Molecules and Cells

TRAF6 Distinctly Regulates Hematopoietic Stem and Progenitors at Different Periods of Development in Mice

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Tumor necrosis factor receptor-associated factor 6 (TRAF6) is identified as a signaling adaptor protein that regulates bone metabolism, immunity, and the development of several tissues. Therefore, its functions are closely associated with multiple diseases. TRAF6 is also involved in the regulation of hematopoiesis under steady-state conditions, but the role of TRAF6 in modulating hematopoietic stem and progenitor cells (HSPCs) during the developmental stages remains unknown. Here, we report that the deletion of TRAF6 in hematopoietic lineage cells resulted in the upregulation of HSPCs in the fetal liver at the prenatal period. However, in the early postnatal period, deletion of TRAF6 drastically diminished HSPCs in the bone marrow (BM), with severe defects in BM development and extramedullary hematopoiesis in the spleen being identified. In the analysis of adult HSPCs in a BM reconstitution setting, TRAF6 played no significant role in HSPC homeostasis, albeit it affected the development of T cells. Taken together, our results suggest that the role of TRAF6 in regulating HSPCs is altered in a spatial and temporal manner during the developmental course of mice.

Keywords: developmental stage, hematopoiesis, HSPC, TRAF6

INTRODUCTION

Hematopoiesis is a crucial mechanism for making all blood lineage cells and maintaining tissue homeostasis (Kondo et al., 2003; Weissman, 2000). The lifelong production of blood cells is exclusively dependent on the self-renewal activity of hematopoietic stem cells (HSCs) in the bone marrow (BM), which differentiate into multipotent, lineage-committed progenitors and finally into lineage cells (Weissman, 2000). As all types of blood cells have a finite life span, production of blood cells should be constantly replenished from HSCs (Orkin and Zon, 2008). Hematopoiesis occurs differently depending on the stage of organogenesis (Cumano and Godin, 2007). In general, vertebrate hematopoiesis is largely categorized into two waves: the primitive wave and the definitive wave (Gao et al., 2018). In mice, the primitive wave, also referred to as primitive hematopoiesis, begins at embryonic day (E) 7 to produce red blood cells for oxygenation and growth of the embryo, but with little HSC activity (Orkin and Zon, 2008). After E10.5, HSCs appear within the aorta-gonad-mesonephros (AGM) region and migrate to the fetal liver (Cumano et al., 1996; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Definitive hematopoiesis is initiated in the fetal liver and spleen with production of erythroid-myeloid progenitors from HSCs, which then migrate to the BM where hematopoiesis continues throughout adult life (Cumano and Godin, 2007). During fetal development, the yolk sac, AGM, fetal liver, and spleen serve as sites of extramedullary hematopoiesis (EMH), which is defined as hematopoiesis occurring outside of the BM and

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plays a crucial role in initiating hematopoiesis. The major function and production of the differentiated progeny of HSCs are different at each stage during development (Copley and Eaves, 2013). For example, fetal liver HSCs primarily produce erythroid and myeloid cells, but adult HSCs generate a balanced production of all blood lineage cells (Copley and Eaves, 2013; Pietras et al., 2011). These observations raise the question of whether specific regulation of different developmental stages is required in order to modulate HSC properties. Previous studies have shown that hematopoiesis during fetal versus adult periods is differently regulated by altered gene expression programs. For example, transcriptome factors Gfi1, Tel/Etv6, and C/EBPα are required for the maintenance of adult HSCs, but not fetal HSCs (Hock et al., 2004a; 2004b; Kim et al., 2007; Park et al., 2003; Ye et al., 2013). By contrast, Sox17 is necessary for the maintenance of fetal HSCs, but not adult HSCs (Kim et al., 2007). These studies suggested that cell-intrinsic factors may regulate HSCs in a manner whereby the activity of HSCs fits the specific period of development, for example, fetal versus adult hematopoiesis. In addition to intrinsic factors, extrinsic factors such as growth factor, chemokine, cytokine, and niche cell composition may affect the various developmental stages of hematopoiesis (Chou and Lodish, 2010). Gut microbiota, their composition, and high-fat diets can also affect BM hematopoiesis in adults (Balmer et al., 2014; Khosravi et al., 2014; Kwon et al., 2015; Luo et al., 2015), and maternal obesity could restrict the expansion of fetal HSCs (Kamimae-Lanning et al., 2015).

Tumor necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase, is involved in the nuclear factor-κB signaling pathway induced by the tumor necrosis factor receptor family, toll-like receptor family, and interleukin-1 receptor, which consequently regulates immune responses (Walsh et al., 2015). In addition, deletion of TRAF6 in mice showed osteopetrosis with defective bone remodeling and impaired osteoclast function, as well as perinatal death with multiple organ abnormalities (Armstrong et al., 2002; Lomaga et al., 1999). TRAF6 has an important role in the pathogenesis of many human diseases such as cancers, autoimmune diseases, chronic inflammation, and infection (Fang et al., 2017; Xie, 2013). However, the role of TRAF6 in HSCs has not been fully identified. Recent studies have shown that overexpression of TRAF6 in HSCs induced increased hematopoietic stem and progenitor cells (HSPCs) in the BM (Fang et al., 2017), while deletion of TRAF6 from HSCs caused impaired HSC self-renewal activity (Fang et al., 2018). Yet, despite the significance of TRAF6 in adult HSCs, the role of TRAF6 in the homeostasis of HSPCs during development remains unknown. We report herein that TRAF6 regulates HSPCs differently during the timeline of hematopoiesis from fetal liver to adult hematopoiesis.

