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Impaired NK Cytolytic Activity and Enhanced Tumor Growth in NK Lytic-Associated Molecule-Deficient Mice\textsuperscript{1,2}

Richard G. Hoover,\textsuperscript{†} Gail Gullickson,\textsuperscript{†} and Jacki Kornbluth\textsuperscript{3,*†}

NK lytic-associated molecule (NKLAM) is a protein involved in the cytolytic function of NK cells. It is weakly expressed in resting NK cells but upon target cell stimulation or after incubation with cytokines that enhance NK killing, NKLAM mRNA levels increase and protein is synthesized and is targeted to cytoplasmic granule membranes. We have previously shown that NKLAM plays a role in perforin/granzyme-mediated cytolysis in vitro. To further investigate the function of NKLAM in NK cell-mediated cytotoxicity, we generated, by gene targeting, NKLAM-deficient mice. These mice have normal numbers of NK cells and other lymphoid populations in the spleen. They also have no alterations in NK maturation or NK receptor repertoire. NK cells from NKLAM-deficient and WT mice have comparable amounts of perforin, granzyme B, and lysosomal membrane-associated protein 1 (CD107a) in their cytolytic granules and comparable levels of granule exocytosis are induced by PMA and calcium ionophore A23187. However, NKLAM-deficient NK cells display significantly less NK cytotoxic activity in vitro than WT NK cells. They also secrete less IFN-γ upon target cell stimulation. In addition, NKLAM-deficient mice exhibit greater numbers of pulmonary metastases after i.v. injection with B16 melanoma cells. These studies indicate that NKLAM-deficient mice have diminished capacity to control tumor metastases and support the role for NKLAM in NK function both in vitro and in vivo. The Journal of Immunology, 2009, 183: 6913–6921.

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\textsuperscript{4}Abbreviations used in this paper: NKLAM, NK lytic-associated molecule; IBR, in between RING; KO, knockout; LAMP-1, lysosomal membrane-associated protein 1; neo, neomycin resistance; poly(LC), polyinosinic:polycytidylic acid; RING, really interesting new gene; WT, wild type; TK, thymidine kinase.

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B16 melanoma cells. These studies indicate a significant role for NKLAM in NK cytotoxic function in vitro and in vivo.

Materials and Methods

Constructs

NKLM genomic clones were isolated from a 129 SvJ mouse liver genomic library (λ Fix II; Stratagene) by hybridization using mouse NKLM cDNA clones as previously described (10). Five overlapping phage clones were obtained and the exon/intron boundaries were characterized by PCR and sequencing. The targeting construct was generated using the pKO scrambler NTKV-1905 vector (Stratagene). This vector contains a neomycin resistance (neo) cassette for positive selection and a HSV thymidine kinase (TK) cassette outside the region of homology to allow for negative selection. The 5′ arm of the targeting construct was a 3.5-kb EcoRI/KpnI fragment located within intron 1 of NKLM. The 3′ arm was a 6-kb EcoRI/EcoRI fragment extending from intron 5 to intron 8. This produced a targeting vector of 15 kb, which upon homologous recombination would replace exons 2–5 of NKLM with the neo cassette. This construct was linearized with Ncol and electroporated into 129Sv/Ev embryonic stem cells (inGenious Targeting Lab). The neo-expressing embryonic stem cells were selected in medium containing G418; DNA from G418-resistant clones was isolated and evaluated for homologous recombination. Cells were transfected with Nol restriction endonuclease and PCR with forward primer SMINTR1D (inside intron 1 within the 5′ arm) and reverse primer A1570 (inside exon 6 within the 3′ arm) generated a WT product of 7.4 kb and a KO product of 3 kb (PCR 1). Primers were SMINTR1D: GCACTTGTGTTCAAGCCTTGAG and AM1570: CACCTC CACCGCAAAAGAAGATTG. Clones showing the 3-kb KO band were confirmed as recombinant by two additional PCR using neo primers combined with NKLM-specific primers on either side of the targeting arms (data not shown).

Production of NKLM-deficient mice

Two recombinant embryonic stem cell clones were microinjected into C57Bl/6 blastocysts by inGenious. Coat color chimeric mice (90–100% agouti) were then mated with C57Bl/6 female mice and the progeny were screened for germine transmission of the KO allele. Crossing of heterozygous mice produced viable homozygous KO offspring. Pups were genotyped using DNA extracted from tail tissue by PCR 1. Heterozygous mice have subsequently been backcrossed for 11 generations onto a C57Bl/6 background. C57Bl/6 mice were obtained from the National Cancer Institute. Early in the backcross process, progeny were selected for the C57Bl/6 NK gene complex alleles by PCR so that the NK cells from these mice were genotypically C57Bl/6 (20). Primers for NKRPa1 were forward, TGCTGTAGCTAATTCTAGG; reverse, ATTTATCTGA CAACGTTGGA, B6 allele 110 nt, 129 allele 133 nt. Primers for Ly49a were forward, GCCTCTGAGCTTTGATGGT; reverse, TTCCTTC CCTGTTGAGACTG, B6 allele 340 nt, 129 allele 321 nt. We observed no significant differences in the results obtained from non-backcrossed and fully backcrossed NKLAM homozygous KO mice. All mice were maintained in specific pathogen-free barrier housing and all experiments were conducted in accordance with institutional animal care and use guidelines.

