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Authors

Williams, Noelle Sevilir
Moore, Thomas A
Schatzle, John D
et al.

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Brief Definitive Report

Generation of Lytic Natural Killer 1.1$^+$, Ly-49$^-$ Cells from Multipotential Murine Bone Marrow Progenitors in a Stroma-free Culture: Definition of Cytokine Requirements and Developmental Intermediates

By Noelle Sevilir Williams,* Thomas A. Moore,‡ John D. Schatzle,* Igor J. Puzanov,* P.V. Sivakumar,* Albert Zlotnik,‡ Michael Bennett,* and Vinay Kumar*

*From the *Laboratory of Molecular Pathology, Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9072; and ‡Immunology Department, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California 94304-1104*

Summary

We have developed a stroma-free culture system in which mouse marrow or thymus cells, known to be enriched for lymphoid progenitors, can be driven to generate natural killer (NK) cells. Culture of lineage marker (Lin)$^-$, c-kit$^+$, Sca2$^+$, interleukin (IL)-2/15Rβ (CD122)$^-$ marrow cells in IL-6, IL-7, stem cell factor (SCF), and flt3 ligand (flt3-L) for 5–6 d followed by IL-15 alone for an additional 4–5 d expanded the starting population 30–40-fold and gave rise to a virtually pure population of NK1.1$^+$, CD3$^-$ cells. Preculture in IL-6, IL-7, SCF, and flt3-L was necessary for inducing IL-15 responsiveness in the progenitors because the cells failed to significantly expand when cultured in IL-15 alone from the outset. Although culture of the sorted progenitors in IL-6, IL-7, SCF, and flt3-L for the entire 9–11-d culture period caused significant expansion, no lytic NK1.1$^+$ cells were generated if IL-15 was not added, demonstrating a critical role for IL-15 in NK differentiation. Thus, two distinct populations of NK progenitors, IL-15 unresponsive and IL-15 responsive, have been defined. Similar results were obtained with Lin$^-$, CD44$^+$, CD25$^-$, c-kit$^+$ lymphoid progenitors obtained from adult thymus. The NK cells generated by this protocol lysed the NK-sensitive target YAC-1 and expressed markers of mature NK cells with the notable absence of Ly-49 major histocompatibility complex (MHC) receptors. However, despite the apparent lack of these inhibitory MHC receptors, the NK cells generated could distinguish MHC class I$^+$ from class I$^-$ syngeneic targets, suggesting the existence of novel class I receptors.

NK cells express two families of receptors, which in the murine system are known as NKR-P1 and Ly-49 (1). The NKR-P1 molecule is expressed on all NK cells of certain mouse strains and may play a role in triggering NK cytotoxicity. Conversely, the Ly-49 receptors, which recognize class I MHC molecules, are expressed on subsets of NK cells and inhibit NK-mediated lysis.

Despite increasing knowledge of NK cell function and target cell recognition, differentiation of NK cells from hematopoietic stem cells is poorly understood. NK cells are known to be bone marrow derived, and early work suggested an important role for the marrow microenvironment in generating mature lytic NK cells (2). To dissect the steps of NK differentiation, several in vitro systems have been established that allow the development of lytic NK cells from CD34$^+$ human cells (3–6) or unseparated rodent bone marrow (reviewed in reference 7). However, in many of these systems, the starting populations were heterogeneous, containing pluripotent stem cells and progenitors at different stages of development. In addition, the NK cells that developed were not fully characterized, especially with respect to the expression and function of class I MHC receptors.

Although IL-2 has frequently been used to support NK development, the failure to detect the IL-2 gene product within bone marrow stroma (8) and the presence of NK cells in IL-2$^{-/-}$ mice (9) strongly suggests that cytokines other than IL-2 participate in NK cell differentiation in vivo. The newly described cytokine IL-15 has been shown to use both the common γ chain (γc) and IL-2Rβ as components of its receptor (10), and it is produced by bone marrow stromal cell cultures (8). In addition, IL-15 supports development of NK cells from human CD34$^+$ stem cells and murine fetal thymic cells (8, 11) and causes terminal differ-

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entiation of immature, nonlytic NK1.1$^+$ cells found in the spleen of marrow ablated mice (12).

