The Generation of Natural Killer (NK) Cells from NK Precursor Cells in Rat Long-Term Bone Marrow Cultures

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Summary

In this report, we describe a novel long-term bone marrow culture (LTBMC) system to study the origin and generation of natural killer (NK) cells from NK precursors. Rat bone marrow was cultured for 4 wk in RPMI 1640 with 5% fetal calf serum and 2-mercaptoethanol to allow the formation of an adherent stromal cell layer containing NK precursor cells. After addition of interleukin 2 (IL-2), the LTBMC generated high numbers (up to 100-fold expansion in 7 d) of pure $3.2.3^+$ large granular lymphocytes with lytic activity against NK-sensitive and -resistant tumor targets, as well as antibody-dependent cellular cytotoxicity. NK activity in LTBMC could be detected 3 d after addition of as little as 1 U/ml rIL-2, whereas lymphokine-activated killer activity was found 5 d after addition of at least 10 U/ml rIL-2. In vivo depletion and in vitro complement lysis studies showed that the NK precursor cells in LTBMC did not express the NK-associated surface markers asialo GM_1 or 3.2.3. We also found that LTBMC cells did not exhibit colony growth in granulocyte/macrophage or spleen colony-forming unit assays. The generation of NK cells from NK precursors required, in addition to IL-2, a second growth/maturation factor(s), which was present in the conditioned medium of the LTBMC. This LTBMC system provides a unique in vitro model to study the development of NK cells from precursor cells, the role of the bone marrow stromal microenvironment in this development, and the lineage relationship of NK cells to other hematopoietic cells.

N K cells are a subpopulation of LGL with the capacity to recognize and lyse certain neoplastic or virally infected cells, as well as certain microorganisms (1–3) without previous sensitization (4, 5) or MHC restriction (6, 7). NK cells do not express TCR or the CD3 complex on their cell surface and do not rearrange functional TCR (8–11), but do express a characteristic pattern of differentiation markers such as CD16 and CD56 (humans) (12–14), NK1.1 and asialo GM_1 (mice) (15, 16), and 3.2.3 and OX8 (rats) (17–19). It is well established that NK cells are derived from bone marrow $(BM)^1$ progenitor cells and require the BM microenvironment for their maturation (20–23). The exact lineage relationship of NK cells to other hematopoietic cells such as T cells or myeloid cells has been controversial. Although NK cells express certain characteristic surface markers such as CD56 or NK1.1, they also share certain markers with T cells (CD2, CD8) (19,

24) and neutrophils (CD16, 3.2.3) (12, 17). Studies with both nude (25, 26) and SCID (27, 28) mice, each of which are genetically incapable of generating mature T cells, have suggested that NK cells do not require thymic processing for their maturation. However, it has not been possible to unequivocally rule out that NK cells are pre-T cells before entry into the thymus. Other studies have proposed that NK cells differentiate along a myeloid differentiation pathway and may represent promonocyte-like cells (29). Finally, it has been suggested that NK cells derive from pluripotent stem cells or a common lymphoid progenitor cell in an independent NK lineage (28). To clarify the lineage relationship of NK cells to other hematopoietic cells, the current study has examined the characteristics of NK precursor cells, enriched in vitro using a modified Whitlock-Witte long-term bone marrow culture (LTBMC) system. The addition of rIL-2 to LTBMC resulted in the rapid expansion of virtually pure populations of LGL that exhibited high levels of NK and antibody-dependent cellular cytotoxicity (ADCC) activity and a surface marker phenotype characteristic of activated rat NK cells. No evidence could be obtained that cells in the LTBMC could

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differentiate along myeloid lineages or contained early progenitor cells, as they were incapable of forming myeloid colonies in vitro or spleen colonies in vivo. Although IL-2 induced the growth and differentiation of mature NK cells from the NK precursors, this response required the concomitant presence of a stromal-derived growth factor(s). Further studies using this LTBMC culture system should be useful in the analysis of NK precursor cells and their lineage characteristics.

Materials and Methods

Animals. Male Fischer 344 rats (75–100 g) were purchased from Taconic Farms, Inc. (Germantown, NY) and were housed in a specific pathogen-free animal facility at the Pittsburgh Cancer Institute for at least 10 d before use. The 5.19/SB mice, previously bred by Dr. Beverly White (NIH, Bethesda, MD), were brother-sister line bred since 1978 by Dr. Sallie S. Boggs in the animal facility of the Department of Radiation Oncology (University of Pittsburgh). They were selected for this study because they were able, after lethal irradiation, to support spleen colony formation (S-CFU) by rat BM cells.

Culture Medium (CM) and Tumor Cells. Standard CM consisted of RPMI 1640 supplemented with 5% FCS, 2 mM glutamine, and antibiotics (penicillin/streptomycin). For LTBMC and for the growth of BM in IL-2, CM was additionally supplemented with freshly prepared 5×10^{-5} M 2-ME. The lysis of the NK-sensitive Moloney virus–induced mouse T cell lymphoma YAC-1 was used as an indicator of NK activity (30). The NK-resistant mastocytoma P815 (31) was used as an indicator of lymphokine-activated killer (LAK) activity and as the target in ADCC assays. Tumor cell lines were grown in CM and subcultured two to three times per week.

IL-2. Human rIL-2 was kindly provided by the Cetus Corp. (Emeryville, CA) and contained 2.5×10^6 U of IL-2/mg of protein (as defined by [³H]thymidine incorporation into CTLL-2 cells).

LTBMC. BM was harvested from the femurs and tibias of F344 rats in CM and pressed through a fine wire mesh to remove particulate debris. Generally, 1.5–2×10^8 BM cells were recovered from both femurs and tibias of a 100 g F344 rat. The BM cells were washed once in CM and cultured at 2×10^6 cells/ml in T25 flasks in CM containing 2-ME. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2/95% air without agitation for 4–6 wk, and were then designated LTBMC. When LTBMC were used to generate NK cells, IL-2 was added. The cultures were then incubated at 37°C for 1–10 d, and the cells were harvested by decanting the medium and adding 3 ml of 5 mM cold EDTA for 2 min. The EDTA-detachable cells were added to the decanted cells and washed twice to remove the EDTA. These cells were then used for surface marker analysis, morphology, and cytotoxicity assays.

