# c-kit$^{+}$ Stem Cells and Thymocyte Precursors in the Livers of Adult Mice

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## Summary

Livers of the adult mice contain c-kit$^{+}$ stem cells that can reconstitute thymocytes, multiple lineage cells, and bone marrow (BM) stem cells. Transfer of $1 \times 10^7$ hepatic mononuclear cells (MNC) and $5 \times 10^4$ hepatic c-kit$^{+}$ cells of BALB/c mice induced DP thymocytes within a week in four Gy-irradiated CB17/-SCID mice, but 2 wk were required for BM cells or BM c-kit$^{+}$ cells to produce DP thymocytes. Moreover, B cell–depleted BM cells or liver MNC of SCID mice that had been rescued by hepatic MNC of BALB/c mice again reconstituted thymus and B cells of other irradiated SCID mice. CD3$^{-}$ IL-2R$\beta^{-}$ populations of both BM cells and hepatic MNC of C57BL/6 (B6) mice could generate T cells with intermediate TCR (mostly NK1.1$^{-}$) in the liver of irradiated B6 SCID mice before thymic reconstitution (extrathymic T cells). Furthermore, transfer of liver c-kit$^{+}$ cells of B6-Ly 5.1 mice into irradiated B6 SCID (Ly5.2) mice revealed that liver c-kit$^{+}$ cells can reconstitute myeloid and erythroid lineage cells. These results strongly suggest that the liver contains pluripotent stem cells and serves an important hematopoietic organ even into adulthood.

BM cells contain c-kit$^{+}$ stem cells (1, 2) which can give rise to multiple leukocyte lineages. Murine T cell precursor from bone marrow (BM)[1] migrate to the thymus and differentiate into mature thymocytes, which are the origin of most peripheral T cells. Recently, however, it has been demonstrated that T cell can differentiate in extrathymic sites. Several groups of researchers proposed that the intestine is such a site (3–7), and we demosntrated that the liver is also a candidate (8–13). Extrathymically developed $\alpha\beta$ T cells in the liver are NK1.1$^{+}$ or NK1.1$^{-}$ T cells with intermediate TCR (13). However, it was not known whether the liver contains hematopoietic precursor cells. During an investigation as to how liver mononuclear cells (MNC) of normal mice transferred into SCID mice migrate and repopulate, we found that the liver MNC from normal adult mice could transiently reconstitute thymus accompanied by the appearance of double positive (DP) thymocytes in non-irradiated SCID mice. Since SCID mice can not rearrange either the TCR gene or the immunoglobulin gene (14), these mice are a proper model to examine whether or not a population of cells has a capacity to reconstruct T and B cells by radiation bone marrow chimera. In addition, B6-Ly5.1 mice (15) enabled us to determine whether or not myeloid and erythroid lineage cells are reconstituted by liver c-kit$^{+}$ cells. Here, we demonstrate that hepatic MNC of adult normal mice contain c-kit$^{+}$ cells which can reconstitute thymocytes, multiple lineage cells and BM stem cells of irradiated SCID mice.

[1]*Abbreviations used in this paper:* B6, C57BL/6; BM, bone marrow; DP, double positive; MNC, mononuclear cells.

## Materials and Methods

*Mice.* Male CB17/-SCID mice (H-2$^{d}$), BALB/c mice (H-2$^{d}$) and C57BL/6 mice (H-2$^{b}$), 6-8 weeks of age, were purchased from CLEA Japan Inc. (Tokyo, Japan). C57BL/6-SCID (H-2$^{b}$) were purchased from Central Institute for Experimental Animals (Kanagawa, Japan). C57BL/6-Ly5.1 (B6-Ly5.1, H-2$^{b}$) mice were kindly provided by Dr. K. Kishihara (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan). All mice were fed under the specific pathogen-free condition.

*Cell Preparations.* Mice were euthanized by exsanguination from the subclavian artery and vein, and liver and spleen were removed. The spleen was pressed on a 200-gauge stainless steel mesh and washed. The pellet was treated with RBC lysis solution (155 mM NH4Cl, 10 mM $KHCO_3$, 1 mM EDTA, 170 mM Tris, pH 7.3). Hepatic MNC were prepared as previously described (16, 17). Briefly, the liver was pressed through a stainless steel mesh and suspended in Eagle's MEM medium supplemented with 5 mM Hepes and 2% FCS. After one washing, the cells were resuspended in 30–35% Percoll solution containing 100 U/ml heparin and centrifuged at 2,000 rpm for 15 min at room temperature. An appropriate density of Percoll solution should be determined between 30 and 35% in each laboratory. The pellet was resuspended in RBC lysis solution, then washed twice with medium. BM cells were obtained by flushing femurs with medium.

