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ORIGINAL ARTICLE

# Altered Gut Microbiota Composition in *Rag1*-deficient Mice Contributes to Modulating Homeostasis of Hematopoietic Stem and Progenitor Cells

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Hematopoietic stem and progenitor cells (HSPCs) can produce all kind of blood lineage cells, and gut microbiota that consists of various species of microbe affects development and maturation of the host immune system including gut lymphoid cells and tissues. However, the effect of altered gut microbiota composition on homeostasis of HSPCs remains unclear. Here we show that compositional change of gut microbiota affects homeostasis of HSPCs using *Rag1*$^{-/-}$ mice which represent lymphopenic condition. The number and proportions of HSPCs in *Rag1*$^{-/-}$ mice are lower compared to those of wild types. However, the number and proportions of HSPCs in *Rag1*$^{-/-}$ mice are restored as the level of wild types through alteration of gut microbiota diversity via transferring feces from wild types. Gut microbiota composition of *Rag1*$^{-/-}$ mice treated with feces from wild types shows larger proportions of family *Prevotellaceae* and *Helicobacterceae* whereas lower proportions of family *Lachnospiraceae* compared to unmanipulated *Rag1*$^{-/-}$ mice. In conclusion, gut microbiota composition of lymphopenic *Rag1*$^{-/-}$ mice is different to that of wild type, which may lead to altered homeostasis of HSPCs.

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Keywords: Hematopoietic stem and progenitor cells, gut microbiota, lymphopenic state

## INTRODUCTION

Hematopoietic stem and progenitor cells (HSPCs) are multipotent cells that capable of producing all adult blood cells throughout life (1,2). Among HSPCs, HSCs (hematopoietic stem cells) have long-term reconstruction capacity and can reconstruct whole circulation system over the lifespan (3, 4). Most of HSCs in the bone marrow require a dormant state to preserve their self-renewal capacity and to prevent stem cell exhaustion (5-7). Dormant HSCs stay on $G_0$ state in cell cycle and have very low cell metabolism (4), thus these cells do not contribute to maintenance of the homeostasis of the circulation and the immune system in steady states (8). Otherwise, homeostatic HSCs differentiate once every 28～36 days, which is comparatively shorter than dormant HSPCs (3,9). Only homeostatic HSCs are capable of keeping circulation and immune system at homeostasis during steady state via hematopoiesis that the generation of blood cell lineages. During heavy bleeding or in infectious or irradiated state, dormant HSCs are awakened and turn into an injury-activated state. Injury-activated

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Abbreviations: BM, bone marrow; HSPCs, hematopoietic stem and progenitor cells; HSC, hematopoietic stem cells; MPPs, multipoptent progenitors; LSK, Lineage$^-$ Sca-1$^+$ c-Kit$^+$; MPs, myeloid progenitors

HSCs have very high metabolism and have continuous cell cycle to compensate for massive blood loss (4).

Human intestine is colonized by 100 trillion microorganisms, thus interaction with microbiota shapes host immune system (10-13). Although there are lots of evidences suggesting that gut microbiota affects host immune system, whether altered composition of gut microbiota affects the homeostasis of HSPCs remained unknown. In this study, we show that compositional change of gut microbiota can alter the number of HSPCs. Our results suggest that gut commensal microorganism community is one of the important factors for regulating homeostasis of HSPCs in the bone marrow.

## MATERIALS AND METHODS

### Mice

Female C57BL/6 *Rag-1*-deficient mice (6 to 8 weeks of age) were purchased from the Charles River Breeding Laboratories (Japan). Mice were kept under specific pathogen free (SPF) conditions in animal care facility in POSTECH (Pohang, Republic of Korea). The procedure of animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at the POSTECH Biotech Center.

### Bacterial transference

Feces from wild type mice or colonic feces from *H. muridarum* mono-associated mice were diluted to 12 g/ml of PBS. Suspensions were placed for 3 min at room temperature. 400 $\mu$l of supernatant was injected to each *Rag-1*-deficient mice 2 times per week for 4 weeks by oral gavage.

