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Quiescence of hematopoietic stem cells and maintenance of the stem cell pool is not dependent on TGF-β signaling *in vivo*

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Abstract

Maintained quiescence of hematopoietic stem cells (HSCs) is of critical importance to prevent premature exhaustion of the stem cell pool under conditions of hematopoietic stress. Transforming growth factor β (TGF-β) plays an important role in maintaining the quiescent state of HSCs *in vitro*. Here, we have assessed this *in vivo* using conditional knockout mice for the TGF-β type I receptor (TβRI). Surprisingly, we found that TGF-β receptor-deficient HSCs have similar susceptibility, compared to controls, to repeated treatments with the cell cycle-specific cytotoxic drug 5-fluorouracil, indicative of normally maintained quiescence. Furthermore, when exhaustion of HSCs was provoked through serial bone marrow transplantations, hematopoietic failure occurred at similar stages in recipients of TβRI null and control BM respectively, demonstrating normal consumption of the stem cell pool. We conclude that TGF-β does not provide the necessary signal, which induces the quiescent state of HSCs and maintains the stem cell pool *in vivo*.

Introduction

Despite their enormous proliferative capacity, most hematopoietic stem cells (HSCs) reside in a non-cycling quiescent state, at any given time point[1]. This is of outmost importance to ensure life-long hematopoiesis and to protect the HSC pool from myelotoxic insult and premature exhaustion under conditions of hematopoietic stress[2,3]. The naturally quiescent state of HSCs is believed to be controlled by negative regulators of cell proliferation. Studies using knockout mice have shown that endogenous levels of the cyclin dependent kinase (CDK) inhibitor p21 are crucial in order to maintain quiescence of HSCs and to protect the stem cell pool from exhaustion during stressed conditions[4]. The extrinsic signals that mediate these functions *in vivo* have not been identified. However, a main candidate based on a number of studies *in vitro* is the inhibitory cytokine transforming growth factor-β (TGF-β). TGF-β has been shown to induce cell cycle arrest in many cell types through upregulation of CDK inhibitors, including p21[5]. Moreover, active TGF-β induces quiescence of primitive hematopoietic progenitor cells in culture while TGF-β neutralization releases these cells from quiescence[6-11]. In order to study how TGF-β regulates HSCs *in vivo* we generated conditional knockout mice for the TGF-β type I receptor (TβRI). We recently reported that TGF-β receptor-deficient HSCs, unexpectedly, had normal ability to regenerate the hematopoietic system following bone marrow transplantation[12]. However, when assayed *in vitro*, these cells had significantly enhanced proliferative capacity consistent with an important, yet highly context-dependent, role for TGF-β in regulation of HSCs[12]. In the present study we have specifically asked how TGF-β signaling influences the quiescent state of HSCs under conditions of hematopoietic stress to determine whether TGF-β plays a role in protection of the stem cell pool *in vivo*. The susceptibility of TGF-β receptor deficient

HSCs to cell cycle-specific myelotoxic injury has been tested as well as their ability to reconstitute serially transplanted mice. Our results clearly demonstrate that quiescence of HSCs and maintenance of the stem cell pool is not dependent on TGF-β signaling *in vivo*.

Materials and Methods

Mice

Genotyping and polyIC induction of TβRI floxed mice carrying Mx*Cre* has been described previously[12]. In order to overcome the inflammatory disease associated with lack of TGF-β signaling in T cells the mice were bred on to a αβ T-cell deficient background[13-15]. In addition, mice used in this study had been backcrossed on C57BL/6 background for at least six generations.

Competitive transplantation and 5-fluorouracil treatment

Bone marrow (BM) cells were harvested from femurs and tibiae and 5 x 10^5 cells (Ly5.2) were transplanted by tail vein injections into lethally irradiated (950 cGy) C57BL/6 X BL6SJL (Ly5.1/5.2) recipient mice in competition with equal numbers of B6SJL (Ly5.1) BM cells. After 12 weeks, half of the recipients were given two intravenous injections of 5-fluorouracil (5-FU) (150mg/g mouse) five days apart. All recipients were subsequently sacrificed, and a half femur equivalent of BM was transplanted together with 2 x 10^5 support BM cells (Ly5.1/Ly5.2) into secondary recipients.

