External and Internal Calibration of the MHC Class I-Specific Receptor Ly49A on Murine Natural Killer Cells

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*J Immunol* 1998; 161:6133-6138; 
http://www.jimmunol.org/content/161/11/6133

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Expression of the H-2D\textsuperscript{d}-specific inhibitory receptor Ly49A on murine NK cells is subject to MHC class I-dependent modulation in vivo. As a result, NK cells in H-2D\textsuperscript{d}-transgenic mice express low cell surface levels of Ly49A, whereas NK cells from non-transgenic C57BL/6 (B6) mice express high levels. The purpose of this study was to assess the role of MHC class I molecules on the NK cell itself vs those on surrounding cells in this calibration and to test whether the Ly49A levels are subject to regulation in mature NK cells also. Analysis of transgenic mice with mosaic expression of an H-2D\textsuperscript{d}/L\textsuperscript{d} transgene showed that MHC class I molecules on surrounding cells (external ligands) and on the NK cell itself (internal ligands) played distinct roles in the determination of Ly49A levels. External ligands were involved in down-regulation of Ly49A levels in vivo, whereas internal ligands kept the down-regulated levels of Ly49A low upon NK cell activation in vitro. Furthermore, in an experimental system based on adoptive transfer of spleen cells, receptor down-regulation of Ly49A occurred as a rapid adaptation process in mature NK cells after interaction with the H-2D\textsuperscript{d} ligand in vivo. This suggests that Ly49A levels are not fixed but can be changed in mature NK cells when they are exposed to a changed MHC class I environment.


External and Internal Calibration of the MHC Class I-Specific Receptor Ly49A on Murine Natural Killer Cells\textsuperscript{1}
of adoptive transfer experiments, we demonstrate that Ly49A levels is not a fixed characteristic of mature NK cells. As early as 10 h after transfer of H-2D^d-negative, Ly49^high NK cells to H-2D^d-positive recipients, Ly49A levels were strongly down-regulated on transferred NK cells. Taken together, our results suggest distinct roles for H-2D^d on the NK cell itself and in the environment of the NK cell for Ly49A receptor levels. They also show that NK cells can rapidly adjust their cell surface levels of Ly49A in response to changes in the MHC class I expressed by surrounding cells, suggesting that receptor calibration against external ligands may be a continuous process that occurs throughout the life span of the NK cell.

Materials and Methods

Mice

All mice were kept and bred at the Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden. Mosaic DL6 mice and control DL1 mice have been described previously (22). The transgene in both DL1 and DL6 mice is a chimeric gene containing the α1/α2 domains of H-2D^d coupled to the α3 domain of H-2L^d. However, in control DL1 mice the transgene is expressed in all cells of the mouse, whereas in DL6 mice it is expressed in a mosaic fashion (22). The chimeric H-2D^d/L^d protein, when expressed in all cells of the mouse, has an effect on NK cell specificity similar to that of the entire H-2D^d gene (22). We will therefore, for the sake of simplicity, use H-2D^d in reference to the H-2D^d/L^d transgene throughout this report.

Flow cytometry

The mAbs 3-25.4 (anti-α1/α2 domains of H-2D^d; FITC conjugated), PK136 (anti-NK1.1; phycoerythrin (PE) conjugated), and A1 (anti-Ly49A; biotinylated) were purchased from PharMingen, San Diego, CA, and streptavidin-RED670 was purchased from Life Technologies AB, Täby, Sweden. The percentage of H-2D^d-positive cells in DL6 mice was determined using FACS analysis of peripheral blood cells stained with FITC-conjugated mAb against the α1/α2 domains of H-2D^d (3-25.4). To analyze for expression of NK cell receptors, nonactivated nylon wool-nonadherent (NWNA) spleen cells, or IL-2-activated spleen cells, were incubated with biotinylated mAb against Ly49A, washed, and in a second step incubated with a mixture containing streptavidin-RED670, 3-25.4-FITC, and PK136-PE. Incubations were performed on ice for 30 min, and washes were done in PBS supplemented with 1% FCS. Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

Activation of NK cells in vitro

Spleen cells were cultured at 37°C in complete medium (α-MEM containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, and 2 × 10^{-3} M 2-ME) in the presence of 1000 U/ml of rIL-2 and in 10% CO_2/90% air mixture as described (23, 24). After 4 days, both adherent and nonadherent cells were removed and used for flow cytometry analysis as described in the previous section. The starting material was either erythrocyte-depleted spleen cells or separated H-2D^d-negative DL6 spleen cells.

