# Thiopurine-mediated impairment of hematopoietic stem and leukemia cells in *Nudt15*$^{R138C}$ knock-in mice

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**Running title:** Mouse model of human NUDT15 R139C polymorphism

**Abstract**

Thiopurines are widely used as antileukemia agents and immunosuppressants. Recent large-scale clinical studies revealed a strong association between the *NUDT15* p.Arg139Cys (*NUDT15*$^{R139C}$) polymorphism and severe thiopurine-induced leukocytopenia. We established knock-in mice harboring p.Arg138Cys (*Nudt15*$^{R138C}$), which corresponds to the human polymorphism. A clinically relevant dose of mercaptopurine (MP) induced lethal cytopenia in *Nudt15*$^{R138C}$-harboring mice. MP dose reduction attenuated the hematopoietic toxicity, phenocopying clinical observations and providing *Nudt15* genotype-based tolerable doses of MP. High-dose MP induced acute damage to hematopoietic stem and progenitor cells (HSPCs) in *Nudt15*$^{R138C/R138C}$ mice. A competitive transplantation assay revealed that not only *Nudt15*$^{R138C/R138C}$ HSPCs, but also *Nudt15*$^{+/R138C}$ HSPCs suffered stronger damage than *Nudt15*$^{+/+}$ HSPCs, even by lower-dose MP, after long-term administration. In a *Nudt15* genotype-based posttransplantation leukemia recurrence model generated by bone marrow replacement with congenic wild-type cells and a small number of leukemia stem cells, MP prolonged the survival of mice with posttransplantation *Nudt15*$^{R138C/R138C}$ leukemia recurrence. In conclusion, our model will facilitate *NUDT15* genotype-based precision medicine by providing safer estimates for MP dosing, and our

findings highlighted the high susceptibility of hematopoietic stem cells to MP and suggested that exploiting thiopurine toxicity might be a novel treatment approach for leukemia in *NUDT15*$^{R139C}$-harboring patients.

## Introduction

Thiopurines are classical drugs that are widely used as cytotoxic agents for many cancers[1, 2] and as immunosuppressants for autoimmune or inflammatory diseases[3]. Mercaptopurine (MP) plays an important role in maintenance therapy for acute leukemia to achieve long-term remission[4, 5]. Azathioprine remains a key drug for inducing and maintaining remission of inflammatory bowel disease (IBD), although many novel agents have been developed[6]. However, patients are frequently forced to reduce or cease thiopurines because of toxicity, particularly, myelosuppression[7].

Thiopurines are enzymatically converted to thio-guanine nucleotides (TGNs), comprising thio-guanosine-5'-triphosphate (TGTP), thio-guanosine-5'-diphosphate (TGDP), and thio-guanosine-5'-monophosphate (TGMP)[8]. TGTP is an active thiopurine metabolite that can exert cytotoxicity by incorporation into RNA or DNA directly or after further conversion to thio-deoxyguanosine-5'-triphosphate (TdGTP), respectively[9, 10]. On the other hand, TGMP is detoxified by conversion to inactive metabolites by thiopurine S-methyltransferase (TPMT)[11, 12]. Single-nucleotide polymorphisms (SNPs) in *TPMT* have been reported to attenuate TPMT enzymatic activity and lead to the accumulation of excess TGNs, inducing predisposition to hematopoietic thiopurine toxicity[13-15]. However, recent

retrospective studies have shown that SNPs in *TPMT* are not significantly associated with leukocytopenia in Japanese IBD patients[16-18].

Recently, we and other groups have identified a nonsynonymous SNP in the *nucleoside diphosphate-linked moiety X-type motif 15* gene (*NUDT15*), rs116855232 (c.415C>T), as a strong risk factor of thiopurine-induced severe leukopenia in patients with IBD and pediatric acute lymphoblastic leukemia[17-22]. NUDT15 is a nucleotide diphosphatase that can convert TGTP to TGMP and is thus responsible for the inactivation of thiopurine metabolites; therefore, NUDT15 functions as a negative regulator of cytotoxic effects[10]. The c.415C>T SNP induces a p.Arg139Cys (R139C) amino acid change. The allele with this SNP (termed $NUDT15^{R139C}$ hereafter) is present in approximately 25% of the Japanese population[18] and 20% of the East Asian population[20]. NUDT15 R139C likely has lost enzyme activity *in vivo* because of the negative influence of this SNP on protein stability and loss of catalytic efficiency[10, 20]. Other SNPs in *NUDT15* causing p.Arg139His, p.Val18Ile, p.Val18_Val19insGlyVal, p.Arg34Thr, p.Lys35Glu, p.Gly17_Val18del, p.Met1Thr, and p.Gly47Arg have also been uncovered[18, 20]. The allele encoding p.Val18_Val19insGlyVal is the second most frequent and co-occurs with R139C in most cases. Other SNPs are present at very low frequency and appear to attenuate catalytic

efficiency. Finally, according to a large retrospective study in Japanese IBD patients, the *NUDT15*$^{R139C}$ allele has a significant impact on thiopurine-induced leukopenia, as the odds ratio for severe leukopenia is 13.4 and 807 in R139C heterozygous patients and homozygous patients, respectively[18]. Therefore, thiopurine administration requires precision medicine based on the *NUDT15* genotype.

To achieve precision medicine, a physiological model to estimate the tolerable thiopurine doses and thoroughly investigate the mechanism of hematopoietic system damage is needed. Although a *Nudt15* knock-out mouse model was recently established[23], it is unclear whether complete loss of Nudt15 is equivalent to the protein instability or catalytic efficiency loss induced by R139C, as two recent papers reported conflicting results; Valerie et al. reported that R139C influences protein stability, but not catalytic efficacy, whereas Moriyana et al. reported opposite findings[10, 20]. In addition, tolerable doses of thiopurines in heterozygous mice have not been addressed, although *NUDT15*$^{R139C}$ heterozygous patients frequently suffer from leukopenia[18, 24]. Further, it remains unclear how NUDT15 R139C affects the susceptibility of hematopoietic stem and progenitor cells (HSPCs) to thiopurines, although severe HSPC damage directly causes critical adverse effects, especially in leukemia treatment. In the current study, we address these unresolved

issues by generating a more physiologically relevant mouse model of human NUDT15 R139C, i.e., a knock-in mouse model harboring a c.412C>T allele in the murine *Nudt15* coding region (termed $Nudt15^{R138C}$ hereafter) that causes the p.Arg138Cys (R138C) substitution.

## Materials and methods

### Mice

C57BL/6 and C57BL/6-Ly-5.1 mice were obtained from Charles River Laboratories Japan (Yokohama, Japan) or CLEA Japan, Inc. (Tokyo, Japan), and the RIKEN BioResource Center (Tsukuba, Japan), respectively. To generate *Nudt15*$^{+/R138C}$ mice, single-guide (sg)RNAs were designed to target near c.412 in the coding sequence of mouse Nudt15 (Fig. 1a) using the optimized CRISPR design tool provided by the Zhang lab (http://crispr.mit.edu/)[25]. One hundred-twenty-base pair donor oligoDNAs with c.412C>T in the center were created for homology-directed repair (Supplementary Table 1). Zygotes from C57BL/6 mice received coinjection of sgRNAs (5 ng/μl) and oligoDNAs (100 ng/μl) and were transferred into the oviducts of pseudopregnant female mice. Based on sequencing analysis (primers shown in Supplementary Table 1), F0 generation mice that harbored *Nudt15* c.412C>T, but not the *Cas9* gene from the gene editing system nor mutations in the pseudogenes *Gm13534* and *Gm5919* with the same sequence as sgRNA targets, were selected and bred with wild-type C57BL/6 mice. Two F1 generation mice were reconfirmed to exhibit transmission of the *Nudt15* c.412C>T allele and were named strain 69 and strain 115, respectively. For analyses, 7- to 12-week-old littermates from

breeding pairs from the *Nudt15*$^{+/R138C}$ F2, F3, and F4 generations were used. Generation and selection of F0 mice were carried out at TransGenic Inc. (Fukuoka, Japan). All mice after F1 generations were maintained under specific pathogen-free conditions at the Research Center for Animal Life Science of Shiga University of Medical Science. All animal experiments were approved by the Animal Research Committee of Shiga University of Medical Science.

**Fluorescence-activated cell sorting (FACS) analysis**

Peripheral white blood cells and bone marrow (BM) cells were stained with the antibodies indicated in each experiment after brief lysis of erythrocytes by ammonium-chloride-potassium buffer. We used antibodies directed against CD3e [145-2C11], CD4 [GK1.5], CD8a [53-6.7], CD19 [6D5], Mac1 [M1/70], Gr-1 [RB6-8C5], B220 [RA3-6B2], Ter119 [TER-119], Ly-6G [1A8], CD34 [RAM34], CD16/32 [93], CD135 [A2F10], c-kit [2B8], and Sca-1 [D7]. To distinguish donor cells from competitor and recipient cells, the cells were also stained with anti-CD45.1 [A20] and anti-CD45.2 [104] antibodies. Ly-6G$^{+}$MAC-1$^{+}$, B220$^{+}$CD3e$^{-}$, and B220$^{-}$CD3e$^{+}$ cells were defined as neutrophils, B lymphocytes, and T lymphocytes, respectively. Lineage markers used in

analysis of HSPCs included CD3e, CD4, CD8a, CD19, Mac1, Gr-1, B220 and Ter119. Each HSPC population was defined as described elsewhere[26, 27]; hematopoietic stem cells (HSCs; $CD34^{-/low}Flt3^{-}Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$), multipotent progenitors (MPPs; $CD34^{+}Flt3^{-}Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$), lymphoid-primed multipotent progenitors (LMPPs; $CD34^{+}Flt3^{+}Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$), common myeloid progenitors (CMPs; $Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}CD16/32^{low}CD34^{low}$), granulocyte-monocyte progenitors (GMPs; $Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}CD16/32^{+}CD34^{+}$), and megakaryocyte-erythrocyte progenitors (MEPs; $Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}CD16/32^{-}CD34^{-}$). The antibodies were purchased from eBioscience (San Diego, CA, USA), BD Biosciences (San Jose, CA, USA), or BioLegend (San Diego, CA, USA). Flow-cytometric data were acquired using a FACSCanto™ II or FACSAria (BD Biosciences) and were analyzed using the FACSDiva and FlowJo (BD Biosciences) software.

