A Subpopulation of B220$^+$ Cells in Murine Bone Marrow Does Not Express CD19 and Contains Natural Killer Cell Progenitors

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Summary

Bone marrow of both normal and rearrangement-deficient mice contains a small population of B220(CD45R)$^+$ cells, which do not express the B lineage marker CD19. Instead, part of this population coexpresses the surface marker CD43 and lacks or expresses very low levels of heat stable antigen (HSA) and BP-1, thus representing a part of Hardy's fraction A (B220$^+$-CD43$^+$HSA$^-$, BP-1$^-$) of B lineage development. However, some 20–40% of these B220$^+$-CD19$^-$ cells also coexpress the NK1.1 surface molecule and do not express genes like V$_{preB}$ or B29 restricted to the B cell lineage. These cells respond to recombinant interleukin 2 in vitro, and develop into killer cells that can lyse the prototypic NK target tumor cell, YAC-1, as well as syngeneic normal lipopolysaccharide or concanavalin A blasts, providing they lack the surface expression of major histocompatibility complex class I molecules. The implications of these findings for studies on B lymphopoiesis are discussed. It is suggested that the CD19-specific monoclonal antibody is more reliable, as in humans, than B220(CD45R) to detect B lineage cells in mice.

In the mouse, B lineage cells express the surface marker B220(CD45R) (1). From the earliest identified c-kit$^+$ pro–B cell in fetal liver and bone marrow, all the way through the mature sIgM$^+$sIgD$^+$ peripheral B cell as well as activated B cell blasts, all B lineage cells express the B220 marker often in upregulated forms. Only at the latest stage of B cell development, the fully differentiated plasma cell, is B220 downregulated. Even in recombinase-deficient (SCID and RAG-T) mice, the B220 surface marker is expressed on early progenitors (2–4). Therefore, B220(CD45R) is commonly used as a pan–B cell marker for studies in the mouse. In humans, however, another cell surface molecule, CD19, is used as a pan–B cell marker, since reagents specific for a possible B220-like isoform of human CD45R do not exist (5, 6). We have used a newly generated mAb specific for murine CD19 to compare the expression of B220 with that of CD19 on cells of bone marrow of normal as well as of rearrangement-deficient (RAG-2T)[1] animals. We identify

here a fraction of bone marrow B220$^+$ cells that does not express CD19 and does not belong to cells committed to the B lineage, but that has both phenotypic and functional properties of NK cell precursors.

Materials and Methods

Mice. 4–5-wk-old female BALB/c, C57BL/6 and (C57BL/6 × DBA/2)F$_1$ (BDF$_1$) mice were purchased from Biological Research Laboratories, Ltd. (Füllinsdorf, Switzerland). RAG-2T mice (4) originally obtained from Dr. F. Alt (The Children's Hospital, Boston, MA) and B$_2$ microglobulin–deficient (B$_2$MT) mice (7) originally obtained from Dr. R. Jaenisch (Massachusetts Institute of Technology, Cambridge, MA) were bred in our own animal facilities.

Antibodies. The FITC-, PE- and APC-labeled mAb RA3.6B2 (anti-CD45R, B220), the biotin-, FITC- or PE-conjugated mAbs 145-2C11 (anti-CD3-ε-chain), RM4-5 (anti-CD4), M1/70 (anti-CD11b [Mac-1]), J11d (anti-CD24 [heat stable antigen] [HSA]), 7D4 (anti-CD25 [TAC, IL-2R α-chain]), 2.4G2 (anti-CD32/CD16 [FcγII/IIIR]), S7 (anti-CD43), TM-β1 (anti-CD122 [IL-2R β-chain]), 6C3 (anti-BP-1), PK136 (anti-NK1.1), 2B4 (anti-NK cells) and 5E6 (anti-NK cells) were all obtained from PharMingen (San Diego, CA). The mAbs ACK$_4$ (anti-c-kit) (8), A7R34 (anti-IL-7R) (9), and 1D3 (anti-CD19, see below) are IgG of rat origin

and were purified from hybridoma culture supernatants on protein G–Sepharose columns (Pharmacia, Uppsala, Sweden) as recommended by the supplier. They were FITC or biotin labeled according to standard procedures. FITC-conjugated goat anti–mouse IgM and streptavidin–PE were purchased from Southern Biotechnology Associates (Birmingham, AL).

Production of Anti-CD19 mAbs. The human erythroleukemia cell K562 was transfected (10) with expression vector pZipNeo containing the full-length cDNA encoding murine CD19 (11). G418-selected K562 cells expressing high levels of murine CD19 were used to immunize Lewis rats, from which hybridomas were produced after fusion of splenocytes with NS-1 myeloma cells following standard procedures. One clone, 1D3, is a rat IgG$_{2a}\kappa$ and was selected for its ability to bind murine CD19-transfected K562, but not wild-type K562, cells. The antibody immunoprecipitates the expected protein of 110 kD from transfected cells (12).

