Mouse Ly-49A Interruptr Events in Natural Killer Cell Cytotoxicity and Functionally Associates with the SHP-1 Tyrosine Phosphatase

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Summary

The lytic activity of natural killer (NK) cells is inhibited by the expression of class I major histocompatibility complex (MHC) antigens on target cells. In murine NK cells, Ly-49A mediates inhibition of cytotoxicity in response to the class I MHC antigen H-2D<sup>d</sup>. In this report, we studied the function of mouse Ly-49A in both the rat NK cell tumor line, RNK-16, transfected with Ly-49A cDNA, and in primary NK cells. We show that ligation of Ly-49A by H-2D<sup>d</sup> inhibits early signaling events during target cell stimulation, including polyphosphoinositide turnover and tyrosine phosphorylation. We also show that Ly-49A directly associates with the cytoplasmic tyrosine phosphatase SHP-1, and that Ly-49A function is impaired in NK cells from SHP-1 mutant viable motheaten mice and from SHP-1–deficient motheaten mice. Finally, we demonstrate that mutational substitution of the tyrosine within the proposed SHP-1 binding motif in Ly-49A completely abrogates inhibition of NK cell cytotoxicity through this receptor. These results demonstrate that Ly-49A interrupts early activating signals in NK cells, and that SHP-1 is an important mediator of Ly-49A function.

NK cells and some T cells express a variety of type II transmembrane receptors characterized by extracellular C-type lectin domains (1). In mice, these proteins include the members of the Ly-49 family (2–8), which recognize MHC class I molecules on target cells (6–10), and the NKR-P1 family, whose physiologic ligands have yet to be determined (11–13). While the NKR-P1 family activates NK cell cytotoxicity (14–16), at least three members of the Ly-49 family inhibit NK cell function (6–8).

Susceptible targets stimulate phosphoinositide turnover, calcium mobilization, and the induction of protein tyrosine phosphorylation in NK cells. These signals have been associated with activation of NK cell cytotoxic responses (17–20). The NKR-P1 lectin-like receptor also transduces these activating signals in NK cells (14–16). In contrast, mouse Ly-49A inhibits NK cell cytotoxicity upon ligation by the target cell MHC class I molecules H-2D<sup>d</sup> or H-2D<sup>k</sup> (6, 9, 10). The mechanisms by which Ly-49A interrupts NK cell activation are poorly understood, but important clues can be derived from structural motifs within the Ly-49A molecule.

The Ly-49A cytoplasmic domain contains the amino acid sequence VxYxxV, which constitutes a proposed binding motif for the cytoplasmic tyrosine phosphatase, SHP-1 (21, 22). SHP-1 is an SH2 domain containing tyrosine phosphatase, expressed in hematopoietic cells that can inhibit specific cellular functions. SHP-1 negatively modulates signaling through the erythropoietin receptor (23, 24), and it inhibits activation of B cells through its association with FcyRIIB1 (25–28). In human NK cells, SHP-1 has been implicated in inhibition of cytotoxicity through its association with the killer inhibitory receptors (KIRs), members of the Ig family that bind to human MHC class I molecules (21, 29, 30). The presence of a proposed SHP-1 binding motif in the cytoplasmic domain of Ly-49A suggests that this murine receptor may also functionally associate with SHP-1. A tyrosine-phosphorylated synthetic tridecapeptide derived from the cytoplasmic domain of Ly-49A has recently been shown to bind to SHP-1 and to the related phosphatase SHP-2, but the functional relevance of these findings to intact Ly-49A has not yet been examined (22). In this report, we demonstrate that ligation of Ly-49A interrupts early signals for NK cell activation, inhibiting tyrosine phosphorylation and polyphosphoinositide turnover.

1Abbreviations used in this paper: APT, phosphotyrosine; InsP<sub>3</sub>, inositol triphosphates; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIRs, killer inhibitory receptors; LAK, IL-2-activated NK cells.
We also demonstrate that intact Ly-49A directly associates with SH P-1. Moreover, we show that the full inhibitory effect of Ly-49A in NK cells requires intact SH P-1 function as well as the tyrosine residue within the proposed SH P-1 binding site of Ly-49A.

Materials and Methods

Cells. R N K-16, a spontaneous NK cell leukemia from F344 rats, was the gift of C. R. Reynolds (National Cancer Institute, Frederick, MD) and was adapted for in vitro growth in R PMI-1640 supplemented with 10% heat-inactivated FCS, 25 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete R PMI) (31). Tumor target cell lines cultured in complete R PMI included YAC-1 (mouse lymphoma, H-2d), P815D1 (mouse macrophage, H-2b), and C1498 (mouse monocyte, H-2b) from the American Type Culture Collection (Rockville, MD). D12 (C1498 transfected with H-2Db, C1498D1), a gift from W. Yokoyama, was described previously (6). B-16.S, a mouse melanoma (H-2d), was a gift from K. Kärre (Karolinska Institute, Stockholm, Sweden).