Antibodies. The following anti-rat mAbs were used: OX7 (Thy-1.1), OX8 (CD8), OX19 (CD5), OX34 (CD2), OX39 (CD25; Tac), OX41 (macrophages, granulocytes, and dendritic cells), and W3/25 (CD4) (all purchased from Bioproducts for Science [Indianapolis, IN]). R73 (which identifies the rat TCR-α/β) was kindly provided by T. Hunig (Ludwig Maximillians University, Munich, FRG) (32). IF4 (which recognizes rat CD3) was a gift from Y. Hashimoto (Tohoku University, Sendai, Japan) (33). Rabbit anti–asialo GM₁ serum was obtained from Wako Chemical (Dallas, TX) and anti-rat IgG was obtained from Cappel Laboratories, Malvern, PA. mAb

3.2.3 (LGL/NK cells, PMN cells) was produced as previously described (17).

For fluorescence staining, each of the various antibodies was used at an optimal dilution based on preliminary dose-response titrations. All second-step antibodies were R-phycoerythrin (RPE)- or FITC-labeled F(ab')₂ fragments of goat antibody against the primary antibody (Cappel Laboratories).

Flow Cytometry. For surface marker analysis, 2.5×10^5 cells in 0.1 ml of staining buffer (0.1% sodium azide and 2% FCS in PBS, pH 7.3) were incubated with various antibodies for 30 min at 4°C. The cells were washed twice with staining buffer and resuspended in FITC-labeled second antibody. After 30 min at 4°C, the cells were again washed twice with staining buffer, resuspended in 1% paraformaldehyde, and analyzed for fluorescence intensity on a FACStar flow cytometer (Becton Dickinson & Co., Mountain View, CA).

For two-color analysis, cells were first stained with the primary antibody and washed. They were then incubated with PE-labeled second-step antibody and again washed. Finally, cells were incubated with FITC-labeled F(ab')₂ fragments of mAb 3.2.3 and then washed, resuspended in paraformaldehyde, and analyzed.

For complement lysis, cells (3×10^7 cells/ml in RPMI 1640) were incubated with anti–asialo GM₁ (1:25 dilution) for 40 min at 4°C, washed once, and then incubated for 40 min at 37°C in the presence of a 1:8 dilution of baby rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada). Cells were washed two times in CM and subsequently cultured as described for LTBMC. Complement lysis with established LTBMC (4 wk) was performed in the culture flask as described above. The efficacy of the anti–asialo GM₁ antisera + C' to eliminate NK activity was tested by treating spleen cells with anti–asialo GM₁ + C', which resulted in virtually complete elimination of NK activity, as described previously (34).

In Vivo Treatment with 3.2.3 or Anti–asialo GM₁. In vivo NK depletion was performed with mAb 3.2.3 (35) or anti–asialo GM₁ (34, 36), as described previously. Briefly, F344 rats were injected intraperitoneally with 1 ml of mAb 3.2.3 ascites (1:10 in HBSS; containing a total protein concentration of 1 mg/ml) once a day for three consecutive days or with 0.04 ml of anti–asialo GM₁ antisera (1:25 in HBSS) once a day for two consecutive days. On day 4, rats were killed and spleen and BM cells were harvested. The efficacy of the in vivo NK depletion was shown by the elimination of splenic NK activity as tested in a 4-h ^{51}Cr-release assay against YAC-1 target cells (data not shown).

Evaluation of Cell Morphology. Morphologic differentials were determined microscopically from Giemsa-stained cytocentrifuge slides. At least 200 cells were examined by two independent observers for each slide.