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The cell suspensions were filtrated through a 200-gauge nylon mesh to remove debris. Thymocytes were obtained by forcing thymus through a 200-gauge steel mesh. Peripheral blood cells were used after lysing red blood cells.

*mAbs, Flowcytometric Analysis and Cell Sorting.* Anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD3 (145-2C11), anti-IL-2Rβ chain (TM-β1), anti-NK1.1 (PK136), anti-B220 (RA3-6B2), anti-Mac-1 (M1/70), anti-mouse IgM (R6-60.2), anti-Gr-1 (RA3-8C5), TER119 (erythroid lineage marker), and anti-c-kit (3C1) Abs were all purchased from PharMingen (San Diego, CA). Mouse anti-Ly5.1 Ab(A20. 1.7) was kindly provided by Dr. T. Kina (Chest Disease Research Institute, Kyoto University, Kyoto, Japan). All mAbs were used with FITC-, PE-, or biotin-conjugated form. Biotinylated reagents were developed with FITC or PE-conjugated streptavidin (Becton-Dickinson Co., Mountain View, CA) or TRI-COLOR-conjugated streptavidin (CALTAG Lab., San Francisco, CA). To prevent nonspecific binding of mAbs, CD32/16 (24G2) was added before staining with labeled mAbs. Cell suspensions ($10^5$ to $2 \times 10^6$) were stained with mAbs and analyzed by FACScan (Becton-Dickinson). Dead cells were excluded by forward scatter, side scatter, and PI gating. c-kit$^+$ Lin$^-$ (CD3$^-$, B220$^-$, Mac-1$^-$, Gr-1$^-$, and TER119$^-$) cells of hepatic MNC and BM cells were sorted by FACS® Vantage (Becton-Dickinson). In some cases, CD3$^+$ or B220$^+$, and/or IL-2Rβ$^+$ cells were also depleted from hepatic MNC or BM cells by sorting.

*Cell Transfer.* After 4 Gy irradiation (18), equal number ($1 \times 10^7$) of BM cells, hepatic MNC and splenocytes of BALB/c mice were injected intravenously into CB17/-SCID mice. In some experiments, $5 \times 10^4$ sorted c-kit$^+$ Lin$^-$ cells of hepatic MNC or of BM cells, sorted $2 \times 10^6$ CD3$^-$ B220$^-$ cells, or $2 \times 10^6$ c-kit$^-$ cells were injected into different mice groups. $2 \times 10^6$ sorted CD3$^-$IL-2Rβ$^-$ cells of liver or BM cells of C57BL/6 mice were also transferred into C57BL/6-SCID mice in another experiment. Further, $2 \times 10^5$ c-kit$^+$ Lin$^-$ cells of liver of B6-Ly5.1 mice were transferred into irradiated B6 SCID mice.

## Results

*Thymus Reconstruction in Irradiated SCID Mice.* Liver MNC could transiently reconstitute thymocytes of non-irradiated SCID mice (Fig. 1). Since it was reported that low-dose irradiation of SCID mice is required for effective BM reconstitution (18), 4 Gy irradiation CB17/-SCID mice were transferred with liver MNC or BM cells of BALB/c mice. The results demonstrated that liver MNC as well as BM cells could fully reconstitute thymocytes (Fig. 1, Table 1). In addition, hepatic MNC could reconstruct thymus more rapidly than BM cells. After transfer of hepatic MNC from normal mice, DP thymocytes emerged in the thymus of SCID mice within 1 wk, whereas 2 wk were required for BM cells to induce DP thymocytes (Fig. 1). 4 wk after liver or BM MNC transfer, T cells (CD3$^+$) and B cells (B220$^+$) were detected in the spleen. In the case of liver MNC transfer, IL-2Rβ$^+$ intermediate CD3$^+$ cells as well as IL2Rβ$^-$ bright CD3$^+$ cells appeared in the spleen, while BM cells transfer induced only a small population of IL-2Rβ$^+$ intermediate CD3$^+$ cells. These T and B cells may include cells expanded from T and B cells contained in transferred liver MNC or BM cells or normal mice as well as cells from their precursors. CB17/-SCID mice that received either BM cells or hepatic MNC of BALB/c mice were still alive 4 mo later, whereas mice that received splenocytes died within 2 wk.