Mice. Viably mothetaten mice C57BL/6 (me/me) mice were obtained from the Jackson Laboratory at 6 wk of age along with littermate heterozygotes (obtained from the Jackson Laboratory at 6 wk of age along with factor cells at the indicated effector to target ratios. Plates were was added to each well of 96-well plates containing 0.1 ml of ef...

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Tumor target cell lines cultured in complete RPMI supplemented with 10% heat-inactivated FCS, 25 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete R PMI) (31). Tumor target cell lines cultured in complete R PMI included YAC-1 (mouse lymphoma, H-2d), P815D1 (mouse macrophage, H-2b), and C1498 (mouse monocyte, H-2b) from the American Type Culture Collection (Rockville, MD). D12 (C1498 transfected with H-2Db, C1498D1), a gift from W. Yokoyama, was described previously (6). B-16.S, a mouse melanoma (H-2d), was a gift from K. Kärre (Karolinska Institute, Stockholm, Sweden).

Mice. Viably mothetaten mice C57BL/6 (me/me) mice were obtained from the Jackson Laboratory at 6 wk of age along with littermate heterozygotes (+/me) and age-matched wild-type C57BL/6 (+/+) mice. Mootethaten C57BL/6 (me/me) mice and littermate heterozygotes (+/me) were killed at 4–5 wk of age at the Jackson Laboratory, and isolated spleen cells were obtained.

Antigens and Flow Cytometry. mAbs to mouse Ly-49A (A1, mouse IgG2a), NK1.1 (PK136, mouse IgG2a), gp42 (3G7, mouse IgG2a), and phosphotyrosine (A PT) (4G10, mouse IgG2b) were produced from their respective hybridoma lines. Antibodies were partially purified from ascites by ammonium sulfate precipitation. Tissue culture supernatant of anti-mouse Fc receptor antibody (2.4G2, rat IgG2b) was a gift from P. Linnemeyer (University of California at San Francisco, CA). Fluorescein conjugation of protein A-purified mAb utilized standard methods (32). F(ab')2 fragments were generated by pepsin digestion, and undigested mAb was absorbed over protein A-Sepharose columns as described (32). Purity of F(ab')2 fragments was verified by SDS-PAGE and silver staining. For fluorescence analysis, mAbs were used at a concentration of 1 μg/10⁶ cells. Staining of IL-2–activated NK cells was performed using directly conjugated mAb in the presence of 1 mg/ml active G418 (Boehringer, Indianapolis, IN). G418-resistant cells grew out in 10–14 days in 5–10% of the wells. Transfected cells were isolated as previously described (6). In each sample, 10⁶ labeled effectors were incubated with 1 mCi/ml [³²P]orthophosphate in phosphate-free RPMI supplemented with 10% dialyzed FCS, at 2 × 10⁶ cells/ml for 90 min at 37°C. After labeling, cells were washed once in phosphate-free RPMI and used immediately for stimulation. In each sample, 10⁶ labeled effector cells were stimulated with 10⁷ unlabeled target cells in a total volume of 1 ml complete phosphate-free RPMI. Cell suspensions were immediately centrifuged for 10 s at 50 g, then incubated at

Preparation of fresh splenocytes as previously described (33). Spleen cells harvested from me/me and +/+ mice were transported from Jackson Laboratories in complete RPMI, after red cell lysis, and received within 24 h of death. Spleen cells were then passaged through nylon wool and placed in culture with IL-2, as with fresh splenocytes (33). Ly-49A positive and negative IL-2–activated NK cells were isolated as previously described (6). In brief, day 6 IL-2–activated NK cells were panned with the anti-Ly-49A mAb, A1. The purity of the Ly-49–NK cell population was ensured by treatment with anti-Ly-49A and rabbit anti-mouse Ig (Cappel, Malvern, PA), followed by rabbit complement (Cedarlane, Westbury, NY) for 1 h at 37°C. Ly-49– and Ly-49+ cell populations were then cultured overnight in complete R PMI supplemented with 1,000 U/ml human IL-2 (National Cancer Institute, Frederick, MD). Cells were washed extensively with BSS with 3% FCS on day 7, replated and used for assays on day 9. The result in populations of NK cells that were >95% pure as assessed by their expression of NK1.1.