Cell lines

YAC-1 and B16-F10 melanoma cells were purchased from the American Type Culture Collection. The NK- sensitive mouse lymphoma YAC-1 cell line was maintained in medium containing RPMI 1640 supplemented with 7.5% FBS and 1% glutamine. They were split twice a week and maintained at a density of 2 × 10^6 cells/ml. B16 melanoma cells were propagated in DMEM containing 7.5% FBS and 1% glutamine. Subconfluent adherent cells were trypsinized and split twice a week.

Isolation of spleen cells

Spleens were harvested, rinsed in RPMI 1640, and gently homogenized using the blunt end of a 3-ml syringe. Released spleen cells were pelleted and then layered over a Lympholyte-M density gradient (Cedarlane Laboratories/Accurate Chemical Corporation) according to the manufacturer's instructions to remove RBC, dead cells, and debris. Lymphocytes at the interface were collected, washed twice with RPMI 1640, and counted. These cells were analyzed by flow cytometry and/or used for the purification of NK cells.

Flow cytometry

Flow cytometry was performed to analyze spleen cell subpopulations and NK cells from WT and NKLAM-deficient mice. Briefly, tubes containing 2 × 10^5 – 2 × 10^6 cells in 0.05 ml of flow buffer (ice-cold PBS plus 1% FBS plus 0.1% sodium azide) were incubated with 1 μl of Fe block (BD Pharmingen) for 10 min at 4°C. Fluorescent-conjugated cell surface-specific primary Abs were then added and incubated for an additional 30 min at 4°C. Cells were washed twice with flow buffer and then either fixed with 0.2 ml of PBS plus 1% formaldehyde or prepared for intracellular staining using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit with GolgiStop according to the manufacturer’s directions. Cells were analyzed on a BD FACSCalibur flow cytometer. Abs purchased from BD Biosciences were FITC-CD3 (no. 553061), Alexa Fluor 700-CD3 (no. 557984), allotypyocyanin-NK-1.1 (no. 550607), PE-Cy7-KLH (no. 553111), CD25 (no. 553866), biotin-KLRG1 (no. 5500863), PE- NKGD2 (no. 558403), FITC-LY49C1 (no. 553278), PE-LY49A (no. 557424), and control Abs were FITC-IgG1 (no. 553971), PE-IgG2b (no. 553987), and allotypycyanin-IgG2a (no. 553932). Abs purchased from cBiosciences were FITC-NKp46 (no. 11-3351-80), PE-LY49I (no. 12-3895), PE-granzyme B (no. 12-8899-41), and FITC-perforin (no. 11-3932-82). Ly49H, Ly49A, and Ly49I Abs were generously provided by Dr. M. Buller (St. Louis University). Samples were analyzed using FlowJo software (Tree Star).

Purification of NK cells and stimulation

NK cells were isolated from spleen cells using magnetic CD49b (DX5) negative selection, a MACS separator, and MS columns according to the manufacturer’s recommendations (Miltenyi Biotech) (21). These clones were routinely >92% DX5 by FACS analysis. For cytokine stimulation, NK cells were cultured at 5 × 10^6 cells/ml in RPMI 1640 containing 10% FBS, 1% glutamine, 5 × 10^-5 M 2-ME, and 500 U/ml rIL-2. IL-2 was a gift from Chiron. For some experiments, unseparated spleen cells were similarly cultured in IL-2 in parallel.

Cells were activated by i.p. injection of mice with 200 μg of polynosinic-polycytidylic acid (polyI:C), sodium salt; Sigma-Aldrich). Control mice were injected with PBS. After 24 h, spleen cells or purified NK cells were assayed for NK activity in vitro.

Killing assays and granule exocytosis

Unstimulated and IL-2-stimulated purified NK cells were used as effectors in cytotoxicity assays against the 51Cr-labeled NK-sensitive mouse lymphoma cell line YAC-1, as previously described (10). Assay conditions were set up in triplicate wells at various E:T ratios in 96-well round-bottom microtiter plates. After incubation for 1 h at 37°C, supernatants (0.1 ml) were harvested and the amount of 51Cr release from YAC-1 target cells was calculated as previously described. The SD of triplicate values never exceeded 10% in all experiments.