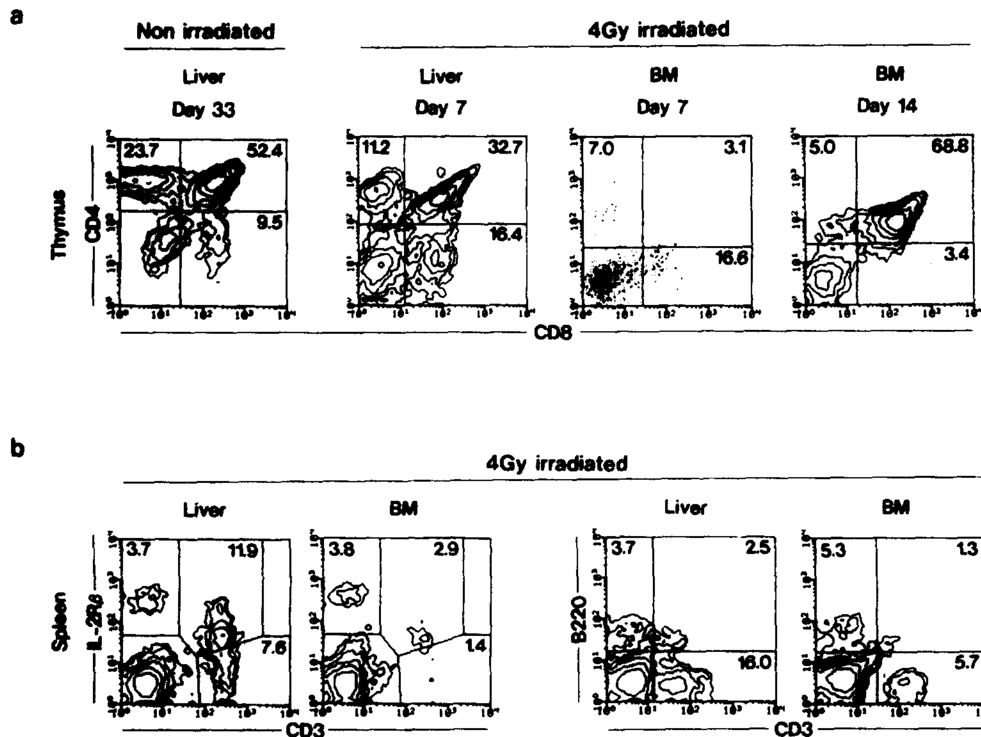


**Figure 1.** (*a*) Liver MNC induce DP thymocytes in non-irradiated and irradiated SCID mice. $10^7$ hepatic MNC of BALB/C mice were injected into non-irradiated SCID mice (*1st panel*). CD4 and CD8 expression of thymocytes of 4 Gy irradiated SCID mice 7 d after injection of $10^7$/hepatic MNC of BALB/C mice (*2nd panel*). CD4 and CD8 expression of thymocytes of irradiated SCID mice 7 and 14 d after injection of $10^7$ BM cells of BALB/C mice (*3rd and 4th panels*). (*b*) Transfer of liver and BM MNC of normal mice could induce T cells and B cells in the spleen. 4 wk after transfer, spleens were examined.

**Table 1.** *Number of Cells Obtained from Irradiated CB17/-SCID Mice Reconstituted with Liver MNC or BM Cells of BALB/c Mice*

| Mouse | Transferred cells | Days after transfer | Number of cells in organs | | |
|---|---|---|---|---|---|
| | | | Liver | Spleen | Thymus |
| | | | | $\times 10^6 \pm SD$ | |
| BALB/c | None | | 4.8 ± 0.8 | 157.9 ± 59.9 | 225.0 ± 61.3 |
| SCID | None | | 1.1 ± 0.2 | 10.0 ± 3.6 | 5.0 ± 1.6 |
| SCID (4Gy) | Liver MNC | 7 | 0.8 ± 0.1 | 21.7 ± 5.7 | 0.3 ± 0.0 |
| | | 14 | 2.1 ± 0.1 | 31.5 ± 4.5 | 33.5 ± 9.5 |
| | | 21 | 2.7 ± 0.4 | 45.0 ± 5.1 | 220.0 ± 45.0 |
| | | 82 | 10.4 ± 0.9 | 227.0 ± 55.3 | 121.0 ± 20.0 |
| | BM | 7 | 0.8 ± 0.2 | 15.8 ± 2.7 | 0.1 ± 0.0 |
| | | 14 | 1.2 ± 0.1 | 18.5 ± 3.9 | 8.8 ± 2.9 |
| | | 21 | 9.0 ± 1.0 | 17.4 ± 2.3 | 284.0 ± 38.2 |

$10^7$ liver MNC and BM cells of BALB/c mice were transferred into 4 Gy irradiated SCID mice, the cell numbers in livers, spleens and thymus were examined on indicated days after transfer. Data shown are mean ± SD of five individual mice.