DNA Constructs and Transfections. The construct for expression of Ly-49A in R NK-16 cells was prepared by subcloning the Ly-49A cDNA into the EcoRI site of the expression vector BSR e EN (A. Shaw and M. Olsowy, Washington University, St. Louis, MO). The Ly-49A/γ8 mutation was generated using site-directed mutagenesis by PCR with the oligonucleotide 5'-ATA- TAGAATTCAAGTAGAGT GAC GAGAGGTC ACTTTT- CAAATGTGAG-3', which was cloned into the EcoRI site of BSR e EN. Constructs were confirmed by sequencing in both directions before transfection. Transfections were performed using cesium-purified plasmids or plasmids purified over two sequential Qiagen tips according to the instructions of the manufacturer (Qiagen, Chaddxford, CA). R NK-16 cells in exponential growth were transfected with 20 μg of Sca-1–linearized plasmid DNA using a BTX-600 Electro Cell manipulator. Electroporation was performed using 3 × 10⁶ cells/ml in 2-mm cuvettes in a total volume of 400 μl of complete RPMI, at 115 V, 850 μF, 129.9 μA. Cuvettes were incubated on ice for 15 min after electroporation. Cells were cultured overnight, then plated in 96-well plates at a density of 10⁶ cells/well in complete RPMI containing 1 mg/ml active G418 (Boehringer, Indianapolis, IN). G418-resistant cells grew out in 10–14 days in 5–10% of the wells. Transfected cells were maintained in 1 mg/ml active G418, but were grown in complete RPMI without G418 for at least 2 d before use in functional assays.

Measurement of Insitol-T riphosphate. To measure changes in water-soluble inositol triphosphates (InsP₃), cells were washed into inositol-free medium containing [³²P]myoinositol (20 μCi/ml, 80–120 Ci/mmol, Amersham, Arlington Heights, IL) at a concentration of 5 × 10⁶ cells/ml. After incubation at 37°C for 3 h, cells were washed in complete RPMI. Then 5 × 10⁶ labeled effector cells were stimulated with 10⁷ target cells in a total volume of 1 ml. Cells were immediately centrifuged at 50 g for 10 s and incubated at 37°C for the appropriate interval. [³²P]InsP₃ was extracted and resolved by ion exchange chromatography on Dowex AG-50, 1X-8 (Bio-Rad, Hercules, CA) as previously described (32).

Target Stimulation of [³²P]–phosphate-labeled E f ector Cells. For ³²P–metabolic labeling, effectors were incubated with 1 μCi/ml [³²P]orthophosphate in phosphate-free RPMI supplemented with 10% dialyzed FCS, at 2 × 10⁶ cells/ml for 90 min at 37°C. After labeling, cells were washed once in phosphate-free RPMI and used immediately for stimulation. In each sample, 10⁶ labeled effector cells were stimulated with 10⁷ unlabeled target cells in a total volume of 1 ml complete phosphate-free RPMI. Cell suspensions were immediately centrifuged for 10 s at 50 g, then incubated at
37°C for the indicated time, after which cells were rapidly centrifuged at 500 g and cell pellets were resuspended in cold HNTG lysis buffer (50 mM Heps, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM MgCl₂, 10% glycerol, 1 mM sodium orthovanadate, and protease inhibitors) with 1% Triton X-100. Lysates were precleared with protein A–Sepharose beads (Pharmacia, Piscataway, NJ) that had been previously coated with 1 ml 2.4G2 supernatant, for 2 h at 4°C, then immunoprecipitated with 30 µl of protein A beads coated with 5 mg APT mAb (4G10) overnight at 4°C. After washing with cold HNTG buffer with 1% Triton X-100, immunoprecipitates were resolved by 8% SDS-PAGE under reducing conditions. Gels were stained, fixed, and dried, and labeled proteins were resolved by autoradiography.

Immunoprecipitations and Western Blotting. Where indicated, transfected and wild-type RNK-16 cells (1.5 × 10⁵ cells/sample) were incubated for 5 min at 37°C in complete RPMI (6 × 10⁵ cells/ml) with 0.03% H₂O₂ and 100 μM sodium orthovanadate (per- vanadate), which pharmacologically increases protein tyrosine phosphorylation by inhibiting phosphatase activity (34). Cell pellets were lysed at 4°C in complete HNTG lysis buffer containing 1% Triton X-100. Lysates were then immunoprecipitated overnight at 4°C on 30 µl protein A–Sepharose beads loaded with anti–Ly-49A (A1) or isotype-matched control mAb (anti-NK1.1). Precipitates were washed four times with complete HNTG buffer containing 0.1% Triton X-100. Precipitated samples were resolved by 8% SDS-PAGE under nonreducing conditions and transferred to PVDF membranes (Immobilon-P, Millipore, Marlborough, MA). After blocking with TBS-T (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween-20) and 3% milk, the membranes were incubated with 0.5 µg/ml of anti–SHP-1 polyclonal rabbit antibody (UBI, Lake Placid, NY) in TBS-T with 3% milk for 1 h at room temperature. After extensive washing in TBS-T, blots were developed using 125I–protein A (Amersham, Arlington Heights, IL) followed by autoradiography.

