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PO Box 117
221 00 Lund
+46 46-222 00 00

Tumor necrosis factor restricts hematopoietic stem cell activity in mice: involvement of two distinct receptors

Cornelis J.H. Pronk,^{1,2,3} Ole Petter Veiby,⁴ David Bryder,² and Sten Eirik W. Jacobsen^{1,5,6}

¹Hematopoietic Stem Cell Laboratory, Lund Strategic Research Center for Stem Cell Biology and Cell Therapy; and ²Immunology Unit, Institute for Experimental Medical Science; Lund University, 221 84 Lund, Sweden

³Department of Pediatric Oncology/Hematology, Skåne University Hospital, 221 85 Lund, Sweden

⁴Millennium Pharmaceuticals, The Takeda Oncology Company, Cambridge, MA 02139

⁵Haematopoietic Stem Cell Biology Laboratory and ⁶Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS, England, UK

Whereas maintenance of hematopoietic stem cells (HSCs) is a requisite for life, uncontrolled expansion of HSCs might enhance the propensity for leukemic transformation. Accordingly, HSC numbers are tightly regulated. The identification of physical cellular HSC niches has underscored the importance of extrinsic regulators of HSC homeostasis. However, whereas extrinsic positive regulators of HSCs have been identified, opposing extrinsic repressors of HSC expansion in vivo have yet to be described. Like many other acute and chronic inflammatory diseases, bone marrow (BM) failure syndromes are associated with tumor necrosis factor- α (TNF) overexpression. However, the in vivo relevance of TNF in the regulation of HSCs has remained unclear. Of considerable relevance for normal hematopoiesis and in particular BM failure syndromes, we herein demonstrate that TNF is a cell-extrinsic and potent endogenous suppressor of normal HSC activity in vivo in mice. These effects of TNF involve two distinct TNF receptors.

CORRESPONDENCE

Sten Eirik W. Jacobsen:
sten.jacobsen@imm.ox.ac.uk

Abbreviations used: 5-FU, 5-fluorouracil; CRU, competitive repopulating unit; HSC, hematopoietic stem cell; PB, peripheral blood.

Loss of function studies have implicated cell-intrinsic factors such as p21 (Cheng et al., 2000), GFI1 (Hock et al., 2004), and LNK (Buza-Vidas et al., 2006) as physiological negative regulators of hematopoietic stem cell (HSC) maintenance. Several extrinsic factors, including TGF- β (Larsson and Karlsson, 2005), IFN- γ (Yang et al., 2005), and IL-3 (Yonemura et al., 1996), as well as TNF (Bryder et al., 2001; Dybedal et al., 2001), have been demonstrated to negatively affect HSC growth and maintenance in vitro. However, the in vivo relevance of these cytokines, as nonredundant cell-extrinsic endogenous negative regulators of the HSC compartment, has not been strongly supported through in vivo lack of function studies (Seita and Weissman, 2010).

TNF, a pleiotropic cytokine exerting both inhibitory and stimulatory effects on a diversity of cellular processes, is a key regulator of immunological responses, and aberrant production of TNF underlies the pathogenesis of many human diseases, in particular acute and chronic inflammatory diseases (Bradley, 2008),

for which TNF-blocking agents have become an established treatment (Wiedmann et al., 2009). Also, several BM failure syndromes such as Fanconi anemia (Dufour et al., 2003; Milsom et al., 2009) are associated with TNF overexpression (Bagby and Meyers, 2007). TNF signals through two distinct TNF receptors, Tnfrsf1a (TNF receptor super family 1a; or TNFR-p55) and Tnfrsf1b (or TNFR-p75; Aggarwal, 2003), that have been ascribed largely diverging roles. Apart from TNF and lymphotoxin (or TNF- β), no other ligands have been shown to activate the TNF receptors (Aggarwal, 2003). Tnfrsf1a has an intracellular death domain implicated in apoptosis signaling, whereas Tnfrsf1b has often been implicated in the promotion of cellular proliferation (Aggarwal, 2003). However, mechanisms of cross talking, including ligand passing between Tnfrsf1a and Tnfrsf1b receptors

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(Tartaglia et al., 1993), implicate that some functions of TNF might be facilitated by or even strictly depend on the expression of both receptors, although evidence for this is limited.

