

# TNF- $\alpha$ /Fas-RIP-1–induced cell death signaling separates murine hematopoietic stem cells/progenitors into 2 distinct populations

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**We studied the effects of TNF- $\alpha$  and Fas-induced death signaling in hematopoietic stem and progenitor cells (HSPCs) by examining their contributions to the development of bone marrow failure syndromes in *Tak1*-knockout mice (*Tak1*<sup>-/-</sup>). We found that complete inactivation of TNF- $\alpha$  signaling by deleting both of its receptors, 1 and 2 (*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup>), can prevent the death of 30% to 40% of *Tak1*<sup>-/-</sup> HSPCs and partially repress the bone marrow failure phenotype of *Tak1*<sup>-/-</sup> mice. Fas deletion can prevent the death of 5%**

**to 10% of *Tak1*<sup>-/-</sup> HSPCs but fails to further improve the survival of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs, suggesting that Fas might induce death within a subset of TNF- $\alpha$ -sensitive HSPCs. This TNF- $\alpha$ /Fas-induced cell death is a type of receptor-interacting protein-1 (RIP-1)–dependent programmed necrosis called necroptosis, which can be prevented by necrostatin-1, a specific RIP-1 inhibitor. In addition, we found that the remaining *Tak1*<sup>-/-</sup> HSPCs died of apoptosis mediated by the caspase-8–dependent extrin-**

**sic apoptotic pathway. This apoptosis can be converted into necroptosis by the inhibition of caspase-8 and prevented by inhibiting both caspase-8 and RIP-1 activities. We concluded that HSPCs are heterogeneous populations in response to death signaling stimulation. *Tak1* mediates a critical survival signal, which protects against both TNF- $\alpha$ /Fas-RIP-1–dependent necroptosis and TNF- $\alpha$ /Fas-independent apoptosis in HSPCs. (*Blood* 2011;118(23):6057-6067)**

## Introduction

In hematopoietic stem and progenitor cells (HSPCs), the survival and death machinery is tightly controlled by a complex interplay between intrinsic signals and stimuli from the surrounding bone marrow (BM) microenvironment, inducing a dynamically balanced network of pro-survival and anti-survival influences. Disruption of this balance can result in hematopoietic disorders, such as myeloproliferative disorders and bone marrow failure (BMF).<sup>1-5</sup>

Prosurvival signaling in HSPCs has been very well studied. Genetic studies clearly demonstrate the role of the survival machinery in regulating numbers and functions of HSPCs.<sup>1-4</sup> Transgenic overexpression of *Bcl-2*, an important pro-survival gene, increases HSPC numbers and improves hematopoietic reconstructive capacity in mice. Mice with deletions of pro-survival genes, such as *Bcl-xl*, *Mcl-1*, or *survivin*, develop severe BMF because of the apoptotic loss of hematopoietic cells, including HSPCs.<sup>1-4</sup> Importantly, the expression of these pro-survival genes is stimulated by hematopoietic cytokines in HSPCs. Hematopoietic cytokines, such as SCF, IL-3, thrombopoietin, IL-6, and IL-11 stimulate the survival of HSPCs as shown by both in vitro culture and genetic studies. Via Jak/Stat, Raf/Mapk, and PI3K/Akt, multiple signaling pathways can be activated by hematopoietic cytokines in HSPCs, resulting in self-renewal, proliferation, differentiation, and survival. Among these, the PI3K/Akt pathway has been proposed to be the mediator of the major survival signal.<sup>6</sup> On activation, PI3K inactivates proapoptotic signals, such as Bad and FoxO3 via a serine/threonine phosphorylation cascade,<sup>7,8</sup> or stimu-

lates the activation of pro-survival signals, such as Nf- $\kappa$ B,<sup>9</sup> which in turn up-regulate the expression of pro-survival genes.

Anti-survival signaling in HSPCs is still not well understood. Previous studies suggested that proinflammatory cytokines, including TNF- $\alpha$ ,<sup>10</sup> IL-1 $\beta$ ,<sup>11</sup> IFN- $\alpha/\gamma$ , as well as FasL (Fas ligand), exercise negative regulation over hematopoiesis and thus were implicated in a number of BMF syndromes.<sup>12-15</sup> However, recent studies suggested that these proinflammatory cytokines stimulate dual signals for HSPC functions.<sup>16-19</sup> A negative signal provokes programmed cell death and/or differentiation by inducing the production of reactive oxygen species (ROS) and by activating cell-extrinsic apoptotic signaling, mediated by caspase-8.<sup>20,21</sup> A positive signal, however, promotes proliferation and survival by inducing the activation of TGF- $\beta$ –activated kinase-1 (Tak1)–mediated signaling.<sup>22-25</sup> Therefore, under normal homeostatic conditions, because of high levels of Tak1 activity, HSPCs are relatively resistant to proinflammatory cytokine-induced cell death.<sup>16,26</sup> The role of apoptotic cell death induced by these factors in BMF syndromes remains somewhat ambiguous. In addition, recent studies have demonstrated that some of these proinflammatory factors also induce a receptor-interacting protein-1 (RIP-1)–dependent programmed cell death called necroptosis in other cell types. It was suggested that RIP-1 and caspase-8 exist within the same protein complex, called a ripoptosome, induced by inflammatory factors that functionally antagonize each other. Caspase-8 represses necroptosis by degrading RIP-1 and stimulates type I or type II apoptosis by directly activating caspase-3 or by promoting

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mitochondrial-dependent caspase-9 activation. Caspase-8–induced apoptosis can be converted into RIP-1-dependent necroptosis when caspase-8 activity is repressed.<sup>27-32</sup> The role of RIP-1–dependent necroptosis in hematopoietic cells has not been studied.

Tak1 is a member of the Map3k family of enzymes and is a key mediator of inflammatory cytokine-induced survival signals.<sup>33</sup> On activation, Tak1 phosphorylates downstream signaling molecules, including Ikk $\beta$ , Mkk3/6, and Mkk4/7; these in turn activate NF- $\kappa$ B, p38, and c-Jun N-terminal kinase (Jnk) signaling cascades, respectively.<sup>33</sup> As a consequence, Tak1 signaling is involved in regulating cell proliferation and survival by inducing the production of several cytokines and the expression of many survival genes (including *Bcl-xl*, *Mcl-1*, and *cIAP*). Gene knockout studies done in animals suggested that Tak1 mediates a major survival signal in almost all types of cells studied, including skin keratinocytes,<sup>34</sup> lymphocytes,<sup>35</sup> intestinal enterocytes,<sup>36</sup> and mouse embryonic fibroblasts.<sup>37</sup> This Tak1-mediated survival signaling plays an essential role in protecting these cells from apoptosis induced by inflammatory cytokines and other unfavorable insults. *Tak1* deletion leads to excessive activation of these cells in response to inflammatory cytokine stimulation, resulting in deregulation of the inflammatory reaction and apoptotic injury in corresponding tissues. We have found that Tak1 is also essential for the survival of hepatocytes and hematopoietic cells, including HSPCs.<sup>38</sup> Interferon-inducible *Tak1*-knockout mice (*MxCre<sup>+</sup>Tak1<sup>fl/fl</sup>*, *Tak1<sup>-/-</sup>* hereafter) developed severe BMF and liver failure because of the massive death of their mutant HSPCs and hepatocytes after induction of *Tak1* deletion.<sup>38</sup>

To study the molecular mechanisms underlying BMF syndromes in our inducible *Tak1<sup>-/-</sup>* mice and also to investigate the factors responsible for inducing the death of *Tak1<sup>-/-</sup>* HSPCs, we generated *Tak1/Tnfr1*, *Tak1/Tnfr2*, *Tak1/Tnfr1/r2*, *Tak1/Fas*, and *Tak1/Tnfr1/r2/Fas* compound-mutant mice to examine whether inactivation of TNF- $\alpha$  signaling, Fas signaling, or the simultaneous inactivation of both of these was able to prevent the BM damage seen in *Tak1<sup>-/-</sup>* mice, and if so, to what degree. In addition, using in vitro assays, we also examined other potential causes of cell death in *Tak1<sup>-/-</sup>* HSPCs. We found that death signaling induced by TNF- $\alpha$  and Fas, via activation of RIP-1, accounted for only 30% to 40% of HSPC loss in *Tak1<sup>-/-</sup>* mice (based on CFU data). Other unknown factor(s), acting through the caspase-8–dependent extrinsic apoptotic pathway, induce the death of the remaining 60% to 70% of *Tak1<sup>-/-</sup>* HSPCs.