Cold-target Inhibition Studies. Cytotoxicity assays to measure cold-target inhibition were performed at an E/T ratio of 10:1. 10⁵ cold targets were added to effectors as the same time as 10⁴ labeled targets. Effectors were preincubated with F(ab')₂ fragments at 25 µg/10⁶ cells for 15 min at room temperature before addition of targets when indicated. Results are expressed as percent inhibition = (1 – [percent cytotoxicity with cold target/percent cytotoxicity without cold target]) × 100.

Results

Mouse Ly-49A Inhibits Killing of H-2D<sup>d</sup> Targets by RNK-16.

To examine the intracellular signaling pathways that mediate the inhibitory function of mouse Ly-49A, we transfected mouse Ly-49A into RNK-16, a rat cell line with phenotypic and functional characteristics of rat NK cells (31). Nine RNK-mLy-49A clones expressing Ly-49A at different levels were obtained, three of which are represented in Fig. 1. Clones 2, 8, and 9 are representative of clones with low, medium, or high expression of Ly-49A, respectively (Fig. 1, B, C, D). Wild-type RNK-16 effector cells lysed P388D1 (H-2D<sup>d</sup>) tumor cells (Fig. 1 E), but RNK-16 cells transfected with Ly-49A (RNK-mLy-49A cells) demonstrated reduced lysis of P388D1 (F, G, H). Inhibition of lysis of P388D1 cells was proportional to the level of Ly-49A expression on the RNK-16 transfectants. The various levels of Ly-49A expression are shown in FACS<sup>®</sup> histograms (A – D). Cells were incubated with anti-Ly-49A (solid line) with FITC–goat anti–mouse Ab (FITC–GAM) or FITC–GAM alone (dotted line). Standard 4-h cytotoxicity assays were performed with either P388D1 cells (E – H) or YAC-1 targets (I – L). Effectors were wild-type RNK-16 cells (closed symbols) or RNK-16 transfected with Ly-49A (open symbols). Effectors used were RNK-16 (A, E, and I) and clones of RNK-16 expressing Ly-49A: low expression, RNK-mLy-49A.2 (B, F, J); intermediate expression, RNK-mLy-49A.8 (C, G, K); and high expression, RNK-mLy-49A.9 (D, H, L). Assays were carried out in the absence of antibody (squares), or in the presence of anti-Ly-49A F(ab')₂ fragments (diamonds) and control F(ab')₂ (anti-NK1.1) (circles). The dotted line in F – H is the killing curve for wild-type RNK-16 without mAb (from E for comparison).
Inhibitory Mechanisms of Ly-49A

The data indicate that the clonal RNK-mLy-49A transfectants behave similarly to freshly isolated murine Ly-49A+ NK cells, and that they are a valid model in which to study Ly-49A function.

Ly-49A Inhibits Phosphoinositide Turnover in Response to H-2D+ Target Cells. Using the RNK-mLy-49A.9 cell line, we examined the inositol phosphate response following stimulation by YAC-1 (sensitive) and P388D1 (resistant) targets. Wild-type RNK-16, but not RNK-mLy-49A.9 cells, responded to the H-2D+ target P388D1 with a rapid increase in InsP3 at 2 min, as shown in Fig. 2. YAC-1, which is susceptible to lysis by both wild-type RNK-16 and RNK-mLy-49A.9, stimulates an InsP3 response in both cell types. The lack of phosphoinositide turnover in RNK-mLy-49A.9 in response to H-2D+ target cells indicates that Ly-49A interrupts proximal signaling events in RNK-16.