**MP administration and toxicity analyses**

MP (Sigma, St. Louis, MO, USA) was orally administered as described elsewhere[28, 29]. As mice drink approximately 5 ml daily according to a previous report[30] and our observations, MP-containing water was adjusted to 0.4 μg MP × mouse body weight (g) × $ml^{-1}$ for the

administration of 2 mg/kg MP. Drinking bottles were exchanged every three days. Control mice were given drinking water containing the same amount of DMSO. Peripheral blood counts were determined with Celltacα (Nihon Koden, Tokyo, Japan). Differential leukocyte counts were determined by multiplying the number of WBCs by the percentage of each population. For BM histology, formalin-fixed femurs were decalcified with K-CX (FALMA, Tokyo, Japan) according to the manufacturer's procedure and sectioned.

**Competitive transplantation and chimerism analysis**

Two million BM cells from *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice (Ly5.2$^{+}$) and from age-matched C57BL/6-Ly5.1 mice (Ly5.1$^{+}$) were transplanted into lethally irradiated (10.5 Gy) age-matched recipient mice (Ly5.1$^{+}$Ly5.2$^{+}$) that were generated by breeding C57BL/6 and C57BL/6-Ly5.1 mice. Chimerism in each population was calculated as the ratio of targeted donor cells (Ly5.1$^{-}$Ly5.2$^{+}$) to competitor cells (Ly5.1$^{+}$Ly5.2$^{-}$).

**Leukemia model**

MLL-AF9 retrovirus-containing supernatant was generated and transduced into Lineage$^{-}$ c-Kit$^{+}$ cells from BM cells of *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice as

described previously[26, 31]. To develop leukemic mice, after culture in viral-free medium, MLL-AF9-transduced cells were transplanted into congenic C57BL/6-Ly5.1 mice after 6-Gy sublethal irradiation. Leukemia stem and progenitor cells were purified as the Ly5.2$^{+}$Lineage$^{-}$c-Kit$^{+}$ population from the BM of leukemic mice[32, 33]. To establish a posttransplantation leukemia recurrence model, 5 000 or 250 leukemia stem and progenitor cells and $2 \times 10^{6}$ BM cells from congenic C57BL/6-Ly5.1 mice were transplanted into concordant *Nudt15* genotype mice after 10.5-Gy lethal irradiation. The mice were euthanized when they became moribund for analysis of chimerism and cytology.

**Cytology**

Cells ($1–5 \times 10^{4}$) were suspended in phosphate-buffered saline and attached to glass slides by centrifugation at 800 rpm for 4 minutes using Cytospin 4 (Thermo, Waltham, MA, USA). The glass slides were stained with a traditional Wright-Giemsa staining.

**Statistical analysis**

ANOVA followed by Tukey's tests was adopted for multiple comparison analyses of toxicity and hematopoiesis among mouse genotypes. The Kaplan Meier test was adopted

for survival analyses. All analyses were conducted in GraphPad Prism version 6 (GraphPad software, La Jolla, CA, USA). All tests were two-sided, and a *p*-value <0.05 was considered significant.

## Results

### Establishment of knock-in mice harboring the *Nudt15*$^{R138C}$ allele

The human and murine NUDT15 proteins share 89% sequence identity. The arginine residue at position 139 of human NUDT15 is conserved at position 138 of murine Nudt15 (Supplementary Figure 1). Overexpression of the murine Nudt15 R138C mutant enhanced susceptibility to MP in a murine blood cell line (Supplementary Figure 2). Thus, we newly established knock-in mice harboring the *Nudt15*$^{R138C}$ allele on the C57BL/6 background by converting c.412 C to T utilizing CRISPR-CAS9 genome editing and homology-directed repair (Fig. 1a). Using two different sgRNAs to minimize off-target effects caused by sgRNAs, we generated two independent F0 mice, mouse number 115 and mouse number 69, that harbored the *Nudt15*$^{R138C}$ allele. By crossing the two F0 mice to wild-type C57BL/6 mice, we generated F1 mice that inherited the *Nudt15*$^{R138C}$ allele, but did not harbor mutations in the two pseudogenes *Gm13534* and *Gm5919*, which have the same sequence as the sgRNA targets (Supplementary Figure 3), and we termed them strain 115 and strain 69. Finally, *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, and *Nudt15*$^{R138C/R138C}$ mice were viably generated by mating *Nudt15*$^{+/R138C}$ mice (Fig. 1b) in a Mendelian fashion, and these mice exhibited no differences in appearance. Next, we assessed transcript and protein levels of Nudt15 in each

genotype mouse and found no difference between wild-type Nudt15 and Nudt15 R138C. This data indicates that intrinsic expression of Nudt15 R138C does not influence protein stability (Supplementary Figure 4a/b). Furthermore, we measured TGMP after incubation of TGTP with extract from packed red blood cells collected from *Nudt15*$^{+/+}$ or *Nudt15*$^{R138C/R138C}$ mice. TGMP production was reduced by approximately 35% in *Nudt15*$^{R138C/R138C}$ mice compared to *Nudt15*$^{+/+}$ mice, indicating that R138C partially, but not completely, impairs the enzyme activity of Nudt15.

**_Nudt15_$^{+/R138C}$ and _Nudt15_$^{R138C/R138C}$ mice are highly susceptible to MP**

To evaluate the association of Nudt15 R138C with thiopurine toxicity, *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, and *Nudt15*$^{R138C/R138C}$ mice were orally administered MP at 0.2, 1, or 2 mg/kg daily, which is equivalent to the clinical dose. In the 2 mg/kg MP arm, *Nudt15*$^{R138C/R138C}$ mice exhibited early death (median survival of 15 days and 21 days in strains 115 and 69, respectively) (Fig. 2a/b; $p < 0.0001$ by log-rank test in both strains). *Nudt15*$^{+/R138C}$ mice also died earlier than *Nudt15*$^{+/+}$ mice, although their median survival time was approximately twice as long as that of *Nudt15*$^{R138C/R138C}$ mice (median survival of 30 days in both strains 115 and 69). By reducing the MP dose to 1 mg/kg, the survival time

of *Nudt15*$^{R138C/R138C}$ mice and *Nudt15*$^{+/R138C}$ mice was prolonged by up to twofold (median survival of 26 days and 66 days, respectively, in strain 115, and 21 days and 48 days, respectively, in strain 69). Upon administration of 0.2 mg/kg MP, all mice survived longer than 70 days. These data indicated that *Nudt15*$^{R138C}$-harboring mice exhibit high susceptibility to MP, with susceptibility depending on the MP dose and the number of *Nudt15*$^{R138C}$ alleles.

**MP induces cytopenia in *Nudt15*$^{+/R138C}$ and *Nudt15*$^{R138C/R138C}$ mice**

To evaluate hematological toxicity, we determined peripheral blood cell counts every week after starting MP administration. *Nudt15*$^{R138C/R138C}$ mice exhibited severe pancytopenia 14 days after starting 2 mg/kg MP orally (Fig. 3a and Supplementary Figure 6a). *Nudt15*$^{+/R138C}$ mice also showed pancytopenia, to the midpoint level between *Nudt15*$^{+/+}$ and *Nudt15*$^{R138C/R138C}$ mice. Pancytopenia was alleviated by reducing the MP dose to 1 mg/kg and was hardly observed upon administration of 0.2 mg/kg MP for 70 days. Next, we conducted differential counts of leukocytes, including neutrophils, T lymphocytes, and B lymphocytes, by FACS analysis (Fig. 3b). Especially, neutrophils were moderately or severely depleted in *Nudt15*$^{+/R138C}$ mice or *Nudt15*$^{R138C/R138C}$ mice, respectively, after

administration of 2 mg/kg MP for 14 days (Fig. 3c and Supplementary Figure 6b). Severe neutropenia was observed in *Nudt15*$^{R138C/R138C}$ mice after 1 or 2 mg/kg MP administration for 7 days, but not after 0.2 mg/kg MP administration for 70 days. These data clearly indicated that our mouse model phenocopies the clinical observations of severe or moderate acute leukopenia in *NUDT15*$^{R139C}$ homozygous patients or heterozygous patients, respectively, and that dose reduction relieves the hematologic toxicity of MP.

**MP rapidly impairs HSPCs in *Nudt15*$^{R138C/R138C}$ mice**

The rapid occurrence of neutropenia and anemia at high doses of MP predicts the development of BM suppression. Thus, we investigated BM histology in the steady state and after treatment with 5 mg/kg MP for 5 days. HE staining revealed no histological differences among mice with different genotypes in the steady state (Supplementary Figure 7a), but we observed severe decreases in nucleated BM cells in *Nudt15*$^{R138C/R138C}$ mice after MP exposure (Fig. 4a). Similarly, HSCs, uncommitted hematopoietic progenitor cells (HPCs) including MPPs and LMPPs, and myeloid-committed HPCs, including CMPs, GMPs, and MEPs, were stored in all genotypes in the steady state (Supplementary Figure 7b), whereas the frequencies of HSCs, LMPPs, CMPs, GMPs, and MEPs were significantly

decreased in *Nudt15*$^{R138C/R138C}$ mice after oral administration of 2 mg/kg MP for 5 days (Fig. 4b/c). Although MP is also known to exert toxicity to organs other than BM[13, 18], no gross abnormalities were observed in other organs, including the esophagus, stomach, intestines, liver, lungs, and kidneys, after administration of 5 mg/kg MP (Supplementary Figure 8). These data suggest that both HSCs and HPCs can be impaired at clinically relevant doses of MP in *NUDT15*$^{R139C}$ homozygous patients.

