Essential Role for the p55 Tumor Necrosis Factor Receptor in Regulating Hematopoiesis at a Stem Cell Level

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Summary

Hematopoietic stem cell (HSC) self-renewal is a complicated process, and its regulatory mechanisms are poorly understood. Previous studies have identified tumor necrosis factor (TNF)-α as a pleiotropic cytokine, which, among other actions, prevents various hematopoietic progenitor cells from proliferating and differentiating in vitro. However, its role in regulating long-term repopulating HSCs in vivo has not been investigated. In this study, mice deficient for the p55 or the p75 subunit of the TNF receptor were analyzed in a variety of hematopoietic progenitor and stem cell assays. In older $p55^{-/-}$ mice (>6 mo), we identified significant differences in their hematopoietic system compared with age-matched $p75^{-/-}$ or wild-type counterparts. Increased marrow cellularity and increased numbers of myeloid and erythroid colony-forming progenitor cells (CFCs), paralleled by elevated peripheral blood cell counts, were found in p55-deficient mice. In contrast to the increased myeloid compartment, pre-B CFCs were deficient in older $p55^{-/-}$ mice. In addition, a fourfold decrease in the number of HSCs could be demonstrated in a competitive repopulating assay. Secondary transplantations of marrow cells from primary recipients of $p55^{-/-}$ marrow revealed impaired self-renewal ability of p55-deficient HSCs. These data show that, in vivo, signaling through the p55 subunit of the TNF receptor is essential for regulating hematopoiesis at the stem cell level.

Key words: hematopoietic stem cell • cell differentiation • cytokine receptors

Tumor necrosis factor (TNF)-α, originally identified for its cytotoxic effect on certain murine tumors (1), is a pleiotropic cytokine that plays a pivotal regulatory role in immune and inflammatory responses (2). Its effects are mediated through two functionally distinct cell surface receptors, designated as the p55 and the p75 TNFR (3). The activities of TNF-α are predominantly mediated by the p55 receptor, and it has been postulated that the role of the p75 receptor is primarily to enhance signaling through the p55 receptor by recruiting and delivering TNF-α to the p55 receptor (4). However, p55 TNFR–independent signaling through the p75 receptor has been implicated in thymocyte proliferation, apoptosis of activated mature T cells, and various other host defense mechanisms (5–9). In hematopoiesis, TNF-α has been shown to both inhibit and stimulate proliferation, depending on the maturation stage of the cell population and other cytokines involved (10–13). Besides its direct effect on hematopoietic cells (10, 13), TNF-α can exert its influence indirectly through the upregulation of other cytokine receptors (10, 14–17). Furthermore, TNF-α can stimulate other hematopoietic cells or cells of the microenvironment to produce cytokines involved in hematopoietic cell proliferation and/or differentiation (10, 17–24).

Hematopoietic stem cells (HSCs)[1] are responsible for the steady state continuous generation of lineage-committed progenitor cells, which then give rise to the different types of mature blood cells. In response to various stimuli in situations that require rapid generation of mature blood cells, such as myeloablation, HSCs are able to increase the production of progenitor cells dramatically (25, 26). Despite the enormous dynamic proliferative nature of HSCs, the incidence of malignant transformation or bone marrow (BM) failure is very low, indicating that the proliferation of these cells is under tight control. One of the control mechanisms is to prevent HSCs from entering the cell cycle. It has been demonstrated that an autocrine production of

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[1]*Abbreviations used in this paper:* BFU-E, erythroid burst-forming unit; BM, bone marrow; CFC, colony-forming cell; CFU-GEMM, multilineage CFU; CFU-G/M, granulocyte/macrophage CFU; CFU-S, CFU in the spleen; CRU, competitive repopulating unit(s); HSC, hematopoietic stem cell; Lin, lineage; PB, peripheral blood; PI, propidium iodide; SSC, side scatter; WBC, white blood cell; WT, wild-type.

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TGF-β negatively regulates the cycling activity of early hematopoietic progenitors (27–29), including HSCs (30). TNF-α has been shown to have a similar regulatory effect on Sca-1^+Lin$^-$c-Kit$^+$ murine cells in culture (31). Moreover, TNF-α cooperates with TGF-β in suppressing the proliferation of primitive Lin$^-$Thy-1^+ progenitors of the mouse (32).

Although there is ample evidence to support the notion that TNF-α plays an inhibitory role in the regulation of proliferation of primitive progenitors, little is known about its activity on true long-term repopulating HSCs. Such knowledge may be critical for ex vivo stem cell manipulation protocols, e.g., gene therapy. The in vitro studies of the effects of TNF-α on HSCs have been limited by the difficulty in obtaining functionally uniform populations of these cells. However, the availability of mice lacking the p55 or the p75 TNFR makes it possible to circumvent this problem. We have now studied the role of TNFR signaling in HSCs by transplanting BM cells from wild-type (WT), p55$^{-/-}$, or p75$^{-/-}$ donor mice into lethally irradiated recipients and directly assessing the ability of the graft to reconstitute all hematopoietic lineages long-term. Because the turnover time of the HSC pool is approximated at 30 d or more (33, 34), we hypothesized that lack of signaling through a TNFR would likely appear later in life. Therefore, we compared hematopoietic cell populations from old and young knockout mice with that of age-matched WT mice. Except for a higher number of myeloid colony-forming cells (CFCs) in the BM of p55$^{-/-}$ mice, we found no significant differences in any aspect of hematopoiesis between knockout and WT mice at a young age (<3 mo). In contrast, the BM of older p55$^{-/-}$ mice (>6 mo) showed significantly lower numbers of HSCs compared with WT mice, whereas progenitor cells and the more mature cell populations had increased in size. The only cell compartment that did not increase was the B cell lineage. Thus, these findings demonstrate an important role for signaling through the p55 TNFR in maintaining the HSC pool.