Granule exocytosis was monitored by the release of β-glucuronidase using the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (22). Reagents used in this assay were purchased from Sigma-Aldrich. Four × 10^5 spleen cells or 2.5 × 10^5 purified NK cells were incubated with 0.025 ml of RPMI 1640 containing 4% FBS in 96-well plates. PMA (40 ng/ml) plus calcium ionophore A23187 (2 μg/ml) was used to induce granule exocytosis. Total release was obtained by incubation of cells with 1% Triton X-100. After 4 h at 37°C, cells were pelleted and supernatants were harvested. Briefly, 0.005 ml of supernatant was added to triplicate wells of 96-well black microtiter plates and mixed with 0.05 ml of β-glucuronidase substrate (1.5 mM 4-methylumbelliferyl-β-D-glucuronide in 0.05 M sodium acetate, 0.01% BSA, and 0.1% Triton X-100, pH 4). Plates were incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.175 ml of 0.25 M sodium tetaborate. The fluorescence intensity was measured with excitation at 355 nm and emission of 460 nm in a Wallac Victor 1420 multilabel counter.

IFN-γ production

Unstimulated and IL-2-stimulated purified NK cells were cocultured with YAC-1 target cells in triplicate wells at E:T ratios of 25:1 and 10:1, respectively, in 96-well round-bottom microtiter plates. After incubation for 4 h at 37°C, supernatants (0.1 ml) were harvested and the amount of IFN-γ was quantitated by ELISA (Endogen and Pierce Biotechnology) according to the manufacturer’s protocol.
A mouse NKLAM targeting construct replaced exons 2–5 of the NKLAM gene, encoding the second and third cysteine-rich domains and two of the three predicted transmembrane domains, with the neomycin resistance (neo) gene. The targeting vector also contained a TK cassette outside the region of homology. This targeting construct was transfected into 129SvEv embryonic stem cells and neo-expressing clones were selected in medium containing G418. Homologous recombinant clones were identified by a combination of Southern blotting and PCR. Progeny were genotyped from tail DNA using multiple PCR. In the representative PCR shown on the left, the primers are on either side of knocked out exons 2–5 of the NKLAM gene. The WT band is 7.4 kb while the KO band is 3 kb. On the right, RT-PCR confirmed the absence of NKLAM mRNA in IL-2-stimulated spleen cells in NKLAM−/− mice. These PCR primers were located within the KO region of NKLAM, β-Actin served as a positive PCR control.

Immunoblot analysis

Rat monoclonal anti-LAMP-1 (CD107a) mAb 1D4B, provided by Dr. J. T. August, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa (Department of Biological Sciences, Iowa City, IA) (23). It was used at 1/100. Anti-perforin Ab (no. 3693, 1/1,000) and anti-granzyme B Ab (no. 4275, 1/1000) were purchased from Cell Signaling. Anti-β-actin mAb AC-15 (1/10,000) was purchased from Sigma-Aldrich. NKLAM mAb 35 kb were replaced with a neo cassette (Fig. 1). This removed the start codon, because intron 1 is extremely large, over 14 kb. There-fore, a targeting vector was constructed in which exons 2–5 (~6 kb) were replaced with a neo cassette (Fig. 1A). This removed the second and third cysteine-rich domains of NKLAM, which together form a RING-IBR-RING structure, important in the E3 ubiquitin ligase activity of NKLAM. It also removed two of the three predicted transmembrane domains. This construct was transfected into 129SvEv embryonic stem cells. The neo clones were selected in medium containing G418; homologous recombinant clones were identified by a combination of Southern blotting and PCR. Two recombinant clones were subsequently microinjected into C57BL/6 blastocysts. Chimeric heterozygotes that displayed germline transmission were intercrossed to produce NKLAM−/− progeny (Fig. 1B). Heterozygotes were simultaneously backcrossed to C57BL/6 mice for 11 generations to generate NKLAM-deficient mice on a C57BL/6 background. Early in the backcross process, heterozygous progeny were selected for the C57BL/6 NK gene complex alleles by PCR so that the NK cells from these mice were genotypically C57BL/6. Progeny were evaluated by a combination of both Southern blotting and PCR. RT-PCR confirmed the absence of NKLAM mRNA in IL-2-stimulated spleen cells from NKLAM−/− mice compared with heterozygous (+/−) and WT (+/++) mice (Fig. 1B).

Homozygous NKLAM-deficient (−/−) mice were born at the expected Mendelian ratio. Overall, the mice appeared normal and were fertile. Histological examination of the major organs of 8-wk-old NKLAM−/− and heterozygous (+/−) mice revealed no overt abnormalities by light microscopy. NKLAM−/− mice were observed for over 18 mo. They developed normally and there was no obvious difference in gross morphology, size, or body weight between WT and NKLAM-deficient mice during the 18-mo period (data not shown).

Lung metastasis studies

B16 melanoma cells were injected i.v. through the tail vein into NKLAM-deficient KO and WT mice. Cells were washed with PBS and each mouse was injected with 300,000 cells in 0.2 ml of PBS. Fifteen days later, animals were sacrificed and lungs were harvested and fixed in Fekete’s solution. Individual lobes were dissected and black B16 melanoma metastatic nodules were enumerated on each lobe surface in a blinded fashion. Data were analyzed using a two-tailed Student’s t test.

