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A Novel Stromal Cell-Dependent B Lymphoid Stem-Like Cell Line That Induces Immunoglobulin Gene Rearrangement[1]

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A stroma-dependent B lymphoid cell line (B31-1) has been established by coculturing sorted stem cells on a novel bone marrow stromal cell line (TBR31-1). B31-1 cells express B220, but do not express other B lymphoid differentiation markers including CD43, heat stable antigen (HSA), or surface immunoglobulin (Ig) M (sIgM), and their Ig heavy chain (IgH) gene loci are germ-line in configuration. The addition of interleukin (IL)-7 or coculture with another stromal cell line, ST2, induces D-J rearrangement of the IgH gene and B lymphocyte differentiation markers. B31-1 cells restore an *in vivo* repopulation activity to lethally irradiated mice, and the repopulated cells differentiate to HSA$^+$ pre-B cells. Continuous coculture results in two distinct populations, B220$^-$ c-Kit$^+$ cells and B220$^+$ c-Kit$^+$ cells; B220$^-$ c-Kit$^+$ cells are self-renewed and differentiate to B220$^+$ c-Kit$^+$ cells, while B220$^+$ c-Kit$^+$ cells produce only B220$^+$ c-Kit$^+$ cells. Both B220$^-$ and B220$^+$ cells similarly express the IgH germ-line transcript ($I\mu$), mRNAs for recombinase (TdT, Rag-1, and Rag-2), and lymphoid-specific transcription factors (Pax-5, EBF, E12/E47, Oct-2, and Ikaros), but the DNA binding activity of Pax-5, EBF, Oct-2, and E2A are low in B220$^-$ cells and while high in B220$^+$ cells. These results suggest the existence of at least two active states in the IgH locus before the induction of IgH gene rearrangement during B lymphopoietic development.

Key words: B-cell differentiation, early B-cell progenitor, hematopoietic stem cell, immunoglobulin gene rearrangement, stromal cell.

B lymphopoietic development from hematopoietic stem cells is thought to be regulated by the bone marrow microenvironment created by stromal cells, as shown by long-term *in vitro* bone marrow culture (*1-5*). Growth factors, cell adhesion molecules, and extracellular matrices, as stromal components, are thought to be required for B lymphopoiesis. While several growth factors secreted by stromal cells regulate B lymphopoiesis, interleukin (IL)-7 and stem cell factor (SCF) are the most critical because the proliferation and differentiation of B cell progenitors are enhanced by their addition and reduced by their functional inhibition (*3-14*). In addition, cell to cell contact mediated by cell adhesion molecules such as very late antigen (VLA)-4 and Pgp-1 and their stromal cellular ligands, vascular cell adhesion molecule (VCAM)-1 and extracellular matrices (*15, 16*), has been shown to be necessary for B cell development. However, there must be as yet unknown molecular mechanisms that regulate the earliest process in B lymphopoiesis *via* direct interaction with stromal cells.

In the stromal cell-dependent phase of B lymphopoietic development, gene segments of the immunoglobulin (Ig) gene loci are rearranged in an ordered fashion, so that D_H segments are first rearranged to J_H segments, followed by V_H to D_HJ_H, then V_κ to J_κ, and finally V_λ to J_λ (*17, 18*). Commitment of the B lymphoid lineage is thought to include D-J rearrangement of the Ig heavy chain (IgH) gene, because it occurs first in the earliest developmental stage (*18*). Understanding the regulatory mechanism of B lymphopoietic development, especially the commitment process of stem cells to the B lymphoid lineage, may be possible by obtaining lymphoid stem-like cell lines with a germ-line configuration in the IgH locus. However, in most B lymphoid cell lines, including stromal cell-dependent B cell lines, the IgH loci have already undergone D-J rearrangement (*2, 11, 17, 19*).

Assuming that the variable activities in the stromal cells support B lymphopoiesis (*20-23*), we previously established many bone marrow stromal cell lines from temperature-sensitive (ts) T antigen gene transgenic mice and reconstructed the *in vitro* systems for hematopoietic develop-

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ment from stem cells by coculturing the cells on these stromal cell lines (*4, 5, 24*). Among them, TBR31-1 cells were shown specifically to induce long-term B cell development (*5*). Using this stromal cell line, we established a stromal cell-dependent earliest B lymphoid cell line that is self-renewing in the earliest (IgH germ-line) character and differentiates to more mature progenitor cells in response to several stimuli.

MATERIALS AND METHODS

Antibodies—Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-B220 (RA3-6B2), FITC-conjugated anti-CD3 (145-2C11), FITC-conjugated anti-erythroid (TER119), FITC-conjugated anti-Gr-1 (RB6-8C5), and FITC-conjugated anti-Mac-1 (M1/70) were purchased from Pharmingen (San Diego, CA) and used as lineage markers (*24*). FITC-conjugated anti-IgM (R6-60.2), biotin-conjugated anti-heat stable antigen (HSA) (M169), and FITC-conjugated anti-CD43 (S7) were purchased from Pharmingen and used as B cell differentiation stage-specific markers (*25*). Anti-c-Kit antibody (ACK2, generously supplied by S.-I. Nishikawa, Kyoto University) (*10*) was coupled with biotin. PE-conjugated anti-Sca-1 (E13-161.7) was purchased from Pharmingen (*24*). Indirect Microbeads goat-anti-rat IgG (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for magnetic cell sorting.

Cell Lines and Culture Conditions—The TBR31-1 cell line was maintained in RITC 80-7 (Kyokuto Pharmaceutical, Tokyo) supplemented with 2% fetal bovine serum (FBS), 10 μg/ml transferrin, 10 ng/ml epidermal growth factor, and 1 μg/ml insulin. Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 33°C (*26*). A bone marrow stromal cell line, ST2 (*2, 3*), was maintained in RPMI 1640, 10% FBS at 37°C.

Establishment of the B31-1 Cell Line—One hundred highly enriched stem cells (Lin^- c-Kit^+ Sca-1^+) obtained from the bone marrow of 6-8 week old C57BL/6 mice were sorted onto TBR31-1 cell layers by a FACStar (Becton Dickinson, San Jose, CA) as described (*4, 5*). One day before coculture, the medium of the sub-confluent TBR31-1 cell layers formed in 60 mm culture dishes was replaced with α-minimum essential medium (α-MEM, Flow Laboratories, Irvine, CA) supplemented with 10% FBS, 50 μM 2-mercaptoethanol (2-ME), 10 U/ml penicillin, and 10 μg/ml streptomycin. The cultures were incubated at 37°C, and half the volume of the medium was changed every 3 days. Hematopoietic cells that crawled onto the surface of the stromal layer were recovered by gentle pipetting, and 1×10^6 of these cells were reseeded on fresh stromal layers every two weeks. Six months after sorting, a stromal cell dependent cell line (B31-1) was established.