Materials and Methods

Mouse Strains. p55- and p75-deficient mice, originally supplied by Immunex Corp. to J.L.M. Ferrara, and C57BL/6 WT animals (The Jackson Laboratory) were bred and maintained at the Redstone Animal Facility of the Dana-Farber Cancer Institute. Congenic Ly5.1^+ C57BL/6 mice were purchased from the National Institutes of Health. All animals were kept under microisolators and provided with food and water ad libitum.

Cell Preparations. To obtain peripheral blood (PB) samples, mice were first anesthetized by metaphane inhalation. For white blood cell (WBC) and RBC counts, the mice were terminally bled via a cardiac puncture. The counts were performed on a Coulter MaxM. A small blood sample was obtained by puncture of the venous sinus in the eye for analysis for the presence of Ly-5.2^+ cells (see below). BM cells were obtained by flushing both tibiae and femurs of donor mice with PBS containing 2% FCS. The cell suspension was layered on a cushion of FCS and washed once in PBS, 2% FCS. Nucleated cells were counted with a hemocytometer. Assuming that the cell content of a tibia is ~0.6 that of a femur (35), the total cell number was divided by 3.2 to calculate the cell number per femur.

FACS® Analysis and Cell Sorting. To determine the presence of the different lineages in the BM, cells were incubated on ice for 35 min with the FITC-conjugated lineage (Lin) markers B220 (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5), Ly-1 (53-7.3), or PE-labeled Ter119 (PharMingen). Cells were then washed twice with PBS, 2% FBS. During the last wash, the medium contained propidium iodide (PI, 2 μg/ml; Sigma Chemical Co.) to stain the dead cells. Cells were analyzed on a single laser FACScan™ (Becton Dickinson).

For purification, cells were labeled with a cocktail of mAbs, including Sca-1, derivatized with cyanine 5 succinimidyl ester (a gift from Dr. P. Lansdorp, Terry Fox Laboratory, Vancouver, British Columbia, Canada), FITC-labeled Lin markers, and (PE)-conjugated c-Kit (2B8; PharMingen). Where indicated, CD34-FITC (RAM34; PharMingen) was used to further subdivide the Sca-1^+Lin$^{-/lo}$c-Kit$^+$ subpopulation into CD34^+ and CD34^- cells. A preenrichment step was included in the purification protocol when cells were sorted; total BM cells were depleted of cells that expressed mature Lin markers by magnetic separation according to the manufacturer's directions (StemCell Technologies). Multicolor analysis to identify and sort Sca-1^+Lin$^{-/lo}$c-Kit$^+$ progenitor cells was performed on a Coulter Epics® Elite ESP.

To calculate the absolute number of each cell type per femur (i.e., the different mature cell lineages and Sca-1^+Lin$^{-/lo}$c-Kit$^+$ cells), the total number of cells per femur was multiplied by the fraction of each cell population in total BM cells as obtained by FACS® analysis.

Cell Cycle Analysis. Unseparated BM cells or highly purified progenitor cells were fixed in ice-cold 80% ethanol and kept on ice overnight. The next day, cells were spun down and washed once in PBS with 0.1% Triton X-100. Cells were resuspended in 0.5 ml PBS containing 50 μg/ml PI, incubated at 37°C for 30 min, and then kept on ice until analysis, which was within 1–2 h of finishing the staining. The sample was analyzed on a Coulter Epics® XL analyzer.

In Vitro Assays for Clonogenic Cells. To assay for erythroid (BFU-E), granulocyte and/or macrophage (CFU-G/M), multilineage (CFU-GEMM), and pre-B clonogenic progenitors, cells were resuspended in IMDM containing 2% FCS. A portion of the cell suspension was resuspended in methylcellulose containing medium that was supplemented with the appropriate cytokines (StemCell Technologies). Pre-B cell colonies were scored in situ after 7 d of culture, the other colonies after 12–14 d of culture. The number of each clonogenic progenitor per femur was calculated by multiplying the number of nucleated cells per femur with the frequency of each progenitor, i.e., the number of respective colonies counted divided by the number of cells plated (5×10^4 cells for the pre-B cell assay and 1.5×10^4 cells for the erythroid/myeloid colony assay).

In Vivo Assay for CFU in the Spleen and Competitive Repopulating Units. The BM content of CFU-S day 12 cells was determined as originally described (36). In brief, 7×10^4 unseparated BM cells were injected into C57BL/6 recipients that were irradiated with 950 cGy given in two equally split doses (5 h apart) from a ^{137}Cs source. The spleens were harvested 12 d later, and the macroscopic surface colonies were counted. The spleens of control animals that were irradiated but not transplanted with BM cells showed none or one to two pin-point size colonies. This size of colony was not included in the colony counts of the spleens from test animals. The number of CFU-S per femur was calculated by multiplying the number of nucleated cells per femur with the frequency of CFU-S, i.e., number of colonies counted divided by 7×10^4.

A slightly modified competitive repopulating units (CRU) assay was used to determine the number of HSCs in BM (37, 38). In brief, varying doses of unseparated test cells ($Ly5.2^+$) were injected together with 1×10^5 unseparated adult BM "helper" cells ($Ly5.1^+$) into lethally irradiated (as for CFU-S) recipients ($Ly5.1^+$). The allelic difference at the Ly5 locus between the recipient/helper cells and the test cell population was used to follow hematopoietic reconstitution from the test cells. 4, 8, and 16 wk after the transplantation, a small blood sample was taken from each mouse and analyzed for the presence of $Ly5.2^+$ cells. A mouse was considered to be reconstituted by CRU present in the test cell population ("positive") when, 16 wk after the transplantation, >1% of the PB cells were $Ly5.2^+$ and included both myeloid and lymphoid cells, as determined by their respective distinct side scatter (SSC) properties. CRU frequencies for every type of BM tested were calculated by analysis of the proportion of negative mice using Poisson statistics (39). The number of CRU per femur was calculated by multiplying the number of nucleated cells per femur with the frequency of CRU.