Results

Generation of NKLAM-deficient mice

Genomic clones containing mouse NKLAM were isolated from a 129SvJ library. The NKLAM gene contains nine exons, spanning over 29 kb. It was not possible to knock out exon 1, containing the start codon, because intron 1 is extremely large, over 14 kb. Therefore, a targeting vector was constructed in which exons 2–5 (~6 kb) were replaced with a neo cassette (Fig. 1A). This removed the second and third cysteine-rich domains of NKLAM, which together form a RING-IBR-RING structure, important in the E3 ubiquitin ligase activity of NKLAM. It also removed two of the three predicted transmembrane domains. This construct was transfected into 129SvEv embryonic stem cells. The neo clones were selected in medium containing G418; homologous recombinant clones were identified by a combination of Southern blotting and PCR. Two recombinant clones were subsequently microinjected into C57BL/6 blastocysts. Chimeric heterozygotes that displayed germline transmission were intercrossed to produce NKLAM−/− progeny (Fig. 1B). Heterozygotes were simultaneously backcrossed to C57BL/6 mice for 11 generations to generate NKLAM-deficient mice on a C57BL/6 background. Early in the backcross process, heterozygous progeny were selected for the C57BL/6 NK gene complex alleles by PCR so that the NK cells from these mice were genotypically C57BL/6. Progeny were evaluated by a combination of both Southern blotting and PCR. RT-PCR confirmed the absence of NKLAM mRNA in IL-2-stimulated spleen cells from NKLAM−/− mice compared with heterozygous (+/−) and WT (+/++) mice (Fig. 1B).

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Normal distribution of lymphoid cells in the spleens of NKLAM-deficient mice

The distribution of lymphoid cells in the spleens of NKLAM−/− and NKLAM+/+ mice was analyzed by flow cytometry. There was no significant difference in the absolute numbers of lymphoid cells in NKLAM-deficient and WT mice; the total number of spleen cells was 87.5 ± 9.2 × 10⁶ in NKLAM-deficient mice and...
Previous studies have shown that NKLA-M is located in NK cytolytic granule membranes (9). In addition, treatment of NK cells with NKLA-M antisense oligonucleotides reduces both NKLA-M levels and NK-mediated cytotoxicity, indicating a role for NKLA-M in NK killing. Therefore, NK cells from NKLA-M-deficient mice were predicted to have lower NK activity. To test this hypothesis, DXS5^+ NK cells from the spleens of NKLA-M^+/+, +/−, and +/+ WT mice were isolated by magnetic bead column purification. Total numbers of NK cells were comparable among the three groups. Freshly isolated NK cells were tested for killing of NK-sensitive YAC-1 tumor target cells in 4-h ^51Cr release assays. As shown in Fig. 3, NK cells from NKLA-M^−/− mice had significantly lower spontaneous NK activity in vitro compared with NKLA-M^+/− heterozygous mice or WT mice. This represents a 60% reduction in NK killing of YAC-1 targets by NKLA-M-deficient NK cells.

NKLA-M expression is greatly increased in NK cells after treatment with cytokines that enhance NK killing, such as IL-2 and IFN. We therefore compared the cytotoxic activity of resting and in vitro IL-2-stimulated NK cells from NKLA-M^−/− and WT mice. As seen previously, resting NK cells from NKLA-M-deficient mice had lower NK activity than cells from WT littermates. Interestingly, IL-2 enhanced the killing activity of both NKLA-M-deficient and WT NK cells. However, the level of killing by IL-2-stimulated NK cells from NKLA-M^−/− mice was still much less than the killing mediated by IL-2-stimulated WT NK cells (Fig. 4). Cumulatively, these results indicate that NKLA-M plays a role in NK cytotoxicity in vitro. However, it is not absolute in that there is residual killing displayed by NKLA-M-deficient NK cells, which can be enhanced by exposure to IL-2. This suggests that NKLA-M is one of multiple factors contributing to the total amount of cytotoxicity mediated by DXS5^+ NK cells.

NKLA-M-deficient NK cells display normal granule exocytosis and granule contents

Since NKLA-M is found in the membrane of cytolytic granules, it is possible that it plays a role in the migration and/or fusion of granules to the cell membrane during the process of granule exocytosis when NK cells are activated to kill. NKLA-M levels are low in resting NK cells and unlike other granule membrane proteins like CD107a (LAMP-1), there is little to no preformed NKLA-M in cytolytic granules (9, 30). Upon target cell stimulation, NKLA-M levels increase. To determine whether NKLA-M plays a role in granule exocytosis, spleen cells and purified NK cells from NKLA-M^−/− and WT mice were treated with PMA and calcium ionophore A23187 to trigger granule exocytosis. Four hours later, the supernatants were monitored for ^β-glucuronidase, a soluble component of the granules and a measure of granule release. Total levels of ^β-glucuronidase in the cells were determined by detergent (Triton X-100) lysis. As shown in Fig. 5, the total and released amounts of ^β-glucuronidase in NKLA-M-deficient and WT cells were comparable. This indicates that NKLA-M is likely not required for granule exocytosis and/or release of soluble granule components.