The physiological relevance of the ability of TNF to suppress mouse and human HSC maintenance in vitro (Zhang et al., 1995; Bryder et al., 2001; Dybedal et al., 2001) can rightfully be questioned, among other reasons because the investigated concentrations of TNF might be physiologically irrelevant, because TNF exists in two isoforms (TNF and TNF- β ; Aggarwal, 2003), and because the in vitro experiments could not mimic the physiological balance and potentially distinct roles of soluble and membrane-bound TNF in vivo (Grell et al., 1995). Furthermore, TNF is likely to have pleiotropic direct and indirect effects on HSCs in vivo. In fact, previous in vivo TNF loss of function experiments did not only fail to support a suppressive role of TNF in HSC regulation, they rather implicated a stimulatory role of TNF in HSC maintenance in vivo (Rebel et al., 1999), in apparent discrepancy with studies demonstrating a potent suppressive effect of TNF on normal HSCs in vitro (Bryder et al., 2001; Dybedal et al., 2001), as well as HSCs in Fanconi anemia models for BM failure syndromes being hyperresponsive to TNF suppression in vitro (Milsom et al., 2009). Herein, we sought to reconcile these previous findings and to establish the in vivo role of TNF in the regulation of HSC maintenance and expansion in mice lacking expression of either or both TNF receptors.

RESULTS AND DISCUSSION

TNF restricts HSC activity in vivo

We first investigated HSC numbers and function in mice deficient for both *Tnfrsf1a* (*Tnfrsf1a*^{-/-}) and *Tnfrsf1b* (*Tnfrsf1b*^{-/-}) receptors, hereafter referred to as *Tnfrsf1* double KO (*Tnfrsf1-dKO*; Peschon et al., 1998). Unlike previous studies of *Tnfrsf1a*^{-/-} mice (Zhang et al., 1995; Rebel et al., 1999), we did not observe any differences in phenotypically enriched HSCs (LSKFLT3⁻; Fig. 1, b and c; Adolfsson et al., 2001) nor in frequencies of long-term reconstituted mice when unfractionated BM cells were transplanted at limiting numbers from steady-state BM of WT and *Tnfrsf1-dKO* (Fig. 1 d), suggesting that HSCs are not affected by TNF in steady-state. Steady-state cell cycle analyses of cells within the LSKFLT3⁻ HSC-enriched compartment revealed only a minor increase of cells in active cell cycle (G1 and S/G2/M) in *Tnfrsf1-dKO* mice as compared with WT mice, and in both cases LSKFLT3⁻ cells were predominantly found to be highly quiescent (Fig. 1, e and f).

In contrast to the steady-state analysis, *Tnfrsf1-dKO* BM cells revealed a considerable competitive advantage over WT BM cells in their ability to long-term reconstitute myeloid and lymphoid cell lineages in myeloablated WT recipients (Fig. 2, a–f). This competitive advantage of *Tnfrsf1-dKO* BM cells was further enhanced after three rounds of serial transplantation and maintained for a total of at least five rounds of transplantation (Fig. 2 b). An enhanced *Tnfrsf1-dKO* donor contribution to the phenotypically defined LSK compartment

(enriched for multipotent stem and progenitor cells; Fig. 2 f) mimicked the increased multilineage peripheral blood (PB) reconstitution levels (Fig. 2, b and d), supporting the idea that the suppressive effect of TNF is targeted to multipotent HSCs. Although we did not specifically address the homing of HSCs, altered HSC homing as a consequence of TNF receptor deficiency is unlikely to explain the increased long-term reconstitution, as short-term (3 wk) reconstitution levels were unaffected in recipients of *Tnfrsf1-dKO* BM cells (Fig. 2 b).

TNF-mediated suppression of HSC activity is dependent on expression of both TNF receptors

As previous studies of the specific hematopoietic actions of TNF mediated through *Tnfrsf1a* and *Tnfrsf1b* have been conflicting (Zhang et al., 1995; Rebel et al., 1999), we next evaluated the consequence of loss of either of the two TNF receptors by studying mice deficient for either *Tnfrsf1a* (*Tnfrsf1a*^{-/-}) or *Tnfrsf1b* (*Tnfrsf1b*^{-/-}) receptors. Notably, we observed a competitive multilineage PB and BM LSK reconstitution advantage of BM cells from *Tnfrsf1a*^{-/-} (Fig. 2, g and i) as well as *Tnfrsf1b*^{-/-} (Fig. 2, h and j) mice. This advantage was sustained after secondary transplantations (Fig. 2, g–j), although the competitive advantage was more pronounced with BM cells from *Tnfrsf1-dKO* mice (Fig. 2, a–f), implicating the importance of both TNF receptors in restricting HSC expansion.