## Methods

### Mice

All experiments using mice were performed according to the guidelines of Loyola University Medical Center and were approved by the Loyola University Institutional Animal Care and Use Committee. *MxCre<sup>+</sup>Tak1<sup>fl/fl</sup>* mice<sup>37,38</sup> were crossed with *Fas<sup>-/-</sup>* mice (B6.MRL-*Fas<sup>lpr</sup>/J* from The Jackson Laboratory) and *Tnfr1<sup>-/-</sup>r2<sup>-/-</sup>* mice (B6.129S-*Tnfrsf1a<sup>tm1Imx</sup>Tnfrsf1b<sup>tm1Imx</sup>/J* from The Jackson Laboratory), respectively, to generate heterozygous compound-mutant mice. These heterozygous compound-mutant mice were further crossed to produce *MxCre<sup>+</sup>Tak1<sup>fl/fl</sup>Fas<sup>-/-</sup>*, *MxCre<sup>+</sup>Tak1<sup>fl/fl</sup>Tnfr1<sup>-/-</sup>*, *MxCre<sup>+</sup>Tak1<sup>fl/fl</sup>Tnfr2<sup>-/-</sup>*, *MxCre<sup>+</sup>Tak1<sup>fl/fl</sup>Tnfr1<sup>-/-</sup>r2<sup>-/-</sup>* mice. *MxCre<sup>+</sup>Tak1<sup>fl/fl</sup>Fas<sup>-/-</sup>* and *Tak1<sup>fl/fl</sup>Tnfr1<sup>-/-</sup>r2<sup>-/-</sup>* mice were further crossed to generate *MxCre<sup>+</sup>Tak1<sup>fl/fl</sup>Tnfr1<sup>-/-</sup>r2<sup>-/-</sup>Fas<sup>-/-</sup>* mice. Genotypes of all mice were determined by PCR assay using primers listed in supplemental Figure 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). At 6 to 8 postnatal weeks, all mice, including WT controls (*Cre<sup>-</sup>*) and heterozygous compound-mutant mice, were injected with 5  $\mu$ g/g weight polyriboinosinic acid/polyribocytidylic

acid (poly I:C; GE Healthcare Life Sciences) every other day for a total of 3 injections to induce deletion of the *Tak1* gene.

### Mouse hematopoietic phenotype analysis

Mice were killed 7 to 8 days after the first poly I:C injection. Peripheral blood (PB), spleens, and BM were collected. PB were analyzed for WBC, platelet, and RBC counts by Hemavet 950FS (Drew Scientific). After RBC lysis, mononucleated cells (MNCs) in PB, spleens, and BM samples were counted and stained with surface markers for flow cytometric analysis as described previously.<sup>38</sup>

### Histologic analysis

Femurs from different genotypes of mice were fixed in zinc formaldehyde at room temperature for 3 days. Femurs were then decalcified by 10% EDTA buffer for 2 weeks; tissues were then transferred into 70% ethanol until embedding. Embedding and cutting of sections were performed according to standard protocols from the Pathology Department, Loyola University Medical Center. Slides were then stained with H&E.

### Reagents

Lipopolysaccharide (LPS), as well as ROS scavengers N-acetyl-L-cysteine and butylated hydroxyanisole, were purchased from Sigma-Aldrich. RIP-1 inhibitor necrostatin-1 (Nec-1) was purchased from Santa Cruz Biotechnology. IL-3, IL-6, and SCF were purchased from eBioscience. TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\alpha$ , FasL, TRAIL, and IFN- $\gamma$  were purchased from BD Biosciences.

### HSPC isolation, retroviral infection, and in vitro culturing

High-titer retrovirus was produced by cotransfecting Phoenix cells with a retroviral vector containing the indicated genes together with packaging vectors using *Calphos* Mammalian Transfection Kit (Clontech). Retroviral supernatants were harvested 24 and 48 hours after transfection. Retroviral plasmids used in this study include: MSCV-Cre-GFP and MSCV-Cre-YFP vectors; MSCV-Bcl-xl-GFP, MSCV-cIAP1-GFP, and MSCV-SOD-GFP vectors, which were generated by subcloning *Bcl-xl*, *cIAP*, and *SOD* genes from pMIG-Bcl-xl, pcdna3.1-hciap1, and pF151-pcDNA3.1(+)-SOD1WT plasmids (Addgene) into the MSCV-GFP retroviral vector; MSCV-Tak1-GFP vector, which was generated by subcloning the *Tak1* gene from pCMV-HA-Tak1 plasmid<sup>39</sup> into the MSCV-GFP vector; and MSCV- $\Delta$ -caspase-8-GFP, MSCV- $\Delta$ -caspase-9-GFP, and MSCV- $\Delta$ -FADD-GFP vectors (kindly provided by Dr Strasser of the Walter and Eliza Hall Institute of Medical Research).<sup>40</sup>

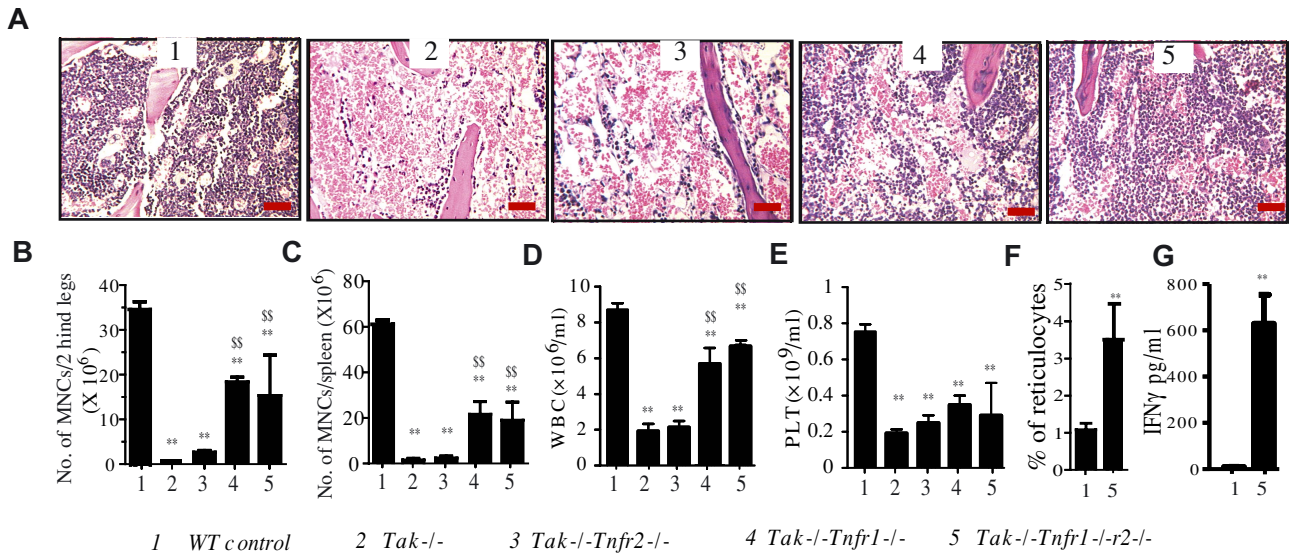
The c-kit<sup>+</sup> HSPCs from indicated genotypes of mice were enriched by *EasySep* Mouse CD117 Positive Selection Kit (StemCell Technologies). HSPCs were cultured in 6-well plates in HSPC culture medium consisting of RPMI 1640 supplemented with 10% FBS, 100 ng/mL mSCF, 50 ng/mL mL-6, and 10 ng/mL mL-3 and were infected with high-titer retrovirus. Infected cells were either incubated in HSPC medium for analysis of changes in the percentage of GFP<sup>+</sup> cells or were purified by FACS for CFU and apoptosis analysis as described in Figures 3, 5 and 6.

### CFU assay

BM MNCs directly isolated from the indicated genotypes of mice or purified infected HSPCs were seeded into MethoCult GF M3434 medium (StemCell Technologies) and incubated at 37°C, 100% humidity, and 5% CO<sub>2</sub> for 10 days. Numbers of colonies were counted according to the manufacturer's instructions.

### Annexin V and 7-AAD staining to analyze for apoptosis

Infected c-kit<sup>+</sup> HSPCs were purified by FACS and incubated in HSPC medium for 24 hours. Cells were then stained with allophycocyanin-conjugated annexin V followed by 7-amino-actinomycin D (7-AAD) staining in binding buffer following the manufacturer's instructions (BD Biosciences). Death of infected cells was examined for the percentages of annexin V<sup>+</sup> and annexin V<sup>+</sup>/7AAD<sup>+</sup> cells by flow cytometry.



**Figure 1. BMF phenotype in *Tak1*<sup>-/-</sup> mice is partially prevented by inactivation of TNF- $\alpha$  signaling.** Femurs, tibias, spleens, and PB of mutant mice with indicated genotypes were collected on day 8 after induction of *Tak1* gene deletion. (A) H&E-stained BM sections from indicated genotypes of mice (20  $\times$  0.7 air). Bar represents 100  $\mu$ m in length. (B-C) Numbers of MNCs from 2 hind legs (B) and spleen (C) of each mouse were counted. (D-E) WBC counts and plt (platelet) numbers in PB of mice were analyzed by CBC. (F) The percentage of reticulocytes was examined in PB by methylene blue staining. (G) Concentration of IFN- $\gamma$  in PB of mice was measured by ELISA. Data are the average of 4 mice from each genotype. \*\*Significant difference compared with WT control mice ( $P < .01$ ). \$\$Significant increase compared with *Tak1*<sup>-/-</sup> mice ( $P < .01$ ).

### Statistical analysis

All data were analyzed using the 2-tailed Student *t* test with GraphPad Prism Version 5.04 software to identify significant differences between groups. Values are expressed as means  $\pm$  SEM. Differences were regarded as significant at  $P < .05$ .