Ly-49A Inhibits an Early Increase in Protein Tyrosine Phosphorylation during Target Cell Stimulation. To examine the effect of Ly-49A–mediated inhibition on protein tyrosine phosphorylation, [32P]orthophosphate-labeled RNK-16 and RNK-mLy-49A.9 cells were stimulated with YAC-1 (H-2a) and P388D1 (H-2D+) target cells. Because Ly-49A interrupts InsP3 turnover, an early signaling event, we examined the effect of target stimulation at 30 s, 1 min, and 5 min time points. Lysates from cells stimulated for these time intervals were immunoprecipitated with APT (4G10) and resolved by SDS-PAGE (Fig. 3). RNK-16 cells stimulated with P388D1 (H-2D+) showed a rapid increase in protein tyrosine phosphorylation at 30 s to 1 min, which diminished toward basal levels at 5 min (Fig. 3, left). In contrast, RNK-mLy-49A.9 cells stimulated with P388D1 failed to show an increase in protein tyrosine phosphorylation at 30 s to 1 min, but showed a minimal increase at 5 min (Fig. 3, center). Nonetheless, RNK-mLy-49A.9 cells demonstrated rapid protein tyrosine phosphorylation in response to YAC-1.
after 30 s, demonstrating that this signaling pathway was intact (Fig. 3, right). Wild-type RN K-16 showed a similar response to YAC-1 target cells (data not shown). Thus, susceptible, but not resistant, targets induce very brisk increases in protein tyrosine phosphorylation, and mouse Ly-49A specifically interrupts rapid tyrosine phosphorylation, and mouse Ly-49A–mediated inhibition of NK cell function might be mediated by SHP-1. Therefore, we first investigated the binding of SHP-1 to Ly-49A in RN K-16 cells by performing immunoprecipitation experiments. RN K-16 and RN K-mLy-49A.9 cells were stimulated with pervanadate, a phosphatase inhibitor that pharmacologically increases protein tyrosine phosphorylation (34). Lysates from stimulated and unstimulated cells were precipitated with anti–Ly-49A, and precipitates were examined for the presence of Ly-49A–associated SHP-1 by Western blot analysis. As shown in Fig. 4, SHP-1 was present only in anti-Ly-49A immunoprecipitates from pervanadate-stimulated RN K-mLy-49A.9 cells. SHP-1 was not detected in anti-Ly-49A immunoprecipitates from unstimulated RN K-mLy-49A.9 cells or in isotype-matched control mAb immunoprecipitates from unstimulated or stimulated RN K-mLy-49A.9 cells. SHP-1 was not detected in anti-Ly-49A or control mAb immunoprecipitates from RN K-16 cells, regardless of stimulation.

Ly-49A Function Is Impaired in NK Cells from Viable Motheaten (me/me) Mice, which have an incomplete defect in SHP-1, and the completely SHP-1–deficient motheaten (me/me) mice. We first examined NK cells from SHP-1 mutant viable motheaten (me/me) mice because they survive to 8–9 wk of age, when NK cell development is complete. These mice contain a point mutation in the SHP-1 gene that creates a donor splice site. This mutation results in aberrant splicing, creating either an in-frame insertion or deletion in the catalytic domain of the SHP-1 phosphatase (35–37). Although SHP-1 activity is reduced, some residual SHP-1 function remains and me/me mice have an attenuated motheaten phenotype.

We isolated Ly-49A+ and Ly-49A– NK cells from homozygous me/me, heterozygous +/me, and wild-type C 57BL/6 +/+ mice. Using these effectors, we tested Ly-49A function in cytotoxicity assays against C 1498 (H-2b) cells and D 12 (C 1498.Dd) cells as targets. Ly-49A+ cells from all mice expressed Ly-49A at similar levels and >95% of all cells were positive for NK 1.1 and negative for CD3 by FACSC (data not shown).

In each of four experiments, with homozygous me/me mice the function of Ly-49A was impaired in that C 1498.Dd targets were lysed by Ly-49A+ effectors, albeit less effectively than by Ly-49A– effectors. However, Ly-49A remained partially effective in cells from me/me mice in that addition of anti–Ly-49A still increased lysis of the C 1498.Dd targets. Fig. 5 shows a representative experiment in which Ly-49A+ cells from wild-type C 57BL/6 +/+ or from heterozygous +/me mice were unable to lyse C 1498.Dd targets (A and B), but Ly-49A+ cells from homozygous mice were able to lyse these targets (C). Ly-49A+ cells from all mice were able to lyse C 1498.Dd equally well (Fig. 5, D–F). Addition of anti–Ly-49A mAb reversed the Ly-49A–mediated inhibition to levels similar to those of Ly-49A– cells from all mice, whereas isotype-matched control anti-gp42 mAb had no effect. Ly-49A+ and Ly-49A– cells from all
mice were able to lyse the H-2b target C1498 and addition of mAb anti-Ly-49A or anti-gp42 had no effect (data not shown). These findings indicate that the function of Ly-

49A is partially impaired in IL-2-activated NK cells isolated from homozygous me/me mice.