We next undertook a series of in vitro experiments to try to corroborate the requirement of both TNF receptors for the observed TNF-induced restriction of HSC activity. As expected, the clonal growth of WT BM cells was strongly inhibited in response to TNF (Fig. 3, a–c). Notably, TNF had no discernable effect on the clonal growth of different myeloerythroid progenitors from *Tnfrsf1a*^{-/-} or *Tnfrsf1b*^{-/-} mice (Fig. 3, a–c), and the same observation was made with regard to the effect of TNF on the growth of HSC-enriched LSK cells (Fig. 3 d), which is compatible with both *Tnfrsf1a* and *Tnfrsf1b* being required for the suppressive effects of TNF on HSCs and hematopoiesis. Importantly, TNF-mediated growth inhibition could not be rescued in cells overexpressing the antiapoptotic protein BCL2 (Fig. S1, a–c; Domen et al., 1998), suggesting that induction of apoptosis plays little or no role in TNF-induced growth inhibition and HSC expansion. However, as these experiments were performed in vitro and as there are alternative, BCL2-resistant pathways for apoptosis, we cannot rule out the possibility that the suppressive effects of TNF on HSCs might involve apoptosis.

In vivo administration of TNF targets HSCs

Although no other ligands than TNF and TNF- β are known for the two TNF receptors, we could not rule out the possibility that the HSC phenotype in *Tnfrsf1a*^{-/-} and *Tnfrsf1b*^{-/-} mice is not mediated by TNF. Thus, we next investigated the effects of TNF administered in vivo on HSCs. Furthermore, as the suppressive impact of TNF on HSC activity after transplantation but not in steady-state hematopoiesis could in part be related to HSCs being recruited from quiescence

into active proliferation upon transplantation (Allsopp et al., 2001), we also sought to address the effect of TNF on cycling versus quiescent HSCs. Of relevance, we previously reported that HSCs up-regulate cell surface expression of TNF receptors and become TNF responsive upon ex vivo proliferation (Bryder et al., 2001). To address whether cell cycle activation also enhances TNF-induced suppression of HSCs in vivo, we took advantage of the cytotoxic drug 5-fluorouracil (5-FU), which effectively eliminates cycling hematopoietic progenitors but not HSCs, resulting in rapid recruitment of dormant HSCs into active proliferation (Harrison and Lerner, 1991). TNF treatment of adult WT steady-state mice (Fig. 4 a)

induced a reduction in total BM cellularity (Fig. 4 b) and a reduction in HSC activity as determined in a competitive transplantation assay (Fig. 4, c–f). This suppressive effect of TNF on HSC activity was enhanced in 5-FU-treated mice (Fig. 4, b–f). These findings suggest that actively cycling rather than quiescent HSCs are the primary targets of TNF suppression, which is of relevance for the enhanced reconstitution after transplantation.

This study provides the first in vivo evidence of a non-redundant, cell-extrinsic factor involved in negative regulation of the HSC compartment, specifically in a setting relevant for BM transplantation. Importantly, our experiments of mice