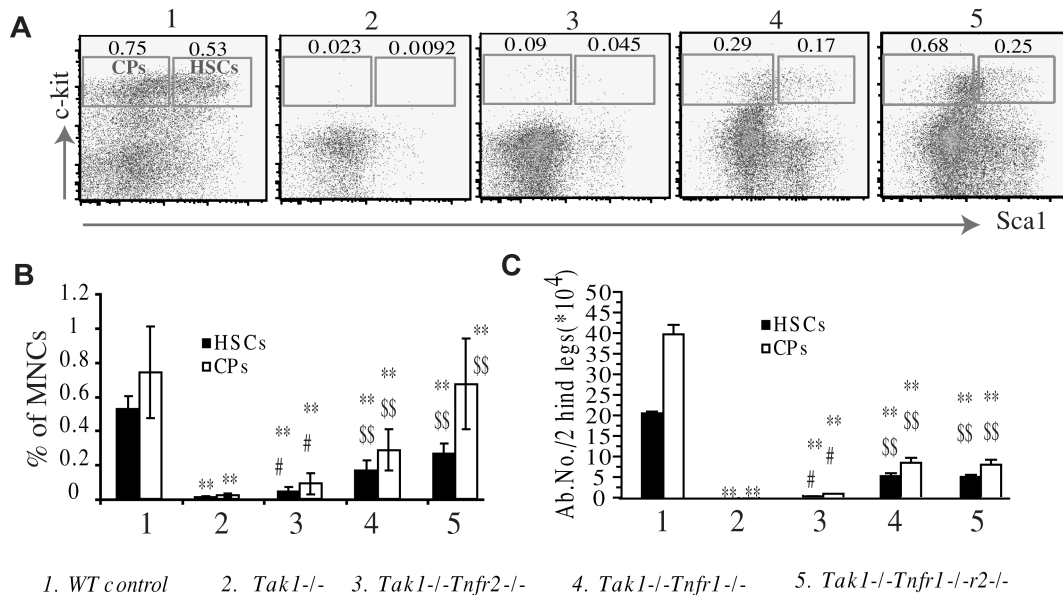
## Results

### The BMF phenotype of *Tak1*<sup>-/-</sup> mice is partially suppressed by the inactivation of TNF- $\alpha$ signaling

TNF- $\alpha$  acts by binding to and activating its specific receptors. Two TNF- $\alpha$ -specific receptors, Tnfr1 (p55) and Tnfr2 (p75), have been identified.<sup>41</sup> To investigate whether the BMF phenotype of *Tak1*<sup>-/-</sup> mice can be prevented by TNF- $\alpha$  signal inactivation, we generated *Mx1Cre<sup>+</sup>Tak1<sup>fl/fl</sup>Tnfr1<sup>-/-</sup>*, *Mx1Cre<sup>+</sup>Tak1<sup>fl/fl</sup>Tnfr2<sup>-/-</sup>*, and *Mx1Cre<sup>+</sup>Tak1<sup>fl/fl</sup>Tnfr1<sup>-/-</sup>r2<sup>-/-</sup>* compound-mutant mice (*Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>, *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup>, and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> hereafter, respectively). Genotypes of the mice were verified by PCR assay, as described in supplemental Figure 1. We previously found that mice with heterozygous *Tak1* deletion did not display phenotypic hematopoietic changes.<sup>38</sup> Therefore, we focused on *Tak1* homozygous knockouts in our studies. Six to 8 weeks after birth, all mice, including Cre-negative *Tak1<sup>fl/fl</sup>* wild-type controls, were injected with poly I:C to induce *Tak1* gene deletion. High efficiency *Tak1* gene deletion was confirmed in hematopoietic cells from *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice by PCR assay (supplemental Figure 1). We found that, as was the case with *Tak1*<sup>-/-</sup> mice, *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> mice, *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> mice, and most *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice died on days 8 to 10 after injection. Only 2 of 10 *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice were able to survive up to 15 and 20 days, respectively, and these showed hunched backs and significantly reduced body weights. Substantial hematopoietic cell infiltration was observed in the livers of these 2 surviving mice when examined on day 15 and day 20, respectively, suggesting excessive inflammatory reactions. However, the death of hepatocytes was obviously reduced compared with

*Tak1*<sup>-/-</sup> mice (supplemental Figure 2). In addition, we found that BM from these 2 mice was hypocellular, as shown by a reduction in the number of total MNCs ( $1.2 \times 10^7$  and  $0.96 \times 10^7$  respectively, approximately one-third of WT mice). We speculated that the *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice might die of super-inflammatory reactions induced by overproduced inflammatory cytokines, such as IFN- $\gamma$  (Figure 1G).

To compare changes in hematopoiesis, mice were killed on day 8 after the first poly I:C injection. Histologic sections showed a significant disruption in BM structure and a hypocellular phenotype for *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> mice, which was comparable to that of *Tak1*<sup>-/-</sup> mice. This *Tak1*<sup>-/-</sup> BM defect was substantially prevented in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> mice and was improved still further in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice (Figure 1A). Consistent with this finding, we also found that the number of MNCs in BM and spleens of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice was significantly increased compared to *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> mice (Figure 1B-C). As a consequence, WBC counts and reticulocyte percentages were also significantly increased in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice compared with *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> mice (Figure 1D,F). Interestingly, the number of platelets in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice was comparable to what was seen in *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> mice, suggesting that the defects in *Tak1* deletion-related thrombopoiesis were not prevented by the inactivation of TNF- $\alpha$  signaling (Figure 1E). It needs to be noted here that, although the BM structure and cellularity of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice were much improved compared with *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> mice, as shown by histologic section studies (Figure 1A), still, because of the relatively smaller body size of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice, the absolute numbers of MNCs in BM and spleens of these mice are still comparable with those in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> mice. These data suggested that TNF- $\alpha$ -induced signaling contributes significantly to the BMF phenotype of *Tak1*<sup>-/-</sup> mice. Both Tnfr1 and Tnfr2 are involved in mediating TNF- $\alpha$ -induced repressive signaling in hematopoietic cells. Consistent with previous studies, we found



**Figure 2. The depletion of *Tak1*<sup>-/-</sup> HSPCs is partially prevented by inactivation of TNF- $\alpha$  signaling.** BM of mutant mice was collected on day 8 after induction of *Tak1* gene deletion. (A-B) Percentages of phenotypic HSCs (Lin<sup>-</sup>Sca<sup>+</sup>c-Kit<sup>+</sup>) and CPs (Lin<sup>-</sup>Sca<sup>-</sup>c-Kit<sup>+</sup>) in total BM MNCs were analyzed by flow cytometry. BM MNCs were stained with FITC-lineage cocktail, PE-Sca1, and allophycocyanin-c-kit antibodies. HSCs and CPs were analyzed by gating first on the lin<sup>-lo</sup> population (lineage<sup>negative/low</sup>); percentages of HSCs and CPs in total BM MNCs were then examined. (C) Absolute numbers of HSCs and CPs were counted. Data are the average of 4 mice from each genotype. \*\*Significant reduction compared with WT control mice ( $P < .01$ ). #Significant increase compared with *Tak1*<sup>-/-</sup> mice ( $P < .05$ ). \$\$Significant increase compared with *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> mice ( $P < .01$ ).

that *Tnfr1* is the major mediator of TNF- $\alpha$ -induced signaling in hematopoietic cells.<sup>10,41-43</sup>

