Immunity, Vol. 15, 659–669, October, 2001, Copyright ©2001 by Cell Press

Upregulation of Flt3 Expression within the Bone Marrow Lin$^-$Sca1$^+$c-kit$^+$ Stem Cell Compartment Is Accompanied by Loss of Self-Renewal Capacity

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Summary

Flt3 has emerged as a potential regulator of hematopoietic stem cells (HSC). Sixty percent of cells in the mouse marrow Lin$^-$Sca1$^+$c-kit$^+$ HSC pool expressed flt3. Although single cell cloning showed comparable high proliferative, myeloid, B, and T cell potentials of Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ and Lin$^-$Sca1$^+$c-kit$^+$flt3$^-$ cells, only Lin$^-$Sca1$^+$c-kit$^+$flt3$^-$ cells supported sustained multilineage reconstitution. In striking contrast, Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ cells rapidly and efficiently reconstituted B and T lymphopoiesis, whereas myeloid reconstitution was exclusively short term. Unlike c-kit, activation of flt3 failed to support survival of HSC, whereas only flt3 mediated survival of Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ reconstituting cells. Phenotypic and functional analysis support that Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ cells are progenitors for the common lymphoid progenitor. Thus, upregulation of flt3 expression on Lin$^-$Sca1$^+$c-kit$^+$ HSC cells is accompanied by loss of self-renewal capacity but sustained lymphoid-restricted reconstitution potential.

Introduction

All myeloid and lymphoid blood cell lineages derive from common hematopoietic stem cells (HSC). HSC fate decisions between self-renewal and commitment toward lineage-restricted development are tightly regulated, but the molecular mechanisms regulating these processes remain largely unknown (Domen and Weissman, 1999).

Most cytokines and their receptors appear to have no or redundant roles in regulating the HSC pool (Metcalf, 1993). However, thrombopoietin (TPO) and c-kit ligand (KL) and their corresponding receptors c-mpl and c-kit have been demonstrated through gene targeting studies to be important for regulating HSC numbers and/or function (Kimura et al., 1998; Solar et al., 1998; Alexander et al., 1996; Miller et al., 1996; Geissler and Russell, 1983). Like c-kit, fms-like tyrosine kinase-3 (flt3) is a cytokine tyrosine kinase receptor selectively expressed in early stages of hematopoiesis (Lyman and Jacobsen, 1998). Mice deficient in expression of flt3 or its ligand (FL) have reductions in B and T lymphopoiesis, with a preferential reduction in the earliest proB cell progenitors (Mackarehtschian et al., 1995; McKenna et al., 2000). FL has also been implicated to be a key and potent stimulator of growth of candidate murine and human HSC (Lyman and Jacobsen, 1998) and as a consequence is frequently used to promote HSC ex vivo expansion and retroviral mediated gene transfer (Bryder and Jacobsen, 2000; Dao et al., 1997; Miller and Eaves, 1997; Yonemura et al., 1997; Petzer et al., 1996; Elwood et al., 1996). Furthermore, bone marrow (BM) HSC from mice deficient in flt3 expression reveal reduced reconstitution potential in vivo (Mackarehtschian et al., 1995). However, whether this reflects a role of flt3 in HSC generation or maintenance during steady-state conditions or potentially in the HSC homing, engraftment, and differentiation process following transplantation has not been established.

In the present study, we evaluated the potential role of flt3 and its ligand in regulating HSC maintenance during steady-state hematopoiesis by investigating the expression of flt3 within the HSC compartment. Although representing only 0.1% of total BM cells, Lin$^-$Sca1$^+$c-kit$^+$ (LSK) cells possess virtually all long-term multilineage reconstituting activity in adult BM (Ikuta and Weissman, 1992; Li and Johnson, 1995; Okada et al., 1991; Orlic et al., 1993). In agreement with others (Zeigler et al., 1994), we found 60% of LSK cells to express detectable levels of cell surface flt3. Although revealing a combined myeloid, B, and T cell potential, LSKflt3$^+$ cells failed to long-term reconstitute myelopoiesis in vivo, but rather reconstituted common lymphoid progenitors (Kondo et al., 1997) and B and T cells. Furthermore, whereas FL (unlike KL) failed to support survival of long-term repopulating HSC (LTRC), it efficiently supported survival of "lymphoid-restricted" LSKflt3$^+$cells. Thus, c-kit and flt3 appear to have distinct roles in early hematopoiesis. Whereas c-kit expression in adult HSC appears crucial for normal HSC function, upregulation of flt3 expression appears to be accompanied by loss of self-renewal capacity but sustained lymphoid restricted reconstitution potential.

Results

The Lin$^-$Sca1$^+$c-kit$^+$ HSC Pool Consists of Flt3$^-$ Multipotent Long-Term Repopulating Stem Cells and Flt3$^+$ Cells with Efficient but Lymphoid-Restricted In Vivo Reconstitution Potential

Lin$^-$Sca1$^+$c-kit$^+$ cells contain most long-term multilineage reconstituting activity in murine BM (Ikuta and Weissman, 1992; Li and Johnson, 1995). However, LTRC can be further enriched using additional markers such as Thy-1, CD34, and CD38, and the LSK population also contains short-term reconstituting multipotent progenitor cells (Morrison and Weissman, 1994; Osawa et al., 1996; Randall et al., 1996; Spangrude et al., 1988). Since flt3 has been implicated to play a nonredundant role in early hematopoietic development (Lyman and Jacobsen, 1998; Mackarehtschian et al., 1995; McKenna et al., 2000;

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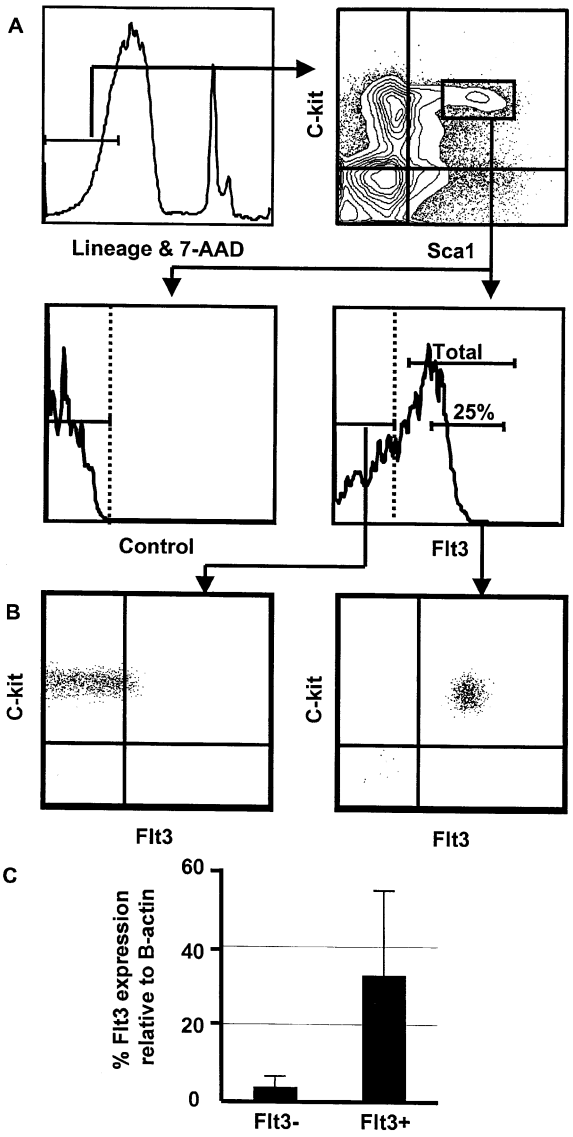

Figure 1. Identification and Purification of Lin$^-$Sca1$^+$c-kit$^+$flt3$^-$ and Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ Cells

(A) Lin$^-$ BM cells coexpressing Sca1 and c-kit were investigated for expression of flt3 or stained with an irrelevant control antibody. LSKflt3$^-$ and LSKflt3$^+$ cells were sorted using the indicated sort gates. In most experiments, the 25% highest flt3 expressing LSK cells were sorted. However, sorting of virtually all (total) LSKflt3$^+$ cells was also performed, resulting in similar purity and functional data.