Some transplanted mice were used as donors for secondary BM transplantations. Marrow cells from both femurs and tibiae were isolated, and a proportion of these cells together with $Ly5.1^+$ helper cells was injected into three to five irradiated $Ly5.1^+$ recipients (analogous to a normal CRU assay).

Statistical Analysis. The Student's *t* test was used for the comparison of the mean of various hematological parameters from WT animals with that from $p55^{-/-}$ or $p75^{-/-}$ animals, unless otherwise stated.

Results

Young Mice Lacking a p55 TNFR Show an Increase in Myeloid Progenitors in Their BM, but No Other Hematopoietic Differences. Several assays were performed to assess the various lineages and subpopulations of the hematopoietic system (Table I). In young TNFR-deficient mice (<3 mo), the WBC and RBC counts in the PB did not differ from those of WT mice, nor did the relative contributions to the several myeloid and lymphoid lineages (data not shown). The PB indices were mirrored by similar BM cellularities in these three strains of mice. Functional analysis of marrow cell suspensions revealed a significantly higher number of myeloid clonogenic cells in $p55^{-/-}$ mice compared with WT mice ($P < 0.01$), but no significant differences were observed in the number of erythroid and pre-B clonogenic cells, or CRU.

Old $p55^{-/-}$ Mice Show Significant Differences in Their Hematopoietic System Compared with WT Mice. We hypothesized that an effect of perturbed TNFR signaling in HSCs may only be measurable with increased age. Therefore, we performed the same series of hematopoietic cell assays with BM cells from older mice (>6 mo). Neither the WT nor the $p75^{-/-}$ mice showed any significant change with aging, aside from the normalization of the WBC counts in $p75^{-/-}$ mice. In contrast, a profound disturbance of hematopoiesis in older $p55^{-/-}$ mice could be demonstrated (Table II). Nearly twice as many WBCs were found in the blood ($P < 0.0005$), and this was paralleled by the BM cell counts ($P < 0.005$). Moreover, FACS® analysis of the various mature lineages (Fig. 1 A) showed a tendency to increased cell numbers in all lineages except the B cell lineage. The same tendency was observed for clonogenic progenitors (Fig. 1 B). When all myeloid CFCs, i.e., BFU-E, CFU-G/M, and CFU-GEMM, were combined, the total number of colonies in $p55^{-/-}$ marrow was shown to be significantly higher than in WT or $p75^{-/-}$ marrow ($P < 0.01$): 128,000 ± 38,300 vs. 85,400 ± 25,000 and 78,300 ± 28,000, respectively. To assess the status of immature progenitors, we performed CFU-S day 12 assays and immunolabeled BM cells to identify the $Sca\text{-}1^+Lin^{-/lo}$ $c\text{-}Kit^{++}$ subpopulation. Similar to the mature myeloid clonogenic progenitors, the number of CFU-S day 12 in $p55^{-/-}$ BM cells was higher compared with WT (not significant) and $p75^{-/-}$ ($P < 0.03$) BM cells: 2,700 ± 500 vs. 2,100 ± 260 and 1,700 ± 450, respectively (Fig. 1 B). Fig. 2 depicts a representative FACS® profile of BM cells showing the parameters used to calculate the absolute number of $Sca\text{-}1^+$ $Lin^{-/lo}c\text{-}Kit^{++}$ cells per femur. There is a considerably larger, although not statistically significant subpopulation of

Table I. *The Numbers of Various Hematopoietic Subpopulations in the Blood and Marrow of Young (<3 mo) $p55^{-/-}$ or $p75^{-/-}$ Mice Do Not Differ from Those of Young WT Mice*

	WT	$p55^{-/-}$	$p75^{-/-}$
PB WBCs ($\times 10^3/\mu l$)	6.5 (2.3)	7.6 (2.1)	3.7 (0.9)*
PB RBCs ($\times 10^6/\mu l$)	7.7 (1.3)	8.4 (0.6)	7.5 (0.8)
BM cellularity			
($\times 10^6$/femur)	15.6 (3.8)	16.8 (2.9)	16.0 (4.8)
BM CFCs (per femur)			
BFU-E ($\times 10^3$)	6.6 (1.5)	5.4 (1.4)	4.4 (2.0)
CFU-G/M ($\times 10^4$)	6.8 (1.2)	9.4 (1.3)‡	6.0 (0.8)
CFU-GEMM ($\times 10^3$)	3.0 (2.1)	2.8 (2.1)	3.2 (1.8)
Pre-B ($\times 10^3$)	31.7 (33.1)	9.4 (7.2)	21.6 (16.2)
CRU per femur	600	700	ND
(95% confidence interval)	(250–1,450)	(350–1,400)	

The numbers indicating the blood and BM cell counts and CFCs represent the mean (± SD) of three separate experiments. The CRU assay was performed once.
*WT vs. $p75^{-/-}$, not significant; $p55^{-/-}$ vs. $p75^{-/-}$, $P < 0.02$.
‡WT vs. $p55^{-/-}$, $P < 0.01$.