MATERIALS AND METHODS

Animal studies

TRAF6$^{fl/fl}$ mice (C57BL/6) were kindly donated by Dr. Yongwon Choi in University of Pennsylvania (Han et al., 2013). TRAF6$^{fl/fl}$ mice were crossed with Vav1Cre mice (Jackson Laboratory, 008610) to generate TRAF6-ΔH (TRAF6$^{fl/fl}$Vav1Cre+) mice. Analysis of fetal liver cells was performed on E14.5 of the mice. For the early postnatal period study, TRAF6-ΔH mice were analyzed at day 19 after birth. All mice were C57BL/6 (B6) background and housed in a pathogen-free animal facility at POSTECH. Animal experiments were performed according to the guidelines of POSTECH institutional animal care and use committee.

Preparation of BM and fetal liver cells

To collect BM cells, leg bones (one tibia and one femur per mice) were flushed with RPMI1640 media (Welgene) containing 0.5% FCS (Hyclone). Cell suspensions were passed through mesh filter (BD Biosciences) before use. To analyze and collect fetal liver cells from E14.5 embryo, fetal livers were dissected from embryos and single cell suspension was made by using syringe in PBS (Welgene) supplemented with 2% FCS. Cell suspensions were passed through mesh filter (BD Biosciences) before use.

Flow cytometry

For flow cytometry analysis of hematopoietic stem and progenitor cells (HSPCs), whole BM cells or fetal liver cells were stained with following monoclonal antibodies (eBioscience, BioLegend or BD Biosciences). Antibodies for lineage marker were used with TER119 (clone TER-119), CD11b/Mac-1 (clone M1/70), CD3e (clone 145-2C11), B220 (clone RA3-6B2), NK1.1 (clone PK136), Gr-1 (clone RB6-8C5), MHC class II (I-A/I-E, clone M5/114.15.2). For HSPC analysis, cells were stained with antibodies against c-Kit (clone 2B8), Sca-1 (clone D7), CD150 (clone mShad150) and CD48 (clone HM48-1). HSPCs were identified by staining for the following markers: LSK cells (LIN$^-$Sca-1$^+$c-Kit$^+$); myeloid progenitors (MPs; LIN$^-$Sca-1$^-$c-Kit$^+$); hematopoietic stem cells (HSCs; LIN$^-$Sca-1$^+$c-Kit$^+$CD150$^+$CD48$^-$); short-term HSCs (ST-HSCs; LIN$^-$Sca-1$^+$c-Kit$^+$CD150$^-$CD48$^-$); hematopoietic progenitor cells-2 (HPC-2; LIN$^-$Sca-1$^+$c-Kit$^+$CD150$^+$CD48$^+$); multipotent progenitors (MPPs; LIN$^-$Sca-1$^+$c-Kit$^+$CD150$^-$CD48$^+$). For fetal liver HSPC analysis, TER119, CD3e, B220, NK1.1, Gr-1, and MHC class II were used for lineage markers. Fetal liver HSPCs were identified by following markers: LSK cells (LIN$^-$Sca-1$^+$c-Kit$^+$Mac-1$^+$), HSCs (LIN$^-$Sca-1$^+$c-Kit$^+$Mac-1$^+$CD150$^+$CD48$^-$); ST-HSCs (LIN$^-$Sca-1$^+$c-Kit$^+$Mac-1$^+$CD150$^+$CD48$^+$); MPPs (LIN$^-$Sca-1$^+$c-Kit$^+$Mac-1$^+$CD150$^-$CD48$^+$). Flow cytometry data were collected with LSR Fortessa (BD Biosciences), LSR Fortessa 5 lasers (BD Biosciences) or Canto II (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.). LSK cells from the spleen and CD11b+ myeloid cells from the BM were sorted on a Moflow-XDP (Beckman Coulter) sorter.

Generation of BM chimeras

For generating mixed BM chimeras, adult recipient mice were lethally irradiated (9.6 Gy) with an X-RAD 320 irradiator (Precision X-Ray, Inc). 1 × 10^6 whole CD45.1$^+$ B6 BM cells were mixed with FACS-sorted LSK cells (2.2 × 10^3) from the spleens of CD45.2$^+$TRAF6-WT (TRAF6$^{fl/fl}$Vav1Cre-) or CD45.2$^+$ TRAF6-ΔH (TRAF6$^{fl/fl}$Vav1Cre+) mice, and then cell mixtures were injected into the recipient mice a day after irradiation. Blood lineage cells of the chimeric mice were

analyzed at 4, 6, 8, and 10 weeks after transplantation, and BM cells were analyzed at 10 weeks. Data shown are sum of two independent experiments and two-tailed Student's t-test was used to assess statistical significance.