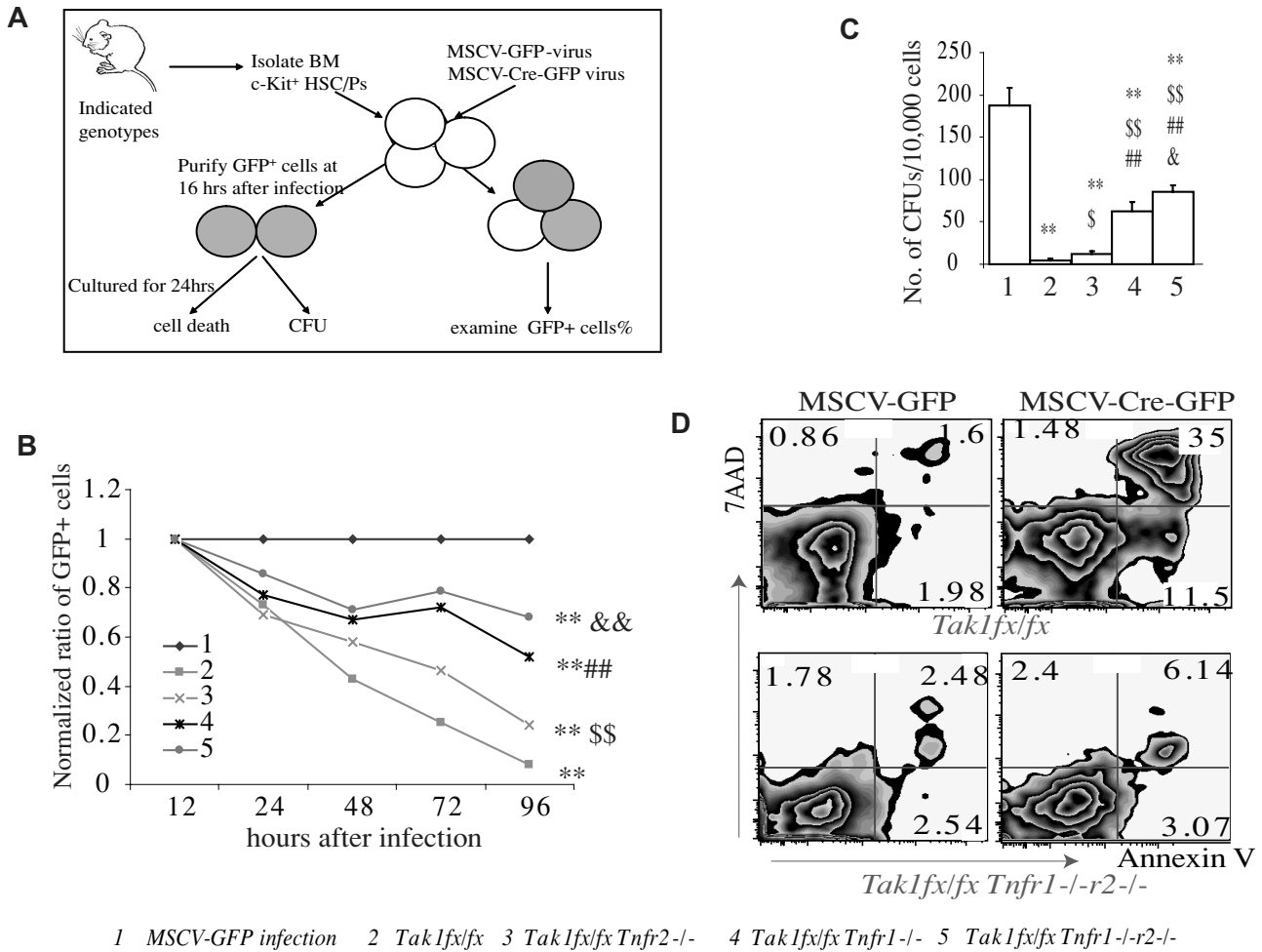
#### The depletion of HSPCs in *Tak1*<sup>-/-</sup> mice is partially prevented by the inactivation of TNF- $\alpha$ signaling

To investigate whether the depletion of *Tak1*<sup>-/-</sup> HSPCs could also be prevented by inactivating TNF- $\alpha$  signaling, we analyzed the percentages and absolute numbers of phenotypic HSCs (lineage<sup>-</sup>c-kit<sup>+</sup>Sca1<sup>+</sup>) and committed hematopoietic progenitors (CPs, lineage<sup>-</sup>c-kit<sup>+</sup>Sca1<sup>-</sup>) in the BM of mutant mice on day 8 after the induction of *Tak1* deletion. We found that the percentages of HSCs and CPs were significantly increased in *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> mice compared with *Tak1*<sup>-/-</sup> mice. Such percentages were further increased in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice, although they had still not returned to normal levels. However, no significant difference was observed between *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice. Consistently, the absolute numbers of HSCs and CPs were significantly increased in *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> mice compared with those in *Tak1*<sup>-/-</sup> mice. These numbers were significantly further increased in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice; no significant difference was observed between the latter 2 types of mice. The absolute numbers of HSCs and CPs in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice were only 25% to 35% of those observed in WT mice (Figure 2).

Out of concern that increased inflammatory cytokines, such as IFN- $\gamma$ , might affect hematopoietic phenotype, to study the role of *Tnfr1* and *Tnfr2*-mediated signaling in the survival of *Tak1*<sup>-/-</sup> HSPCs, we isolated c-kit<sup>+</sup> HSPCs (including HSCs and CPs) from *Tak1*<sup>flx/flx</sup>*Tnfr1*<sup>-/-</sup>, *Tak1*<sup>flx/flx</sup>*Tnfr2*<sup>-/-</sup>, and *Tak1*<sup>flx/flx</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice and infected them in vitro with MSCV-Cre-GFP retrovirus to induce *Tak1* deletion. c-kit<sup>+</sup> HSPCs from *Tak1*<sup>flx/flx</sup> mice were infected with MSCV-Cre-GFP and MSCV-GFP virus (empty vector only) separately and were studied in parallel as positive and negative controls, respectively. Infected cells were cultured in HSPC culture medium with daily medium changes (Figure 3A). The percentages of GFP<sup>+</sup> cells

(representing infected cells) were examined at the indicated time points. These percentages from each sample were first normalized to the percentages of GFP<sup>+</sup> cells at 12 hours in the same sample to obtain a ratio of increased or decreased GFP<sup>+</sup> cells in each sample at the indicated time points. These ratios from each sample were further normalized to the ratio from the MSCV-GFP-infected control group samples at the corresponding time points. This then set the ratio of GFP<sup>+</sup> cells at 1 for the 12 hour time point for all experimental samples, and the ratio of GFP<sup>+</sup> cells at 1 at all time points for MSCV-GFP control samples. The capacity of *Tnfr1* and *r2* deletions to prevent the depletion of *Tak1*<sup>-/-</sup> HSPCs was evaluated by examining the dynamic changes in GFP<sup>+</sup> cell ratios in infected cells (Figure 3B). We found that this dynamic analysis of infected cells ratio assay was very sensitive, reliable, and reproducible in studying gene transduction-related cell loss (such as Cre transduction-related depletion of *Tak1*<sup>-/-</sup> HSPCs in this study). As expected, the ratio of GFP<sup>+</sup> cells in Cre-infected *Tak1*<sup>flx/flx</sup> HSPCs was reduced rapidly during culturing, whereas this reduction in GFP<sup>+</sup> cell ratio was significantly retarded by deletion of *Tnfr2*, *Tnfr1*, or both of these simultaneously. The ability of *Tnfr1* deletion to prevent depletion of *Tak1*<sup>-/-</sup> HSPCs was significantly less than that observed for the deletion of both *Tnfr1* and *r2* but was significantly greater than for the deletion of *Tnfr2* only. However, this ratio in the *Tak1*<sup>flx/flx</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> group was still significantly reduced compared to the MSCV-GFP control group. These data were consistent with in vivo data, supporting the conclusion that inactivation of TNF- $\alpha$  signaling can only partially prevent the death of *Tak1*<sup>-/-</sup> HSPCs. Both *Tnfr1* and *r2* contribute to the TNF- $\alpha$ -induced depletion of *Tak1*<sup>-/-</sup> HSPCs (Figure 3B). In agreement with previous studies,<sup>43,44</sup> we found *Tnfr1* to be the major mediator of TNF- $\alpha$  signaling in HSPCs. This conclusion was further confirmed by CFU assays (Figure 3C).

To study whether TNF- $\alpha$  signaling inactivation can rescue functional HSPCs from *Tak1* deletion-induced depletion, we used FACS to purify the GFP<sup>+</sup> cells from the MSCV-Cre-GFP-infected BM HSPCs with indicated genotypes 16 hours after infection. Purified



**Figure 3. Both *Tnfr1* and *Tnfr2* are involved in mediating the TNF- $\alpha$ -induced death of *Tak1*<sup>-/-</sup> HSPCs.** (A) Schematic diagram of the experimental design. c-kit<sup>+</sup> HSPCs were isolated from BM of mice with indicated genotypes. These HSPCs were infected with MSCV-Cre-GFP virus to induce *Tak1* deletion in vitro. Infected cells were cultured in HSPC medium with medium changes every day. MSCV-GFP-infected *Tak1<sup>lox/flox</sup>* HSPCs were studied in parallel as controls (group 1 in B-C). (B) The percentages of infected cells (GFP<sup>+</sup>) from each sample were examined by flow cytometry at indicated time points and normalized to the percentage of MSCV-GFP-infected control group cells at corresponding time points. (C-D) Infected cells were purified by FACS and seeded for CFU assay. Exactly 30 000 GFP<sup>+</sup> cells from each genotype were sorted and seeded into 3 plates. Numbers of colonies for each plate were counted on day 10 after cell seeding. Data shown in the figure are representative of 3 independent experiments (C). Purified infected cells with indicated genotypes were incubated in HSPC medium for 24 hours, followed by annexin V and 7-AAD staining for cell death analysis (D). \*\*Significant reduction compared with MSCV-GFP-infected control group ( $P < .01$ ). \$Significant increase compared with *Tak1<sup>lox/flox</sup>* group ( $\$P < .05$ ;  $\$\$P < .01$ ). ##Significant increase compared with *Tak1<sup>lox/flox</sup> Tnfr2<sup>-/-</sup>* group ( $P < .01$ ). &Significant increase compared with *Tak1<sup>lox/flox</sup> Tnfr1<sup>-/-</sup>* group ( $\&P < .05$ ;  $\&\&P < .01$ ).