Cytotoxicity Assay. Cytotoxicity was measured in a standard 4-h ^{51}Cr-release assay using 96-well, round-bottomed microplates (Costar, Cambridge, MA). The target cells were labeled with 100 μCi of Na₂^{51}CrO₄ per 2×10^6 cells, washed, and seeded at 5×10^3 cells/well. Suspensions of effector cells were then added to triplicate wells to give various E/T ratios in a final volume of 200 μl. After an incubation at 37°C for 4 h, 100 μl of supernatant was removed from each well and was counted in a gamma counter to determine experimental release. Spontaneous release was obtained from wells receiving target cells and medium only, and total release was obtained from wells receiving 1% Triton X-100. The spontaneous release never exceeded 15% of the total release. Percent cytotoxicity was calculated by the following formula: percent cytotoxicity = 100 × [(experimental release − spontaneous release)/(total release − spontaneous release)]. Assays of ADCC were per-
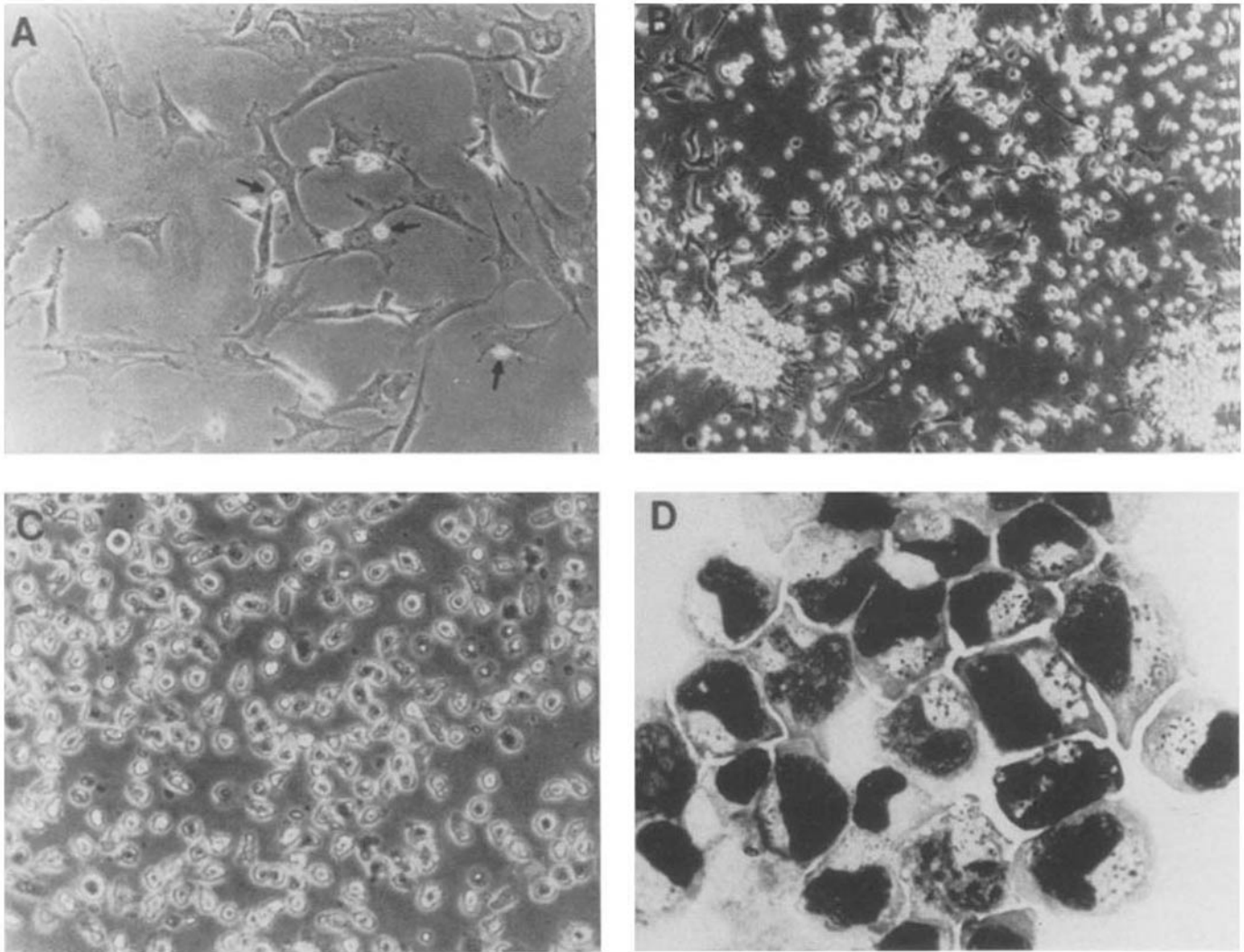
Figure 1. Photomicrographs of LTBMC (4 wk) before and after additional culture for 1 wk with 1,000 U/ml IL-2. (*A*) A characteristic LTBMC (4 wk) with adherent stromal layer cells and highly luminescent small lymphocyte-like cells (*arrows*), which are either attached to the stromal cells or floating in the culture medium. After 5 d of culture with IL-2, cell nests have formed in the stromal layer and many adherent or floating LGL are present (*B*). After 10 d of culture with IL-2, the stromal layer is no longer detectable and the culture consists mainly of LGL (*C*). (*D*) A photomicrograph of a Giemsa-stained cytospin preparation from a LTBMC after 10 d in IL-2. All cells have the characteristic LGL morphology (reniform nucleus and azurophilic granules). Photographs were taken at the following magnifications: 100× (*B*), 200× (*A* and *C*), and 1,000× (*D*).

formed as described above, with additional pretreatment of the P815 targets with 10 μl of rat anti-P815 serum for 30 min.

LUs of cytotoxic activity were determined from linear regression curves plotted from cytotoxicity values at various E/T ratios. In all cases, 1 LU was defined as the number of effector cells required to cause 20% specific ^{51}Cr release from 5×10^3 target cells.

Granulocyte/Macrophage-CFU (GM-CFU) Assay. Various quantities of fresh BM cells or LTBMC cells were cultured in 1 ml enriched RPMI 1640 medium (37) containing 20% FCS, 10% leukocyte-conditioned medium (from 5-d cultures of Con A–stimulated spleen cells), and 40% methylcellulose (2.25%) in 35-mm plastic petri dishes (5% CO_2, 37°C). GM-CFU colonies, defined as granulocytic, monocytic, or eosinophilic aggregates of >20 cells, were scored on day 7 under an inverted microscope.

S-CFU Assay. The spleen colony formation assay with rat BM cells in lethally irradiated 5.19/SB mice was performed as previously

described (38). Briefly, mice were exposed to lethal irradiation (950 rad) and injected intravenously with rat BM cells or LTBMC cells within 3 h. Spleens were harvested 10 d later and subsequently weighed and placed in Bouin's fixative for 24 h before the numbers of visible colonies were counted.

Statistics. All statistical analysis was performed using the student's *t* test.

Results

Morphological Analysis of the LTBMC. We and others have previously reported that the addition of rIL-2 to unfractionated populations of fresh BM results in the generation of cells mediating NK activity (39–41). This occurred in BM specifically depleted of mature NK cells, indicating that the generation of NK cells by rIL-2 was dependent upon the ac-

tivation of NK precursor cells in the bone marrow. To further investigate the generation of mature NK cells from NK precursor cells, as well as to determine the role of the BM microenvironment in this process, we established a modified Whitlock-Witte LTBMC. BM from F344 rats was cultured for 4 wk in RPMI 1640 with 5% FCS and 5×10^{-5} M 2-ME to allow the formation of an adherent stromal layer that consisted primarily of macrophages, endothelial cells, large blanket cells, and undefined highly luminescent small round cells (Fig. 1 *A*).

The addition of rIL-2 to the 4-wk LTBMC (without removing its conditioned medium) resulted in the formation of nests of proliferating cells within the stromal cell layer (Fig. 1 *B*). The first signs of cellular proliferation (as determined microscopically) were noted ~3 d after the addition of IL-2. After 9–10 d, the LTBMC contained high numbers of actively proliferating cells (Fig. 1 *C*). Note that by this time no stromal layer cells were detectable. These proliferating cells had the characteristic LGL morphology, including a reniform nucleus and azurophilic granules (Fig. 1 *D*).