Flowcytometry—B31-1 cells were recovered by gentle pipetting and passed through 70 mm nylon mesh. The cells were washed with phosphate-buffered saline (PBS) containing 0.02% BSA and incubated with FITC- or PE-conjugated anti-B220, FITC-conjugated anti-CD3, FITC-conjugated anti-erythroid, FITC-conjugated anti-Gr-1, FITC-conjugated anti-Mac-1, FITC-conjugated anti-IgM, biotin-conjugated anti-HSA, FITC-conjugated anti-CD43, and/or biotin conjugated anti-c-Kit antibodies for 30 min on ice. After incubation, the cells were washed twice and incubated with allophycocyanin (APC)-conjugated streptavidin (Becton Dickinson Immunocytometry Systems, San Jose, CA) for 30 min on ice. The cells were again washed twice and resuspended in PBS supplemented with 0.02% BSA. Unstained bone marrow cells were used as a negative control. Stained cells were analyzed and sorted by a FACStar^{Plus} (Becton Dickinson).

PCR Analysis of Ig Gene Rearrangement—Genomic DNAs were prepared by incubating 1×10^5 cells at 55°C for 60 min in 200 ml of lysis buffer (10 mM Tris-Cl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 60 mg/ml proteinase K) as described by Schlissel *et al.* (*27*). The primers used to detect D_HJ_H recombination and to estimate input genomes (Thy1 gene) have been described previously (*28, 29*). PCR protocols were optimized by amplifying the genomic DNA of sorted $B220^+$ splenocytes using a PCR optimizer (Invitrogen, Leek, Netherlands). Genomic DNAs were amplified for 25-30 cycles in a volume of 50 ml denaturation at 95°C for 60 s, annealing at 65°C for 60 s, and polymerization at 72°C for 150 s as described by Ehlich *et al.* (*29*). One-fifth of the PCR products were analyzed by Southern blots using oligonucleotide probes recognizing the J_{H4} region of the heavy chain or the Thy1 gene (*29*).

RT-PCR Assay—$B220^-$ and $B220^+$ cells were sorted with MACS (Miltenyi Biotec) using anti-B220 and goat-anti-rat IgG coupled with magnetic bead antibodies. Total RNAs from 1.0×10^7 of each cell line were extracted with Isogene (Nippongene, Tokyo) and dissolved in 30 ml of diethylpyrocarbonate (DEPC)-treated water. cDNAs were synthesized from 5 μg of total RNA in a 20 μl reaction volume after treatment with 0.2 μl of RQ1 RNase-Free DNase (1,000 U/ml) (Promega, Madison, WI). The DNase treated RNAs were primed with 0.6 μl of random hexamers (500 μg/ml) (Promega), incubated at 70°C for 10 min, and quickly chilled on ice; 0.6 μl of 28 U/μl RNase inhibitor (Toyobo, Tokyo) and 5 μl of 2 mM dNTP-mix (PerkinElmer, Branchburg, NJ) were added, and the reaction was pre-incubated at 37°C for 2 min. Then 1 μl of 200 U/μl moloney-murine leukemia virus (M-MLV) reverse transcriptase (RT) was added, and the reaction was incubated at 37°C for 60 min, heated to inactivate at 70°C for 10 min, then quickly chilled on ice and diluted to a final volume of 1.25 ml with 10 mM Tris-HCl (pH 8.0)/0.1 mM EDTA (pH 8.0). cDNA was amplified by PCR using different primers. PCR reactions were performed in a volume of 10 μl containing 1/500 of cDNA sample, 1×PCR buffer (Gibco BRL, Gaithersburg, MD), 0.2 mM of each dNTP mix (PerkinElmer), 0.1 μl [α-^{32}P]dCTP (370 MBq/ml) (Amersham, Arlington Heights, IL), 10 pmol of each sense and antisense primer, 1 μl (2 U) of TaqStart Antibody (Clontech, Palo Alto, CA), and 0.1 μl of TaqPolymerase (Gibco BRL). After an initial 2 min incubation at 94°C, the PCR reaction was carried out for 25 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and polymerization at 72°C for 90 s. To verify that equal amounts of RNA were added to each of the PCR reactions, the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was amplified. Five microliters of PCR samples were then separated by 6% polyacrylamide gel electrophoresis and imaged on X-ray film. Primers for amplifying Ikaros (*30*), E12 (*31*), E47 (*32*), EBF (*33*), Pax-5 (*32*), Oct-2 (*27*), TdT (*18*), Rag-1

(*18*), Rag-2 (*18*), and Iμ (*27, 34*) genes have been described previously. The GAPDH primers used were as follows: forward, 5′-CTTCACCACCATGGAGAAGG-3′; reverse, 5′GGTTGTCTCCTGCGACTTCA-3′.

Induction of Differentiation in B31-1 Cells—Conditioned media (CM) from a murine IL-7 gene transfected NIH3T3 cell line (kindly provided by Dr. H. Karasuyama, Tokyo Metropolitan Medical Institute), cultured in RPMI1640 (Nissui Pharmaceutical, Tokyo) supplemented with 10% FBS, was used as an IL-7 CM. Fifty thousand of B31-1 cells were seeded on a TBR31-1 cell layer in the presence of 20% IL-7 CM. One million of B31-1 cells were cultured on a confluent ST2 cell layer (*2, 3*) formed in RPMI1640 (Nissui Pharmaceutical) supplemented with 5% FBS, 50 mM 2-ME, 10 U/ml penicillin, and 10 μg/ml streptomycin in 60 mm culture dishes. The cultures were incubated at 37°C and half the volume of the medium was changed every 3 days. Two weeks after cocultivation, the expression of surface markers on the lymphoid cells was analyzed by flowcytometry, and the cells were lysed to analyze D_H-J_H recombination.

Repopulation of B31-1 Cells in Lethally Irradiated Mice—Ten million of B31-1 cells were injected intravenously into lethally irradiated C57BL/6-Ly5.1 mice. At various times (2, 3, and 4 weeks) after injection, cells from the bone marrow and spleen were stained with anti-Ly5.1 (specific for the recipient mouse cells), anti-Ly5.2 (specific for the donor B31-1 cells), and monoclonal antibodies for B lymphoid markers (B220, CD43, HSA, and sIgM). The $Ly5.1^-$ $Ly5.2^+$ fractions were gated with a FACStar-plus as repopulated fractions and the expression of B-lymphoid markers was analyzed.

