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Positive Recognition of MHC Class I Molecules by the Ly49D Receptor of Murine NK Cells

Thaddeus C. George,* Llewellyn H. Mason,† John R. Ortaldo,† Vinay Kumar,* and Michael Bennett²∗

Members of the murine Ly49 family of receptors have been shown to inhibit and activate NK cell function. Subsets of Ly49-expressing NK cells mediate the rejection of bone marrow cell allografts and the lysis of allogeneic lymphoblasts. In this report we have studied Ly49-mediated positive and negative signaling in an in vitro cytotoxicity assay using sorted NK cell subsets as effectors and a panel of ⁵¹Cr-labeled Con A lymphoblasts as targets in the presence or the absence of Abs to Ly49 and/or class I molecules. Our results demonstrate that the activating receptor Ly49D delivers stimulatory signals for target cell lysis upon interacting with H₂-Dᵈ, Dʰ, and Dʰ⁹², but not H₂-b or H₂-k class I Ags. On the other hand, the inhibitory receptor Ly49G₂ delivers negative signals for target cell lysis upon interacting with Dʰ, Dʰ, and H₂, but not H₂-b or Dʰ⁹², class I Ags. Furthermore, Ly49-mediated negative signaling dominates Ly49D-mediated positive signaling. Thus, lysis of class I MHC-bearing targets by NK cells is not merely the consequence of the absence of an Ly49-mediated negative signal, but also requires positive recognition of class I molecules by certain Ly49 receptors. Activation of NK cells by nonself class I molecules was not predicted by the missing self hypothesis. The Journal of Immunology, 1999, 162: 2035–2043.

Abbreviations used in this paper: BMC, bone marrow cell; ITIM, immunoreceptor tyrosine-based inhibitory motif; PE, phycoerythrin; LAKs, lymphokine-activated killer cells.

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† Abbreviations used in this paper: BMC, bone marrow cell; ITIM, immunoreceptor tyrosine-based inhibitory motif; PE, phycoerythrin; LAKs, lymphokine-activated killer cells.
we have studied the alloreactivity of the Ly49D\(^{−}\) and Ly49D\(^{+}\) subsets from B6 hosts in an in vitro cytotoxicity assay.

**Materials and Methods**

**Mice**

All mice were bred and maintained in the Microbiology Colony at the University of Texas Southwestern Medical Center (Dallas, TX). Derivation of the D\(^{d}\) transgenic D8 strain from C57BL/6 mice and of class I-deficient TAP\(^{−}\) mutant mice has been described previously (30, 31). The haplotypes of the various strains used are given in Table I.

### Purification and Modification of mAbs

The unconjugated and biotinylated 4E5 (anti-Ly49D) and FITC-conjugated 4D11 (anti-Ly49G2) mAbs as well as the 4D11 salt cut and 12A8 mAbs were prepared as previously described (16, 17, 32). Biotinylated 4LO3311 (anti-Ly49C) was a gift from Dr. Suzanne Lemieux (University of Quebec, Montreal, Canada). FITC-conjugated anti-NK1.1 and biotin-conjugated anti-Ly49A and S6E (anti-Ly49C/I) were purchased from Pharmingen (San Diego, CA). The anti-H2-D\(^{d}\) mAb, which is specific for the a\(_2\)a\(_2\) domain (33) was derived from hybridoma 34-5-8S purchased from American Type Culture Collection (Manassas, VA; catalog no. HB 102). The S6E mAb was derived as previously described (27). The supernatants from hybridoma cells grown in serum-free medium (HyClone, Logan, UT) were purified by affinity chromatography using Affigel protein A-agarose (Bio-Rad, Hercules, CA) for mouse 34-5-8S and S6E, or protein G-Sepharose 4 Fast Flow (Pharmacia LKB Biotechnology, Piscataway, NJ) for rat 12A8 according to the instructions of the manufacturer. F(ab\(^{9}\))\(_2\) reagents were then generated. Briefly, the purified mAbs were dialyzed twice against 0.5X PBS buffer. Pilot digestions using pepsin (Sigma, St. Louis, MO) dissolved in 200 mM sodium citrate buffer (pH 3.5) at a 1:40 pepsin to mAb ratio were performed at 37°C for each Ab preparation to determine the optimal length of digestion. F(ab\(^{9}\))\(_2\) were then dialyzed against PBS. The efficiency of digestion was checked by 4–20% gradient SDS-PAGE.

### Enrichment of splenic NK cells

Single cell suspensions of splenocytes were prepared aseptically in complete RPMI 1640 (10% FBS, 100 U/ml streptomycin, 100 \(\mu\)g/ml penicillin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 0.1 mM nonessential amino acids) by gently crushing spleens between the frosted edges of two glass slides. The cells were then washed, resuspended at 50 \(\times\) 10\(^6\) cells/ml in PBS containing 2% FBS (PBS/FBS), and incubated with 5 \(\mu\)g/ml anti-FcR-H11 (2.432) mAb to block the FcR. After washing, the cells were resuspended at 60–100 \(\times\) 10\(^6\) cells/ml in PBS/FBS, then incubated with StemSep murine NK enrichment mixture containing mAbs CD5, CD22, Gr-1, and TER-119 (Stem Cell Technologies, Vancouver, Canada). After washing and resuspending in PBS/FBS at the same concentration, the cells were incubated with StemSep anti-biotin tetramer, then incubated with magnetic colloid. All incubations were performed for 15 min at 4°C. The cells were then filtered onto a PBS-washed StemSep 0.6-in. column placed inside a VarioMACS magnetic field (Miltenyi Biotec, Auburn, CA). The cells collected in the flow-through typically stained 50–70% NK1.1 positive. The cells were then washed and resuspended at 3 \(\times\) 10\(^8\) cells/ml in complete DMEM supplemented with 2.25 \(\times\) 10\(^{-3}\) M 2-ME and 500 U/ml recombinant human IL-2 (Chiron, Wapole, MA) and were cultured overnight in a 24-well plate at 37°C in a 1% CO\(_2\)/air mixture.