Kinetics of the Generation of NK Cells from LTBMC. The generation of NK cells from LTBMC was monitored by determination of cytolytic activity against the NK-sensitive tumor target YAC-1 at various intervals after the addition of 1,000 U/ml rIL-2 to the cultures. In addition, LAK activity was also measured as cytolytic activity against the NK-resistant tumor target P815. Fig. 2 shows that NK activity was detectable after 3 d of culture with 1,000 U rIL-2/ml, whereas LAK activity was first detectable after 5 d of culture with rIL-2. Cytolytic activity peaked by day 5 and remained present thereafter. As many as 10^7 cells could be generated from a single T-25 flask (which contained at the time of addi-

tion of rIL-2 ~10^5 total cells) representing at least a 100-fold expansion in 7 d. After refeeding and further culture of the LTBMC, we were able to generate in 12 d up to 10^8 cells with high levels of cytolytic activity from a single T-25 flask. NK activity could be generated in LTBMC by the addition of as little as 1 U/ml rIL-2, and LAK activity by the addition of as little as 10 U/ml rIL-2 (Fig. 3). The extent of proliferation was also dependent on the level of added IL-2. While 100 U/ml induced maximal cellular expansion and cytolytic activity, significant levels of cell proliferation were also noted at 1 U/ml IL-2. ADCC activity was also found after stimulation of LTBMC with only 1 U/ml rIL-2, and was detectable after 3 d of culture with IL-2, concomitant with the generation of NK activity (data not shown).

Phenotypic Analysis of the Induced NK Cells from NK Precursors in LTBMC after 7 d of Culture with IL-2. 4-wk-old LTBMC, which were cultured for an additional 7 d in the presence of 1,000 U/ml IL-2, were analyzed for surface phenotype by flow cytometry and morphology in Giemsa-stained cytocentrifuge slides. We found that virtually all cells generated in these LTBMC cultures brightly expressed the NK-associated 3.2.3 antigen (17) and had characteristic LGL morphology (Table 1). No evidence for surface Ig+ B cells was obtained. Two-color FACS analysis (Fig. 4) showed that the 3.2.3-positive cells were negative for T cell–specific antigens (CD3, CD4, CD5, or TCR-α/β) and macrophage/granulocyte–related antigens (OX41, not shown). Only a low expression (8%) of OX39, which recognizes the p55 low affinity IL-2-R (CD25, Tac), was found. However, the 3.2.3-positive cells did coexpress characteristic NK-associated antigens (18, 19), such as asialo GM₁ (48%), CD2 (64%), and CD8 (40%). We noted that these NK-associated antigens showed a de-
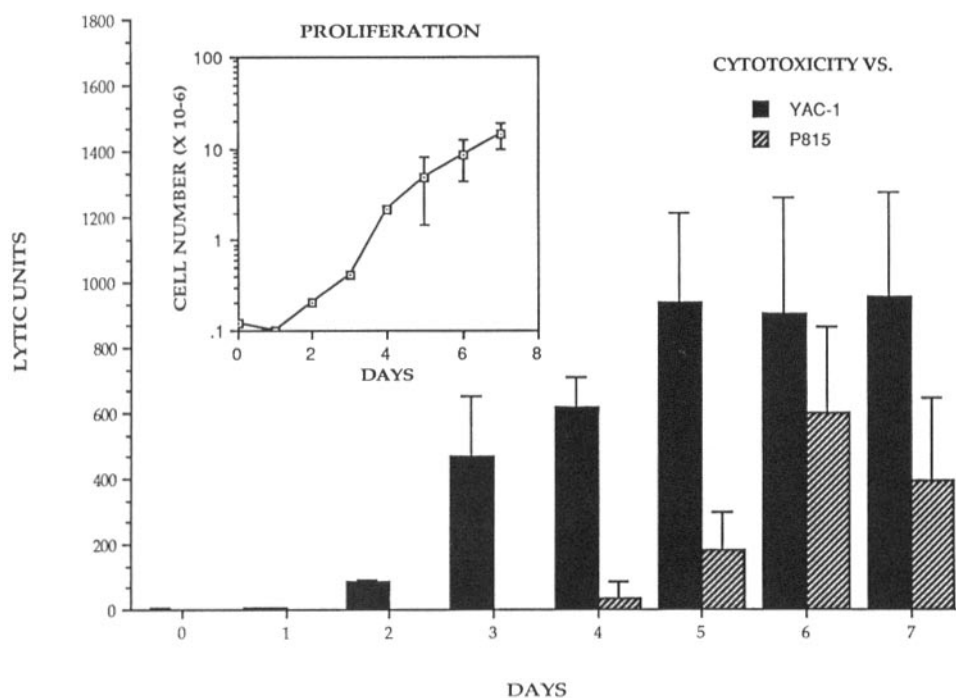