Flow Cytometry. Flow cytometric analyses were performed as described below (13, 14). For cell sorting, bone marrow cells were stained with APC-labeled B220 together with biotin-labeled anti-CD19 revealed by PE–streptavidin. Stained cells were resuspended in PBS containing 2% FCS and 1 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) to exclude dead cells by gating in FL3 as described (15). Sorted cells were either used for in vitro cell culture or for further flow cytometric analyses. For in vitro cell culture, B220$^+$NK1.1$^+$, B220$^+$CD4$^+$, and B220$^+$CD19$^-$NK1.1$^-$CD4$^-$ cell populations were sorted. Therefore, bone marrow cells were stained with FITC-labeled anti-B220 together with either PE-conjugated anti-NK1.1, PE-conjugated anti-CD4, or a cocktail of PE-conjugated anti-NK1.1, anti-CD4, and anti-CD19. Sorted cell populations were routinely reanalyzed and showed >95% purity in all experiments showed herein. Cell sorting was performed on a FACStar® (Becton Dickinson & Co., Mountain View, CA), while a FACScan® (Becton Dickinson & Co.) was used for flow cytometric analyses.

Cell Culture and Generation of LAK Cells. All cell cultures were performed using IMDM (GIBCO-BRL, Life Technologies Ltd., Paisley, Scotland) containing 1× nonessential amino acids, 1× kanamycin (both from GIBCO-BRL), 5 × 10^{-5} M β-mercaptoethanol (Fluka AG, Buchs, Switzerland), 5 μg/ml porcine insulin (Sigma Chemical Co.), 0.03% Primatone RL (Quest International, Naarden, The Netherlands), and 2% FCS (GIBCO-BRL) as described earlier (16). For the generation of LAK cells, sorted cells were plated at 10^5 cells/ml in 1 ml culture medium supplemented with 10^3 U/ml of rIL-2 obtained from a transfected X63-Ag8.653 cell line (17) generated in our laboratory. After 5 d of culture, cells were harvested, washed, and counted for subsequent use either in FACS analyses or ^{51}Cr release assays. The recovery of rIL-2–activated NK1.1$^+$ cells after 5 d was generally 10–20 times the number of input cells.

Preparation of NK Targets and ^{51}Cr Release Assay. Target cells from normal or β$_2$mT mice were prepared by culturing spleen cells at 10^6 cells/ml in the presence of 2 μg/ml of Con A (Pharmacia) or 20 μg/ml of LPS (from *Escherichia coli* EH100, kindly provided by Dr. C. Galanos, Max Planck Institute for Immunobiology, Freiburg, Germany). After 2 d, the cells were harvested and enriched for viable blast cells by centrifugation onto a cushion of Ficoll-Paque (Pharmacia) according to the instructions of the manufacturer. Spleen cell blasts as well as YAC-1 Moloney leukemia virus–induced lymphoma cells (18) were adjusted to 10^7 cells/ml in medium containing FCS, of which 100 μl was labeled with 1 mCi of sodium–^{51}chromate (CJS 4, sp. act. 12.5 GBq/mg; Amersham Life Science, Amersham, UK) for 2 h at 37°C (19). After three washes with prewarmed medium, labeled target cells

were counted and concentrations adjusted to 10^5 cells/ml. Target cells were distributed in 50-μl aliquots (5,000 targets/well) into wells of V-bottom microtiter plates, which previously had received 50-μl replicas containing twofold dilutions in medium of effector cells so as to obtain different E/T cell ratios. To allow immediate contact between targets and effector cells, plates were spun for 1 min at 1,000 rpm followed by a 4-h incubation period at 37°C. After incubation, plates were spun at 1,200 rpm for 5 min, and 50 μl of supernatant was aspirated for counting in a gamma counter (Cobra 5010; Packard Instrument Company, Meriden, CT). Specific lysis was calculated as % specific lysis = $(E - S)/(T - S) \times 100$, where E was the experimental ^{51}Cr release, S was the count released when target cells were cultured alone, and T was the total counts contained in the target. SD of triplicate samples never exceeded 2.5%.

Limiting Dilution of Sorted Bone Marrow Cells. After sorting bone marrow cells into B220$^+$NK1.1$^+$, B220$^+$CD4$^+$, and B220$^+$CD19$^-$-CD4$^-$ cells, threefold dilutions of cells in 200 μl of medium containing 10^3 U/ml of rIL-2 were cultured in wells of round-bottom microtiter plates at 37°C in a 10% CO$_2$/air atmosphere. 48 cultures were set up with each dilution of sorted cells. After 5 d of incubation, all cultures received 25 μl of medium containing an average of three YAC-1 lymphoma cells. Plates were spun for 1 min at 1,000 rpm and incubated for an additional 10 d. Cultures containing growing YAC-1 cells were then scored by visual inspection and considered not to have contained any cell capable of developing into lytic NK cells. In control cultures, initially not containing any bone marrow cells, YAC-1 cells plated at three cells/culture showed growth in >95% of the wells. The frequency of functional NK cells responding to rIL-2 was calculated according to Poisson's distribution.