In the present report, we describe an in vitro culture system yielding virtually pure populations of lytic NK1.1$^+$ cells from lineage (Lin)$^-$, c-kit$^+$, and Sca2$^+$ multipotent progenitors in the absence of stroma. The data show that a mixture of early acting cytokines and IL-15 play sequential and important roles in the differentiation of NK cells from these marrow progenitors.

Materials and Methods

Animals. 7–12-wk C57BL/6 mice were bred at The University of Texas Southwestern Medical Center (Dallas, TX) and used for bone marrow progenitor experiments. 4–5-wk (C57BL/6 × BALB/C)F1 mice (Jackson Labs., Bar Harbor, ME) were used for thymic progenitor experiments.

mAbs. Except as noted below, all mAbs and their isotype controls were obtained from PharMingen (San Diego, CA). Anti-gp49B1 FITC (B23.1) was the gift of Dr. H. Katz (Harvard University, Boston, MA), anti-Ly-49G2 (4D11) and anti-Ly-49D (12A8) were provided by Dr. J. Ortaldo (National Cancer Institute, Frederick, MD), anti-Ly-49A (JR9-318) was the gift of Dr. J. Roland (Institut Pasteur, Paris, France), and anti-Sca2 hybridoma supernatant was provided by Dr. G. Spangrude (University of Utah, Salt Lake City, UT). Goat anti–rat γ Texas red (Southern Biotechnology Assoc., Birmingham, AL) or streptavidin-Red670 (GIBCO BRL, Gaithersburg, MD) was used to detect some primary antibodies.

Cell Preparation, Isolation, and Analysis of Precursor Cells. Cell suspensions of bone marrow and thymocytes were prepared as previously described (13, 14). Staining was performed on ice in PBS/5 mM EDTA at 3×10^6 cells/ml. For three-color sorting of bone marrow progenitors, cells were incubated with anti-FcγRIII/II (2.4G2) and then with a mixture of lineage-specific biotinylated antibodies: anti-B220 (RA3-6B2), anti-CD2 (RM2-5), anti-Gr1 (RB6-8C5), anti-CD11b (M1/70), and anti-NK1.1 (PK136) followed by streptavidin magnetic beads (Miltenyi Biotec Inc., Auburn, CA). After washing, the cells were passed over a CS column (Miltenyi Biotec Inc.) to remove Lin$^+$ cells. The Lin-depleted population was then stained with streptavidin-Red670, c-kit (2B8) PE, and Sca2 (thymic shared antigen [TSA]1) FITC. Lin-Red670$^-$, c-kit PE$^+$, Sca2 FITC$^+$ cells of lymphoid and blast size were then sorted on a FACStar Plus® flow cytometer (Becton Dickinson, San Jose, CA) using Lysis II software. The identification and isolation of CD4^{lo} (CD44$^+$, CD25$^-$, c-kit$^+$, Lin$^-$) has been previously described (14).

For four-color analysis of marrow progenitors, cells were stained sequentially with anti-Sca2 hybridoma supernatant, goat anti–rat γ Texas red, 10% normal rat serum, the cocktail of biotinylated Lin-specific mAbs described above, and finally a mixture of streptavidin-Red670, c-kit FITC, or PE and either CD44 (IM7) PE, IL-2/15Rβ (TM-β1) PE, HSA (M1/69) FITC, or CD4 (H129.19) FITC. Analysis of cultured cells was performed by sequential staining with 2.4G2, a biotinylated mAb, and finally streptavidin-Red670 plus an FITC- and/or PE-conjugated mAb.