Figure 2. Kinetics of proliferation and NK and LAK activity in LTBMC after addition of 1,000 U/ml rIL-2. NK activity was determined as cytolytic activity against the NK-sensitive tumor target YAC-1 in a 4-h ^{51}Cr-release assay, and LAK activity as cytolysis of the NK-resistant P815 tumor target. Cytolysis is expressed as $LU_{20}/10^7$ cells (see Materials and Methods). Proliferation was determined by counting the number of floating cells in culture at different intervals. Results are shown as mean cytolysis or cell number ± SD.
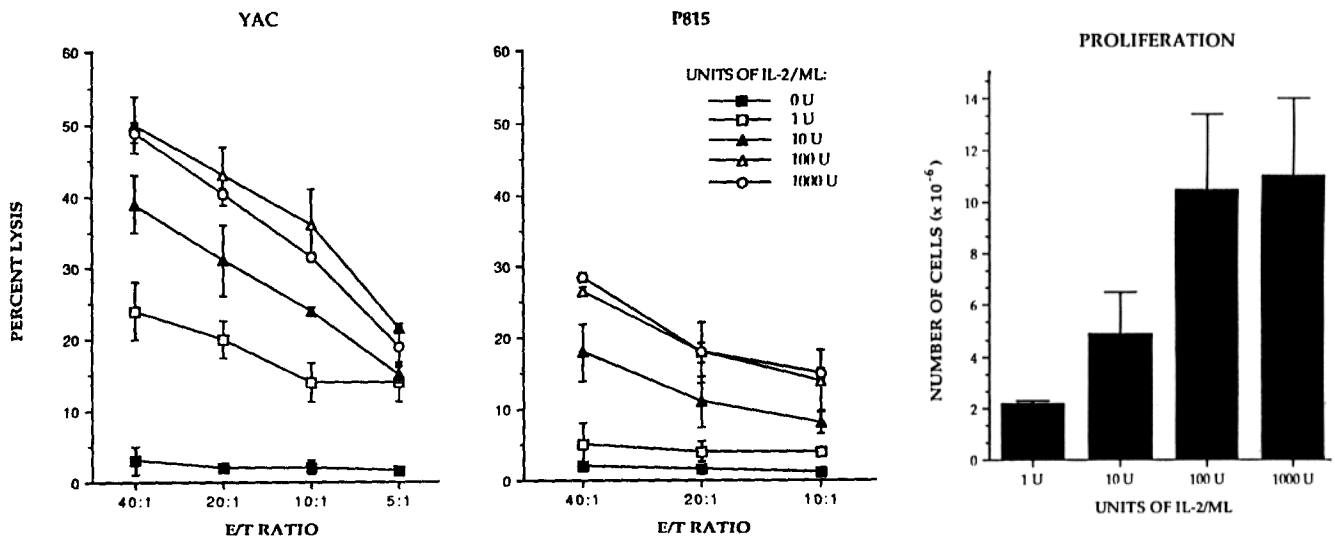
Figure 3. Dose-response relationship for rIL-2 and the generation of NK and LAK activity from LTBMC. After addition of various doses of rIL-2, LTBMC (4 wk) were cultured for 1 wk and subsequently counted and tested for NK and LAK activity against YAC-1 and P815 tumor targets, respectively, in a 4-h ^{51}Cr-release assay. Mean cytotoxicity ± SD and mean number of cells per culture ± SD from at least two cultures is shown.

Table 1. *Phenotype and Percentage of LGL in Cells Generated from LTBMC after 7 d of Culture with rIL-2*

Surface marker	CD equivalent	Percentage of positive cells
3.2.3	NK cells	96 ± 3*
Asialo GM₁	NK cells, subpopulations of macrophages and cytotoxic T cells	61 ± 12
OX 34	CD2	48 ± 16
OX 8	CD8	37 ± 6
IF4	CD3	1 ± 1
R73	TCR-α/β	2 ± 2
W3/25	CD4	0.8 ± 0.5
OX 19	CD5	1 ± 1
OX 39	CD25	6 ± 5
OX 7	Thy-1.1	6 ± 1
OX 41	Macrophages, granulo-cytes, and dendritic cells	1 ± 1
sIgG	B cells	0 ± 0
LGL		98.5 ± 0.7‡

4-wk-old LTBMC were cultured for 1 wk with 1,000 U/ml rIL-2, and then all floating and EDTA-detachable cells were analyzed for the expression of cell surface markers by flow cytometric analysis and for the percentage of LGL in Giemsa-stained cytospin preparations.
* Mean percentage ± SD from 2-10 experiments.
‡ Mean percentage ± from two experiments.

crease in expression when the IL-2-induced NK cells were cultured for a longer time, whereas the expression of the 3.2.3 antigen remained constant (data not shown).

Characteristics of the NK Precursor Cell in the LTBMC. To determine the phenotype of the NK precursor cell in LTBMC, we treated normal BM with anti–asialo GM₁ plus C' before initiation of the LTBMC, or we treated 4-wk-old LTBMC with anti–asialo GM₁ plus C'. Each of these cultures was subsequently incubated for 7 d in the presence of 1,000 U/ml IL-2. In all cases, treatment with anti–asialo GM₁ + C' did not significantly affect the development of cytolytic activity compared with nontreated or C'-treated control cultures (Table 2). We also established LTBMC from rats that were depleted of endogenous NK activity by in vivo treatment with anti–asialo GM₁ (34, 36, 42) or 3.2.3 (35). Table 3 shows that NK and ADCC activity present in fresh BM, as well as in spleen cells (data not shown), from these NK-depleted rats was completely eliminated. However, LTBMC established from these rats were not impaired in their ability to generate NK and LAK activity after 7 d of culture with 1,000 U/ml IL-2. From these experiments, we conclude that the NK precursor cells in the LTBMC do not express 3.2.3 or asialo GM₁ markers.

Role of the LTBMC-conditioned Medium in the Generation of NK Cells. To further analyze the role of the LTBMC culture conditions in the generation of NK cells from precursor cells, we replaced the medium in the LTBMC (which we have termed LTBMC-conditioned medium) with fresh medium before the addition of IL-2. In these experiments, the nonadherent cells in the LTBMC-conditioned medium were always spun down and added back to the culture, to ensure that LTBMC with either conditioned medium or fresh medium
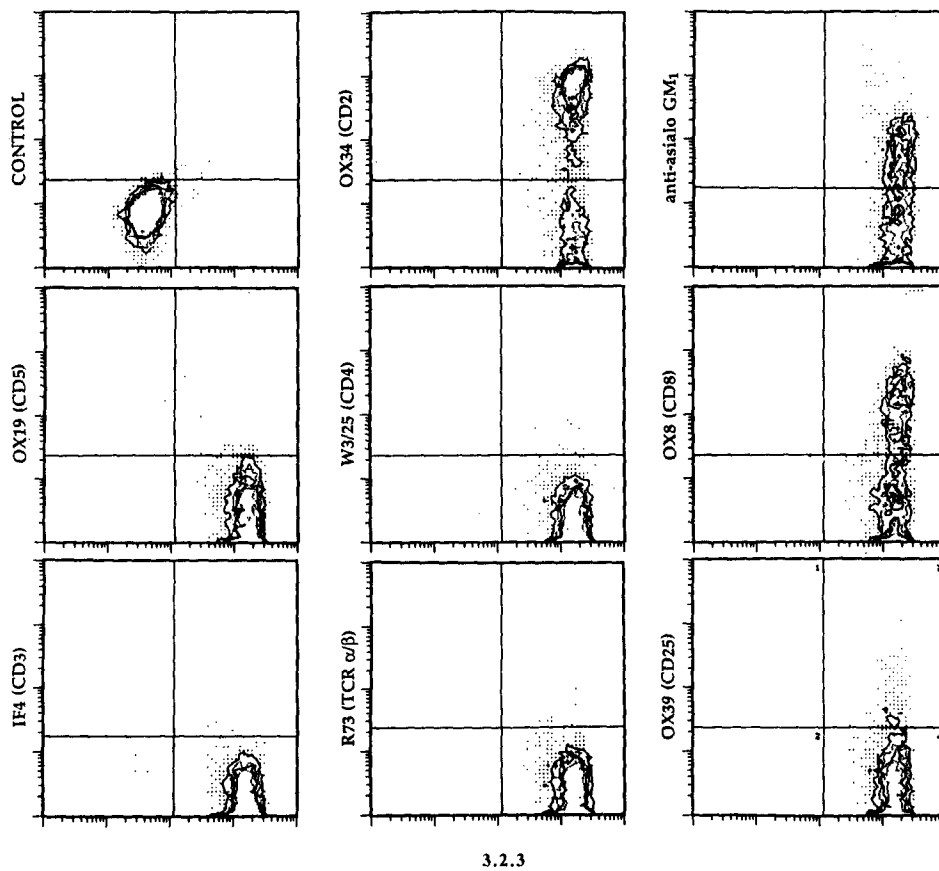
Figure 4. Two-color cytofluorometric analysis of cells from a LTBMC (4 wk) after culture for 1 wk with 1,000 U/ml IL-2. This experiment was repeated five times and representative results are depicted. In each panel, fluorescence intensity of the cells for 3.2.3-FITC is shown on the abscissa and for various subset markers with a second-step RPE-conjugated antibody on the ordinate. Virtually all cells were positive for 3.2.3 (98%) and double-staining cells were only found with OX34 (64%), anti-asialo GM_1 (48%), and OX8 (40%).