Isolation of RNA and Reverse Transcriptase PCR. Total RNA for cDNA synthesis was prepared from different fractions of freshly sorted bone marrow cells as well as 5-d cultured LAK cells using RNAzol B (Biotecx Laboratories, Inc., Houston, TX) according to the manufacturer's recommendations. RNA was reverse-transcribed using Superscript II reverse transcriptase (RT; GIBCO Life Technologies, Gaithersburg, MD), 1 mM dNTPs, 1 μg random hexameric oligonucleotides, and the supplied RT buffer. PCR assays were carried out using the following primer pairs: β-actin (20): 5′ GAA GTC TAG AGC AAC ATA GCA CAG CTT CTC 3′, 5′ GTG GGA ATT CGT CAG AAG GAC TCC TAT GTG 3′; V$_{preB}$ (21): 5′ GTC TGA ATT CCT CCA GAG CCT AAG ATC CC 3′, 5′ CAG GTC TAG AGC CAT GGC CTG GAC GTC TG 3′; B29 (22): 5′ TAA GTC TAG AAG TTC CGT GCC ACA GCT GTC 3′, 5′ CAC TGA ATT CCC AAG GAA GCC CTT GTT CCC 3′; perforin (23): 5′ ACC GAT GCT GAC CTG GGC CTC 3′, 5′ AGG TTC CTG AGG CCT GAC CGC 3′. Amplification of the cDNA was carried out with 1 cycle at 94°C for 30 s followed by 30 cycles at 94°C for 20 s, 55°C for 20 s, and 72°C for 60 s. The expected sizes of the PCR products are 535 bp for β-actin, 400 bp for V$_{preB}$, 320 bp for B29, and 610 bp for perforin. RT-PCR products were electrophoresed through a 1% agarose gel and visualized by UV light after ethidium bromide staining. In addition, after electrophoresis, the products of the perforin PCR were analyzed by Southern blotting after transfer to a Genescreen Plus (NEN Dupont, Boston, MA) membrane in 0.4 M NaOH. After neutralization with 0.2 M phosphate buffer (pH 7.2) and prehybridization at 42°C for 4 h, the membranes were hybridized at 42°C overnight with a ^{32}P-labeled oligonucleotide probe (protocol and probe kindly provided by Dr. H.-R. Rodewald, Basel Institute for Immunology, Basel, Switzerland). Both prehybridiza-

tion and hybridization were performed in 4 × SSC, 1 × Denhart's solution, and 0.1% SDS. Membranes were washed four times in 4 × SSC, 0.1% SDS, at 42°C for 10 min and exposed to x-ray film (X-OMAT; Eastman Kodak Co., Rochester, NY).

Results and Discussion

The Expression of B220(CD45R) and CD19 on Mouse Bone Marrow Cells. A mAb recognizing mouse CD19 has been used to identify cells in the mouse bone marrow that express this B lineage–related surface marker. Double staining, together with an antibody directed against the other mouse B lineage–related surface marker B220(CD45R), identified three populations in FACS® analyses of normal BDF$_1$ bone marrow: B220$^-$CD19$^-$ cells, B220$^+$CD19$^+$ cells, and B220$^+$CD19$^-$ cells. No B220$^-$CD19$^+$ cells could be detected by FACS® analyses (Fig. 1). The B220$^+$CD19$^+$ cells in normal mouse bone marrow contain all the cells belonging to different stages of B cell development, that is, c-kit$^+$CD43$^+$CD25$^-$ pro-pre–B-I cells (14), c-kit$^-$CD25$^+$ pre–B-II cells (24, 25), sIgM$^+$IgD$^-$ immature B cells, and sIgM$^+$IgD$^+$ mature B cells (26) (data not shown). Thus, all B lineage–committed cells in normal bone marrow identified so far express both B220 and CD19.

In bone marrow of RAG-2T mice, the same three populations are found: B220$^-$CD19$^-$ cells, B220$^+$CD19$^+$ cells, and B220$^+$CD19$^-$ cells (Fig. 1). Again, no B220$^-$CD19$^+$ cells could be detected, as in normal bone marrow. The B220$^+$CD19$^+$ fraction of cells in RAG-2T mice is reduced in size compared with bone marrow of normal mice, which is in agreement with a block of B cell differentiation at the transitional stage from pro/preB-I to pre–B-II cells due to the rearrangement deficiency. The size of the B220$^+$CD19$^-$ fraction of cells in the RAG-2T bone mar-