Nucleic acid extraction and RT-PCR

Total RNA was isolated and cDNA was synthesized with a QuantiTect Rev.Transcription Kit (QIAGEN) according to manufacturer's protocol. For DNA extraction, genomic DNA extraction kit (iNtRON) was used according to manufacturer's protocol. Real-time PCR was performed with 7300 Fast Real Time PCR System (Applied Biosystems) using Power SYBR Green PCR master mix (Life technology) and primer sequences (listed below). Relative expression was normalized to the internal control L32 and presented as relative to WT. Forward TRAF6 primer: GCG CTG TGA AGT CTC TAC CC. Reverse TRAF6 primer: GCT CGT GAC CTC ACT GAT GA. Forward L32 primer: GAA ACT GGC GGA AAC CCA. Reverse L32 primer: GGA TCT GGC CCT TGA ACC TT.

Statistical analysis

Statistical analyses were performed with two-tailed unpaired Student's t-test as indicated in the legends. P values are indicated by asterisks in the figures: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Gehan-Breslow-Wilcoxon Test was used for survival analysis. GraphPad Prism 5.0 was used for data analysis.

RESULTS

Deletion of TRAF6 in hematopoietic cells resulted in high postnatal mortality

To investigate whether TRAF6 plays a role in regulating hematopoiesis in the BM, we first analyzed the mRNA expression of TRAF6 in various subsets of HSPCs. HSCs, which are the most primitive stem cell populations, expressed higher levels of TRAF6 than hematopoietic progenitor cells-2 (HPC-2), multipotent progenitors (MPPs), and myeloid progenitors (MPs) did (Fig. 1A). To determine the role of TRAF6 in HSPCs, we next crossed TRAF6$^{fl/fl}$ mice with Vav1Cre mice to nullify TRAF6 in hematopoietic cells (TRAF6-ΔH). We verified the deletion of TRAF6 at both genomic DNA and mRNA levels in FACS-sorted BM myeloid cells and total BM cells, respectively (Supplementary Figs. S1A and S1B). TRAF6-ΔH mice showed a defect in tooth eruption compared to their littermate controls (TRAF6-WT) (Fig. 1B). Moreover, TRAF6-ΔH mice displayed abnormal development of the femurs with defective formation of the hematopoietic marrow, resulting in faint color of the marrow (Fig. 1C). These results are consistent with the previous finding that TRAF6 whole knock out mice showed hematologic defects and failure of tooth eruption (Fang et al., 2018; Naito et al., 1999). Taken together, these data suggest a critical role of TRAF6 in hematopoietic lineage cells in bone remodeling and marrow development. Surprisingly, TRAF6-ΔH mice showed the short survival, succumbing to death around 2-3 weeks of age (Fig. 1D). It remains to uncover the exact causes of the premature death in TRAF6-ΔH mice. However, it is worth to note that TRAF6 defects induced abnormal blood generation such as red blood cells and/or platelets (Fang et al., 2018; Lomaga et al., 1999), which might be part of reason for the short survival of TRAF6-ΔH mice in this study.

TRAF6 negatively regulated HSPCs in the fetal liver

As the principal site of hematopoiesis switches from the yolk sac to the fetal liver, definitive hematopoiesis begins with the production of myeloid and erythroid lineage cells (Mikkola and Orkin, 2006). The functional properties and transcrip-