contained the same cells. While optimal growth of NK cells was obtained with 100% LTBMC-conditioned medium, exchanging the LTBMC-conditioned medium with fresh medium almost completely abrogated the ability to generate NK and LAK activity in a 5-d culture with IL-2 (Fig. 5). Additional experiments showed that the growth-promoting effects of LTBMC-conditioned medium were concentration depen-

dent (Table 4). This indicates that the NK precursor cells in LTBMC require (in addition to IL-2) another factor(s) that is contained in the LTBMC-conditioned medium and supports their responsiveness to IL-2. Further studies indicated that this soluble factor(s) appears to be actively secreted in the LTBMC, since it could be shown that LTBMC, in which the LTBMC-conditioned medium had been replaced by fresh

Table 2. NK and LAK Activity from LTBMC after Treatment with Anti-asialo GM_1 and C'

Pretreatment of cells				Lytic activity after 7-d culture with rIL-2			
Fresh BM		LTBMC (4 wk)		YAC-1		P815	
Anti-asialo GM_1	C'	Anti-asialo GM_1	C'	20:1*	10:1	20:1	10:1
−	+			46 ± 10‡	38 ± 11	41 ± 7	35 ± 9
+	+			51 ± 10	46 ± 8	40 ± 9	32 ± 5
		−	+	48 ± 7	35 ± 7	24 ± 2	17 ± 4
		+	+	46 ± 10	38 ± 10	25 ± 5	17 ± 7

Rat BM cells were either first treated with anti-asialo GM_1 antiserum (1:25) and C' (1:8) and subsequently cultured as LTBMC, or first cultured as LTBMC and then treated with anti-asialo GM_1 and C'. Finally, all cells were cultured for 7 d with 1,000 U/ml rIL-2 and tested for cytolytic activity in a 4-h ^{51}Cr-release assay against YAC-1 and P815 tumor targets.
* E/T ratio.
‡ Mean percentage ± SD of at least three cultures.

Table 3. *NK, ADCC, and LAK Activity in LTBMC after In Vivo NK Depletion*

| Treatment of rats | Lytic activity of fresh bone marrow | | | | Lytic activity of LTBMC + rIL-2 | | | |
| | YAC-1 | | ADCC* | | YAC-1 | | P815 | |
	100:1‡	50:1	– Ab	+ Ab	10:1	5:1	10:1	5:1
Control IgG	7 ± 5§	5 ± 3	0 ± 0	8 ± 4	34 ± 6	24 ± 5	17 ± 4	10 ± 2
3.2.3	0 ± 0	0 ± 0.6	0 ± 1	0 ± 1	35 ± 2	29 ± 2	21 ± 4	10 ± 3
Asialo GM₁	ND	ND	ND	ND	34 ± 8	31 ± 7	18 ± 4	8 ± 4

Rats were treated for three consecutive days with 0.1 ml i.p. of mAb 3.2.3 ascites or control IgG ascites in HBSS. On day 4, BM cells were isolated and cultured as LTBMC. After 4 wk, 1,000 U/ml rIL-2 was added and the cells were cultured for another week. NK, ADCC, and LAK activity of fresh BM cells and LTBMC + rIL-2 cells was determined in a 4-h ^{51}Cr-release assay against YAC-1 and P815 tumor targets.
* ADCC was determined as cytolytic activity against P815 in the presence of rat anti-P815 antiserum at an E/T ratio of 100:1.
‡ E/T ratio.
§ Mean percentage ± SD of at least two cultures.

medium, would also begin to generate appreciable levels of NK activity when the incubation with IL-2 was prolonged (e.g., 8 d instead of 5 d).