**_Nudt15_$^{+/R138C}$ and _Nudt15_$^{R138C/R138C}$ HSPCs are exhausted after long-term exposure to low-dose MP**

We investigated the tolerance of *Nudt15*$^{+/R138C}$ and *Nudt15*$^{R138C/R138C}$ HSPCs to long-term exposure to a low dose of MP. To enable precise comparison with wild-type HSCs, a competitive transplantation method was adopted. Briefly, BM cells from *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, or *Nudt15*$^{R138C/R138C}$ mice (Ly5.2-positive) were transplanted with the same number of BM cells from congenic mice (Ly5.1-positive) into recipient mice (Ly5.1- and Ly5.2-positive), which were orally treated with various doses of MP from 5 weeks after transplantation (Supplementary Figure 9). Peripheral leukocytes and BM cells were analyzed by FACS to assess the chimeric state, which was calculated as the ratio of

Ly5.2-positive donor cells to Ly5.1-positive competitor cells. Neutrophil chimerism analysis showed that *Nudt15*$^{R138C/R138C}$ neutrophils were nearly completely depleted by day 14 after starting administration of 1 or 2 mg/kg MP (Fig. 5a and Supplementary Figure 10). Even in the case of 0.2 mg/kg MP, the chimeric ratio in *Nudt15*$^{R138C/R138C}$ neutrophils decreased to approximately half of that in *Nudt15*$^{+/+}$ neutrophils after administration for 28 days. Half or complete depletion of *Nudt15*$^{+/R138C}$ neutrophils was also observed after administration of 1 mg/kg MP for 14 or 28 days, respectively. HSPC chimerism analysis revealed that *Nudt15*$^{R138C/R138C}$ uncommitted HSPCs, such as Lineage$^{-}$Sca-1$^{+}$c-Kit$^{+}$ cells (LSKs), and myeloid-committed progenitors, such as CMPs and GMPs, were completely depleted after administration of 1 mg/kg MP for 28 days (Fig. 5b and Supplementary Figure 11). Even when the MP dose was reduced to 0.2 mg/kg, all *Nudt15*$^{R138C/R138C}$ HSPC fractions were depleted by more than 70%. In *Nudt15*$^{+/R138C}$ HSPC fractions, nearly complete or approximately half depletion was observed after administration of 1 mg/kg MP or 0.2 mg/kg MP, respectively, for 28 days. Finally, we calculated the reduction rate of each *Nudt15*$^{+/R138C}$ or *Nudt15*$^{R138C/R138C}$ HSPC fraction to the corresponding congenic wild-type HSPC fraction (Fig. 5c). The MP dose required for a 50% reduction was 0.23 or 0.12 mg/kg in *Nudt15*$^{+/R138C}$ LSKs or *Nudt15*$^{R138C/R138C}$ LSKs, respectively, 0.19 or 0.099 mg/kg

in *Nudt15*$^{+/R138C}$ CMPs or *Nudt15*$^{R138C/R138C}$ CMPs, respectively, and 0.23 or 0.11 mg/kg in *Nudt15*$^{+/R138C}$ GMPs or *Nudt15*$^{R138C/R138C}$ GMPs, respectively. These data suggested that *Nudt15*$^{+/R138C}$ HSPCs can tolerate twice the amount of MP tolerated by *Nudt15*$^{R138C/R138C}$ HSPCs, but they may be seriously damaged after long-term administration of relatively low-dose MP.

**MP impairs *Nudt15*$^{+/R138C}$ or *Nudt15*$^{R138C/R138C}$ leukemia cells in a posttransplantation leukemia recurrence model**

We examined whether MP could be a possible option for *NUDT15*$^{R139C}$ homozygous patients with hematopoietic malignancies by exploiting the high susceptibility to MP. To this end, we generated a posttransplantation leukemia recurrence model by applying the MLL-AF9 leukemia model[32, 33]. Briefly, 5 000 *Nudt15*$^{+/+}$ or *Nudt15*$^{R138C/R138C}$ leukemia stem and progenitor cells (Ly5.2$^{+}$Lineage$^{-}$Kit$^{+}$) were sorted from mice that had developed leukemia by transplantation of MLL-AF9-transduced cells, and were transplanted into genotypically concordant mice together with two million normal BM cells from congenic mice. In these mice, peripheral blood was dominated by cells (Ly5.1$^{+}$Ly5.2$^{-}$) derived from congenic BM cells two weeks after transplantation, but by recipient phenotype cells (Ly5.1$^{-}$

Ly5.2$^{+}$) approximately four weeks after transplantation, and the mice exhibited splenomegaly due to leukemic cell infiltration (Fig. 6a). Therefore, these mice reflect patients who experience leukemia relapse after transplantation from a NUDT15 wild-type donor. The mice were randomly assigned to a control arm and an MP arm on day 15 after transplantation. In the control arm orally administered vehicle, posttransplantation *Nudt15*$^{+/+}$ and *Nudt15*$^{R138C/R138C}$ leukemia recurrence mice died due to leukemia development within 30 days after transplantation (Fig. 6a). Oral administration of 1 mg/kg MP significantly prolonged the survival of posttransplantation *Nudt15*$^{R138C/R138C}$ leukemia recurrence mice (median: 21 days vs. 34 days in control vs. MP, $p$ = 0.0002), but not posttransplantation *Nudt15*$^{+/+}$ leukemia recurrence mice (median: 26 days vs. 26 days in control vs. MP). These data demonstrated that support by normal wild-type hematopoiesis allows the administration of a lethal dose of MP for *Nudt15*$^{+/+}$ mice and that the difference in susceptibility to MP between *Nudt15*$^{R138C/R138C}$ leukemia cells and wild-type normal donor HSPCs can be exploited for leukemia treatment. Moreover, to evaluate the effect of early intervention, we transplanted a smaller number of leukemia stem and progenitor cells and analyzed spleens 12 days after starting administration. Splenomegaly due to leukemic cell infiltration was suppressed by administration of 1 mg/kg MP in posttransplantation

*Nudt15*$^{R138C/R138C}$ leukemia recurrence mice (Fig. 6b). The survival time of posttransplantation *Nudt15*$^{R138C/R138C}$ leukemia recurrence mice was prolonged in the MP arm (median: 40 days vs. 63 days in control vs. MP, $p = 0.0002$). These data suggested that the antileukemia effect of MP may be more effective if treatment is initiated earlier.

## Discussion

We generated *Nudt15*$^{R138C}$ knock-in mice that exhibit thiopurine-mediated cytopenia in a *Nudt15*$^{R138C}$ allele number-dependent manner, which is alleviated by reducing the dose of MP. The enzyme activity involved in thiopurine metabolism is known to vary across races. Particularly, TPMT enzyme activity is reported to be lower in Japanese patients than in Caucasians[34-37]. This may reduce the impact of TPMT and explains why an association between *TPMT* SNPs and leukopenia is found in Caucasians, but not Japanese patients. Similarly, TPMT enzyme activity varies across mouse backgrounds[38, 39]. Therefore, to more precisely reflect the patient background, we aimed to establish a physiological knock-in mouse model with relatively low TPMT enzyme activity. In this context, we established *Nudt15*$^{R138C}$ knock-in mice in the C57BL/6 background with low TPMT activity, not in the FVB/N background, which exhibits high TPMT activity and is adopted in the *Nudt15* knock-out mouse model. Finally, our model showed no failure in any organ other than the BM; we did not find esophagus injury, which is observed in *Nudt15* knock-out mice[23], nor hepatotoxicity, which is frequently caused by thiopurines and significantly associated with *TPMT* SNPs[13, 18]. In addition, our model successfully provided safer estimates of MP dose for *NUDT15*$^{R139C}$ homozygous or heterozygous patients. *Nudt15*$^{R138C/R138C}$ mice survived

for at least 70 days when orally administered 0.2 mg/kg MP. A dose of 0.2 mg/kg in mice is equivalent to approximately 0.016 mg/kg in an adult human[40]. This estimate clearly supports the previous report that 1–2 mg per whole body was estimated as a safer initial daily dose of MP for *NUDT15*$^{R139C}$ homozygous adult patients[18]. Furthermore, although the susceptibility to MP in *Nudt15*$^{+/-}$ mice was not reported[23], in our study, half of the *Nudt15*$^{+/R138C}$ mice exhibited late-onset death at 1 mg/kg MP, suggesting that it is presumably safer to start treatment at 0.08 mg/kg or less in *NUDT15*$^{R139C}$ heterozygous adult patients. Together, these findings indicate that our model sufficiently phenocopies thiopurine-induced myelosuppression in patients with the *NUDT15*$^{R139C}$ allele to contribute to *NUDT15* genotype-based precision medicine.

Our data indicated that not only HPCs, but also HSCs were impaired in *Nudt15*$^{R138C/R138C}$ mice treated short-term with a clinically relevant dose of MP, implying that HSCs suffer acute MP damage in *NUDT15*$^{R139C}$ homozygous patients. Previous reports demonstrated that NUDT15 R139C or loss of Nudt15 enhanced thiopurine-induced cytotoxicity[20, 23], but did not clarify whether HSPCs with the SNP allele were more severely impaired by thiopurines. As HPCs are highly proliferative whereas HSCs are generally dormant, cytotoxic agents that act during DNA synthesis can rapidly injure HPCs, but

require a longer time to impair HSCs[41, 42]. Therefore, acute damage of *Nudt15*$^{R138C/R138C}$ HSCs by MP may be caused in a cell cycle-independent manner. Further studies will be needed to clarify the mechanism underlying the acute damage in *Nudt15*$^{R138C/R138C}$ HSCs and the alterations of thiopurine metabolism. On the other hand, our competitive transplantation assay demonstrated that LSKs, which include HSCs and uncommitted progenitors, were impaired by long-term administration of low-dose MP. The MP dose for a 50% reduction in *Nudt15*$^{+/R138C}$ or *Nudt15*$^{R138C/R138C}$ LSKs was 0.23 mg/kg or 0.12 mg/kg, respectively, which was equivalent to that in genotypically concordant CMPs and GMPs. It is known that the majority of HSCs exit the $G_0$ phase and enter the cell cycle in response to long-term BM injury by a cytotoxic agent, such as 5-fluorouracil[42]. As cycling HSCs become sensitive to cytotoxic agents, long-term exposure of MP could lead to *Nudt15*$^{+/R138C}$ or *Nudt15*$^{R138C/R138C}$ HSC damage, even at low doses. Together, our data suggest that MP should be carefully administered to *NUDT15*$^{R139C}$ heterozygous or homozygous patients in long-term maintenance therapy.