(B) Reanalysis of sorted populations. FACS plots presented are all in log-scale.

(C) LSKflt3$^-$ cells and LSKflt3$^+$ cells were subjected to global mRNA amplification. The signals for flt3 and β-actin were detected by PhosphorImager quantitation and flt3 signals normalized against the signals of the constitutively expressed β-actin gene. Results are from analysis of five samples (mean ± SD) containing ten cells each. Samples of 25 cells from either subset amplified without addition of reverse transcriptase served as a negative control.

Zeigler et al., 1994), adult BM cells were labeled with antibodies against mature blood cell lineage markers (Lin), Sca1, c-kit, and flt3 to investigate flt3 expression within the LSK HSC compartment (Figure 1). In agreement with previous studies (Zeigler et al., 1994), as much as 60% of Lin$^-$ cells expressing Sca1 and c-kit at high levels were flt3$^+$ (frequency of Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ cells was 0.06% in total BM).

To obtain the Lin$^-$Sca1$^+$c-kit$^+$flt3$^-$ (LSKflt3$^-$) and Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ (LSKflt3$^+$) candidate HSC populations (as defined by FACS analysis) at high purity, a double sort strategy was adopted (Experimental Procedures), resulting reproducibly in more than 95% purity with regard to Sca1, c-kit, and flt3 expression (Figure 1A). LSKflt3$^+$ cells were either sorted to include the 25% highest flt3 expressing cells or all LSKflt3$^+$ cells, resulting in comparable purity (Figure 1B) and biological data (see below).

Since flow cytometric detection of low levels of cytokine receptor expression is not possible, we also applied a highly sensitive RT-PCR method to investigate whether FACS-purified LSKflt3$^-$ cells might express flt3 mRNA. By representing a semiquantitative RT-PCR method, we could also get an impression about differences in flt3 mRNA expression between the sorted LSKflt3$^-$ and LSKflt3$^+$ cells. With 10 LSKflt3$^-$ cells, we could detect low but significant expression of flt3 mRNA in only three out of five samples, whereas all five samples of 10 LSKflt3$^+$ cells showed very high levels of flt3 mRNA expression. On average, there was a 10-fold difference in flt3 mRNA between LSKflt3$^-$ and LSKflt3$^+$ cells (Figure 1C). Thus, at least a fraction of purified LSKflt3$^-$ cells express low levels of flt3 mRNA.

LSKflt3$^-$ and LSKflt3$^+$ donor cells (Ly5.1) were transplanted at only 500 to 1000 cells into each lethally irradiated congenic (Ly5.2) recipient along with a 200- to 300-fold excess of unfractionated (Ly5.2) BM competitor cells. Four weeks posttransplantation, high levels of donor reconstitution were observed in peripheral blood (PB) (Figure 2A) of mice receiving LSKflt3$^-$ cells and LSKflt3$^+$ cells (mean reconstitution of 32.5% ± 6.6% and 20.1% ± 3.1%, respectively). However, whereas mice transplanted with LSKflt3$^-$ cells sustained high levels of donor reconstitution throughout the 16 weeks of analysis (44% ± 15%), the level of reconstitution contributed by LSKflt3$^+$ cells rapidly declined, although 4.8% ± 1.5% donor reconstitution was maintained following 16 weeks (Figure 2A).

LSKflt3$^-$ cells supported long-term multilineage reconstitution of B220$^+$ B cells (Figure 2B), CD$^+$ T cells (Figure 2C), and Gr1/Mac1$^+$ (B220$^-$CD3$^-$) myeloid cells (Figure 2D). In striking contrast, LSKflt3$^+$ reconstitution of PB appeared to be almost exclusively restricted to B and T cells (Figures 2B and 2C). After 4 weeks, most (25 of 30) mice had low levels of Gr1/Mac1$^+$ PB myeloid cells, but by 12 weeks only one out of 20 mice were positive for myeloid reconstitution (Figure 2D).

We next addressed whether the low frequency of LSKflt3$^+$ recipient mice showing long-term myeloid reconstitution could be due to the presence of a low frequency of contaminating LSKflt3$^-$ HSC. One group of mice was transplanted with 1000 LSKflt3$^+$ cells with a purity of 97%. Since the remaining 3% of transplanted cells were almost exclusively LSKflt3$^-$, we transplanted a second group of mice with 30 LSKflt3$^-$ cells (n = 5). Notably, the mice in the two groups showed comparable levels of test cell-derived reconstitution at 16 weeks (mean of 1.6% and 1.5%, respectively). Furthermore,