Table II. *Cell Counts of the Blood and Marrow from Old WT and Knockout Mice (>6 mo) Reveal an Increased Cell Output in $p55^{-/-}$ Mice*

	WT	$p55^{-/-}$	$p75^{-/-}$
PB WBCs ($\times 10^3/\mu l$)	5.5 (1.1)	9.4 (1.9)*	5.5 (1.1)
PB RBCs ($\times 10^6/\mu l$)	8.4 (0.7)	9.1 (0.5)	7.8 (0.4)
BM cellularity ($\times 10^6$/femur)	15.8 (2.2)	26.2 (5.9)‡	15.9 (2.6)

Presented is the mean (± SD) from four separate experiments.
*WT vs. $p55^{-/-}$, $P < 0.0005$.
‡WT vs. $p55^{-/-}$, $P < 0.005$.

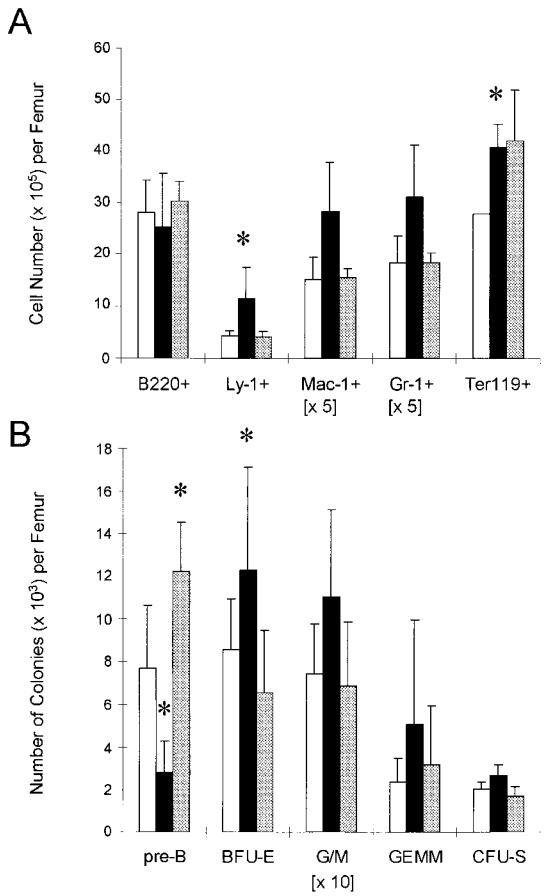

Figure 1. The numbers of mature and clonogenic progenitor cells, except pre-B cells, are increased in the BM from old $p55^{-/-}$ mice. (A) The mature cell compartment was assayed by immunolabeling the marrow cells with mAbs directed against mature lineage markers. (B) Clonogenic progenitors were measured in vitro (methylcellulose assay) and in vivo (CFU-S). For specific details, see Materials and Methods. The results represent the mean ± SD of data combined from three separate experiments. The values for WT cells are indicated by white bars, $p55^{-/-}$ cells by black bars, and $p75^{-/-}$ cells by gray bars. For the purpose of resolution of the other parameters shown in this figure, the observed values for Mac-1^+ cells, Gr-1^+ cells, and G/M CFCs are divided by 5, 5, and 10, respectively. *Significant compared with WT values: Ly-1^+, $P < 0.002$; Ter119$^+$, $P < 0.05$; pre-B ($p55^{-/-}$), $P < 0.02$; pre-B ($p75^{-/-}$), $P < 0.05$.

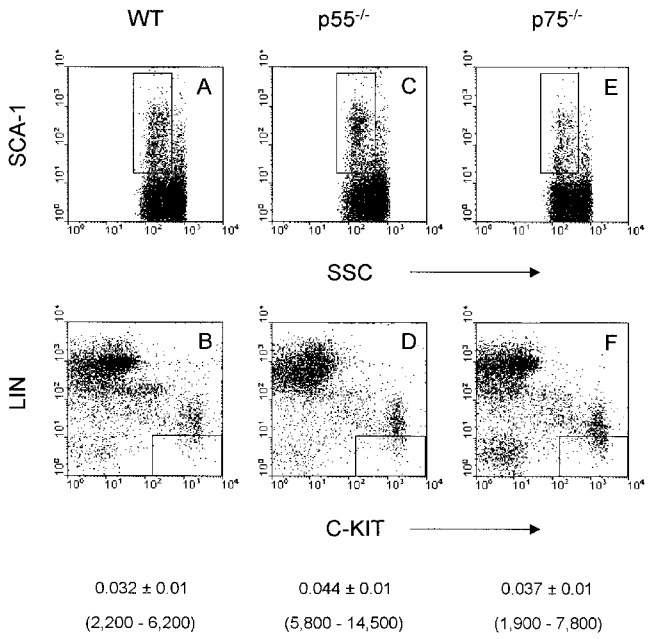

Figure 2. FACS® analysis of BM cells from old WT and knockout mice reveals an enlarged Sca-1^+Lin$^{-/lo}$c-Kit^{++} compartment in $p55^{-/-}$ mice. WT cells are shown in A and B, $p55^{-/-}$ in C and D, and $p75^{-/-}$ in E and F. Viable cells with low SSC properties that express Sca-1 (indicated by the rectangles in A, C, and E) were further analyzed for expression of mature Lin markers and c-Kit (B, D, and F). The proportion of primitive Sca-1^+Lin$^{-/lo}$c-Kit^{++} cells in each mouse strain was calculated by multiplying the percentage of Sca-1^+ cells (rectangles in upper panel) by the percentage of cells within the Sca-1^+ gate that showed high levels of c-Kit expression but lacked the expression of mature Lin markers (rectangles in the lower panel). Indicated below the profiles are the respective mean percentages (± SD) of Sca-1^+Lin$^{-/lo}$c-Kit^{++} cells obtained from combining the data from three to four separate experiments. In parentheses is shown the range of the numbers of Sca-1^+Lin$^{-/lo}$c-Kit^{++} cells per femur.