### Cell sorting and generation of effector cells

The NK-enriched cells described above were harvested, washed, then resuspended at 30 \(\times\) 10\(^6\) cells/ml in PBS/FBS. The FcR was blocked as described. Without washing, the cells were incubated with a 1/1000 dilution of biotinylated anti-Ly49D-specific 4E5 mAb. After washing, the cells were resuspended and incubated with 1 \(\mu\)g/ml PE-conjugated streptavidin (PharMingen, San Diego, CA) alone or with a 1/1000 dilution of FITC-conjugated 4D11. For generation of Ly49D\(^{+}\)/Ly49A/C/G2/T and Ly49D\(^{−}\)/Ly49A/C/G2 and/or 1\(^{\prime}\) subsets, cells were incubated with FcR block as described. Without washing, the cells were incubated with 5 \(\mu\)g/ml 5 \(\mu\)g/ml anti-Ly49A, 5 \(\mu\)g/ml 4LO3311 (anti-Ly49C) and 4D11 (anti-Ly49G2) mAbs as well as the 4D11 salt cut and 12A8 mAbs. All incubations were performed for 15 min at 4°C. After washing, cells with forward and side scatter characteristics of lymphocytes were sorted on the FACStar Plus device (Becton Dickinson, Mountain View, CA). The recovered cells were cultured at 2.5 \(\times\) 10\(^3\) cells/ml and 5 \(\times\) 10\(^3\) cells/well in 96-well U-bottom plates in complete DMEM supplemented with 2.25 \(\times\) 10\(^{-3}\) M 2-ME and 500 U/ml human rIL-2 for 5 days at 37°C in a 10% CO\(_2\)/air mixture.

### Generation of target cells

Splenocytes were prepared as described and resuspended at 4–6 \(\times\) 10\(^6\) cells/ml in complete RPMI 1640 with 6 \(\mu\)g/ml Con A (Sigma). The cells were cultured 2 ml/well in 24-well plates for 2 days at 37°C in a 5% CO\(_2\)/air mixture. The cells were then harvested, washed once, resuspended in 2 ml complete RPMI 1640, layered on top of 4 ml of Ficoll-Hypaque solution (Pharmacia LKB Biotechnology), and centrifuged at room temperature for 20 min at 1300 \(\times\) g. Theuffy coat containing viable lymphoblasts was removed and washed once in complete RPMI 1640 before radiolabeling.

### Cytotoxicity assay

Target Con A lymphoblasts (1.5–2 \(\times\) 10\(^6\)) were incubated for 1.5 h at 37°C in a total volume of 0.6 ml with 150–250 \(\mu\)Ci of sodium chromium (\(^{51}\)Cr; Amersham, Arlington Heights, IL). Radiolabeled cells were washed once, resuspended in 5 ml of complete RPMI 1640, and incubated an additional 1 h at 37°C. The cells were washed twice, and diluted to 500 targets/100 \(\mu\)l of medium. Effectors at constant or variable E/T cell ratios in a final volume of 100 \(\mu\)l were added first to the wells of 96-well V-bottom plates. An identical volume of targets, except as noted below, was added to the appropriate wells. The coculture was performed in triplicate groups at 37°C in a 5% CO\(_2\)/air mixture. Target cells were preincubated with mAb 34-5-8S (anti-a\(_2\)a\(_2\) domain-specific H2-D\(^{d}\)) F(ab\(^{9}\))\(_2\), at 4°C for 15 min, while effector cells were preincubated with 4E5 salt cut, 4D11 salt cut, S6E F(ab\(^{9}\))\(_2\), and/or 12A8 F(ab\(^{9}\))\(_2\), for 30–60 min at 37°C. For the cold target competition studies, unlabeled Con A lymphoblasts were added to the plated effector cells in a volume of 50 \(\mu\)l before the addition of labeled (hot) D8 targets at various cold target to hot target ratios. The 500 hot D8 targets were added in a total volume of 50 \(\mu\)l of complete RPMI 1640. After 4 h of incubation, 100 \(\mu\)l of supernatant was removed, and \(^{51}\)Cr radioactivity was measured in a liquid scintillation counter. Specific lysis, represented as the mean \(\pm\) SEM, was calculated as follows: percent specific lysis = \(^{51}\)Cr cpm ([E/R] – [SR]/[MR – SR]) \times 100, where ER is the experimental \(^{51}\)Cr release in the presence of effector cells, SR is the spontaneous \(^{51}\)Cr release in the presence of medium, and MR is the maximum \(^{51}\)Cr release in the presence of 1.0% Triton X-100. The percent inhibition of lysis is calculated as follows: ([percent specific lysis of D8 hot target alone] – [percent specific lysis of D8 target in the presence of cold targets])/[percent specific lysis of D8 hot target alone]. Each cytotoxicity assay was performed at least three times.

