Interactions Between c-kit and Stem Cell Factor Are Not Required for B-Cell Development In Vivo

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The receptor-type tyrosine kinase, c-kit, is expressed in hematopoietic stem cells (HSC), myeloid, and lymphoid precursors. In c-kit ligand-deficient mice, absolute numbers of HSC are mildly reduced suggesting that c-kit is not essential for HSC development. However, c-kit-/- HSC cannot form spleen colonies or reconstitute hematopoietic functions in lethally irradiated recipient mice. Based on in vitro experiments, a critical role of c-kit in B-cell development was suggested. Here we have investigated the B-cell development of c-kit-null (W/W) mice in vivo. Furthermore, day 13 fetal liver cells from wild type or W/W mice were transferred into immunodeficient RAG-2/- mice. Surprisingly, transferred c-kit-/- cells gave rise to all stages of immature B cells in the bone marrow and subsequently to mature conventional B2, as well as B1, type B cells in the recipients to the same extent as transferred wild type cells. Hence, in contrast to important roles of c-kit in the expansion of HSC and the generation of erythroid and myeloid lineages and T-cell precursors, c-kit-/- HSC can colonize the recipient bone marrow and differentiate into B cells in the absence of c-kit.

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was identified as designated as day 0 to isolate fetal liver cells. Homozygous mutant mice were identified by lack of c-kit expression on fetal liver cells by flow cytometry or by the pale appearance of skin of newborn mice. All animal experiments were done complying with standard guidelines of the Basel City.

Monoclonal antibodies (MoAbs). The following primary antibodies were used in this study: phycoerythrin (PE)-coupled and biotinylated ACK-4 (anti-c-kit); Pharmingen, San Diego, CA), fluorescein isothiocyanate (FITC)-coupled 145-2C11 (anti-CD3ε; Pharmingen, Mountain View, CA), Red613-coupled 53-6.7 (anti-CD8α; GIBCO BRL, Gaithersburg, MD), PE-coupled R3A-6B2 (anti-CD45R [B220]ε; Caltag), APC-coupled RA3-6B2 (Pharmingen), biotinylated LO-MM (anti-IgM; Caltag), PE-coupled anti-CD43ε (Pharmingen), biotinylated 6C3 (anti-BP-1ε; Pharmingen), PE-coupled anti-HSA (M1/69ε; Pharmingen), PE-conjugated 53-7.3 (anti-Ly-1 [CD5]ε; Pharmingen), FITC-conjugated 104-2.1 (anti-Ly-5.1ε) and FITC-labeled and biotinylated A20-1.7 (anti-Ly-5.2ε) (both hybridomas kindly provided by Dr S. Kimura, Sloan-Kettering Cancer Center, New York, NY). Second step reagents were streptavidin-PE, streptavidin-FITC (both from Southern Biotechnology, Birmingham, AL) and streptavidin-APC (Molecular Probes, Eugene, OR).

Transfer of fetal liver cells or bone marrow cells into RAG-2ε−/− mice. Pregnant females were killed at indicated times of gestation. All subsequent handling was done under sterile conditions. The uterine horns were removed and dissected in phosphate-buffered saline (PBS). Each fetus was separated from uterine decidua and extra embryonic membranes and transferred to a 100 × 15-mm petri dish filled with PBS. Each fetal liver was removed and placed in 1 mL of PBS/5% fetal calf serum (FCS). A cell suspension was prepared by repeated pipetting of the liver through a 23-gauge needle attached to a 1-mL pipette. The nucleated cells were separated by centrifugation at 50g for 10 minutes and filtered through a Nitex filter with a 41-μm pore size (Nitex HC3-41; Tekto, Elmsford, NY.). The absence of c-kit expression in W/W fetal liver was verified by fluorescence-activated cell sorter (FACS) analyses. The final viable cell count (usually greater than 80%) was performed in PBS/5% FCS at a concentration of 8 × 10^6/0.3 mL. Subsequently, 8 × 10^6 fetal liver cells (Ly5.1ε) were injected intravenously into 4Gy irradiated RAG-2ε−/− (Ly5.2ε) mice. The irradiation was done by GammaCell 40 (Atomic Energy of Canada Limited, Ontario, Canada).

Collection of blood and tissues. Peripheral blood was obtained by incision of the tail tip with a scalpel. Blood was collected into 1.5 mL endopore tubes containing 10 μL heparin (Roche, Basel, Switzerland). Nucleated cells were purified using Percoll (Pharmacia, Sweden) following the recommendations of the manufacturer. Bone marrow cells were obtained by flushing femurs and tibias with PBS/5% FCS using a 1-mL disposable syringe; 27- and 22-gauge needles were used for 5-day old and adult mice, respectively. Thymus and spleen were removed using micro scissors and placed in petri dishes with PBS. A cell suspension were made by grinding tissues between frosted ends of microscope slides. To isolate peritoneal cells, the peritoneal cavity was flushed 5 times with 1 mL of PBS/5% FCS.