The partial function of Ly-49A in me/me mice could reflect the residual activity of SHP-1 in these mice. To examine the effect of the complete absence of SHP-1, we next examined Ly-49A function in IL-2-activated NK cells isolated from me/me mice. Using spleen cells harvested from me/me mice (sacrificed just before their natural demise), +/me littermate heterozygote controls, and wild-type +/+ mice, we isolated Ly-49A+ and Ly-49A− cells. As shown in Fig. 6 (B, D, F), Ly-49A expression was equivalent on cells isolated from all mice. Ly-49A+ cells (Fig. 6, C and E) contained <5% Ly-49A+ cells, whereas the +/+ Ly-49A− population (A) contained ~10% Ly-49A+ cells. >95% of all cells were positive for NK1.1 and negative for CD3 by FACS® analysis was performed on day 9 LAK cells using FITC anti-Ly-49A (A1). Staining was performed in the presence of unlabeled blocking antibodies (IgG2a mouse myeloma protein, 1 μg/106 cells in 0.1 ml 2.4G2 supernatant). Ly-49A expression is shown in FACS® histograms (A-F). Dotted lines represent cells incubated with saline; solid lines represent FITC anti-Ly-49A staining.

Figure 5. Ly-49A function is impaired in me/me LAK cells. 9-d Ly-49A+ and Ly-49A− LAK cells were tested in 4-h cytotoxicity assays against D12 (C1498.Dd) targets against Ly-49A+ (A−C) or Ly-49A− effector cells (D−F) from +/+ (A and D), +/me (B and E), or me/me mice (C and F). Assays were done in the absence of antibody (open squares), or in the presence of anti-Ly-49A (A1, closed diamonds), or isotype-matched control antibody (anti-gp42, 3G7, open circles).

Ly-49A is functionally impaired in NK cells isolated from homozygous me/me mice. However, even in the complete absence of SHP-1, Ly-49A had some remaining inhibitory activity.

The Position 8 Tyrosine Is Required for Mouse Ly-49A Function. To examine further the role of SHP-1 in the function of Ly-49A, we mutated the tyrosine residue within the proposed SHP-1 binding motif in the Ly-49A cytoplasmic domain. R NK-16 cells transfected with this tyrosine mutant, (RNK-mly-49A/Y8F) stained with anti-Ly-

49A mAb at levels similar to those seen in RNK-mly-49A.9 (Fig. 8, D, F, H). Despite this level of Ly-49A expression, RNK-mly-49A/Y8F clones 1 and 4, derived from separate transfections, were not inhibited in their capacity to lyse P388D1 (H-2Dk) cells (Fig. 8, E and G). Con-

Figure 6. Ly-49A expression on Ly-49A+ and Ly-49A− LAK cells isolated from +/+ , +/me, me/me mice. 6-d LAK cells were separated into Ly-49A+ and Ly-49A− populations by panning with anti-Ly-49A Ab. Ly-49A− cells were additionally treated with rabbit anti-mouse Ab and complement depletion. FACS® analysis was performed on day 9 LAK cells using FITC anti-Ly-49A (A1). Staining was performed in the presence of unlabeled blocking antibodies (IgG2a mouse myeloma protein, 1 μg/106 cells in 0.1 ml 2.4G2 supernatant). Ly-49A expression is shown in FACS® histograms (A-F). Dotted lines represent cells incubated with saline; solid lines represent FITC anti-Ly-49A staining.
consistent with our earlier results, RNK-mLy-49A.9 cells could not lyse P388D1 (Fig. 8C), whereas wild-type RNK-16 lysed P388D1 efficiently (A). Notably, neither anti–Ly-49A nor control mAb (anti–NK1.1) had any effect on lysis of P388D1 by RNK-mLy-49A/Y8F (Fig. 8, E and G), but anti–Ly-49A reversed the inhibition of P388D1 lysis by RNK-mLy-49A.9 (C). RNK-mLy-49A/Y8F clones 1 and 4 effectively lysed YAC-1 targets (data not shown). These data show that the tyrosine within the proposed immunoreceptor tyrosine-based inhibitory motif (ITIM) is required for the inhibitory effect of Ly-49A on RNK-16 cytotoxicity. These experiments complement the studies of motheaten mice, demonstrating a functional role for SHP-1 in the inhibitory activity of Ly-49A.

Addition of H-2Dd (Resistant) Targets Does Not Affect the Killing of Non-H-2Dd (Susceptible) Targets by RNK-mLy-49A.9.