### Results

The mAb 4E5 divides B6 NK cells into two subsets of similar sizes

The 12A8\(^{+}\) NK cell subset from B6 recipients reject BALB/c (H2\(^{d}\)) and D8 (H2\(^{d}\), D\(^{d}\)) BMC grafts, and blocking experiments suggest that the 12A8 receptor delivers positive signals from H2\(^{d}\), especially D\(^{d}\), in vivo (29). mAb 12A8 reacts with at least two receptors, Ly49D and Ly49A (16). Since Ly49A is a known inhibitory receptor for D\(^{d}\) (12), the in vivo data imply that the Ly49D receptor sends positive signals to host NK cells, resulting in the rejection of H2-D\(^{d}\) BMC grafts. Another Ly49D-reactive mAb, 4E5, does not cross-react with Ly49A (17). Two-color FACSscan analysis of magnetically enriched day 1 cultured splenocytes using anti-NK1.1 and 4E5 revealed that approximately 50% of B10 NK cells express the 4E5 Ag (Fig. 1A). Based on the previous in vivo
data, we hypothesized that the 4E5+ subset is responsible for the rejection of H2-Dd+ BMC and lysis of H2d targets in vitro.

The Ly49D+ subset preferentially lyse H2d Con A lymphoblast targets in vitro

If Ly49D delivers activating signals to NK cells as a result of interactions with specific H2d class I ligands expressed on the surface of target cells, we postulated that Ly49D+ NK cells would lyse H2d target cells more efficiently than Ly49D- cells. To test this hypothesis, freshly purified B6 (H2b) NK cells were sorted into 4E5+ and 4E5− subsets, cultured in IL-2, then tested for their ability to lyse 51Cr-labeled B6, B10.D2, and B10.BR Con A lymphoblasts. These three targets share a similar background but differ at the MHC region. The results shown in Fig. 1, A and B, demonstrate that 4E5+ NK cells lyse B10.D2 (H2d) targets significantly more efficiently than 4E5− NK cells do. The increased cytolytic activity against these targets does not reflect a general increase in lytic potential of 4E5+ NK cells, since neither the syngeneic B6 target nor the allogeneic B10.BR (H2k) targets are lysed by this subset compared with the 4E5− subset. Furthermore, 4E5− NK cells lyse target cells deficient in the transporter associated with Ag processing (TAP2/2) as efficiently as 4E5+ NK cells do, suggesting that the 4E5− subset has intact lytic machinery. This result also demonstrates that while NK cells may recognize MHC molecules, the presence of class I is not required for NK-mediated lysis. It is presumed that the efficient lysis of H2-identical TAP−/− targets is due to undefined triggering structures on Ly49D+ or Ly49D− NK cells in the absence of any negative signals from the target cells. Thus, these data show that the Ly49D+ subset specifically lyses H2d targets in vitro.

Dd+ and Dsp2+ targets compete for lysis of the H2-Dd+ (D8) target by the 4E5+ subset

Previous in vivo data suggest that Dd, Dr, and Ld share a cross-reactive antigenic motif that is positively recognized by B6 NK cells (28). Since the Ly49D+ cells are involved in the rejection of D8 BMC grafts by B6 hosts (29), we predicted that Ly49D+ NK cells would lyse D8 lymphoblasts and that Dr and Ld might also be stimulatory ligands for 4E5+ NK cells. To test this possibility, we assayed the ability of a panel of unlabeled cold Con A lymphoblasts to inhibit the lysis of 51Cr-labeled D8 targets by B6 4E5− NK cells. Cold targets susceptible to lysis by Ly49D+ cells would be expected to inhibit cytolysis of the D8 target more efficiently than cold targets resistant to lysis. D8 target cells were lysed efficiently (30%) at an E:T cell ratio of 15:1 (data not shown). Unlabeled TAP2/2 blasts, previously shown to be susceptible to lysis by Ly49D+ NK cells, fully compete for lysis of the hot D8 target (data not shown). Unlabeled H2-Dd+ targets, such as B10.D2 and D8 Con A lymphoblasts, also effectively compete for lysis of the D8 target, while syngeneic B6 targets do not (Fig. 2). IntraH2 recombinants B6.R4 (Kk, Dr) and B10.R40 (Kk, Dsp2) both effectively inhibit lysis of D8 targets by 4E5− B6 NK cells, suggesting that Dr and Dsp2 may also serve as ligands for positive signaling to this subset. H2k (C3H) and Ld transgenic H2k (C3H.Ld) targets did not compete for lysis of D8, suggesting that H2k- and H2Ld-expressing targets are not positively recognized by the 4E5+ subset.

**FIGURE 1.** Ly49D+ NK cells preferentially lyse allogeneic B10.D2 (H2b) and D8 (H2b, Dd) Con A lymphoblasts. A, Splenic NK cells from B10 mice were enriched by magnetic depletion of T cells, B cells, granulocytes, and erythroid cells and were cultured overnight in IL-2. The cells were then stained with FITC conjugated anti-NK1.1 and biotin-conjugated 4E5 followed by streptavidin-PE. B and C, B6 cells were enriched and cultured as described in A. After sorting into 4E5+ (B) and 4E5− (C) subsets, the cells were cultured for 5 days in IL-2 to generate LAKs. These LAKs were coincubated with a panel of 51Cr-labeled Con A lymphoblasts at various E:T cell ratios, and the percent specific lysis was determined.
FIGURE 2. D8+ (B6.R4) and D92+ (B10.R40) Con A blasts compete for lysis of the D8 target by the 4E5+ subset. 4E5+ B6 LAKs were generated as described and coincubated at a 12.5:1 E:T cell ratio with 51Cr-labeled Con A blasts in the presence or the absence of unlabeled (cold) lymphoblasts at various cold to hot (C:H) ratios. The data are presented as the percent inhibition of lysis. Lysis of the D8 hot target in this experiment was 30.1 ± 3.7%.