*c-kit$^+$ Cells are Present Not Only in BM but Also in the Liver.* The fact that liver MNC can reconstitute thymus and extrathymic T cell development takes place in the liver (8–13) led us to search for precursors in the liver. It was found that a small proportion of c-kit$^+$ cells are also present in the liver of normal mice, which were mainly detected in a large blastic population (Fig. 2). These c-kit$^+$ cells in the liver are lineage marker negative (CD3$^-$, B220$^-$, Mac-1$^-$, Gr-1$^-$, and TER119$^-$) (Fig. 2). The population of hepatic c-kit$^+$ cells was unaffected by perfusion of the liver with PBS from portal vein, as was previously demonstrated in the case of other lymphoid cells in the liver (16) (not shown).

*Induction of DP Thymocytes by Hepatic c-kit$^+$ Cells and CD3$^-$B220$^-$ Fraction but Not c-kit$^-$ Cells.* Transfer of hepatic c-kit$^+$ cells and CD3$^-$B220$^-$ cells could induce production of DP thymocytes within 1 wk, while BM c-kit$^+$ cells took 2 wk to generate DP thymocytes (Fig. 3). It is noteworthy, however, that hepatic c-kit$^+$ cells alone could not effectively reconstitute thymocytes; hepatic c-kit$^-$ cells were also needed to efficiently reconstruct the thymus, especially at early period of reconstitution, as revealed by a greatly reduced thymus size when c-kit$^-$ cells were absent (Table 2). Interestingly, splenocytes could not fulfill the supporting role played by hepatic c-kit$^-$ cells (Table 2).

*Increase of c-kit$^+$ Cells in the Liver and BM of SCID Mice after Transfer of Liver MNC.* 2 wk after transfer of hepatic MNC into irradiated SCID mice, c-kit$^+$ cells markedly increased not only in the liver but also in BM, which suggest that c-kit$^+$ cells were actively proliferating in both sites (Fig. 4).

*Hepatic MNC Can Reconstitute BM Stem Cells.* To test whether or not hepatic MNC can reconstitute BM stem cells that are committed to T and B cells, and to confirm that B cells found in the periphery of SCID mice was not merely the result of expansion of B cells contained in hepatic MNC of normal mice, BM cells as well as liver MNC from SCID mice, which had been rescued with hepatic

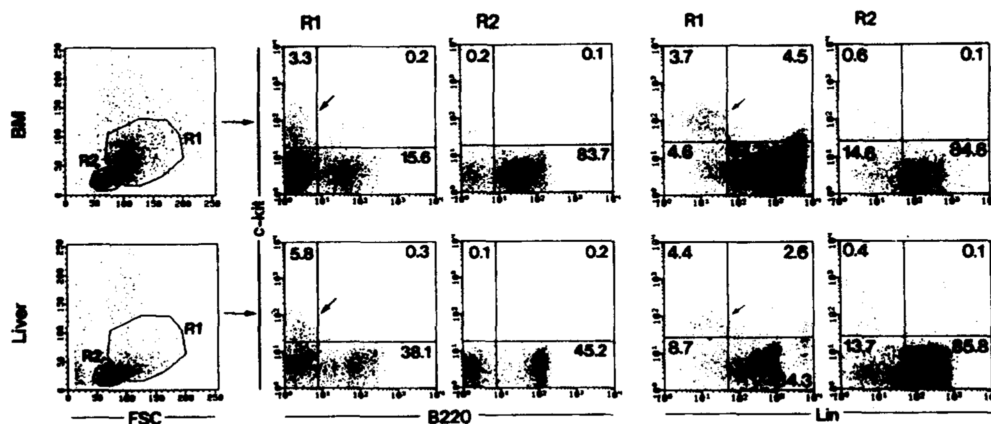


**Figure 2.** c-kit$^+$ cells are present in hepatic MNC as well as in BM cells of BALB/c mice. R1 gated a large blastic population and R2 gated a population of smaller cells. These c-kit$^+$ cells are Lin$^-$.

