Self-Renewal of Multipotent Long-Term Repopulating Hematopoietic Stem Cells Is Negatively Regulated by Fas and Tumor Necrosis Factor Receptor Activation

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Abstract

Multipotent self-renewing hematopoietic stem cells (HSCs) are responsible for reconstitution of all blood cell lineages. Whereas growth stimulatory cytokines have been demonstrated to promote HSC self-renewal, the potential role of negative regulators remains elusive. Receptors for tumor necrosis factor (TNF) and Fas ligand have been implicated as regulators of steady-state hematopoiesis, and if overexpressed mediate bone marrow failure. However, it has been proposed that hematopoietic progenitors rather than stem cells might be targeted by Fas activation. Here, murine $Lin^-Sca1^+c\text{-}kit^+$ stem cells revealed little or no constitutive expression of Fas and failed to respond to an agonistic anti-Fas antibody. However, if induced to undergo self-renewal in the presence of TNF-α, the entire short and long-term repopulating HSC pool acquired Fas expression at high levels and concomitant activation of Fas suppressed in vitro growth of $Lin^-Sca1^+c\text{-}kit^+$ cells cultured at the single cell level. Moreover, $Lin^-Sca1^+c\text{-}kit^+$ stem cells undergoing self-renewal divisions in vitro were severely and irreversibly compromised in their short- and long-term multilineage reconstituting ability if activated by TNF-α or through Fas, providing the first evidence for negative regulators of HSC self-renewal.

Key words: hematopoietic stem cells • bone marrow transplantation • tumor necrosis factor • Fas • Fas ligand

Introduction

Self-renewing hematopoietic stem cells (HSCs)* are responsible for replenishing all blood cell lineages through life. Although representing only 1 in 10,000 bone marrow (BM) cells, sophisticated methods have been developed to isolate HSCs to high purity (1), allowing meaningful studies of candidate stem cells in vitro. However, HSCs cannot be purified to homogeneity, and must thus ultimately be identified through their unique ability to long-term multilineage reconstitute ablated recipients (1).

Hematopoiesis is regulated in part by a large number of hematopoietic growth factors supporting proliferation, differentiation, and survival of hematopoietic cells at different levels of differentiation (2, 3). Most evidence support that the primary role of such cytokines in early hematopoiesis is to prevent programmed cell death, rather than to induce programs for growth and differentiation (4–7).

Much less is known about the potential role of negative regulators in hematopoiesis (8–11). Receptors for TNF-α and Fas ligand have been implicated to be involved in suppressing hematopoiesis. A growth-inhibitory effect of TNF-α in early murine hematopoiesis is supported by in vitro studies of purified primitive murine progenitors (11, 12), but it has not been investigated whether this is associated with a negative or positive effect on HSC function. Studies in TNF receptor–deficient mice have suggested that the number of phenotypically defined ($Lin^-Sca1^+c\text{-}kit^+$) stem cells is increased (11). However, more recent studies

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*Abbreviations used in this paper: BM, bone marrow; GVHD, graft versus host disease; HSC, hematopoietic stem cell; LTRC, long-term repopulating cell; PB, peripheral blood.

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have shown that this increase does not necessarily correlate with enhanced in vivo HSC function (13), emphasizing that true long-term repopulating cells (LTRCs) only can be evaluated through their unique ability to long-term multilineage reconstitute conditioned recipients in vivo (1, 14).

Fas has been implicated to play a role in regulation of myelopoiesis and as a potential tumor suppressor of myeloid leukemic development (10). However, the status of its expression and potential function on HSC remains elusive. Fas-mediated apoptosis of committed myeloid progenitors has been demonstrated to be involved in the myelosuppression associated with acute graft versus host disease (GVHD; reference 9). Accumulating evidence also support a role of TNF-α and Fas in a number of chronic disorders of blood cell formation associated with enhanced apoptosis (15–22). However, as candidate murine BM stem cells, unlike myeloid progenitors, have been reported to lack expression of cell surface Fas (10, 23), HSCs have been suggested to represent unlikely targets for Fas-induced BM suppression.

We have recently, through HSC division tracking and serial transplantation experiments, demonstrated that murine $Lin^-Sca1^+c\text{-}kit^+$ long-term reconstituting stem cells can undergo multiple self-renewing divisions in vitro (24, 25). Others and we have demonstrated that this self-renewal process is positively affected by cytokines such as IL-3, IL-6, IL-11, c-kit ligand, and thrombopoietin (24–27). Thus, this allowed us to investigate how TNF-α and Fas might potentially affect HSC self-renewal.

In these studies we demonstrate that purified $Lin^-Sca1^+c\text{-}kit^+$ repopulating BM stem cells (28–30) have little or no constitutive expression of Fas and fail to respond to an agonistic anti-Fas antibody. However, TNF-α efficiently induces Fas expression and responsiveness of cycling $Lin^-Sca1^+c\text{-}kit^+$ HSCs, resulting in severely compromised ability to short- and long-term multilineage reconstitute the hematopoietic system.

Materials and Methods

Hematopoietic Growth Factors and Antibodies. Recombinant rat (rr) KL (stem cell factor) and recombinant human (rh) G-CSF were provided by Amgen Corp. (Thousand Oaks, CA). Recombinant murine (rm) IL-3 was from PeproTech. rhIL-6 was a gift from Genetics Institute (Cambridge, MA). rmTNF-α was supplied by Genentech (San Francisco, CA) and rh flt3 ligand (FL) was a gift from Immunex (Seattle, WA). Unless otherwise indicated, all cytokines were used at the following predetermined optimal concentrations: rrKL and rhIL-6: both at 50 ng/ml; rmIL-3 and rmTNF-α: 20 ng/ml. When a cocktail of KL plus IL-3 plus IL-6 plus FL plus G-CSF was used, the concentrations of the cytokines were 25 ng/ml, except for IL-3 which was used at 10 ng/ml. Purified monoclonal hamster anti–mouse Fas antibody (Jo2) and irrelevant isotype-matched hamster IgG antibody (both from BD PharMingen) were used at 0.2 to 0.5 μg/ml, based on initial titration experiments showing similar growth-inhibitory effects of Jo2 at 0.05, 0.1, 0.2, or 0.5 μg/ml. Jo2 as well as the isotype-matched antibody contained no sodium azide and low endotoxin levels (≤0.01 ng/μg of protein).

Enrichment and Purification of $Lin^-Sca1^+c\text{-}kit^+$ Murine BM Cells. All animal procedures were performed with consent from the local ethics committee at Lund University. Lineage-depleted (Lin^-) BM cells were isolated from normal 6–14-wk-old C57BL/6 mice (CD45.2), congenic B6.SJL-$Ptprc^aPep^b$/BoyJ (CD45.1), or Fas-deficient (lpr) mice inbred to C57BL/6 background (B6.MRL-Fas<lpr&gt mice; CD45.2) according to previously described protocols (24, 25, 30, 31). When phenotyping or sorting $Lin^-Sca1^+ckit^+CD34^-$ HSCs, donor mice were always 12–14 wk old, as it has been shown that HSCs from younger mice are mostly $CD34^+$ (32, 33). HSC populations were isolated as described previously (24) and sorted on a FACSVantage™ (Becton Dickinson). Reanalysis of sorted cells reproducibly showed a purity of 96–99%.