Culture Conditions. Sorted Lin$^-$, c-kit$^+$, Sca2$^+$ marrow cells were cultured in 96-well U-bottomed plates (Falcon, San Jose, CA) at 8,000–10,000 cells/well in 0.2 ml of complete RPMI-1640 (10% FBS, 100 U/ml streptomycin, 1 mM sodium pyruvate, 2 mM glutamine, 1× nonessential amino acids, 50 μg/ml gentamicin, 5×10^{-5} M 2-mercaptoethanol, and 1 μg/ml Indomethacin) plus 20 ng/ml murine IL-15 (a gift from Dr. Tony Trout, Immunex R&D Corp., Seattle, WA) or a mixture of 20 ng/ml murine IL-6 (PharMingen), 0.5 ng/ml murine IL-7 (PeproTech, Rocky Hill, NJ), 50 ng/ml rat stem cell factor (SCF; a gift from Amgen, Thousand Oaks, CA), and 100 U/ml murine flt3 ligand (flt3-L; DNAX, Palo Alto, CA). The cells were refed with the same media on day 3, and then on day 5 or 6 the cultures were harvested, washed, counted, and replated at 10,000–15,000/well in complete RPMI containing IL-15 alone or the mixture of cytokines described above. After an additional 3 d, the cultures were refed with the same media, and on day 11 or 12 of total culture time, the cells were harvested for analysis. Sorted Lin$^-$, CD44$^+$, CD25$^-$, c-kit$^+$ thymic progenitors were cultured as previously described (15), and the two-step culture protocol described above for marrow progenitors was followed.

Target Cells and Cytotoxicity Assays. Tumor targets used were YAC-1 (H-2^a); RMA (H-2^b); a transport associated with antigen processing (Tap)-2 mutant derivative of RMA, RMA-S (H-2^b); and a Tap-2 transfectant of RMA-S, Q11 (H-2^b; reference 16), which was a kind gift of Dr. J. Monaco (University of Cincinnati, Cincinnati, OH). All cell lines were maintained in supplemented RPMI-1640 (17); Q11 additionally received 1 mg/ml G418. Target cells were used in a 4-h ^{51}Cr cytotoxicity assay, and percent specific lysis was expressed as the mean ± SEM of triplicate wells and calculated as described earlier (17).

RNA Extraction, PCR Amplification, and Southern Analysis. Total RNA was prepared from whole cells using a modification of the Chomczynski and Sacchi method. Reverse transcription was performed using the Superscript II system (GIBCO BRL) and PCR using the GIBCO BRL PCR kit with standard PCR buffer (1.5 mM $MgCl_2$) for 35 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 1 min). Primers were designed from the published IL-15Rα sequence (18). Under these conditions, the forward primer (CTCCAGGCTGACACCATC) and reverse primer (CATGGTTTCCACCTCAACACGGCA) yield a 282-bp product. PCR products were transferred to Hybond N plus (Amersham Life Science Corp., Arlington Heights, IL) and probed with an end-labeled internal IL-15Rα oligo (GAGAACGTCGTTGTTACTG) at 55°C. IL-2–cultured NK cells served as a positive control for IL-15Rα message, and RNA integrity was confirmed by amplification of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH).

Results and Discussion

Isolation and Characterization of a Lin$^-$, c-kit$^+$, Sca2$^+$ Bone Marrow Population. We previously identified a multipotential bone marrow population characterized as Ly6 (Sca1)$^+$, Lin$^-$, c-kit (CD117)$^+$, CD43^{hi}, Fall-3^{hi}, Sca2 (TSA1)$^-$, AA4^{lo}, and Rh123^{hi} with both lymphoid and myeloid repopulating abilities in vivo (13). Upon adoptive transfer, this population also gives rise to NK cells (13). To define a population capable of giving rise to NK cells in vitro, a marker more restricted to the lymphoid lineage was sought. TSA-1, also known as Sca2 (19), was previously found to delineate a population of Lin$^-$, Thy-1^{lo}, HSA (CD24)$^+$ bone marrow cells enriched for lymphoid (T and B cell) repopulating ability, but essentially devoid of pluripotent stem cells (20). Because NK cells may share a common progenitor with T and B cells (21) and may indeed derive from an NK/T precursor (11, 22), we hypothesized that a marrow population expressing Sca2 may also contain NK progenitors. Thus,

Table 1. *Expansion and Generation of NK Cells from Lin$^-$, c-kit$^+$, Sca2$^+$ Bone Marrow Progenitors Cultured in IL-6, IL-7, SCF, and flt3-L ± IL-15*

Primary culture		Secondary culture			
Cytokines	Yield*	Cytokines	Yield*	Total Yield‡	NK1.1$^+$
					%
IL-15	0.3	IL-15	1.0	0.3	ND
6/7/SCF/flt3-L	9.4	6/7/SCF/flt3-L	8.7	81.8	2
6/7/SCF/flt3-L	9.4	IL-15	3.8	35.7	85

Sorted progenitors were cultured in the two-part culture system as described in Materials and Methods. NK1.1 expression was assessed using PE-labeled anti-NK1.1 mAb (PK136) from PharMingen. The data are representative of six experiments.