Cell separation and coculture experiments

Dynabeads conjugated to a rat anti-mouse IgM Ab (M-450; Dynal, Oslo, Norway) were preincubated with an IgM mAb specific for H-2D^d α1/α2 (34-4-21S) for 30 min at 4°C. A total of 1.5 μg Ab/long beads were used. Erythrocyte-depleted DL6 splenocytes were incubated with the precoated beads for 30 min at 4°C, and beads with bound cells were subsequently collected using a magnetic particle concentrator. The procedure was repeated once to completely deplete the sample of H-2D^d-positive cells. Incubations and washes were made in PBS containing 0.1% BSA. After separation, H-2D^d-negative DL6 cells were cultured either alone or together with increasing numbers of H-2D^d-positive spleen cells in rIL-2, and the expression of Ly49A receptor was analyzed 4 days later. For the coculture experiments, we could not use separated H-2D^d-positive DL6 cells since they were attached to the beads. Separated H-2D^d-negative DL6 cells were therefore mixed with increasing numbers of spleen cells from DL1 mice treated with NK1.1 Ab to deplete NK cells. NK cell depletion was

Abbreviations used in this paper: PE, phycoerythrin; NWNA, nylon wool nonadherent.
The number of H-2D\textsuperscript{d}-positive cells in the mouse affects cell surface levels of Ly49A on both H-2D\textsuperscript{d}-positive and H-2D\textsuperscript{d}-negative NK cells

The number of H-2D\textsuperscript{d}-positive cells in DL6 mice varies between individual mice but is stable within a given animal (22). To investigate whether the percentage of H-2D\textsuperscript{d}-positive cells in the environment would influence Ly49A levels, we compared NK cells from several DL6 mice containing different numbers of H-2D\textsuperscript{d}-positive cells. When the Ly49A expression from all mice were plotted against the percent H-2D\textsuperscript{d}-positive cells in DL6 mice, we observed a linear correlation ($p < 0.001$) between these two parameters. Down-regulation of Ly49A was less pronounced in mice with a low percentage of H-2D\textsuperscript{d}-expressing cells and became more marked as the percentage of H-2D\textsuperscript{d}-positive cells increased (Fig. 2). Interestingly, this correlation was observed for both H-2D\textsuperscript{d}-positive and negative cells. From this comparison we draw three conclusions: 1) down-regulation of Ly49A is an efficient process that occurs in mice in which only 1 cell of 10 expresses H-2D\textsuperscript{d}; 2) the extent of down-regulation of Ly49A may depend on the frequency with which an NK cell interacts with a surrounding cell expressing the ligand; 3) H-2D\textsuperscript{d} expression on surrounding cells may determine Ly49A levels even if H-2D\textsuperscript{d} is present on the cell surface of the NK cell itself.

The H-2D\textsuperscript{d} molecules of the NK cell itself, but not H-2D\textsuperscript{d} on surrounding cells, maintain down-regulation of Ly49A levels on activation in IL-2