Figure 1. HSC numbers are normal in steady-state adult BM of *Tnfrsf1*-dKO mice. (a) In six separate experiments with three mice in each experiment, BM cells from two tibiae and two femurs per mouse were individually isolated from age (8–12 wk old)- and sex-matched WT ($n = 18$) and *Tnfrsf1*-dKO ($n = 18$) mice, and mean (SD) cellularities were determined. (b and c) In two separate experiments, age (8–12 wk old)- and sex-matched adult WT C57BL/6 and *Tnfrsf1*-dKO mice, five of each genotype, were analyzed individually for frequencies of LSKFLT3⁺ cells by FACS. (b) Representative FACS analysis for each of the genotypes. Displayed percentages of population frequencies of total BM cells are mean values for five mice in each group, each mouse analyzed individually. (c) BM cell numbers and absolute numbers of LSKFLT3⁺ cells were determined for each mouse individually (see Materials and methods). Mean (SD) values of five mice are shown. (d) In two separate experiments with 14 recipients in each, 20,000 unfractionated BM cells from either WT (CD45.1^{+/2+}) or *Tnfrsf1*-dKO (CD45.1^{-/2+}) mice were transplanted, together with 200,000 competitor BM cells (CD45.1^{+/2-}), into lethally irradiated congenic WT recipients (CD45.1^{+/2-}). 16 wk after transplantation, recipients were analyzed individually for multilineage reconstitution. The percentage of PB donor myeloid reconstitution in positively reconstituted mice is indicated. Horizontal lines represent median reconstitution levels. The frequencies of reconstituted mice (as defined in Materials and methods) out of total transplanted mice are indicated. The indicated p-value is for difference in mean donor reconstitution levels of mice transplanted with *Tnfrsf1*-dKO and WT BM cells. (e and f) CD45.1⁺ BM cells from four WT mice and CD45.2⁺ BM cells from four *Tnfrsf1*-dKO mice were individually isolated, and subsequently cells from each of the WT replicates were mixed with cells from one of the *Tnfrsf1*-dKO mice. Cells from each genotype were mixed at equal numbers, and each cell mixture was analyzed individually. Cell mixtures were analyzed by FACS for G0 (DAPI^{low}/Ki67⁻), G1 (DAPI^{low}/Ki67⁺), and S/G2/M (DAPI^{high}/Ki67⁺) cell cycle stages within the LSKFLT3⁺ HSC compartment. Percentages represent mean (SD) values of all mice.

Figure 2. Enhanced activity of TNF receptor-deficient HSCs after transplantation. (a–f) BM cells were pooled from three *Tnfrsf1*-dKO (CD45.2⁺) or three WT (CD45.1⁺) mice (8–12 wk old), and for each of the genotypes 10^6 unfractionated BM cells were competitively cotransplanted into lethally irradiated congenic WT recipients (CD45.1⁺/2⁺). Recipient mice were evaluated for PB multilineage reconstitution levels at the indicated time points. (a) Representative FACS analysis illustrating PB myeloid chimerism evaluation. (b) PB myeloid reconstitution derived from transplanted *Tnfrsf1*-dKO and WT cells was analyzed at the indicated time points. At the time points indicated by the vertical dashed lines, mice were sacrificed, and 0.5 femur equivalent of BM cells were serially transplanted into newly irradiated (CD45.1⁺/2⁺) recipients. Displayed are PB myeloid chimerism levels. Results are mean (SEM) values from six separate experiments with five to seven recipients in each experiment. (c–f) 16 wk after the primary transplantation, PB analysis of T (CD4/CD8), myeloid (MAC1), and B (B220) cell reconstitution was performed (d), BM was analyzed for LSK chimerism (f), and mean percentages for all recipients are presented. (c and e) Representative FACS analysis for reconstitution levels within the PB and BM are shown. (d and f) Results are from six separate experiments with five to seven recipients in each experiment. Mean (SEM) values are shown. (g–j) BM cells from 8–12 wk old *Tnfrsf1a*^{-/-} (g and i) and *Tnfrsf1b*^{-/-} (h and j) mice (CD45.2⁺, backcrossed for 10 generations with C57BL/6 mice) were transplanted in competition with WT BM cells (CD45.1⁺) into lethally irradiated WT (CD45.1⁺/2⁺) recipients. PB myeloid chimerism levels (g and h) and BM LSK chimerism levels (i and j) at the indicated time points after primary and secondary (indicated by dashed line) transplantations are shown. All results are mean (SEM) values from two separate experiments with five to seven recipients in each experiment. PB and BM reconstitution analysis were performed as illustrated in panels a and e, respectively.

mice of each indicated genotype were single cell sorted into Terasaki plates containing SCF and G-CSF, $\pm$ TNF. After 11 d of culture, the number of colonies (>50 cells) was scored. Mean (SD) values from two experiments with two replicate plates each and each plate containing 60 cells are shown.

deficient in *Tnfrsf1a* or *Tnfrsf1b* expression, corroborated by in vitro TNF response experiments, indicate that the ability of TNF to suppress HSCs is dependent on expression of both TNF receptors. This clearly implicates the importance of both receptors in eliciting the signaling required to suppress

HSCs, either through signaling through both receptors or potentially through the suggested role of the *Tnfrsf1b* in ligand passing to the *Tnfrsf1a* receptor (Aggarwal, 2003).