*Yield represents the fold increase in total cell number.

‡Total yield is the product of the yield in the primary and secondary cultures.

a Lin (CD2, B220, Gr1, CD11b, NK1.1)$^-$, c-kit$^+$, Sca2$^+$ population, representing ~1% of whole bone marrow, was isolated. Four-color analysis revealed that these cells were CD44^{hi}, HSA^{int}, and CD4$^-$ (data not shown). The phenotype of this population is similar to a CD4^{lo}, CD8$^-$, CD3$^-$, CD44$^+$, Sca2$^+$, c-kit$^+$, CD25$^-$ lymphoid progenitor from thymus (23) and to a related CD4$^{-/lo}$, Lin$^-$, Thy1^{lo}, HSA^{int}, CD44^{hi}, and Sca2$^+$ population isolated from bone marrow with predominant lymphoid and limited myeloid repopulating abilities (20).

Culture of Lin$^-$, c-kit$^+$, Sca2$^+$ Multipotent Progenitors. To determine the ability of this "lymphoid-enriched" progenitor population to give rise to NK cells, sorted Lin$^-$, c-kit$^+$, Sca2$^+$ cells were cultured with IL-15 for 9–11 d. However, these cells failed to expand significantly in IL-15 alone (Table 1). Cell recovery on average (n = 6) was only 1.8-fold over the input numbers of cells, but these cells did lyse the NK-sensitive tumor YAC-1 (Fig. 1). It is possible that a small number of the progenitors are truly capable of responding to IL-15 alone. We do not favor this hypothesis because cell surface expression of IL-2/15Rβ was not detected on the sorted population (Fig. 2 *B*), and the β chain is usually required for signal transduction (24). Alternatively, this growth may represent contamination by rare mature NK cells.

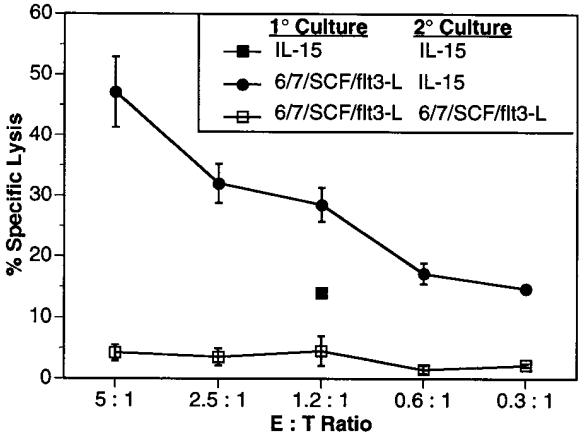

Figure 1. Lytic activity of Lin$^-$, c-kit$^+$, Sca2$^+$ cells cultured with a cocktail of early acting cytokines ± IL-15. Sorted cells were cultured in the indicated cytokines as described in Materials and Methods. Cytotoxic activity was assessed on YAC-1 tumor cells. The data are representative of six experiments.