Granule exocytosis-mediated killing of tumor targets requires functional granules containing perforin and granzymes. If NKLA-M alters the expression and/or function of these proteins, it could alter the killing activity of NK cells. Therefore, the levels of perforin, granzyme B and the granule membrane protein CD107a in NKLA-M^−/− and WT NK cells were evaluated. A representative immunoblot is shown in Fig. 6. The amount of perforin, granzyme B, and CD107a was quantitated by densitometry and normalized to the levels of ^β-actin. We found no significant differences in perforin, granzyme B, or CD107a expression between NKLA-M^−/− and WT NK cells, suggesting that NKLA-M does not alter the

### Table I. Analysis of spleen cell subpopulations in NKLA-M-deficient mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Designation</th>
<th>NKLA-M^+/+</th>
<th>NKLA-M^−/−</th>
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<tr>
<td>T cell</td>
<td>CD3^+</td>
<td>36.9 ± 3.1</td>
<td>33.6 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>CD4^+</td>
<td>19.6 ± 1.7</td>
<td>19.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>CD8^+</td>
<td>14.8 ± 2.0</td>
<td>11.8 ± 2.5</td>
</tr>
<tr>
<td>Activated T</td>
<td>CD3^+/CD25^+</td>
<td>3.2 ± 0.9</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>B cell</td>
<td>CD19^+</td>
<td>44.0 ± 5.2</td>
<td>48.3 ± 7.3</td>
</tr>
<tr>
<td>NK cell</td>
<td>CD3^+DX5^+</td>
<td>3.5 ± 1.0</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>DX5^+CD62L^+</td>
<td>2.3 ± 0.9</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>CD3^−NK1.1^+</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.4</td>
</tr>
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<td>NKp46</td>
<td>90.9 ± 1.5</td>
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<td>92.0 ± 3.8</td>
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<td>NKG2A/C/E</td>
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<td>Ly49A</td>
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<td>Ly49C/I</td>
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<td>35.9 ± 2.4</td>
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<td>Ly49D</td>
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<td>52.4 ± 3.0</td>
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<td>Ly49H</td>
<td>67.8 ± 2.0</td>
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<td></td>
<td>Ly49I</td>
<td>60.0 ± 4.3</td>
<td>60.3 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>KLRG1</td>
<td>33.5 ± 3.7</td>
<td>33.1 ± 0.5</td>
</tr>
<tr>
<td>Total spleen cells (×10^6)</td>
<td>84.0 ± 7.8</td>
<td>87.5 ± 9.2</td>
<td></td>
</tr>
</tbody>
</table>

^ Results represent the percentage of spleen cells positive for the indicated markers by flow cytometry. The mean ± SD of eight individual mice per genotype is shown. The percentage of NK cells bearing individual NK receptors is derived from the gated CD3^−NK1.1^+ population. There were no significant differences in the absolute number or distribution of lymphocyte or NK subsets between WT (+/+) and NKLA-M-deficient (−/−) mice.

84 ± 7.8 × 10^6 in WT mice. The percentages of CD3^+^, CD4^+^, and CD8^+^ T cells, CD3^+^CD25^+^ activated T cells, CD19^+^ B cells, and CD3^+^DX5^+^ NK cells were evaluated. We also looked at the proportion of DX5^+^ NK cells coexpressing CD62L (L-selectin). In humans, L-selectin expression is associated with an immature NK cell phenotype (24). In mice, L-selectin expression on NK cells may influence the migration of NK cells to various lymphoid organs (25). As shown in Table I, there were no differences in any of the cell populations between NKLA-M-deficient and WT mice.

Analysis of the number of CD3^−NK1.1^+ NK cells and the NK receptor repertoire was also performed. NKLA-M-deficient and WT mice had equivalent levels of CD3^−NK1.1^+ cells (Table I). Examination of gated CD3^−NK1.1^+ splenic NK cells for inhibitory and activating receptors showed no differences in the expression of NKp46, NKG2D, NKG2A/C/E, Ly49A, Ly49C/I, Ly49D, Ly49H, Ly49L, and KLRG1 between KO and WT NK cells (Table I and Fig. 2). Therefore, the lack of NKLA-M expression does not significantly alter the numbers or distribution of NK populations or other lymphocyte subsets in the spleen.