### Flow cytometry

Bone marrow was collected by flushing femurs with RPIM containing 2% FBS. Cells were stained with a combination of anti-lineage (CD3e, B220, Gr-1, CD11b, NK1.1, and MHC II) APC, anti-c-Kit PE-cy7, anti-CD150 PerCP-eFluor710, anti-CD48 PE (eBioscience, USA), and anti-Sca-1 BV421 (Biolegend, USA). Flow cytometry was performed on a FACS Fortessa (BD Bioscience, USA) and Canto II and the data were analyzed using Flow Jo 3.0 (Tree Star, Ashland, OR).

### DNA extraction and meta 16S rRNA sequencing

Each 2.5 g fecal samples were placed in FastDNA SPIN Kit lysing matrix E tubes. 978 ml of sodium phosphate buffer and 122 ml MT buffer were added to each tube and mixed. Tubes were centrifuged at 14,000 g, 10 min. Supernatant was transferred to new tubes and added 250 $\mu$l Protein Precipitation Solution (PPS) and shaken 10 times by hand. Tubes were centrifuged at 14,000 g, 5 min. Supernatant was transferred to new tubes and added 1,000 $\mu$l Binding Matrix Suspension buffer. Tubes were placed on rotator for 2 min. Tubes were placed on a rack for 3 min. 500 $\mu$l of supernatant were removed Remaining amount of supernatant were suspended and transferred to a SPIN FILTER and centrifuged at 14,000 g, 5 min. The catch tubes were emptied. 500 $\mu$l of SEWS-M were added to each tube and resuspended gently. Tubes were centrifuged at 14,000 g, 2 min twice. Catch tubes were replaced with new tubes. 50 $\mu$l of DNase/Pyrogen-free water were added to each tube and resuspended gently. Tubes were centrifuged at 14,000 g, 1 min. DNA in catch tubes were stored at −20°C. PCR was performed with *Taq* DNA polymerase and primers to the V1 − V3 region of the 16S rRNA gene. Then, the amplified DNA was used as template for 454 GS Junior (Roche Diagnosis, Indianapolis, IN) pyrosequencing. Filter-passed 3,000 reads were subjected to operational taxonomic unit (OTU) analysis with the cutoff similarity of 97% identity by using CLcommunity™ softwere (14).

## RESULTS

### Lymphopenic condition down-regulates the number of HSPCs

Gut microbiota direct innate immune cell development via promoting hematopoiesis (15). In addition, *Rag1*$^{-/-}$ mice that lack all mature lymphocytes have different compositions of gut bacterial community compared to wild type mice (16,17). To check the number and proportion of the HSPCs in response to change of commensal microbial composition via a lymphopenia, we measured population of the bone marrow HSPCs of C57BL/6 female wild type and *Rag1*$^{-/-}$ mice. The absolute number of total bone marrow cells was decreased in *Rag1*$^{-/-}$ mice than wild type (Fig. 1A). As assessed by flow cytometry, the absolute number and proportions of LSK cells (Lineage$^-$ Sca-1$^+$ c-Kit$^+$), LT-HSCs (Long-term HSCs; Lineage$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^+$ CD48$^-$), and ST-HSCs (Short-term HSCs; Lineage$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^+$ CD48$^+$) were dramati-