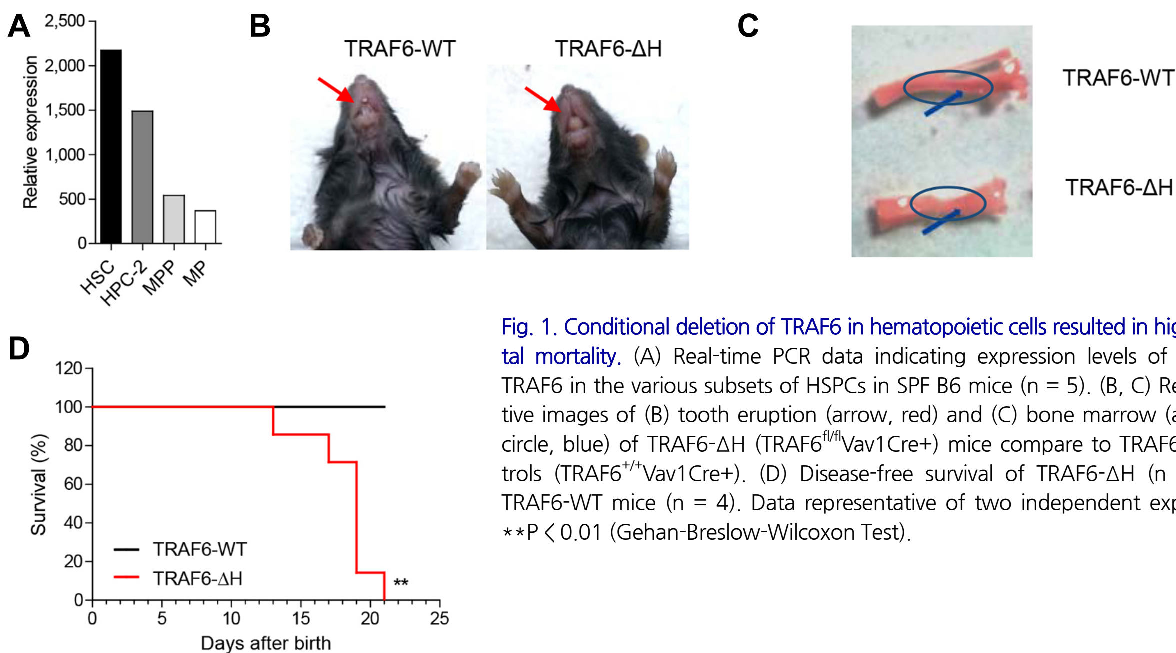

Fig. 1. Conditional deletion of TRAF6 in hematopoietic cells resulted in high postnatal mortality. (A) Real-time PCR data indicating expression levels of mRNA of TRAF6 in the various subsets of HSPCs in SPF B6 mice (n = 5). (B, C) Representative images of (B) tooth eruption (arrow, red) and (C) bone marrow (arrow and circle, blue) of TRAF6-ΔH (TRAF6$^{fl/fl}$Vav1Cre+) mice compare to TRAF6-WT controls (TRAF6$^{+/+}$Vav1Cre+). (D) Disease-free survival of TRAF6-ΔH (n = 7) and TRAF6-WT mice (n = 4). Data representative of two independent experiments. $**P < 0.01$ (Gehan-Breslow-Wilcoxon Test).

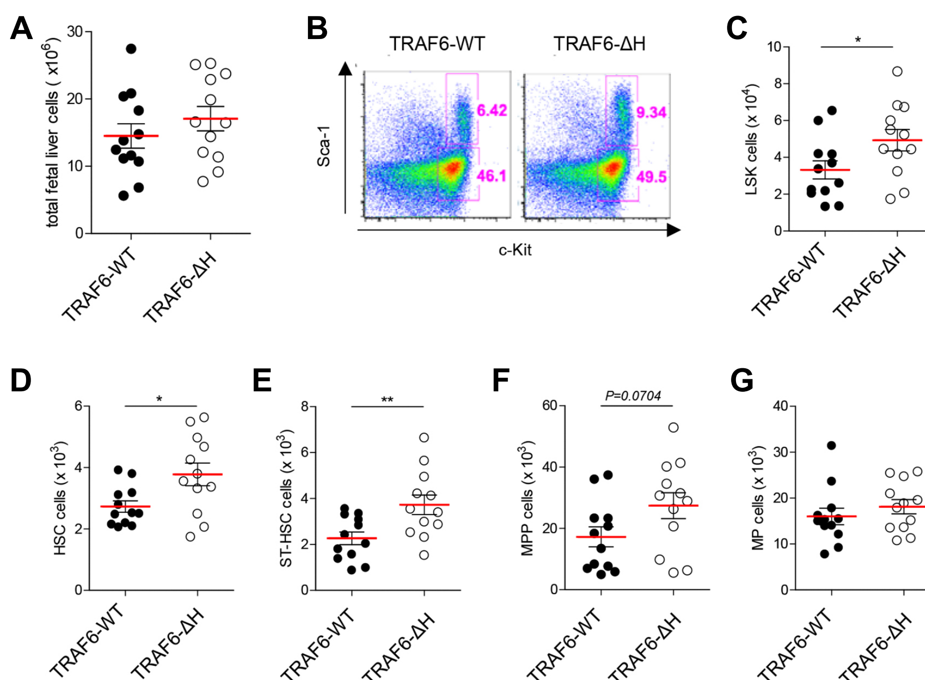

Fig. 2. TRAF6-ΔH mice showed downregulated HSPCs in the fetal liver. (A) Absolute numbers of total fetal liver cells of TRAF6-WT and TRAF6-ΔH mice (n = 12). (B) Flow cytometry of LSK cells in the fetal liver of TRAF6-WT and TRAF6-ΔH mice. (C-G) Numbers of (C) LSK cells and further separation of HSPCs into (D) HSC, (E) ST-HSC, (F) MPP, and (G) MP cells of TRAF6-WT (n = 12) and TRAF6-ΔH mice (n = 12). Data are sum of two independent experiments. Data are displayed as mean ± SEM. *P < 0.05, **P < 0.01 (Student's t test).