Maturation of NK cells was first shown to be accompanied by an up-regulation of CD11b (Mac-1) expression (26). It was subsequently demonstrated that mouse NK cells could be dissected into four subsets based on cell surface density of CD27 and CD11b (27–29). This developmental program begins with CD11b^low^CD27^low^ NK cells and progresses through CD11b^low^CD27^high^, CD11b^high^CD27^high^, and CD11b^high^CD27^low^ NK cells. The distribution of these subsets in CD3^−NK1.1^+ NK cells from the spleens of NKLA-M-deficient and WT mice was evaluated. As shown in Fig. 2, NKLA-M KO and WT mice have similar levels of these subsets. In addition, as described, the percentage of NK cells expressing Ly49C/I in the CD11b^low^ subsets of both KO and WT mice was much lower (8%) than in the CD11b^high^ subsets (30%) (data not shown). Overall, these results suggest no NK maturation alterations in NKLA-M-deficient mice.

NKLA-M-deficient mice display lower splenic NK activity in vitro

Previous studies have shown that NKLA-M is located in NK cytolytic granule membranes (9). In addition, treatment of NK cells

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FIGURE 2. Normal NK cell subsets and receptor repertoire in NKLAM-deficient mice. Representative flow cytometry results from eight independent experiments are shown. Cells isolated from the spleens of NKLAM-deficient (KO) and WT mice were stained for the markers indicated. A. Electronically gated NK cells (CD3^- NK1.1^; left panel) were evaluated for expression of the NK maturation markers CD27 and CD11b (middle panel). The dot plots indicate the percentage of each NK subset. The right panel depicts the intracellular staining levels of granzyme B and perforin in electronically gated CD3^- NK1.1^ cells from purified NK cells stimulated with IL-2 for 3 days in vitro from KO and WT mice. B and C. The histogram plots represent the expression levels of the indicated NK activating and inhibitory receptors electronically gated on CD3^- NK1.1^ spleen cells from KO and WT mice. Numbers indicate the percentage of positive cells for each subset.
expression of these proteins. However, we cannot rule out the possibility that there may be functional differences in the proteins between these mice. NKLAM was undetectable in KO cells.

Intracellular levels of perforin and granzyme B were also analyzed by flow cytometry. Fig. 2A (third panel) depicts the intracellular expression of these proteins in NKLAM KO- and WT-purified NK cells stimulated for 3 days with IL-2. Cells from NKLAM KO and WT mice have comparable levels of granzyme B and perforin; ~97% of the CD3⁻ NK1.1⁺ NK cells express granzyme B and ~70% express perforin. Similar results were seen in three independent experiments. Total splenocytes from NKLAM-deficient and WT mice were also cultured for 3 days in IL-2. Granzyme B and perforin levels in the KO and WT CD3⁻ NK1.1⁺ cells in these cultures were also comparable, with ~97% of the cells expressing granzyme B and ~45% expressing perforin (data not shown). The flow cytometry results correlate with the protein levels seen in immunoblot analysis. Consistent with studies by Fehninger et al. (8), granzyme B and perforin were undetectable in purified, unstimulated NK cells.

**NKLAM-deficient mice produce less IFN-γ upon target stimulation in vitro**

In addition to cytotoxic activity against YAC-1 tumor cells, we compared the production of IFN-γ by NKLAM-deficient and WT

![FIGURE 3](image-url) **In vitro splenic NK activity is lower in NKLAM-deficient mice.** DX5⁺ NK cells from the spleens of NKLAM KO (−/−), heterozygous (+/−), and WT (+/+) mice were isolated by magnetic bead column purification. These cells were assessed for cytolytic activity against ⁵¹Cr-labeled YAC-1 target cells in 4-h killing assays at the E:T ratios shown. Results are expressed as the percent specific lysis of YAC-1 cells. This represents one of six experiments with similar results. SDs of triplicate values in each experiment did not exceed 10%.

![FIGURE 4](image-url) **IL-2 partially restores the killing activity of NKLAM-deficient NK cells in vitro.** DX5⁺ NK cells from the spleens of NKLAM KO (−/−) and WT (+/+) mice were isolated by magnetic bead column purification. Cells were then cultured at 5 × 10⁶ cells/ml in RPMI 1640 containing 10% FBS, 1% glutamine, and 5 × 10⁻³ M 2-ME for 3 days with and without 500 U/ml rIL-2. These cells were assayed for cytolytic activity against ⁵¹Cr-labeled YAC-1 target cells in 4-h killing assays at the E:T ratios shown. Error bars represent the SDs from five independent experiments.

![FIGURE 5](image-url) **NK cells and spleen cells from NKLAM KO and WT mice display comparable levels of granule exocytosis.** Granule exocytosis was monitored by the release of β-glucuronidase using the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide. Four × 10⁵ spleen cells or 2.5 × 10⁶ purified NK cells from NKLAM KO or WT mice were incubated with 0.025 ml of RPMI 1640 containing 4% FBS in 96-well plates. PMA (40 ng/ml) plus calcium ionophore A23187 (2 µg/ml) (P + I) was used to induce granule exocytosis. Total release was obtained by incubation of cells with 1% Triton X-100. After 4 h at 37°C, 0.005 ml of supernatant was added to triplicate wells of 96-well black microtiter plates and mixed with 0.05 ml of β-glucuronidase substrate. Plates were incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.175 ml of 0.25 M sodium tetraborate. The fluorescence intensity was measured in a Wallac Victor 1420 multilabel counter. Error bars represent the SEM from five independent experiments. unstim, Unstimulated.