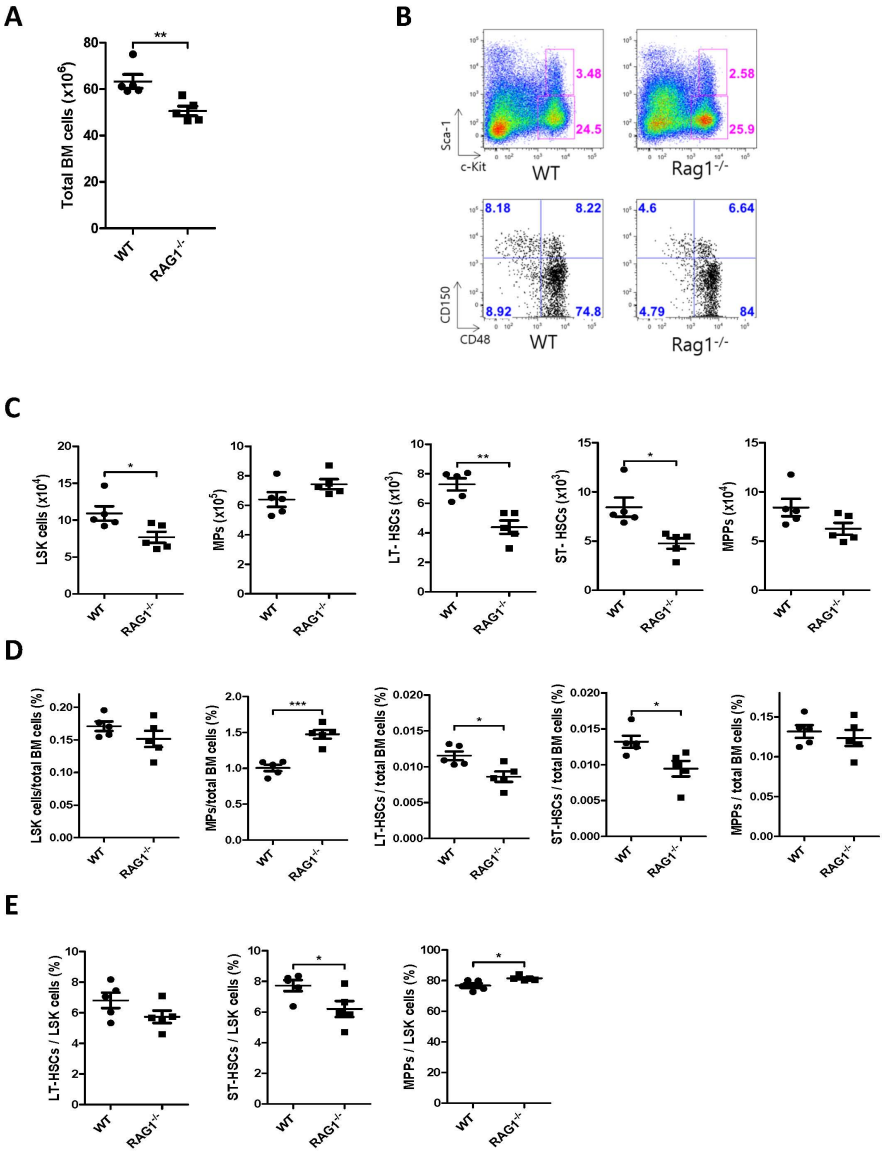


**Figure 1.** Lymphopenic condition reduces the number of HSPCs. (A) Absolute number of total bone marrow (BM) cells from wild type and *Rag1* $^{-/-}$ mice. (B) Flow cytometry of LSK cells (Lin$^-$ Sca-1$^+$ c-Kit$^+$) and MPs (Lin$^-$ Sca-1$^-$ c-Kit$^+$), and LT-HSCs (Lin$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^+$ CD48$^-$), ST- HSCs (Lin$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^+$ CD48$^+$), and MPPs (Lin$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^-$ CD48$^+$). Absolute number (C) and the frequency (D) of LSK cells, MPs, LT-HSCs, ST-HSCs, and MPPs among total BM cells. (E) The percentage of LT-HSCs, ST-HSCs and MPPs among LSK cells. Mean values±s.e.m. are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with drinking water controls. (Student's t-test).

cally reduced (Fig. 1B), while MPs (myeloid progenitors; Lineage$^-$ Sca-1$^-$ c-Kit$^+$) were increased in the bone marrow of *Rag1* $^{-/-}$ mice (Fig. 1C and D). Similarly, percentage of ST-HSCs among LSK cells was reduced in the bone marrow of *Rag1* $^{-/-}$ mice (Fig. 1E). On the contrary, percentage of MPPs (multipotent progenitors; Lineage$^-$ Sca-1$^+$ c-Kit$^+$ CD150$^-$CD48$^+$) was relatively up-regulated among LSK cells. Taken together, lymphopenic condition reduces the number of LT-HSCs and ST-HSCs and raises the number of MPs within the bone marrow.