Immunofluorescence staining and analysis. For phenotypic analysis, single cell suspensions from fetal liver, bone marrow, peritoneal, peripheral blood, splenocytes, or thymocytes were prepared in PBS + 2% FCS. Splenocytes and bone marrow cells were treated with Tris-buffered ammonium chloride at room temperature for 10 minutes to remove red blood cells for staining. A total of 10^6 to 10^8 cells were stained with MoAbs as indicated in the figure legends. Flow cytometric analysis was performed on a FACScan (Becton Dickinson) after gating on viable cells. Fluorescence data are displayed as logarithmic contour or dot plots or logarithmic histograms using LYSIS software (Becton Dickinson).

RESULTS

B lymphocyte development in W/W mice. Early stages of B-cell development were analyzed in FL (fetal day 16 and 18, Fig 1A), bone marrow and spleen (day 5 after birth, Fig 1B) comparing wildtype and W/W mice. For the dissection of distinct stages of B-cell differentiation, cell surface expression of B220 (CD45R), CD43, BP-1, heat stable antigen (HSA), and IgM was analyzed according to Hardy et al.37-39. The majority of precursors in the A fraction (B220ε CD43ε BP-1ε HSAε) are not apparently B-cell precursors: these cells do not express c-kit, although both HSC and more differentiated precursors in the B and C fractions express c-kit.22,40 Proliferation of B220ε CD43ε early B-cell precursors in fractions B and C can be stimulated by c-kit ligand in vitro. The expression of cytoplasmic μ-chain in early B-cell precursors inhibits c-kit expression and drives their differentiation into B220ε CD43ε IgM− late precursors (fraction D).22,41,42

The comparison of fetal livers, bone marrows and spleens from W/W and wildtype mice showed, as expected, severely reduced total cellularity in all examined organs (Fig 1A and B). However, flow cytometric analysis showed that all stages of B-cell development were phenotypically indistinguishable between W/W and wildtype cells: All stages of B-cell development were analyzed in FL (fetal day 16 and 18, Fig 1A), bone marrow and spleen (day 5 after birth, Fig 1B) comparing wildtype and W/W mice. For the dissection of distinct stages of B-cell differentiation, cell surface expression of B220ε CD45Rε CD43ε BP-1ε, heat stable antigen (HSAε), and IgMε was analyzed according to Hardy et al.37-39. The majority of precursors in the A fraction (B220ε CD43ε BP-1ε HSAε) are not apparently B-cell precursors: these cells do not express c-kit, although both HSC and more differentiated precursors in the B and C fractions express c-kit.22,40 Proliferation of B220ε CD43ε early B-cell precursors in fractions B and C can be stimulated by c-kit ligand in vitro. The expression of cytoplasmic μ-chain in early B-cell precursors inhibits c-kit expression and drives their differentiation into B220ε CD43ε IgM− late precursors (fraction D).22,41,42
Fig 1. Phenotypic analysis of B-cell compartments in FL and day 5 bone marrow and spleen from wildtype and W/W mutant mice. (A) Analysis of B lineage subpopulations present in day 16 and day 18 fetal liver (FL) of wildtype and W/W mutant mice. Total cell numbers are indicated on top of each column. B lineage cells were analyzed by two-color flow cytometry for expression of B220 versus IgM (top row) and by four-color flow cytometry for expression of B220 versus CD43 versus BP-1 versus HSA. Data are presented as expression of B220 versus CD43 of the whole population of nucleated cells (middle row) and BP-1 versus HSA gated on B220^-CD43^ cells (bottom row). Numbers given in the individual quadrants indicate percentages. (B) Analysis of B lineage subpopulations present in bone marrow (BM) and spleen (SP) of day 5 wildtype and W/W mutant mice. The data are presented in the same way as for (A).
Fig 2. Analysis of the contribution of transferred wildtype or W/W FL cells to B-cell and myeloid development in the bone marrow of reconstituted RAG-2^-/- mice. A total of 8 x 10^5 FL cells from day 13 wildtype or W/W mice were injected intravenously into 4Gy irradiated RAG-2^-/- mice, and reconstituted mice were analyzed 3 weeks post-transfer. Bone marrow cells from Ly5.2^- RAG-2^-/- mice reconstituted with Ly5.1^- wildtype FL cells (A through F) or with Ly5.1^- W/W FL cells (G through L) were analyzed by three-color flow cytometry for the expression of Ly5.2, CD43, and B220. Total cell counts are shown on top of (A) and (G). Bone marrow cells are gated, according to forward/side scatter analysis, into nonlymphoid (R1) and lymphoid (R2) gates. Cells in the R1 and R2 gates are further analyzed for the expression of B220 versus CD43 (B, C, H, and I). Percentage of donor-type cells (Ly5.2^-) are determined among the nonlymphoid (D and J), B220^-CD43^- early B-cell precursor (E and K) and B220^-CD43^- late B-cell precursor (F and L) populations. Numbers given on the gates indicate percentages.