BDF1   RAG-2$^{-/-}$
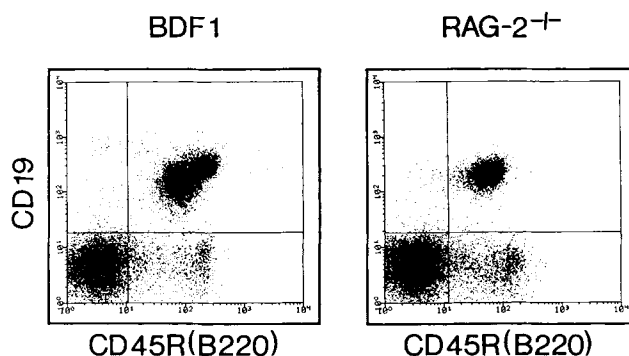
Figure 1. Both normal and RAG-2T bone marrow contain CD45R$^+$(B220)CD19$^-$ cells. FACS® analyses of normal BDF$_1$ and RAG-2T bone marrow after double staining for surface expression of CD45R(B220)CD19. Biotinylated anti-CD19 was revealed with PE–streptavidin in conjunction with FITC-labeled anti-B220.

row was determined and compared with that of normal mice. Therefore, bone marrow cells pooled from five RAG-2T mice and five age-matched normal mice at different times after birth (4, 10, and 15 wk) were analzyed. In all samples, independent of age and strain, 3–6% of all nucleated bone marrow cells were B220$^+$CD19$^-$ (data not shown).

Analysis of the Function(s) of B220$^+$CD19$^-$ Cells in Mouse Bone Marrow. It appeared reasonable to expect that the B220$^+$CD19$^-$ cell fraction in bone marrow (Fig. 1) could contain earlier B lineage precursors, before the expression of CD19. Therefore, we used culture conditions similar to those used for cloning and long-term proliferation of pro-pre–B-I cells (16), that is, a layer of stromal cells and added recombinant cytokines, in an attempt to convert the B220$^+$CD19$^-$ cells to B220$^+$CD19$^+$ stromal cell/IL-7–
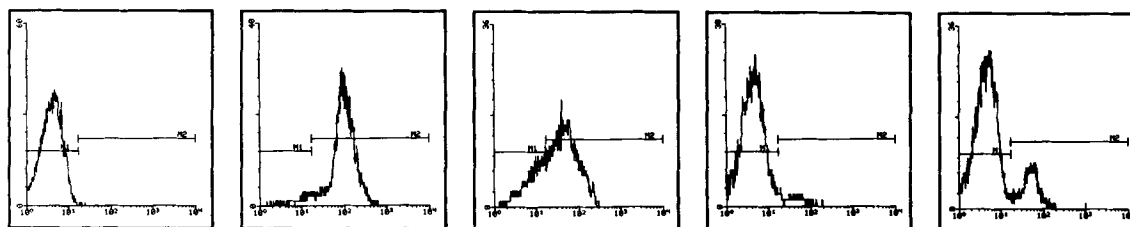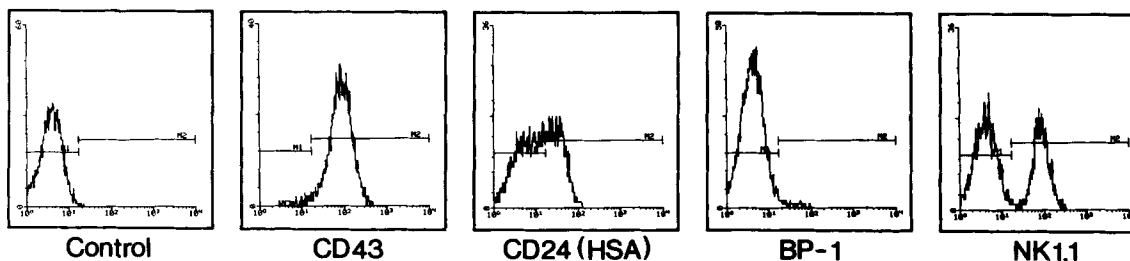
Figure 2. FACS® analyses of sorted B220$^+$CD19$^-$ bone marrow cells from BDF$_1$ and RAG-2T mice. Cells were double stained for B220 and CD19 and subjected to cell sorting as described in Materials and Methods. The sorted cells were restained and analyzed by FACScan® for the surface markers CD43, CD24 (HSA), BP-1, and NK1.1, as described in Materials and Methods. Since the restaining was done using biotinylated antibodies revealed by streptavidin–PE, the control graphs indicate background staining with PE–streptavidin only.

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reactive pre–B-I cells (27). rIL-2, rIL-3, rIL-4, rIL-5, rIL-6, rIL-7, and rIL-10 were tested in these experiments (17). IL-3, IL-5, IL-6, IL-7, and IL-10 did not change the phenotype or reactivity of the B220$^+$CD19$^-$ cells in the expected way. However, to our surprise, cells in cultures containing rIL-2 or rIL-4 within 5 d developed into apparent killer cells, destroying the coculture stromal cells. This occurred with B220$^+$CD19$^-$ cells from bone marrow of both normal and RAG-2T mice, excluding the participation of conventional cytotoxic T killer cells.