Flow cytometry

Flow cytometry for detection of Ly5.1 and Ly5.2 markers was carried out as previously described[12].

Serial transplantations

For each consecutive BM transplantation 2 x 10^6 BM cells were injected into lethally irradiated recipients. Mice were monitored for survival for 2-3 months and then sacrificed for subsequent BM transfers.

Results and discussion

To investigate the role of TGF-β signaling in maintaining quiescence of HSCs *in vivo* we analyzed conditional knockout mice with an inducible deletion of TβRI. We have previously demonstrated that polyIC mediated induction of MxCre in these mice deletes TβRI and blocks TGF-β signaling with practically 100% efficiency in bone marrow and HSCs[12]. Here, we first asked to what extent TβRI deficient HSCs would retain quiescence under stressed conditions by measuring their susceptibility to repeated treatment with the cell cycle-specific antimetabolite 5-fluorouracil (5-FU). BM cells from induced TβRI null (flox/flox Mx*Cre*) and control (flox/+ Mx*Cre*) mice, all expressing the Ly5.2 cell surface marker, were transplanted along with equal numbers of competitor cells (Ly5.1) into lethally irradiated recipients (Fig 1A). As expected from previous work TβRI null and control BM cells reconstituted primary mice with a similar efficiency after 12 weeks (Fig 1B). We next administered two doses of 5-FU to half of the reconstituted mice with a five-day interval (Figure 1A). 5-FU kills a majority of cycling progenitors causing a recruitment of HSCs into proliferation[16]. Upon the second treatment, the fraction of HSCs that had been recruited into cell cycle would be killed. We hypothesized that TGF-β signaling deficient HSCs would be more prone to enter cell cycle following the first 5-FU treatment and that a larger fraction of the stem cell pool would be killed by the second treatment. This would then be read out as lower competitive repopulation ability when bone marrow from the 5-FU treated primary recipients was transplanted into secondary recipients (Fig 1A). However no significant difference in Ly5.2 contribution between TβRI null and control groups was observed when these mice were analyzed after 14 weeks (Figure 1C). Thus, TGF-β receptor-deficient HSCs were equally susceptible, compared to controls, to repeated treatments with 5-FU

suggesting that quiescence and the relative restriction to cell cycle entry is not dependent on TGF-β.

Since quiescence of HSCs is associated with maintenance of the stem cell pool we performed serial BM transplantations in order to ask whether the subsequent consumption of the stem cell pool would be altered in recipients of TGF-β signaling-deficient BM. BM from five donor mice of each genotype was transplanted into three lethally irradiated recipients for each donor. Mice were monitored for survival for two to three months and then sacrificed for subsequent BM transfers. A total of five consecutive BM transplantations were performed in order to provoke exhaustion of the stem cell pool. After the 4th serial transplantation 50% of the mice died within 4 weeks in both groups, while less than 20 % remained after the 5th transplant in both TβRI null and control groups (Fig 2). This clearly demonstrates a normal consumption of the HSC pool in recipients of TβRI null BM. Thus, even under extremely forced conditions *in vivo* we could not recapitulate the increased release from quiescence observed when TGF-β signaling is neutralized in primitive hematopoietic cells *in vitro*. Our results are also unexpected in light of the previously reported role for p21 in maintaining quiescence of HSCs and the direct connection between TGF-β signaling and p21 activity observed in several cell types[4,5]. However, recent work by Cheng and colleagues support our findings as TGF-β mediated inhibition of primitive progenitors *in vitro* could be executed independently of p21[17]. Similarly, Dao *et al* reported that neutralization of TGF-β in cooperation with reduced levels of p27, leads to increased cell-cycle entry supporting the view that p27 is not downstream of TGF-β[9]. Other signals such as interferon-γ and tumor necrosis factor-α (TNF-α) have been associated with negative regulation of HSCs and may therefore be responsible for p21 mediated quiescence in HSCs[18-21]. An alternative possibility is

that the specific epigenetic constitution of HSCs, rather than extrinsic signals, would determine their quiescent state. Nevertheless, as the use of HSCs in the clinic promises to expand and new cell therapy-based strategies are being developed, it will certainly be an important task to get a better understanding of the molecular biology of HSC quiescence. In summary, we have demonstrated, in contrast to prevailing views, that TGF-β signaling is dispensable for maintaining quiescence of HSCs and does not play an essential role to protect the stem cell pool from premature consumption during stressed conditions *in vivo*.