We established a *Nudt15* genotype-based posttransplantation leukemia recurrence model. In this model, BM of *Nudt15*$^{+/+}$ or *Nudt15*$^{R138C/R138C}$ mice was replaced with normal wild-type BM cells from congenic mice and a small number of genotypically concordant

MLL-AF9 leukemia stem and progenitor cells. Consequently, these mice died due to leukemia development after acquiring the engraftment of normal BM-cells dominance, reflecting leukemia recurrence after transplantation from a *NUDT15* wild-type donor. However, MP significantly prolonged the survival of mice with posttransplantation *Nudt15*$^{R138C/R138C}$ leukemia recurrence. Furthermore, lethal doses of MP for *Nudt15*$^{R138C/R138C}$ mice were tolerable upon normal wild-type hematopoiesis. A previous study using a different leukemia model based on combination of *Arf*$^{-/-}$ and *BCR-ABL1* reported that MP might be administrable in *Nudt15*$^{-/-}$ leukemia mice[23]; however, safety has remained a major issue because the observation period was too short. In fact, several reports have referred to the impossibility of MP administration to safely treat leukemia patients with NUDT15 R139C[20, 43]. In addition, the high susceptibility of *Nudt15*$^{+/R138C}$ or *Nudt15*$^{R138C/R138C}$ HSCs to low-dose MP implies that MP does not preferentially kill leukemia cells and consequently, might eliminate HSCs impaired by leukemia. Together, our data indicate that NUDT15-based thiopurine myelotoxicity can be safely and maximally exploited in leukemia treatment with the support of normal wild-type HSCs in *NUDT15*$^{R139C}$-harboring patients. Further clinical studies are warranted to investigate whether MP is effective for *NUDT15*$^{R139C}$ heterozygous or homozygous leukemia patients

who have recurrence or a high risk of relapse after allogenic hematopoietic stem cell transplantation from *NUDT15* wild-type donors.

In conclusion, our novel *Nudt15*$^{R138C}$ knock-in mouse model provided safer estimates for MP dosing and highlighted the high susceptibility of HSPCs to MP. The conceptual finding that *NUDT15* genotype-based hematopoietic toxicity caused by MP can be converted to a leukemia treatment is expected to contribute to the development of novel leukemia treatments. Finally, our model will facilitate *NUDT15* genotype-based precision medicine for patients receiving thiopurines.

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**Author contributions**

G.T. maintained the mouse strains and performed transplantation and FACS sorting and analyses. M.K. designed the mouse model, conducted all experiments, analyzed data, provided funding and wrote the paper. T.I. maintained the mouse strains and performed histology and cytology experiments. A.N. designed the mouse model. A.A-N. performed some cytology experiments and FACS analyses. O.I. edited the paper. A.Y. provided the MLL-AF9 vector and helped the establishment of leukemia model. Y.K., K.K., and A.A wrote and edited the paper.

**Conflict of interest**

We have no conflicts of interest.

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## Figure legends

**Fig. 1.**

**Establishment of *Nudt15*$^{R138C}$ harboring mice.**

(A) Schematic diagram of the establishment of mice harboring the *Nudt15*$^{R138C}$ allele by gene editing. The target sites of different sgRNAs for strain 69 and strain 115 and the donor oligoDNA for converting c.412 C to T are indicated. (B) Validation of Nudt15 c.412 C>T by sequencing in strains 115 and 69. The base at c.412 is indicated in red.

**Fig. 2.**

**Overall survival of *Nudt15*$^{+/+}$, *Nudt15*$^{+/R138C}$, and *Nudt15*$^{R138C/R138C}$ mice upon MP administration.**

OS upon oral administration of 0.2, 1, or 2 mg/kg MP in strain 115 (top) and in strain 69 (bottom). In each arm, 7–11 mice were analyzed. The treatment and observation period was 70 days. $p < 0.0001$ for both strains (log-rank test).

**Fig. 3.**

**MP enhances cytopenia in *Nudt15*$^{+/R138C}$ and *Nudt15*$^{R138C/R138C}$ mice.**

(A) Peripheral blood cell counts after oral administration of 2 mg/kg MP for 7 or 14 days, 1 mg/kg MP for 7 or 14 days, or 0.2 mg/kg MP for 14, 28, 42, 56, or 70 days. Data are presented in box and whiskers with 10 to 90 percentile. Horizontal lines in boxes indicate the means. (B) Representative FACS plots of neutrophils, B lymphocytes, and T lymphocytes after oral administration of 2 mg/kg MP for 14 days. Populations and percentages are indicated by red boxes and in red font, respectively. (C) Summarized data of the number of neutrophils, B lymphocytes and T lymphocytes. Data are presented in box and whiskers with 10 to 90 percentile. Horizontal lines in boxes indicate the means. The data were obtained from strain 115. The data from strain 69 are presented in Supplementary Figure 6. In (A) and (C), 6–9 mice were analyzed in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (ANOVA followed by Tukey's test).

**Fig. 4.**

**BM failure in *Nudt15*$^{R138C/R138C}$ mice after short-term MP treatment.**

(A) BM histology after intraperitoneal injection of 5 mg/kg MP. HE staining after decalcification is presented. The genotype is indicated at the top of the panel. Scale bars indicate 50 μm. (B) Representative FACS plots of LK (Lineage$^{-}$Sca-1$^{-}$c-Kit$^{+}$), LSK

(Lineage$^{-}$Sca-1$^{+}$c-Kit$^{+}$), CMP (Lineage$^{-}$Sca-1$^{+}$c-Kit$^{+}$CD16/32$^{low}$CD34$^{low}$), GMP (Lineage$^{-}$Sca-1$^{+}$c-Kit$^{+}$CD16/32$^{+}$CD34$^{+}$), MEP (Lineage$^{-}$Sca-1$^{+}$c-Kit$^{+}$CD16/32$^{-}$CD34$^{-}$), HSCs (CD34$^{-/low}$Flt3$^{-}$Lineage$^{-}$Sca-1$^{+}$c-Kit$^{+}$), MPP (CD34$^{+}$Flt3$^{-}$Lineage$^{-}$Sca-1$^{+}$c-Kit$^{+}$), and LMPP (CD34$^{+}$Flt3$^{+}$Lineage$^{-}$Sca-1$^{+}$c-Kit$^{+}$). Each population is indicated and labeled by red boxes and red characters. Two million BM cells were analyzed by FACS after oral administration of 2 mg/kg MP for 5 days. (C) Summarized data of cell numbers in each HSPC population. The X- and Y-axis indicate the *Nudt15* genotype and the number of cells in each population, respectively. For each genotype, 5–6 mice were analyzed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (ANOVA followed by Tukey's test).

**Fig. 5.**

**HSPC impairment assessed by competitive transplantation assay.**

(A) Neutrophil chimerism (Ly-6G$^{+}$MAC1$^{-}$). Chimerism was calculated by dividing the number of Ly5.2$^{+}$ cells by the number of Ly5.1$^{+}$ cells. The X-axis indicates the number of days of administration of MP. The Y-axis indicates the ratio of neutrophil chimerism on indicated days compared to that on day 0. Mean and SD are presented. Representative FACS plots are shown in Supplementary Figure 10. (B) Chimerism in LSKs, CMPs, and

GMPs after MP administration for 28 days. The X-axis indicates the number of MP doses. The Y-axis indicates the ratio of each HSPC chimerism for indicated MP doses compared to that for 0 mg/kg. The mean and SD are presented. Representative FACS plots are shown in Supplementary Figure 11. In each group, 3–5 mice were analyzed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ (ANOVA followed by Tukey's test). (C) The reduction curves for *Nudt15*$^{+/R138C}$ or *Nudt15*$^{R138C/R138C}$ LSKs, CMPs, and GMPs. The X- and Y-axis indicate the same as in (B). In each group, 2–3 mice were analyzed. The curve was drawn with GraphPad Prism version 6.