Initial reports demonstrated that ligation of H-2Dd targets by the Ly-49A receptor resulted in inhibition of natural killing, of antibody-dependent cytotoxicity (ADCC), and of lectin-induced cytotoxicity by NK cells (6, 38). These studies, performed on bulk populations of Ly-49A+ NK cells, suggested that Ly-49A might transduce signals that globally inhibit NK cell function. Using our RNK-Ly-49A transfectants, we were able to examine mouse Ly-49A function in a clonal cell population. To examine the effect of inhibitory H-2Dd targets on the lysis of labeled bystander non-H-2Dd targets, we performed cold-target competition experiments with wild-type RNK-16 or RNK-mLy-49A.9 as effectors, and 32P-labeled B-165 (H-2d) melanoma tar-
B-16S lysis by RNK-mLy-49A. In the presence of F(ab')2 anti-Ly-49A, was relatively ineffective as a cold-target inhibitor of lysis of labeled B-16S targets on lysis of labeled B-16S was determined for each effector (Fig. 9). As expected, unlabeled B-16S (H-2a) were ineffective as cold-target competitors for the lysis of 51Cr-labeled B-16S targets. Four-hour cytotoxicity assays were performed with RNK-16 (left) or RNK-mLY-49A.9 cells (right) as effectors. B-16S (H-2a) target cells were labeled with 51Cr and tested as an effector to labeled target ratio of 10:1. Cold targets were either unlabeled B-16S cells (H-2a) or P388D1 cells (H-2Dd). Cold targets were added in 10-fold excess to labeled targets. 105 cold targets and 104 labeled targets were added at the same time to 105 effectors in a total volume of 0.2 ml. Effectors were preincubated with no antibody, F(ab')2 anti-Ly-49A or F(ab')2 anti-NK1.1. Results are expressed as percent inhibition = (1 – [percent cytotoxicity with cold target/percent cytotoxicity without cold target]) × 100.

The effect of unlabeled H-2Dd or non-H-2Dd targets on lysis of labeled B-16S was determined for each effector (Fig. 9). As expected, unlabeled B-16S (H-2a) were effective cold-target competitors for the lysis of 51Cr-labeled B-16S by both RNK-16 and by RNK-mLy-49A.9. Unlabeled P388D1 (H-2Dd) targets, which are sensitive to lysis by RNK-16, effectively inhibited B-16S lysis by RNK-16. In contrast, P388D1, which was not killed by RNK-mLy-49A.9, was relatively ineffective as a cold-target inhibitor of B-16S lysis by RNK-mLy-49A.9. In the presence of F(ab')2 anti-Ly-49A, P388D1 became sensitive to lysis by RNK-mLy-49A.9. This allowed cold P388D1 cells to compete effectively with labeled B-16S for the lytic machinery of RNK-mLy-49A.9. Control F(ab')2 anti-NK1.1 fragments had no effect on cold-target competition. Thus, the expression of H-2Dd on bystander targets does not globally inhibit the Ly-49A+ effector cell response towards a non-H-2Dd cell. Rather, because H-2Dd cells are not susceptible to NK cell lysis by Ly-49A+ effectors, they compete less well than susceptible (B-16S) cold targets for the lysis of labeled B-16S. These data indicate that there is no bystander inhibition through Ly-49A. They suggest that inhibitory effects mediated through this receptor are spatially oriented toward ligand-bearing H-2Dd targets on the NK cell membrane, and this does not affect killing of susceptible targets recognized by the same NK cell. Alternatively, the duration of Ly-49A–mediated inhibition could be brief, and temporally limited to periods of NK cell contact with H-2Dd targets. Temporal restriction would allow the subsequent lysis of susceptible H-2D targets by the same NK cell.

**Discussion**

In an attempt to elucidate the pathways through which Ly-49 molecules inhibit natural killing, we examined mouse Ly-49A, which prevents NK cell lysis of targets expressing H-2Dd or H-2Dk (6). To study Ly-49A function in a uniform clonal NK cell population, we transfected the rat NK cell line RNK-16 with the mouse Ly-49A cDNA. Ly-49A was functional in these RNK-16 transfectants, specifically inhibiting lysis of the H-2Dd target P388D1.

Because activation of cytotoxicity is associated with phosphoinositide turnover and an increase in protein tyrosine phosphorylation in NK cells, we examined the effect of Ly-49A ligation on these early signaling events during target-induced NK cell activation. Rapid rises in InsP3 were studied in wild-type RNK-16 cells in response to P388D1 (H-2Dd) targets, but the expression of Ly-49A on RNK-16 cells prevented this response. Ligation of Ly-49A also inhibited target cell–induced protein tyrosine phosphorylation in response to P388D1 targets. In contrast, YAC-1 targets stimulated a prompt increase in protein tyrosine phosphorylation in wild-type RNK-16 cells and in RNK-mLy-49A.9 cells.