Limiting Dilution Assay. $Lin^-Sca1^+c\text{-}kit^+$ cells were seeded in Terasaki plates (Nunc) at a density of 1 cell/well in 20 μl IMDM (BioWhittaker) supplemented with 20% FCS (BioWhittaker) containing 100 U/ml penicillin (BioWhittaker), 100 U/ml streptomycin (BioWhittaker), 2 mM L-glutamine (BioWhittaker), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), and various cytokines. Wells were scored for cell growth following 10–12 d of incubation at 37°C in a humidified atmosphere with 5% CO_2 in air. In some experiments, cells were deposited by a single cell depositor coupled to a FACSVantage™ (Becton Dickinson), and subsequently carefully visualized by microscopy to only include wells containing a single cell. The cytokine response pattern was similar with these two methods.

Semisolid Clonogenic Assay. $Lin^-Sca1^+c\text{-}kit^+$ cells were plated in duplicates in 1 ml IMDM supplemented with 20% FCS, 1.2% (final concentrations) methylcellulose (Methocel™; Fluka Chemie), and cytokines at predetermined optimal concentrations in 35-mm Petri dishes. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air for 10–12 d, at which time colonies (>50 cells) were visualized and scored with an inverted microscope.

Flow Cytometric Analysis. Cell surface phenotypings was performed on freshly isolated Lin^- BM cells or $Lin^-Sca1^+c\text{-}kit^+$ cells, incubated for different periods of time with cytokines at predetermined optimal concentrations in a serum-free medium: X-Vivo 15™ (BioWhittaker) supplemented with 1% detoxified BSA (StemCell Technologies, Inc.), 100 U/ml penicillin (BioWhittaker), 100 U/ml streptomycin (BioWhittaker), 2 mM L-glutamine (BioWhittaker), and 0.1 mM 2-mercaptoethanol (Sigma-Aldridge) or in IMDM supplemented as described for the limiting dilution assay above. Cells were stained with a monoclonal hamster anti–mouse Fas (Jo2) PE-conjugated antibody (BD PharMingen), and monoclonal rat anti–mouse antibodies against CD34 (FITC), Sca1 (biotin, FITC, or PE), c-kit (allophycocyanin [APC]), TNF receptors p55 and p75 (biotin; HyCult Biotechnology), and lineage-specific antigens (FITC-conjugated or purified antibodies detected with a secondary goat anti–rat PE-Tricolor™ antibody; Caltag): B220, Gr-1, Mac-1, CD8, CD4, and Ter-119 (all BD PharMingen). Cells were incubated with antibodies for 15–20 min on ice after blocking unspecific binding with hamster IgG (Jackson ImmunoResearch Laboratories) or Fc block (purified CD16/CD32 monoclonal antibody; BD PharMingen). Cells were washed, incubated with Streptavidin-PE (BD PharMingen), -PerCP (Becton Dickinson), or -CyChrome™ (BD PharMingen) to visualize biotin-conjugated antibodies, and washed again. Finally, cells were analyzed for forward light scatter, orthogonal light scatter, and the four fluorescence signals on a FACSCalibur™ (Becton Dickinson). Flow cytometry analysis was performed us-

ing CELLQuest™ (Becton Dickinson) or FloJo™ (Treestar Inc.) software.

Detection of TNFR-p55 and TNFR-p75 Transcripts. $Lin^-Sca^+kit^+34^-$ cells were subjected to global mRNA amplification and subsequent Southern analysis essentially as described previously (34, 35). Briefly, 25 $Lin^-Sca^+kit^+34^-$ cells were transferred into PCR tubes using an automated micromanipulator device. Subsequently, cells were lysed in first strand buffer followed by reverse transcription (RT) of mRNA using an oligo(dT)-primer. In a second step the first strand cDNA was polyadenylated by terminal transferase to generate a 5′-oligo(dT)-transcript-poly(A)-3′ cDNA that finally was amplified by PCR using a sequence independent X-$(dT)_{24}$ primer. The resulting cDNA (2 μg) was run on a 1% agarose gel, transferred to a nylon membrane by capillary blotting and fixed by UV irradiation. Probes used for Southern hybridization were generated by PCR from sequence verified EST clones (TNFR-p75 GenBank/EMBL/DDBJ accession no. BE635232, forward primer 5′-AGG CTG CCA TAC CTG CCC-3′, reverse primer 5′-GGT ATG TGA GTG TGT GTG C-3′; TNFR-p55 GenBank/EMBL/DDBJ accession no. AI267050, forward primer 5′-AGC CGC CGA GGA CTG CC-3′, reverse primer 5′-AGA GTC GAG GGC TCC AGC-3′). Probes (50 ng) were radiolabeled with ^{32}P-dCTP (Random Primers DNA labeling system; GIBCO BRL/Life Technologies), purified (QIAaquick Nucleotide removal kit; QIAGEN), hybridized to the membranes in ULTRAhyb buffer, and washed according to the manufacturer (ULTRAhyb; Ambion). After washing, blots were analyzed by PhosphorImager quantitation (Fuji Imaging Plate, Fuji Imaging Analyzer BAS-2500, Image Reader V1.4E, and Image Gauge V.3.01 software; Fuji Photo Film Europe).

Competitive Repopulation Assay for Murine BM Stem Cells. C57BL/6 recipient mice (CD45.2) were lethally irradiated by a single exposure to 9.5 Gy of gamma irradiation from a ^{137}Cs source (Instrument AB Scanditronix). Irradiated recipients were transplanted intravenously (0.5 ml/mouse) by tail-vein injection with 1,000 freshly isolated $Lin^-Sca1^+c\text{-}kit^+$ cells (from B6.SJL mice, CD45.1) or the expansion equivalent (EE) of 1,000 $Lin^-Sca1^+c\text{-}kit^+$ cells cultured in the absence or presence of TNF-α and Jo2 (or an isotype-matched control antibody). In vitro cultures were performed in two to six parallels for each group of treatment. At the day of transplantation, parallels were individually counted, and subsequently pooled, washed, and transplanted. Donor cells were cotransplanted with 200,000 unfractionated CD45.2 (recipient type) BM cells to ensure survival, but also to promote a standard competitor source of HSCs, thereby allowing a better quantification of HSC activity (36). To address the effect of TNF-α and Jo2 on single HSC, a limiting number of HSCs were transplanted, in that only six $Lin^-Sca1^+kit^+CD34^-CD45.2^+$ cells were directly sorted (by a single cell depositor) into 0.1 ml serum-free media supplemented with SCF, IL-3 plus IL-6 with or without TNF-α and Jo2 in a U-bottomed 96-well plate. After 9 d incubation, individual wells were harvested and washed with a total of 0.5 ml PBS plus 1% FCS, mixed with 240,000 unfractionated $CD45.1^+$ competitor BM cells, and subsequently, 0.5 ml was injected into each irradiated $CD45.1^+$ mouse (cell numbers injected corresponded to the expansion equivalent of five $Lin^-Sca1^+c\text{-}kit^+CD34^-$ cells and 200,000 unfractionated BM cells as competitor). All mice were kept in individually ventilated cages throughout the experiment, and given sterile food and autoclaved acidified water. Mice were bled from the retroorbital sinus venous plexus and peripheral blood (PB) analyzed 4–6 wk and 14–18 wk after transplantation for donor reconstitution. PB samples were lysed with ammonium chloride before being stained with antibodies against CD45.1, CD45.2, and lineage-specific antigens (all from BD PharMingen) and analyzed on a FACSCalibur™ (Becton Dickinson). Serial transplantations were performed by pooling BM from primary recipients 16–18 wk after transplantation and injecting 0.5 femur equivalent into each new lethally irradiated secondary recipient. Analysis of secondary recipients was performed 3 mo after secondary transplantation.