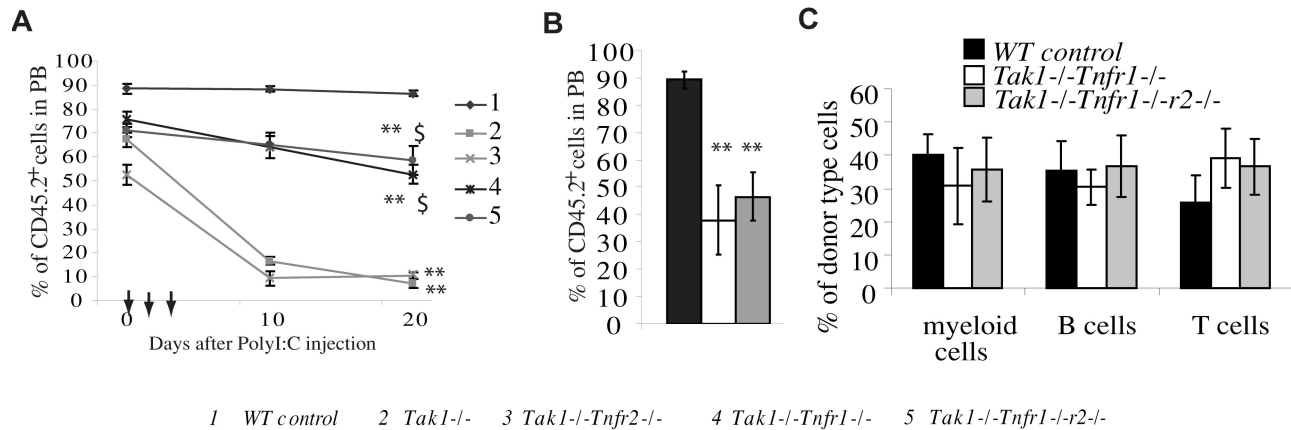
cells were seeded into methylcellulose medium for CFU assay. Because exactly the same number of infected cells was sorted by FACS in each sample for CFU assay, the accuracy of experimental results (accuracy of cell numbers seeded) was assured. We found that deletion of *Tnfr1*, *r2*, or both, as well as Cre overexpression, did not affect the colony-forming ability of *Tak1*-intact HSPCs (supplemental Figure 3). However, deletion of *Tnfr1* and *r2* was able to rescue 20% to 30% and 5% to 10% of the colony-forming capacity of *Tak1*<sup>-/-</sup> HSPCs, respectively. Such rescue was further enhanced by the codeletion of both *Tnfr1* and *r2* (30%-40% of MSCV-GFP-infected control HSPCs; Figure 3C). The phenomenon of prevention of cell death in *Tak1*<sup>-/-</sup> HSPCs by inactivated TNF- $\alpha$  signaling is mainly accomplished through the repression of cell death, as shown by annexin V staining (Figure 3D).

***Tak1*<sup>-/-</sup> *Tnfr1*<sup>-/-</sup> *r2*<sup>-/-</sup> HSPCs have long-term engraftment ability and multipotent differentiation capacity**

To study whether HSPCs from *Tak1*<sup>-/-</sup> *Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup> *Tnfr1*<sup>-/-</sup> *r2*<sup>-/-</sup> mice have multipotent differentiation and long-term engraftment capacities, BM MNCs were isolated from *Tak1*<sup>-/-</sup> *Tnfr2*<sup>-/-</sup>,

*Tak1*<sup>-/-</sup> *Tnfr1*<sup>-/-</sup>, and *Tak1*<sup>-/-</sup> *Tnfr1*<sup>-/-</sup> *r2*<sup>-/-</sup> mice (CD45.2<sup>+</sup>) before *Tak1* deletion was induced. Cells for each group were mixed at a 10:1 ratio with support BM MNCs from WT mice (CD45.1<sup>+</sup>). These mixed BM cells were transplanted into lethally-irradiated recipient mice, each mouse receiving  $2 \times 10^6$  mutant MNCs and  $2 \times 10^5$  WT support MNCs. BM MNCs from WT (Cre<sup>-</sup>) littermates and *Tak1*<sup>-/-</sup> mice were transplanted in parallel as positive and negative controls, respectively. One month after transplantation, the contribution of donor-derived cells (CD45.2<sup>+</sup>) in the PB of recipient mice was examined. Recipient mice were then injected with poly I:C (every other day) for a total of 3 injections to induce *Tak1* gene deletion. The percentages of donor-derived cells in the PB of recipient mice were examined at the indicated time points (Figure 4A).

As was expected, the percentage of donor cells in WT control-transplanted mice remained consistent at > 90%, whereas this percentage was significantly reduced in mice receiving *Tak1*<sup>-/-</sup> BM cells. We found that donor-derived cells in mice receiving *Tak1*<sup>-/-</sup> *Tnfr1*<sup>-/-</sup>, *Tak1*<sup>-/-</sup> *Tnfr2*<sup>-/-</sup>, and *Tak1*<sup>-/-</sup> *Tnfr1*<sup>-/-</sup> *r2*<sup>-/-</sup> BM



**Figure 4. Long-term engraftment ability and multipotent differentiation capacity of compound-mutant HSCs.** BM MNCs (CD45.2<sup>+</sup>) were collected separately from each genotype of mice before *Tak1* deletion was induced. These BM cells were mixed with competitor BM MNCs (CD45.1<sup>+</sup>) in a 10:1 ratio and transplanted into lethally-irradiated recipient mice (CD45.1<sup>+</sup>). Each recipient mouse received  $2 \times 10^6$  BM MNCs of the indicated genotype and  $2 \times 10^5$  competitor BM cells. One month after transplantation, the contribution of donor cells to hematopoiesis in recipient mice was examined by analyzing the percentage of CD45.2<sup>+</sup> cells in their PB. Mice were then injected with poly I:C every other day for a total of 3 injections to induce *Tak1* deletion. The donor cell contribution to recipients' PB composition was analyzed on day 10 and day 20 (A), as well as 3 months (B) after poly I:C injections. (C) The myeloid and lymphocyte differentiation of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs was analyzed 3 months after the induction of *Tak1* deletion. \*\*Significant reduction compared with WT control group ( $P < .01$ ). \$\$Significant increase compared with *Tak1*<sup>-/-</sup> group ( $P < .01$ ).

cells were also reduced significantly compared with WT control transplantation. However, this reduction in the *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> groups was significantly less than that for *Tak1*<sup>-/-</sup> transplanted animals, whereas the *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> group data were comparable with those for the *Tak1*<sup>-/-</sup> group. The reduction in donor-derived cells observed in all experimental groups compared with WT controls before poly I:C injection was due to the spontaneous deletion of the *Tak1* gene caused by Mx1Cre leakage. This result further supports the suggestion of a partial rescue of the depletion of *Tak1*<sup>-/-</sup> HSPCs by inactivation of TNF- $\alpha$  signaling. The donor-derived cells of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> compound-mutant mice were maintained for more than 3 months in recipient mice, at which time the *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr2*<sup>-/-</sup> transplantations were no longer detectable (Figure 4B). Cell surface marker staining suggested that the compound-mutant HSCs had multipotent differentiation capacity with a minor T lymphocyte bias (Figure 4C).

#### Fas inactivation partially prevents the apoptosis of *Tak1*<sup>-/-</sup> HSPCs in vitro but not in vivo

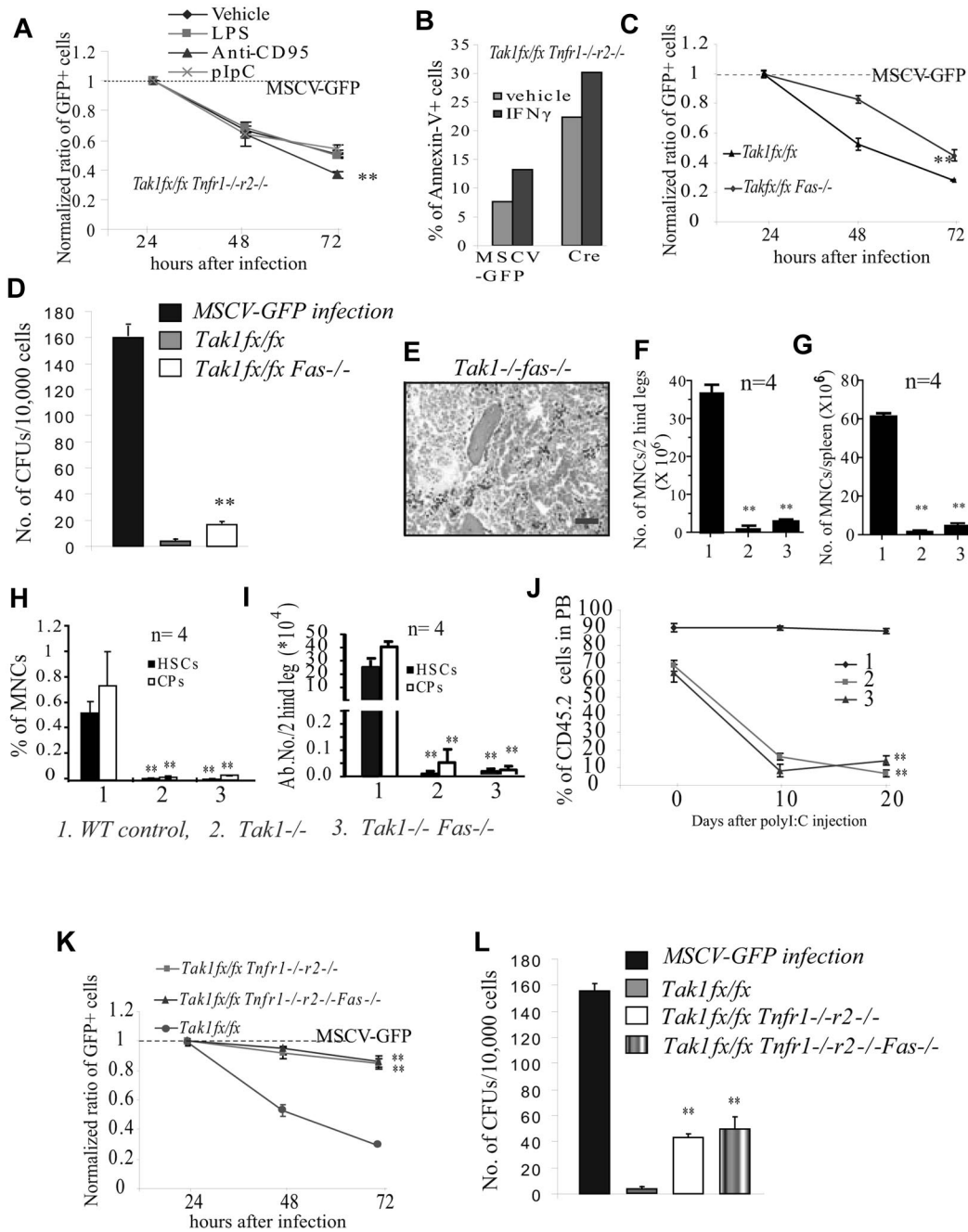
To search for other factors that might potentially be involved in inducing depletion of *Tak1*<sup>-/-</sup> HSPCs, we examined the expression levels of receptors specific for other inflammatory-related factors, including Tnfr2, Tlr-1 (Toll-like receptor-1), Tlr-4, Tlr-5, Tlr-9, Dr-5 (TRAIL receptor) and IFN- $\gamma$ -receptor, in normal and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> hematopoietic cells to define which of these are expressed in normal HSPCs and which are induced in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> HSPCs. We found that the expression levels of Tnfr2, Tlr-1, Tlr-4, Tlr-5, Tlr-9, Dr-5, and IFN- $\gamma$ -r are higher in HSPCs and CPs than in lineage<sup>+</sup> mature blood cells. We also found that the levels of these receptors are elevated in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> HSPCs compared to normal HSPCs. In addition, we found that, although Fas (CD95) is not expressed in normal HSPCs, it is expressed in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup> HSPCs (supplemental Figure 4).