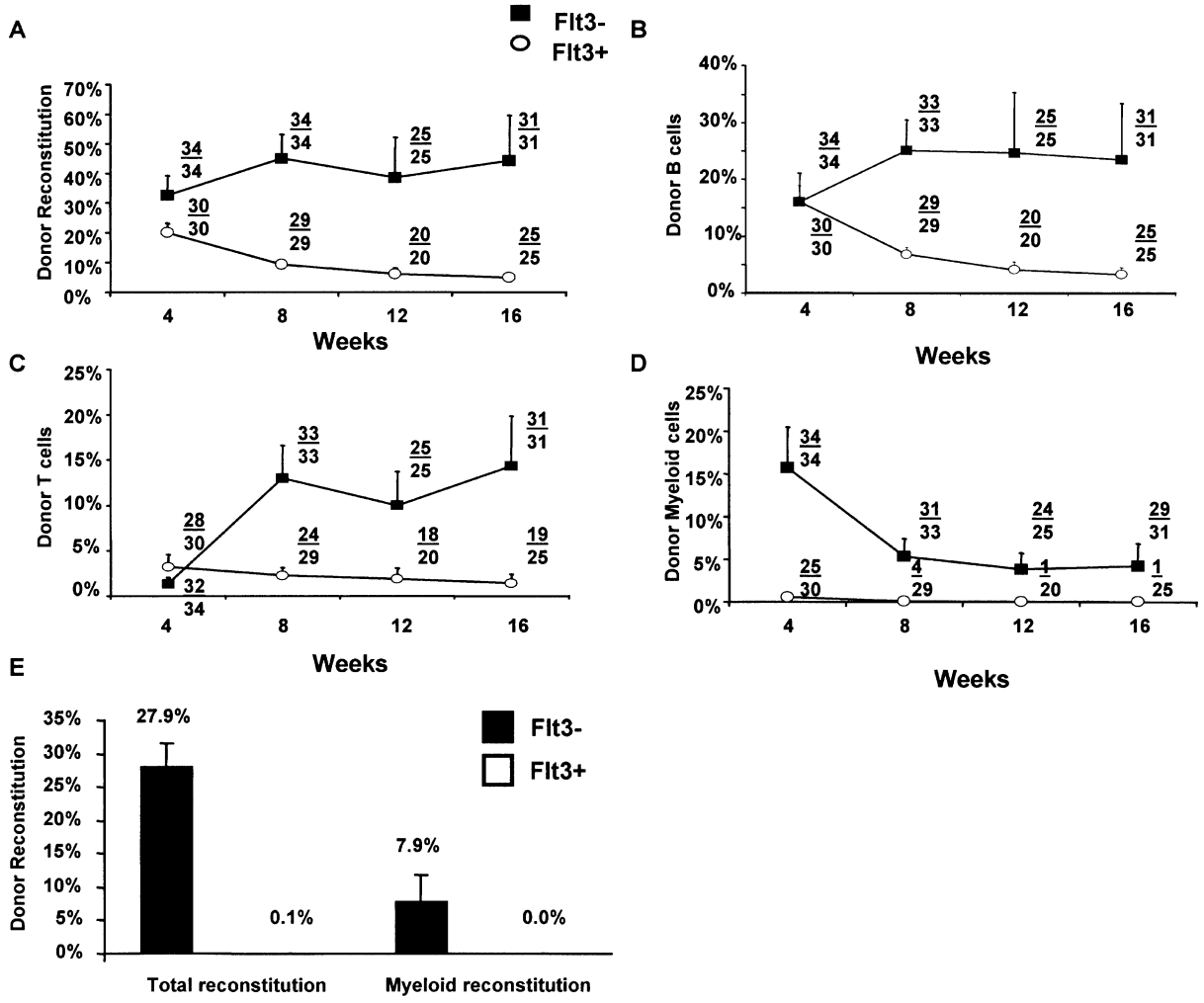

Figure 2. In Vivo Reconstituting Ability of Lin$^-$Sca1$^+$c-kit$^+$flt3$^-$ and Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ Cells

Lethally irradiated (Ly5.2) mice were transplanted with 500–1000 LSKflt3$^-$ or LSKflt3$^+$ (Ly5.1) test cells and 150,000–200,000 congeneic (Ly5.2) BM cells. Following 4, 8, 12, and 16 weeks, mice were investigated for percent test/donor cell contribution to total PB reconstitution.

(A) Total cell reconstitution. (B) B cell reconstitution. (C) T cell reconstitution.

(D) Myeloid reconstitution. Each time point represents the mean (SEM) from four to six experiments. Numbers above each time point indicate frequency of positive mice.

(E) 18 weeks posttransplantation, BM cells were pooled from primary recipients of LSKflt3$^+$ and LSKflt3$^-$ cells and transplanted (1/2 femur equivalent) into lethally irradiated secondary recipients. 6 weeks later, PB was investigated for % donor (Ly5.1) reconstitution (total and myeloid). Data presented are means (SD) of five mice from each group.

none of the mice transplanted with 1000 LSKflt3$^+$ cells and only one out of five mice transplanted with 30 LSKflt3$^-$ cells showed detectable myeloid reconstitution (J.A. and S.J., unpublished data).

One week posttransplantation, high levels of myeloid reconstitution were observed in the spleen (Figure 3A) and BM (J.A. and S.J., unpublished data) of mice transplanted with 5000 LSKflt3$^+$ cells. However, similarly to PB, myeloid reconstitution in BM and spleen dropped below detection level following 6 weeks (Figure 3B). Furthermore, LSKflt3$^+$ cells failed to multilineage reconstitute secondary recipients, whereas LSKflt3$^-$ cells sustained high levels of multilineage reconstitution upon serial transplantation (Figure 2E). LSKflt3$^+$-derived lymphoid cells differentiated normally as demonstrated through the presence of mature B220$^+$IgM$^+$ B cells as well as CD4 and CD8 single positive T cells in PB (Figure 3C). Notably, T cell reconstitution by LSKflt3$^+$ cells was more rapid than that observed with the same number of LSKflt3$^-$ stem cells, and 4 weeks following posttransplantation, the T cell chimerism was higher in mice transplanted with LSKflt3$^+$ than LSKflt3$^-$ cells (Figure 2C).

The possibility that potential LSKflt3$^+$ HSC might be negatively affected in their reconstitution potential by the flt3 antibody was addressed by injecting 1000 LSK (Ly5.1) cells which had been incubated either with the flt3 antibody or with an isotype-matched control antibody. The level of reconstitution and lineage distribution was comparable in the two groups, demonstrating that the flt3 antibody has little or no effect on HSC reconstitu-

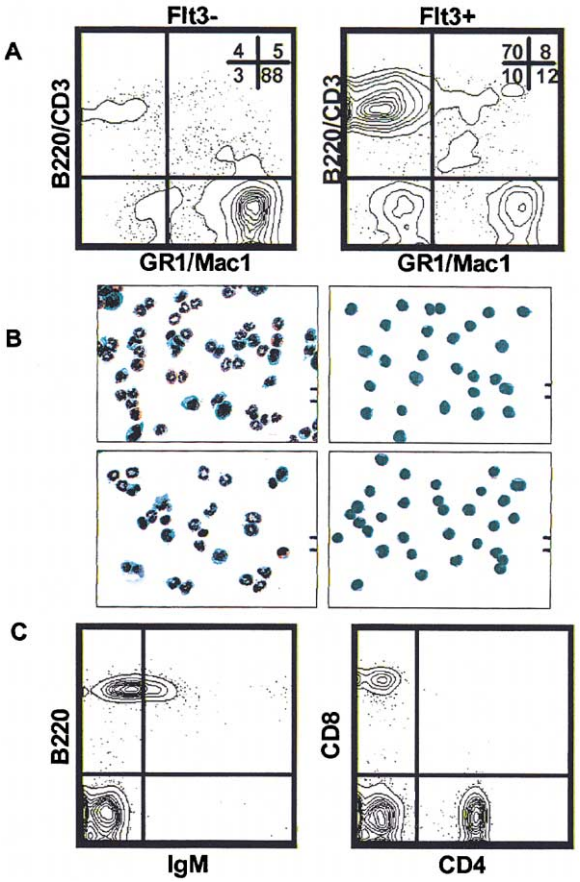

Figure 3. In Vivo Short-Term Multilineage Reconstituting Ability of Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ and Lin$^-$Sca1$^+$c-kit$^+$flt3$^-$ Cells

(A) Short-term myeloid engraftment in spleen from (Ly5.1) donor LSKflt3$^-$ (left panel) and LSKflt3$^+$ (right panel) cells 1 week following transplantation of 1000–5000 cells into Ly5.2 recipients without competition. Plots show myeloid versus lymphoid engraftment of gated Ly5.1$^+$ cells. Similar data were observed in BM (not shown) after 1 week.

(B) May-Grünwald/Giemsa-stained donor (Ly5.1$^+$GR1/MAC1$^+$)-derived cells from LSKflt3$^-$ (left panels) and LSKflt3$^+$ (right panels) cells from BM (upper panels) and PB (lower panels) 6 weeks following transplantation (from one representative experiment out of four total). A 10 μm scale is inserted on the right side of each photo.

(C) Donor-derived mature B and T cells 6 weeks following transplantation of LSKflt3$^+$ cells. Plots show expression of B220 versus IgM (left panel) and CD8 versus CD4 (right panel) on Ly5.1$^+$ gated cells.

tion potential (Table 1). Thus, whereas LSKflt3$^-$ HSC support long-term multilineage reconstitution, LSKflt3$^+$ cells rapidly and efficiently reconstitute B and T lymphopoiesis but fail to long-term reconstitute myelopoiesis.

Myeloid and Lymphoid Differentiation Potential of Single Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ Cells

The limited ability of LSKflt3$^+$ cells to in vivo reconstitute myelopoiesis might reflect that upregulation of flt3 expression within the LSK HSC compartment is accompanied by loss of self-renewal capacity. Alternatively, it might be a consequence of only a small fraction of single

Table 1. HSC Reconstitution Potential Is Not Affected by an Anti-flt3 Antibody

		4 Weeks		8 Weeks	
		% Donor Reconstitution		% Donor Reconstitution	
Cells	Antibody	Total	Myeloid	Total	Myeloid
LSK	flt3	24(2)	5(2)	33(4)	5(3)
LSK	control	23(9)	4(1)	32(6)	6(5)

1000 freshly isolated Lin$^-$Sca1$^+$c-kit$^+$ (Ly5.1) cells were incubated with the anti-flt3 antibody (as in the purification experiments) or with an isotype control antibody and then transplanted directly to irradiated (Ly5.2) recipients with 200,000 unfractionated congeneic (Ly5.2) BM cells. Peripheral blood was analyzed at the indicated time points for presence of donor (Ly5.1)-derived reconstitution. Mean data (SD) from one out of two representative experiments.