Statistical Analysis. All results were expressed as the mean (SEM) of data obtained from three or more separate experiments, and else as the mean (SD). The statistical significance of differences between group means were determined using the Student's *t* test when $n \geq 3$.

Results

$Lin^-Sca1^+c\text{-}kit^+$ Candidate Stem Cells Have Little or No Constitutive Expression of Fas and Lack Responsiveness to an Agonistic Fas-activating Antibody. $Lin^-Sca1^+c\text{-}kit^+$ cells, although constituting only 0.05–0.1% of total BM cells, have been demonstrated to contain most if not all LTRCs and represent a virtually pure population of multipotent progenitors. In agreement with previous studies (23), $Lin^-Sca1^+c\text{-}kit^+$ cells lacked detectable cell surface Fas expression (Fig. 1 A). In comparison, a small fraction of $Lin^-Sca1^-c\text{-}kit^+$ progenitor cells expressed low levels of Fas, whereas a larger fraction of more mature $Lin^-Sca1^-c\text{-}kit^-$ cells were Fas^+.

In vitro clonogenic growth of $Lin^-Sca1^+c\text{-}kit^+$ cells cultured in the presence of KL plus IL-3 or a combination of multiple early-acting cytokines (KL plus IL-3 plus IL-6 plus FL plus G-CSF), was not affected by stimulation with a Fas-activating antibody (Jo2; Fig. 1 B). In contrast, murine thymocytes underwent apoptosis in response to Jo2 (37). Thus, $Lin^-Sca1^+c\text{-}kit^+$ candidate murine BM stem cells express little or no cell surface Fas, and remain unresponsive to Fas activation after activation with growth-promoting cytokines.

Effects of In Vitro Cycling and TNF-α on Fas Expression and Fas Responsiveness of Candidate Murine Stem Cells. As $Lin^-Sca1^+c\text{-}kit^+$ cells cultured in the presence of growth-promoting cytokines remained unresponsive to Fas activation (Fig. 1 B), we next investigated whether or not $Lin^-Sca1^+c\text{-}kit^+$ cells remained Fas^- after cytokine stimulation. Such cytokine stimulation is associated with proliferation as well as differentiation and as expected, Fas expression increased with differentiation as assessed by acquisition of lineage-specific antigens (Fig. 2 A). In contrast, cells maintaining a Lin^- phenotype after cytokine stimulation were heterogeneous with regard to Fas expression. Thus, Fas expression was also specifically investigated on cells that maintained a $Lin^-Sca1^+c\text{-}kit^+$ phenotype, as virtually all short- and long-term repopulating stem cells have been demonstrated to have this phenotype (28, 30, 38, 39). After 5 d of culture in c-kit ligand, IL-3, and IL-6 (K36), cells had expanded 54-fold, of which 12% remained $Lin^-Sca1^+c\text{-}kit^+$ (Fig. 2 A; means of three experiments). Whereas >50% of $Lin^-Sca1^-c\text{-}kit^+$ progenitor cells ex-

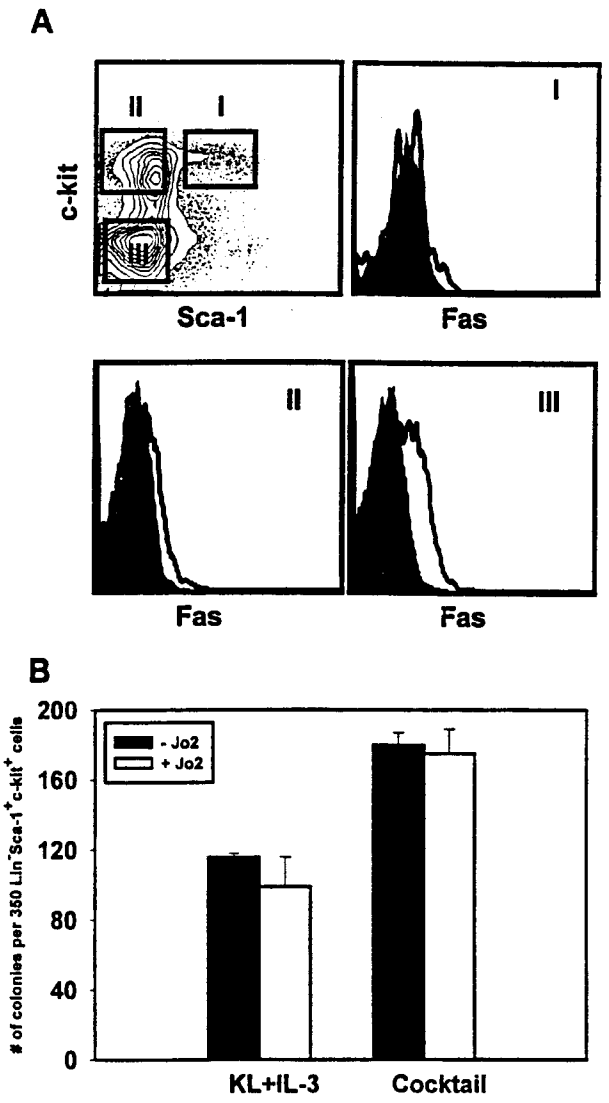

Figure 1. Fas expression and responsiveness of Lin$^-$Sca1$^+$c-kit$^+$ candidate stem cells. (A) Freshly isolated unfractionated BM cells from wild-type mice (open histograms) or lpr mice (closed histograms), were stained with antibodies against lineage markers (CD4, CD5, CD8, B220, Gr-1, Mac-1, Ter-119), Sca1, c-kit, and Fas (Jo2), and analyzed by flow cytometry. Lineage-negative cells were gated and investigated for Fas expression in the Sca1$^+$c-kit$^+$ (I), Sca1$^-$c-kit$^+$ (II), or Sca1$^-$c-kit$^-$ (III) fraction as shown. (B) Number of colonies generated from Lin$^-$Sca1$^+$c-kit$^+$ cells plated in semisolid medium supplemented with KL + IL-3 or a cocktail of cytokines (KL + IL-3 + IL-6 + FL + G-CSF) in the absence or presence of Jo2 (0.2 μg/ml). Colonies were scored after 10 to 12 d of incubation and are presented as means (SD) of two out of totally five experiments.