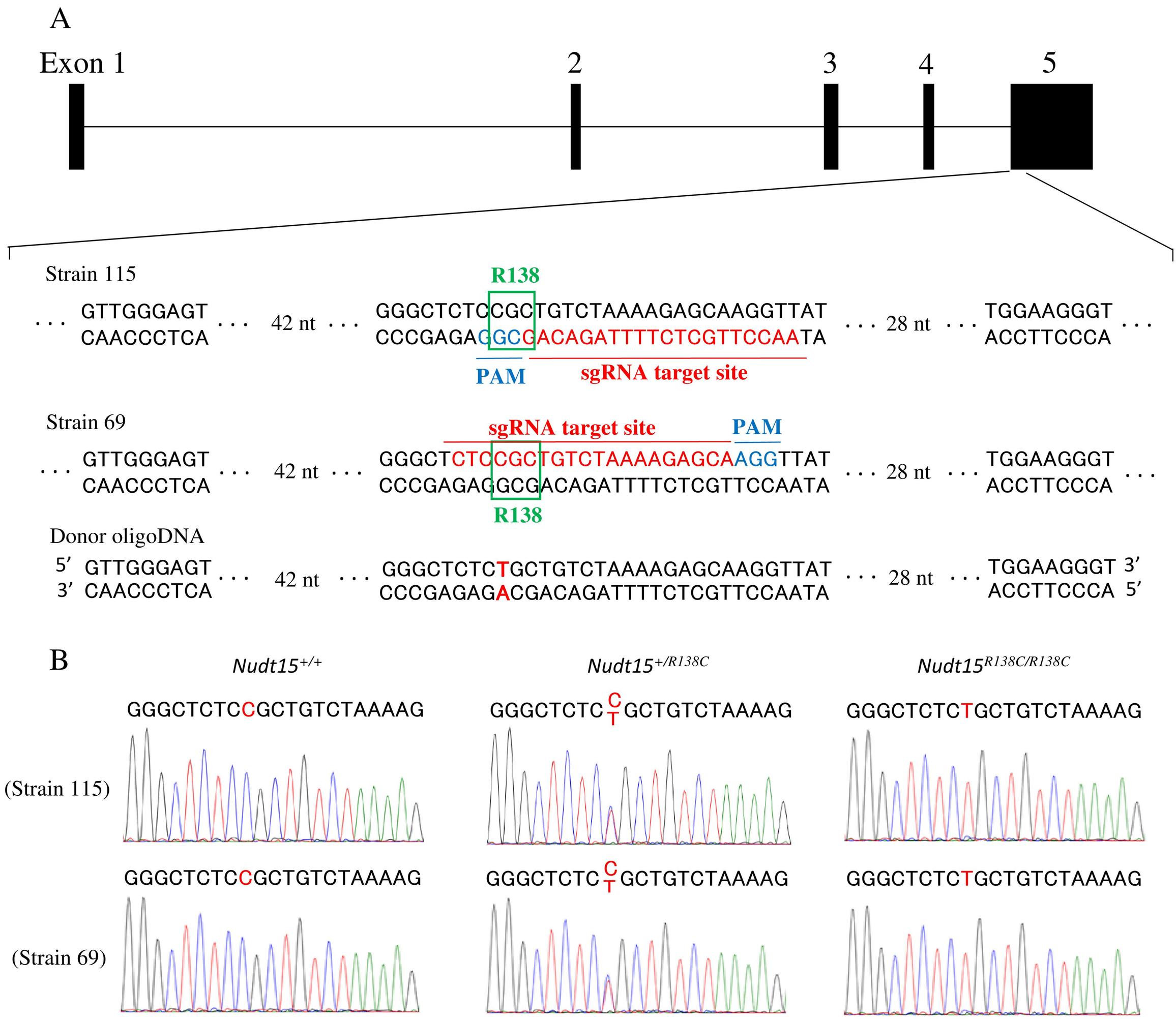
**Fig. 6.**

**Antileukemic efficacy of MP in posttransplantation *Nudt15*$^{+/+}$ or *Nudt15*$^{R138C/R138C}$ leukemia recurrence mice.**

(A) A posttransplantation *Nudt15*$^{+/+}$ or *Nudt15*$^{R138C/R138C}$ leukemia recurrence mouse model was generated by injection of 5 000 MLL-AF9 leukemia stem cells and progenitors. In upper figures, FACS data in the left panels indicate that congenic (Ly5.1$^{+}$Ly5.2$^{-}$) cells were dominant in peripheral blood at 14 days after transplantation, indicating normal BM engraftment, while recipient phenotype (Ly5.1$^{-}$Ly5.2$^{+}$) cells were dominant when mice

became moribund approximately two weeks later. Right panels present spleen size and cytology of spleen cells as assessed by Wright–Giemsa staining. Bottom figures present survival curves of posttransplantation *Nudt15*$^{+/+}$ and *Nudt15*$^{R138C/R138C}$ leukemia recurrence mice by starting at 1 mg/kg MP or DMSO from day 15. (B) A posttransplantation *Nudt15*$^{+/+}$ or *Nudt15*$^{R138C/R138C}$ leukemia recurrence mouse model was generated by injection of 250 MLL-AF9 leukemia stem cells and progenitors. Upper figures present spleen size and FACS plot of spleen cells 12 days after starting 1 mg/kg MP. Bottom figures present survival curves of posttransplantation *Nudt15*$^{+/+}$ and *Nudt15*$^{R138C/R138C}$ leukemia recurrence mice by starting 1 mg/kg MP or DMSO from day 15. In each arm, 7–12 mice were analyzed. *p*-Values were calculated by the log-rank test. One scale of the ruler indicates 1 mm.

Fig. 1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Exon 1
2
3
4
5
Strain 115
R138
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28 nt
TGGAAGGGT
ACCTTCCCA
PAM
sgRNA target site
Strain 69
sgRNA target site
PAM
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R138
Donor oligoDNA
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3' CAACCCTCA
GGGCTCTCTGCTGTCTAAAAGAGCAAGGTTAT
CCCGAGAGACGACAGATTTTCTCGTTCCAATA
TGGAAGGGT 3'
ACCTTCCCA 5'
B
Nudt15+/+
Nudt15+/R138C
Nudt15R138C/R138C
GGGCTCTCCGCTGTCTAAAAG
GGGCTCTC C/T GCTGTCTAAAAG
GGGCTCTCTGCTGTCTAAAAG
(Strain 115)
GGGCTCTCCGCTGTCTAAAAG
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GGGCTCTCTGCTGTCTAAAAG
(Strain 69)

Fig. 2

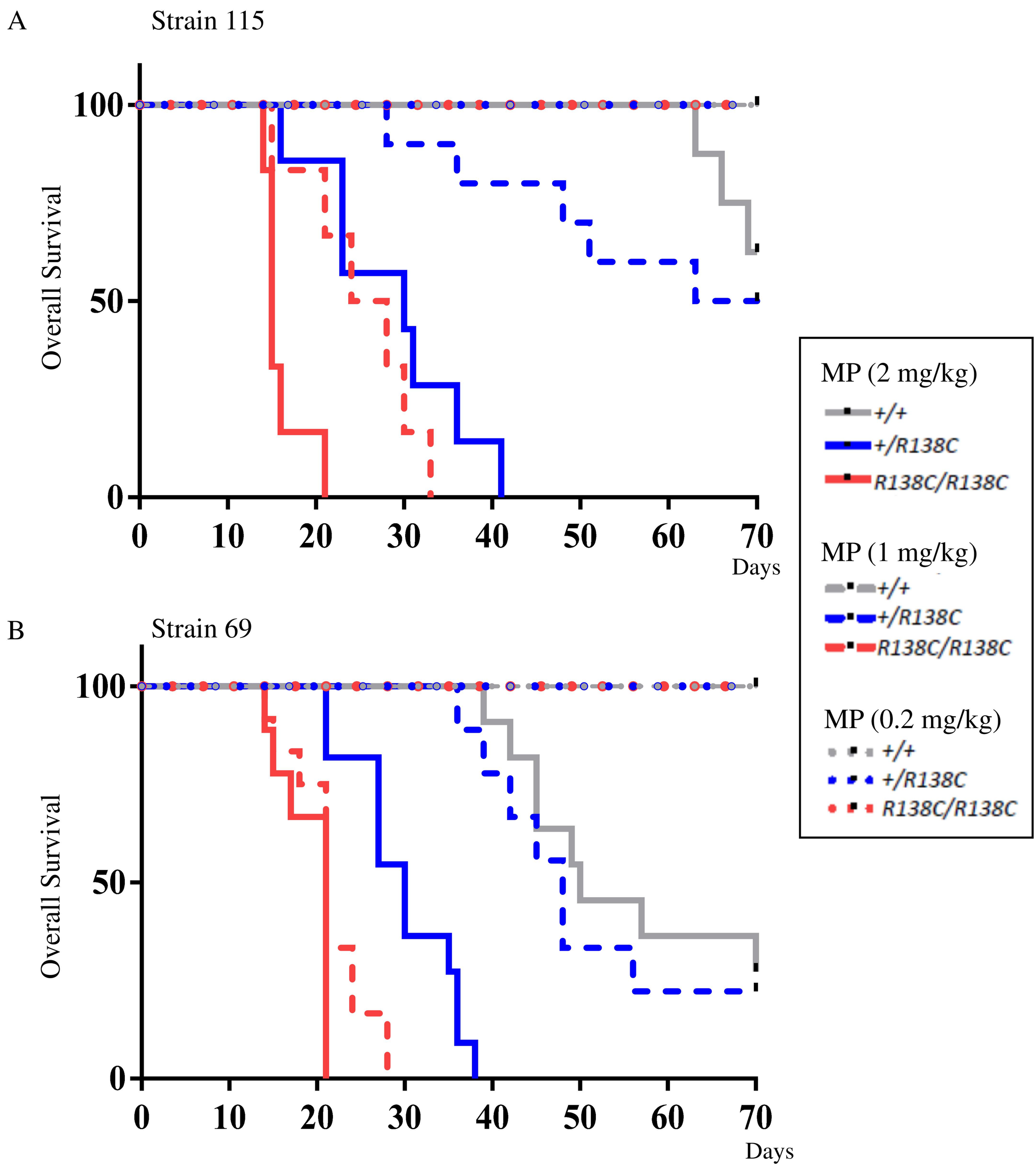
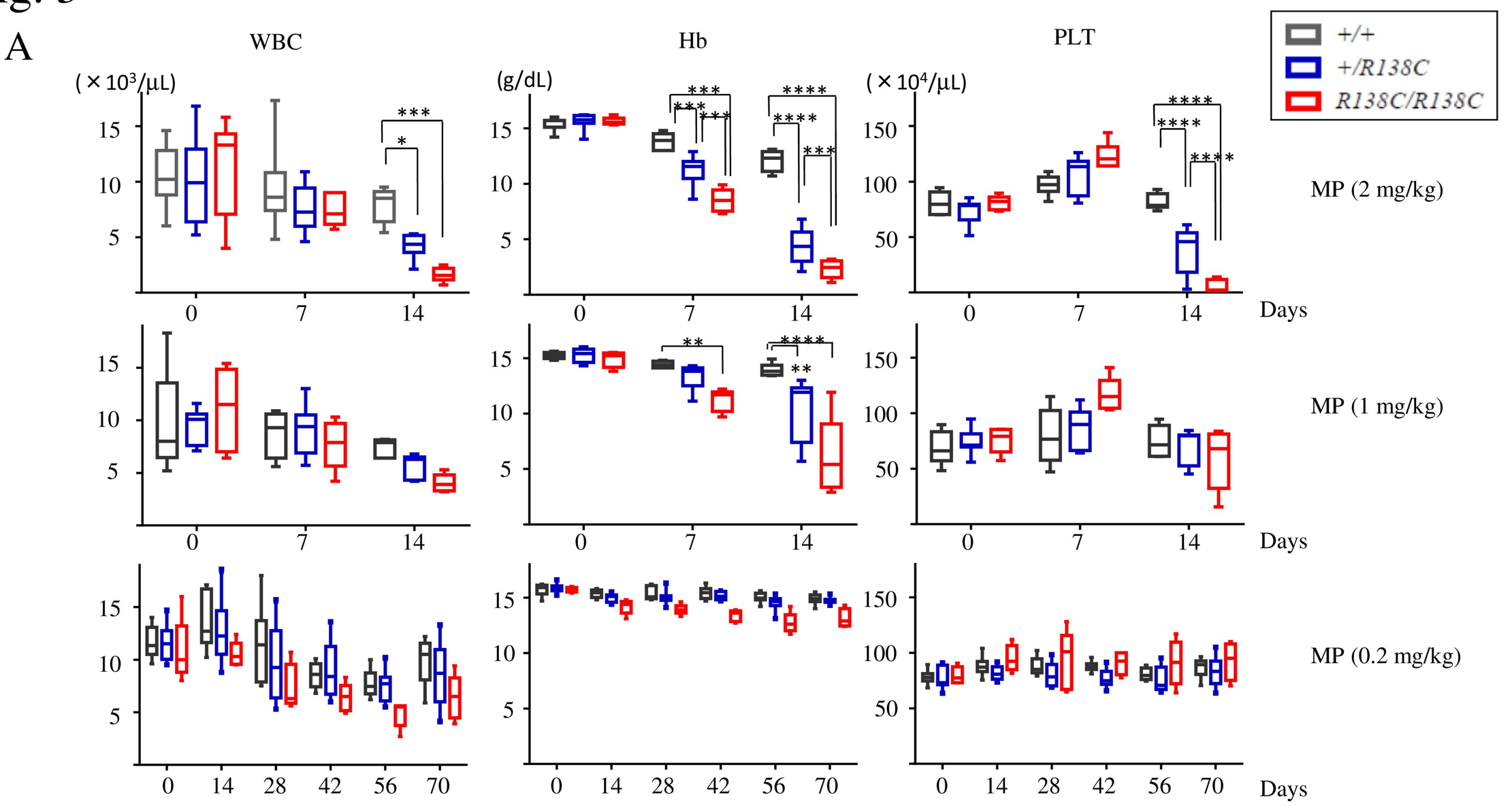