To test whether Ly49A levels could be changed in mature NK cells when the MHC environment was changed, we separated DL6 spleen cells into an H-2D\textsuperscript{d}-positive and an H-2D\textsuperscript{d}-negative population and cultured them either separately or together for 4 days in medium supplemented with IL-2. Under both these culture conditions, Ly49A expression in the H-2D\textsuperscript{d}-positive subset only changed marginally, if at all. In contrast, Ly49A expression increased dramatically in the H-2D\textsuperscript{d}-negative population. Since these changes were observed both in cultures where the mosaic population was left without separation (Fig. 3A) and in cultures where the H-2D\textsuperscript{d}-negative cells were cultured separately (Fig. 3B), we conclude that in the presence of IL-2, the influence of surrounding cells was minimal. Instead, the MHC ligand of the NK cell itself acted to maintain low receptor expression. This was also the case when conditions were pushed such that most of the cells in the environment were H-2D\textsuperscript{d} positive. Mosaic DL6 mice with >50% H-2D\textsuperscript{d}-positive cells are rare, so this was achieved by coculturing separated H-2D\textsuperscript{d}-negative DL6 cells with increasing numbers of H-2D\textsuperscript{d}-positive cells. Despite a ninefold excess of H-2D\textsuperscript{d}-positive over -negative cells, the Ly49A levels on H-2D\textsuperscript{d}-negative NK cells increased after IL-2-activation (Fig. 3B). This result showed that the H-2D\textsuperscript{d} molecules of the NK cell itself acted to maintain stability of the down-regulated Ly49A expression upon cellular activation. Without cell surface expression of H-2D\textsuperscript{d}, Ly49A expression rapidly rose to levels comparable to those in B6 mice, despite a large number of H-2D\textsuperscript{d}-expressing surrounding cells.

Down-regulation of Ly49A expression by surrounding cells occurs in mature H-2D\textsuperscript{d}-negative NK cells after adoptive transfer to H-2D\textsuperscript{d}-positive mice

To test whether Ly49A levels could be down-regulated in mature NK cells after exposure to surrounding H-2D\textsuperscript{d}-positive cells, we mixed H-2D\textsuperscript{d}-positive and -negative mature spleen cells and cultured them together in IL-2 in vitro. This attempt failed; Ly49A expression on B6 NK cells was high from the beginning and remained high during the coculture (data not shown). We therefore went back to the in vivo situation, where the influence of surrounding cells seemed dominant, and asked whether Ly49A levels could be down-regulated in mature NK cells if they were exposed to a changed MHC class I environment in vivo. To test this, we developed an adoptive transfer system, in which mature splenocytes were inoculated into sublethally irradiated mice of various MHC genotypes. The spleens of recipient mice were recovered 10–18 h after inoculation, and donor NK cells were analyzed for Ly49A expression. We first transferred B6 splenocytes to BALB/c (H-2\textsuperscript{b}) and BALB.B (H-2\textsuperscript{b}) mice, which differ only with respect to the MHC genotype but share the same non-MHC background. The advantage of this donor/recipient combination is that donor-derived NK cells can be positively identified both by their unique expression of the NK1.1 marker and by the Ly49A allele recognized by the A1 Ab (Fig. 4A). Eighteen hours after transfer, injected B6-derived NK cells demonstrated greatly reduced Ly49A levels in BALB/c recipients (expressing the H-2D\textsuperscript{d} ligand) but not in H-2\textsuperscript{a}-expressing BALB.B mice (Fig. 4B). The levels of Ly49A on Ly49A on the donor-derived NK cells in BALB/c recipients (Fig. 4B) was similar to the levels expressed by NK cells in DL1 mice (data not shown).

To confirm that it was in fact H-2D\textsuperscript{d} itself and just not any foreign MHC class I molecule that induced down-regulation after transfer, we analyzed a set of MHC congenic mouse strains on the A/Sn background (also lacking the NK1.1 marker). The A/Sn MHC is a natural recombinant haplotype (H-2\textsuperscript{a}), which contains the MHC class I molecules H-2K\textsuperscript{a}, D\textsuperscript{a}, and L\textsuperscript{a}, thus including the major Ly49A ligand. A/Sn was compared with three of its injected A/Sn mice demonstrated greatly reduced Ly49A levels in BALB/c recipients (expressing the H-2D\textsuperscript{d} ligand) but not in H-2\textsuperscript{a}-expressing BALB.B mice (Fig. 4B). The levels of Ly49A on Ly49A on the donor-derived NK cells in BALB/c recipients (Fig. 4B) was similar to the levels expressed by NK cells in DL1 mice (data not shown).
A.Ca mice were both statistically significant relative the A.By recipients (Fig. 5). We also inoculated B6 spleen cells into H-2D\textsuperscript{d}-transgenic B6 mice (D\textsuperscript{8}). B6 and D\textsuperscript{8} NK cells cannot be distinguished using the NK1.1 marker, but gating on H-2D\textsuperscript{d}-negative cells in the D\textsuperscript{8} recipient 10 h after transfer revealed strong down-regulation of Ly49A on the B6-derived NK cells (data not shown). Together, these findings show that rapid down-regulation of Ly49A can take place in mature H-2D\textsuperscript{d}-negative NK cells when the environment changes with respect to MHC class I expression.