This prompted an examination of the expression of additional markers on sorted B220$^+$CD19$^-$ bone marrow cells. In Fig. 2, part of the extra markers expressed on B220$^+$-CD19$^-$ cells in the bone marrow of BDF$_1$ and RAG-2T mice are shown. In both strains, these cells express CD43 but not or only low levels of CD24 (HSA) and no BP-1 (Fig. 2) and therefore might contain cells defined by Hardy et al. (28) as fraction A in their scheme of B cell development. About 20–40% of the B220$^+$CD19$^-$ cells in the bone marrow of both BDF$_1$ and RAG-2T mice express the NK cell–related marker NK1.1 (29). This marker is not detectable on the B220$^+$CD19$^+$ cells (data not shown). The number of B220$^+$NK1.1$^+$ cells was in several experiments slightly higher (maximally twofold) in RAG-2T mice compared with age matched normal mice (see Fig. 2).

Sorting the B220$^+$CD19$^-$NK1.1$^+$ and the B220$^+$CD19$^-$-NK1.1$^-$ populations and subsequently analyzing their IL-2 reactivity revealed that only the B220$^+$CD19$^-$NK1.1$^+$, but not the NK1.1$^-$, cells developed into killer cells (see below). The B220$^+$CD19$^-$NK1.1$^+$ cells lack the expression of CD25 (the IL-2 receptor α chain), but do express CD122 (the IL-2 receptor β chain).
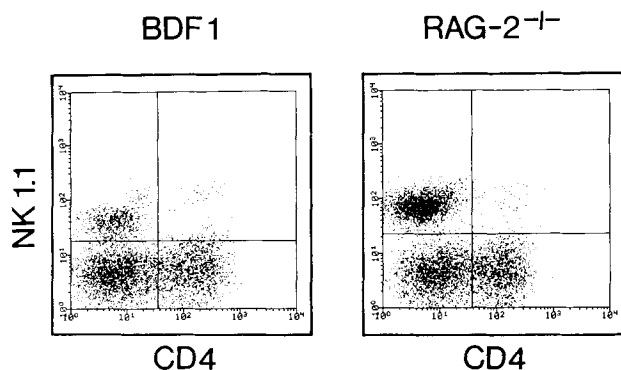
After culture in rIL-2, these cells remain B220$^+$CD19$^-$-NK1.1$^+$, but lose the expression of CD43 (Fig. 3). They are homogeneously large and granular as revealed by forward and sideward scatter analyses. All these cells coexpress the newly described NK cell surface marker 2B4 (30) and

low levels of CD122 (IL-2R β chain), but not CD25 (IL-2R α chain). A fraction of the cells express Mac-1 and another NK cell surface marker, 5E6 (31). In the bone marrow of normal mice, a fraction of the B220$^+$CD19$^-$-NK1.1$^+$ cells coexpress CD3ε, and their relative number stays constant during culture in rIL-2. Since no CD3ε-expressing B220$^+$CD19$^-$NK1.1$^+$ cells are present in the bone marrow of RAG-2T mice, these cells most likely represent the previously described NK1.1$^+$ T cells (32, 33).