A
Strain 115
Overall Survival
100
50
0
0
10
20
30
40
50
60
70
Days
B
Strain 69
Overall Survival
100
50
0
0
10
20
30
40
50
60
70
Days
MP (2 mg/kg)
+/+
+/R138C
R138C/R138C
MP (1 mg/kg)
+/+
+/R138C
R138C/R138C
MP (0.2 mg/kg)
+/+
+/R138C
R138C/R138C

Fig. 3


A
WBC
Hb
PLT
(× 10³/μL)
(g/dL)
(× 10⁴/μL)
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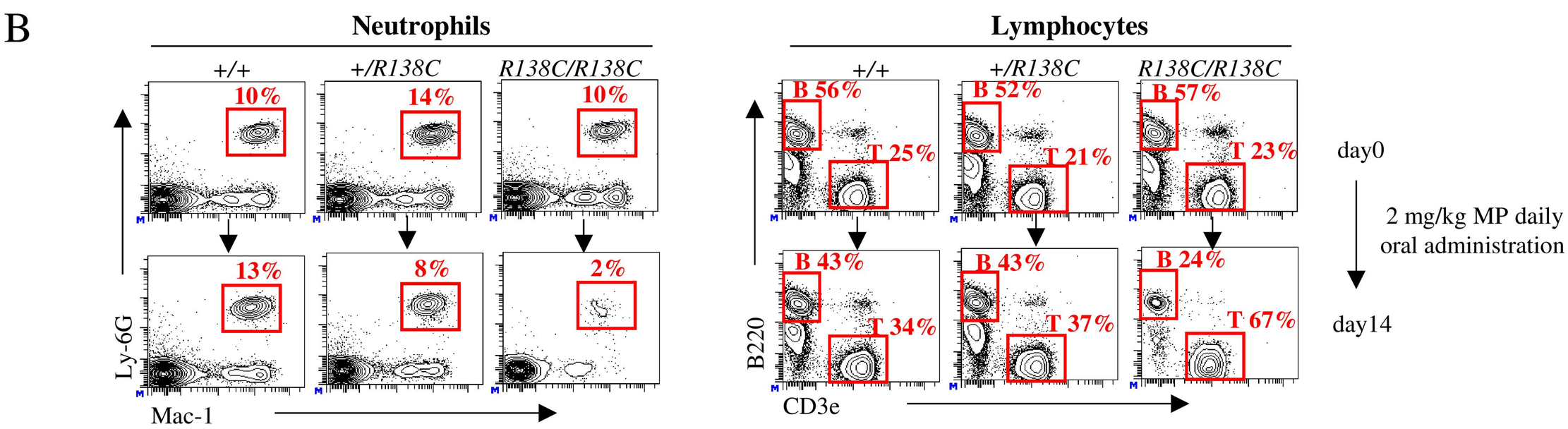
R138C/R138C
MP (2 mg/kg)
MP (1 mg/kg)
MP (0.2 mg/kg)
Days


B
Neutrophils
Lymphocytes
+/+
+/R138C
R138C/R138C
10%
14%
10%
13%
8%
2%
B 56%
T 25%
B 52%
T 21%
B 57%
T 23%
B 43%
T 34%
B 43%
T 37%
B 24%
T 67%
Ly-6G
Mac-1
B220
CD3e
day0
2 mg/kg MP daily
oral administration
day14