The failure of C3H.L4 lymphoblasts to compete with the lysis of the D8 target was unexpected (28). This observation is discussed below.

The Ly49D receptor specifically activates cytotoxicity against H2-Dd+, Dr-, and Dsp2-positive Con A lymphoblasts

In vivo administration of F(ab')2 of the anti-Ly49D mAb 12A8 reversed rejection of H2-Dd+ BMC grafts by B6 hosts (29). This suggests that the Ly49D molecule is involved in the recognition of H2-Dd. The previous data presented here demonstrate that Ly49D+ cells specifically recognize H2-Dd+, Dd+, and D92+ targets in vitro (Fig. 2). To test the prediction that the Ly49D receptor delivers stimulatory signals to NK cells upon interaction with H2-Dd+, Dr+, or D92+ in vitro, we preincubated 4E5+ effector NK cells with anti-Ly49D/A 12A8 F(ab')2 to block the interaction between Ly49D and its target cell ligands (Fig. 3). Preincubation with 12A8 F(ab')2 significantly reduced the cytotoxic activity of 4E5+ NK cells against B10.D2, D8, B6.R4, and B10.R40 targets. Such preincubation resulted in a similar level of lysis of these targets as seen with 4E5+ effectors. Because the 12A8 mAb recognizes not only Ly49D but also Ly49A, it could be argued that corecognition of the Ly49A subset within the Ly49D population may have influenced the results. To remove this variable, a similar experiment was performed with the Ly49D-specific 4E5+ mAb (Table II). Preincubation of 4E5+ cells with the Ly49D-specific 4E5+ mAb also reduced the lysis of D8 targets to the same extent that 12A8 F(ab')2 preincubation did, while such preincubation did not affect the lysis of TAP−/− lymphoblasts (Table II). This result also demonstrates that 4E5+ mAb or 12A8 F(ab')2 preincubation was not cytotoxic to the effectors, because such treatment had no effect on the lysis of syngeneic B6 or NK-sensitive TAP−/− targets. Taken together, these data suggest that preincubation of effector cells with an anti-Ly49D mAb interrupts the interaction between Ly49D and specific target cell ligands, resulting in a blockage in the delivery of positive signals. The significant reduction in the lysis of D8, B6.R4, and B10.R40 target cell by 12A8-preincubated 4E5+ cells strongly suggests that Ly49D stimulates lysis upon interaction with Dd+, Dr+, and D92+. The differential sensitivity of the H2-Dd+ targets (B10.D2 and D8) to lysis by these subsets will be addressed in later experiments and in Discussion.

4D11 and 4E5 mAbs define partially overlapping subsets of B6 NK cells

Because it has been reported that negative signals often dominate positive signals delivered to NK cells, it is likely that the subset responsible for the lysis or rejection of D92+ cells in vivo receives only positive signals from the target cell alloantigen. Thus, to detect lysis of H2d targets, there should exist a subset of B6 Ly49D+ cells that does not coexpress Ly49G2 or Ly49A, both of which are known to inhibit NK cytolysis upon interaction with D9. Two-color FACScan analysis of NK-enriched B6 splenocytes using 4E5 and anti-Ly49G2 (4D11) identifies two partially overlapping subsets of NK cells (Fig. 4A). We predicted that the 4E5+ 4D11− subset would preferentially lyse targets that expressed the Dd alloantigen.

The Ly49D+ G2− subset is responsible for the lysis of Dd+ and other class I allogeneic target cells

Approximately 50% of B6 Ly49D+ NK cells coexpress Ly49G2, a known inhibitory receptor for class I Ags, in particular Dd. Since Table II. 12A8 F(ab')2 fragments and 4E5 mAb reduce the lysis of D8 but not TAP−/− targets by 4E5+ B10 NK cells*

<table>
<thead>
<tr>
<th>Effector Pretreatment</th>
<th>ConA Lymphoblast Target</th>
<th>D8</th>
<th>TAP−/−</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>29.5 ± 4.3</td>
<td>53.4 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>12A8 F(ab')2*</td>
<td>6.7 ± 1.7</td>
<td>51.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>4E5 mAb*</td>
<td>6.9 ± 4.5</td>
<td>47.7 ± 1.9</td>
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</tbody>
</table>

* 4E5+ B10 LAKs were generated as described and coincubated at a 15:1 E:T ratio with 51Cr-labeled target cells. Percent specific lysis values ± SEM values are given.

**Effectors were preincubated with 12A8 (F(ab')2) fragments at a concentration of 30 µg/ml.

† Effectors were preincubated with 4E5 mAb at a concentration of 15 µg/ml.
The Ly49D<sup>G2</sup> subset is responsible for the lysis of D<sup>+</sup> and other class I allogeneic target cells. A. Splenic NK cells from B6 mice were enriched and cultured overnight as described. The cells were stained with FITC-conjugated 4D11 and biotin-conjugated 4E5 followed by streptavidin-PE. B. LAKs were generated from sorted 4E5<sup>+</sup> and 4E5<sup>−</sup> subsets, then coincubated with a panel of 51 Cr-labeled Con A lymphoblasts at a 10:1 E:T cell ratio.