The current findings are of considerable relevance for several BM failure syndromes, in which enhanced expression of TNF has been implicated in the pathogenesis of hematopoietic failure

Figure 4. In vivo administration of TNF suppresses cycling HSCs. (a) Experimental design. Age (8–12 wk old) and sex-matched C57BL/6 WT mice received 150 mg/kg 5-FU or PBS 3 d before intravenous injections of $3 \times 2 \mu\text{g}$ TNF or PBS. (b) BM cellularity in mice treated with PBS, PBS and TNF, 5-FU, or 5-FU and TNF. Mean (SD) values of three experiments, each with three individual mice in each treatment group, are shown. (c–e) At day 0, 1/50 of unfractionated BM cells (CD45.2⁺) in two femurs and two tibias from treated mice in each of the indicated groups were transplanted in competition with 10^6 unfractionated WT BM (CD45.1⁺) cells into congenic lethally irradiated WT recipients (CD45.1⁺/2⁺). 16 wk after transplantation, PB reconstitution levels were analyzed for percentage of CD45.2⁺ contribution within the T cell (c), B cell (d), and myeloid blood cell (e) lineages. (f) Bars show calculated numbers (see Materials and methods) of total CRUs in PBS-, PBS + TNF-, 5-FU-, and 5-FU + TNF-treated donor BM cells based on PB levels of myeloid chimerism 16 wk after transplantation. (c–f) Results are mean (SD) values from three separate experiments, each with five to seven recipients/group.

(Bagby and Meyers, 2007; Aalto et al., 2011). For instance, in the case of Fanconi anemia, TNF has not only been demonstrated to be overexpressed in the BM (Dufour et al., 2003), but HSCs have also been found to be hyperresponsive to TNF suppression in vitro (Milsom et al., 2009). The physiological relevance of these in vitro findings, as well as previous findings demonstrating that TNF has potent suppressive effects on WT mouse and human HSCs in vitro (Bryder et al., 2001; Dybedal et al., 2001), are supported by our demonstration of a suppressive role of *Tnfrsf1a* and *Tnfrsf1b* on HSCs in vivo, as well as an HSC-suppressive effect of TNF administration in vivo. The reason for the opposite conclusions reached in the experiments by Rebel et al. (1999) remains unclear. In that study, *Tnfrsf1a* was rather suggested to play a critical positive role in maintaining HSCs. This role of TNF appeared to be restricted to older mice (>6 mo old), although phenotypic analysis of the HSC compartment in older mice showed no significant difference from WT mice (Rebel et al., 1999). Thus, it was only in long-term transplantation experiments that reduced multilineage reconstitution was observed from *Tnfrsf1a*^{-/-} BM cells as an indication of reduced HSC activity (Rebel et al., 1999), although phenotypic analysis of the HSC compartment itself was not performed in transplanted mice. It is not obvious how the age of the TNF receptor-deficient HSCs could explain the different conclusions reached between this previous study and our study, as we in our study performed serial transplantation experiments for up to 76 wk with HSCs with an intrinsic TNF receptor deficiency. Moreover, in our study, the enhanced long-term multilineage reconstitution levels of TNF receptor-deficient BM cells were corroborated by correspondingly enhanced chimerism also in the phenotypic HSC compartment. Although the mice used herein carry the same mutated alleles, the genetic background of the TNF receptor-deficient strains and the nature of the WT control mice were not described in detail in the study of Rebel et al. (1999), and therefore the apparent discrepancy could potentially be explained by this. In that regard, our study included experiments in which experimental mice had been backcrossed to a C57BL/6 genetic background and in which WT littermates were used as controls.

This study establishes the in vivo relevance of the suppressive effects of TNF on HSCs in hematopoiesis and BM failure syndromes and supports the further exploration of anti-TNF treatment in such conditions (Dufour et al., 2009; Scott et al., 2010; Aalto et al., 2011). The in vivo conditioning models used in this study, 5-FU treatment and irradiation, have been shown to cause increases in blood levels of TNF (Xun et al., 1994; Okamoto et al., 2000), suggesting that anti-TNF treatment might also be beneficial to enhance posttransplantation reconstitution (Ferrara et al., 2009).