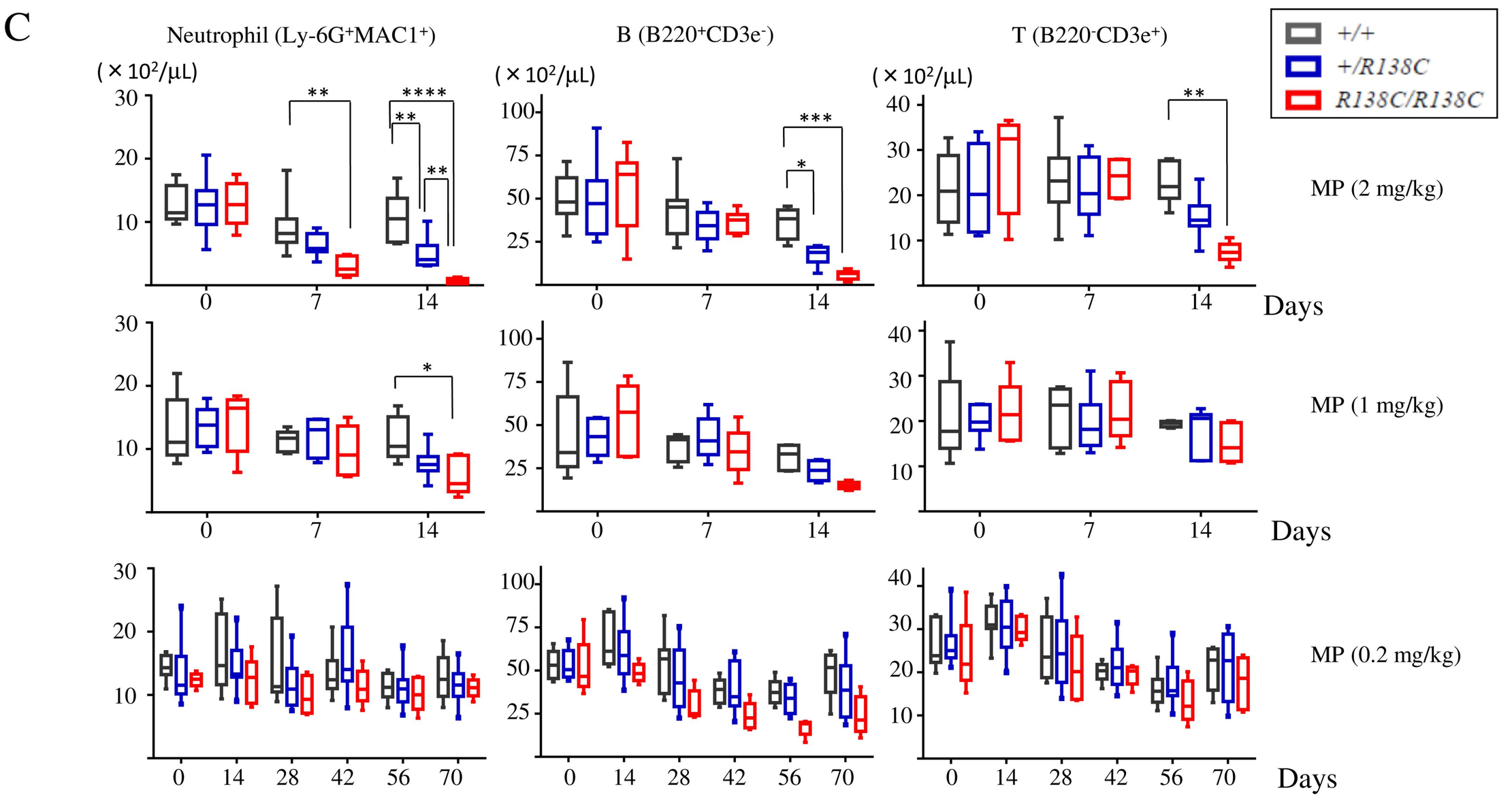
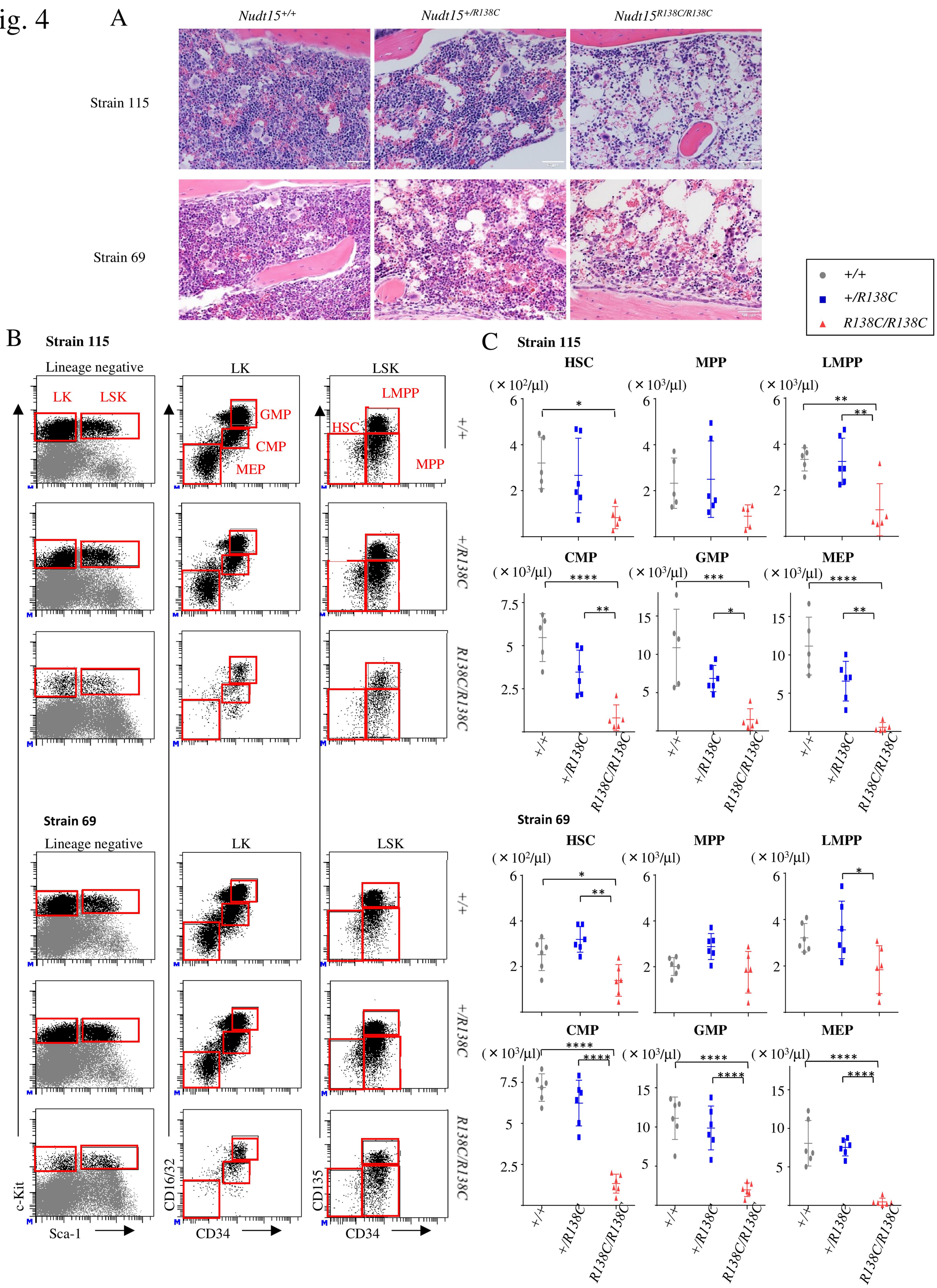

C
Neutrophil (Ly-6G⁺MAC1⁺)
B (B220⁺CD3e⁻)
T (B220⁻CD3e⁺)
(× 10²/μL)
+/+
+/R138C
R138C/R138C
MP (2 mg/kg)
MP (1 mg/kg)
MP (0.2 mg/kg)
Days

Fig. 4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Nudt15+/+
Nudt15+/R138C
Nudt15R138C/R138C
Strain 115
Strain 69
+/+
+/R138C
R138C/R138C
B
Strain 115
Lineage negative
LK
LSK
GMP
CMP
MEP
LMPP
HSC
MPP
Strain 69
c-Kit
Sca-1
CD16/32
CD34
CD135
C
HSC
MPP
LMPP
CMP
GMP
MEP
(× 10²/μl)
(× 10³/μl)
*
**
***
****