To study whether these inflammatory-related factors are also involved in inducing HSPC death and BMF in *Tak1*<sup>-/-</sup> mice, c-kit<sup>+</sup> HSPCs were isolated from *Tak1*<sup>fl/fl</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice and infected with MSCV-Cre-GFP retrovirus. Infected cells were treated with these

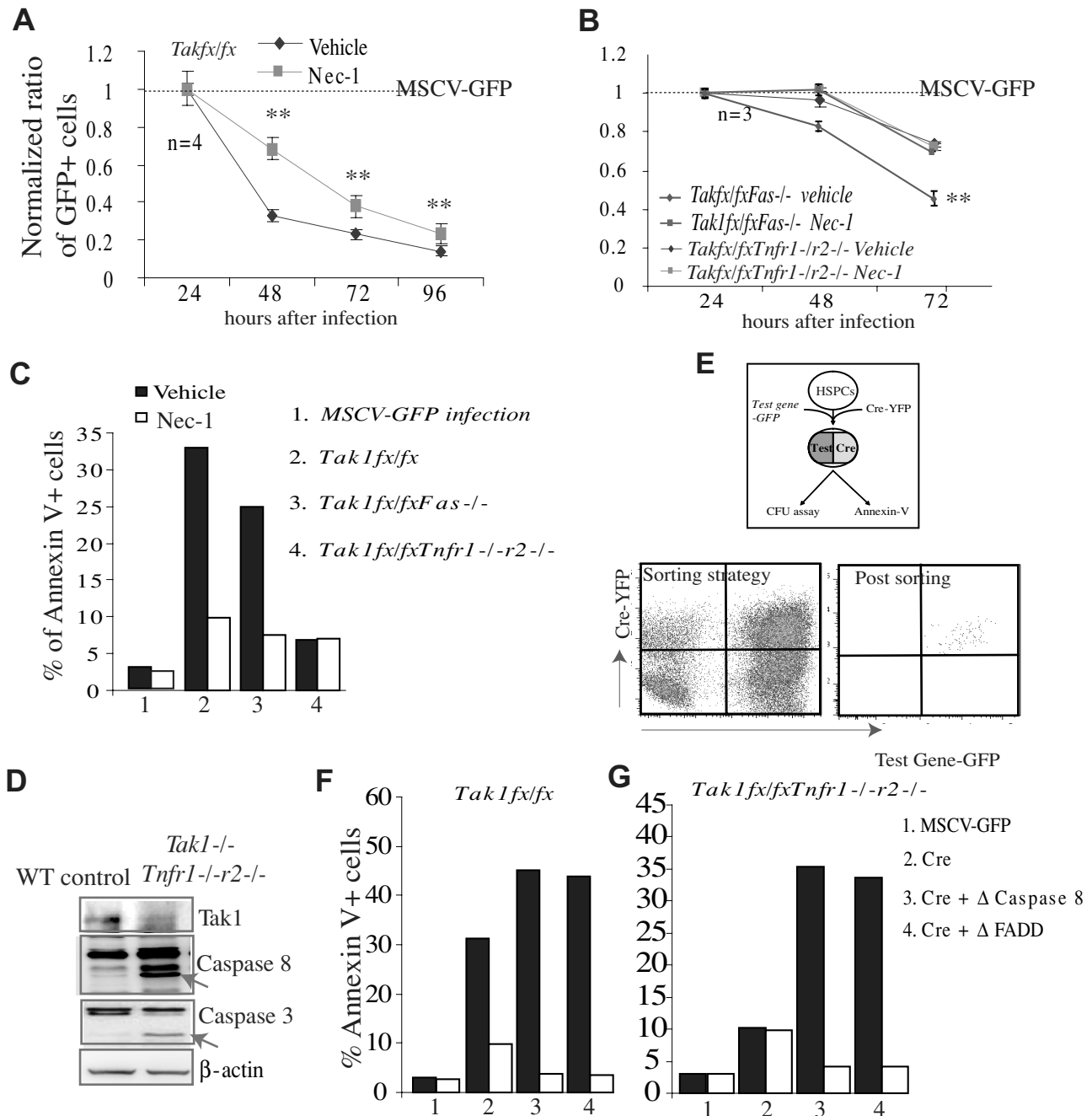
factors individually to test which of them could further enhance the elimination of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs by examining the ratio of reduction of GFP<sup>+</sup> cells. MSCV-GFP viral infection was studied in parallel as a control. We found that, among all the factors we tested, only FasL further enhanced the death of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs (Figure 5A; supplemental Figure 5). IFN- $\gamma$ , on the other hand, induced the death of both WT and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs, which seems to be independent of *Tak1* (Figure 5B). To confirm this finding further, c-kit<sup>+</sup> HSPCs were isolated from *Tak1*<sup>fl/fl</sup>*Fas*<sup>-/-</sup> mice and infected with MSCV-Cre-GFP or MSCV-GFP retrovirus separately. Infected cells were cultured in HSPC culture medium. The effects of Fas deletion on the survival of *Tak1*<sup>-/-</sup> HSPCs were evaluated by examining the dynamic ratio of changes in GFP<sup>+</sup> cells or by CFU assay. We found that *Fas* deletion could also partially prevent the depletion of *Tak1*<sup>-/-</sup> HSPCs in vitro (Figure 5C-D). Interestingly, we found that the depletion of HSPCs and the BMF phenotype of *Tak1*<sup>-/-</sup> mice could not be protected by *Fas*<sup>-/-</sup> in vivo (Figure 5E-J). This difference in *Fas*<sup>-/-</sup> phenotype in *Tak1*<sup>-/-</sup> HSPCs in vitro and in vivo suggests that there might be an additional in vivo factor, which can induce the depletion of the same population of cells, rendering the effects of Fas signaling undetectable. This notion was confirmed by a comparative study of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs with *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup>*Fas*<sup>-/-</sup> HSPCs in vitro. We found that *Fas*<sup>-/-</sup> did not further improve the survival of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs, suggesting that Fas and TNF- $\alpha$  were operating on the same population of cells. In vivo, the death induced by high levels of TNF- $\alpha$  obscures the effects of Fas signaling (Figure 5K-L).