We conclude that by marker expression and morphology these activated B220$^+$CD19$^-$NK1.1$^+$ bone marrow cells are similar to NK cells found in the periphery and classified as LGL (34). It has been shown previously that stimulation of spleen cells with IL-2 (35) or IL-4 (36) may generate lytically active NK1.1$^+$ cells that coexpress B220. However, fresh ex vivo, sorted B220$^+$CD19$^-$NK1.1$^+$ bone marrow cells do not cause lysis of the stroma cells in a short-term assay, nor do they kill classical NK targets, like YAC-1 (see below). We were concerned that the anti-NK1.1 mAb (PK136) used for sorting these cells would inhibit spontaneous killer activity, as has been reported previously (37). Therefore, freshly isolated total bone marrow cells from RAG-2T animals were used in the standard ^{51}Cr release assay. At E/T cell ratios up to 300:1 we observed no significant lysis of YAC-1 tumor cells, in accordance with previously published data (38, 39). Also, in assays with rIL-2–activated NK1.1 cells, addition of up to 100 μg/ml of PK136 mAb would not inhibit lysis of YAC-1 tumor targets or normal LPS- or Con A–activated blasts of β$_2$mT origin. We emphasize, though, that we used bone marrow of healthy, unmanipulated mice, whereas most other investigators demonstrating NK activity of cells freshly isolated ex vivo used spleen cells from animals pretreated with interferon inducers like polyinositol:polycytidine (37) or tilorone (40). It therefore appears that the 5-d cell culture period in the presence of rIL-2 or rIL-4 differentiated NK activity from more undifferentiated, inactive precursor cells.
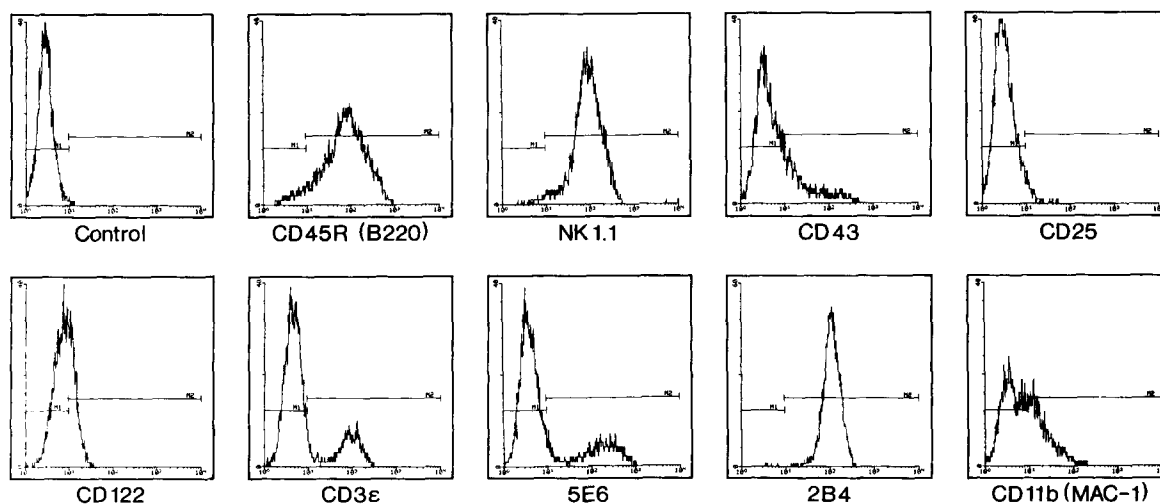
Figure 3. Cell surface phenotype of rIL-2–activated NK1.1$^+$ bone marrow cells from BDF$_1$ mice. Cells sorted into the B220$^+$CD19$^-$NK1.1$^+$ fraction was cultured for 5 d in the presence of 10^3 U/ml of rIL-2. Staining and FACScan® analyses were performed as described in Materials and Methods.

BDF1 **RAG-2⁻/⁻**

NK 1.1 (vertical axis) / CD4 (horizontal axis)

Killer activity of IL-2 activated B220⁺(CD45R)

a NK1.1⁺ bone marrow cells

b Number of sorted cells plated

Figure 4. A large fraction of B220⁺CD19⁻NK1.1⁻ bone marrow cells express CD4. Bone marrow cells from normal BDF₁ and RAG-2T mice were sorted for B220⁺/CD19⁻ cells as described in Materials and Methods. The collected cells were restained with FITC-labeled anti-CD4 and PE-labeled anti-NK1.1 and analyzed by FACScan®.

The subpopulation of the B220⁺CD19⁻NK1.1⁻ cells in bone marrow that do not give rise to NK cells were further analyzed. As shown in Fig. 4 in both normal BDF₁ as well as RAG-2T mice, this population can be subdivided based on the expression of CD4. Thus, ~50% of the B220⁺-CD19⁻NK1.1⁻ cells express CD4. The level of CD4 expression is comparable to that found on T cells. Since these cells do not express CD3 or any other component of TCR (data not shown) and moreover are present in identical numbers in RAG-2T mice, they are not B220⁺ T cells. So far we have not observed any effects of rIL-2, rIL-3, rIL-4, rIL-5, rIL-6, rIL-7, or GM-CSF on these cells in vitro. Currently the potential of these cells is analyzed by "in vivo" transfer systems.

The B220⁺CD19⁻NK1.1⁻CD4⁻ population might contain some early B cell progenitors, since preliminary RT-PCR analyses revealed low expression of λ_5, V_{preB}, mb-1, and B29 genes in these cells (data not shown). Moreover, limiting dilution experiments showed that 1/100 to 1/200 of these cells can form pre–B cell colonies in the presence of rIL-7. Also, the potential of these cells is currently being analyzed by in vivo transfer systems.