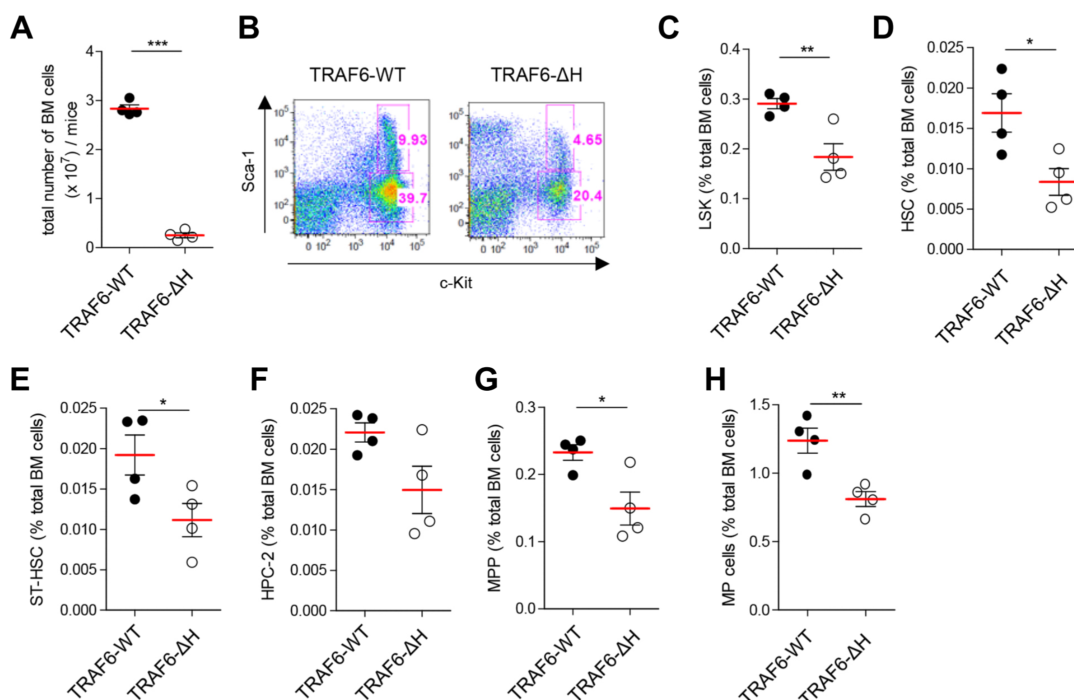

Fig. 3. TRAF6-ΔH mice displayed reduced number of HSPCs of the BM in the early postnatal period. (A) Numbers of total BM cellularity of TRAF6-ΔH (n = 4) and control mice (n = 4). (B) Representative flow cytometry plot of LSK cells in the BM of TRAF6-WT and TRAF6-ΔH mice. (C-H) Frequency of (C) LSK cells and further separation of HSPCs into (D) HSC, (E) ST-HSC, (F) HPC-2, (G) MPP, and (H) MP cells of TRAF6-WT (n = 4) and TRAF6-ΔH mice (n = 4). Data representative of two independent experiments. Data are displayed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (Student's t test).

tional regulations are different between fetal liver HSCs and adult BM HSCs, consequently affecting their cell cycling activity and potential for lineage cell differentiation (Babovic and Eaves, 2014). This led us to investigate the role of TRAF6 during fetal liver hematopoiesis. Although the total numbers of fetal liver cells were comparable between control and TRAF6-ΔH mice (Fig. 2A), we found that the number of Lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells in the fetal liver was increased in TRAF6-ΔH mice. As LSKs include heterogeneous populations of HSPCs, we separated cells on the basis of surface expression of the signaling lymphocyte activation molecule family. Based on previous studies, we distinguished HSCs (CD150$^+$CD48$^-$LSK), short-term HSCs (CD150$^+$CD48$^+$LSK, ST-HSCs), and MPPs (CD150$^-$CD48$^+$LSK) from LSK cells (Kim et al., 2006). An increased number of LSK cells in TRAF6-ΔH mice resulted from the expansion of HSCs, ST-HSCs, and MPPs (Figs. 2B-2F). However, the number of MPs (Lineage$^-$Sca-1$^-$c-Kit$^+$) was comparable (Fig. 2G). These data indicated that TRAF6 negatively regulated fetal liver HSPCs.

Deletion of TRAF6 showed impaired HSPC maintenance and induced EMH in early postnatal period

HSCs leave the liver and colonize in special microenvironments in the BM within two weeks of postnatal life (Mikkola and Orkin, 2006). Thus, the early postnatal period is a turning point when hematopoiesis takes place at the BM (Copley and Eaves, 2013). As TRAF6-ΔH mice succumb to defects in bone development and die at around 2-3 weeks of age, we investigated the role of BM hematopoiesis at this early postnatal period. TRAF6-ΔH mice showed a highly decreased number of total BM cells at day 19 (Fig. 3A). Unlike HSPCs in the fetal liver (Fig. 2), the total numbers and percentages of HSCs, ST-HSCs, HPC-2, MPPs, and MPs decreased severely (Figs. 3B-3H). As EMH actively appears under conditions of BM failure (Schuettpelz and Link, 2013), we hypothesized that the decreased number of HSPCs in the BM of TRAF6-ΔH mice might induce EMH. To test this possibility, we analyzed HSPCs in the spleen. The size of the spleen and the total number of cells in TRAF6-ΔH mice were comparable to