#### Inhibition of RIP-1 mimics the effects of inactivation of TNF- $\alpha$ signaling and partially represses the death of *Tak1*<sup>-/-</sup> HSPCs

It was known that TNF- $\alpha$  and Fas-induced death signaling is mediated by FADD/caspase-8 (known as the traditional extrinsic apoptotic pathway) and RIP-1/RIP-3 (known as the necroptotic pathway), as shown by many cell studies.<sup>27-32</sup> We asked which of these 2 pathways transduces TNF- $\alpha$  and Fas-induced death signaling in *Tak1*<sup>-/-</sup> HSPCs. We found that the death of *Tak1*<sup>-/-</sup> HSPCs was significantly repressed by treatment with the RIP-1 specific inhibitor Nec-1. Interestingly, Nec-1 can only partially prevent the



**Figure 5. Fas inactivation can partially prevent the depletion of *Tak1*<sup>-/-</sup> HSPCs in vitro but not in vivo.** (A-B) c-kit<sup>+</sup> HSPCs were isolated from *Tak1<sup>lox/fx</sup>Tnfr1<sup>-/-</sup>r2<sup>-/-</sup>* mice. (A) These cells were infected with MSCV-Cre-GFP virus to induce *Tak1* deletion and cultured in HSPC medium with or without inflammatory cytokine treatment. MSCV-GFP infection was studied in parallel as a control. The ratios of GFP<sup>+</sup> cells (normalized to MSCV-GFP infection) were compared among different treatment groups. \*\*Significant reduction compared with vehicle-treated group ( $P < .01$ ). (B) These infected cells were purified by FACS and cultured in HSPC medium with or without IFN- $\gamma$  treatment. Cell death was examined by annexin V staining. Data shown in this figure are representative of 3 independent experiments. (C-D) c-kit<sup>+</sup> HSPCs were isolated from *Tak1<sup>lox/fx</sup>* and *Tak1<sup>lox/fx</sup>Fas<sup>-/-</sup>* mice. These cells were infected with MSCV-Cre-GFP virus to induce *Tak1* deletion and cultured in HSPC medium to examine dynamic changes in the ratios of infected (GFP<sup>+</sup>) cells (C) or seeded for CFU assay (D). MSCV-GFP infections were studied in parallel as a control. Normalized ratios of GFP<sup>+</sup> cells were compared between *Tak1<sup>lox/fx</sup>* and *Tak1<sup>lox/fx</sup>Fas<sup>-/-</sup>* HSPCs. \*\*Significant increase compared with *Tak1<sup>lox/fx</sup>* group ( $P < .01$ ). (E-J) *Tak1<sup>-/-</sup>Fas<sup>-/-</sup>* mice were injected with poly I:C every other day for a total 3 injections; BM and spleens were collected 8 days hence. WT and *Tak1<sup>-/-</sup>* mice were treated and studied in parallel as controls. BM histology (20  $\times$  0.7 air; bar represents 100  $\mu$ m in length; E), MNCs in spleen (G), and absolute numbers (I) of HSCs and CPs in BM were compared among WT, *Tak1<sup>-/-</sup>*, and *Tak1<sup>-/-</sup>Fas<sup>-/-</sup>* groups. J. As described in Figure 4, BM MNCs from *Tak1<sup>-/-</sup>Fas<sup>-/-</sup>* (CD45.2<sup>+</sup>) mice (before *Tak1* deletion was induced) were mixed with WT (CD45.1<sup>+</sup>) competitor cells (10:1 ratio) and transplanted into lethally-irradiated recipient mice (CD45.1<sup>+</sup>). After examining the percentages of donor-derived cells in PB of recipient mice, these mice were injected with poly I:C every other day for a total of 3 injections to induce *Tak1* deletion. BM MNCs from WT and *Tak1<sup>-/-</sup>* mice were transplanted and studied in parallel as controls. The percentages of donor-derived cells in PB of the recipients were examined 10 and 20 days later. (F-J) \*\*Significant reduction compared with WT control ( $P < .01$ ). (K-L) c-kit<sup>+</sup> HSPCs were collected from *Tak1<sup>lox/fx</sup>Tnfr1<sup>-/-</sup>r2<sup>-/-</sup>*, *Tak1<sup>lox/fx</sup>Tnfr1<sup>-/-</sup>r2<sup>-/-</sup>Fas<sup>-/-</sup>*, and *Tak1<sup>lox/fx</sup>* mice and infected with MSCV-Cre-GFP to induce *Tak1* deletion. Infected cells were incubated in either HSPC medium to examine the dynamic changes in ratios of infected cells (K), or sorted for CFU assay (L). MSCV-GFP-infected *Tak1<sup>lox/fx</sup>* HSPCs were studied in parallel as controls. (K-L) \*\*Significant increase compared with *Tak1<sup>lox/fx</sup>* group ( $P < .01$ ).



**Figure 6.** The death of *Tak1<sup>-/-</sup>* HSPCs is nearly completely prevented by the inhibition of both RIP-1 and caspase-8 activity. (A-C) c-kit<sup>+</sup> HSPCs were isolated from *Tak1<sup>fx/fx</sup>*, *Tak1<sup>fx/fx</sup>Tnfr1-r2<sup>-/-</sup>*, and *Tak1<sup>fx/fx</sup>Fas<sup>-/-</sup>* mice and separately infected with MSCV-Cre-GFP and MSCV-GFP virus. (A-B) Infected cells were incubated in HSPC medium with or without addition of 30  $\mu$ M Nec-1. The dynamic changes in GFP<sup>+</sup> cell ratios were examined at the indicated time points. Data were normalized to MSCV-GFP infected HSPCs. Dashed line represents the normalized data for MSCV-GFP infected HSPCs. \*\*Significant difference between Nec-1-treated groups and vehicle-only groups. (C) Infected HSPCs were purified by FACS 16 hours after infection and incubated in HSPC medium with or without addition of Nec-1. Death of infected cells was analyzed 24 hours later by annexin V staining. (D) WT and *Tak1<sup>-/-</sup>Tnfr1-r2<sup>-/-</sup>* mice were injected with poly I:C on days 1 and 2 to induce *Tak1* deletion. BM MNCs were isolated on day 3 to examine the activity of caspase-8 and caspase-3 by Western blotting. The bands indicated by arrows are active forms of caspase-8 or caspase-3. (E-G) c-kit<sup>+</sup> HSPCs were isolated from *Tak1<sup>fx/fx</sup>* and *Tak1<sup>fx/fx</sup>Tnfr1-r2<sup>-/-</sup>* mice and coinfected with MSCV-Cre-YFP and MSCV- $\Delta$ -caspase-8-GFP or MSCV- $\Delta$ -FADD-GFP virus. Coinfected cells were purified by FACS to sort for GFP<sup>+</sup>YFP<sup>+</sup> cells (D) and incubated in HSPC medium for 24 hours with or without Nec-1 treatment. Death of coinfecting cells was analyzed by annexin V staining (F-G). MSCV-GFP and MSCV-Cre-YFP infections were studied in parallel as controls. (C,F-G) Data are a representative of 3 independent experiments.

death of *Tak1<sup>-/-</sup>* and *Tak1<sup>-/-</sup>Fas<sup>-/-</sup>* HSPCs; it failed to further improve the survival of *Tak1<sup>-/-</sup>Tnfr1-r2<sup>-/-</sup>* HSPCs (Figure 6A-C). These results suggest that the RIP-1 pathway mediates TNF/Fas-induced death signaling in *Tak1<sup>-/-</sup>* HSPCs.

#### The death of *Tak1<sup>-/-</sup>* HSPCs can be nearly completely prevented by the inhibition of both RIP-1 and caspase-8 activities

To study whether the remaining *Tak1<sup>-/-</sup>* HSPCs died of apoptosis, we examined whether caspases are activated in



*Tak1*<sup>-/-</sup> cells and whether inhibition of caspase activity can prevent the death of these cells. We found that the activities of caspase-8 and caspase-3 are increased in *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> BM MNCs compared with WT counterparts, as shown by Western blotting (Figure 6D, data from *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> BM MNCs only are shown; similar results were observed in *Tak1*<sup>-/-</sup> cells). However, inhibition of FADD/caspase-8 signaling by transduced overexpression of dominant-negative ( $\Delta$ )-FADD or  $\Delta$ -caspase-8 did not repress but rather promoted the death of *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs (necroptosis occurs faster than apoptosis). Interestingly, inhibition of both caspase-8 and RIP-1 activities can nearly completely prevent the death of *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs (Figure 6F-G). These data suggested that the death of the remaining *Tak1*<sup>-/-</sup> HSPCs could be attributed to caspase-8–dependent apoptosis because it can be converted into RIP-1–dependent necroptosis by the inhibition of caspase-8.<sup>27-32</sup>

It was known that active caspase-8 can promote either type I apoptosis by directly inducing the activation of executive caspase-3 or type II apoptosis by inducing mitochondrial-related caspase-9 activation.<sup>45</sup> The increased percentages of *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> BM MNCs with reduced mitochondrial membrane potential ( $\Delta\psi_m$ ) and the activation of caspase-9 in the same cells suggested that at least a subset of *Tak1*<sup>-/-</sup> HSPCs died of type II apoptosis (supplemental Figure 6A-B). This notion was further supported by a partial prevention of the apoptosis of *Tak1*<sup>-/-</sup> and *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs by the overexpression of Bcl-x1 or  $\Delta$ -caspase-9, as shown by both annexin V staining and CFU assays (supplemental Figure 6C,E).