Electrophoretic Mobility Shift Assay (EMSA)—Cells were harvested, rinsed with PBS, and resuspended in 1.5 volumes of lysis buffer (20 mM Hepes pH 7.9, 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF), then kept on ice for 20 min, frozen at −80°C for 15 min, and thawed on ice. The suspension was then vigorously vortexed and centrifuged for 10 min at 13,000 rpm. The supernatant was used as the extract. Synthetic oligonucleotides were 5′ end-labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP and annealed in annealing buffer (20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl). The DNA was gel purified and diluted to 5,000 cpm/ml. One milliliter of the probe DNA was incubated in a final volume of 10 ml with 1.5 mg poly(dIdC), 1 mM DTT, 50 mM KCl, 20 mM Hepes-KOH pH 8.0, 4% Ficoll, 2 mM EDTA, pH 8.0, and 1 ml of cell extract for 30 min on ice. The mixture was separated on a native 6% polyacrylamide gel developed in 0.25×TBE for 120 min in a cold room. The gel was dried and exposed for autoradiography. Competition experiments were performed by mixing an excess amount of an appropriate competitor DNA to the binding reaction prior to the addition of the cell extract protein. The oligonucleotides used were derived from the intron enhancer region of the IgH locus, and comprised binding sites for E2A (μE4) (*35*), Oct (octamer) (*35*), EBF (mb-1) (*36*), and Pax-5 (3′ α-hs4) (*37*), which were described previously.

RESULTS

Establishment of a Stromal Cell-Dependent B Cell Line—We previously reported that a bone marrow stromal cell line, TBR31-1, specifically induces B lymphopoiesis by forming a cobblestone (*5*), a characteristic of primitive hematopoietic cells (*38-42*), from Lin^- c-Kit^+ $Sca\text{-}1^+$ bone marrow hematopoietic stem cells (*24*). In this coculture, the cobblestone formation continued for more than 2 months with a concomitant release of hematopoietic cells from the surface of the stromal layer. Most of the released

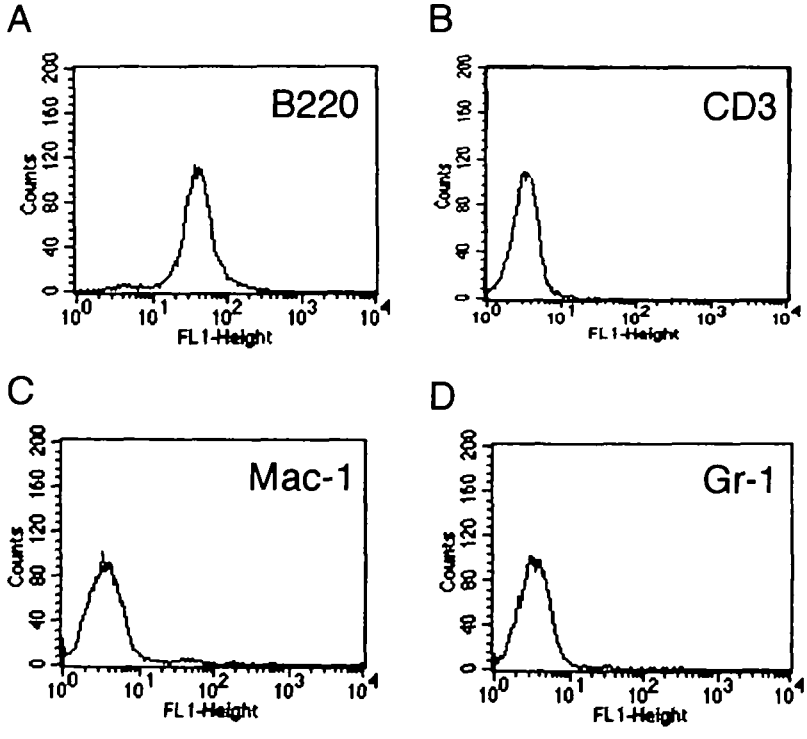

Fig. 1. **Expression of lineage markers on B31-1 cells.** After 2 weeks of coculture with TBR31-1 cells, 1×10^6 B31-1 cells were stained with FITC-conjugated anti-B220 (A), anti-CD3 (B), anti-Mac-1 (C), or anti-Gr-1 (D) monoclonal antibodies and analyzed by flow cytometry.

cells expressed B220, but did not express myeloid lineage markers. When the released hematopoietic cells were recovered by gentle pipetting and transferred to a fresh TBR31-1 stromal cell layer, the cell number decreased, possibly due to cell death, during the initial stages of culture, but after about one week the cells began to proliferate by forming cobblestones by kinetics similar to the primary culture of the sorted stem cells. After repeated transfers for 6 months, a continuously proliferating stromal cell-dependent cell line was established called B31-1. Although the cells were not recloned by a limiting dilution method, B31-1 could be maintained with the same properties for 2 years.

Flowcytometry analysis to determine whether B31-1 cells consist of only B lymphoid lineage cells showed the B31-1 cells to express B220, but not markers for other lineages (CD3 for T cells, Mac-1 for monocytes, Gr-1 for granulocytes) (*24*) (Fig. 1). The expression of B cell differentiation stage specific surface markers (CD43, HSA, and sIgM) (*25*) was analyzed by flowcytometry to determine the developmental stage of the B31-1 cell line (Fig. 2). CD43 and sIgM were not detectable and HSA was detected in only 1.2% of the cells, suggesting that most cells are maintained at a very early developmental stage. After the $B220^+$ $CD43^-$ HSA^- $sIgM^-$ cells were sorted from the culture to remove a minor population of mature cells, their Ig gene D_H-J_H rearrangement was analyzed by the genomic PCR method (Fig. 3). If the gene is rearranged, 4 fragments should be amplified as detected in $B220^+$ splenocytes. D_H-J_H rearrangement was not detected in most of the B31-1 cells. Since D_H-J_H rearrangement occurs from pre-pro-B stage (*18*), the majority of B31-1 cells appear to be very early B cells.

In Vitro Differentiation Capacities of B31-1 Cells—We examined the differentiation capacity of B31-1 cells. Since IL-7 is an important factor for B lymphoid development, we examined the effect of IL-7 on the induction of B31-1. Seven days after the addition of IL-7, the proportion of HSA^+ cells increased to 61% from 1.2%, while no expression of either CD43 or sIgM was detected (Fig. 4A). When B31-1 cells were cocultured for 2 weeks on a ST2 cell layer that can support the whole process of B lymphopoiesis by producing IL-7 (*2, 3*) for 2 weeks, 68% of the cells expressed HSA while some expression of either CD43 (12.5%) or sIgM (1.7%) was also observed (Fig. 4B).