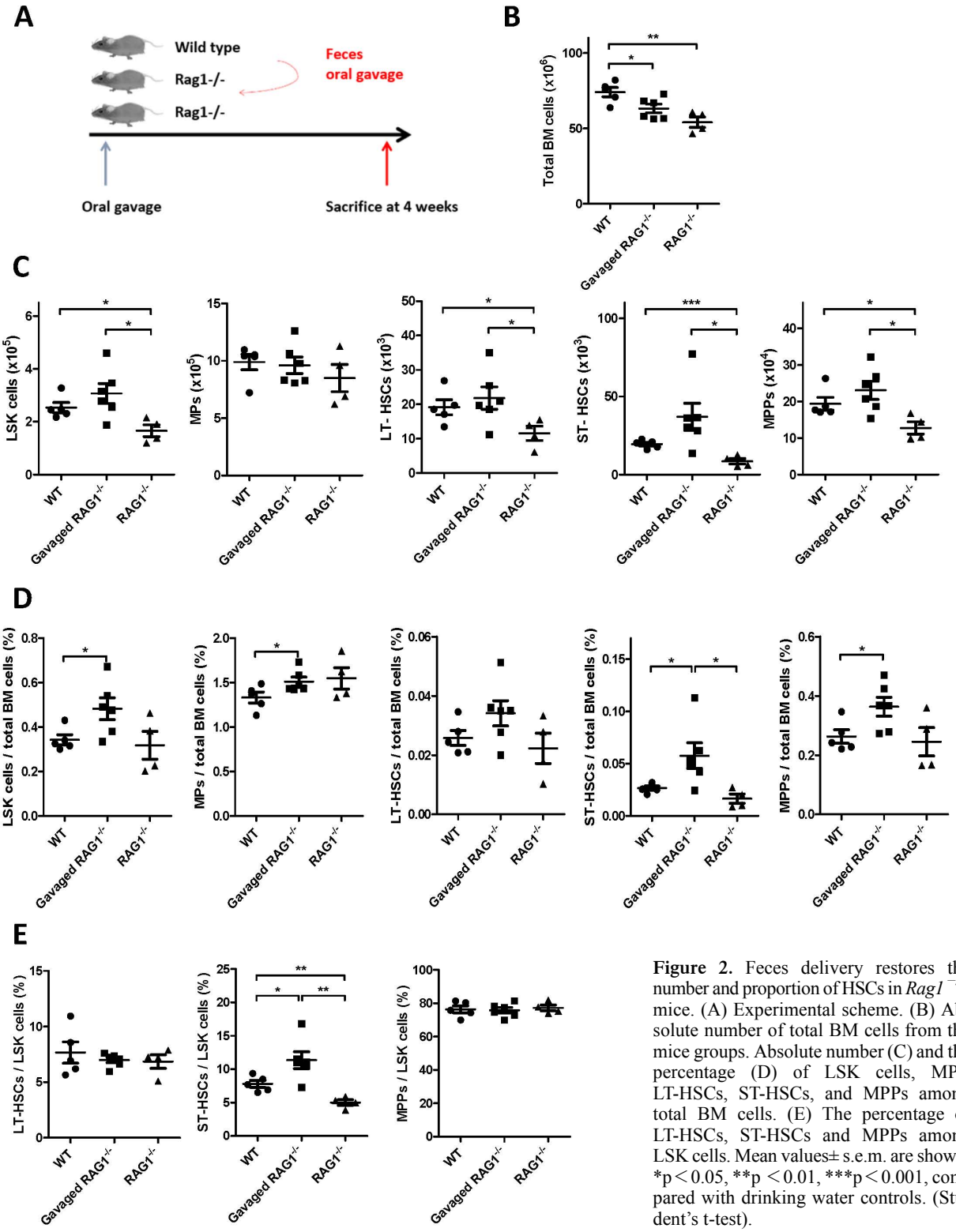


**Figure 2.** Feces delivery restores the number and proportion of HSCs in *Rag1* $^{-/-}$ mice. (A) Experimental scheme. (B) Absolute number of total BM cells from the mice groups. Absolute number (C) and the percentage (D) of LSK cells, MPs, LT-HSCs, ST-HSCs, and MPPs among total BM cells. (E) The percentage of LT-HSCs, ST-HSCs and MPPs among LSK cells. Mean values± s.e.m. are shown. *p < 0.05, **p < 0.01, ***p < 0.001, compared with drinking water controls. (Student's t-test).