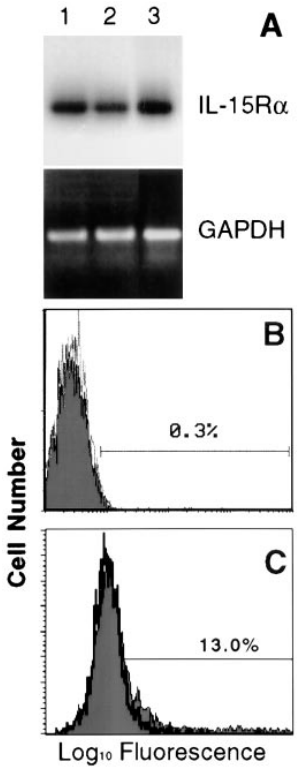

Figure 2. Expression of IL-15Rα messenger RNA and IL-2/15Rβ on Lin$^-$, c-kit$^+$, Sca2$^+$ progenitors and an IL-15–responsive population derived by culture of the progenitors in IL-6, IL-7, SCF, and flt3-L. (*A*) IL-15Rα message levels were determined with RNA isolated from sorted Lin$^-$, c-kit$^+$, Sca2$^+$ progenitors in two experiments (lanes *1* and *2*) and from a population of splenic NK cells (45% NK1.1$^+$ by flow cytometry) derived by culture of spleen cells in 500 U/ml of recombinant human IL-2 for 4 d (lane *3*). (*B*) IL-2/15Rβ expression was examined by flow cytometry on gated Lin$^-$, c-kit$^+$, Sca2$^+$ cells using PE-labeled anti–IL-2/15Rβ mAb TM-β1. The data are representative of three experiments. (*C*) Sorted Lin$^-$, c-kit$^+$, Sca2$^+$ cells, cultured for 5 d in IL-6, IL-7, SCF, and flt3-L, were stained for IL-2/15Rβ expression using FITC-labeled TM-β1. Shaded curves represent staining with TM-β1, whereas open curves represent staining with isotype control mAbs. The data are representative of two experiments.

Although the sorted marrow progenitor cells failed to expand significantly when cultured in IL-15 only, when cultured for 5–6 d in IL-6, IL-7, SCF, and flt3-L and then placed in IL-15 alone for an additional 4–5 d, significant expansion occurred (Table 1), and the majority of cells generated were NK1.1$^+$ and lytic (Table 1 and Fig. 1). If the cultures were maintained in the original cocktail instead of switching to IL-15, significant expansion again occurred, but no lytic activity and few, if any, NK1.1$^+$ cells were detectable. Together these data indicate that culture of sorted Lin$^-$, c-kit$^+$, Sca2$^+$ progenitors in the early acting cytokines causes expansion and primes at least some cells within this population to respond to IL-15 alone. The identity of other cells generated in the presence of the cytokine cocktail and absence of IL-15 is still being explored, but a small fraction (~13%) are CD19$^+$, sIgM$^-$, indicative of immature B cells, whereas a larger fraction (~63%) express high levels of CD11b. Thus, it appears that this population may be multipotential. The data also point to a critical role for IL-15 in NK differentiation because culture of the multipotential progenitors, "primed" by early cytokines in IL-15 only, generated a population of predominantly NK1.1$^+$ cells.

Table 2. *Expansion and Generation of NK Cells from Lin$^-$, CD44$^+$, CD25$^-$, c-kit$^+$ Thymic Progenitors Cultured in IL-3 or flt3-L and IL-6, IL-7, and SCF ± IL-15*

Primary culture (6–7 d)		Secondary culture (6–7 d)		Total Yield	% NK1.1$^+$
Cytokines	Yield	Cytokines	Yield		
IL-15	0				
3/6/7-SCF	10.4	IL-15	5.8	60.3	93
6/7/SCF/flt3-L	ND	6/7/SCF/flt3-L	–	40*	1.8
6/7/15/SCF/flt3-L	ND	6/7/15/SCF/flt3-L	–	381*	86

Sorted Lin$^-$, CD44$^+$, CD25$^-$, c-kit$^+$ thymic progenitors were cultured for the indicated number of days and analyzed as described in Table 1.
*In these experiments, only the total yield was determined after the secondary culture.

Similar experiments were performed using immature thymic progenitors (Lin$^-$, CD44$^+$, CD25$^-$, c-kit$^+$, CD4^{lo}) previously shown to generate T, B, and NK cells (14, 23). When these cells were cultured with IL-15 alone, all cells died within 72 h (Table 2). However, like the marrow progenitor population, when this thymic population was cultured in a cocktail of early acting cytokines and then switched to culture with IL-15 only, significant expansion was observed and a virtually pure population of NK1.1$^+$ cells was generated. In initial experiments, the primary culture was performed in IL-3, IL-6, IL-7, and SCF because this mixture is known to maintain and expand lymphoid progenitors (14). Because IL-3 is a T cell–derived cytokine that should not be required for NK differentiation, we hypothesized that the NK progenitors would develop in the absence of IL-3. Indeed, culture in IL-6, IL-7, IL-15, SCF, and flt3-L for 13 d generated large numbers of an 86% pure population of NK cells, whereas culture in the absence of IL-15 failed to give rise to NK1.1$^+$ cells but did result in significant expansion (Table 2).