Discussion

NK cell expression of Ly49 receptors is strongly influenced by the MHC class I environment in which the NK cell matures. For example, the presence of the MHC class I ligand H-2D\textsuperscript{d} has profound consequences for the level of Ly49A receptor on each Ly49A-positive NK cell. In mice lacking H-2D\textsuperscript{d}, Ly49A levels expressed by individual NK cells are high. In contrast, much lower levels of Ly49A are observed in H-2D\textsuperscript{d}-expressing mice (15). The nature of the Ly49A/H-2D\textsuperscript{d} interaction that results in this ligand-induced down-regulation in vivo is not known. Sykes et al. (25), with the

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Ly49A expression on H-2D\textsuperscript{d}-negative and -positive NK cells before and after IL-2 activation. NWNA spleen cells from B6, DL1, and DL6 (five mice with different fractions of H-2D\textsuperscript{d}-positive cells) were triple-stained for NK1.1, H-2D\textsuperscript{d}, and Ly49A before and after activation in 1000 U/ml rIL-2 for 4 days (A). Each data point represents median fluorescence intensity of Ly49A on NK1.1-positive cells, and the two values connected with a line shows Ly49A levels on H-2D\textsuperscript{d}-negative and/or H-2D\textsuperscript{d}-positive NK cells before and after activation. B, Ly49A expression on H-2D\textsuperscript{d}-negative and -positive NK cells in mixed cultures with increasing numbers of H-2D\textsuperscript{d}-positive DL1 cells. H-2D\textsuperscript{d}-positive cells were removed from a DL6 spleen cell preparation with the use of immunomagnetic beads. The remaining H-2D\textsuperscript{d}-negative cells were then activated in IL-2 either alone or together with increasing numbers of DL1 spleen cells. Median fluorescence intensity of Ly49A on NK1.1-positive cells is plotted. The ratio indicated for each mix is the number of H-2D\textsuperscript{d}-negative to H-2D\textsuperscript{d}-positive cells.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Adoptive transfer of mature H-2D\textsuperscript{d}-negative NK cells to BALB/c (H-2\textsuperscript{b}) or BALB.B (H-2\textsuperscript{h}) recipients. B6 spleen cells (15 × 10\textsuperscript{6}) were injected i.v.; 18 h later, the recipients were killed and NK1.1-positive splenocytes were gated (A) and analyzed for expression of Ly49A (B). The donor/recipient combination is shown above each histogram, and the number above each region is the median fluorescence intensity.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Transfer of mature H-2D\textsuperscript{d}-negative NK cells to A/Sn (H-2\textsuperscript{a}), A.By (H-2\textsuperscript{b}), A.Sw (H-2\textsuperscript{h}), and A.Ca (H-2\textsuperscript{f}) recipients. B6 spleen cells (15 × 10\textsuperscript{6}) were injected i.v.; 18 h later, NK1.1-positive recipient splenocytes were analyzed for expression of Ly49A. Median values as percentage of B6 are plotted. The graph shows mean ± SD of four mice in each group, except for A/Sn, which includes three recipients, derived from two independent experiments. ***, p < 0.005; ****, p < 0.001 compared with A.By recipients by Student’s t test.
use of bone marrow chimeric mice, previously demonstrated that down-regulation of Ly49A did not require NK cell expression of H-2D\(^d\). A similar conclusion was reached by Fahlden et al. (20) in a study of T cells expressing an Ly49A transgene. Our data from mosaic DL6 mice confirm those findings in that they demonstrate that H-2D\(^d\) on surrounding cells could down-regulate NK cell expression of Ly49A even if the NK cell itself lacked H-2D\(^d\).