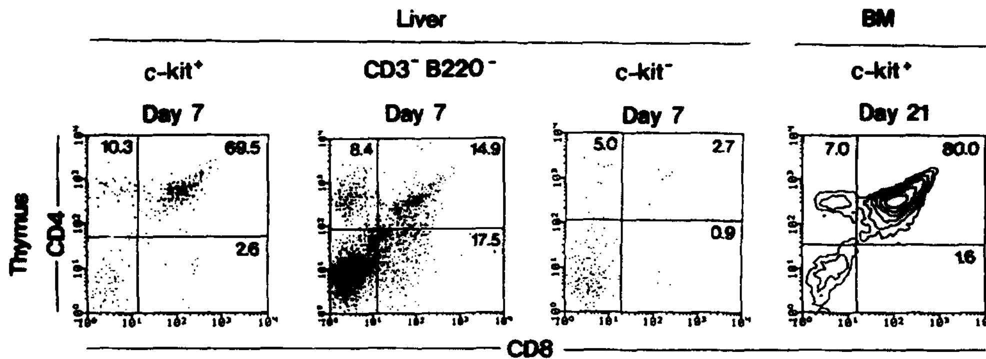


**Figure 3.** Transfer of c-kit$^+$ cells and the fraction of non-T and non-B cells of BALB/c mice, but not c-kit$^-$ cells, in the liver induced DP thymocytes within a week. Transfer of BM c-kit$^+$ cells of BALB/c mice requires 2 wk or more to induce DP thymocytes. 5 × $10^4$ sorted c-kit$^+$ Lin$^-$ cells, 2 × $10^6$ CD3$^-$B220$^-$ cells and 2 × $10^6$ c-kit$^-$ Lin$^-$ cells in the liver of BALB/c mice were transferred into 4Gy irradiated SCID mice (*1st to 3rd panels*) and CD4 and CD8 staining of thymocytes was examined at indicated day. 5 × $10^4$ BM c-kit$^+$ cells were also injected and thymocytes were examined on day 21 (*4th panel*).

MNC from normal mice 12 wk before, were further transferred into other irradiated SCID mice after depletion of B cells. The result showed that thymus was efficiently reconstituted and B cells (B220$^+$ as well as surface Ig M$^+$) could again emerge within 4 wk (Fig. 5).

*Hepatic c-kit$^+$ Cells Can Reconstitute Myeloid as Well as Erythroid Lineage Cells.* To examine the capability of hepatic c-kit$^+$ cells to produce multiple lineage cells, hepatic c-kit$^+$ cells of B6-Ly5.1 mice were transferred into irradiated B6 SCID (Ly5.2) mice. Donor derived cells (except mature erythrocytes) can be descriminated by anti-Ly5.1 Ab from host Ly5.2$^+$ cells. Within 4 wk after transfer, Ly5.1$^+$ myeloid as well as erythroid lineage cells appeared in BM or peripheral blood (Fig. 6). Significant populations of BM Gr-1$^+$ cells (granulocyte lineage) and TER-119$^+$ cells (erythroid lineage) appeared in the BM and peripheral blood. Ly5.1$^+$ Mac-1$^+$ Gr-1$^-$ cells (macrophage lineage) were also detected in BM by three-color flowcytometric analysis. These results strongly suggest that liver c-kit$^+$ cells are indeed pluripotent stem cells.

*Liver Intermediate TCR Cells Differentiate Extrathymically in the Liver.* Next, CD3$^-$IL-2R$\beta^-$ populations of hepatic MNC or BM cells of C57BL/6 mice were transferred into irradiated B6 SCID mice. 10 d after transfer, many CD3$^+$ IL-2R$\beta^+$ cells (mostly NK1$^-$) appeared in the liver. Here, the intensity of TCR is intermediate level, as compared to thymus derived IL-2R$\beta^-$ bright TCR cells; intermediate

**Table 2.** *Number of thymocytes in CB17/-SCID Mice Reconstituted with Liver c-kit$^+$ Cells of BALB/c Mice*

| Transferred cells | Days after transfer | Mean no. of thymocytes |
|---|---|---|
| | | × $10^5$ |
| Liver c-kit$^+$ | 7 | 1.4 |
| | 14 | 2.0 |
| | 21 | 9.7 |
| Liver c-kit$^-$ | 7 | 1.0 |
| | 10 | 1.9 |
| Liver c-kit$^+$ + c-kit$^-$ | 7 | 1.8 |
| | 14 | 14.5 |
| | 21 | 140.0 |
| Liver c-kit$^+$ + splenocytes | 7 | 2.0 |
| | 14 | 1.8 |
| | 21 | 5.2 |

5 × $10^4$ sorted hepatic c-kit$^+$ cells, and 5 × $10^4$ c-kit$^+$ cells with either 2 × $10^6$ hepatic c-kit$^-$ cells or 2 × $10^6$ splenocytes were transferred into irradiated SCID mice. The number of liver MNC, splenocytes and thymocytes were counted on indicated days after transfer. SCID mice reconstituted by liver c-kit$^-$ cells died within 2 wk. Repeated experiments showed similar results.