Colony Formation of LTBMC Cells in GM-CFU and S-CFU Assays. To determine whether LTBMC contained myeloid progenitor cells, we cultured these cells in methylcellulose in the presence of 10% leukocyte-conditioned medium containing GM-CSF. Colony growth was determined 7 d later. As shown in Table 5, LTBMC contained no detectable GM-CFU progenitor cells, whereas whole rat BM consistently
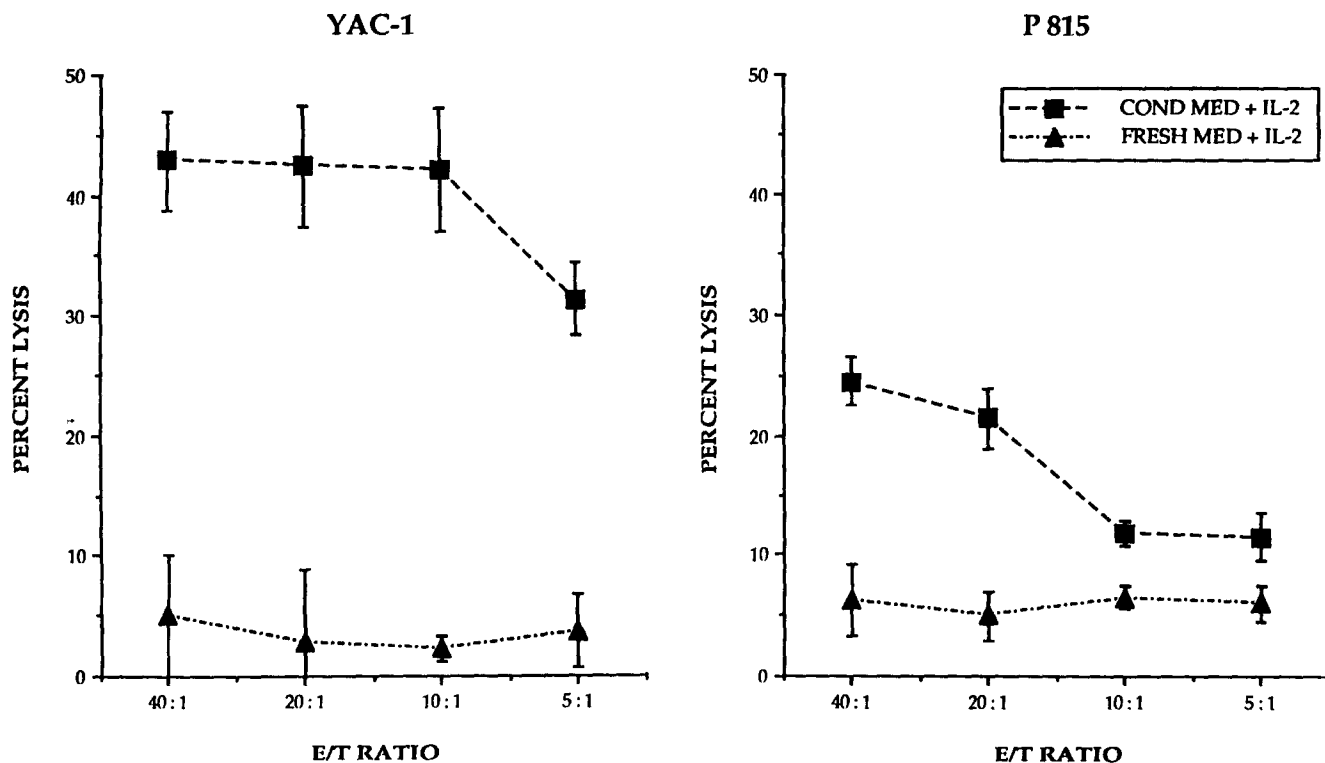

Figure 5. Effect of replacement of conditioned medium by fresh medium on the generation of NK cells from LTBMC. LTBMC (4 wk) were established, and in one group of cultures, the conditioned medium was replaced by fresh medium before adding 1,000 U/ml rIL-2. All nonadherent cells in the LTBMC-conditioned medium were spun down and added back. The remaining cultures received IL-2 together with their conditioned medium. After 5 d, NK and LAK activity was determined against YAC-1 and P815 tumor targets, respectively, in a 4-h ^{51}Cr-release assay. Mean cytotoxicity ± SD from four cultures is shown.

Table 4. *Requirement of Conditioned Medium for the Generation of NK Cells from LTBMC in Response to IL-2*

Percentage of conditioned medium	No. of cells in LTBMC after culture with IL-2	LU$_{20}$/10^7 cells YAC-1	LU$_{20}$/10^7 cells P815	LU$_{20}$/culture YAC-1	LU$_{20}$/culture P815
	× 10^{-5}				
0	4.5*	24‡	0	1	0
10	9	38	9	3	1
25	12	16	0	2	0
50	19	75	12	14	2
100	42	280	252	118	106

Conditioned medium was collected from 4-wk-old LTBMC (LTBMC-CM) and passed through 0.22-μm filters to remove cells and debris. Subsequently, the CM from other 4-wk-old LTBMC was collected, and the cells were pelleted by centrifugation. The pelleted cells were then suspended in varying concentrations of LTBMC-CM, diluted in fresh RPMI 1640 with 5% FCS and 2-ME (ranging from 100% to 0% in a total volume of 10 ml), and added back to the original LTBMC with IL-2. The cultures were then incubated for 5 d in the presence of 1,000 U IL-2/ml before all cells were harvested, counted, and tested for cytolytic activity against YAC-1 and P815 tumor targets in a 4-h ^{51}Cr-release assay. The results of a representative experiment of six experiments are shown.
* Mean number of cells from two cultures.
‡ Mean LU from two cultures.

contained detectable levels of GM-CFU, which was at least 100 times higher than that present in the LTBMC cultures.

The possible presence of early progenitor cells in LTBMC was examined by performing S-CFU assays in lethally irradiated mice. As shown in Table 6, no spleen colonies were found after injection of LTBMC cells, in contrast to appreciable colony formation by fresh BM cells.

Table 6. *Colony Formation of LTBMC Cells in S-CFU Assays*

Cells injected Type	No.	No. of animals injected	S-CFU
	× 10^{-5}		
Fresh BM	5	17	27 ± 13*
	1	5	13 ± 6
LTBMC cells	1	16	0 ± 0
	0.1	7	0.4 ± 1
	0.01	6	0 ± 0
No cells‡		20	0 ± 0

S-CFU assays were performed as described in Materials and Methods. Varying doses of fresh rat BM cells or LTBMC (4 wk) cells were injected intravenously in lethally irradiated (950 rad) 5.19.SB mice, and spleen colonies were counted 10 d later.
* Mean number of colonies ± SD.
‡ Irradiated control animals.

Discussion

Several studies have shown that mature NK cells are derived directly from progenitor cells present in the BM without the obligatory need for extramedullary tissue sites, and that they are dependent on an intact marrow environment for their maturation from these progenitor cells (20–23, 25–28).