However, with the mosaic DL6 mice, we were able to ask further questions. The first was whether the number of H-2D\(^d\)-positive cells in the environment affected the final level of Ly49A. This analysis was possible because individual DL6 mice have different percentages of H-2D\(^d\)-positive cells (22). When data from a large cohort of DL6 mice were pooled, we found a highly significant inverse correlation between the number of H-2D\(^d\)-positive cells in the mouse and the Ly49A levels, suggesting that Ly49A levels may be determined by the frequency by which an NK cell encounters an H-2D\(^d\)-expressing cell. An interesting possibility is that this correlation reflects a calibration process dependent on continuous contacts with H-2D\(^d\)-positive cells in vivo. In mice in which H-2D\(^d\)-positive cells are rare, such as in DL6 mice with few H-2D\(^d\)-expressing cells, the frequency by which an Ly49A-positive NK cell interacts with an H-2D\(^d\)-positive cell is likely reduced. Thus, the time elapsing between two H-2D\(^d\) contacts would in this case be long enough to allow Ly49A protein to accumulate to higher levels at the cell surface of NK cells. This hypothesis remains to be tested, but it fits with the suggestion that Ly49A receptor levels are regulated by a posttranscriptional event, triggered after contact with H-2D\(^d\)-expressing cells (6, 20). It is also consistent with our data from the adoptive transfer experiments in this report, which show that the Ly49A levels on mature NK cells rapidly change after contact with H-2D\(^d\)-positive cells in vivo (see below). Our study is not the first to describe a quantitative relationship between the amount of H-2D\(^d\) in the environment and Ly49A expression. Using bone marrow chimeric mice, Sykes et al. (25) reported that the Ly49A level on donor cells was down-regulated to a larger extent in recipient mice homozygous for the H-2D\(^d\)-gene than in recipients heterozygous for H-2D\(^d\). This result indicated that the level of H-2D\(^d\) expressed by individual calibrating cells in the environment was an important factor for the extent of Ly49A down-regulation. These results are not mutually exclusive with the results in this report, which imply the number of surrounding H-2D\(^d\)-positive cells as a critical factor. The overall strength of the Ly49A/H-2D\(^d\) interaction is likely affected both by the level of H-2D\(^d\) on individual cells (as in the study by Sykes et al.) and by the frequency of encounters with H-2D\(^d\)-positive cells (our study). Alterations in either parameter would thus be expected to affect Ly49A levels.

The second question in which DL6 mice were useful related to the role of the MHC class I of the NK cell itself. Since H-2D\(^d\)-positive and -negative NK cells coexist in the same environment in DL6 mice, we reasoned that a comparison between these two NK cell populations would reveal differences in Ly49A expression in situations where the MHC class I of the NK cell itself was important and no difference when it was not. As has already been discussed, higher Ly49A levels were found on both the H-2D\(^d\)-positive and -negative NK cell populations in DL6 mice with a low percentage of H-2D\(^d\)-expressing cells than in mice with a high percentage. This result suggests that calibration in vivo may be exclusively dependent on contacts with H-2D\(^d\) expressed by cells in the environment, even if the NK cell itself expresses H-2D\(^d\). However, in contrast to the in vivo situation, a difference between H-2D\(^d\)-positive and negative NK cells were found in vitro, in response to activation with IL-2. High doses of this cytokine activates NK cells and leads to the development of MHC class I-specific killing responses (23, 24). We found that the low Ly49A levels on H-2D\(^d\)-negative DL6 NK cells (down-regulated by H-2D\(^d\) on surrounding cells) were highly unstable, and Ly49A expression rapidly rose to high levels after activation in IL-2. In contrast, if the NK cell itself expressed H-2D\(^d\), Ly49A levels remained low during activation. Thus, in this case, H-2D\(^d\) on the NK cells were necessary to maintain low Ly49A levels, and H-2D\(^d\) molecules expressed by surrounding cells were unable to influence Ly49A expression.