#### Antioxidant treatment fails to prevent the apoptosis of HSC/PS and BMF in *Tak1*<sup>-/-</sup> mice

It has been shown that ROS is the major cause of apoptotic death in intestinal enterocytes and skin keratinocytes in *Tak1*<sup>-/-</sup> mice.<sup>34,36</sup> To study whether ROS-induced cell death also contributes to the BM damage observed in *Tak1*<sup>-/-</sup> mice, we first examined whether ROS levels are increased in *Tak1*<sup>-/-</sup> HSPCs using CM-H<sub>2</sub>DCFDA staining. We found that ROS levels in *Tak1*<sup>-/-</sup> HSPCs are comparable to those in WT counterparts (supplemental Figure 7A). In addition, we found that repression of ROS by treatment of cells with ROS scavengers, such as N-acetyl-L-cysteine or butylated hydroxyanisole, or by overexpression of superoxide dismutase, did not protect the death of *Tak1*<sup>-/-</sup> HSC/PS in vitro (supplemental Figure 7B-E). Moreover, we found that treatment of in vivo with N-acetyl-L-cysteine can significantly prevent liver damage but not the BMF phenotype in *Tak1*<sup>-/-</sup> mice (supplemental Figures 7F-I and 8). We concluded that ROS-induced cell damage might not be significantly involved in the *Tak1*<sup>-/-</sup> deletion-induced apoptotic death of HSPCs.

## Discussion

Studies have suggested that proinflammatory factors are the major negative regulators of hematopoiesis in the BM microenvironment, inducing the depletion of HSPCs. However, studies also suggested that, under certain conditions, most of these inflammatory factors can enhance the growth of HSPCs.<sup>16-19</sup> The molecular mechanism underlying this phenomenon has not been explored.

TNF- $\alpha$  is a major proinflammatory cytokine produced by activated macrophages and lymphocytes. Physiologic levels of TNF- $\alpha$  can be detected in the BM microenvironment.<sup>46</sup> Classically,

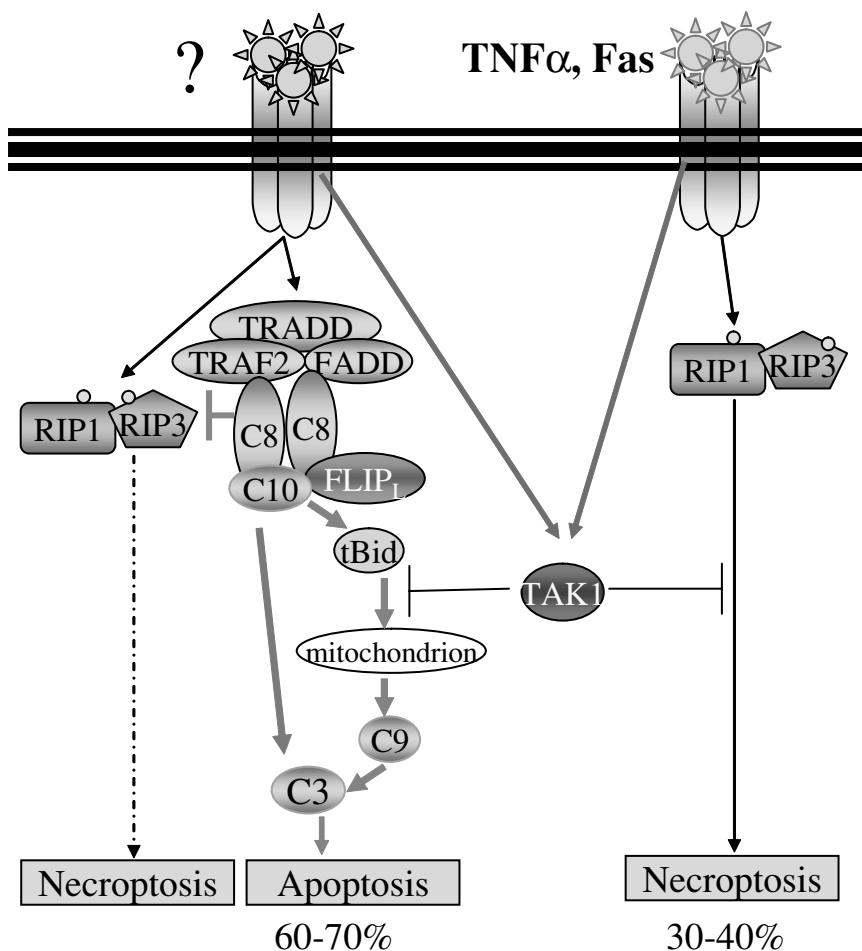
TNF- $\alpha$  has been understood to be an inhibitor of growth and an inducer of apoptosis in HSPCs.<sup>5,10,43</sup> However, increased evidence suggests that TNF- $\alpha$  can also have a positive stimulatory effect on hematopoiesis and is required for the self-renewal and BM engraftment of HSPCs. The significant reduction in both competitive reconstitutive capacity and success in secondary transplantation of *Tnfr1*<sup>-/-</sup> HSPCs suggests that a certain physiologic baseline level of this inflammatory factor is crucial for the maintenance of proper HSC function.<sup>47</sup>

The bidirectional effects of TNF- $\alpha$  on HSPCs observed in previous experiments have been speculated to be the result of the concentration used, effects mediated by different receptors, or other cytokines generating effects at subsequent stages of differentiation.<sup>18,48</sup> Recent evidence suggests that the hematopoietic system is actually maintained by multiple HSC subtypes, each possessing distinct functional characteristics. Based on the ratio of myeloid to lymphoid cells they produced in recipient animals, HSCs can be further distinguished into myeloid-biased or lymphoid-biased HSC categories.<sup>49</sup> Studies indicated that different subsets of HSCs might respond differently to stimulation by various environmental factors. For example, TGF- $\beta$  stimulates the proliferation of myeloid-biased HSCs while repressing the proliferation of lymphoid-biased HSCs.<sup>50</sup> This might explain why certain lineages of hematopoietic cells become selectively activated and expand measurably in certain disease situations, as well as during the aging process.<sup>50</sup>

Our studies suggested that HSPCs are also heterogeneous in terms of their responses to death signal stimulation. HSPCs can be clearly separated into 2 distinct populations based on their response to TNF- $\alpha$ /Fas-induced death signaling (Figure 7). TNF- $\alpha$ /Fas induces a RIP-1–dependent necroptosis in 30% to 40% of *Tak1*<sup>-/-</sup> HSPCs. Whether TNF- $\alpha$ -RIP-1 signaling selectively induces the death of lymphoid-biased HSCs or myeloid-biased HSCs needs further study. In addition, our studies suggest that the remaining *Tak1*<sup>-/-</sup> HSPCs died of traditional extrinsic apoptotic pathway-mediated FADD/caspase-8 cascade–dependent apoptosis. The apoptosis-to-necroptosis conversion of *Tak1*<sup>-/-</sup> HSPCs by inhibition of caspase-8 activity supports the mutual inhibition concept of FADD/caspase-8 and RIP-1/RIP-3 signaling.<sup>27-32</sup>

The factor(s) which induce(s) the apoptosis of these remaining cells need(s) further investigation. Nevertheless, our studies demonstrate that Fas-induced signaling is definitely not the cause. Our data suggest that Fas might be operative in the same population of hematopoietic cells on which TNF- $\alpha$  acts. Inflammatory factors, such as IL-1 $\beta$ , TRAIL, and LPS, also induce death receptor-related apoptosis and Tak1-mediated survival signaling, as demonstrated in studies of other tissues.<sup>33,37</sup> These factors have also been found to repress hematopoiesis and are implicated in the pathogenesis of some BMF syndromes.<sup>11,19</sup> Although we found that the addition of IL-1 $\beta$ , TRAIL, poly I:C, or LPS in vitro did not further enhance the death of *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> HSPCs, there is a possibility that *Tak1*<sup>-/-</sup> HSPCs might be super-sensitive to the apoptosis induced by these factors. Even a low level of these factors in the culture medium might be sufficient to induce the maximum elimination of *Tak1*<sup>-/-</sup> HSPCs. Therefore, we cannot completely exclude the contribution of death signals induced by these factors to the apoptosis of *Tak1*<sup>-/-</sup> HSPCs.

Inflammatory factors, such as IFN- $\alpha$  and IFN- $\gamma$ , have been found to inhibit the growth of HSPCs and promote the proliferation of quiescent HSCs; this scenario has been implicated in many BMF syndromes.<sup>5</sup> However, these factors do not induce death receptor-related signaling, nor do they stimulate Tak1 activity. We found that IFN- $\alpha$  has no significant effect on the apoptosis of WT or *Tak1*<sup>-/-</sup> HSPCs, whereas IFN- $\gamma$  was able to induce the death of both WT



**Figure 7. Schematic model of the mechanism of Tak1 protection of HSPC survival.** Two distinct populations of HSPCs exist in BM hematopoietic tissue based on their response to TNF- $\alpha$ /Fas-induced death signaling. TNF- $\alpha$ /Fas, acting through RIP-1, stimulates necroptosis in 30% to 40% of HSPCs, whereas (an) unknown factor(s) induce(s) caspase-8-dependent type I or II apoptosis in the remaining HSPCs. Tak1-mediated survival signaling, stimulated by proinflammatory cytokines, prevents both types of cell death.

and *Tak1*<sup>-/-</sup> HSPCs. This suggests that IFN- $\gamma$  might induce HSPC death through a mechanism that is independent of Tak1 and TNF- $\alpha$ /Fas. The significant increase of IFN- $\gamma$  concentration in *Tak1*<sup>-/-</sup>*Tnfr1*<sup>-/-</sup>*r2*<sup>-/-</sup> mice suggests that Tak1 might repress the expression of IFN- $\gamma$ . The mechanism behind this phenomenon needs further investigation.

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