![FIGURE 6](image-url) **Immunoblot analysis of granule proteins in NKLAM-deficient and WT mice.** Purified NK cells from the spleens of NKLAM-deficient KO and WT mice were stimulated with IL-2 (500 U/ml) in vitro for 3 days. Cell lysates were then prepared as described and subjected to Western blot analysis. The amount of perforin, granzyme B and CD107a from four independent experiments was quantitated by densitometry and normalized to the levels of β-actin. Levels of expression between KO and WT cells varied by <25% for each protein and were not significantly different. NKLAM protein was undetectable in KO cells.

NK cells after target cell stimulation. Freshly isolated, purified, unstimulated NK cells or NK cells stimulated with IL-2 for 3 days in vitro were cocultured with YAC-1 target cells at E:T ratios of
NK activity in NKLAM-deficient mice is stimulated in vivo by poly(I:C)

NK activity is enhanced by in vivo exposure to poly(I:C). Twenty-four hours after i.p. injection of mice with poly(I:C) or PBS, spleen cells or purified NK cells from NKLAM-deficient KO mice and WT mice were assayed for NK activity in vitro. Poly(I:C) increased the killing activity of splenocytes and purified NK cells from both NKLAM KO and WT mice to the same extent (Fig. 8).

Enhanced lung metastases in NKLAM-deficient mice

The B16 melanoma model of experimental lung metastasis has been widely used in studies of NK function (31–33). In these experiments, NKLAM−/− and WT mice were injected i.v. with 300,000 B16 melanoma cells. Fifteen days later, lungs were harvested, fixed, and the individual lobes dissected. Black metastatic melanoma colonies were counted on each lung lobe surface using a dissecting microscope. Colonies were counted in a blinded fashion. A. Photographic shows representative lungs from two WT (+/+) and two NKLAM-deficient KO (−/−) mice. B. Lung tumors were quantitated in individual NKLAM KO (−/−) and WT (+/+) male and female mice. Results indicate the mean number of lung colonies. Error bars indicate the SEM (n = 8). The statistical significance is P < 0.001 using the Student t test.

FIGURE 9. NKLAM-deficient mice exhibit greater pulmonary metastasis of B16 melanoma cells than WT mice. NKLAM-deficient KO (−/−) and WT (+/+ or +/−) mice were injected i.v. through the lateral tail vein with 300,000 B16 melanoma cells. Fifteen days later, lungs were harvested, fixed, and the individual lobes dissected. Black metastatic melanoma colonies were counted on each lung lobe surface using a dissecting microscope. Colonies were counted in a blinded fashion. A. Photograph shows representative lungs from two WT (+/+) and two NKLAM-deficient KO (−/−) mice. B. Lung tumors were quantitated in individual NKLAM KO (−/−) and WT (+/+) male and female mice. Results indicate the mean number of lung colonies. Error bars indicate the SEM (n = 8). The statistical significance is P < 0.001 using the Student t test.

Discussion

This report describes the generation and initial characterization of mice with a targeted genetic deletion of NKLAM. NKLAM is a RING-IBR-RING finger-containing transmembrane protein found in NK cytolytic granules. It is weakly expressed in resting NK cells; upon target cell stimulation or after incubation with cytokines that enhance NK killing, NKLAM mRNA levels in NK cells increase and protein is synthesized and becomes targeted to cytoplasmic granule membranes. Previous studies have shown a role for NKLAM in perforin/granzyme-mediated cytotoxicity. Treatment of NK cells with NKLAM antisense oligonucleotides inhibits their killing activity, indicating that NKLAM participates in the cytotoxic process.

To further evaluate the role of NKLAM in NK function in vivo, NKLAM-deficient mice were generated. These mice were initially
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129Sv/Ev × C57BL/6 and have been backcrossed for 11 generations onto a B6 background. During this process, we observed no significant differences in the results obtained from non-backcrossed and fully backcrossed NKLAM homozygous KO mice. NKLAM-deficient mice were monitored for >18 mo. Overall, the mice appeared normal and were fertile. Histological examination of the major organs of NKLAM−/− and +/+ mice revealed no overt abnormalities by light microscopy. NKLAM−/− mice developed normally and there was no obvious difference in gross morphology, size, or body weight between WT and NKLAM-deficient mice during the 18-mo period.