Sca-1^+Lin$^{-/lo}$c-Kit^{++} cells in $p55^{-/-}$ BM compared with WT or $p75^{-/-}$ BM: 11,300 ± 4,800 vs. 4,800 ± 2,200 and 5,200 ± 2,400 cells, respectively. Since this subpopulation includes long-term repopulating HSCs (40), this phenotypic finding may indicate an increase in the number of HSCs in $p55^{-/-}$ BM. However, it has been clearly shown that phenotype does not always predict function (38, 41). Therefore, we performed competitive repopulating experiments under limiting dilution conditions to determine the number of CRU per femur (Table III). To our surprise, we found a significant decrease in the frequency of CRU among $p55^{-/-}$ BM cells compared with WT ($P < 0.001$). When corrected for BM cellularity, this translated into a near fourfold reduction of the absolute number of CRU in $p55^{-/-}$ mice (350 vs. 1,275 in WT mice). Taken together,

Table III. *Marrow Cells from Older $p55^{-/-}$ Mice Contain Fewer CRU Than BM Cells from Age-matched WT or $p75^{-/-}$ Mice*

		HSCs		
Donor cells	Experiment	Frequency (in total BM)	(95% confidence interval)	Average no. per femur
WT	1	1:12,500	(5,700–27,500)	1,000
	2	1:12,200	(5,600–26,400)	1,550
$p55^{-/-}$*	1	1:55,100	(22,900–132,300)	300
	2	1:70,700	(32,750–152,500)	400
$p75^{-/-}$	1	1:20,700	(7,250–59,000)	700
	2	1:14,800	(7,000–31,400)	1,100

Graded doses of unseparated BM cells from each mouse strain were injected into three groups of animals, five mice each. Presented are the results from two separate experiments, analyzed 16 wk after transplantation. For more details, see Materials and Methods.

*The combined data from $p55^{-/-}$ mice (i.e., CRU frequency of 1:64,200 [36,350–113,100]) compared with the combined data from WT mice (i.e., CRU frequency of 1:12,350 [7,100–21,500]) are significantly different (χ^2 test; $P < 0.001$).

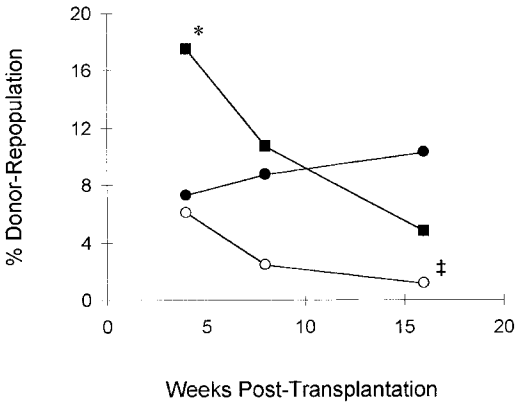

Figure 3. Transplantation of $p55^{-/-}$ marrow cells into irradiated recipients results in less donor repopulation than equal numbers of WT marrow cells. Depicted is the average percentage of donor-derived nucleated blood cells (% Donor-Repopulation) at 4, 8, and 16 wk after the transplantation in three groups of animals. Group 1 (●) recipients received 16,000 unseparated WT BM cells. Groups 2 (○) and 3 (■) received $p55^{-/-}$ cells: group 2 16,000 cells per mouse, and group 3 64,000 cells per mouse. *Significantly different from the WT value at the corresponding time point, $P < 0.01$. ‡Significantly different from the WT value at the corresponding time point, $P < 0.05$.

these data show a tendency with aging in p55-deficient mice to increased sizes of most mature and immature hematopoietic (progenitor) cell populations, which could explain the overall increase in marrow cellularity found in these animals. At the same time, the number of HSCs that give rise to this variety of cell populations is reduced.

HSCs That Lack the p55 Receptor Display Impaired Proliferative and Self-renewal Abilities. To further elucidate the extent of the stem cell defect in old $p55^{-/-}$ mice, we analyzed in detail the hematopoiesis of three sets of mice transplanted with low numbers of BM cells (Fig. 3, and Table IV). Groups 1 and 2 represent animals transplanted with 16,000 total BM cells (WT and $p55^{-/-}$, respectively). A comparison of their donor repopulation kinetics as measured at various time points after transplant illustrates a dramatic decline in donor reconstitution over a 4-mo time period by $p55^{-/-}$ BM cells, whereas the donor reconstitution by WT cells showed a slight increase over the same time period tested (Fig. 3). 16 wk after transplantation, the level of donor cells in recipients of $p55^{-/-}$ BM cells (group 2) was significantly lower ($P < 0.05$) compared with that in recipients of WT BM cells (group 1) (1.1 ± 1.0 vs. 10.2 ± 12.2, respectively). The difference in repopulating ability between WT and $p55^{-/-}$ HSCs becomes even more apparent when individual mice are analyzed (Table IV). Between 4 and 16 wk after the transplantation, $Ly5.2^+$ donor reconstitution did not increase in any of the recipients of $p55^{-/-}$ ($Ly5.2^+$) BM cells. In contrast, more than half of all the animals that had received WT ($Ly5.2^+$) BM cells showed an increase in $Ly5.2^+$ cells, by as much as 38% in one of the recipients. Moreover, only 22% of the group 2 animals that were multilineage reconstituted at week 4 remained so at week 16, compared with nearly 80% of the animals in group 1 (Table IV).

