 0.05\) significance threshold. Orange triangles plot the locations of genes reported in the literature as being involved in the metabolism or transport of each drug. The peaks on distal chromosome 4 in (d) and (e) are 20 Mb apart and do not overlap. CSF, colony-stimulatin factor; NEUT, neutrophil; SNP, single-nucleotide polymorphism. |
(rs254540780) that removes a lysine from the translated protein. 

Therefore, our data are inconclusive regarding whether Abcb4 also has a role in DOX-induced neutropenia.

There are many peaks that are below the significance threshold that may harbor genes with smaller effects. Although looking under these peaks increases the risk of false positives, we found two genes previously linked to DOX’s mechanism of action. A peak

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on chromosome 13 contains tyrosyl-DNA phosphodiesterase 2 (Tdp2, Supplementary Figure S2A), a gene involved in the release of topoisomerase II from the 5’ end of DNA, and a target for inhibition in cancers that are also treated with DOX. Another peak on chromosome 14 contains topoisomerase (DNA) II beta (Top2b, Supplementary Figure S2B), which is consistent with DOX’s role as a topoisomerase inhibitor.

Figure 5. Effect of knocking out ATP-binding cassette transporters on neutropenia. (a) The knockout alleles of Abcb1a are plotted on the x axis versus the log2-ratio of post-dose over pre-dose NEUT. Each point represents one mouse. The red line is the least-squares fit and the pink shading is the 95% prediction interval for the fit. The P-value indicates whether the slope differs from zero. (b) Same as (a), but for the Abcb1a and Abcb1b combined knockout. Each functional allele of Abcb1b produces a 2.2-fold increase in log2-ratio NEUT. (c) Same as (a), but for the Abcb4 knockout. Each functional allele of Abcb4 produces a 1.2-fold increase in log2-ratio NEUT. NEUT, neutrophil.

Genetic influences on doxorubicin myelosuppression with CSF3

We dosed 179 DO mice with DOX followed by CSF3. We mapped the change in NEUT before and after dosing and found a peak on chromosome 5 at 6.51 Mb with a P-value of 2.78 × 10^{-17} (PGW < 0.001) that explained 11.4% of the variance (Figure 3c). We found 232 SNPs in linkage disequilibrium with that SNP and mapped the change in NEUT. The Bayesian credible interval for the GW peak covers almost 10 Mb (4.3–13.9 Mb) and there were no clear candidate genes.

Genetic influences on cyclophosphamide myelosuppression

We dosed 191 DO mice with CYC and mapped the change in NEUT before and after dosing. We found a peak on chromosome 11 at 70.82 Mb with a P-value of 2.78 × 10^{-17} (PGW < 0.001) that explained 15.7% of the variance (Figure 3c). We found 232 SNPs with P-values below 1 × 10^{-8} (Figure 7a). Mice carrying the A/J, CAST, NZO/HILtJ (NZO) or PWK alleles at these SNPs had lower post-dose NEUT. These SNPs intersect with the exons or untranslated regions of several genes (Supplementary Table S3). Two of these genes, dynein axonemal heavy chain 2 (Dnah1) and SRY-box 15 (Sox15), contain missense SNPs. Although there are no high significance SNPs that intersect with the exons of transformation-related protein 53 (Trp53), this genes is within the confidence interval and is a promising functional candidate because it is a DNA checkpoint gene that induces cell cycle arrest and apoptosis in the presence of DNA damage. Trp53 may be influenced by nearby regulatory SNPs. Trp53 protein levels are upregulated in a rat model of CYC-induced liver injury and CYC increases the levels of activated TP53 in HepG2 cells and thus may be associated with the survival of replicating cells.

Genetic influences on docetaxel myelosuppression

We dosed 154 DO mice with TAX and mapped the change in NEUT before and after dosing. We mapped a peak on chromosome 4 at 143.3 Mb with a P-value of 5.12 × 10^{-7} (PGW < 0.001) that explained 11.4% of the variance (Figure 3d). The lowest P-value for pre-dose NEUT at this locus was not significant (P = 0.011). There was one SNP at 142 870 495 bp on chromosome 4 with a P-value below the significance threshold (rs225386879) and it did not overlap with the exons or untranslated regions of any genes. This SNP is not located in a conserved region but is in a CpG island and thus may have a regulatory role on one or more genes nearby. The closest gene is PR domain containing 2, with ZNF domain (Prdm2), which is a nuclear protein methyltransferase that has tumor-suppressing functions that may impact cell survival in the context of chemotherapy. There are several genes with plausible functional roles in the QTL interval. Kazrin (Kazr) is a protein that co-localizes with acetylated microtubules. Capsase 9 (Caps9) is part of the apoptosis cascade and has been associated with increased DNA damage in hematopoietic cells after exposure to an alkylating agent. TAX also activates Caps9 and increases apoptosis in prostate cancer cell lines. Another functional candidate is kinesin family member 1B (Kif1b), a molecular motor that is involved in vesicle transport and axon growth. The peak on chromosome 4 also weakly affected MONO (Figure 2d) and we hypothesize that the causative gene(s) acts in the direct precursor of NEUT and MONO. We regressed out the most significant SNP and did not find any other significant SNPs.