Expression of IL-15Rα and IL-2/15Rβ by Sorted Population and Developmental Intermediates. The ability of IL-6, IL-7, SCF, and flt3-L to induce IL-15 responsiveness in sorted Lin$^-$, c-kit$^+$, Sca2$^+$ cells suggested that this mixture of cytokines may be inducing expression of functional IL-15 receptors. Sorted Lin$^-$, c-kit$^+$, Sca2$^+$ bone marrow cells were thus examined for IL-15Rα expression by PCR and IL-2/15Rβ expression by FACS® analysis. Interestingly, the freshly sorted cells did express transcripts of IL-15Rα (Fig. 2 *A*), but they failed to show cell surface expression of IL-2/15Rβ (Fig. 2 *B*). Furthermore, preliminary evidence has indicated surface expression of the γc by a small proportion of these cells (data not shown). These data suggest that the failure of sorted Lin$^-$, c-kit$^+$, Sca2$^+$ cells to expand significantly in IL-15 alone is most likely due to the absence of IL-2/15Rβ expression. We, therefore, anticipated that culture of Lin$^-$, c-kit$^+$, Sca2$^+$ cells in IL-6, IL-7, SCF, and flt3-L would induce IL-2/15Rβ expression. Indeed, after primary culture, ~13% of the progenitors expressed IL-2/15Rβ (Fig. 2 *C*), but not NK1.1 (data not shown). Upon further culture in IL-15, the vast majority of the NK1.1$^+$ cells generated also expressed IL-2/15Rβ (data not shown). These data thus suggest that IL-2/15Rβ expression may precede that of NK1.1, and its acquisition may be a critical event in NK cell differentiation. This idea is supported by the absence of NK cells in IL-2/15Rβ$^{-/-}$ mice (25) and isolation of an IL-2/15Rβ$^+$, NK1.1$^-$ population from SCID bone marrow that can give rise to lytic NK1.1$^+$ cells upon culture in IL-15 (our unpublished data).

Cell Surface Profile of In Vitro Generated NK Cells. The NK cells generated by culture of marrow progenitor cells were

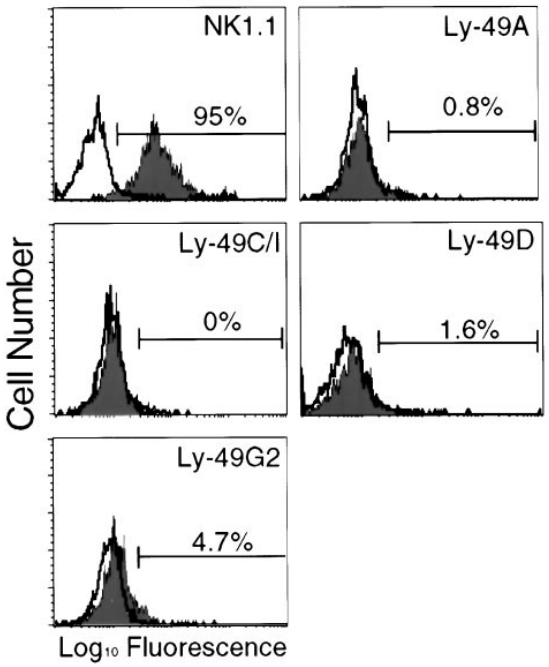

Figure 3. Ly-49 profile of NK cells generated from culture of Lin$^-$, c-kit$^+$, Sca2$^+$ progenitors. Expression of NK1.1 was analyzed by setting a "live" gate based on forward and side scatter of the cells, whereas expression of the remainder of the markers was analyzed on NK1.1$^+$, live cells. Shaded curves represent staining with the NK1.1 or Ly49 mAbs, whereas open curves represent staining with isotype control mAbs. The data are representative of four separate experiments.