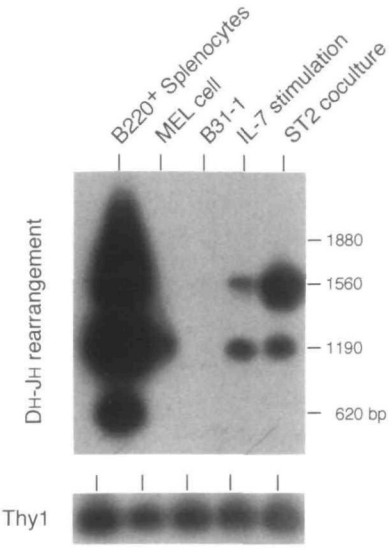

Fig. 3. **D-J rearrangement of the IgH gene in B31-1 cells.** $B220^+$ $CD43^-$ HSA^- $sIgM^-$ cells (B31-1) were collected by a cell sorter as undifferentiated B31-1 cells from B31-1 cells co-cultivated for 2 weeks. To induce their differentiation for further maturation, B31-1 cells were cocultured with TBR31-1 stromal cells in the presence of IL-7 (20% of conditioned medium of IL-7-producing NIH3T3 cells) (IL-7 Stimulation), or cocultured with another stromal cell line (ST2 Coculture). D-J rearrangement of the IgH gene was analyzed in 1×10^5 frastinated cells by the genomic PCR method as described in "MATERIALS AND METHODS." When the IgH gene was rearranged, 4 fragments (1,880, 1,560, 1,190, and 620 bp) were amplified as shown in the lane for sorted $B220^+$ splenocytes. The mouse erythroleukemia cell line B8/3 (MEL) was used as a negative control. The Thy-1.2 (Thy1) gene was amplified to estimate the amounts of input genomes.

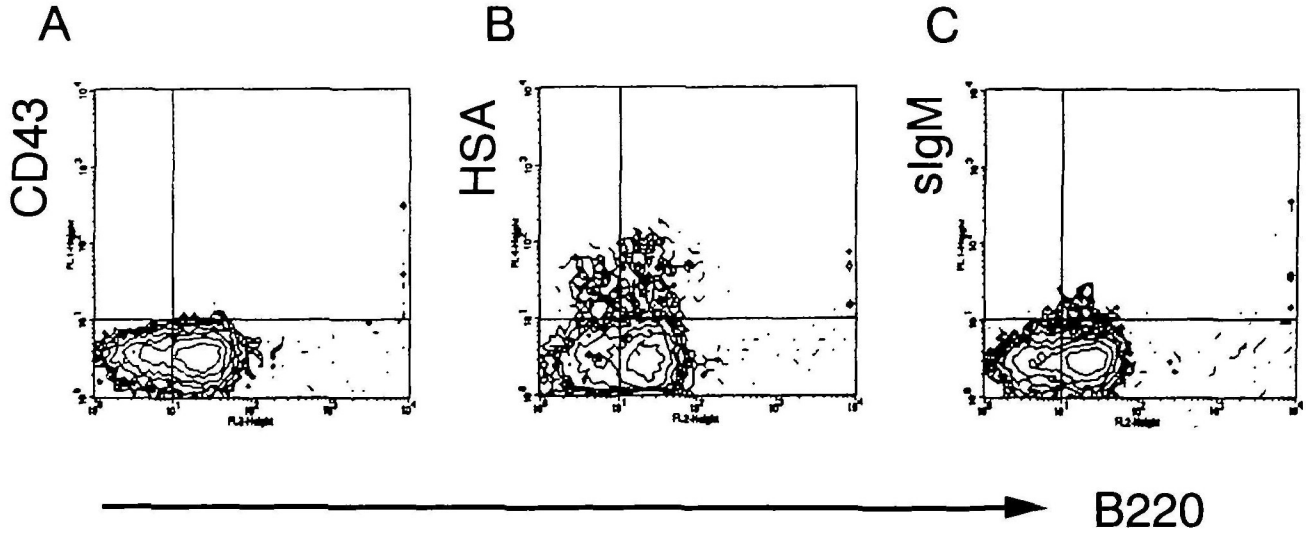

Fig. 2. **Expression of B cell differentiation stage specific markers.** B31-1 cells were stained with anti-CD43 (A), anti-heat stable antigen (HSA) (B), anti-surface IgM (sIgM) (C), or anti-B220 (A-C) monoclonal antibodies.

Then, we examined whether D-J rearrangement of IgH gene is accompanied by the expression of surface markers induced by both stimuli. The results clearly showed that the rearrangement is induced by both stimuli; the population of cells with a rearranged D_H-J_H gene was greatly increased in coculture with ST2 cells (10.5%), and significantly higher in the presence of IL-7 stimulation (1.9%) than in coculture without IL-7 (0.27%) (Fig. 3).

B31-1 Cells Consist of 2 Cell Populations—We observed 2 cell populations ($B220^+$ c-Kit^+ and $B220^-$ c-Kit^+ cells) in the coculture of B31-1 cells on TBR31-1 stromal cells. After 2 weeks of coculture following transfer, 78% of B31-1 cells were $B220^+$ c-Kit^+ and 6.8% were $B220^-$ c-Kit^+, but, interestingly, the latter population increased to 52.0% after 4 weeks (Fig. 5A). Expecting that $B220^-$ c-Kit^+ cells are the precursor of $B220^+$ c-Kit^+ cells and may differentiate into $B220^+$ cells, both cells were sorted and cocultured with TBR31-1 cells. Both cell populations formed cobblestones and proliferated with similar kinetics, however, $B220^-$ c-Kit^+ cells generated both $B220^-$ c-Kit^+ and $B220^+$ c-Kit^+ cells, while $B220^+$ c-Kit^+ cells generated only $B220^+$ c-Kit^+ cells after 2 weeks of coculture (Fig. 5B). Thus, the $B220^-$ c-Kit^+ cells have the capacity to both self-renew and to differentiate to $B220^+$ c-Kit^+ cells.

Characteristic Differences between $B220^-$ Cells and $B220^+$ Cells—Since the IgH locus appears to be activated at a very early stage of B cell development and the level of the germ-line IgH transcript ($I\mu$) is one of the earliest indications of B lineage commitment (*43-45*), we examined the expression of $I\mu$ by RT-PCR after the $B220^-$ c-Kit^+ cells and $B220^+$ c-Kit^+ cells were sorted. The $I\mu$ transcript was found to be expressed in both $B220^-$ and $B220^+$ cells to the same extent (Fig. 6).

TdT, Rag-1, and Rag-2 are involved in the rearrangements of the immunoglobulin or T cell receptor genes (*18, 46-50*). The transcription of these recombinase genes is initiated before or together with IgH rearrangement (*18*). Recombinase gene expression was detected in both $B220^-$ and $B220^+$ cells, although the levels of Rag-1 and Rag-2 were higher in $B220^+$ cells than in $B220^-$ cells, while TdT levels were higher in $B220^-$ cells (Fig. 6). These results are consistent with previous reports that TdT is expressed earlier than Rag-1 (*18*).