References

1. Cheshier SH, Morrison SJ, Liao X, Weissman IL. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci U S A. 1999;96:3120-3125

2. Hodgson GS, Bradley TR, Radley JM. The organization of hemopoietic tissue as inferred from the effects of 5-fluorouracil. Exp Hematol. 1982;10:26-35

3. Mauch P, Constine L, Greenberger J, Knospe W, Sullivan J, Liesveld JL, Deeg HJ. Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. Int J Radiat Oncol Biol Phys. 1995;31:1319-1339

4. Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, Scadden DT. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science. 2000;287:1804-1808

5. Massague J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. Cell. 2000;103:295-309

6. Keller JR, Mantel C, Sing GK, Ellingsworth LR, Ruscetti SK, Ruscetti FW. Transforming growth factor beta 1 selectively regulates early murine hematopoietic progenitors and inhibits the growth of IL-3-dependent myeloid leukemia cell lines. J Exp Med. 1988;168:737-750

7. Keller JR, McNiece IK, Sill KT, Ellingsworth LR, Quesenberry PJ, Sing GK, Ruscetti FW. Transforming growth factor beta directly regulates primitive murine hematopoietic cell proliferation. Blood. 1990;75:596-602

8. Hatzfeld J, Li ML, Brown EL, Sookdeo H, Levesque JP, O'Toole T, Gurney C, Clark SC, Hatzfeld A. Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor beta 1 or Rb oligonucleotides. J Exp Med. 1991;174:925-929

9. Dao MA, Taylor N, Nolta JA. Reduction in levels of the cyclin-dependent kinase inhibitor p27(kip-1) coupled with transforming growth factor beta neutralization induces cell-cycle entry and increases retroviral transduction of primitive human hematopoietic cells. Proc Natl Acad Sci U S A. 1998;95:13006-13011

10. Soma T, Yu JM, Dunbar CE. Maintenance of murine long-term repopulating stem cells in ex vivo culture is affected by modulation of transforming growth factor-beta but not macrophage inflammatory protein-1 alpha activities. Blood. 1996;87:4561-4567

11. Cashman JD, Eaves AC, Raines EW, Ross R, Eaves CJ. Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. I. Stimulatory role of a variety of mesenchymal cell activators and inhibitory role of TGF-beta. Blood. 1990;75:96-101

12. Larsson J, Blank U, Helgadottir H, Bjornsson JM, Ehinger M, Goumans MJ, Fan X, Leveen P, Karlsson S. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. Blood. 2003;102:3129-3135

13. Mombaerts P, Clarke AR, Rudnicki MA, Iacomini J, Itohara S, Lafaille JJ, Wang L, Ichikawa Y, Jaenisch R, Hooper ML, et al. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. Nature. 1992;360:225-231

14. Leveen P, Larsson J, Ehinger M, Cilio CM, Sundler M, Sjostrand LJ, Holmdahl R, Karlsson S. Induced disruption of the transforming growth factor beta

type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. Blood. 2002;100:560-568

15. Gorelik L, Flavell RA. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity. 2000;12:171-181

16. Harrison DE, Lerner CP. Most primitive hematopoietic stem cells are stimulated to cycle rapidly after treatment with 5-fluorouracil. Blood. 1991;78:1237-1240

17. Cheng T, Shen H, Rodrigues N, Stier S, Scadden DT. Transforming growth factor beta 1 mediates cell-cycle arrest of primitive hematopoietic cells independent of p21(Cip1/Waf1) or p27(Kip1). Blood. 2001;98:3643-3649

18. Jacobsen SE, Ruscetti FW, Dubois CM, Keller JR. Tumor necrosis factor alpha directly and indirectly regulates hematopoietic progenitor cell proliferation: role of colony-stimulating factor receptor modulation. J Exp Med. 1992;175:1759-1772