LSKflt3$^+$ cells having a myeloid differentiation potential, as demonstrated for the recently identified common lymphoid progenitor (CLP) (Kondo et al., 1997). Thus, clonal in vitro and in vivo assays were employed next to investigate the myeloid and lymphoid differentiation potential of single LSKflt3$^+$ cells.

Most if not all LSKflt3$^+$ as well as LSKflt3$^-$ cells (98% and 97%, respectively) proliferated in response to a defined cocktail of cytokines, and a very high proliferative potential was revealed for both populations (Figure 4A). Morphological evaluation revealed that virtually all LSKflt3$^-$- and LSKflt3$^+$-derived clones produced granulocytes as well as macrophages (Figure 4B). In the same experiments, using a recently developed in vitro assay for proB cell development (Veiby et al., 1997), as much as 60% of LSKflt3$^+$ cells revealed an in vitro B cell differentiation potential (Figure 4C). These data demonstrated that virtually all in vitro clonogenic LSKflt3$^+$ cells (as LSKflt3$^-$ stem cells) have a myeloid differentiation potential and that most of these also have a B cell potential. However, to unequivocally demonstrate the existence of LSKflt3$^+$ progenitor cells with a combined myeloid, B, and T cell differentiation potential, it was paramount to also demonstrate the development of B and T cells from single clonogenic cells. Toward this aim, intrathymic (i.t.) injections were employed, since T cell precursor homing and reconstitution can be rather inefficient following i.v. injections (Goldschneider et al., 1986). Single LSKflt3$^+$ progenitors were individually plated and cultured in KL+FL+IL-7 for 11–13 days, at which time half the content of the resulting clones were individually picked and transplanted i.t. in sublethally irradiated mice, whereas the other halves were cultured for prolonged time for detection of B cell potential in vitro. A total of 18 mice were i.t. injected with single colonies, of which eight revealed development of flt3$^+$-derived T cells (Figure 4D). All 18 colonies also generated B220$^+$CD19$^+$ B cells in vitro (data not shown), and the T cell reconstituted thymi also demonstrated presence of low numbers of B220$^+$IgM$^+$ B cells (Figure 4D). LSKflt3$^+$ (as well as LSKflt3$^-$) cells also efficiently reconstituted CD3$^-$NK1.1$^+$ NK cells (Figure 4E). Thus, although LSKflt3$^+$ cells fail to long-term reconstitute myelopoiesis in vivo, they possess a common myeloid, NK-, B, and T cell differentiation potential.

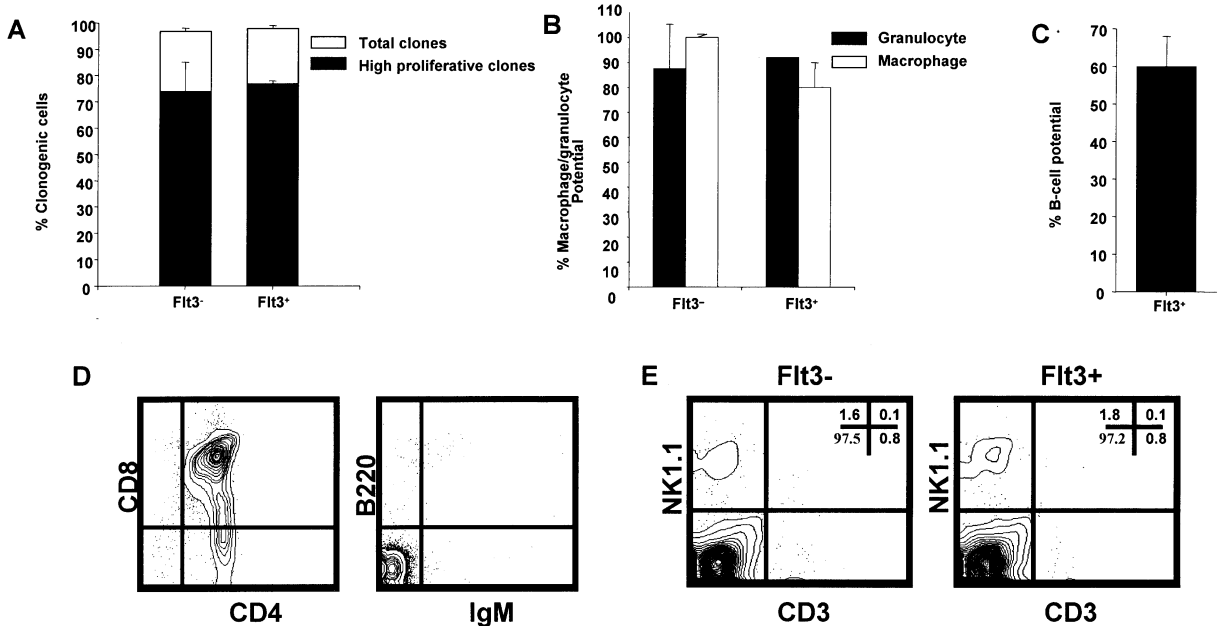

Figure 4. Lineage Potential of Lin$^-$Sca1$^+$c-kit$^+$flt3$^-$ and Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ Cells

Single LSKflt3$^-$ and LSKflt3$^+$ cells were seeded directly (either by a single cell depositor coupled to FACSVantage or manually, resulting in similar results) into medium supplemented with different cytokine combinations and evaluated for clonogenic growth and differentiation potential.

(A) Cloning frequency (white bars) and clone size (black bars represent percent of clones covering >50% of the well) were evaluated 10 days after initiation of cultures supplemented with KL, FL, MGDF, IL-3, G-CSF, and CSF-1. Results represent the mean (SEM) of three individual experiments.

(B) Contents of individual wells were transferred to glass slides, May-Grünwald/Giemsa-stained, and evaluated for the presence of macrophages and granulocytes. Results represent the means (SD) of two individual experiments.

(C) ProB cell potential of LSKflt3$^+$ cells evaluated by plating cells at a density of one cell per well in a serum-depleted medium supplemented with KL, FL, and IL-7. 14–28 days after initiation of culture, individual colonies were picked and analyzed by flow cytometry to verify the presence of proB cells, as defined by combined B220 and CD19 expression. Results represent the mean (SEM) of three individual experiments and are presented as percent LSKflt3$^+$ cells forming proB cell colonies. LSKflt3$^-$ cells, although having a high B cell reconstitution potential, do not form proB cells in response to FL, KL, and IL-7 in this assay (Borge et al., 1999), probably due to the lack of flt3 receptor expression, and were therefore not investigated for in vitro proB cell formation in the present study.

(D) Cells were individually deposited and cultured for 11–13 days in a medium supplemented with KL, FL, and IL-7 before half the content of individual colonies were picked and injected intrathymically in congenic (Ly5.2) mice, whereas the other halves were individually analyzed for in vitro proB cell generation 6–8 days later by flow cytometry. Thymi were analyzed 20 days after transplantation for donor (Ly5.1)-derived cells. LSKflt3$^+$-derived thymocytes stained with CD4 and CD8 (left panel) and with B220 and IgM in a representative mouse (right panel). All clones transplanted intrathymically produced B220$^+$CD19$^+$ proB cells in vitro (data not shown).