Next, the expression of lymphoid-specific transcription factors [E2A (*32-34*), EBF (*30, 51, 52*), Pax-5 (*53-55*), and Oct-2 (*56, 57*)] involved in lymphoid development was examined. The EBF, Pax-5, and Oct-2 genes show no significant differences between $B220^+$ and $B220^-$ cells. The expression of all Ikaros (*58, 59*) splicing isoforms except Ik-5 was detected and showed slight differences between

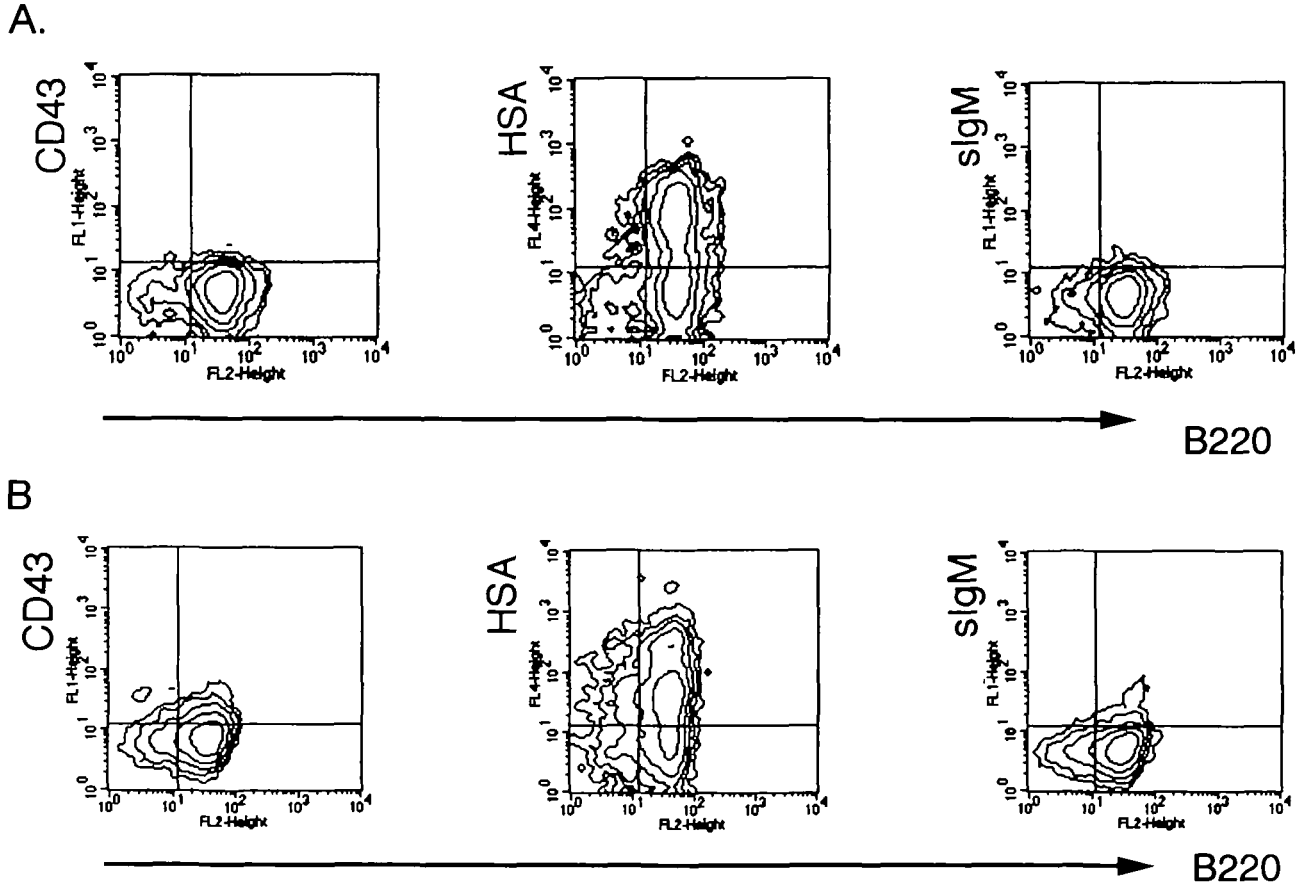

Fig. 4. **Induction of B31-1 cell differentiation.** To induce their differentiation for further maturation, B31-1 cells were cocultured with TBR31-1 stromal cells in the presence of IL-7 (20% of conditioned medium of IL-7-producing NIH3T3 cells) (A), or cocultured with another stromal cell line (ST2) (B). After 1 week (A), or 2 weeks (B) of culture, the expressions of CD43, HSA, sIgM, and B220 were examined by FACS after staining with the respective antibody.

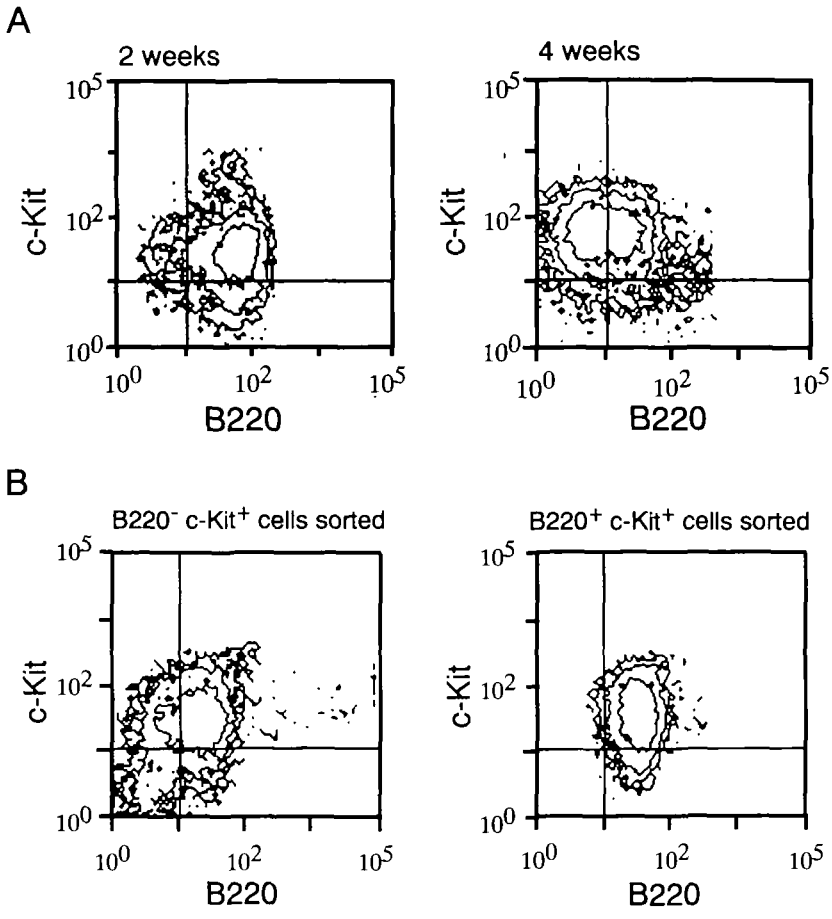

Fig. 5. **Self-renewal and differentiation of B31-1 cells.** B31-1 cells were recovered from 2- or 4-week cocultures and stained with anti-B220 and anti-c-Kit antibodies. Cell populations ($B220^+$ c-Kit^+ and $B220^-$ c-Kit^+ cells) were analyzed by two-color flowcytometry (A), and each gated fraction was sorted. One hundred of the sorted $B220^-$ c-Kit^+ cells or $B220^+$ c-Kit^+ cells were cocultured on TBR31-1 cell layers. After 2 weeks of culture, the cells released from the cobblestones were stained with anti-B220 and anti c-Kit antibodies and analyzed by two color flowcytometry (B). $B220^-$ c-Kit^+ and $B220^+$ c-Kit^+ cells were generated from the sorted $B220^-$ c-Kit^+ cells, while only $B220^+$ c-Kit^+ cells were generated from the sorted $B220^+$ c-Kit^+ cells by coculture with TBR31-1 stromal cells.

these two cell types. Both E21 and E47 were found to be expressed in $B220^-$ and $B220^+$ cells, but the levels of E47 were higher in $B220^-$ cells (Fig. 6).