Overlap with human neutropenia loci

We obtained data from a Japan-based GWAS of chemotherapy-induced neutropenia that dosed patients with DOX, CYC and TAX. We looked for overlap between the genes that they identified for each drug and found none consistent with our study. Variants in ABCB1 were associated with severity of DOX-induced bone marrow toxicity in a Danish pediatric cohort. In another study of breast cancer patients treated with CYC and DOX along with filgrastim, variants in ABCB1 were not associated with hematotoxicity.
DISCUSSION

In this study, we dosed DO mice with three chemotherapy drugs, each with distinct modes of action, as well as CSF3, a stimulator of NEUT production. As seen in humans, we observed differences in the neutropenic response between subjects. For both CYC and DOX, we observed neutropenia levels on par with Grade 4 neutropenia in humans. Although mean NEUT did not decrease significantly in the TAX group, it did decrease in a subset of mice, recapitulating the variation in response seen in human patients. The combination of DOX with CSF3 increased neutrophil counts in many mice, but some still experienced neutropenia. This variation in response to CSF3 may have a genetic basis and further research in this area is warranted.

The genetic architecture of hematotoxicity was quite different between drugs. DOX-induced hematotoxicity was influenced by a pleiotropic locus on chromosome 5 that affected all cell types.
populations as well as BW. This suggests that the gene or genes in this region are acting in a manner that protects the entire hematopoietic system and one of them may be a therapeutic target to protect patients from hematotoxicity. There also appear to be at least two genes in this region with different allelic effects, as shown in Figure 4. We showed that Abcb1b influenced DOX-induced hematotoxicity and Abcb4 or Sfr may have a role as well. One possible explanation for the lack of replication of the Abcb4 knockout experiment may be due to diet and the influence of the microbiome on xenobiotic metabolizing enzymes. We have included a comparison of the two diets in Supplementary Table S4.

When we dosed DO mice with DOX, we identified a pleiotropic locus on chromosome 5 that influenced most hematopoietic cell populations. The DOX-associated locus contains Abcb1a and Abcb1b, which are the murine orthologs of ATP-binding cassette, sub-family B, member 1 (ABCB1), an efflux transporter that has been associated with DOX resistance in human cancers.74 However, there is currently no evidence from epidemiological or pharmacogenomics studies that ABCB1 variants affect susceptibility to myelosuppression. We showed that the presence of functional copies of Abcb1b is positively correlated with resistance to neutropenia after treatment. The protective effect of Abcb1b on the hematopoietic system suggests that it is acting in the liver or in one of the early hematopoietic progenitor cells. If these genes are functioning in the hematopoietic system, they may be affecting the survival of uncommitted progenitor cells in the hematopoietic hierarchy. Abcb1b is constitutively expressed in hematopoietic progenitor cells and may be acting at that level.51 Abcb1b may also be induced in liver and may be acting to protect the entire body by excreting DOX metabolites directly into the bile. When we dosed mice with both DOX and CSF3, we found an association for neutropenia in the same region of chromosome 5 as DOX alone. Although CSF3 attenuated neutropenia in many mice, the chromosome 5 locus still affected susceptibility to neutropenia. It is interesting to note that the loss in BW did not correlate with the degree of neutropenia. This suggests that different mechanisms of toxicity may affect different organ systems. For example, the NOD allele of Abcb1b may confer protection from DOX-induced neutropenia, but it does not appear to protect mice from weight loss. Conversely, mice carrying the CAST allele on proximal chromosome 5 had the most severe neutropenia, but the least weight loss. If Abcb1b is acting in the liver, then it should export DOX metabolites into the bile and out of the body, precluding any other toxicity. Alternately, if Abcb1b is protecting cells in the hematopoietic system, then it is exporting DOX metabolites back into the circulation, where they may have a deleterious effect on other organ systems.