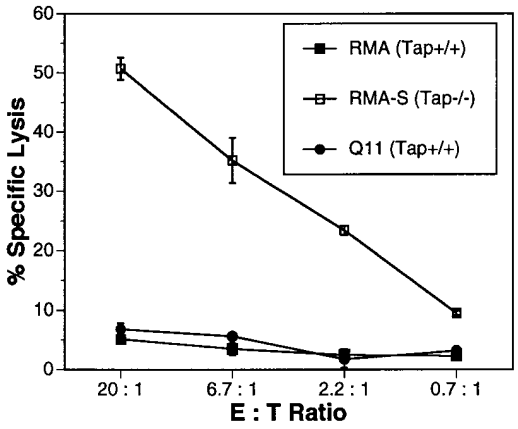

Figure 4. Lysis of syngeneic $Tap^{+/+}$ and $Tap^{-/-}$ tumor cells by in vitro generated NK cells. Target cells were class I^+ RMA ($Tap^{+/+}$) and Q11 ($Tap^{+/+}$) and class I^{lo} RMA-S ($Tap^{-/-}$). The data are representative of three separate experiments.

phenotypically quite similar to mature IL-2–activated splenic NK cells (12, 26, 27). They were $CD3^-$, $CD11b^+$, $Fc\gamma RIII/II^+$, $IL\text{-}2/15R\beta^+$, $c\text{-}kit^-$, and $2B4^+$ (data not shown) and lysed the NK-sensitive target YAC-1 (Fig. 1). Interestingly, they also expressed gp49B1, an inhibitory receptor of the Ig superfamily shared by mast cells (28). However, they failed to express the Ly-49 family of MHC receptors (Fig. 3). In four experiments, expression of Ly-49A, C/I, and D was undetectable or <3% over isotype controls, whereas mature C57BL/6 splenic $NK1.1^+$ cells are 20% $Ly\text{-}49A^+$, 50% $Ly\text{-}49C/I^+$, and 50% $Ly\text{-}49D^+$. Expression of Ly-49G2 was slightly more variable ranging from 2–8% over isotype controls, whereas nearly 50% of splenic NK cells express this receptor. We have been unable to ascertain whether the in vitro–derived NK cells truly express low levels of Ly-49G2 or whether this antibody simply shows nonspecific binding. In any case, these data suggest that NK cells acquire expression of NK1.1 before expression of Ly-49 receptors. The factors required for induction of Ly-49 molecules are unknown, but it is clear that IL-15 alone is not sufficient.

Cytotoxic Activity of In Vitro–generated NK Cells Against Class I^+ and Class I^- Tumor cells. It has been demonstrated that interaction of Ly-49 receptors with their appropriate MHC class I ligands sends an inhibitory signal to NK cells and prevents NK-mediated lysis of the class I^+ target (1). However, despite the failure to express significant levels of Ly-49 MHC receptors, these in vitro–derived NK cells differentiated class I^+ from class I^- syngeneic tumor cells. They lysed class I^{lo} Tap-deficient RMA-S cells but failed to lyse RMA, the class I^+ parent of RMA-S (Fig. 4). That this difference in lysis was due to the absence of class I on the surface of RMA-S is supported by the observation that the culture-derived NK cells failed to lyse Q11, a class I^+ Tap-2 transfectant of RMA-S (16).

In summary, we have developed a stroma-free culture system capable of generating lytic $NK1.1^+$ cells from a defined multipotential progenitor population contained in the bone marrow. This system has allowed identification of a putative ($NK1.1^-$) developmental intermediate in NK differentiation, characterized by expression of $IL\text{-}2/15R\beta$ and the ability to respond to IL-15. One or all of a mixture of cytokines including IL-6, IL-7, SCF, and flt3-L was shown to be critical in reaching this developmental stage. This study also provided additional evidence for the critical role that IL-15 plays in NK differentiation, because no $NK1.1^+$ cells were detected in its absence. IL-15 was not, however, capable of inducing expression of the Ly-49 family of MHC molecules, suggesting that some stimulus necessary to induce Ly-49 expression was missing from this culture system. Finally, these data suggested that development of lytic activity and expression of NK1.1 can precede expression of Ly-49 molecules; however, despite the absence of these receptors, the in vitro–derived NK cells could distinguish class I^+ from class I^- syngeneic tumor cells, suggesting the existence of novel class I receptors.

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Address correspondence to Dr. Noelle S. Williams, Laboratory of Molecular Pathology, Department of Pathology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9072. Phone: 214-648-4081; FAX: 214-648-4033; E-mail: williams.n@pathology.swmed.edu

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