In the recipient reconstituted with W/W FL cells, donor-type cells accounted for 23.1% of the early B precursors in the bone marrow (Fig 2K), indicating that early B precursors from c-kit^-/- mice are indeed capable of competing with a larger number of wildtype precursors. In contrast to the observed B-cell development, the reconstitution of nonlymphoid precursors (gate R3 in Fig 2H) by W/W FL cells was poor and accounted for only 1.2% of the CD43^-B220^- population (Fig 2J). In contrast, injected wildtype FL cells gave rise to 15.6% of the CD43^-B220^- population (Fig 2D). These observations are in agreement with previous studies demonstrating that W/W bone marrow cells can neither make sizable spleen colonies nor reconstitute hematopoiesis in lethally irradiated recipients (for review see Russell15).

Reconstitution of peripheral B-cell compartments. In our preceding experiments, c-kit^-/-B220^- cells, which contain HSC,13,45,46 were purified from adult bone marrow and injected into RAG-2^-/- mice. The injected cells were found to give rise to surface IgM^- cells in the recipient bone marrow
Failure of W/W FL cells to reconstitute donor-type thymocytes in RAG-2−/− recipients. In previous experiments, intrathymically expressed c-kit ligand was shown to drive the expansion of c-kit− intrathymic precursors. To analyze whether donor-type thymocytes can be generated from c-kit−/− deficient HSC, thymopoiesis was analyzed in RAG-2−/− recipients injected with either wildtype or W/W FL (Fig 4). RAG-2−/− recipients were analyzed at 3 weeks posttransfer. Transferred wildtype FL cells yielded $5.5 \times 10^6$ donor-type (Ly5.2−) thymocytes (Fig 4d), which contained >90% CD4−CD8− double-positive thymocytes, and at this time point, very few mature single-positive thymocytes (Fig 4e). In contrast, no donor-type (Ly5.2+) thymocytes were detectable in the recipient mice that had received W/W FL cells (Fig 4b and c). This finding is in marked contrast with the observed normal B-cell development following transfer of W/W FL.

DISCUSSION

Interactions between c-kit and SCF are known to play important roles in the generation of various hematopoietic
Fig 4. W/W FL cells are not capable of reconstituting thymocytes in RAG-2−/− recipient mice. Thymocytes from Ly5.2−/− RAG-2−/− mice (first row), Ly5.2−/− RAG-2−/− mice reconstituted with day 13 Ly5.1−/− W/ W FL cells (second row) and Ly5.2−/− RAG-2−/− mice reconstituted with day 13 Ly5.1−/− W/W FL cells (third row) were analyzed by three-color flow cytometry for the presence of Ly5.2, CD4, and CD8. Total cell counts are shown at the left of each panel. Two parameters are shown at a time: Ly5.2 versus CD4 (left column) and CD4 versus CD8 (right column). The contour plots of CD4 versus C8 of the reconstituted mice (c and e) are gated on donor-derived (Ly5.2−/−) cells only. Numbers given in quadrants and gates indicate percentages.

lineages. Based on the findings that (1) W/W-derived HSC fail to compete against wildtype HSC following in vivo cell transfers, and (2) most colony-forming units-spleen (CFU-S) are eliminated following administration of antagonistic anti-c-kit antibody, a critical role for SCF/c-kit in the proliferation of HSC had been demonstrated (Fig 5A).12,13,45,46 Also, very rapid and complete elimination of erythroid and myeloid precursors by anti-c-kit antibody indicates that the expansion of committed hematopoietic precursors in steady-state hematopoiesis is strongly dependent on c-kit.10 (Fig 5B and C; for review see Galli et al10 and Russell10). In T-cell development, SCF–c-kit interactions were previously shown to drive early thymocyte proliferation at early intrathymic stages preceding T-cell receptor (TCR) V(D)Jβ chain rearrangements (Fig 5F).44 In addition, it is possible that c-kit plays a role in the generation of thymic precursors (Fig 5D) and/or the thymus colonization (Fig 5E).