Thus, when equal numbers of BM cells are transplanted, the repopulating ability of $p55^{-/-}$ marrow cells is inferior to that of WT cells. However, when the CRU frequencies (Table III) were used to calculate the average number of CRU received by individual transplanted animals (i.e., the total number of cells transplanted divided by the frequency of CRU), it was calculated that group 1 had received on average 1 HSC, whereas group 2 had received on average 0.3 HSC. This could potentially explain, or at least contribute to, the observed differences. Therefore, we compared group 1 WT mice with a group of mice that received on

Table IV. *Analysis of the Changes in the Percentage of Donor-derived Nucleated Blood Cells in Recipients of WT or $p55^{-/-}$ Marrow Cells Identifies a Rapid Loss of Hematopoietic Reconstitution by $p55^{-/-}$ Cells*

	Transplanted cells		Change in donor repopulation from week 4 to week 16‡					
Group (*n*)	Donor	Total cell no.* (HSC)	Increase		Decrease		Multilineage donor repopulation	
			%§	*mean ± SD (range)*	*%*§	*mean ± SD (range)*	*wk 4 (%)*‖	*wk 16 (%)*‖
1 (9)	WT	16,000 (1)	55	11.3 ± 15.4 (2.1–38.8)	45	7.5 ± 5.5 (2.2–14.2)	100	78
2 (9)	$p55^{-/-}$	16,000 (0.3)	0	–	100	5.0 ± 4.2 (0.1–13.7)	89	22
3 (10)	$p55^{-/-}$	64,000 (1)	10	0.4	90	14.0 ± 8.8 (3.5–34.3)	100	70

*Indicated are the total number of $Ly5.2^+$ BM cells transplanted per recipient. The average number of HSCs received per recipient is shown in parentheses. ‡Mice that showed a decrease in donor repopulation between weeks 4 and 16 were combined, and the mean of the decrease ± SD was calculated. The range of the values is shown in parentheses. The same was done with mice that showed an increase in donor repopulation. With "%" is indicated the fraction of animals within a group that showed a decrease/increase in the level of §donor reconstitution or ‖multilineage reconstitution analyzed at the indicated time points after transplantation.

average 1 $p55^{-/-}$ HSC, equivalent to 64,000 total BM cells (group 3). As expected, approximately the same number of mice remained multilineage reconstituted after 16 wk (Table IV; 78% in group 1 and 70% in group 3). However, although 4 wk after the transplantation the level of donor reconstitution in group 3 ($p55^{-/-}$ transplant) recipients was significantly higher than in group 1 (WT transplant) recipients ($P < 0.01$), the percentage of donor $p55^{-/-}$ cells, again, gradually decreased with each subsequent analysis (Fig. 3). 90% of all animals that received $p55^{-/-}$ marrow cells showed as much as a 34% decline in donor reconstitution (Table IV). Thus, not only does $p55^{-/-}$ marrow contain fewer HSCs, these data also indicate that the proliferative potential of $p55^{-/-}$ HSCs is less than that of WT HSCs.

To further stress the proliferative response of the $Ly5.2^+$ transplanted cells, BM cells of some of the primary recipients were transplanted into secondary recipients. The results of these experiments are shown in Table V. Eight primary recipients of WT marrow cells and three of $p75^{-/-}$ marrow cells were used in these experiments. Because we had not been able to demonstrate remarkable differences between WT and $p75^{-/-}$ hematopoiesis in older mice, we grouped these mice together to compare their secondary transplantation results to the results of the serially transplanted $p55^{-/-}$ marrow cells. Of 11 $WT/p75^{-/-}$ primary recipients, 4 animals (36%) contained $Ly5.2^+$ HSCs that generated multilineage progeny upon transplantation into secondary recipients. This proportion is in accordance with previous published data (42). Only 2 (18%) of the 11 primary recipients of $p55^{-/-}$ BM gave secondary reconstitution upon serial passage. As observed in the primary recipients of $p55^{-/-}$ cells, and in contrast to secondary recipients of WT donor cells, the kinetics of Ly-5.2^+ reconstitution in the secondary recipients showed a marked decline over time (data not shown).

Cell Cycle Analysis of $p55^{-/-}$, $p75^{-/-}$, and WT BM Cells Reveals No Gross Differences. One possible explanation for the observed differences in HSC number and proliferative potential between WT and $p55^{-/-}$ mice is a higher cycling rate of $p55^{-/-}$ HSCs. Purified Sca-1^+Lin$^{-/lo}$c-Kit^{++} cells still include large numbers of committed progenitor cells, as confirmed by our finding that between 20 and 30% of these cells (WT and knockout cells) were in the S/G2/M phase of the cell cycle (data not shown). Therefore, we further subdivided this population using an mAb against the CD34 antigen. The Sca-1^+Lin$^{-/lo}$c-$Kit^{++}CD34^-$ cells, which include the long-term reconstituting HSCs (43), comprise ~10% of all Sca-1^+Lin$^{-/lo}$c-Kit^{++} cells. The sorting strategy for obtaining these cells is depicted in Fig. 4. The results of cell cycle analyses of unseparated BM cells and highly purified cells (box 3) are shown in Table VI. As expected, DNA staining of unseparated BM cells did not demonstrate a difference in cycling activity between the WT and either knockout mouse. Moreover, the result obtained with the highly purified $CD34^-$ subpopulation of Sca-1^+Lin$^{-/lo}$c-Kit^{++} cells isolated from $p55^{-/-}$ marrow cells did not strongly suggest that cycling activity of $p55^{-/-}$ HSCs was altered. Therefore, a difference in percentage of cycling HSCs does not seem to explain the observed qualitative and quantitative differences between HSCs from WT and p55-deficient mice.