Negative signaling often dominates positive signaling to NK cells, we hypothesized that targets capable of delivering negative signals through Ly49G2 would be lysed by the Ly49D<sup>G2</sup> subset. By contrast, targets incapable of delivering such negative signals would be lysed equally well by both Ly49G2<sup>−</sup> and Ly49G2<sup>+</sup> subsets of Ly49D<sup>−</sup> cells. To test these predictions, we sorted B6 NK cells into 4E5<sup>−</sup>4D11<sup>−</sup> and 4E5<sup>+</sup>4D11<sup>−</sup> subsets and tested their ability to lyse Con A lymphoblast targets (Fig. 4B). Ly49D<sup>+</sup> cells that coexpress Ly49G2 were unable to lyse H2-D<sup>+</sup> B10.D2 or D8 blasts, while Ly49D<sup>G2</sup><sup>−</sup> cells efficiently lysed these targets. In keeping with the latter, only 25% of Ly49D<sup>G2</sup><sup>−</sup> cells coexpressed Ly49A (data not shown). Similarly, D<sup>+</sup> B6.R4 targets were lysed preferentially by the 4D11<sup>−</sup> subset of Ly49D<sup>+</sup> effector cells. Thus, the presence of Ly49G2 abrogates the ability of Ly49D<sup>+</sup> NK cells to lyse D<sup>+</sup> and D<sup>+</sup> targets. B10.R40 (K<sup>b</sup>, D<sup>+</sup>) targets were lysed by both subsets, demonstrating that Ly49G2 is not a negative signaling receptor specific for H2-D<sup>+</sup> targets. Although in Fig. 4B a difference is seen in the susceptibility of the B10.R40 target, most experiments do not show this difference. Neither of the H2<sup>+</sup>-expressing targets (C3H and C3H.L<sup>d</sup>) was lysed by either subset. Taken together, these data show that expression of the negative signaling Ly49G2 receptor on Ly49D<sup>+</sup> cells impairs the ability to lyse D<sup>+</sup> and D<sup>+</sup>, but not D<sup>+</sup>, targets. It follows that Ly49G2 is an inhibitory receptor for D<sup>+</sup> and D<sup>+</sup>, but not D<sup>+</sup>, targets.

Positive signaling to the 4E5<sup>+</sup>4D11<sup>−</sup> subset

Previous experiments demonstrated that blockade of the Ly49D receptor with mAb 12A8 prevented lysis of targets normally susceptible to killing by 4E5<sup>+</sup> NK cells. Since the 4E5 subset that is responsible for the lysis of these targets does not coexpress Ly49G2 (4E5<sup>−</sup>4D11<sup>−</sup>), we predicted that preincubating this subset with 12A8 F(ab<sup>9</sup>)<sub>2</sub> would inhibit lysis of targets that express positive signaling ligands for Ly49D. The ability of 4E5<sup>−</sup>4D11<sup>−</sup> B6 NK cells to lyse D8, B6.R4, and B10.R40 was abolished when the effectors were preincubated with 12A8 F(ab<sup>9</sup>)<sub>2</sub> (Fig. 5A). Such treatment also greatly reduced the lysis of B10.D2 cells. B6, C3H, and C3H.L<sup>d</sup> targets were not lysed in the absence of blocking reagent, and effector preincubation with 12A8 F(ab<sup>9</sup>)<sub>2</sub> had no effect on the lysis of the NK-sensitive TAP<sup>−/−</sup> target. These data suggest that D<sup>+</sup>, D<sup>+</sup>, and D<sup>+</sup> specifically deliver positive signals to 4E5<sup>+</sup>4D11<sup>−</sup> B6 NK cells, resulting in target cell lysis.