Since both $B220^-$ and $B220^+$ cells express the IgH germline transcript ($I\mu$), the mRNAs for recombinase genes and lymphoid specific transcription factors (Pax-5, EBF, E12/E47, and Ikaros) are similarly expressed, we asked whether the activities of the transcription factors bound to the intron enhancer locus could differ between the cells types (Fig. 7). EMSA showed that the binding activities of E2A (consisting mostly of E12 and E47 proteins bound to μE4), Pax-5, and Oct-2 are low in $B220^-$ cells, but enhanced in $B220^+$ cells, whereas EBF shows no significant difference. Thus, the transcription factors involved in the activation of the intron enhancer of the IgH gene may be more activated in $B220^+$ cells than in $B220^-$ cells.

In Vivo Repopulation Activity of B31-1 Cells—To investigate the repopulating activity of B31-1 cells, the cells were injected intravenously into lethally irradiated C58BL/6-Ly5.1 mice. The repopulated B31-1 cells were monitored in the gated fraction ($Ly5.1^-$ $Ly5.2^+$) after staining with anti-Ly5.2 (specific for the isotype expressed in B31-1 cells) and anti-Ly5.1 (specific for the isotype expressed in the recipient mice) (Fig. 8A). It is evident that B31-1 cells can repopulate in the bone marrow and spleen after 2-4 weeks. The developmental stage of the repopulated cells in bone marrow was monitored by the expression of B220, CD43, HSA, and sIgM (Fig. 8B). Two weeks after injection, half of the repopulated cells were $B220^-$ cells, but most cells became $B220^+$ cells by 4 weeks. The repopulated B31-1 cells showed the patterns of the earlier progenitor cells ($B220^+$ $CD43^+$ HSA^- $sIgM^-$) after 4 weeks in bone marrow, and the same patterns were obtained for repopulated cells in the spleen after 4 weeks. Even after 6 months, most of the B31-1 cells were maintained as $B220^+$ $CD43^+$ HSA^- $sIgM^-$ cells in the bone marrow (data not shown), indicating that B31-1 cells can be repopulated in mice and maintained as early B cell progenitors.

DISCUSSION

In the present work, a novel primitive B lymphoid cell line, B31-1, was established from bone marrow hematopoietic stem cells sorted as Lin^- c-Kit^+ $Sca\text{-}1^+$ cells. In studying B cell development in bone marrow, Hardy *et al.* (*25*) separated B cells into several developmental stages: pre-pro-B, pro-B, pre-B, immature-B, and mature-B, by their patterns of expression of cell surface antigens. For example, CD43 is expressed in the pre-pro to pro-B stages, HSA in stages between pro-B and mature-B, and sIgM from the immature-B to mature-B stages. Li *et al.* (*18*) reported that D-J rearrangement of the IgH gene occurs from the pre-pro-B stage, followed by V-D-J rearrangement from the pro-B stage. B31-1 cells show very immature phenotypes earlier than the pre-pro-B stage; they grow dependent on stroma cells by forming cobblestones, a characteristic of primitive hematopoietic cells (*38-42*), their IgH gene locus is a germ line configuration, and they show no