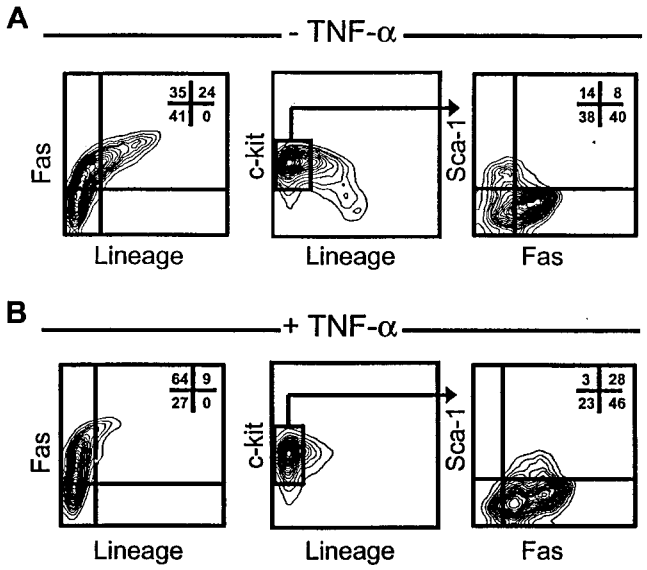

Figure 2. Effects of early-acting cytokines and TNF-α on Fas expression of Lin$^-$Sca1$^+$c-kit$^+$ candidate stem cells. Lin$^-$Sca1$^+$c-kit$^+$ cells were cultured in serum-free medium supplemented with KL + IL-3 + IL-6 in the absence or presence of TNF-α for 5 d, at which time cells were stained with anti-Fas, c-kit, Sca1, and lineage-specific antibodies (or isotype-matched control antibodies) and analyzed by flow cytometry. Profiles show results from one representative experiment. Percentages presented for quadrants are means of totally three experiments.

pressed Fas at high levels, only a small fraction of Lin$^-$Sca1$^+$c-kit$^+$ candidate stem cells expressed Fas, and at very low levels (Fig. 2). After 9 d of incubation, only a small fraction of cells remained Lin$^-$Sca1$^+$c-kit$^+$, on which Fas expression was not further upregulated when compared with day 5 (unpublished data).

As TNF-α has been demonstrated to upregulate Fas expression and induce Fas responsiveness on more committed progenitor cells (18, 37, 40), we next investigated whether TNF-α could upregulate Fas expression on Lin$^-$Sca1$^+$ c-kit$^+$ cells. After 5 d of incubation in K36 plus TNF-α, cell numbers were 57% lower than in the absence of TNF-α. However, a lower fraction of cells became lineage positive in the presence of TNF-α and 20% of the cells remained Lin$^-$Sca1$^+$c-kit$^+$ (Fig 2 B; means of three experiments). Noteworthy, most if not all (>90%) TNF-α exposed Lin$^-$Sca1$^+$c-kit$^+$ cells expressed Fas at high levels (Fig. 2 B), whereas K36 plus TNF-α–treated Lin$^-$Sca$^+$kit$^+$ cells from lpr (Fas deficient) mice, remained negative for Fas (unpublished data). Interestingly, the mean geometric intensity of Fas expression was higher on Lin$^-$Sca1$^+$c-kit$^+$ than Lin$^-$Sca1$^-$c-kit$^+$ cells ($P < 0.05$). Thus, TNF-α in combination with early-acting cytokines induces Fas expression at high levels on candidate murine stem cells.

Next, Lin$^-$Sca1$^+$c-kit$^+$ cells were explored for their TNF-α and TNF-α plus Fas-responsiveness when cultured in KL plus IL-3 or a cocktail of early-acting cytokines (Fig. 3 A). In agreement with previous studies (11, 12), colony formation by Lin$^-$Sca1$^+$c-kit$^+$ cells in response to both cytokine combinations was inhibited by TNF-α. Furthermore, and in striking contrast to cells cultured in the absence of TNF-α (Fig. 1 B), KL plus IL-3 plus TNF-α- and cocktail TNF-α-stimulated colony formation was inhibited by Jo2 by as much as 69 and 59% (Fig. 3 A), respectively. Neither Jo2 in the absence of TNF-α, or an isotype-matched control antibody in the presence of TNF-α showed any effect on colony formation (Fig. 3 A).

As freshly isolated Lin$^-$Sca1$^+$c-kit$^+$ cells displayed little or no Fas expression or responsiveness, we next performed single cell experiments to establish whether stimulation

with TNF-α and Jo2 would be able to block the first cell divisions of Lin$^-$Sca1$^+$c-kit$^+$ cells (Fig. 3 B). Again, Jo2 alone showed no ability to reduce the number or size of clones generated in the absence of TNF-α (unpublished data). Interestingly, TNF-α did not significantly reduce the number of proliferative clones, but rather reduced their size ($P < 0.01$). Likewise, in the presence of TNF-α, Jo2 reduced the size but not number of Lin$^-$Sca1$^+$c-kit$^+$ clones ($P < 0{,}005$; Fig. 3 B), suggesting that TNF-α– and Fas-responsive Lin$^-$Sca1$^+$c-kit$^+$ cells can undergo a limited number of cell divisions before becoming sensitive to TNF-α and Fas-induced growth inhibition. In further support of this, delaying addition of Jo2 for 20–92 h only slightly reduced Jo2-induced growth inhibition (Fig. 3 B). However, when Jo2 was added after 164 h, almost no Jo2-induced growth suppression was observed.

The delayed addition experiments suggested a lag time between TNF-α stimulation and acquisition of Fas responsiveness, which potentially could reflect a requirement for upregulation of TNF receptors in response to K36-induced cycling. In steady-state BM of adult mice, almost all LTRCs reside in the CD34$^-$ fraction, and Lin$^-$Sca1$^+$c-kit$^+$CD34$^-$ cells represent a virtually pure population of HSCs (33, 41). RT-PCR analysis of 25 highly purified Lin$^-$Sca1$^+$c-kit$^+$CD34$^-$ cells demonstrated mRNA expression of both TNF receptors (Fig. 4 A). However, only a small fraction of freshly isolated Lin$^-$Sca1$^+$CD34$^-$ cells had detectable cell surface expression of TNF receptors (Fig. 4 B). In response to cytokine-induced expansion, LTRCs become CD34$^+$ (42), and after 20 h of K36 stimulation (at which time point virtually no LTRCs have divided; unpublished data), TNF receptor expression was dramatically upregulated on Lin$^-$Sca1$^+$c-kit$^+$CD34$^+$ cells (86%; Fig. 4 B). Moreover, after 3 d of K36 stimulation, virtually all Lin$^-$Sca1$^+$c-kit$^+$CD34$^+$ cells expressed TNF receptors. Thus, TNF receptor expression