In this report, we describe an LTBMC that enriches NK precursor cells in order to study the origin and generation of NK cells from these BM precursors. LTBMC, established from rat BM, were generated by incubation of whole BM at 37°C for 4 wk (without refeeding) in Whitlock-Witte-type CM (43). This resulted in death of most cells, leaving only ~1% of the input population. This remaining population of cells consisted of an adherent stromal layer that contained macrophages, endothelial cells, and blanket cells, as described by Whitlock and Witte (43), and undefined highly

Table 5. *Colony Formation of LTBMC Cells in GM-CFU Assays*

Exp.	Cells	No. of cells plated 10^5	10^4	10^3
1	Fresh BM	90, 98, 113* (100 ± 12)	7, 8, 14 (10 ± 4)	2, 2, 2 (2 ± 0)
	LTBMC (4 wk)	0, 0, 0 (0 ± 0)	0, 0, 0 (0 ± 0)	0, 0, 0 (0 ± 0)
2	Fresh BM	18, 29, 32, 38 (29 ± 8)	3, 5, 6, 11 (6 ± 3)	0, 0, 1, 1 (0.5 ± 0.6)
	LTBMC (4 wk)	0, 0, 0, 0 (0 ± 0)	0, 0, 0, 0 (0 ± 0)	0, 0, 0, 0 (0 ± 0)

GM-CFU assays were performed as described in Materials and Methods, using fresh BM cells or LTBMC (4 wk) cells. Various concentrations of cells were plated in 35-mm plastic dishes, and after a 7-d incubation (5% CO$_2$, 37°C), the numbers of colonies were counted in each dish.
* Numbers of colonies per dish (mean ± SD).

luminescent small round cells (Fig. 1 *A*). Although the CM for these LTBMC is analogous to that used for Whitlock-Witte cultures, we found no evidence for spontaneous B cell production. This is most likely due to the nature of our LTBMC as representing starvation cultures, while Whitlock-Witte cultures are refed twice weekly. Previously, lymphoid culture systems have been described for pre-B cells by Whitlock and Witte (43) and for terminal deoxynucleotidyl transferase–positive lymphoid precursor cells by Hayashi et al. (44). Both systems share common features, such as the use of FCS and incubation at 37°C, as opposed to the Dexter culture system (45), which uses horse serum, hydrocortisone and incubation at 33°C. The Dexter culture selectively maintains S-CFU and GM-CFU, and contains only low levels of lymphoid precursor cells (46), whereas the culture systems of Whitlock and Witte (43) and Hayashi et al. (44) allow the maintenance of lymphoid precursor cells and seem not to be able to support S-CFU and GM-CFU (44). Also in our LTBMC, we were not able to detect S-CFU or GM-CFU.

The addition of IL-2 to these LTBMC generated a characteristic and reproducible pattern of cell growth, which consisted of three phases. In the first 3 d (first phase), little proliferative activity was noted. From day 4 to 10 (second phase), active proliferation ensued (2–3 logs of expansion), with the development of cells exhibiting high levels of non-MHC-restricted cytotoxicity. These cells express only NK-associated antigens (3.2.3, asialo GM₁, CD2, and CD8) and had characteristic LGL morphology. Beyond 9–10 d (third phase), the LTBMC contained a virtually pure population of proliferating LGL, with complete loss of the adherent stromal cell layer. The disappearance of the stromal layer could be due to detachment from the plastic or to cytolysis by the highly active LGL in the LTBMC. Studies to distinguish between these two possibilities are currently in progress.

Kinetic studies and IL-2 dose-response studies revealed the following differences in the generation of NK and LAK activity from LTBMC: (*a*) NK activity was detectable by 3 d after addition of IL-2, while LAK activity was not present until day 5; (*b*) the generation of NK activity from LTBMC could be observed after the addition of as little as 1 U/ml rIL-2, whereas LAK activity required at least 10 U/ml rIL-2. Two-color FACS and morphological analysis clearly showed that the LTBMC after culture with IL-2 contained a purely 3.2.3⁺-proliferating LGL, which displayed both NK and LAK activity. These results suggest that NK and LAK activity appears to be derived from a common precursor in the LTBMC and that these cytolytic activities are most likely sequentially related along a common differentiation and/or activation pathway that is controlled by IL-2.

We have identified the NK precursor cells in the LTBMC as asialo GM₁ negative and 3.2.3 negative by complement lysis and in vivo depletion studies. LTBMC cells do not give rise to colony growth in in vitro GM-CFU assays (Table 5) and in in vivo S-CFU assays (Table 6). This suggests that the NK generation in LTBMC is not derived from pluripotent stem cells or myeloid precursor cells.

This is the first study to demonstrate the direct generation of NK cells from enriched precursor cells in a LTBMC system using a modified Whitlock-Witte culture method. Our study also confirms the pivotal role of the stromal microenvironment for NK maturation. The importance of an intact stromal microenvironment of the marrow in vivo for the differentiation of NK cells has previously been shown, since mice treated with high levels of estrogen as well as congenitally osteopetrotic mi/mi mice, all of which have altered marrow stroma, produce deficient maturation of NK cells (21, 23, 47). Moreover, when BM is destroyed with the bone-seeking isotope ⁸⁹Strontium, splenic NK activity is abolished, whereas CTL generation and macrophage-mediated cytotoxicity remain intact (20, 22, 48). We suggest that the role of the stroma in controlling NK cell growth is at least partially due to its ability to produce growth factor(s) that allow the precursor cells to acquire the IL-2-responsive state. This is based upon our findings that replacement of the conditioned medium by fresh medium in the LTBMC, before adding IL-2, markedly delayed or even prevented NK generation. Adding back varying proportions of conditioned medium yielded a dose-dependent proliferation and NK generation.

In summary, we have developed a LTBMC system that allows, upon activation with IL-2, the study of the origin and generation of NK, ADCC, and LAK activity from early 3.2.3-negative and asialo GM₁-negative precursor cells. We believe that this LTBMC system will be useful in further studies regarding the differentiation and lineage of NK cells from BM precursor cells and the role of the BM stromal microenvironment and additional growth factors in the generation of NK cells.

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