# Fig. 5

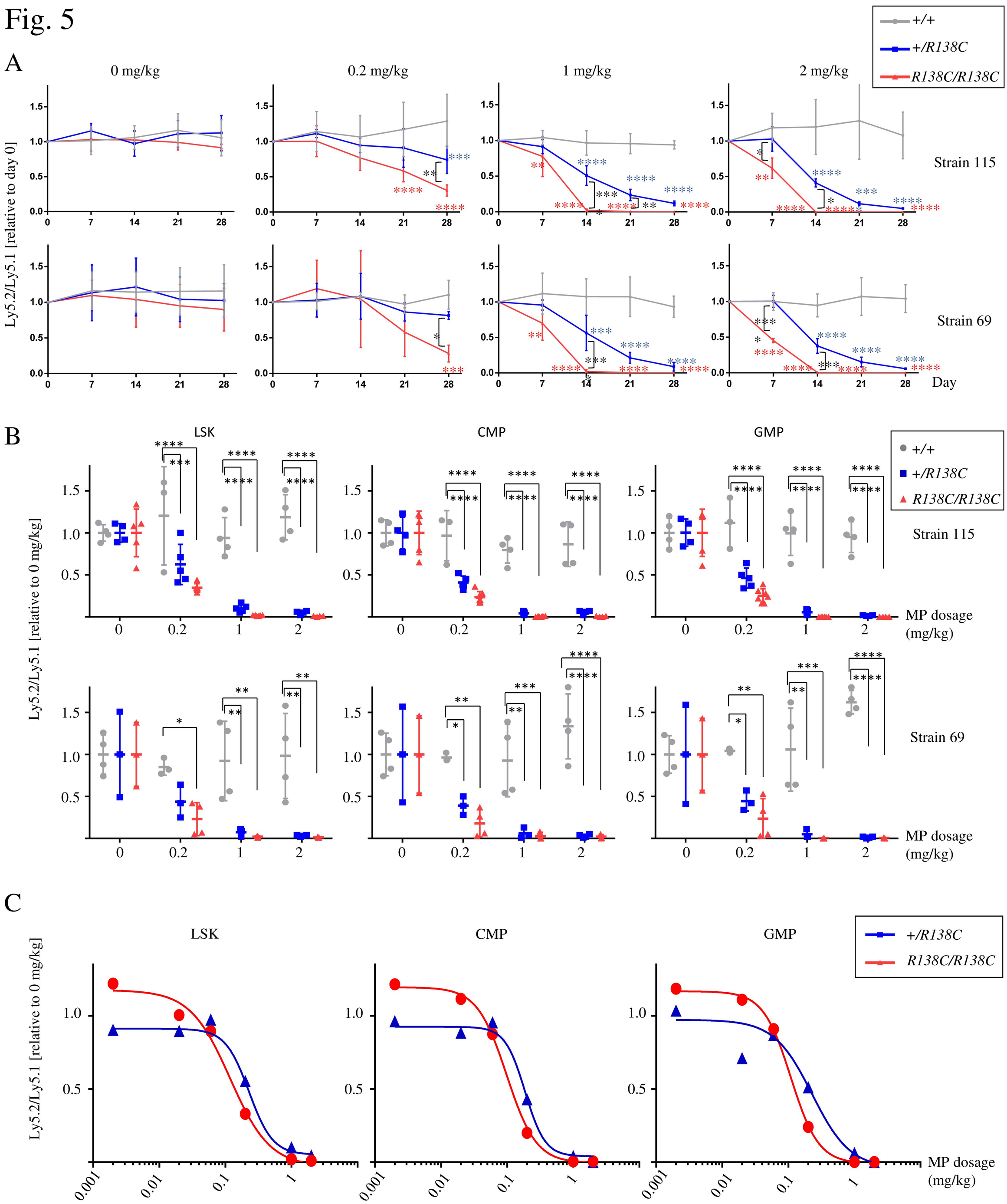
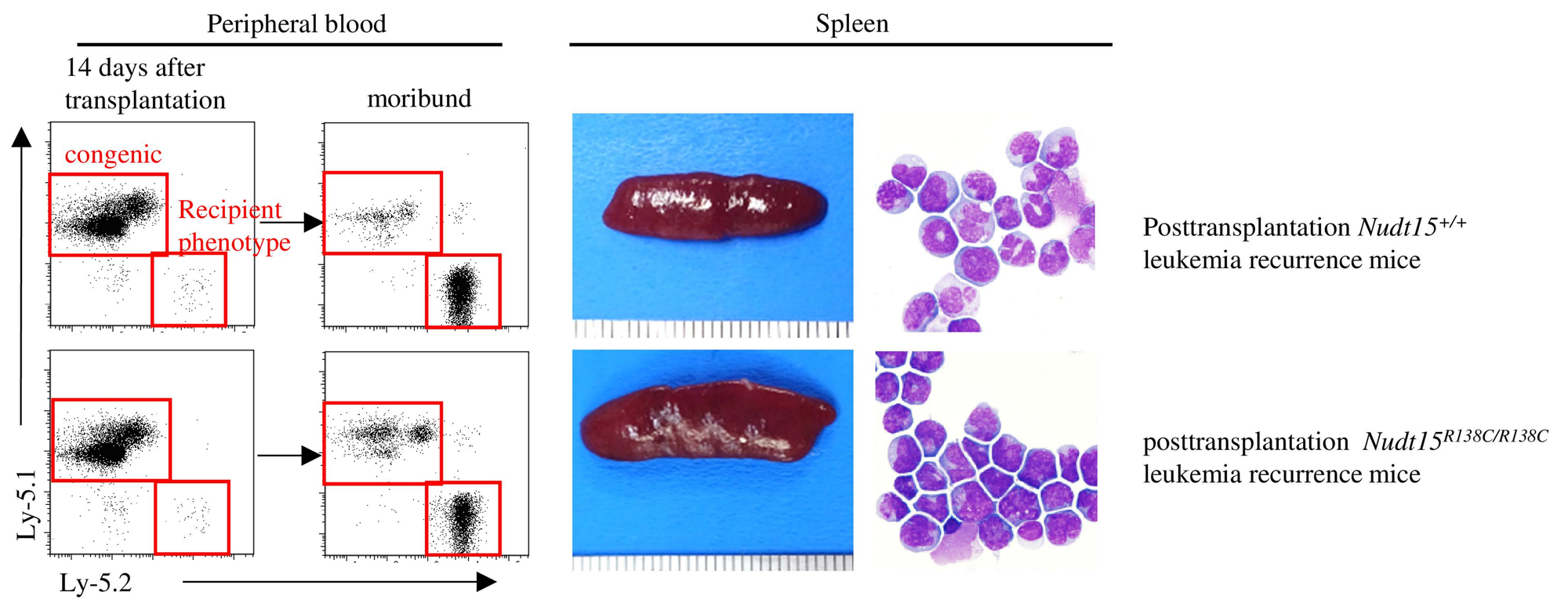
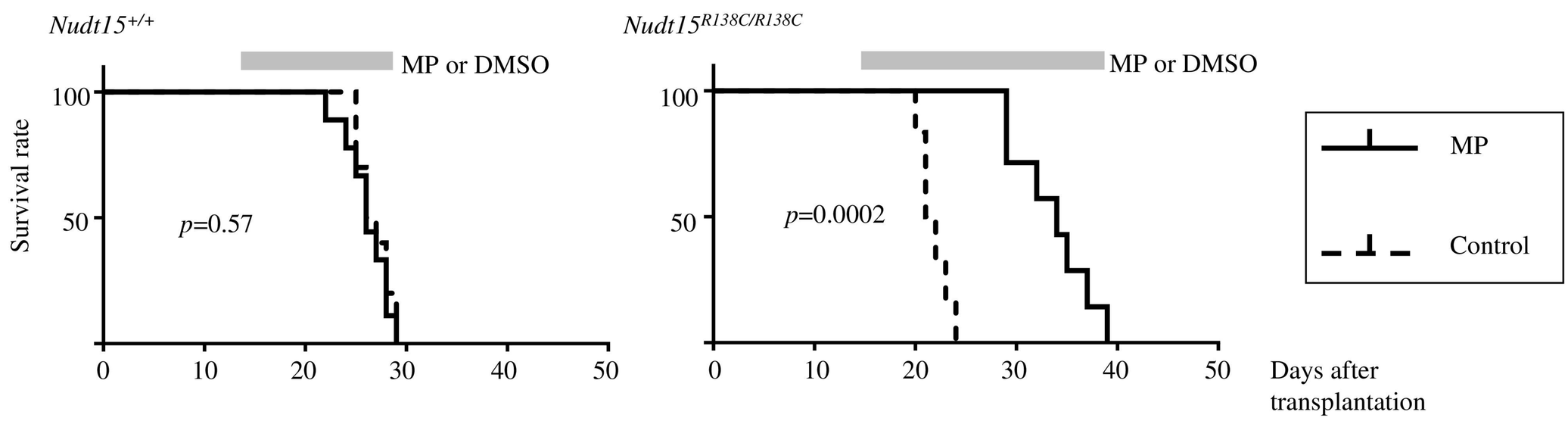

A
0 mg/kg
0.2 mg/kg
1 mg/kg
2 mg/kg
+/+
+/R138C
R138C/R138C
Ly5.2/Ly5.1 [relative to day 0]
Strain 115
Strain 69
Day
B
LSK
CMP
GMP
[relative to 0 mg/kg]
Ly5.2/Ly5.1
MP dosage (mg/kg)
C
LSK
CMP
GMP
Ly5.2/Ly5.1 [relative to 0 mg/kg]
MP dosage (mg/kg)

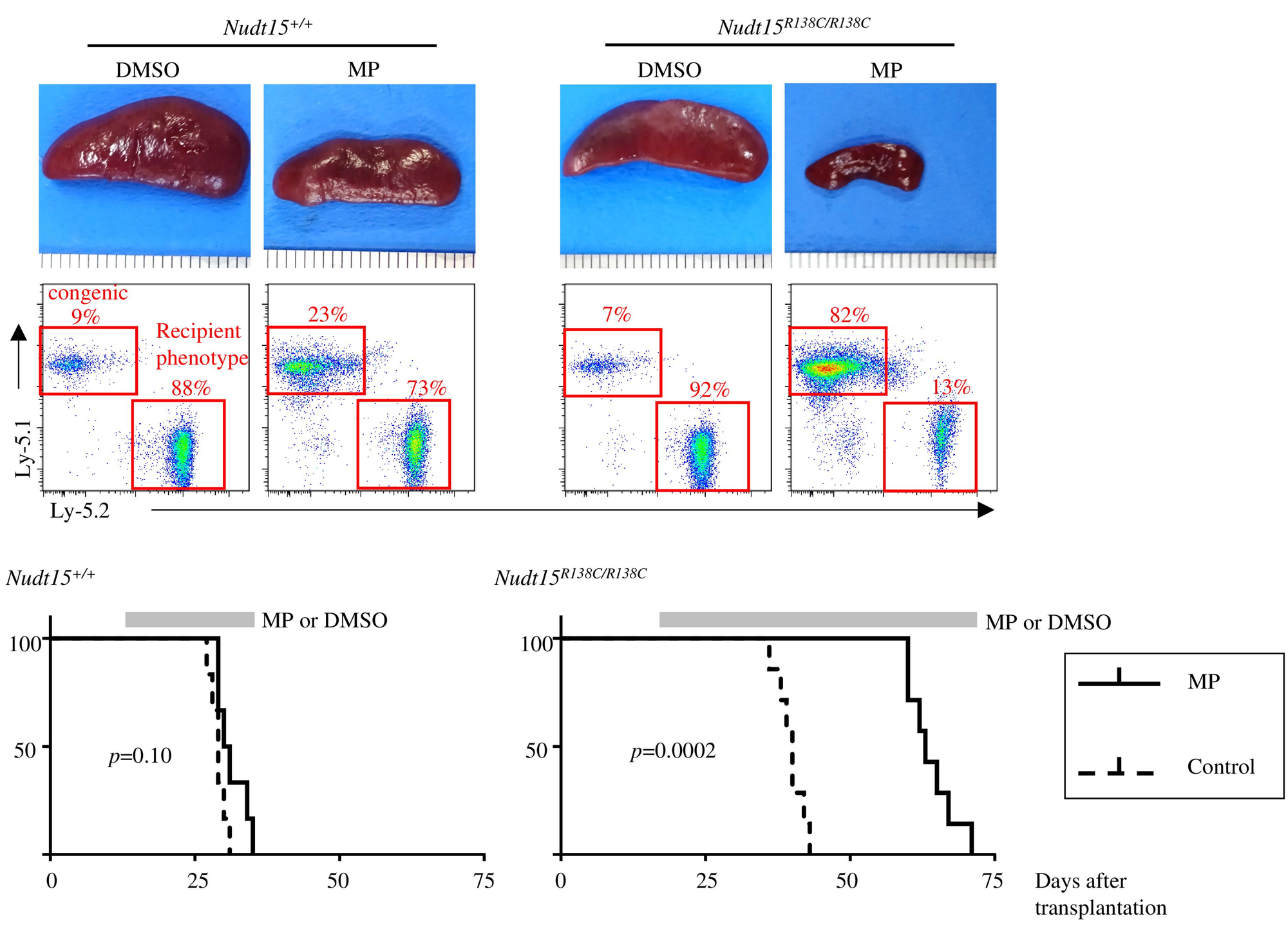
Fig. 6

A


Peripheral blood
Spleen
14 days after transplantation
moribund
congenic
Recipient phenotype
Ly-5.1
Ly-5.2
Posttransplantation Nudt15+/+ leukemia recurrence mice
posttransplantation Nudt15R138C/R138C leukemia recurrence mice


Nudt15+/+
Nudt15R138C/R138C
MP or DMSO
Survival rate
100
50
p=0.57
p=0.0002
0
10
20
30
40
50
Days after transplantation
MP
Control

B


Nudt15+/+
Nudt15R138C/R138C
DMSO
MP
congenic
9%
Recipient phenotype
88%
23%
73%
7%
92%
82%
13%
Ly-5.1
Ly-5.2
MP or DMSO
Survival rate
100
50
p=0.10
p=0.0002
0
25
50
75
Days after transplantation
MP
Control