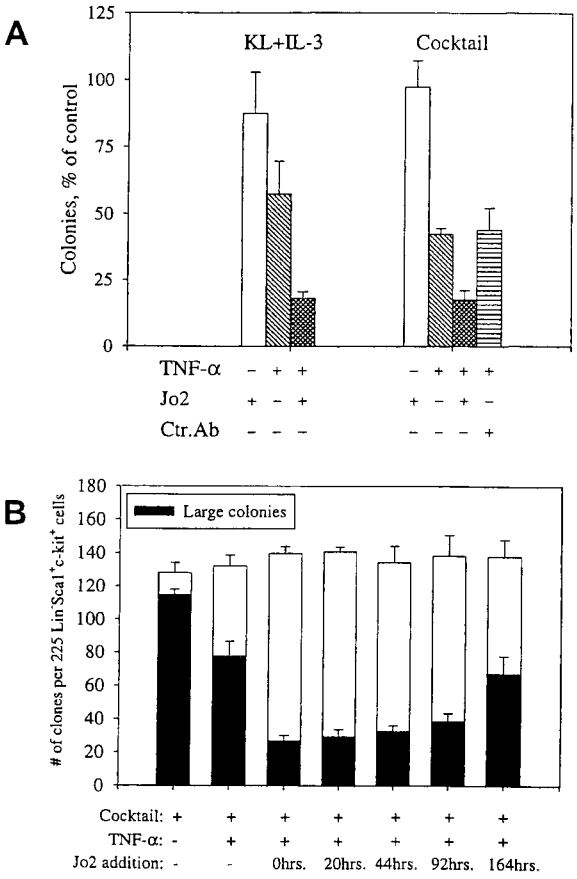

Figure 3. Effects of early-acting cytokines and TNF-a on Fas responsiveness of Lin$^-$Sca1$^+$c-kit$^+$ candidate stem cells. (A) 350 (KL + IL-3) or 150 (cocktail: KL + FL + IL-3 + IL-6 + G-CSF) Lin$^-$Sca1$^+$c-kit$^+$ cells were plated in methylcellulose with the indicated cytokines in the absence or presence of TNF-α, Jo2 (0.2 μg/ml) or an isotype-matched hamster IgG antibody (Ctr. Ab; 0.2 μg/ml). After 10 to 12 d of incubation, colony numbers were evaluated. Results are presented as mean (SD) and represent data from two to four experiments. Controls (no TNF-α or Jo2) were set to represent 100%, and reflected 104 ± 9 and 78 ± 3 colonies for KL + IL-3– and cocktail-stimulated cultures, respectively. (B) Lin$^-$Sca1$^+$c-kit$^+$ cells were plated at 1 cell/well in 20 μl complete IMDM as described in experimental procedures, at a total of 225 cells/group. Jo2 (0.2 μg/ml) was added as indicated to cocktail (KL + IL-3 + IL-6 + FL + G-CSF) + TNF-α–stimulated cultures at initiation of culture (time 0) or after 20-, 44-, 92-, and 164 h preincubation. Wells were scored for clonal growth (total number of clones and clone size) after 10 to 12 d of incubation. Large colonies were defined as clones covering more than 10% of the well. Results are presented as the mean (±SEM) of three separate experiments. Hamster IgG control antibody had no effect on cocktail + TNF-α–induced clonal formation.

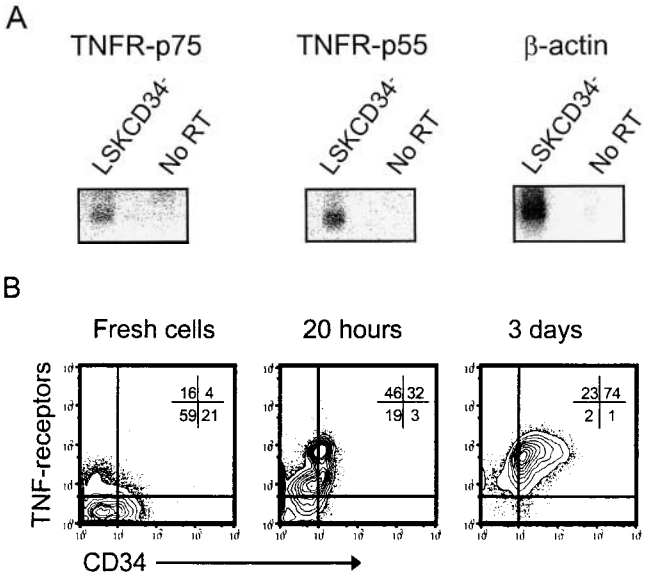

Figure 4. In vitro cycling of candidate HSCs is accompanied by upregulation of TNF receptor expression. (A) 25 Lin$^-$Sca$^+$kit$^+$34$^-$ cells were subjected to global mRNA amplification. 2 μg of the resulting cDNA was run on a 1% agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled probes for TNFR-p75 and TNFR-p55, or β-actin (as a house keeping control gene). Samples of 25 Lin$^-$Sca$^+$kit$^+$34$^-$ cells amplified without addition of reverse transcriptase (no RT) served as negative control. (B) Lineage depleted or Lin$^-$Sca1$^+$c-kit$^+$ BM cells were stained for CD34, Sca1, and TNF receptor expression as well as 7-AAD after indicated times of culture in the presence of K36. Cells shown are gated as "live" (i.e., 7-AAD negative) and Sca-1$^+$. Results are from one representative experiment of four.

is highly upregulated on candidate stem cells upon cytokine-induced cycling.

TNF-α and Fas Receptor Activation Compromise the Reconstituting Ability of Lin⁻Sca1⁺c-kit⁺ Stem Cells. Although highly enriched in LTRC activity, Lin⁻Sca1⁺c-kit⁺ cells are heterogenous, and represent in part cells with only short-term repopulating activity (43). Thus, only in vivo reconstitution experiments could conclusively address whether Fas- and TNF-α–responsive progenitors included true LTRCs. Furthermore, as Lin⁻Sca1⁺c-kit⁺ cells can undergo a limited number of cell divisions in the presence of TNF-α and Fas receptor activation, it remained unclear whether this activation would positively or negatively affect HSC function.

First, to investigate whether Lin⁻Sca1⁺c-kit⁺Fas⁺ cells generated in in vitro cultures included LTRCs, we conducted in vivo reconstitution experiments in which Lin⁻Sca1⁺c-kit⁺ cells were cultured for 5 d with K36 with or without TNF-α. Remaining Lin⁻ cells were subsequently sorted into Fas⁺ and Fas⁻ subpopulations (Fig. 5, A and B) and investigated for their ability to multilineage reconstitute lethally irradiated recipients. From cultures lacking TNF-α, all long-term reconstituting activity was derived from Fas⁻ cells. This was in striking contrast to cells cultured in the presence of TNF-α, in which the Fas⁺ fraction was responsible for all LTRC activity (Table I). Thus, TNF-α efficiently induces high levels of Fas expression on LTRCs.

We have recently demonstrated that Lin⁻Sca1⁺c-kit⁺ cells cultured under serum-free conditions in the presence of K36, undergo self-renewing cell divisions and preserve their ability to short- and long-term multilineage reconstitute lethally irradiated mice (24, 25). Thus, Lin⁻Sca1⁺c-kit⁺ cells were cultured under such self-renewing conditions in the absence or presence of TNF-α and/or Jo2. After 3, 5, and 9 d of incubation in K36, TNF-α reduced the cellular expansion by 14, 46, and 43%, respectively, whereas the addition of Jo2 only slightly reduced cell numbers further (33, 56, and 69% reduction after 3, 5, and 9 d respectively).

Lethally irradiated mice were transplanted with 1,000 freshly isolated Lin⁻Sca1⁺c-kit⁺ CD45.1⁺ cells or their expansion equivalents (EEs) after 3, 5, and 9 d of culture (in competition with 200,000 CD45.2⁺ BM cells; Fig. 6). 3–9 d exposure to TNF-α reduced the short-term (4–6 wk) reconstituting activity of cultured Lin⁻Sca1⁺c-kit⁺ cells by 40% (Fig. 6), whereas Lin⁻Sca1⁺c-kit⁺ cells incubated for 3, 5, or 9 d in the absence of TNF-α showed no drop in reconstituting activity in response to Jo2 (unpublished data). The level of short-term engraftment of cells cultured in the presence of TNF-α plus Jo2 was reduced by as much as 87–95% after culture (Fig. 6). Similarly, TNF-α reduced long-term (14–18 wk) reconstituting activity of cultured Lin⁻Sca1⁺c-kit⁺ cells by 63–71%, and TNF-α plus Jo2 by 90–98% (Fig. 6).