Positive and negative signaling to the 4E5<sup>−</sup>4D11<sup>−</sup> subset

Unlike their Ly49G2<sup>−</sup> counterparts, Ly49D<sup>G2</sup><sup>−</sup> NK cells do not lyse D<sup>+</sup> or D<sup>+</sup> targets efficiently (Fig. 4B). If target cells express inhibitory ligands for Ly49G2 in addition to stimulatory ligands for Ly49D, the net signaling outcome might result in inhibition of target cell lysis. Alternatively, coexpression of Ly49G2 may render Ly49D nonfunctional. To distinguish between these two possibilities, 4E5<sup>−</sup>4D11<sup>−</sup> NK cells were tested for their abilities to lyse a panel of lymphoblast targets in the presence or the absence of 12A8 F(ab<sup>9</sup>)<sub>2</sub>, and/or 4D11 mAbs (Fig. 5B). The resistance of H2-D<sup>+</sup> targets (B10.D2 and D8) to lysis by this subset was reversed by preincubating the effector cells with anti-Ly49G2 4D11 Ab. Cytolysis of B6 targets was not enhanced by such effector treatment, demonstrating that 4D11 preincubation does not nonspecifically activate the lytic machinery of B6 NK cells. Furthermore, when 4D11-blocked effectors were preincubated with 12A8 F(ab<sup>9</sup>)<sub>2</sub>, the boosted lysis of D<sup>+</sup> targets was reduced, demonstrating that Ly49D function is intact in this subset. However, the lysis of the B10.D2 target boosted in the presence of 4D11 is not completely abrogated even when the effectors are preincubated with 12A8 F(ab<sup>9</sup>)<sub>2</sub>. This suggests that there might exist other unidentified non-Ly49D-activating receptors on Ly49G2<sup>−</sup> NK cells. In addition, B6.R4 (K<sup>b</sup>, D<sup>+</sup>) targets were lysed by Ly49D<sup>G2</sup><sup>−</sup> effectors only in the presence of 4D11 Ab, suggesting that interactions between D<sup>+</sup> and Ly49G2 result in inhibition of NK-mediated lysis. 12A8 F(ab<sup>9</sup>)<sub>2</sub> preincubation abrogated the boosted lysis of B6.R4, again demonstrating that functional interactions between D<sup>+</sup> and Ly49D activate lysis. This subset lysed B10.R40 (K<sup>b</sup>, D<sup>+</sup>), targets equally well in the presence or the absence of 4D11; the lysis was reduced if the effectors were preincubated with 12A8 F(ab<sup>9</sup>)<sub>2</sub>, suggesting that neither D<sup>+</sup> nor K<sup>b</sup> delivers inhibitory signals to NK cells upon interaction with Ly49G2, but that D<sup>+</sup> does stimulate lysis through interaction with Ly49D. Conversely, C3H and C3H.L<sup>d</sup> targets were only lysed in the presence of mAb 4D11, and 12A8 F(ab<sup>9</sup>)<sub>2</sub> preincubation of the effectors did not
inhibit their lysis, suggesting that Ly49G2 delivers inhibitory signals, but Ly49D does not deliver stimulatory signals, upon interaction with H2k alloantigens. Overall, these data support the conclusion that Ly49D can function in a subset that coexpresses Ly49G2, and that inhibitory signals delivered from one Ly49 can dominate stimulatory signals from another.

Anti-H2-D^d F(ab')_2 block positive signals to Ly49D^+ NK cells

The previous genetic data strongly suggest that Ly49D interacts with the D^d alloantigen, resulting in stimulation of NK-mediated lysis. However, it remains possible that the D^d Ag serves as a source of peptide that is presented in the context of a different class I, and this complex served as the stimulatory ligand for Ly49D. This would be similar to the ligand for human CD94, which can be HLA-E complexed with various class I leader peptides (34–36).

To test these possibilities, we assessed the cytolytic ability of 4E5^+4D11^+ B10 NK cells against D^k^+ D8 target cells preincubated with α1α2 domain-specific anti-H2-D^d F(ab')_2 (Fig. 6). D8 lymphoblasts were lysed efficiently, and this lysis was diminished when the effectors were preincubated with 12A8 F(ab')_2, and/or the target cells were preincubated with anti-H2-D^d F(ab')_2. Furthermore, the reduction in lysis of D8 cells was similar in all three Ab-treated groups. In control experiments, D8, but not B6, Con A lymphoblasts stained with the anti-Dd reagent (data not shown), and as expected, preincubation of the B6 target with anti-H2-D^d F(ab')_2 had no effect on its lysis by this subset. Taken together, these data strongly suggest that Ly49D interacts with D^d to deliver stimulatory signals to B6 NK cells, resulting in target cell lysis.

The B6 Ly49D^+ A/C/G2/I^+ subset is responsible for the lysis of H2-D^d transgenic D8 target cells

Since we have shown that Ly49-mediated inhibitory interactions can dominate Ly49D-mediated stimulatory interactions, it is likely that the precise B6 subset responsible for the rejection of D8 stem cells in vivo and the lysis of D8 targets in vitro does not coexpress inhibitory receptors specific for H2-D^d (Ly49A or Ly49G2) or H2^b (Ly49C or Ly49I) class I molecules. Two-color FACScan analysis of NK-enriched B6 splenocytes using 4E5 and a mixture of 5E6 (anti-Ly49C/I), 4LO3311 (anti-Ly49C), 4D11 (anti-Ly49G2), and A1 (anti-Ly49A) reagents identified a 4% subset that expresses Ly49D, but not Ly49A, C, G2, or I (data not shown). We predicted that this small subset would be able to efficiently lyse D8 targets in vitro. To test this prediction, we assayed the ability of 4D11^+ B6 NK cells to lyse Con A lymphoblast targets. As shown in Fig. 7, Ly49D^+ NK cells that do not coexpress any of the known inhibitory Ly49 receptors for H2-D^d or H2^b class I Ags lyse B6 targets poorly, but are able to lyse B10.D2, D8, and TAP−/− targets efficiently. Preincubation of these effectors with 12A8 F(ab')_2 reduced lysis of the B10.D2 and D8 targets but not the TAP−/− targets, demonstrating that Ly49D delivers positive signals upon interaction with the H2-D^d alloantigen. It is instructive to compare this experiment with others (Figs. 3, 4B, and 5) in...
which Ly49D+ or Ly49D−G2− effectors were employed. Under the latter conditions, the ability of Ly49D− cells to lyse H2-Dd+ targets was noted, but the lytic activity against B10.D2 targets was always higher than that against D8 T cell blasts. In this experiment (Fig. 7), when Ly49D+ cells were selected in a manner so that they did not express any known negative signaling receptors specific for H2-Dd (shared by B10.D2 and D8) or H2b (expressed only on D8), the previously noted difference in the lysis of B10.D2 and D8 disappeared. These data demonstrate that the B6 NK subset responsible for lysis of the H2-Dd+ transgenic H2 target expresses Ly49D and that the full lytic potential of the Ly49D+ population is revealed only by selecting cells that do not express known inhibitory receptors for class I molecules expressed on target cells.