### Gut microbiota compositional change alters number and proportion of HSPCs

Gut microbial diversity in co-housed animals is equilibrated by coprophagy (18). To examine homeostatic change of the bone marrow HSPCs according to gut microflora alteration, we used an additional bacterial administration to mimic recolonization (Fig. 2A). We extracted gut bacteria of feces from the wild type mice, and then orally injected the bacterial extraction of the feces to *Rag1* $^{-/-}$ mice 4 times for 4 weeks. Another mice group was injected with the same volume of PBS as the bacterial injected *Rag1* $^{-/-}$ mice. As expected, oral gavage treatment of feces increase the absolute number of total bone marrow cells of the *Rag1* $^{-/-}$ mice (Fig. 2B). It also showed that the absolute number of LSK cells, LT-HSCs, ST-HSCs and MPPs were elevated as the level of wild types (Fig. 2C). Moreover, percentage of LSK cells, MPs, and MPPs among total bone marrow cells in gavaged *Rag1* $^{-/-}$ mice were increased than the wild types (Fig. 2D). Especially, a proportion of ST-HSCs in feces-treated *Rag1* $^{-/-}$ mice were significantly increased (Fig. 2D). Taken together, compositional change of gut microbiota in *Rag1* $^{-/-}$ mice via feces transference affects homeostasis of HSPCs within the bone marrow.

### Feces oral treatment changes composition of gut microbiota in lymphopenic mice

To determine whether bacterial transfer induces compositional change of gut microbial community, we obtained bacterial DNA sample from feces of the wild type and *Rag1* $^{-/-}$ mice. Bacterial compositions of the feces were analyzed by metagenomics analysis (Fig. 3A). It was shown that members of phylum *Firmicutes* were relatively less abundant and members of phylum *Bacteriodetes* had larger proportion in the feces-treated *Rag-1*-deficient mice than the PBS-treated *Rag1* $^{-/-}$ mice (Fig. 3B). Family *Lachnospiraceae* in phylum *Firmicutes* were mostly reduced and family *Helicobacteraceae* in phylum *Proteobacteria* and family *Prevotellaceae* in phylum *Bacteroidetes* were relatively increased (Fig. 3B). Next, we tried to find a spe-

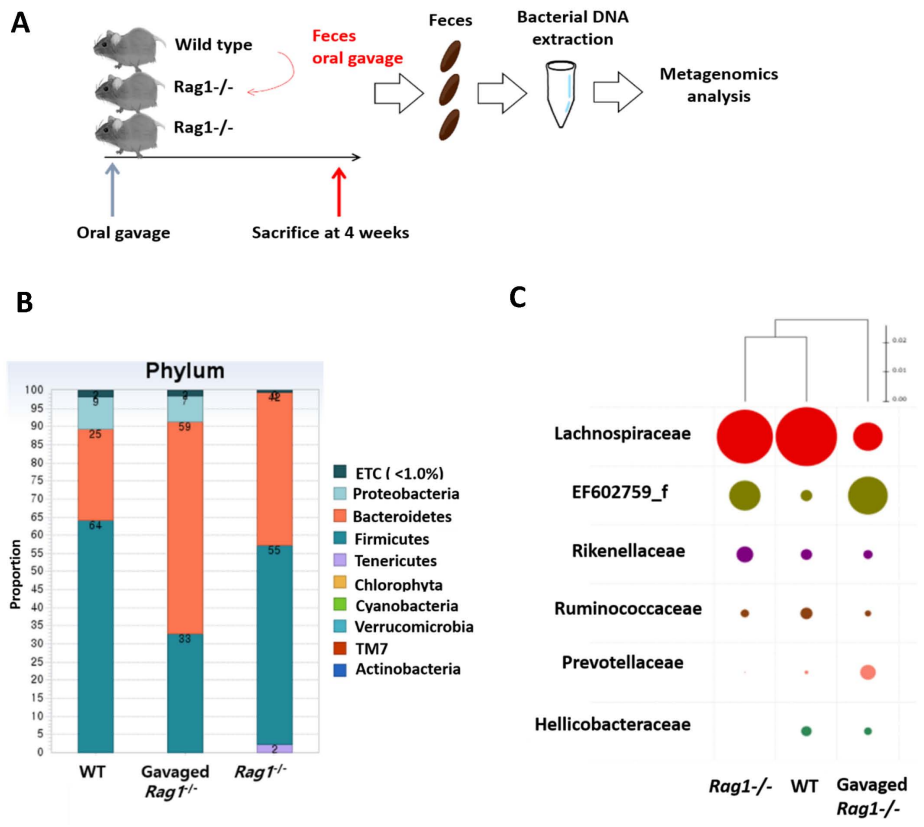


**Figure 3.** Feces delivery alters composition of colon microbial community. (A) Experimental scheme for metagenomics analysis. Gut microbiota composition based on phylum (A), family (B), and species (C) by bacterial DNA metagenomics analysis. Bacterial DNA in pooled feces (from 4 to 5 mice per each group) were identified by standard microbiological method.