Most NK1.1⁺B220⁺CD19⁻ Cells Are Precursors of Classical NK Cells and Respond to IL-2 by Development into Clones of Cytolytic NK Cells. Classical NK cells kill MHC class I⁻ target cells (40, 41), and are inhibited in their killing activity by the expression of MHC class I molecules on target cells (42). Therefore, a variety of LPS- or Con A–activated spleen cells were used as targets in ⁵¹Cr release cytotoxicity assays. Syngeneic, semisyngeneic, or allogeneic targets from normal mice were not significantly lysed by these rIL-2–activated NK1.1⁺ cells at E/T ratios of 10:1 and below. However, when Con A– or LPS-activated targets were generated from β₂mT mice lacking MHC class I surface expression, syngeneic and allogeneic targets were lysed to the same extent irrespective of whether the targets were of T or B cell origin, often reaching 30–60% specific lysis at E/T ratios of 1:1 during the 4-h assay period (Fig. 5 a). Always included in the ⁵¹Cr release assays was the prototypic NK target YAC-1, which typically showed 60–80% spe-
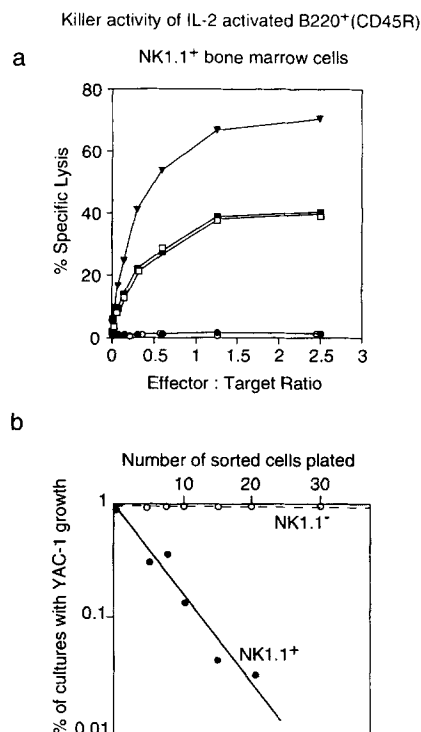
Figure 5. Sorted, rIL-2–activated NK1.1⁺ cells kill the prototypic target YAC-1 as well as normal targets lacking MHC class I expression, but do not kill normal targets expressing MHC class I. (a) Conventional 4-h ⁵¹Cr release cytotoxic assay performed as described in Materials and Methods. The effectors were derived after 5 d of culture in rIL-2 of B220⁺CD19⁻NK1.1⁺ bone marrow cells sorted from normal BDF₁ animals. The targets were YAC-1 (▼-▼), LPS blasts (●, ■) or Con A blasts (○, □) from normal BALB/c spleen (●, ○) or BALB/c–β₂mT spleen (■, □). (b) Limiting dilution analyses of B220⁺CD19⁻NK1.1⁺ (●) and B220⁺CD19⁻NK1.1⁻ (○) bone marrow cells sorted from RAG-2T mice. For each concentration of sorted cells, 48 microwell cultures were seeded. After 5 d of culture in the presence of rIL-2 (10³ U/ml), an average of 3 YAC-1 cells were added to each culture, and the incubation was continued for an additional 10 d. Cultures showing clones of growing YAC-1 tumor cells were scored by visual inspection. The data points represent the fraction of cultures showing growth of tumor cells out of 48 cultures seeded.

cific lysis at similarly low E/T ratios. The fact that we observe a plateau of specific lysis different for the tumor target compared with the splenocyte targets may be due to our way of calculating the percentage of specific lysis: the total cpm of ⁵¹Cr contained within the targets was used rather than attempting to determine total counts releasable by artificial means using agents like SDS, NP-40, or acetic acid. These results support the conclusion reached by analysis of NK1.1 expression that the cytotoxic activity developed from B220⁺CD19⁻NK1.1⁺ cells of bone marrow is conveyed by NK cells.

Since only 60–80% specific ⁵¹Cr release could be obtained in the cytotoxic assay using YAC-1 as a target, we devised a method to determine the viability of the tumor target cells by a test that measures their growth after contact with the effector cells. For that purpose, limiting numbers of YAC-1 targets were distributed into cultures already containing IL-2–activated B220⁺CD19⁻NK1.1⁺ effector