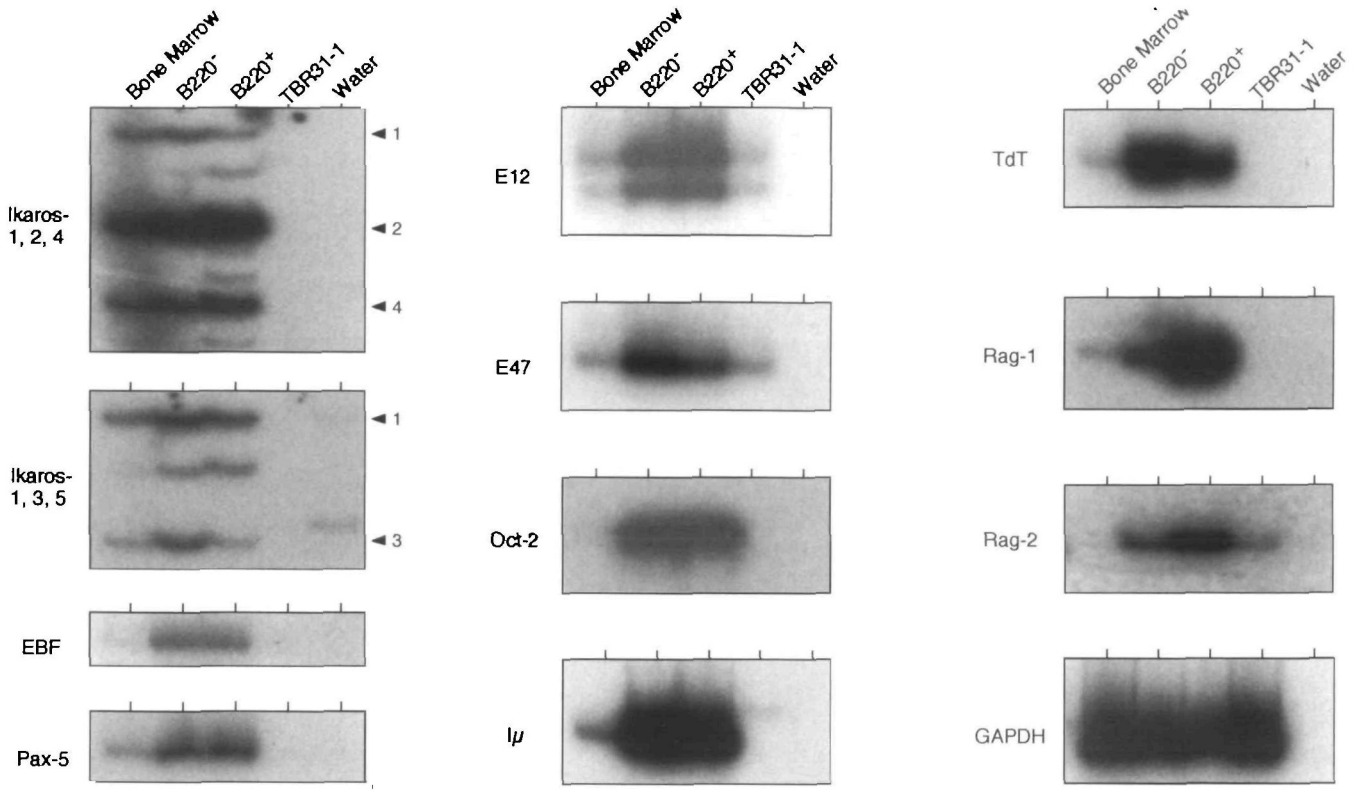

Fig. 6. **Expression of early lymphoid-specific marker genes in fractionated B220⁻ and B220⁺ cells.** In B220⁻ cells, the expressions of the TdT, Rag-1, Pax-5, E12, E47, and EBF genes and the germ-line IgH transcript (Iμ) were analyzed by RT-PCR. The expression of the GAPDH gene was used to estimate the amounts of input cDNA; whole bone marrow cells and TBR31-1 stromal cells were used as positive and negative controls, respectively.

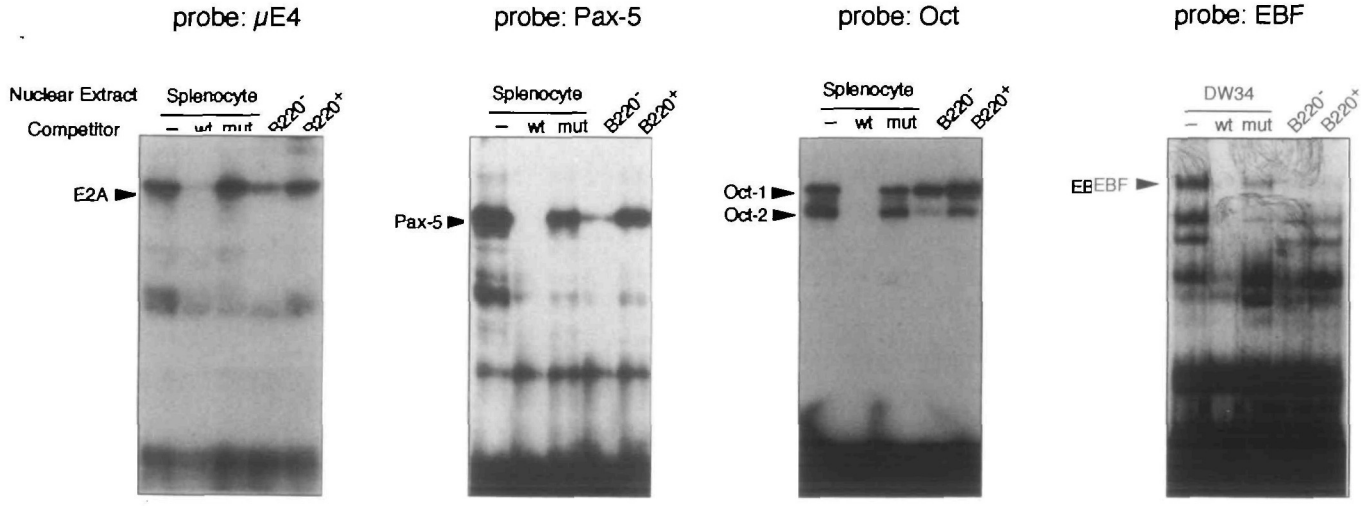

Fig. 7. **Electrophoretic gel mobility shift assay (EMSA) of B lymphoid cell-specific transcription factors in B220⁻ and B220⁺ cells.** EMSA was performed with whole cell extracts from the fractionated B220⁻ and B220⁺ cells by incubation with the ^{32}P-labeled oligonucleotide probe specific for each factor. μE4 for E2A, 3′ a-hs4 for Pax-5, octamer for Oct, and mb-1 for EBF were used as probes. For each probe, spleer extracts were incubated without competitor (−), with an excess amount of the competitor (wt), and a mutant competitor (mut), and the respective probe was incubated with either B220⁻ or B220⁺ nuclear extract without competitor. Arrowheads indicate the positions of the specific DNA-protein complexes.

detectable expression of surface antigens such as CD43, HSA, and sIgM.

Speculating that B31-1 cells form a B lymphoid stem cell-like cell line, we asked how B31-1 cells could be maintained in the early stage and committed to differentiate. We found a significant population of B220⁻ c-Kit⁺