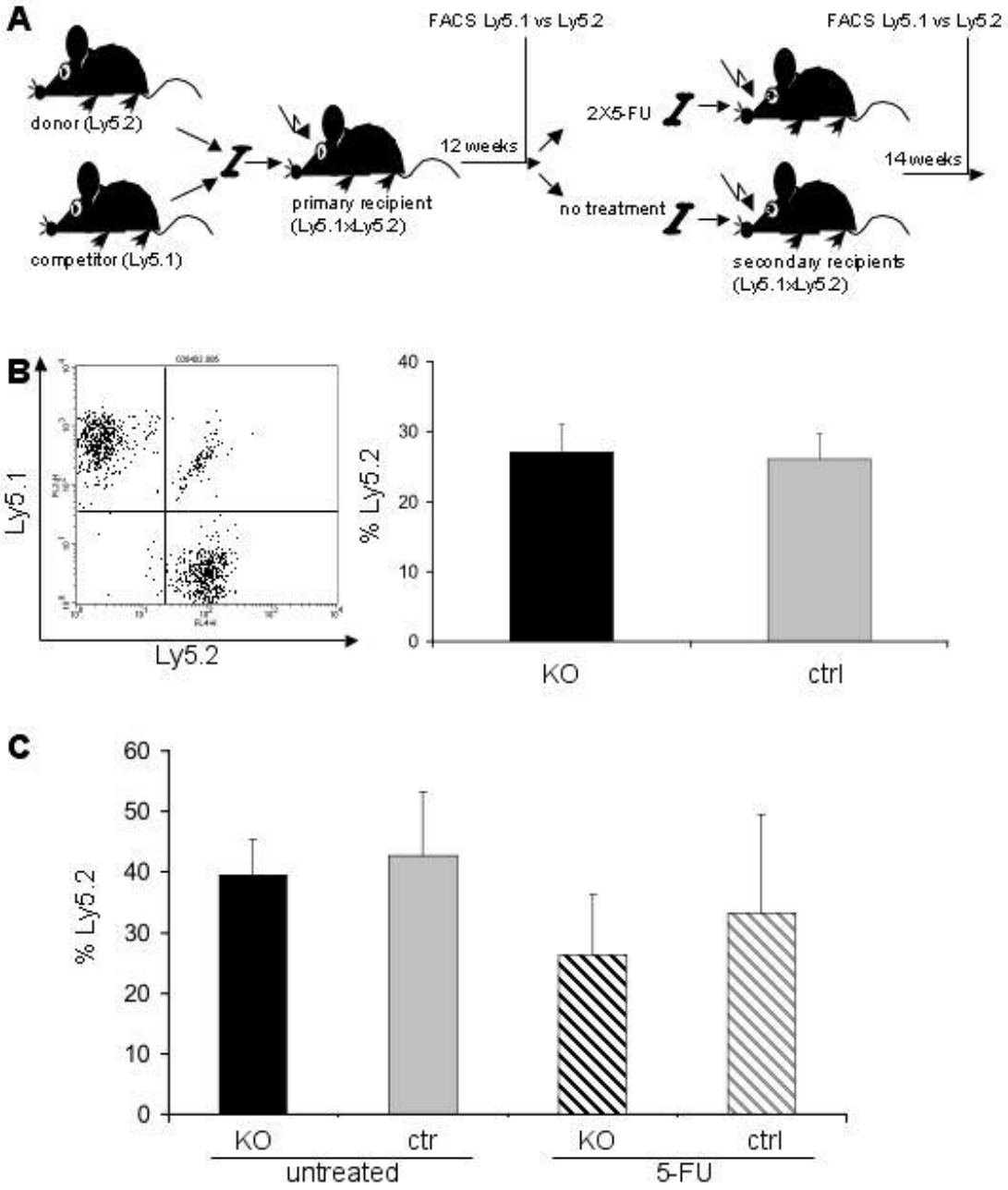
19. Maciejewski J, Selleri C, Anderson S, Young NS. Fas antigen expression on CD34+ human marrow cells is induced by interferon gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro. Blood. 1995;85:3183-3190