MATERIALS AND METHODS

Mice. *Tnfrsf1-dKO* mice (TNF receptor double KO; Peschon et al., 1998) were obtained from the Jackson Laboratory (stock no. 003243). The *Tnfrsf1-dKO* mice were originally generated by interbreeding mice deficient for *Tnfrsf1a* (or *Tnfr-p55*, generated on a C57BL/6 background) with mice deficient for *Tnfrsf1b* (or *Tnfr-p75*, generated on a 129 background),

followed by subsequent backcrossing for five generations to C57BL/6 mice. *Tnfrsf1-dKO* mice were used for phenotyping and competitive transplantation experiments, with WT C57BL/6 mice (CD45.2⁺, CD45.1⁺, or F1 CD45.1⁺/CD45.2⁺) as controls. *Tnfrsf1a*^{-/-} and *Tnfrsf1b*^{-/-} single KO mice were generated by crossing *Tnfrsf1-dKO* to WT C57BL/6 mice to create single KO mice with identical allelic mutations as the original *Tnfrsf1a*^{-/-} and *Tnfrsf1b*^{-/-} mice, respectively. Heterozygous offspring were interbred to generate *Tnfrsf1a*^{+/-} and *Tnfrsf1b*^{+/-} mice for in vitro experimentation (Fig. 3, a–d), and WT littermates were used as controls. Single KO mice were also backcrossed with C57BL/6 mice for an additional four generations to generate 10th generation *Tnfrsf1a*^{-/-} and *Tnfrsf1b*^{-/-} single KO mice. These mice were used in the transplantation experiments. *H2K-BCL-2* mice have been described previously (Domen et al., 1998). Mice were maintained at the Lund University animal facility. All mice procedures were performed with consent from the local ethics committee (Malmö/Lunds djurförsöksetiska nämnd).

Phenotypic analysis and purification of HSCs. BM cells were collected from femurs, tibiae, and sometimes iliac crests. For both phenotypic analysis and FACS purification, unfractionated BM cells were stained with either purified or fluorochrome-conjugated lineage antibodies against B220 (RA3-6B2), GR-1 (RB6-8C5), MAC1 (M1/70), CD8 (53-6.7), TER119, CD4 (H129.19), and sometimes CD5 (53-7), all from BD. FACS purifications were performed as previously described (Adolfsson et al., 2001). In brief, cells were next either lineage depleted using sheep anti-rat IgG(Fc)-conjugated immunomagnetic beads (Invitrogen) or cKIT enriched by using cKIT-conjugated magnetic beads (Miltenyi Biotec). Purified lineage antibodies were visualized with CyChrome (Invitrogen)- or Qdot 605 (Invitrogen)-conjugated goat anti-rat antibodies; in the case of lineage depletion, cells were stained after the lineage enrichment, and in the case of cKIT enrichment, cells were stained before magnetic selection. Subsequently, cells were stained with different combinations of fluorochrome-conjugated antibodies against SCA1 (D7 [BioLegend] or E13-161.7 [BD]), cKIT (or CD117, 2B8; eBioscience or BD), FLT3 (or Flk2/CD135, 2F10.1; BD or eBioscience), and CD150 (or Slamf1, TC15-12F12.2; BioLegend). Propidium iodide (Invitrogen) or 7-amino-actinomycin D (Sigma-Aldrich) was used to exclude dead cells. Gating was performed to exclude doublets from analysis.

For calculation of total numbers of LSK and LSKFLT3⁻ cells, the frequency of LSKFLT3⁻ cells in each individual mouse, as determined by flow cytometric evaluation of unfractionated BM cells, was multiplied by total BM cell numbers. Total BM numbers were calculated based on total BM cells in two femurs and two tibiae, representing 25% of total BM cells present in a mouse. For cell cycle analyses (Thorén et al., 2008), ~30 × 10⁶ CD45.1⁺ (WT) and 30 × 10⁶ CD45.2⁺ (*Tnfrsf1-dKO*) BM cells were mixed and stained with antibodies against lineage markers, SCA1, cKIT, CD150, and FLT3 as described in the previous paragraph, as well as with antibodies against CD45.1⁺ (A20; BioLegend) and CD45.2⁺ (104; BioLegend). Subsequently, cells were fixated in 2% paraformaldehyde (GTF) and permeabilized in 0.1% saponin from quillaja bark (Sigma-Aldrich) and stained with anti-Ki67 (B56; BD) or isotype-conjugated antibodies. Before analysis, cells were incubated in 2.5 µg/ml DAPI (Invitrogen) to visualize DNA content.

Cells were analyzed or sorted on FACS Aria or DIVA cell sorters (BD). All flow cytometry and FACS data were analyzed with FlowJo software (Tree Star).