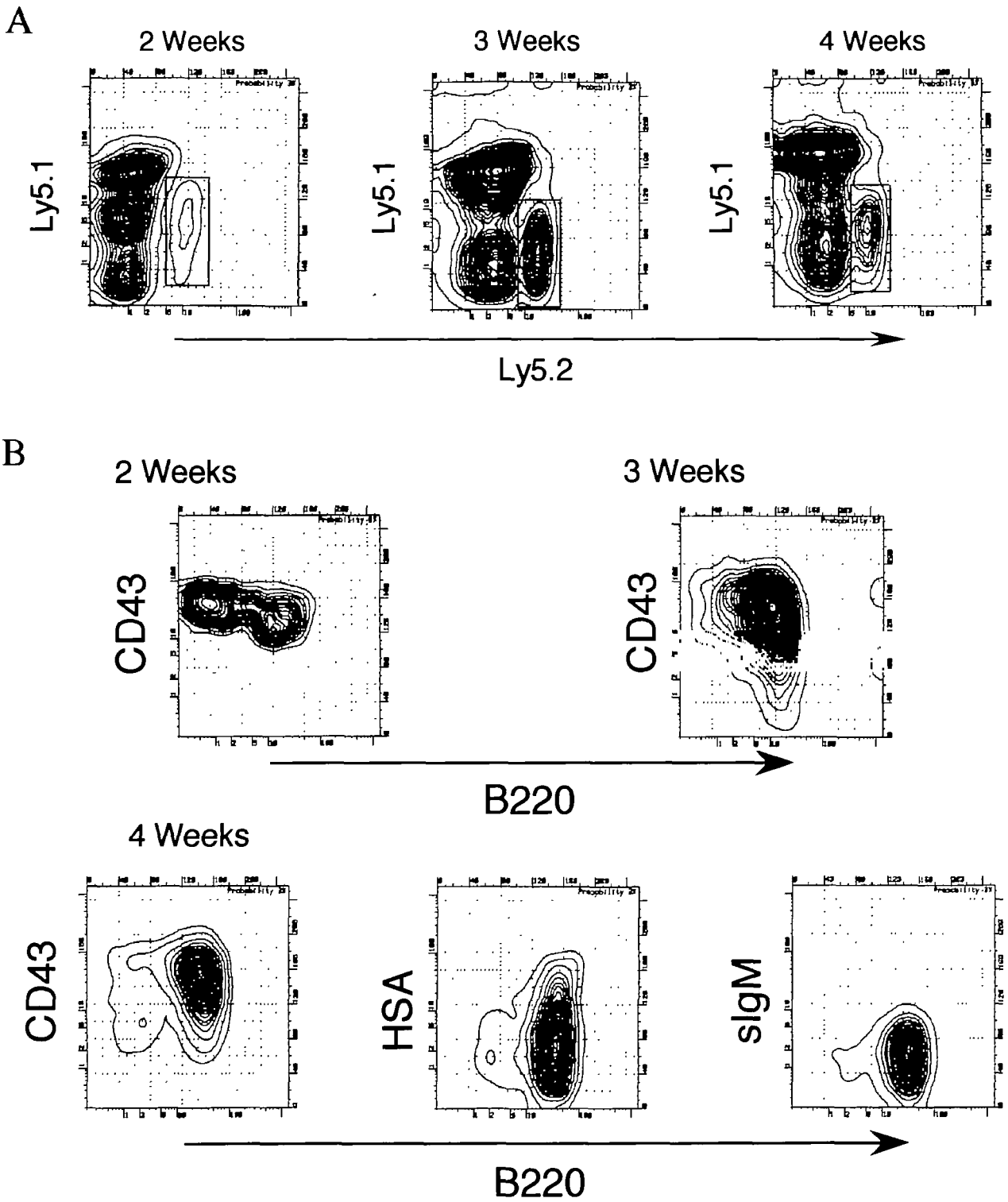

Fig. 8. **Repopulation of B31-1 cells.** B31-1 cells (1×10^7) were injected intravenously into lethally irradiated C57BL/6-Ly5.2 mice. At various times after injection, cells from the bone marrow and spleen were stained with anti-Ly5.1 (specific for the recipient mouse cells), anti-Ly5.2 (specific for the donor B31-1 cells), and monoclonal antibodies for B-lymphoid markers (B220, CD43, HSA, and sIgM), and analyzed with a FACStar plus. (A) Repopulated cells are shown in the gated fractions ($Ly5.1^-$ $Ly5.2^+$). (B) Expression of B lymphoid markers in the repopulated fractions.

cells and observed that this cell population increases with prolonged culture. The maintenance of $B220^-$ cells in culture is intriguing because most lymphoid cell lines in previous studies were $B220^+$ cells with a rearranged Ig gene; thus the B31-1 cell line seems to be one of the earliest B lymphoid cell lines so far obtained. We demonstrated

that B220$^-$ c-Kit$^+$ cells can self-renew like uncommitted stem cells without expressing B220 and were able to differentiate to B220$^+$ c-Kit$^+$ cells, while B220$^+$ c-Kit$^+$ cells generate only B220$^+$ c-Kit$^+$ cells. Thus, B220$^-$ c-Kit$^+$ cells may be the precursors of B220$^+$ c-Kit$^+$ cells and the maintenance of both cell populations is dependent on TBR31-1 stromal cells.

B31-1 cells have been shown to be repopulated in the bone marrow and spleen, and the repopulated B31-1 cells can be maintained as early progenitor cells (B220$^+$ CD43$^+$ HSA$^-$ sIgM$^-$) long term (over 6 months) in bone marrow. It is interesting that most repopulated cells are CD43$^+$ HSA$^-$ whereas most B31-1 cells *in vitro* culture are CD43$^-$ HSA$^+$ after IL-7 addition or coculture with ST-2, suggesting that the stimuli for proliferation and differentiation of B31-1 cells may differ between *in vivo* and *in vitro* culture.

We examined whether B31-1 cells can be induced to differentiate to more mature B cells either by adding excess amounts of IL-7 (*3, 6*) or by coculturing with another stromal cell line (ST2) (*2, 3*). Upon the addition of excess amounts of IL-7 to the coculture, a large increase in the cell population expressing HSA was observed, while only a small population of cells expressed sIgM$^+$; however, the coculture of B31-1 cells on ST2 stromal cells resulted in a significantly higher population of B31-1 cells expressing HSA and sIgM. Thus, ST2 stromal cells support the induction and maintenance of mature B cells much more strongly than IL-7 addition to TBR31-1 stromal cells. Quite importantly, a significantly higher population of cells with the D-J rearranged IgH gene (10.5%) was induced in B31-1 cells with the germline IgH gene by coculturing with ST2 cells. The results indicate that rearrangement of the germline IgH gene can be conditionally induced in B31-1 cells in the *in vitro* culture system. The IgH gene has been shown to be completely D-J rearranged by the pre-B stage *in vivo* (*18*), but D_H-J_H rearrangement in B31-1 cells was infrequent, while HSA$^+$ pre-B cells became abundant in coculture with ST2 cells, suggesting that the appearance of cell surface markers and D_H-J_H rearrangement may be regulated by independent signals during B cell development.

The IgH locus appears to be activated at a very early stage of B cell development and the level of germ-line IgH transcript ($I\mu$) is one of the earliest indications of B lineage commitment (*43-45, 60*). High levels of germ-line IgH transcripts were detected in B220$^-$ c-Kit$^+$cells as well as in B220$^+$ c-Kit$^+$ cells and the expression of recombinase genes (TdT, Rag-1, and Rag-2) and transcription factors (Ikaros, Pax-5, EBF, Oct-2, and E21/E47) were detected similarly in both cells, although IgH rearrangement did not occur in a majority of either cell type. Thus, $I\mu$ expression may be a prerequisite, but not sufficient for IgH rearrangement. Thus, we expected that the activities of the transcription factors bound to the intron enhancer locus might differ between the two cells and, in fact, we found that the activities of E2A, Pax-5, and Oct-2 are low in B220$^-$ cells but enhanced in B220$^+$ cells, whereas that of EBF shows no significant difference. Although a more precise examination, such as *in vivo* footprinting assay, is required, the apparent change in the binding activities of the transcription factors suggests that the activation of the intron enhancer of the IgH gene may be greater in B220$^+$ cells than in B220$^-$ cells, and that least two active states of the IgH locus may exist during B lymphopoietic development before the induction of IgH gene rearrangement. The earliest B lymphoid lineage cells in the B220$^+$ HSA$^-$ pre-pro-B fraction of mouse delineated by the expression of AA4.1, as reported by Li *et al.* (*61*), have the capacity to generate B lymphoid lineage cells rapidly in stroma-dependent culture and express high levels of germ-line IgH transcripts in the absence of Rag-1/2. Their phenotypes are consistent with the characteristics of lymphoid stem cells, and the B220$^-$ population of B31-1 cells seems similar to this fraction.

Our *in vitro* coculture of B31-1 cell line with a germline IgH gene demonstrates that D_H-J_H rearrangement can be induced conditionally, and may thus be very useful in analyzing how the D_H-J_H rearrangement of the Ig gene is initiated.

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