Here we show that B-cell development can occur normally in the absence of c-kit from the following two experiments: (1) Analysis of fetal and early postnatal W/W and wildtype littermates, and (2) reconstitution of immature and mature B lineage cells in RAG-2−/− c-kit−/− recipients by W/W or wildtype FL cells.

The comparison of various stages of B-cell development in W/W and wildtype littermates showed severely reduced hematopoietic cell numbers, but no distortion of B lymphopoiesis. While this analysis shows that B cells can be generated from c-kit−deficient precursors, it does not exclude the possibility that c-kit− B-cell precursors are inferior compared with c-kit− B-cell precursors in a competitive repopulation assay. To address this point, we compared B lymphocyte development following transfer of 8 × 10⁴ day 13 FL derived from W/W or wildtype mice into 4Gy irradiated RAG-2−/− mice. D₁J₁ joints at the IgH locus are hardly detectable in day 13 fetal liver,11 and B220+ B-cell precursors do not appear until day 15/16 of gestation.39 Thus, day 13 fetal liver is expected to contain few, if any, committed B-cell precursors. Since the content of HSC in day 13 fetal liver from SCF null-mutant mice is very similar to that from wildtype mice,14 W/W and wildtype day 13 fetal livers are expected to contain similar percentages of HSC. Upon transfer of W/W or wildtype day 13 FL cells, we could thus follow the initial reconstitution of B-cell development from similar numbers of injected HSC. In addition, the transfer experiment was expected to magnify a defect in the expansion of W/W lymphohematopoietic precursors, because transferred c-kit− precursors need to compete with larger numbers of endogenous c-kit+ precursors.

The data show that comparable reconstitution of both B-1 and B-2 lineages was observed following transfer of W/W and wildtype day 13 FL. In both groups of recipients, similar numbers of donor-type early B220+CD43+ precursors existed 3 weeks posttransfer. Hardy et al39 showed that wildtype B220+CD43+ proB cells injected into severe combined immunodeficient (SCID) mice underwent B-cell development transiently and, at 3 weeks posttransfer, virtually all donor-type cells had already left the recipient bone marrow for the periphery.39 Hence, the presence of donor-type CD43+ B220+ early B precursors as late as 3 weeks posttrans-
fer indicates that injected HSC, rather than committed B-cell precursors, were mostly responsible for B-cell development in both groups of recipients. Thus, injected c-kit positive HSC may have colonized the recipient bone marrow and differentiate into B cells with similar efficiency as injected wildtype HSC (Fig 5G). This conclusion is in agreement with the previous observation showing that injected W/WV bone marrow cells generated the same numbers of spleen colonies, but with much reduced sizes in lethally-irradiated recipient mice, when compared with injected wildtype bone marrow cells.19

A previous study suggested that W/WV bone marrow cells reconstituted B220⁺ B-cell precursors in lethally irradiated wildtype littermates at a sevenfold reduced level compared with bone marrow cells from wildtype mice.23 By contrast, the same study showed that W/VWV day 16 FL cells gave rise to B-cell precursors at an only slightly reduced level compared with day 16 FL cells from wildtype mice, and the present study shows no difference in the reconstitution by day 13 W/W and wildtype FL cells. These different results (reconstitution by bone marrow versus fetal liver) could be explained by the differences in the content of HSC; the content of HSC in adult W/WV bone marrow is much lower than that in wildtype bone marrow,25 while day 13 W/W and wildtype FL may contain HSC at comparable frequencies.14 Alternatively, since injected FL cells, but not adult bone marrow cells, can give rise to B1 cells,39 HSC of FL might be different from HSC of adult bone marrow with respect to the requirement of c-kit-mediated signals.

Since flk2(flt3), another receptor tyrosine kinase, exhibits strong similarities in both sequence and expression pattern to c-kit, the two molecules may play similar and overlapping roles in lymphohematopoiesis.53,54 While flk2 null mutant (flk2⁻/⁻) and W/WV mice showed a slight decrease in the overall cellularity of bone marrow, W/WV/flk2⁻/⁻ double-mutant mice exhibited a very significant reduction.18 This finding demonstrates functional redundancy between c-kit and flk2 in hematopoiesis. Given that W/WV mice exhibit much more severe anemia than flk2⁻/⁻ mice, c-kit plays a more important role than flk2 in the self-renewal of HSC (Fig 5A) and the expansion of early myeloid precursors (Fig 5B and C). On the other hand, flk2⁻/⁻ mice showed specific deficiencies in B-cell development, which were amplified following transfer of Flk2⁻/⁻ bone marrow cells into wildtype mice.18 Taking normal B-cell development from transferred W/W HSC into account, flk2 may play a more important role than c-kit in B-cell development (Fig 5G).

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