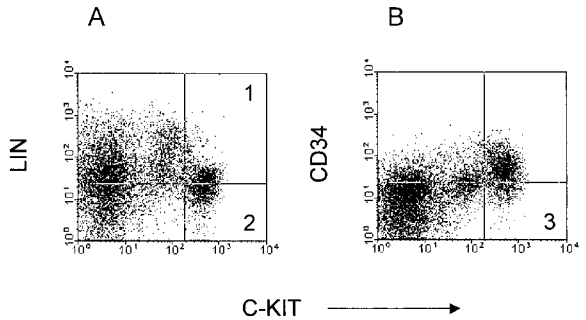

Figure 4. Purification strategy for isolating Sca-1^+Lin$^{-/lo}$c-Kit^{++} $CD34^-$ cells. Cells that express Sca-1 were identified as in the legend to Fig. 2. Depicted are representative profiles of viable Sca-1^+ WT marrow cells after lineage depletion. The Sca-1^+c-Kit^{++} cells, indicated by quadrants 1 and 2, showed no or low levels of mature Lin expression (A). Thus, identifying Lin markers as a means to further purify "Lin-depleted" Sca-1^+c-Kit^{++} cells is not very helpful. In contrast, replacing the mAb cocktail directed against the Lin markers with an anti-CD34 mAb allows the identification of a small subpopulation of the Sca-1^+Lin$^{-/lo}$c-Kit^{++} cells (~10%), the $CD34^-$ cells, that has been shown to include the long-term repopulating HSCs (B, quadrant 3) (reference 43).

Table V. *Self-renewal in $p55^{-/-}$ HSCs Occurs Less Often Than in $WT/p75^{-/-}$ HSCs*

Original $Ly5.2^+$ donor cells	No. of primary recipients used as donors	No. of $Ly5.2^+$ cells transplanted*	No. of primary recipients generating $Ly5.2^+$ progeny in secondary recipients 20 wk after transplant (average ± SD of $Ly5.2^+$ cells)
		($\times 10^5$)	
$WT/p75^{-/-}$	11	4.4 ± 2.0	4 (8.8 ± 8.9)
$p55^{-/-}$	11	5.4 ± 2.2	2 (3.0 ± 1.6)‡

*BM cells of primary recipients were harvested as usual and transplanted in three to five secondary recipients. The number of $Ly5.2^+$ (original) cells that was injected per secondary recipient was calculated by multiplying the total number of BM cells injected with the percentage of $Ly5.2^+$ cells determined in the blood by FACS® analysis. The difference between the number of cells transplanted from $WT/p75^{-/-}$ and $p55^{-/-}$ primary recipients is not significant.
‡The level of $Ly5.2^+$ reconstitution in the $p55^{-/-}$ secondary recipients is significantly different compared with the $WT/p75^{-/-}$ group; $P < 0.03$.

Table VI. *The Proportion of Total BM Cells and Sca-1$^+$Lin$^{-/lo}$ c-Kit^{++}CD34$^-$ Cells in S/G2/M Phases of the Cell Cycle*

	WT	p55$^{-/-}$	p75$^{-/-}$
Total BM cells*	19.6 (4.5)	19.0 (4.2)	20.1 (4.7)
Sca-1$^+$Lin$^{-/lo}$c-Kit^{++}CD34$^{-‡}$	2.3–8.6	9.4	9.2

The mice used for this analysis were all >6 mo.
*Shown are the mean values (± SD) from the combined data of five separate experiments.
‡10–20 mice were killed to obtain enough cells for a reliable PI DNA analysis. With WT marrow, this was done twice; with p55$^{-/-}$ and p75$^{-/-}$ marrow, once.

Discussion

The p55 and p75 TNFRs are ubiquitously expressed on all hematopoietic cells, with the exception of erythroid and unstimulated T cells (2, 44). There is ample evidence from in vitro culture systems that, through one or both of these receptors, TNF-α can promote the expansion and differentiation of various hematopoietic subpopulations, as well as inhibit their proliferative response (10–13). However, the long-term effects of this cytokine in vivo as a possible regulator of steady state hematopoiesis, especially with regard to the stem cell compartment, are not well defined. The present studies were undertaken to address this question by analyzing the hematopoietic system of mice in which TNF-α signaling is impaired. We and others (45) have shown that most hematopoietic subpopulations tested are present in young p55$^{-/-}$ and p75$^{-/-}$ mice at levels comparable to those of WT mice. In older mice, no significant differences in the assays tested between p75$^{-/-}$ and WT mice could be demonstrated. However, when older p55$^{-/-}$ mice were compared with WT mice they showed an interesting phenotype: their HSC compartment was significantly smaller than that of their WT counterparts (approximately fourfold), whereas the Sca-1$^+$Lin$^{-/lo}$c-Kit^{++} progenitor compartment had increased in size (approximately twofold). This was reflected in significant increases in total cellularity of the BM, total myeloid CFCs, and WBC and RBC counts in the PB. Thus, with aging of the mice, the lack of signaling through the p55 TNFR seems to affect the balance between generating new HSCs (self-renewal) and producing committed progenitors (differentiation). Interestingly, only the B cell lineage deviated from this pattern, in that the numbers of B220$^+$ cells in the marrow (Fig. 1 A) and in the blood (data not shown) were similar to their WT counterparts, whereas the number of pre-B CFCs was significantly decreased in p55-deficient mice (Fig. 1 B). Previous studies have shown that the p55 TNFR is essential for the structural organization of lymphoid organs (46–49) and the number of B cells in the Peyer's patch (48). Our data also suggest that the generation of B cells in the marrow depends on p55 TNFR signaling. Whether this is mediated by TNF-α or lymphotoxin α remains to be studied.