We also observed no differences in the numbers or distribution of lymphoid populations in the spleens of NKLAM−/− mice compared with WT animals. The numbers of CD3+ T cells, CD8+ T cells, CD3+CD25+ activated T cells, CD19+ B cells, CD3+ DX5+ NK cells, and CD3+ NK1.1+ NK cells in both groups of mice were identical. We also looked at the proportion of DX5+ NK cells coexpressing CD62L (L-selectin). In humans, L-selectin expression is associated with an immature NK cell phenotype (24). In mice, L-selectin expression on NK cells may influence the migration of NK cells to various lymphoid organs (25). Again, there was no significant difference in the proportion of DX5+ L-selectin+ cells in NKLAM−/− and −/− mice. We also saw no maturational differences between NKLAM KO and WT spleen NK cells based on the levels of coexpression of CD27 and CD11b as described previously (26–29). Analysis of NK inhibitory and activating receptor expression by splenic NK cells in NKLAM-deficient and WT mice also showed no differences. Evaluation of NK populations in other organs in NKLAM-deficient mice, including bone marrow, lymph node, liver, and lungs, is currently in progress.

Although NKLAM-deficient mice have normal numbers of splenic NK cells and a normal NK receptor repertoire, these cells display significantly less cytotoxic activity against YAC-1 target cells in vitro compared with heterozygous or WT control cells. This demonstrates unequivocally that NKLAM plays a role in NK-mediated cytoxicity. However, it also indicates that NKLAM is not absolutely required for all NK-mediated killing. Similarly, when purified NK cells were stimulated with IL-2 in vitro, killing of both NKLAM−/− and +/+ NK cells was increased. However, the cytotoxic activity mediated by NKLAM-deficient NK cells never reached the levels of WT NK cells. This indicates that IL-2 enhances the killing activity of NKLAM-deficient NK cells. It also suggests that there is an NKLAM-dependent and NKLAM-independent pathway used by NK cells. This is further supported by the finding that poly(I:C) treatment in vivo enhances NK activity in both NKLAM-deficient and WT mice to the same extent. Because the analysis of perforin-deficient mice led to the delineation of a perforin-independent, Fas-dependent mechanism of cytotoxicity, studies of NKLAM-deficient mice may similarly unveil alternative killing pathways (35).

NKLAM is found in the membrane of cytolytic granules, suggesting that it might play a role in the migration and/or fusion of granules to the cell membrane during the process of granule exocytosis when NK cells are activated to kill. However, our results indicate comparable release of the soluble granule protein β-granulin by both NKLAM−/− and WT spleen cells and purified NK cells after PMA and calcium ionophore treatment. Experiments are planned to evaluate the potential role of NKLAM in induction of granule exocytosis by other stimuli. We also found no differences in expression of the granule proteins perforin, granzyme B, or CD107a (LAMP-1) by flow cytometry and/or immunoblot analysis between NKLAM−/− and WT NK cells, indicating that NKLAM does not alter the expression of these proteins. Overall, these results suggest that NKLAM is not involved in granule protein expression and the process of granule exocytosis. However, we cannot rule out the possibility that NKLAM deficiency may lead to an alteration in other granule protein expression and/or function.

In addition to decreased cytotoxic activity against YAC-1 tumor cells mediated by NK cells from NKLAM-deficient mice, these cells also secrete less IFN-γ than WT NK cells after target cell stimulation. It has been suggested that IFN-γ is released both symaptically and multidirectionally by NK cells (36). Studies are in progress to determine whether NKLAM is involved in IFN-γ production and/or release by either of these two mechanisms.

We used the B16 melanoma model to evaluate the potential role of NKLAM in tumor metastasis in vivo. B16 melanoma cells express low amounts of MHC class I and are readily killed by NK cells. It has been well established that inhibition of NK activity by various methods in vivo is associated with increased B16 pulmonary metastasis. More recently, Kim et al. (37) demonstrated that NK cell-deficient transgenic mice have more than a 60-fold greater number of B16 lung metastases compared with WT mice, confirming a predominant role of NK cells in B16 lung metastasis following i.v. injection. A gender difference in response to B16 melanoma was reported in mice deficient in the NK receptor 2B4 (34). We found that NKLAM-deficient mice have significantly greater numbers of B16 lung nodules than WT mice (between 3- and 4-fold greater). We observed no gender differences; both male and female NKLAM−/− mice showed greater numbers of tumors than their WT gender-matched controls. In addition to having more metastases, both male and female NKLAM-deficient mice had larger tumor nodules than WT mice. These results indicate that NKLAM-deficient mice have diminished capacity to control tumor metastases in vivo and support the role for NKLAM in NK function in vivo. Again, these results suggest a partial rather than absolute defect in NK activity in NKLAM-deficient NK cells.

These studies point to an important role for NKLAM in NK cell function in vivo and in vitro. These NKLAM-deficient mice are a tool for characterizing the function of NKLAM and its role in various disease processes. It also opens up new investigations into alternate, NKLAM-independent mechanisms used by NK cells to control tumors and viruses in vitro and in vivo.

Disclosures

The authors have no financial conflict of interest.

References