In contrast to DOX, different loci affected each cell population when we dosed mice with CYC or TAX. CYC is a myeloablative drug that reduced all cell counts and BW, yet changes in each cell population were associated with distinct loci. The same pattern of differential association of cell counts with distinct loci was true for TAX. This suggests that different chemotherapy drugs may affect different hematopoietic progenitor cells and their committed cell populations in a distinct manner. There may be lineage-specific genes that are expressed in different progenitor populations that confer resistance to toxicity and a greater understanding of the genetics underlying these alleles may help to improve patient outcomes.

When we mapped the change in NEUT after CYC administration, we found a broad peak on chromosome 11 containing many genes. We narrowed the candidate gene list to Trp53, a plausible candidate gene involved in DNA repair and cell cycle regulation. CYC is a DNA alkylating agent that damages DNA and also leads to cell cycle arrest,75 consistent with the action of Trp53. The protective allele of Trp53 may be acting in committed neutrophil progenitor cells, as the QTL appears only for NEUT and not for other myeloid-derived cells. When we regressed out the effect of the chromosome 11 locus, we did not find significant peaks over any phase I or II metabolizing genes or DNA repair genes that have been shown to influence differential metabolism in humans.75,76 It is important to note that while there may be more than one gene at this locus, we may be unable to dissect it due to the width of the linkage disequilibrium blocks in early generations of the DO.

For each drug that we tested, we identified a different locus with chemotherapy-induced neutropenia. This is consistent with findings in both humans9 and model organisms.28 This result highlights the importance of developing tractable translational models to understand the role of genetic variation in chemotherapy-induced toxicities. Inbred mouse strains are valuable tools, but these results would not have been discovered using a single inbred strain. The broad genetic diversity in DO mice allowed us to sample alleles from a large number of genes to interrogate their effects. DO mice also have the advantage that they are a whole-body system that includes complex interactions between organs that may influence toxicity.

We were initially concerned that our results provide no overlap with the results of a clinical GWAS for chemotherapy-induced neutropenia.21 However, we agree with the authors of the human GWAS that their study had low statistical power and was further weakened by uncontrolled confounding variables. Each of the three drug groups had a small number of cases, ranging from 168 for doxorubicin to 423 for cyclophosphamide. These are very low sample sizes for an unbiased human GWAS. Further, the authors did not use measured neutrophil counts; rather, they used neutropenia grades binned into two groups (grades 1 and 2 or grades 3 and 4). This artificial discretization further reduces power. In our view, this highlights the strength of using whole animal dosing to perform GWAS in murine models. We can afford adequate sample sizes, we can control confounding covariates and we can impute full genome sequences to find candidate genes. Further, we can control the minor allele frequency in DO mice to ensure that we do not have rare variants (that is, with a minor allele frequency < 5%), which is impossible in human GWAS studies. The high minor allele frequency in the DO (an average of 1/8) allowed us to identify large QTL peaks above the α = 0.05 genome-wide significance level for all three drugs and identified plausible and translationally relevant genes for two. This stands in contrast to the clinical GWAS in which the authors 'failed to identify genetic variants that surpassed the genome-wide significance level.'21

One of the limitations of this study was the relatively small numbers of mice in each cohort. We dosed between 200 and 400 mice per drug and, while we identified peaks for each drug, the width and resolution of the peaks was limited by early DO outbreeding generation with low numbers of recombinations. Many chemotherapy drugs have strong effects and this produces correspondingly strong heritabilities and peaks. The mice in this study were also from generations 3 through 5 of outbreeding and, correspondingly strong heritabilities and peaks. The mice in this study were also from generations 3 through 5 of outbreeding and hence they had large recombination blocks (median = 6.47 Mb), which limits the mapping resolution of the experiment. DO mice are currently at generation 23 of outbreeding and we estimate that their median recombination block size is now 2.9 Mb. Investigators who wish to estimate the number of mice required to power a study using DO mice are referred to the power simulations and associated figure in Gatti et al.9

These findings leave us with several questions for future research. In which organ or cell population is Abcb1b acting? What is the other gene on proximal chromosome 5 that makes DO mice susceptible to neutropenia? What are the genes that regulate the response to CSF3? How can we translate these findings to the clinic? Although we do not expect genetic polymorphisms in mice to be identical to polymorphisms in the human population,
the pathways that affect the variation in susceptibility to myelo-suppression may translate between species. An improved mechanistic understanding of the pathways that affect susceptibility to chemotherapy-induced myelosuppression may lead to new treatment options in the clinic.

**CONFLICT OF INTEREST**

DMG and GAC are employed by The Jackson Laboratory, which sells Diversity Outbred mice.

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