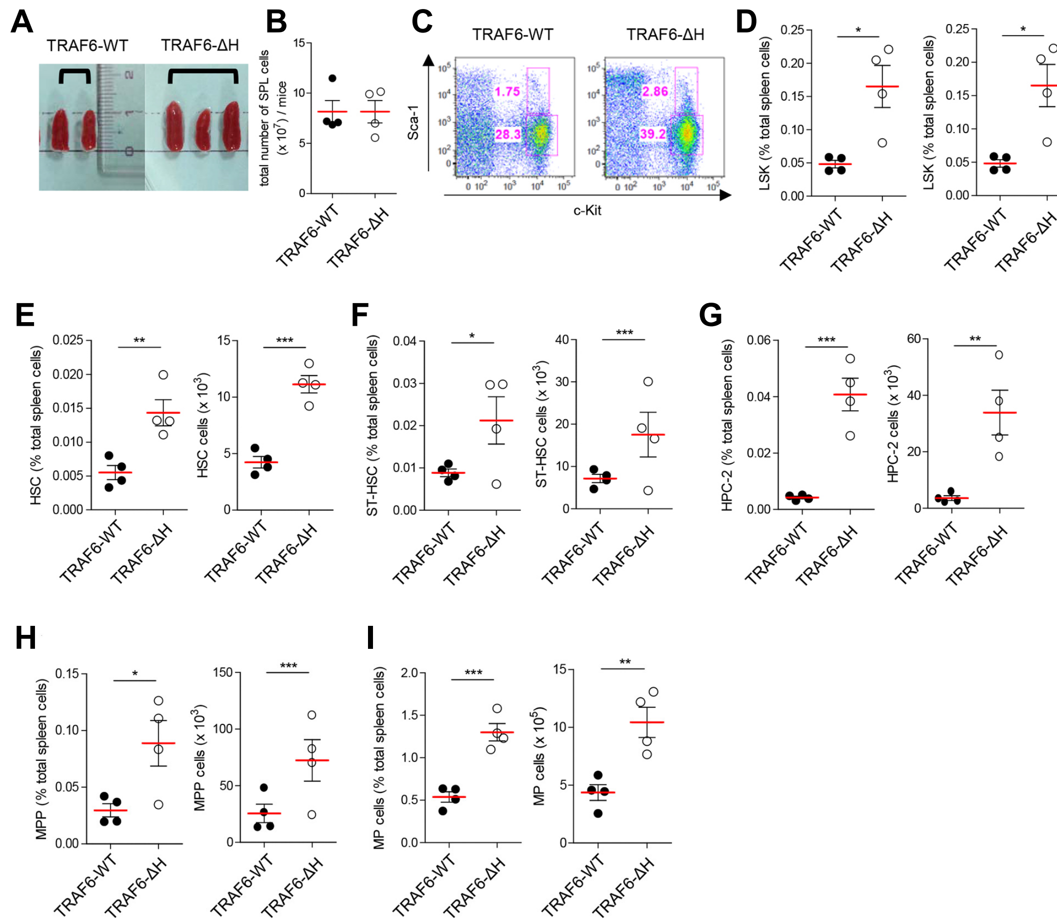

Fig. 4. TRAF6-ΔH mice induced extramedullary hematopoiesis of the spleen in the early postnatal period. (A) Representative images of spleen size of TRAF6-ΔH and control mice. (B) Total number of spleen cells of TRAF6-ΔH (n = 4) and control mice (n = 4). (C) Representative flow cytometry plot of LSK cells in the spleen of TRAF6-WT and TRAF6-ΔH mice. (D-I) Numbers and frequency of (D) LSK cells and further separation of HSPCs into (E) HSC, (F) ST-HSC, (G) HPC-2, (H) MPP, and (I) MP cells of TRAF6-WT (n = 4) and TRAF6-ΔH mice (n = 4). Data representative of two independent experiments. Data are displayed as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's t test).