The ability of mouse Ly-49A to inhibit the generation of InsP3 is similar to the effect previously shown in human NK cells, where inhibition of inositol phosphate turnover parallels the inhibition of NK cell cytotoxicity by class I molecules (39). Although no crosshybridizing human Ly-49 homologues have yet been identified, human NK cells express a different family of KIRs (40). The structure of KIRs, which are type I immunoglobulin-like receptors, is not related to that of Ly-49A. However, like Ly-49A, the KIRs inhibit NK cell cytotoxicity upon ligation by specific MHC class I antigens on target cells (41–46).

Initial reports of human NK cells indicated that protein tyrosine phosphorylation was not inhibited by the expression of MHC class I targets (29, 39). One study examined target-induced phosphorylation in NK cells stimulated for 1, 5, and 30 min, and another study examined NK cells stimulated for 5 min (29, 39). In these human studies, there were no appreciable differences in levels of phosphorylation when NK cells were stimulated with susceptible or resistant targets. In our RNK-mLy-49A.9 transfectant, we also could detect no significant differences in protein tyrosine phosphorylation at 5 min (Fig. 3), or at 15 and 30 min (data not shown) after target cell stimulation with either sensitive or resistant targets. However, we were able to demonstrate clearly that rapid (30–60 s) protein tyrosine phosphorylation is markedly reduced when Ly-49A mediates inhibition of cytotoxicity. The inhibitory effects of Ly-49A on phosphoinositide turnover and on tyrosine phosphorylation suggest that Ly-49A interrupts signaling events early in target cell–induced activation of NK cells.

A possible mechanism for the inhibitory activity of Ly-49A to inhibit the generation of InsP3 is similar to the effect previously shown in human NK cells, where inhibition of inositol phosphate turnover parallels the inhibition of NK cell cytotoxicity by class I molecules (39). Although no crosshybridizing human Ly-49 homologues have yet been identified, human NK cells express a different family of KIRs (40). The structure of KIRs, which are type I immunoglobulin-like receptors, is not related to that of Ly-49A. However, like Ly-49A, the KIRs inhibit NK cell cytotoxicity upon ligation by specific MHC class I antigens on target cells (41–46).

**Inhibitory Mechanisms of Ly-49A**

![Image](60x547 to 300x726)
Ly-49A function in paired. We initially thought that incomplete impairment of activity with C1498 (H-2b), lysis of D12 (C1498.Dd) was almost somewhat diminished compared with lysis by wild-type, eaten (lysis of YAC-1 targets by anti–Ly-49A immunoprecipitates from pervanadate-stimulated R NK-mLy-49A cells (data not shown)).

Site-directed mutational analysis of the Ly-49A molecule confirmed the functional significance of the Ly-49A/SHP-1 interaction. Disruption of the putative ITIM motif in Ly-49A by mutating the tyrosine to phenylalanine at residue 8 (Y8F), completely eliminated Ly-49A-mediated inhibition of cytotoxicity against H-2Dd target cells by R NK-16 transfectants. Our studies demonstrate that Ly-49A interrupts proximal signaling events during natural killing, and they provide evidence that this inhibitory function is largely mediated through the SHP-1 phosphatase. The mechanisms by which SHP-1 inhibits cytotoxicity have not been fully elucidated. Our cold-target inhibition studies suggest that the Ly-49A-mediated inhibitory effect is localized within the N cell, because targets expressing H-2Dd are protected from lysis but fail to inhibit N cell cytotoxicity against non-H-2Dd targets. Thus, Ly-49A does not transduce global inhibitory signals to N cells. Rather, it appears that Ly-49A may locally interrupt activating signals transduced by other receptors during natural killing.

In summary, we have demonstrated that the ligation of Ly-49A by its MHC class I ligand, H-2Dd, interrupts early signaling events stimulated during target-induced activation of N K cells, including phosphoinositide turnover and protein tyrosine phosphorylation. We have also shown the direct association of the cytoplasmic tyrosine phosphatase SHP-1 with Ly-49A. Our studies indicate a requirement for SHP-1 for the optimal function of Ly-49A, as we found that Ly-49A function is markedly impaired in SHP-1 mutant me/me and me/me mice. The finding of partial Ly-49A function despite the complete absence of SHP-1 in me/me mice indicates the possible involvement of other cytoplasmic mediators, such as SHP-2. In addition, we have shown that the tyrosine within the proposed SHP-1 binding motif in Ly-49A is strictly required for the inhibitory effects of this receptor on cytotoxicity. Finally, we have demonstrated that inhibition through Ly-49A is target specific. Although a number of candidate activating receptors on N K cells have been described, including the lectin-like NKR-P1 molecule (54), the specific receptors that bind tumors and activate N K cell cytotoxicity have not yet been identified. Our studies indicate that the inhibitory Ly-49A receptor interrupts target-induced activation signals through its recruitment of inhibitory mediators to the effector/target interface in a spatially or temporally restricted manner.
References