(E) 3 weeks after transplantation of 2000–5000 LSKflt3$^-$ or LSKflt3$^+$ (Ly5.1) cells to lethally irradiated mice, spleens were analyzed for presence of CD3$^-$NK1.1$^+$ NK cells. Percentages in quadrants represent means of three mice from two individual experiments.

Flt3 Ligand but Not c-kit Ligand Promotes Survival of Lin$^-$Sca1$^+$c-kit$^+$ Cells with a Lymphoid-Restricted In Vivo Reconstitution Potential

Next, we investigated whether the LSK HSC compartment might contain FL-responsive LTRC with undetectable levels of cell surface flt3 expression, utilizing the observation that FL similar to KL (as single cytokines) has a strong viability promoting effect on targeted primitive progenitors (Bodine et al., 1992; Veiby et al., 1996). In agreement with previous studies (Bodine et al., 1992; Keller et al., 1995; Li and Johnson, 1994), KL efficiently supported in vitro survival of LSK long-term repopulating HSC, whereas FL failed to support maintenance of LSK cells capable of long-term myeloid reconstitution (Table 2). Notably, although LSKflt3$^+$ cells express high levels of both c-kit and flt3 (Figure 1), FL but not KL promoted in vitro survival of reconstituting LSKflt3$^+$ cells (Table 2). Thus, KL and FL possess distinct abilities to promote survival of LTRC and LSKflt3$^+$ reconstituting cells, respectively.

Relationship between Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ Cells, Lin$^-$Sca1$^+$c-kit$^+$flt3$^-$ Long-Term Repopulating Stem Cells, and Common Lymphoid Progenitor Cells

Previous studies demonstrated that LTRC in adult mice are almost exclusively CD34$^-$ (Osawa et al., 1996; Sato et al., 1999). LSKflt3$^+$ adult BM cells were found to be almost exclusively CD34$^+$, whereas LSKflt3$^-$ cells as expected contained a small but significant (5%) fraction of CD34$^-$ cells (Figure 5A).

A CLP with potent but lymphoid-restricted reconstitution potential was recently identified (Kondo et al., 1997). Although also "lymphoid-restricted" in their in vivo reconstitution potential (Figure 2A), the in vitro myeloid potential of LSKflt3$^+$ cells (Figure 4B) suggested that the LSKflt3$^+$ population is distinct and most likely an

Table 2. Distinct Abilities of KL and FL to Promote Survival of Long-Term Reconstituting Stem Cells and Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ Reconstituting Cells

Donor-Derived Reconsitution							
		4 Weeks		6–8 Weeks		22 Weeks	
Cells	Treatment	% Total	Myeloid	% Total	Myeloid	% Total	Myeloid
LSK	Uncultured	ND		29(4)	7/7	25(22)	4/5
LSK	Medium	ND		0(0)	0/5	2(4)	1/5
LSK	KL	ND		30(15)	12/12	20(22)	4/5
LSK	FL	ND		4(2)	2/13	1(1)	0/5
LSKflt3$^+$	Uncultured	20(8)	6/10	12(2)	3/10	ND	
LSKflt3$^+$	Medium	0(0)	0/3	0(0)	0/3	ND	
LSKflt3$^+$	KL	0(0)	0/9	1(3)	1/9	ND	
LSKflt3$^+$	FL	19(10)	16/18	12(6)	6/17[a]	ND	

1000 freshly isolated Lin$^-$Sca1$^+$c-kit$^+$ or Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ (Ly5.1) cells were transplanted intravenously to irradiated (Ly5.2) recipients either directly (uncultured) or after 4–7 days incubation in serum-free medium in the absence (medium only) or presence of KL or FL (50 ng/ml) each. 200,000 unfractioned congenic (Ly5.2) BM cells were cotransplanted to provide a competitor and survival population. Peripheral blood was analyzed at the indicated time points (Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ recipients were only analyzed at 4 and 6 weeks) for presence of donor (Ly5.1)-derived multilineage reconstitution. Mean data (±SD) from three experiments.

[a] Three mice in this group had a myeloid reconstitution just above detection level (0.1%).

intermediate between LTRC and CLP. In agreement with this, the lymphoid reconstitution potential of 1000 LSKflt3$^+$ cells (in competition with 200,000 BM cells) was considerably higher than that of the same number of CLP (contribution to total PB cellularity of 20% ± 3% and 3.5% ± 1.7%, respectively, at 4–5 weeks posttransplantation; mean of two experiments ± SD, n = 10). Furthermore, whereas the CLP express low levels of Sca1, c-kit, and the IL-7Rα (Figure 5B and Kondo et al., 1997), LSKflt3$^+$ cells, like LTRC, express high levels of Sca1 and c-kit, but lack detectable IL-7Rα expression (Figure 5B).

To unequivocally demonstrate the hierarchical relationship between LSKflt3$^-$ HSC and LSKflt3$^+$ reconstituting cells, LSKflt3$^-$ and LSKflt3$^+$ (Ly5.1) cells were transplanted in competition with 200,000 unfractionated BM (Ly5.2) and analyzed 18 weeks posttransplantation. Whereas LSKflt3$^-$ HSC as expected generated LSKflt3$^-$ as well as LSKflt3$^+$ cells (Figure 5C), LSKflt3$^+$ cells failed to sustain LSK cells over time (Figure 5C). However, both LSKflt3$^-$ and LSKflt3$^+$ cells produced cells with a CLP phenotype (Lin$^-$Sca^{low}c-kit^{low}IL-7Rα$^+$; Figure 5D), supporting the idea that LSKflt3$^+$ reconstituting cells represent an intermediate between LSKflt3$^-$ HSC and CLP.

We have previously shown that FL in vitro promotes the lymphoid commitment process from LSKflt3$^+$ cells and induction of IL-7Rα expression (Borge et al., 1999). Here, FL+IL-7 stimulation in vitro resulted in formation of cells with a CLP phenotype (Figure 5E). Furthermore, when donor-derived Lin$^-$Sca^{low}c-kit^{low}IL-7Rα$^+$ cells were sorted from mice transplanted with LSKflt3$^+$ cells, they produced large numbers of B220$^+$ cells (>99%) in response to FL, KL, and IL-7, whereas only 1% of the cells produced colonies in response to a cocktail of myeloid growth factors (IL-3, Tpo, G-SCF, and GM-CSF; J.A. and S.J., unpublished data).

Discussion

The flt3 receptor was originally identified and characterized based on its preferential expression on candidate HSC in mice (Matthews et al., 1991) and has since emerged as a likely regulator of the HSC pool in mice and human (reviewed in Lyman and Jacobsen, 1998). Of particular relevance, BM from mice deficient in flt3 expression have reduced ability to multilineage reconstitute lethally irradiated recipients (Mackarehtschian et al., 1995). However, such findings are not necessarily a consequence of reduced HSC numbers in steady-state BM and could rather reflect an important (direct or indirect) role of flt3 and its ligand in the process of homing, reconstitution, or differentiation of HSC following transplantation.

Single cell clonogenic assays revealed that LSKflt3$^+$ and LSKflt3$^-$ cells both have a high in vitro proliferative capacity and a combined myeloid, B, and T cell differentiation potential, supporting their primitive nature. Furthermore, as few as 500–1000 LSKflt3$^+$ cells were capable of efficiently reconstituting lethally irradiated recipients in vivo when competing with a 200- to 300-fold excess of unfractionated BM cells. However, surprisingly, and unlike LSKflt3$^-$ HSC, LSKflt3$^+$ reconstituting cells failed to fulfill other key characteristics of self-renewing HSC. First, although significant levels of reconstitution were observed in the long-term with LSKflt3$^+$ cells, their contribution was steadily declining, in contrast to the sustained reconstitution activity of LSKflt3$^-$ HSC. Furthermore, although having an in vitro myeloid differentiation potential, in vivo myeloid reconstitution by LSKflt3$^+$ cells was almost exclusively short term, and experiments suggested that the low frequency (4%–5%) of mice with long-term myeloid reconstitution could be due to infrequent but contaminating LSKflt3$^-$ cells in the sorted LSKflt3$^+$ population.