This result is interesting in relation to the “missing self” hypothesis, which proposes a role of Ly49 receptors in NK cell detection of self cells with down-regulated levels of MHC class I (7). It has been demonstrated that the levels of Ly49A receptors influence the outcome of an interaction between an Ly49A-positive NK cell and an H-2D\(^d\)-positive target cell. If the NK cell is Ly49A high, it can be more easily inhibited than if it is Ly49A low (15, 16). Thus, mechanisms should exist that prevent the apparently unstable Ly49A receptor expression from increasing upon activation. Our results suggest that expression of MHC class I on the NK cell itself may be one such mechanism, possibly through an interaction with Ly49A at the cell surface. Such an interaction should be possible to identify with biochemical methods. A similar effect of the MHC class I of the NK cell itself on another inhibitory receptor, Ly49C, has recently been observed by Andersson et al. (30) suggesting that this phenomenon is not unique for Ly49A.

In adoptive transfer experiments, we found that down-regulation of Ly49A in H-2D\(^d\)-negative NK cells occurred rapidly when they were transferred to H-2D\(^d\)-expressing mice. This down-regulation was specific since it did not occur in mice of the H-2\(^s\) or H-2\(^b\) haplotypes (26). Interestingly, in addition to H-2D\(^d\), a weak, but statistically significant, down-regulation was seen in H-2\(^s\) mice, suggesting that any one of the MHC class I molecules expressed in this haplotype may constitute a ligand for Ly49A, perhaps with lower affinity. The extent of down-regulation in H-2\(^s\) mice parallels the down-regulation we have previously observed in mice expressing an H-2D\(^d\) transgene (our unpublished data). This may be significant in light of the data showing that H-2\(^d\), H-2\(^s\), and H-2\(^p\) share a genetic determinant governing the specificity of NK cell-mediated rejection of bone marrow grafts (27–29).

The rapid down-regulation of Ly49A after transfer demonstrates that Ly49A down-regulation does not require normal NK cell maturation. More importantly, it also suggests that Ly49A levels on mature NK cells are not fixed but can be influenced in mature cells after interactions with surrounding cells. If this is true, it may have important implications for NK cell function. The “missing self” model suggests that NK cells react against cells with down-regulated MHC class I expression (7). If this hypothesis is correct, each NK cell must express a level of inhibitory receptors that makes it capable of distinguishing small changes in self MHC class I expression, the most important consideration being that receptor levels on individual NK cells are not too high. Given the rapid adaption in Ly49A levels that was seen against surrounding cells, we speculate that a continuous calibration against surrounding cells could occur in vivo, serving to optimize the NK cell to varying levels of MHC class I molecules expressed in different tissue compartments, thereby adapting it to detect small changes in MHC class I expression in each compartment. Such a mechanism may also be dangerous, since an NK cell continuously calibrating its levels of inhibitory receptors against the environment would run the risk of being tolerized, rather than activated, by pathologic changes in MHC class I ligand expression. However, this would not occur if calibration switches to internal ligands (the MHC class I of the NK cell itself) under conditions of strong activation, as shown by the data in this study. Further studies should reveal...
whether, in addition to IL-2, other inflammatory cytokines, and perhaps also cell-bound ligands, may release the NK cell from the continuous adaptation of receptor levels to external ligands. At this stage, we conclude that expression of Ly49A receptors is subject to calibration in the mature NK cell according to principles in which the influence of external and internal MHC class I ligands appear to be balanced differentially depending on the cellular activation stage.

Acknowledgments

We thank Maj-Lis Solberg and Margareta Hagelin for expert assistance with in vivo experiments and Ros-Mari Johansson and the staff in the animal house for taking care of the mice.

References