In vivo transplantation experiments. Competitive transplantation experiments using the congenic CD45.1⁺/CD45.2⁺ mouse model were performed as described previously (Szilvassy et al., 1990; Adolfsson et al., 2001). In brief, CD45.2⁺ BM cells from TNF receptor-deficient mice were injected intravenously along with competitor CD45.1 BM cells into lethally irradiated (950 cGy) CD45.1⁺/2⁺ mice. PB was collected at the indicated time points after transplantation and analyzed for donor reconstitution by FACS. PB was obtained from retroorbital bleeding, red blood cells were sedimented with 2% dextran T-500 (Pharmacia), remaining red blood cells

were lysed with ammonium chloride, and leukocytes were subsequently stained with antibodies against CD3, B220, and MAC1 or against CD4, CD8, B220, MAC1, and NK1.1 (PK136; BioLegend).

In limiting dilution experiments, positively multilineage reconstituted mice were defined as having a minimum of 0.1% total donor PB cells and 0.02% donor cells of each of the myeloid (MAC1⁺), B (B220⁺), and T (CD4/CD8⁺) cell lineages. Competitive repopulating unit (CRU) frequencies were calculated using Poisson9 software (Benveniste et al., 2003). For serial transplantations, BM cells from reconstituted recipients were isolated, and cells representing 0.5 femur equivalent were serially transplanted into secondary irradiated recipients.

In *in vivo* TNF treatment experiments, WT (CD45.2⁺) were injected intravenously with 5-FU and/or recombinant mouse TNF (Genentech). After treatment, BM cells were isolated from two femurs and two tibiae and counted, and 1/50 of isolated BM cells (i.e., 1/200 of total mouse BM cells) were transplanted in competition with 10⁶ fresh CD45.1⁺ BM into each lethally irradiated CD45.1⁺ recipient. The total number of CRUs present in a mouse after treatment was calculated as previously described (Szilvassy et al., 1990). Based on the assumption that one CRU resides within 10⁴ whole BM cells (Szilvassy et al., 1990), the competing graft (10⁶ whole BM) contained 100 CRUs. The number of CRUs per transplant from treated mice (X) = 100 × (% myeloid chimerism 16 wk after transplant)/(100% – % myeloid chimerism 16 wk after transplant). The number of BM cells containing 1 CRU (Y) = (total BM cellularity/200) × X. Total CRUs/mouse = total BM cellularity/Y.

In vitro culture assays. Freshly isolated unfractionated BM cells and sorted HSC-enriched cells were cultured using liquid and semisolid culture conditions (at 37°C, 98% humidity, and 5% CO₂) to evaluate their colony-forming and clonogenic capacities. CFU-GM, BFU-E, and CFU-Meg formations from whole BM were evaluated in semisolid methylcellulose medium as previously described (Adolfsson et al., 2001), and cells were cultured in the presence or absence of 20 ng/ml TNF in addition to the indicated cytokines. For evaluation of proliferative capacities, HSC-enriched cells were sorted into serum-free medium (X-vivo15; BioWhittaker), supplemented with 1% detoxified bovine serum albumin (STEMCELL Technologies), 0.1 mM β-mercaptoethanol, 2 mM L-glutamine (Invitrogen), streptomycin/penicillin (Sigma-Aldrich), and cytokines rSCF (25 ng/ml; Amgen or PeproTech), rhIL-6 (50 ng/ml; provided by Genetics Institute Cambridge, San Francisco, CA), and rmlL3 (20 ng/ml; PeproTech), with or without 20 ng/ml TNF. After 7 d of culture, total cell numbers were counted, and expansion equivalents were calculated. For evaluation of clonogenic capacity, HSC-enriched cells were single cell sorted in Terasaki plates into IMDM, supplemented with 10% prescreened FCS (Invitrogen), β-mercaptoethanol, L-glutamine, and streptomycin/penicillin as above, with the addition of 25 ng/ml rSCF and 25 ng/ml rhG-SCF (Amgen), with or without 20 ng/ml TNF. After 11 d in culture, wells were evaluated for colony numbers and size.

Statistics. Statistical analyses were performed using Excel software (Microsoft). Student's *t* test was used to generate all *p*-values.

Online supplemental material. Fig. S1 shows clonogenic capacities of HSCs sorted from WT or *Bcl2* transgenic mice cultured in the presence and absence of TNF. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20110752/DC1>.

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