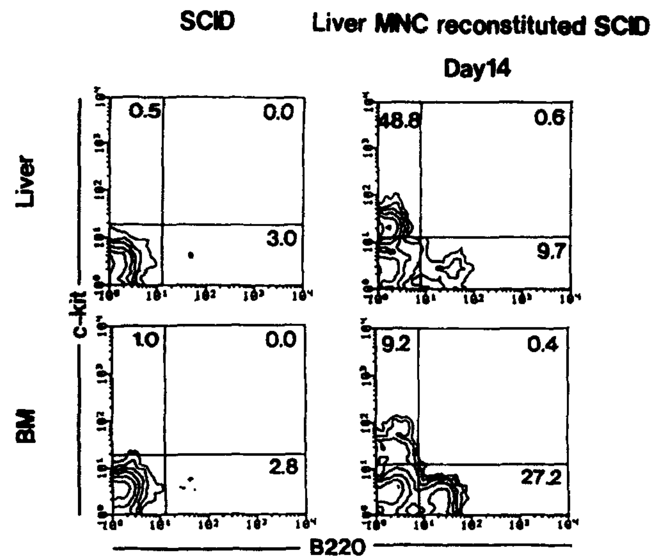


**Figure 4.** Appearance of a large number of c-kit$^+$ cells in the liver and BM of irradiated SCID mice after transfer of hepatic MNC. 2 wk after transfer of 1 × $10^7$ of total hepatic MNC into irradaited SCID mice, BM and liver MNC were examined.

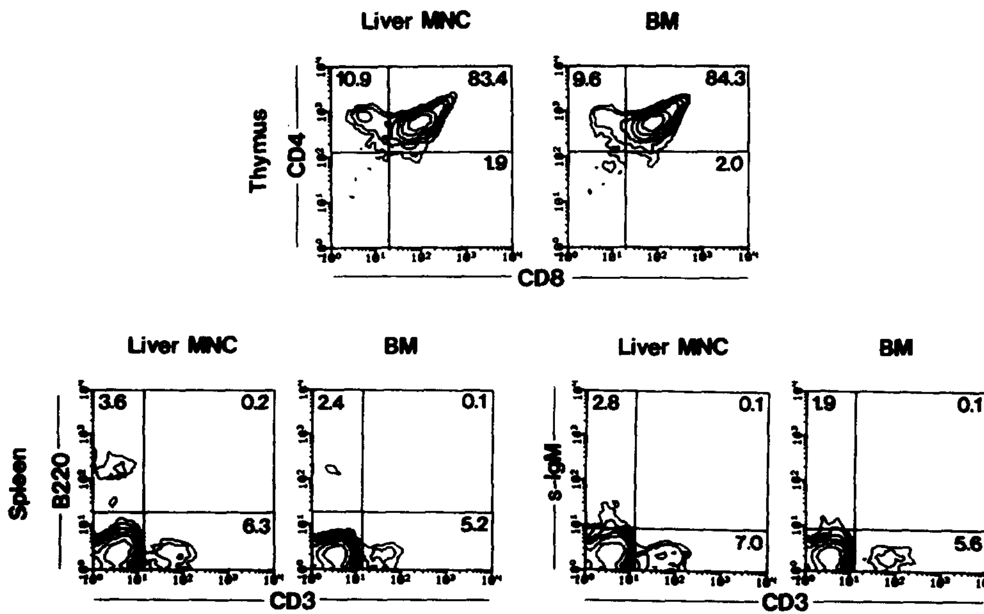


**Figure 5.** Induction of DP thymocytes and B cells in the irradiated SCID mice by hepatic MNC or BM cells of SCID mice, which had been rescued by hepatic MNC. B cell depleted $10^7$ hepatic MNC or BM cells of SCID mice that had been irradiated and rescued by hepatic MNC of BALB/c mice were further transferred into other irradiated SCID mice. 2 wk after transfer, thymocytes were examined for their CD4 and CD8 expression (*1st and 2nd panels*), and splenocytes were examined for their CD3 and B220 or surface IgM expression 4 wk after transfer.