Discussion

Previously, the murine in vitro lymphoblast cytotoxicity assay has been successfully used to elucidate inhibitory interactions between target cell class I and their cognate Ly49 receptors (10–12, 15, 27, 37). We have extended those studies here. Our results show that Ly49D+G2− cells lyse B10.D2, D8, B6.R4, C3H, and C3HLs targets only if preincubated with anti-Ly49G2 mAbs (Fig. 5B). The boost in lysis was not due to a stimulatory effect of the Ab, since the lysis of other targets, such as B6, B10.R40, and TAP−/−, was unaffected. These data demonstrate that Ly49G2 is probably an inhibitory receptor not only for Dd and Ld as previously reported, but also for Dd and H2a. Ly49G2 does not appear to be a negative signaling receptor for H2-Dd+ or H2b class I Ags.

The data presented here argue that Ly49D has at least three class I ligands, Dd, Dd, and Dd; and that interaction with these ligands stimulates NK-mediated lysis. Firstly, Ly49D+ cells specifically and preferentially lyse targets that express these class I molecules (Fig. 3). Secondly, lysis of these targets is significantly reduced in the presence of anti-Ly49D reagents (Fig. 3). Thirdly, lymphoblasts bearing these class I Ags compete for lysis of the Dd+ D8 target cells by Ly49D+ effectors (Fig. 2). Although unlikely, our data cannot rule out the possibility that Ly49D functions as a coreceptor with another molecule that determines the class I specificity of positive signaling. In fact, previous binding studies could not demonstrate direct binding of Ly49D to Dd (38). However, recent studies suggest that Ly49D-expressing rat leukemia cells specifically lyse H2-Dd+ expressing target cells.4 It seems, therefore, that the previous binding data (38) may have failed to detect the Ly49D-Dd interaction.

Although the in vitro cytotoxicity assay system has supported in vivo models of class I-induced negative signaling to NK cells, transplant models for positive allorecognition could not be reproduced in vitro. B6 BMC grafts or lymphoblasts are rejected or lysed, respectively, by NK cells of the D8 strain, which is a B6 mouse transgenic for the Dd alloantigen, presumably because B6 cells do not express all the self class I molecules capable of inhibiting all D8 NK cell subsets (24–26, 39). For a similar reason, H2b/F1 hybrid NK cells reject parental BMC grafts in vivo and lyse parental lymphoblasts in vitro (21–23, 27). Strikingly, however, B6 host NK cells mediate rejection of D8 BMC, a finding that is not consistent with the missing self hypothesis but instead argues that NK cell receptors can be triggered by non-self MHC molecules (25, 26, 28). Administration of anti-Ly49D/A mAbs or F(ab′)2 reverses the rejection, suggesting that Ly49D mediates positive recognition of the Dd alloantigen (29). Surprisingly, despite the fact that approximately 50% of B6 NK cells express Ly49D (Fig. 1a), previous studies failed to show lysis of D8 targets by unseparated B6 lymphokine-activated killer cells (LAKs) (28, 40). Although in vitro positive allorecognition is readily detectable in the rat and human (41–45), no such allorecognition has previously been demonstrated in the murine system. We now provide clear evidence for allorecognition by murine Ly49D molecules in an in vitro system. This became possible by sorting subsets of NK cells.

Although we sorted for Ly49D using the Ly49D-specific 4E5 mAb, approximately 20% of these 4E5+ cells coexpress Ly49A (data not shown). Since the mAb 12A8 cross-reacts with Ly49D and Ly49A, we must consider the possibility that preincubation of effectors with 12A8 F(ab′)2 interrupts the signaling potential of both receptors (16). Since it is known that Ly49A interaction with Dd results in negative signaling, 12A8 blockade might boost the potential of Ly49D+ cells to lyse D8 targets. However, in all cases, 12A8-preincubated Ly49D+ effectors could not lyse D8 targets. It is possible that even if Ly49A Dd− cells can no longer receive negative signals from Dd, blockade of Ly49D prevents positive signaling interactions with the D8 target. This is supported by the fact that preincubating Ly49D− cells with either the Ly49D-specific 4E5 mAbs or the Ly49D/A-specific 12A8 F(ab′)2, reduced lysis of the D8 target to the same extent (Table II).

Preclinical transplant data from our laboratory demonstrated that Dd shares a common antigenic motif with Ld and possibly Dd that is positively recognized by B6 NK cells (28). Preliminary experiments have shown that the rejection of C3HLs (H2a, Ld) and B6.R4 (Kb, Dd) BMC grafts can be reversed by administering 12A8 mAb to B6 hosts, suggesting that the Ly49D− subset is responsible for the positive recognition of Dd and Ld in addition to Dd (data not shown). Because B6 Ly49D− NK cells lyse the B6.R4 target unless the effectors are preincubated with 12A8 (F(ab′)2), we propose that Ly49D is a positive signaling receptor for Dd (Fig. 3). The inability of B6 NK cells to lyse H2-Dd-expressing targets is not

suggesting that the Dsp2 epitope is positively recognized in vivo and responsible for the vigorous rejection of B10.R40 BMC grafts, either does not express the putative stimulatory receptor for H2k, or expresses a positive signaling receptor specific for a ligand expressed on C3H and C3H.Ld lymphoblasts, presumably H2k alloantigens. The inability of 12A8 F(ab')2 to inhibit lysis of either of these two targets suggests that a distinct positive signaling receptor specific for C3H Ags exists within the Ly49D G2− subset. Because Ly49D G2+ cells do not lyse C3H or C3H.Ld targets, this subset either does not express the putative stimulatory receptor for H2k, or negative signals from other inhibitory receptors prevent positive signaling. Our results, therefore, do not support or rule out positive signaling from L4. We are currently backcrossing the L4 background onto an H2k background to prevent the complicating effects of H2k negative signaling to NK cell subsets.