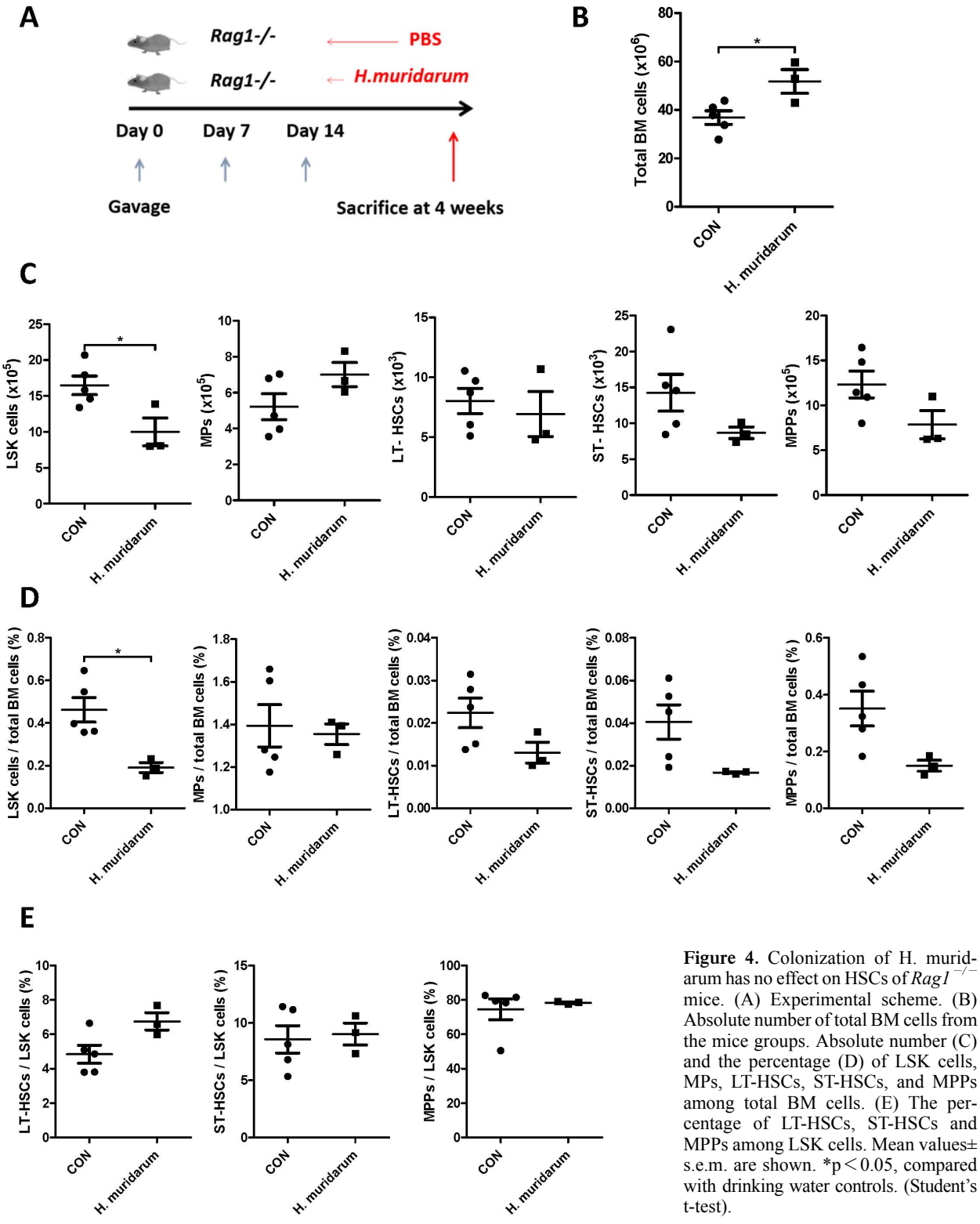


**Figure 4.** Colonization of H. muridarum has no effect on HSCs of *Rag1*$^{-/-}$ mice. (A) Experimental scheme. (B) Absolute number of total BM cells from the mice groups. Absolute number (C) and the percentage (D) of LSK cells, MPs, LT-HSCs, ST-HSCs, and MPPs among total BM cells. (E) The percentage of LT-HSCs, ST-HSCs and MPPs among LSK cells. Mean values± s.e.m. are shown. *$p < 0.05$, compared with drinking water controls. (Student's t-test).

cies that had the most significant changed of the three phyla. We focused on family *Helicobacteraceae* that had only identified bacterial species among the most abundant top 15 species in total microbiota of the feces-treated *Rag-1*-deficient mice (Fig. 3C). These data suggest that gut microbiota composition in *Rag1* $^{-/-}$ mice could be altered by the injection of wild type mice gut bacteria.

### Colonization of H. muridarum has no effect on HSPCs of lymphopenic mice

In our metagenomics results, we found two species of bacteria, such as *Helicobacter muridarum* (*H. muridarum*) and *Helicobacter ganmani* (*H. ganmani*), which were abundant and identified. Since other bacterial species were unidentified or reduced in the feces-treated *Rag1* $^{-/-}$ mice, we focused on two *Helicobacter* bacteria. To examine whether these species of bacteria affect homeostasis of the host HSPCs in the bone marrow, we treated a single-species bacterial injection experiment using male C57BL/6 *Rag1* $^{-/-}$ mice. One group (5 mice) had been treated with *H. muridarum* via oral gavage 3 times for 4 weeks, while the other group (3 mice) had been treated PBS. Both groups were sacrificed at day 28 after first administration (Fig. 4A). Although absolute number of total bone marrow cells were significantly increased in bacteria-treated mice (Fig. 4B), the absolute number and proportions of HSPCs in the *H. muridarum*-treated mice were not up-regulated (Fig. 4C and D). The proportion of LT-HSCs, ST-HSCs, and MPPs among LSK cells had not shown significant change (Fig. 4E). These suggest that a single-species bacteria transfer of *H. muridarum* cannot induce the change in HSPCs homeostasis in the bone marrow in *Rag-1*-deficient mice.