Exhaustion of the HSC pool can be explained by loss of HSCs due to cell death or differentiation. TNF-α has been shown to both induce and suppress apoptosis in murine Sca-1$^+$Lin$^-$ cells. Analogous to the effects of TNF-α on the proliferation of hematopoietic cell populations, its effect on apoptosis seems to depend on which additional cytokine was present: suppression in cultures with IL-1α present, and induction when Steel factor was supplied (50). The apoptotic effects of TNF-α are well documented and mainly exerted through its p55 receptor (51, 52), although an apoptotic role for the p75 receptor has been demonstrated in mature CD8$^+$ cells (9). Increased apoptosis in HSCs will definitely decrease the HSC pool. However, if this were the case in p55$^{-/-}$ mice, one would expect to find an accompanying decrease in the number of progenitor cells. The opposite was found in our p55-deficient mice. The mechanisms that control self-renewal versus commitment decisions in HSCs are not known. However, it is generally accepted that an increase in the number of HSC cell divisions is accompanied by an increased chance of HSCs to commit and differentiate among one of the lineages (53–55). TNF-α has been shown to prevent WT but not p55$^{-/-}$ Sca-1$^+$Lin$^-$c-Kit$^+$ cells from entry into S phase (31). Increased cycling of p55$^{-/-}$ HSCs could explain our results. However, we could not detect a difference in cell cycle activity between Sca-1$^+$Lin$^-$c-Kit$^+$ subpopulations (CD34$^+$ or CD34$^-$) sorted from WT or p55$^{-/-}$ BM cells. The effects of TNF-α on the cell cycle of HSCs may be very subtle, which would explain why HSCs in young mice seem unaffected and why it is not detectable by the methods used in this study. One study has demonstrated the importance of TGF-β in maintaining HSCs in a quiescent, noncycling state in unperturbed steady state hematopoiesis (30). Since TNF-α cooperates with TGF to inhibit the proliferation of primitive progenitor cells (32), it is not inconceivable that more HSCs escape their quiescent state when one of the inhibitory signals is missing.

There are two important considerations with respect to the finding that the phenotype in p55$^{-/-}$ mice manifests only with aging (>6 mo of age). First, TNF-α production and TNF-α signaling cascades seem to change with aging. Aged mice produce more TNF-α in response to LPS (56), and T cells from older humans express more p55 but less p75 TNFR subunits and are more susceptible to TNF-α–induced apoptosis (57). It is possible that TNF-α signaling in HSCs also changes with aging, and that any deficiencies in this signaling pathway have greater consequences later in life. It may be of interest to look at mice of 20 mo or older since the number of cycling HSCs increases dramatically at this age (58). Second, hematopoiesis during fetal and/or neonatal development, which is at that time expanding tremendously, not only contains HSCs with properties different from adult HSCs (42, 59), but is also probably regulated differently than adult, steady state hematopoiesis (60). The regulatory mechanisms of fetal/neonatal hematopoietic cells may not depend on TNF-α signaling at all. This, in addition to the possibility that older hematopoietic cells, and HSCs in particular, may be more sensitive to TNF-α, could explain the lack of a phenotype in young p55$^{-/-}$ mice.

Thus, our findings suggest that, with age, a lack of signaling

through the p55 TNFR increases the number of committed hematopoietic progenitors and their subsequent progeny at a cost to the stem cell pool. The scarcity of secondary recipients with multilineage $p55^{-/-}$ progeny confirms the lack of self-renewal or exhaustion of $p55^{-/-}$ HSCs. However, what is surprising and contrary to our expectations is the lower level of multilineage reconstitution per HSC from $p55^{-/-}$ donors compared with WT. The explanation for this reduction may be that hematopoietic regeneration in myeloablated recipients is different from both adult steady state hematopoiesis and fetal/neonatal hematopoiesis. Although little is known about the cytokines and other regulatory elements in the activation and/or recruitment of transplanted HSCs, there are some important differences compared with steady state hematopoiesis (61). One important difference is that transplanted HSCs must home to an appropriate microenvironment. It is possible that altered TNF-α signaling affects the homing properties of $p55^{-/-}$ cells. In this regard, it is of interest that TNF-α can affect the expression of CXC chemokine receptor 4 (62–64), a chemokine receptor critical for murine BM engraftment and regeneration as well as B cell development (65, 66). Moreover, in our experimental design, the transplanted HSCs are influenced by stress-induced environmental changes associated with the myeloablation of the recipient. Stress can induce elevated levels of glucocorticoid hormones, which, in synergy with other cytokines, make BM cells more susceptible to IL-1 through upregulation of the IL-1 receptors, which favors myeloid differentiation (67, 68). A beneficial effect of IL-1 may be the suppression of apoptosis. However, for this effect to be delivered, the combination of TNF-α may be essential (50). Therefore, it is possible that $p55^{-/-}$ HSCs acquire elevated levels of IL-1 receptors after transplantation but are more likely to differentiate than their WT counterparts when they do not receive the appropriate TNF signal. Thus, the loss of HSCs and all other hematopoietic cell types after transplantation of $p55^{-/-}$ marrow cells may not necessarily reflect steady state hematopoiesis, but rather the inability of these cells to adequately respond to extreme proliferative stimuli.

The need for intact TNFR signaling in HSCs during hematopoietic regeneration of a myeloablated recipient may be more crucial for its survival than during steady state hematopoiesis. The G0/G1 arrest that TNF-α has been shown to induce in Sca-1^{+}Lin^{-}c-Kit^{+} cells may be imperative in restraining the proliferative response of HSCs to external stimuli and thereby preventing their extinction. In this respect, it is of interest that in $TNF^{-/-}$ mice it was shown that TNF is important for the generation of an inflammatory response, but its presence was far more essential in limiting the extent and duration of this response (69). This possible regulatory role in limiting the number of cell divisions in HSCs warrants further investigation, for example in retroviral gene therapy protocols with HSCs as target cells. Future studies of the role of TNF in steady state hematopoiesis should include the analysis of mice older than 20 mo and the generation of allophenic mice, which would allow the tracking of HSC progeny over time, without perturbing the hematopoietic system (70).

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