Although a previous study concluded that flt3 is expressed on a fraction of LTRC in murine BM (Zeigler et al., 1994), all Lin$^-$Sca1$^+$flt3$^+$ BM cells were, as in the present study, found to coexpress CD34, and more importantly, a drop in reconstitution activity was observed with time. That flt3 might not be expressed on LTRC is also supported by recent experiments showing that FL-deficient mice have normal levels of BM LTRC (E.S. and S.J., unpublished data).

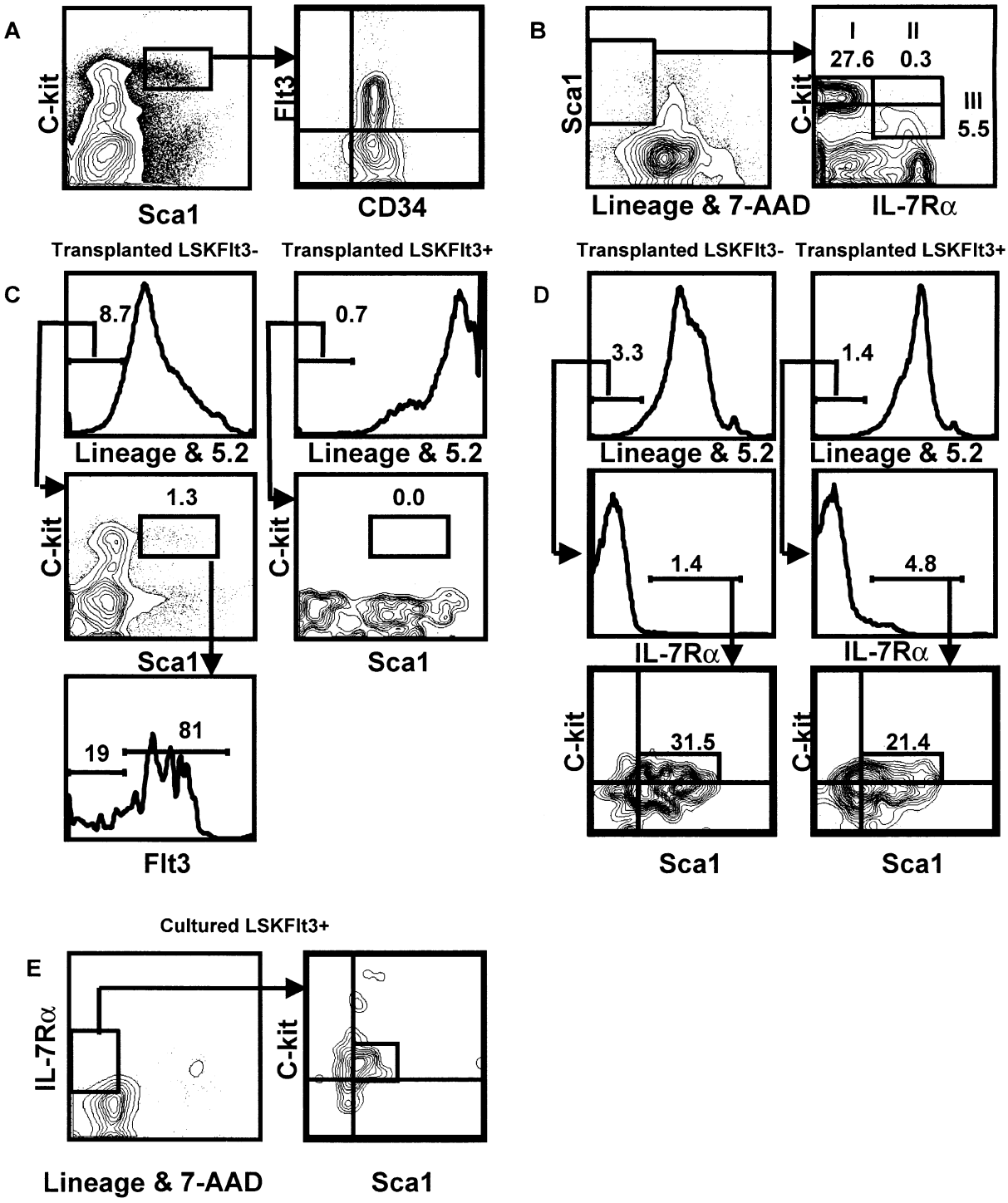

Figure 5. Coexpression Pattern of CD34 and IL-7Rα on Lin$^-$Sca1$^+$c-kit$^+$flt3$^+$ Cells and Relationship to CLP

(A) Lin-depleted BM cells stained for Sca1, c-kit, and flt3 expression together with anti-CD34.

(B) BM cells stained with anti-lineage cocktail together with anti-Sca1, c-kit, and IL-7Rα. 7AAD was included to exclude dead cells. Note that quadrant I is where LSKflt3$^+$ cells reside, whereas quadrant III is where the Lin$^-$Sca1^{lo}c-kit^{lo}IL-7R$^+$ CLP population resides (Kondo et al., 1997). Also note that virtually no LSK(flt3$^+$) cells express IL-7Rα (quadrant II).

(C) 1000 LSKflt3$^-$ and LSKflt3$^+$ (Ly5.1) cells were injected into lethally irradiated Ly5.2 recipients. After 18 weeks, BM cells were investigated for donor-derived LSKflt3$^-$ and LSKflt3$^+$ cells.

(D) 5000 LSKflt3$^-$ and LSKflt3$^+$ (Ly5.1) cells were injected into lethally irradiated Ly5.2 recipients. After 3 weeks, spleens were investigated for donor derived CLP. Boxes identify position of the CLP and represent means of three mice from two individual experiments.

(E) LSKflt3$^+$ cells were cultured in vitro for 7 days in FL + IL-7 and subsequently investigated for presence of cells with a CLP phenotype. Representative analysis from one of two independent experiments.

Although the highly purified LSKflt3$^-$ population contains at least some cells expressing low levels of flt3 mRNA, our data clearly demonstrate that the 60% of cells within the BM LSK compartment expressing detectable cell surface flt3 is functionally distinct from that of LSKflt3$^-$ LTRC, and that LSKflt3$^+$ cells have a prominent but lymphoid-restricted reconstitution potential. The ability of the LSKflt3$^+$ population to reconstitute lymphopoiesis is clearly superior to that of the CLP population (Kondo et al., 1997), and the LSKflt3$^+$ cell population provides a more rapid lymphoid reconstitution than the LSKflt3$^-$ population. Thus, LSKflt3$^+$ cells could potentially be used to rapidly and efficiently correct deficiencies in lymphopoiesis in immunocompromised recipients.