TCR is characteristic of extrathymic T cells, as shown previously (11–13) and demonstrated here (Fig. 7). Namely, although control liver T cells consist of IL-2R$\beta^+$ intermediate TCR cells and IL-2R$\beta^-$ bright TCR cells, virtually all liver T cells of SCID mice reconstituted with CD3$^-$IL-2R$\beta^-$ liver or BM cells were intermediate TCR cells (mainly IL-2R$\beta^+$) (Fig. 7). The number of these T cells in the liver reconstituted by hepatic CD3$^-$IL-2R$\beta^-$ cells of normal mice was much greater than that induced by BM CD3$^-$IL-2R$\beta^-$ cells (Fig. 7), while a larger population of NK cells were induced by the transfer of BM CD3$^-$IL-2R$\beta^-$ cells than the transfer of hepatic CD3$^-$IL-2R$\beta^-$ cells (Fig. 7). A smaller population of intermediate TCR cells (but not bright TCR cells) was also found in the spleen at an early stage of reconstruction, but after thymic reconstitution (beyond 2 wk after liver or BM MNC transfer) accompanied by the appearance of single positive T cells with bright TCR, bright TCR cells gradually increased in the spleen and periphery as well as in the liver (data not shown).

## Discussion

It is well known that the fetal liver is a major hematopoietic organ (19, 20), whereas the hematopoietic function of the liver seems to be abrogated at later stage of the fetus in humans and after birth in mice. In this report, however, we demonstrate that the adult mouse liver MNC contain not only c-kit$^+$ thymocyte precursors but also c-kit$^+$ stem cells that can reconstitute multiple lineage cells in SCID mice.

The thymus of irradiated SCID mice could be fully reconstructed by the transfer of hepatic MNC or BM cells. In addition, hepatic MNC or c-kit$^+$ cells could reconstruct thymus more rapidly than BM cells or BM c-kit$^+$ cells. BM cells as well as liver MNC from SCID mice which had been rescued with hepatic MNC from normal mice could further reconstitute thymus and B cells of other irradiated SCID mice, suggesting that liver MNC can reconstitute BM stem cells. Moreover, when liver c-kit$^+$ cells of B6-Ly5.1 mice were transferred into irradiated B6 SCID mice, Ly5.1$^+$ myeloid and erythroid lineage cells were detected in peripheral blood or BM. Further, transfer of CD3$^-$IL-2R$\beta^-$

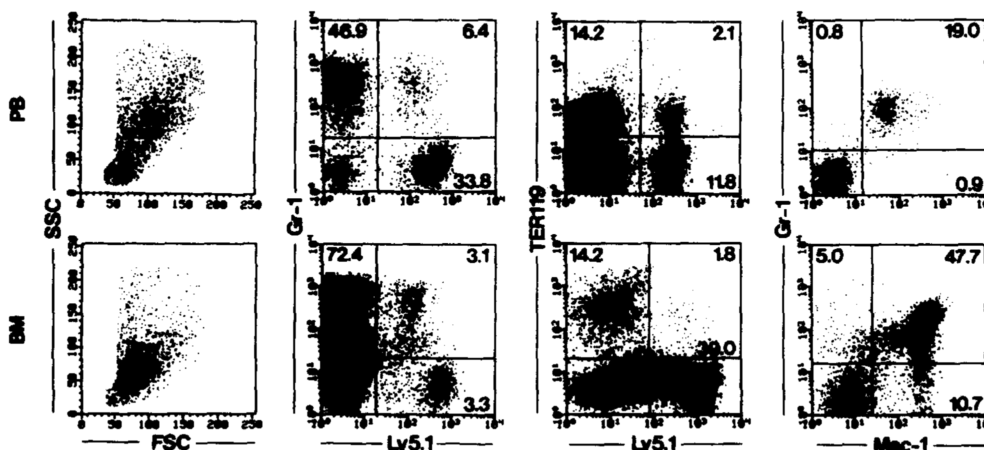


**Figure 6.** Liver c-kit$^+$ cells of B6-Ly5.1 mice could induce myeloid as well as erythroid lineage cells. 4 wk after transfer of 2 × $10^5$ c-kit$^+$ cells of B6-Ly5.1 mice into irradiated B6 SCID mice (*Ly5.2*), peripheral blood cells (*PB*) and BM were stained by anti-Ly5.1, Gr-1, TER119, and Mac-1 mAbs. For Mac-1 and Gr-1 staining, three-color flowcytometric analysis of cells was carried out after staining with anti-Ly5.1, Mac-1 and Gr-1 mAbs.