The ability to self-renew in vivo is an important characteristic of true LTRCs, best established through their ability to multilineage reconstitute lethally irradiated serially transplanted recipients (44, 45). Thus, cells from primary recipients were transplanted into secondary recipients (Table II). Reductions in reconstituting activity were observed for cells originally cultured in the presence of TNF-α, and exposure to TNF-α + Jo2 virtually abrogated the ability to reconstitute secondary recipients (Table II). The relative contribution of TNF-α and/or Jo2-exposed Lin⁻Sca1⁺

Table I.

	KL + IL-3 + IL-6			KL + IL-3 + IL-6 + TNF-α		
	Total	Fas⁻	Fas⁺	Total	Fas⁻	Fas⁺
Percent donor cell reconstitution	33 (9)	25 (17)	<0.1	12 (7)	<0.5	9 (3)

The expansion equivalent of 650 Lin⁻Sca1⁺c-kit⁺ cells were transplanted for both the Fas⁻ and Fas⁺ as well as total Lin⁻ population together with 200,000 unfractionated BM cells (CD45.2) into lethally irradiated C57bl/6 recipients (CD45.2). After 4 mo, PB analysis was performed to determine percentage donor derived reconstitution (SD). All mice with >1% CD45.1 reconstitution showed multilineage reconstitution. Similar results were obtained in a second experiment in which recipients did not receive competitor marrow cells, in that Fas⁻ and Fas⁺ cells were responsible for all LTRC activity in TNF-α–unexposed and exposed cultures, respectively (unpublished data).

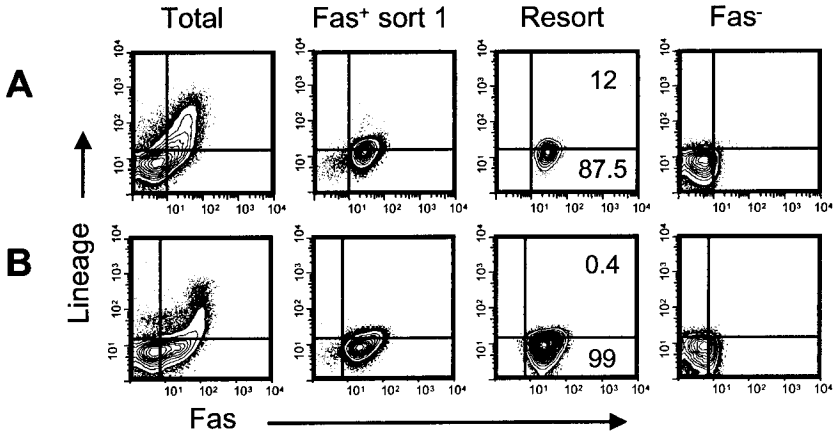

Figure 5. TNF-α induces cell surface Fas expression on long-term reconstituting HSCs. Lin⁻Sca1⁺c-kit⁺ cells (CD45.1) were cultured for 5 d in K36 in the absence (A) or presence (B) of TNF-α. Lin⁻Fas⁺ and Lin⁻Fas⁻ were sorted from both cultures, and Fas⁺ cells resorted to ensure high purity (percentages in quadrants represent percentage of Fas⁺ cells).

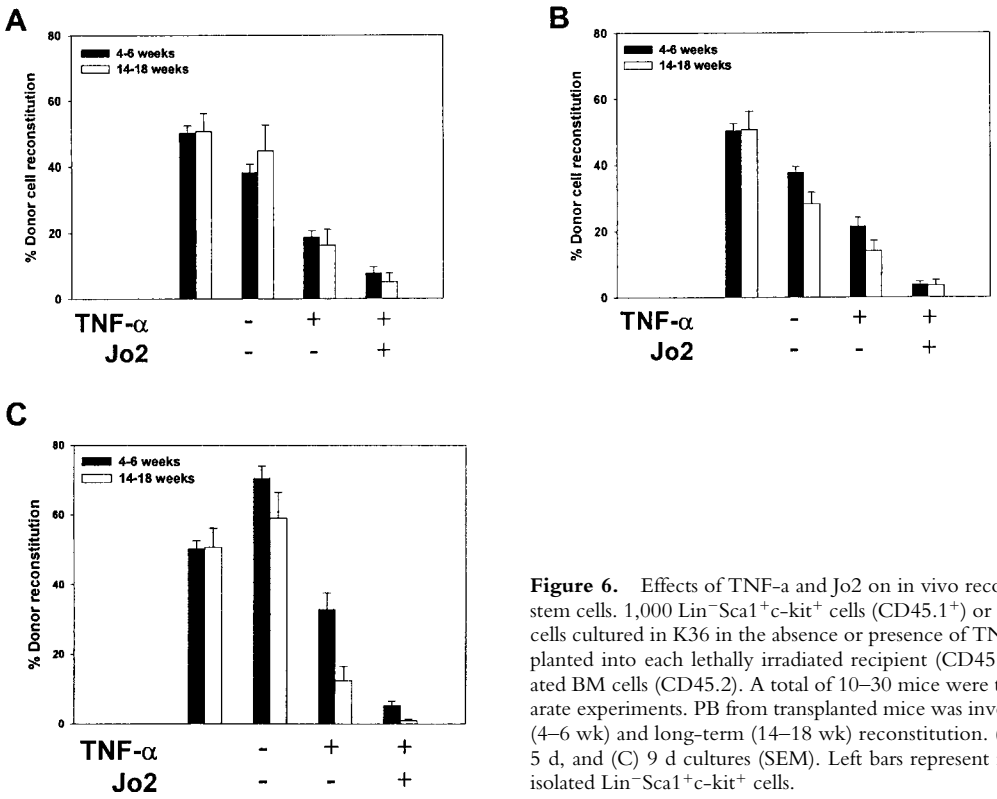

Figure 6. Effects of TNF-a and Jo2 on in vivo reconstitution activity of $Lin^-Sca1^+c\text{-}kit^+$ stem cells. 1,000 $Lin^-Sca1^+c\text{-}kit^+$ cells ($CD45.1^+$) or the progeny of 1,000 $Lin^-Sca1^+c\text{-}kit^+$ cells cultured in K36 in the absence or presence of TNF-α and Jo2, as indicated, were transplanted into each lethally irradiated recipient (CD45.2) together with 200,000 unfractionated BM cells (CD45.2). A total of 10–30 mice were transplanted in each group in four separate experiments. PB from transplanted mice was investigated for donor-derived short-term (4–6 wk) and long-term (14–18 wk) reconstitution. (A) Reconstituting activity of 3 d, (B) 5 d, and (C) 9 d cultures (SEM). Left bars represent reconstituting activity of 1,000 freshly isolated $Lin^-Sca1^+c\text{-}kit^+$ cells.