Both KL and FL do, as single cytokines, effectively promote in vitro survival of primitive hematopoietic progenitors (Bodine et al., 1992; Keller et al., 1995; Veiby et al., 1996). Whereas we confirmed the ability of KL to promote survival of LTRC within the LSK pool, FL failed to have any such effect, in agreement with the absence of detectable flt3 cell surface expression on LTRC. In striking contrast, FL efficiently supported survival of LSKflt3$^+$ reconstituting cells, whereas KL had no effect, although LSKflt3$^+$ and LSKflt3$^-$ cells expressed high and comparable levels of c-kit. As we have reported before (Borge et al., 1999), FL has no viability promoting effect on LSKflt3$^-$ cells. The lack of KL responsiveness of LSKflt3$^+$ cells is not restricted to effects on survival, since KL also has little or no ability to synergistically interact with other cytokines to stimulate growth of LSKflt3$^+$ cells (J.A. and S.J., unpublished data). These findings suggest that the distinct abilities of FL and KL to promote early lymphoid and myeloid development, respectively (Borge et al., 1999; Lyman and Jacobsen, 1998), cannot solely be explained by different expression patterns for flt3 and c-kit and could potentially result from utilization of different signaling pathways as indicated in recent studies (Zhang et al., 2000).

The observation that transplanted LSKflt3$^-$ cells efficiently regenerated LSKflt3$^+$ cells in vivo but not vice versa confirms the hierarchical relationship between these two populations in the LSK HSC pool. Furthermore, the higher reconstitution potential as well as lack of IL-7Rα expression of LSKflt3$^+$ cells clearly implicate the CLP as progeny of LSKflt3$^+$ cells, a conclusion supported by the formation of CLP (Lin$^-$Sca^{low}c-kit^{low}IL-7Rα^+) from LSKflt3$^+$ cells in vitro as well as in vivo. Thus, upregulation of flt3 expression within the BM HSC pool is coupled to loss of self-renewal ability and lymphoid-restricted repopulating ability.

What implications, if any, do these findings have for the recent and considerable interest in utilizing FL to promote retroviral-mediated gene transfer and expansion of reconstituting candidate human HSC (reviewed in Lyman and Jacobsen, 1998; Petzer et al., 1996; Piacibello et al., 1997, Dao et al., 1997)? As a consequence of promising preclinical results in these directions, FL has surfaced as one of the key stimulators of candidate human HSC, and there are a number of other findings suggesting that c-kit and flt3 might have different functions in murine and human HSC. However, it is possible that a human counterpart of the LSKflt3$^+$ reconstituting cells presented here could reconstitute in surrogate human HSC assays, since it is not established how efficiently these surrogate assays can distinguish between short- and long-term reconstituting human HSC. However, most data would suggest a key role of flt3 in human HSC, although the status of flt3 expression and function on human LTRC remains to be explored in more detail. There are also multiple lines of data suggesting that flt3 and its ligand might be involved in regulating murine LTRC numbers or function under conditions other than steady-state hematopoiesis. Although the mechanisms remain unclear, this possibility is supported by reconstitution studies with flt3-deficient BM (Mackarehtschian et al., 1995) and by studies showing that FL can synergize with other early acting cytokines to preserve HSC isolated from 5-fluorouracil-treated mice (Matsunaga et al., 1998), suggesting that flt3 might perhaps be upregulated on cycling HSC.

Experimental Procedures

Hematopoietic Growth Factors

Recombinant human (rh) IL-7 was generously provided by Dr. Natalio Vita, Sanofi Elf (Montpellier, France). Rh megakaryocyte growth and development factor (MGDF), rh granulocyte colony-stimulating factor (G-CSF), and recombinant mouse (rm) granulocyte-macrophage colony-stimulating factor (GM-CSF) were generously provided by Amgen Corp. (Thousand Oaks, CA). Rm mast cell growth factor (C-kit ligand; KL) and rh fms-like tyrosine kinase-3 ligand (Flt3L; FL) were kind gifts from Immunex (Seattle) and Rh interleukin-11 (IL-11) from Genetics Institute (Cambridge, CA). Rh colony-stimulating factor 1 (CSF-1) was from Chiron (Emeryville, CA), and Rm interleukin-3 (IL-3) from PeproTech Inc. (Rocky Hill, NJ). Cytokines were used at predetermined optimal concentrations, and all human cytokines utilized have been shown to be fully crossreactive with murine cells.

Enrichment and Purification of Subpopulations of Lin$^-$Sca1$^+$ BM Cells Based on Expression of c-kit and Flt3

All sorts were performed by an immunomagnetic-based preenrichment followed by multicolor flow cytometric sorting. In brief, lineage-depleted (Lin$^{-/lo}$) BM cells were isolated from 5- to 18-week-old C57Bl/6 (Ly5.2$^+$) from M&B (Ry, Denmark) or offspring of congenic (Ly5.1$^+$) breeders originating from Jackson Laboratories (Bar Harbor, ME). BM cells were incubated at 4°C for 30 min in a cocktail of predetermined optimal concentrations of antibodies: purified RA3-6B2 (B220), RB6-8C5 (Gr1), M1/70 (Mac1), 53-6.7 (CD8), 53-7 (CD5), H129.19 (CD4), and Ter-119, all from PharMingen (San Diego, CA). Cells were incubated at 250 $\times$ 10^6 cells/ml with sheep anti-rat IgG (Fc)-conjugated immunomagnetic beads (Dynal, Oslo, Norway) at a cell:bead ratio of 1:0.3, at 4°C for 45 min. Magnetic beads were removed with a magnetic particle concentrator (MPC-6, Dynal), and unattached cells were exposed to the same absolute amount of magnetic beads and processed as for the first separation.

Lin$^{-/lo}$ cells recovered from the supernatant were further purified based on the expression of Lineage, Sca1, c-kit (Bryder and Jacobsen, 2000; Li and Johnson, 1995), as well as flt3. In brief, Lin$^{-/lo}$ cells were resuspended at 100–400 $\times$ 10^6 cells/ml and incubated for 15 min on ice with CyChrome-conjugated goat anti-rat antibody (Caltag Laboratories, Burlingame, CA) and subsequently stained with Fitc-conjugated rat anti-mouse E13-161.7 antibody (Sca1), APC-conjugated rat anti-mouse 2B8 (c-kit), and PE- or biotin-conjugated rat anti-mouse flt3 (A2F10.1) plus streptavidin-PE or isotype-matched control antibodies (all PharMingen). Cells were stained with 7-amino actinomycin (7-AAD; Sigma-Aldrich Co, St. Louis, MO) to exclude dead cells and Lin$^-$Sca1$^+$c-kit$^+$ (LSK) cells were sorted on a FACSVantage Cell Sorter (Becton Dickinson, San Jose, CA), equipped with an 488 nm argon and a 633 nm He-Ne laser, at a rate of 2000–8000 cells per second. The purity was high after the first sort (>95% with regard to Lin, Sca1, and c-kit expression). LSK cells were in a second sorting separated into flt3$^+$ and flt3$^-$ populations. The sorted

LSKflt3+ subfraction was 95%–99% pure when reanalyzed on a FACS Vantage or FACSCalibur (Becton Dickinson).