20. Zhang Y, Harada A, Bluethmann H, Wang JB, Nakao S, Mukaida N, Matsushima K. Tumor necrosis factor (TNF) is a physiologic regulator of hematopoietic progenitor cells: increase of early hematopoietic progenitor cells in TNF receptor p55-deficient mice in vivo and potent inhibition of progenitor cell proliferation by TNF alpha in vitro. Blood. 1995;86:2930-2937

21. Bryder D, Ramsfjell V, Dybedal I, Theilgaard-Monch K, Hogerkorp CM, Adolfsson J, Borge OJ, Jacobsen SE. Self-renewal of multipotent long-term repopulating hematopoietic stem cells is negatively regulated by Fas and tumor necrosis factor receptor activation. J Exp Med. 2001;194:941-952

Figure 1. Susceptibility to 5-fluorouracil is not altered in TGF-β receptor-deficient HSCs. (A) Experimental design for competitive repopulation experiments. BM cells from 7 donor mice (Ly5.2) of each genotype were transplanted individually along with equal numbers of competitor cells (Ly5.1) into a total of 42 lethally irradiated recipient mice (Ly5.1XLy5.2). After 12 weeks recipients were divided into two cohorts where one cohort was treated twice at 5 days interval with 5-FU and the other was left untreated. All recipients were subsequently sacrificed and their bone marrow transplanted into a total of 60 secondary recipient mice. (B) The frequencies of Ly5.2 donor-derived cells, Ly5.1 competitor-derived cells and Ly5.1XLy5.2 recipient-derived cells were determined by FACS on peripheral blood samples taken from primary recipients after 12 weeks. The percentage Ly5.2 cells out of total single positive Ly5.1 and Ly5.2 is shown. (C) Following 5-FU treatment and BM transplantation the percentage of Ly5.2 cells was determined in peripheral blood from secondary recipients after 14 weeks.

Figure 2. Normal consumption of TβRI null HSCs following serial BMT. BM from 5 donor mice of each genotype was transplanted into three recipients for each individual donor. At the consecutive BMTs, recipient BM from each individual donor group was pooled and transplanted into new groups of recipients with the same numbers of mice. The ratio between the number of surviving animals at each BMT and the total number of animals at the initial BMT is shown as % survival.


A
donor (Ly5.2)
competitor (Ly5.1)
primary recipient
(Ly5.1xLy5.2)
12 weeks
FACS Ly5.1 vs Ly5.2
2X5-FU
no treatment
14 weeks
FACS Ly5.1 vs Ly5.2
secondary recipients
(Ly5.1xLy5.2)
B
Ly5.1
Ly5.2
% Ly5.2
40
30
20
10
0
KO
ctrl
C
% Ly5.2
60
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40
30
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0
KO
ctr
KO
ctrl
untreated
5-FU

Figure 1

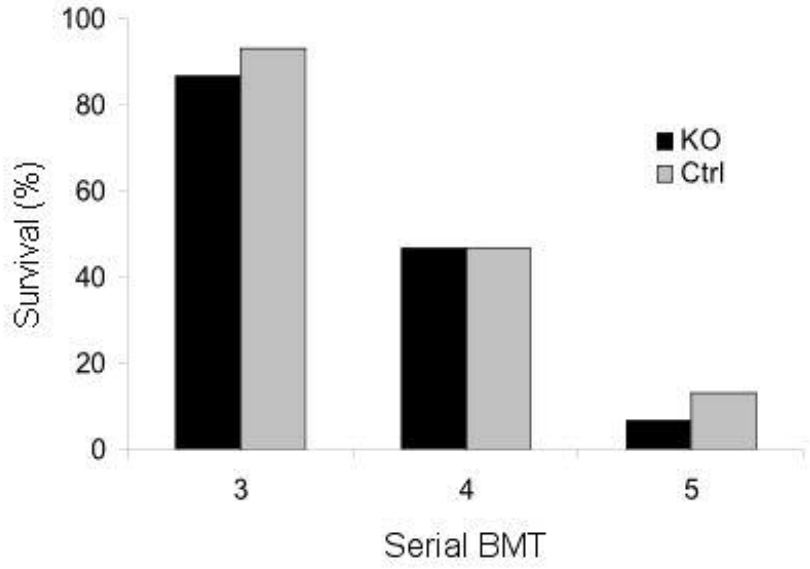

Figure 2