$c\text{-}kit^+$ cells toward reconstitution of various blood cell lineages (B, T, and myeloid) did not differ from that of uncultured or K36-cultured cells (Table III). Thus, exposure of $Lin^-Sca1^+c\text{-}kit^+$ stem cells to TNF-α and Fas activation severely compromise their ability to short- and long-term reconstitute the hematopoietic system.

Finally, to address the direct effect of TNF-α and Jo2 on individual HSC, limiting numbers of $Lin^-Sca1^+c\text{-}kit^+CD34^-$ HSC were used to study the effect of TNF-α and Jo2 in mice primarily repopulated by single LTRCs, also allowing a more accurate assessment of loss of LTRC activity in response to TNF-α and Jo2. Out of a total of 19 recipients transplanted with 5 $Lin^-Sca1^+c\text{-}kit^+CD34^-$ cells stimulated for 9 d with K36, 12 (63%) were found to sustain long-term multilineage donor derived reconstitution (Table IV). In contrast, of mice transplanted with the same number of cells exposed to K36 plus TNF-α plus Jo2, only 1 out of 17 (6%) showed multilineage reconstitution. These data suggested that ~20% of K36-cultured $Lin^-Sca1^+c\text{-}kit^+CD34^-$ cells sustained long-term reconstituting activity, which was reduced to 1–2% if cultured in the presence of TNF-α plus Jo2. Thus, TNF-α and Fas activation severely compromise the ability of $Lin^-Sca1^+c\text{-}kit^+CD34^-$ HSC to short- and long-term multilineage reconstitute the hematopoietic system. In agreement with this, Lin^-

Table II. *TNF and Fas Activation Suppress Self-Renewing HSCs*

	Days of culture	No. of recipients	Percent donor reconstitution (SD)
Fresh cells	–	10	36 (7)
K36	3	5	29 (6)
K36 + TNF-α	3	5	20 (4)
K36 + TNF-α + Jo2	3	4	<0.1
K36	5	5	9 (3)
K36 + TNF-α	5	5	0.5 (0.2)
K36 + TNF-α + Jo2	5	5	<0.1
K36	9	3	58 (25)
K36 + TNF-α	9	5	0.2 (0.1)
K36 + TNF-α + Jo2	9	5	<0.1

Reconstitution analysis of secondary recipients from two experiments shown as mean (SD).

Table III.

	$B220^+$	$CD3^+$	$Gr1^+/Mac1^+$
Fresh cells	49 (6)	38 (9)	17 (6)
K36	51 (8)	37 (11)	13 (4)
K36 + TNF-α	55 (16)	38 (18)	12 (8)
K36 + TNF-α + Jo2	54 (22)	21 (21)	19 (17)

Lineage distribution of $Lin^-Sca1^+c\text{-}kit^+$ cells cultured for 5 d in the absence of TNF-α and/or Jo2. Data represent analysis of 11–20 mice/group from a total of three experiments and shows mean (SD).

Table IV. *TNF-α and Fas Activation Compromise Cycling HSCs*

In vitro treatment	Donor-positive recipients[a]	Percent $CD45.2^+$ reconstitution in positive mice[b]
K36	12/19 (63%)	11 (10)
K36 + TNF-α + Jo2	1/17 (6%)	4

The progeny of five $Lin^-Sca1^+c\text{-}kit^+CD34^-CD45.2^+$ cells directly sorted into serum-free medium and K36 or K36 + TNF-α + Jo2, were transplanted after 9 d of culture together with 200,000 $CD45.1^+$ BM cells into lethally irradiated $CD45.1^+$ recipients. 14 wk after transplantation, PB was analyzed by flow cytometry for the presence of donor-derived ($CD45.2^+$) cells of the B (B220), T (CD3), and myeloid (Gr1/Mac1) cell lineages.

[a]Mice were considered as reconstituted with cultured $Lin^-Sca1^+c\text{-}kit^+$ $CD34^-$ HSCs if >0.5% of PB cells were $CD45.2^+$, and these contributed to B, T, and myeloid cell reconstitution. None of the negative mice contained CD45.2-derived myeloid reconstitution.

[b]Reconstitution levels are shown as mean (SD) values of positive mice.

$Sca1^+c\text{-}kit^+$ cells cultured for 9 d in K36 and TNF-α showed a dramatic reduction in cells with a Lin^-Sca1^+ $c\text{-}kit^+$ HSC phenotype, which were virtually eliminated upon activation with Jo2 (Fig. 7).

The negative effect of TNF-α on LTRC self-renewal might potentially be mediated through its ability to induce Fas expression on HSC, thus rendering HSCs responsive to Fas activation during/after transplantation. However, TNF-α–exposed $Lin^-Sca1^+c\text{-}kit^+$ cells purified from lpr mice (defective in Fas receptor expression) were equally reduced in their long-term repopulating ability as wild-type cells, suggesting that TNF-α–induced reduction in LTRC activity occurs independently of the Fas pathway (Fig. 8).

Discussion

Previous studies have demonstrated that cytokines such as TNF-α, macrophage inhibitory protein 1α (MIP-1α), and TGF-β can inhibit the in vitro growth of primitive hematopoietic progenitors (8, 46, 47). Such an effect of MIP-1α is however associated with enhanced rather than reduced maintenance of candidate HSCs in culture (48, 49). TGF-β has been shown to accelerate depletion of HSCs under in vitro culture conditions which themselves result in loss of HSC activity (50, 51), making it difficult to distinguish between effects on self-renewal and other mechanisms. Recent developments have now made it possible to study HSC self-renewal and its regulation in vitro (24–27). Through such studies, a number of growth stimulatory cytokines have been shown to promote HSC self-renewing cell divisions. Using the same approach, we here demonstrate that TNF-α and Fas can negatively regulate the self-renewal of highly purified HSCs.

TNF receptors and Fas which are members of the same receptor superfamily, have been demonstrated to be nonredundant regulators of the immune system (52–54). Ample evidence also implicate their involvement in the pathophysiology of a number of blood formation disorders associated with enhanced apoptosis, such as myelodysplastic syndromes and aplastic anemia (15–17, 19, 21, 22, 55) as well as in acute GVHD (9, 56).

Whereas TNF-α and Fas have been convincingly demonstrated to mediate growth suppression of in vitro clonogenic progenitor cells (8, 10–12, 18, 40), their receptor expression pattern and potential effects on LTRCs has been unknown. Recently, CD27, another member of the TNF family, was demonstrated to be expressed on HSCs (57), but the biological function of CD27 on HSCs remains to be investigated. The present studies unequivocally demonstrate that self-renewing murine BM HSCs can be targeted by TNF-α and Fas, and consequently become severely compromised in their ability to short- and long-term multilineage reconstitute hematopoiesis.