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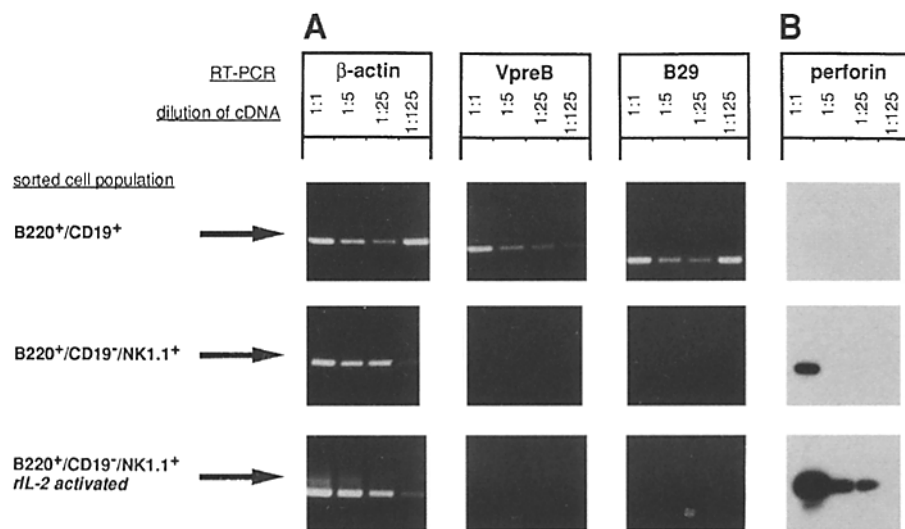
Figure 6. Expression of mRNA for V_{preB}, B29, and perforin in sorted B220$^+$CD19$^+$, B220$^+$CD19$^-$NK1.1$^+$, and rIL-2–stimulated B220$^+$CD19$^-$NK1.1$^+$ cells from bone marrow of RAG-2T animals. Total RNA isolation and RT-PCR assays were performed as described in Materials and Methods. Different dilutions of cDNA were subjected to PCR amplification specific for β-actin, V_{preB}, B29, and perforin. Dilutions of the resulting products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized by UV light illumination (A). In addition, the products of the PCR assay for perforin were blotted and hybridized with an internal probe of the following sequence: 5' AGG CCA GAG GCA AAC ATG CG 3' specific for perforin (B).

The figure labels in panel A:

cells. At estimated E/T ratios of 10–20:1, no YAC-1 tumor cells would grow out. This method was further used as a read-out system in limiting dilution analyses to determine the frequency of sorted B220$^+$CD19$^-$NK1.1$^+$ cells responding to rIL-2 by developing into clones of cells capable of killing limiting numbers of YAC-1 tumor cells. We observe straight lines in semilogarithmic plots scoring the number of cultures yielding growing YAC-1 target tumor cells against dilutions of previously added sorted cells stimulated for 5 d with rIL-2 (Fig. 5 b). According to Poisson's distribution, an average of one NK1.1$^+$ cell is present in a culture and responds to rIL-2 when 37% of the cultures do not show any growing YAC-1 tumor cells. From the recovery of cells in our previous mass culture experiments, we can expect one NK1.1$^+$ cell responding to rIL-2 to yield a clone of 10–30 cells after 5 d of culture. As can be seen in Fig. 5 b, using limiting numbers of sorted B220$^+$-CD19$^-$NK1.1$^+$ cells added to rIL-2–containing cultures for 5 d before adding the YAC-1 cells, an average of five NK1.1$^+$ cells have to be plated in order to achieve that degree of tumoricidal effect. Thus, the NK1.1$^+$ cells respond to rIL-2 and develop into clones of NK cells with lytic capability. In contrast, sorted B220$^+$CD19$^-$NK1.1$^-$ cells did not give rise to cytolytic cells (Fig. 5 b), even when plated at 10^4 cells/well (data not shown).

B220$^+$CD19$^-$NK1.1$^+$ Cells Lack Expression of Genes Specific for B Cell Lineages. Bone marrow cells from RAG-2T animals were sorted into B220$^+$CD19$^+$ and B220$^+$CD19$^-$-NK1.1$^+$ cells. In addition, NK1.1$^+$ cells were activated into killer cells by culture for 5 d in the presence of rIL-2.

RNA was extracted from freshly sorted as well as cultured cells. Subsequently, expression of mRNA for V_{preB}, B29, and perforin was analyzed by RT-PCR. A variety of oligonucleotide primers spanning an intron–exon boundary described in Materials and Methods were used. As can be seen in Fig. 6, only the B220$^+$CD19$^+$ and not the B220$^+$CD19$^-$-NK1.1$^+$ or the rIL-2–activated B220$^+$CD19$^-$NK1.1$^+$ cells express the B cell lineage marker V_{preB} and *B29* genes, whereas only rIL-2–activated, not freshly isolated NK1.1$^+$, cells express abundant levels of perforin mRNA, a distinguishing marker for mature cytolytic T and NK cells (23).

Conclusions. The data presented here show that not all B220$^+$ cells belong to the B cell lineage. Within the B220$^+$CD19$^-$ cells at least three subpopulations can be distinguished. The first, expressing NK1.1, has been identified here in detail to be the precursor of NK cells. This cell is likely to be a product of an earlier NK1.1$^-$ precursor, which, upon transplantation, develops into NK1.1$^+$ cells capable of hybrid resistance (43). The second, expressing CD4, has a potential and/or function that is so far unknown. Within the third group of cells, which are NK1.1$^-$CD4$^-$, are those that, upon culture on stromal cells and IL-7, form pre–B colonies. Precursors for other blood cell lineages might also be contained within this group of cells, but attempts to clone them with the appropriate cytokines have so far failed. All of these B220$^+$ subpopulations of cells express CD43 and are low or negative for HSA and BP-1 expression, markers that define fraction A in Hardy et al.'s (28) scheme of B cell differentiation.

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