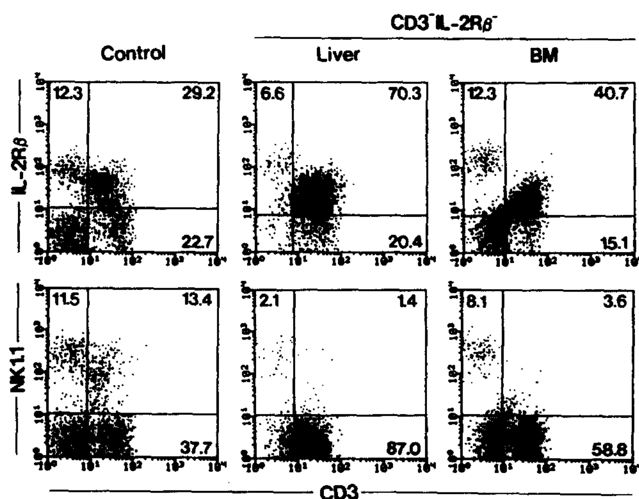


**Figure 7.** Induction of T cells with intermediate TCR and NK cells in the liver of B6 SCID mice from $CD3^-IL\text{-}2R\beta^-$ cells of hepatic MNC or BM cells of B6 mice. $1 \times 10^6$ sorted $CD3^-IL\text{-}2R\beta^-$ cells of hepatic MNC or BM cells of C57BL/6 mice were transferred into irradiated B6 SCID mice. 10 d after transfer, phenotype of liver MNC were examined. Control data is from a normal B6 mouse.

populations of hepatic MNC or BM cells of C57BL/6 mice into irradiated B6 SCID mice resulted in the appearance of many $CD3^+IL\text{-}2R\beta^+$ cells with intermediate TCR in the liver before thymic reconstitution, confirming that liver is the site where extrathymic T cell differentiation occurs.

The proto-oncogene, c-kit, encodes a transmembrane receptor of the tyrosine kinase gene family (21) and is reported to be an essential molecule for constitutive hematopoiesis in BM (22, 23), and pluripotent stem cells in BM are surface c-kit$^+$ (1, 2). This study strongly suggests that these c-kit$^+$ pluripotent stem cells are also present in the liver of adult mice. In addition, the fact that the hepatic c-kit$^+$ cells can generate DP thymocytes faster than BM c-kit$^+$ cells and that hepatic $CD3^-IL\text{-}2R\beta^-$ MNC could more efficiently produce hepatic $CD3^+$ cells with intermediate TCR than BM $CD3^-IL\text{-}2R\beta^-$ cells indicates that the hepatic c-kit$^+$ cells contain more differentiated T cell progenitors of both intrathymic and extrathymic T cell lineages. Further, hepatic MNC transfer could increase c-kit$^+$ cells in BM. These findings suggest that hepatic MNC contain stem cell populations at heterogeneous stages of differentiation. It was reported that c-kit$^+$ cells in the thymus also can reconstitute thymocytes faster than BM c-kit$^+$ cells, although they were not already pluripotential (24–26). It is possible that a population of liver c-kit$^+$ cells preferentially migrate into thymus, while another population mainly migrates into BM to reconstitute BM c-kit$^+$ cells. A detailed comparison of surface markers of c-kit$^+$ cells in the liver, BM and thymus is now underway.

Another interesting point is that hepatic c-kit$^-$ cells are needed to efficiently reconstruct thymus, because hepatic c-kit$^+$ cells alone could only reconstruct a thymus of much smaller size. However, the fact that this supporting effect of c-kit$^-$ cells of the liver could not be assumed by splenocytes indicates that effective thymic reconstruction, especially in early period of reconstitution, depends upon the interaction of thymic epithelial cells with certain cells in the liver different from splenocytes. It is also suggested that donor liver derived c-kit$^+$ cells in host BM do not need liver c-kit$^-$ cells any more, because whole BM cells of SCID mice rescued by liver MNC could effectively reconstitute thymus of other irradiated SCID mice.

The present results raise the possibility that these stem cells may originate in bone marrow and migrate to the liver after birth. Another possibility is that a small number of stem cells in fetal liver remain in the liver even after birth. At present, it can not be decided which is the case. According to a recent report (27) some hepatic MNC which firmly interact with hepatocytes have been detected in Disse's space and hepatocyte damage markedly decreased liver T cells. Considering the fact that the perfusion of the liver do not change the population of c-kit$^+$ cells in the liver, the possibility is raised that the maturation and differentiation of stem cells or precursor cells in the liver could occur in such parenchymal spaces rather than in the liver sinusoids.

Finally, the present results may offer a new insight into the host tolerance after liver transplantation (28–31). Stem cells in the liver of the donor may provide leukocytes to the host and create a donor host chimera that may contribute for decreasing the rate of rejection of other organ transplantations in the host.

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