Whereas TNF-α has been clearly implicated to be involved in a number of BM failure syndromes, the potential role of TNF-α in regulating steady-state hematopoiesis is unclear. Although studies in p55 TNF receptor–deficient demonstrated increased numbers of $Lin^-Sca1^+c\text{-}kit^+$ cells and colony forming unit spleen day 12 (11, 13), older (but not younger) mice revealed reduced HSC numbers and function, suggesting a complex role of TNF-α in regulating HSCs. The interpretation of this finding is further complicated by the existence of two TNF receptors, and the HSC compartment of mice deficient in both TNF receptors has yet to be investigated. In addition, the effects of TNF-α on hematopoiesis are pleiotropic and context dependent (8), and whereas low (physiological) levels of

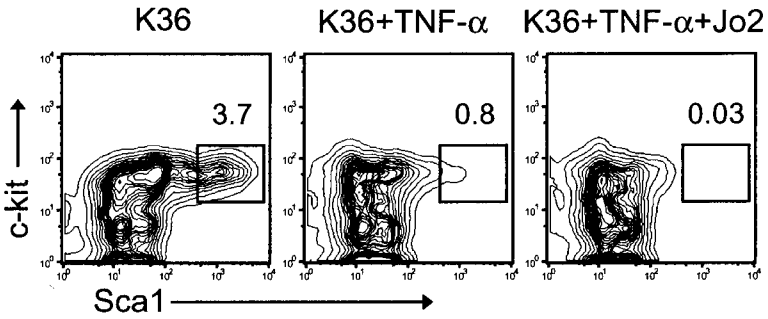

Figure 7. Loss of $Lin^-Sca1^+c\text{-}kit^+$ cells after culture in the presence of TNF-α and Jo2. $Lin^-Sca1^+kit^+$ cells were cultured for 9 d (in indicated culture conditions), after which they were stained against lineage markers, Sca1, c-kit, and 7-AAD. Profiles shown are gated to include only viable, lineage-negative cells. The boxed regions indicate the frequency of live, lineage-negative cells with a $Sca1^+c\text{-}kit^+$ phenotype. Results are from one representative experiment out of four with similar results.

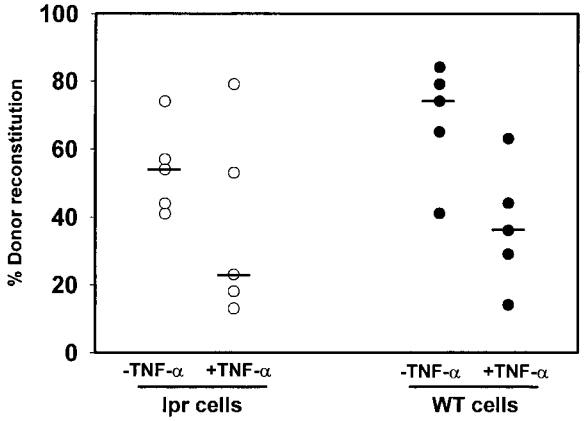

Figure 8. The compromising effect of TNF-α on LTRC activity of cultured $Lin^-Sca1^+c\text{-}kit^+$ cells is independent of Fas. 1,000 $Lin^-Sca1^+c\text{-}kit^+$ cells from lpr or wild-type mice were cultured in K36 in the absence or presence of TNF-α, and transplanted into lethally irradiated recipients together with 200,000 unfractionated BM cells. Data points indicate individual recipients and reference lines shows the median level of donor cell reconstitution in PB 4 mo after transplant.

TNF-α in many systems have been demonstrated to be crucial, high levels can be detrimental.

The present findings suggests that TNF-α is likely to have little or no suppressive effect on quiescent HSC dominating during steady-state hematopoiesis, as supported by low levels of TNF receptor expression and lack of TNF-α suppression of noncycling HSCs. In contrast, under in vitro cycling conditions, TNF receptor expression was upregulated and HSCs became sensitive to TNF-α–induced suppression. In support of this, TNF-α has been shown to stimulate proliferation of mitotically quiescent hematopoietic cell lines, but suppress their growth when rapidly cycling (58). The negative effect of TNF-α on in vitro HSC self-renewal was found to be independent of the Fas pathway, as $Lin^-Sca1^+c\text{-}kit^+$ cells from lpr mice, defective in Fas receptor expression, were equally sensitive to TNF-α–induced suppression of HSCs. Although the lpr mice have a somewhat leaky Fas deficiency, no Fas expression was observed on $Lin^-Sca^+kit^+$ cells exposed to TNF-α.

In a previous study, $Lin^-Sca1^+Thy1.1^+$ HSCs were found to lack expression of Fas (23). In agreement with this, freshly isolated $Lin^-Sca1^+c\text{-}kit^+$ BM cells in the present studies also lacked detectable cell surface expression of Fas. When induced to cycle by combinations of early-acting cytokines, Fas expression was upregulated but only marginally on cells which maintained a $Lin^-Sca1^+c\text{-}kit^+$ HSC phenotype, and in vitro clonogenic and in vivo short- and long-term reconstituting $Lin^-Sca1^+c\text{-}kit^+$ cells remained unresponsive to activation by an agonistic anti-Fas antibody. This unresponsiveness was not altered by using an anti–hamster IgG antibody to further enhance cross-linking (unpublished data). The difference in Fas responsiveness of HSCs and myeloid progenitors (10) could in part be explained by HSCs expressing higher levels of apoptosis-inhibiting genes such as Bcl-2 (59). In fact, when Bcl-2 is overexpressed, committed myeloid progenitors show enhanced Fas resistance (10). Noteworthy, $Lin^-Sca1^+c\text{-}kit^+$ cells became highly Fas-responsive only when costimulated with cytokines promoting cycling and TNF-α, whereas TNF-α alone had little or no ability to induce Fas responsiveness (unpublished data). In support of a key role of TNF-α activation, HSC activity was retained entirely in the Fas negative fraction after in vitro expansion in the absence of TNF-α, whereas in the presence of TNF-α, LTRCs were found exclusively in the Fas-positive fraction. Thus, it appears that the ability of TNF-α to induce Fas responsiveness of HSCs is at least in part a consequence of upregulation of Fas expression on cycling HSCs, which constitutively express no Fas. The ability of TNF-α to upregulate Fas expression has been demonstrated in a number of other systems, and to be critically dependent on nuclear factor (NF)-κB activation (60–62). It is possible that TNF-α might also provide an important co activation signal for Fas activation in HSCs, as implicated for other cell types (63–65).

It is noteworthy that the reduction in HSC numbers (more than 10-fold) in response to TNF-α and Fas activation was much more dramatic than reductions in total cell numbers (threefold). A preferential targeting of HSCs was further supported by the preferential upregulation of Fas and TNF receptors on $Lin^-Sca1^+c\text{-}kit^+$ cells, and the virtual elimination of such cells following prolonged TNF-α and Fas activation.

Whereas HSCs can expand in vivo, extensive efforts at promoting stem cell expansion in vitro have proven less successful (1, 66). One reason for this could be the tendency of in vitro cultured stem cells to undergo apoptosis (1). Based on our findings it is obvious that HSCs will upregulate TNF receptor expression after in vitro stem cell expansion with potent growth-promoting cytokines. If such expanded stem cells are subsequently exposed to TNF-α and/or Fas ligand in vitro, or more likely in the BM microenvironment of ablated recipients, the consequence might be an engraftment defect, as frequently observed after transplantation of ex vivo expanded stem cells (67).

In conclusion, we have demonstrated that although HSCs constitutively express little or no Fas, enforced cycling of HSCs combined with TNF-α activation upregulate Fas expression on HSCs, making them susceptible to Fas-induced suppression. This combined with ample evidence of the involvement of excessive TNF-α and Fas activity in various BM failure syndromes and GVHD (9, 56), suggest that self-renewing HSCs are likely targets for TNF-α– and Fas-induced BM suppression.

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