Detection of Flt3 Transcripts in Lin−Sca1+c-kit+flt3− and Lin−Sca1+c-kit+flt3+ Cells

For semiquantitative detection of flt3 transcripts, LSKflt3+ cells and LSKflt3− cells were subjected to global mRNA amplification and subsequent Southern analysis essentially as described previously (Theilgaard-Mönch et al., 2001; Brady et al., 1990). In brief, cells were transferred into PCR tubes using an automated micromanipulator device. Subsequently, cells were lysed in first strand buffer followed by reverse transcription 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 generatedby PCR from sequence-verified clones containing the 3′ end of the flt3 and β-actin mRNA sequence. The flt3 clone was established from LSKflt3+ cells by PCR and inserted into the pCRII vector according to the manufacturer (InVitrogen, Carlsbad, CA). The following primers were used for flt3 cloning and probe synthesis: forward primer 5′-AGAAGTTAGCGAGGAGGCC-3′, reverse primer 5′-ATT TCCATCCATAAAATATATCAC-3′. The cytoskeletal β-actin IMAGE clone (3377012) was obtained from the HGMP Resource center (Cambridge, UK). The following primers were used for cytoskeletal β-actin probe synthesis: forward primer 5′-TTGCTTCTGTGTAAAT TATGTAC-3′, reverse primer 5′-GTAAGGTGTGCACTTTTATTGG-3′. Probes (50 ng) were radiolabeled with ^{32}P-dCTP (Random Primers DNA labeling system, GIBCO BRL, Life Technologies, Paisley, UK), purified (QIAaquick Nucleotide removal c-kit, Quiagen, Hilden, Germany), and hybridized at 65°C overnight in a 5 × SSPE buffer (5 × Denhardt's = 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% Polyvinylpyrolidon MW 40.000; 5 × SSPE = 0.9 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, and 5 mM EDTA [pH 7.7]; all reagents from Sigma, St. Louis, MO). Following hybridization, membranes were washed 2 × 15 min at 62°C in 2 × standard saline citrate (1 × SSC = 150 mM NaCl and 15 mM sodium citrate [pH 7]), 0.1% SDS followed by 2 × 15 min in 0.2 × SSC, 0.1% SDS. 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, Duesseldorf, Germany). Signals for flt3 and β-actin expression were corrected by subtracting the signals from the negative control, i.e., cells amplified without addition of reverse transcriptase. The corrected flt3 signals were normalized against the corrected β-actin signals to compare the expression levels of flt3 transcripts in LSKflt3+ cells and LSKflt3− cells.

Single Cell Cultures

LSKflt3− and LSKflt3+ cells were seeded in Terasaki plates at a concentration of one cell per well in 20 μl of serum-free medium (all groups in survival experiment and proB cell cultures) or serum-containing medium (for evaluation of in vitro myeloid potential only) plus cytokines, either manually or using a single cell depositor coupled to the FACSVantage (giving similar results). In some experiments, single cells were verified by microscopy 2–12 hr after seeding, and only wells containing one cell were included in the experiments. The serum-free medium (X-vivo 15; BioWhittaker) was supplemented with 1% detoxified Bovine Serum Albumin (BSA; Stem Cell Technologies Inc). A serum-containing medium (IMDM; BioWhittaker), was supplemented with 20% FCS. Wells were scored for cell growth following 10–14 days of culture. Individual colonies were transferred to slides in a cytospin centrifuge and examined morphologically after May-Grünwald/Giemsa-staining or were stained with antibodies and analyzed by flow cytometry.

In Vivo Reconstitution Experiments

All mice experiments were approved by the local ethics committee. 500–1000 LSKflt3−, LSKflt3+, or LSK freshly isolated Ly5.1 cells or their cultured equivalents were injected intravenously to lethally irradiated (950 rad) C57Bl/6 (Ly5.2) mice. All mice were kept in individually ventilated cages throughout the experiment and given sterile food and autoclaved acidified water. Irradiated C57Bl/6 mice (three to five mice per group and experiment) were cotransplanted with 150,000–200,000 unfractionated congeneic (Ly5.2) BM cells to provide a competitor and survival population. Peripheral blood (PB) samples were obtained at different time points. To study short-term (1–2 weeks) reconstitution, Ly5.1 LSKflt3+ and LSKflt3− cells were injected without competition into lethally irradiated Ly5.2 recipients, and spleen and BM were investigated for Ly5.1 and lineage reconstitution. Donor Ly5.1 cells were also sorted for morphological evaluation. In some experiments, Ly5.1 donor cells were injected directly into the thymus of 4- to 8-week-old C57Bl/6 (Ly5.2) mice 2–6 hr after sublethal irradiation (650 rad) as previously described (Goldschneider et al., 1986), and the thymi were isolated and analyzed 20 days later. Serial transplantations were performed by pooling BM from eight primary recipients 18 weeks posttransplantation and injecting 1/2 femur equivalent into secondary irradiated (Ly5.2) recipients. Cells from all in vivo assays were stained with antibodies against Ly5.1 and Ly5.2, as well as lineage markers (CD3ϵ, CD4, CD8a, B220, IgM, Mac1, Gr1, and NK1.1; all from PharMingen), and subsequently analyzed on a FACSCalibur or sorted on a FACSVantage. As adopted in other studies (Domen et al., 2000; Hsu et al., 2000; Morrison and Weissman, 1994), mice were considered as donor/test cell reconstituted if more than 0.5% of PB cells were of donor origin, and positive for myeloid reconstitution if Gr1/Mac1+ cells represented at least 0.1% of the reconstitution.

Phenotypic Analysis of the Lin−/loSca1+c-kit+ HSC Compartment

Lineage-depleted BM cells were stained with anti-Sca1-biotin (detected with Streptavidin-CyChrome) anti-c-kit-APC, anti-flt3-PE in combination with anti-CD34-Fitc (all from Pharmingen). To study IL-7Rα expression on Lin−/loSca1+c-kit+ cells, the same cocktail of purified antilineage antibodies was used as for lin depletion (visualized by CyChrome-conjugated goat anti-rat antibody), together with directly conjugated anti-B220-CyChrome, anti-CD3-CyChrome, anti-Sca1-Fitc, anti-c-kit-APC, and anti-IL-7Rα-Biotin (A7R34; generously provided by Dr. Koichi Akashi; visualized by Streptavidin-PE) antibodies. 7-AAD was included to exclude dead cells. Stained BM cells were analyzed on a FACSCalibur.

Reconstitution of CLP in Spleen and BM of Mice Transplanted with Lin−Sca1+c-kit+flt3− and Lin−Sca1+c-kit+flt3+ Cells

LSKflt3− and LSKflt3+ (Ly5.1) cells were injected into lethally irradiated Ly5.2 recipients with 200,000 unfractionated BM cells of recipient type to investigate their ability to generate LSKflt3+ and LSKflt3− cells in vivo. BM cells were stained with antibodies against lineage (visualized by a CyChrome-conjugated goat anti-rat antibody), Ly5.2-Cy5-PE, Sca1-Fitc, c-kit-APC, and flt3-biotin (visualized by Streptavidin-PE). To study the generation of CLP, spleen cells were incubated with antibodies against lineage (visualized by CyChrome-goat-anti-rat antibody), Ly5.2-Cy5-PE, Sca1-Fitc, c-kit-APC, and IL-7Rα−biotin (visualized by Streptavidin-PE). 7-AAD was included in all samples to exclude dead cells, and samples were analyzed on a FACSCalibur.

Acknowledgments

The authors thank Dr. Corrado Cilio and Karin Petersson for expert advice and assistance in intrathymic injections, Dr. Kris Grzegorzewski for expert evaluation of cellular morphology, and Per Anders Bertilsson, Sverker Segren, Carl-Magnus Högerkorp, and Anna Fossum for essential assistance in cell sorting. The technical assistance of Gunilla Gärdebring, Eva Gynnstam, Irene Persson, and Lilian Wittman is also highly appreciated. We thank Graham Molineux (Amgen) and Stewart Lyman (Immunex) for generously contributing cytokines for these studies, Dr. William Matthews (Genentech) for providing anti-flt3 antibodies, and in particular Dr. Koichi Akashi for fruitful discussions and for sharing important reagents for these studies.

These studies were generously supported by grants from: ALF (Government Public Health Grant); the Berta Kamprad Foundation; the Crafoord Foundation; the Harald and Greta Jeansson Foundation; the Thelma Zoega Foundation; the John and Augusta Persson Foundations; the Medical Faculty, University of Lund; The Swedish Medical Research Council (MFR); the Svensson Siblings Founda-

tion; the O.E. and Edla Johansson Foundation; the Royal Physiographic Society in Lund, and the Swedish Foundation for Strategic Research.

Received February 21, 2001; revised August 17, 2001.

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