## DISCUSSION

The number and a proportion of HSPCs in *Rag1* $^{-/-}$ mice were down-regulated compared to wild types; however, *Rag1* $^{-/-}$ mice treated with wildtype feces restored the absolute number and the proportion of HSPCs in *Rag1* $^{-/-}$ mice as those of wild types. Feces treatment did not construct same composition of gut microbiota of *Rag1* $^{-/-}$ mice as that of wildtype mice. It would be difficult to equilibrate gut microbial diversity between wild types and *Rag1* $^{-/-}$ mice, even if bacteria from wild type are transferred and colonized on gut mucosa of *Rag1* $^{-/-}$ mice. Nevertheless, our data show that composition of wild types and feces-treated *Rag1* $^{-/-}$ mice share specific bacteria species. Interestingly, the most altered bacteria species were unidentified species. Furthermore, *H. muridarum* single colonization has no effect on controlling homeostasis of HSPCs in *Rag1* $^{-/-}$ mice. Therefore, our data indicate that restoration of the number and proportions of HSPCs are dependent on complex gut microbiota composition, not restricted to some of specific bacteria proportion. Inoculating several species of bacteria in *Prevotellaceae* and *Helicobacterceae* families in combination into *Rag1* $^{-/-}$ mice might be required for a future study. HSPCs might be affected by unknown signals from microbial community. Although defined mechanisms of regulating HSPCs by commensal gut microbiota is unknown, this result indicates that composition of gut microbiota have an effect on homeostasis of HSPCs.

In summary, gut microbiota affects the number and proportion of HSPCs, and compositional change of gut microbiota induces change of the homeostasis of HSPCs. Mechanisms of regulating homeostasis of HSPCs via gut microbiota, or specific bacterial signals that key regulators of hematopoiesis would be clearly established after further study.

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## CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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