Another in vivo model suggests that B6 host NK cells positively recognize H2-Dd9+ -bearing BMC grafts. Backcrossing H2d9 onto the B10 background produced several intraH2 recombinant mice, including the B10.R40 (Kb, Dsp2) strain (47). B6 NK cells are responsible for the vigorous rejection of B10.R40 BMC grafts, suggesting that the Dd9 epitope is positively recognized in vivo (48, 49). Furthermore, 12A8 administration reverses the rejection of B10.R40 BMC grafts by B6 hosts, suggesting that the Ly49D+ subset is responsible for the positive allorecognition of Dd9 (data not shown). In vitro, blocking the Ly49D receptor abolishes the preferential lysis of B10.R40 lymphoblasts by the B6 Ly49D+ subset, regardless of Ly49G2 coexpression (Figs. 3 and 6). These data support the conclusion that the rejection of B10.R40 BMC grafts by B6 hosts results from positive signaling interactions between Ly49D and Dd9.

While it is clear that Ly49D+ NK cells positively recognize and efficiently lyse B10.D2, B6.R4, and B10.R40 targets in vitro, this subset lyse the D8 target only slightly better than the syngeneic B6 target. This is not surprising, since, as opposed to the other three susceptible targets, the D8 lymphoblasts express all the MHC class I molecules that the B6 NK cell considers self. In fact, approximately 60% of this subset coexpresses Ly49C and/or Ly49I, which are negative signaling receptors specific for H2b class I molecules (data not shown). Additionally, approximately 20% of these 4E5+ cells coexpress Ly49A, and >50% coexpress Ly49G2, the known negative signaling receptors specific for Dd (Fig. 4A and data not shown). Coexpression of any one of these inhibitory Ly49 receptors can prevent lysis of the D8 target (Fig. 4B). Only 4% of 4E5+ cells do not coexpress known inhibitory ligands specific for D8 class I Ags. This subset efficiently lyse B10.D2, D8, and TAP− targets to a comparable extent, demonstrating that this particular B6 NK cell subset is responsible for the lysis or rejection of D8 cells (Fig. 7).

By virtue of the fact that we sorted for expression of Ly49D and lack of expression of all other Ly49 receptors for which reagents are currently available, we can only hypothesize what other receptors are expressed by this subset. It is possible that other H2k-specific inhibitory receptors are expressed to ensure self tolerance. If this is the case, then the susceptibility of the D8 target to lysis by this subset implies that Ly49D-mediated positive signaling dominates in this instance. Alternatively, this subset may not express any H2k-specific inhibitory or stimulatory receptors. Self tolerance in this case would not require self class I-specific inhibition, and the alloantigenic class I transgenic target is susceptible because it expresses a stimulatory ligand (Dd) but no inhibitory ligand for receptors on the NK cell. Until more reagents become available, either explanation remains possible.

Thus, as previously observed (28, 40), D8 target cells are resistant to lysis by bulk B6 NK cells because the frequency of the single Ly49D+ cells is very low. Selecting for Ly49D+ cells increases the frequency of these effectors to the point where lysis of the D8 target is detectable but not impressive (Fig. 3). Sorting away a major subset bearing inhibitory receptors specific for the Dd transgene (Ly49D G2+ ) again increases the frequency of cells capable of lysing the D8 target, and D8 targets are lysed to a moderate extent (Fig. 4B). Finally, NK cells selected for expression of only the stimulatory H2-Dd-specific Ly49D are able to lyse D8 targets as well as B10.D2 and TAP−/− targets (Fig. 7). Thus, minor subsets of NK cells can be responsible for the lysis of target cells that differ from self only by the additional expression of a single class I molecule.

The existence of functional class I-specific positive signaling Ly49 receptors within an NK cell repertoire adds a second layer of complexity to understanding the alloreactive specificity of a given NK cell. Thus, the resistance to NK-mediated lysis requires that the target cell either must express ligands for negative signaling receptors or must not express ligands for positive signaling receptors of the NK effector. Coexpression of ligands for both types of receptors also probably results in resistance to lysis. What function do class I-recognizing stimulatory receptors serve in physiological NK-mediated events? It is unlikely that the purpose of class I-specific stimulatory receptors is to facilitate rejection of allografts or lysis of allomorphic lymphoblasts. In fact, these types of receptors could theoretically jeopardize self tolerance. As originally suggested by Kärre (20), the requirement for target cells to express ligands for class I inhibitory receptors provides a mechanism by which tumor cells or virally infected cells that evade T cell immunity by down-regulating or altering self class I may be rejected by NK cells. The selective loss of one negative signaling class I motif would allow rejection mediated by positive signaling receptors specific for a different self class I motif. Another, not mutually exclusive, possibility is inhibition of self NK cells requires stimulatory signals to phosphorylate the ITIM on inhibitory Ly49 receptors. Whereas all NK subsets should be self-tolerant, some may not be tolerant to allogeneic cells. Thus, specific NK subsets are responsible for the lysis of allogeneic targets in vitro and for the rejection of BMC allografts in vivo.

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