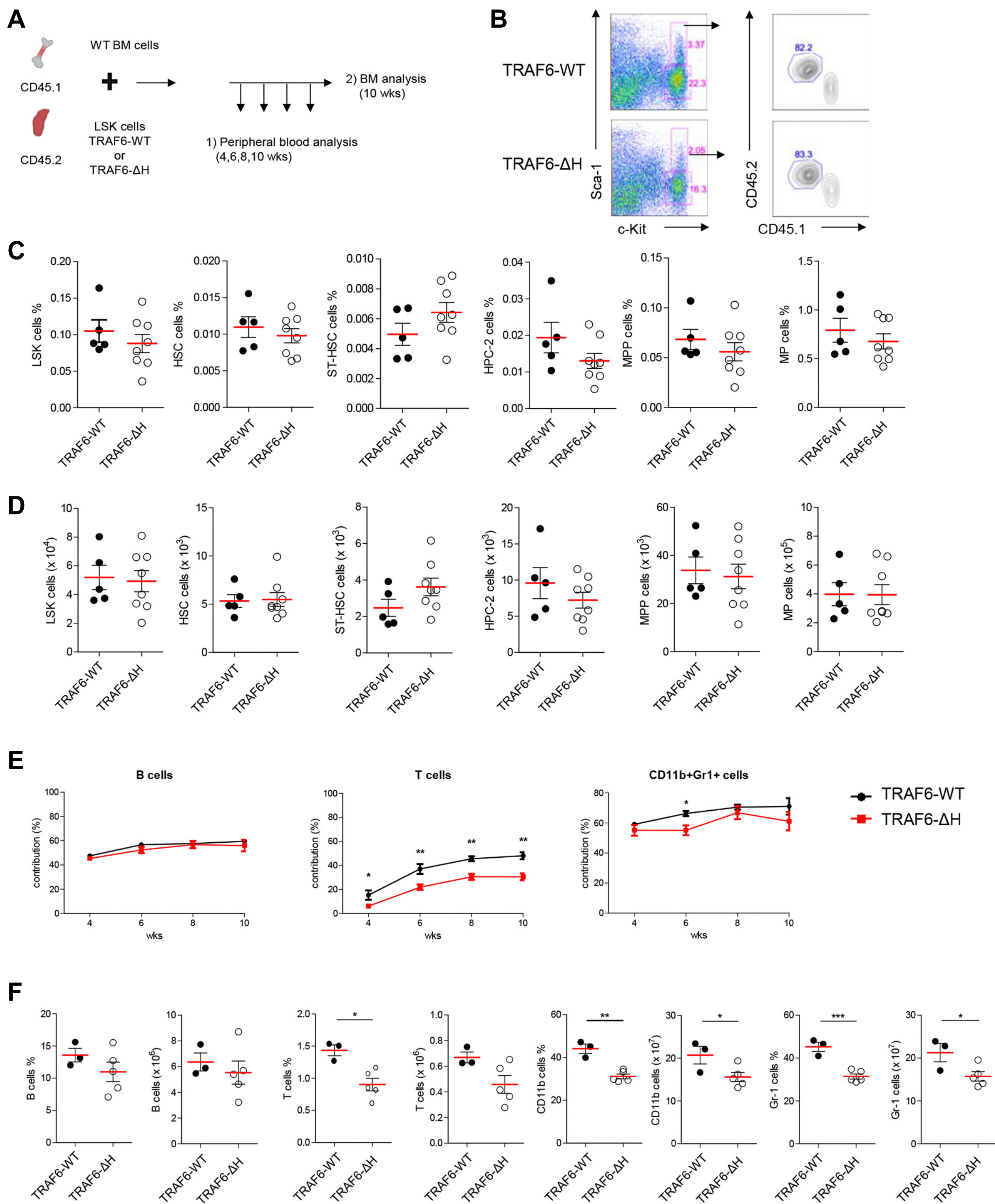

Fig. 5. TRAF6-deficiency displayed normal hematopoietic reconstitution in adult period, but impaired differentiation of T cells and myeloid cells. (A) Experimental outline. (B) Representative FACS plots of LSKs. Donor-derived (CD45.2$^+$) LSKs from recipient mice were analyzed by flow cytometry. (C, D) (C) Frequency and (D) number of LSK cells and further separation of HSPCs into HSC, ST-HSC, HPC-2, MPP, and MP cells in BM recipient mice (n = 5-7). (E) Donor-derived peripheral blood proportion at the indicated time points in BM chimeric mice (n = 5-7). (F) Frequency and absolute numbers of B cells, T cells, and myeloid cells (Gr-1$^+$ CD11b$^+$ cells) in the BM of chimeric mice at 10 weeks after transplantation (n = 5-7). Data is representative of two independent experiments. Data are displayed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (Student's t test).

their controls (Figs. 4A and 4B). However, the absolute number and percentage of HSPCs were elevated in TRAF6-ΔH mice (Figs. 4C-4I), which suggested that downregulated HSPCs of the BM in TRAF6-ΔH mice may accelerate EMH in the spleen.

TRAF6 played no role in HSPC maintenance but contributed to lineage cell development in the BM during adult hematopoiesis

Since we only examined the role of TRAF6 in BM hematopoiesis at an early postnatal age due to the defective bone development and premature death of TRAF6-ΔH mice, we evaluated the role of TRAF6 in BM hematopoiesis at adult ages. To do this, we generated mixed BM chimeras by reconstituting hematopoietic compartments with adoptively transferred HSCs in irradiated recipient mice. Because of the difficulty in gathering enough BM cells in TRAF6-ΔH mice (Fig. 3A), we transferred LSK cells, isolated by fluorescence-activated cell sorting, from the spleen mixed with congenic wild-type total BM cells into lethally irradiated mice and analyzed them ten weeks after the transfer (Fig. 5A). The transferred LSK cells were successfully seeded and maintained as HSPCs in the BM, but TRAF6 deletion in HSCs showed no defects in both absolute number and percentage in most subsets of HSPCs, as compared to TRAF6-intact HSCs (Figs. 5B-5D). Blood cell chimerism showed that T cell ($CD3e^+$ cells) development was decreased in the absence of TRAF6 (Fig. 5E), while B cell and granulocyte development were relatively normal. In contrast, TRAF6 positively contributed to myeloid lineage cell production in the BM (Fig. 5F). Interestingly, T cells but not B cells were also downregulated in the BM in the absence of TRAF6 (Fig. 5F). Collectively, these data suggested that the deletion of TRAF6 did not intrinsically affect HSPC homeostasis but regulated the development of T cells and myeloid cells in the BM at an adult age.

DISCUSSION

In this report, we demonstrate for the first time that TRAF6 differentially regulates HSPC homeostasis at different developmental stages in mice. Our data showed that 1) TRAF6 suppressed the expansion of HSPCs during fetal liver hematopoiesis; 2) TRAF6 upregulated HSPCs in the BM but downregulated HSPCs in the spleen in early postnatal hematopoiesis; and 3) TRAF6 played no dominant role in adult BM hematopoiesis, at least under competitive BM reconstitution.

The altered function of TRAF6 in distinct phases of hematopoiesis may be a result of the different intrinsic mechanisms between fetal liver and postnatal BM hematopoiesis. Fetal liver HSCs rapidly divide and give rise to more rapid reconstitution activity when they are transferred into irradiated recipient mice (Harrison et al., 1997; Morrison et al., 1995). Fetal liver HSCs have higher self-renewal activity than postnatal HSCs, and selective transcriptional factors such as Sox17, Lin28b, Hmga2, and Cebpα regulate self-renewal activity (Copley et al., 2013; He et al., 2011; Kim et al., 2007; Yuan et al., 2012). The self-renewal potential of fetal liver HSCs is down-regulated by the phosphatidylinositol 3'-kinase (PI3K)/protein kinase B (AKT) intracellular signaling pathway through the suppression of polycomb proteins (PcG) (Liu et al., 2013). PcG proteins are necessary for the maintenance of the self-renewal activity of HSCs (Bracken and Helin, 2009) and fetal liver hematopoiesis (Park et al., 2003). Thus, as the activation of PI3K/AKT signaling through TRAF6 was reported previously (Yang et al., 2009), it may be possible that TRAF6 intrinsically downregulates fetal liver HSCs.

During the early postnatal period at around two weeks of age, TRAF6 seemed to play an important role in BM development and hematopoiesis, which showed the dramatic dysregulation of BM HSPCs. We may consider two possible explanations for this phenotype of TRAF6-ΔH mice. 1) In contrast to the negative role of TRAF6 in fetal liver HSPCs, TRAF6 plays a positive role in the maintenance of HSPCs in the BM, as suggested by a recent report, which showed that TRAF6 overexpression in mice elevated HSPCs in the BM (Fang et al., 2017). 2) Otherwise, TRAF6 deletion in hematopoietic lineage cells generated severe defect in bone remodeling and BM development, which consequently prohibited the retention and maintenance of HSPCs in the BM microenvironment. More recently, Feng et al. (2018) reported that TRAF6 deletion in the hematopoietic compartment shows no significant change of HSPC proportions, but only the percent of $CD150^+$LSK cells was decreased in the BM, albeit they analyzed the mice at much later time points of around 2-3 months. Thus, we propose that the defects of BM development in our TRAF6-ΔH mice would be the more likely reason for the reduced HSPCs in the BM at this early time point of development in these mice. The BM microenvironment, termed as the "niche", supports the maintenance and retention of HSCs (Morrison and Scadden, 2014). Several cell types constitute this BM microenvironment for HSPCs such as osteoblasts, whose homeostasis is regulated by the bone resorption activity of osteoclasts. TRAF6 has a critical role in activating mature osteoclasts to resorb bone (Armstrong et al., 2002; Lamothe et al., 2007; Park et al., 2017) and osteoclast dysfunction results in inhibition of BM cavity formation, HSC maintenance, and HSPC mobilization (Kollet et al., 2006; Miyamoto et al., 2011; Winkler et al., 2010). As osteoclasts originate from hematopoietic monocyte/macrophage precursors near the bone surface (Boyle et al., 2003), osteoclast formation in TRAF6-ΔH mice may be defective. In accordance, TRAF6-ΔH mice showed defects in tooth eruption and BM development (Figs. 1B and 1C), which may result in abnormal formation of the BM niche for HSPCs and dysregulation of hematopoiesis. As EMH occurs in conditions of severe BM failure (Johns and Christopher, 2012), it is expected that TRAF6-ΔH mice would exhibit enhanced EMH in the spleen as a compensatory action of reduced medullary hematopoiesis.

Premature death of TRAF6-ΔH mice in our study led us to investigate the role of TRAF6 in adult BM hematopoiesis in the BM reconstitution experiments using HSPCs isolated from the spleen. Overall, the results from these competitive reconstitution experiments suggested no major role of TRAF6 in HSPCs in adult hematopoiesis. In sharp contrast, Fang et al. (2018) recently reported that TRAF6 deletion in hematopoietic lineage cells made with the similar approach to ours using $TRAF6^{fl/fl}$ and Vav1Cre mice played a crucial

role in the self-renewal of HSCs in competitive reconstitution settings. It is difficult to understand why TRAF6 showed such extreme differences in regulating the self-renewal of HSCs in experiments. However, the phenotypic difference in TRAF6-deleted mice developed by Fang et al. may provide some clues. Instead of dying at very early postnatal periods like our mice did, the TRAF6-deleted mice in the study by Fang et al. (2018) survived for around 4-5 months. They showed inflammatory infiltrates in several tissues and impaired blood formation. Therefore, we used LSK cells isolated from the spleens of two-week-old TRAF6-ΔH mice to reconstitute the recipient mice, but Fang et al. used intact BM cells isolated from adult TRAF6-deleted mice. Since the functions of HSCs can be modulated by many environmental factors (Crane et al., 2017), which are mainly produced by niche cells, the self-renewal activity of HSCs may be intrinsically distinct when we isolated HSCs from the spleens versus the BM to reconstitute the recipient mice. Our data suggested that TRAF6 may play distinct roles in HSPCs depending on the developmental stage of the mice, for example, the fetal liver for the primary wave and the BM for the secondary wave of definitive hematopoiesis. Thus, our data in this report and those presented by Fang et al. suggest that the function of TRAF6 in regulating HSPCs may not be fixed but is flexible throughout the course of organ development.

In summary, our study demonstrated that TRAF6 plays different roles in regulating HSPCs during the timeline of hematopoiesis from fetal liver to adult BM hematopoiesis. Future studies will investigate the extracellular signals that converge to TRAF6 for modulating HSPC functions in spatiotemporally different environments that influence vertebrates in their development.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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