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Immunology

Altered phenotype and function of natural killer cells expressing the major histocompatibility complex receptor Ly-49 in mice transgenic for its ligand

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ABSTRACT The Ly-49 molecule has been shown to interact with major histocompatibility complex (MHC) class I molecules, and the lytic function of Ly-49$^+$ natural killer (NK) cells from C57BL/6 (H-2^b) mice is inhibited by the recognition of H-2D^d on tumor target cells. Introduction of a Ly-49 ligand, H-2D^d, into C57BL/6 mice did not alter the percentage of Ly-49$^+$ NK cells (13–18%), but it led to three functional effects on this subset. (*i*) The Ly-49 expression in the positive population was reduced by 30–50% compared to C57BL/6 control mice. (*ii*) While this Ly-49$^+$ subset (Ly-49lo) in the transgenic mice failed to kill BALB/c concanavalin A (Con A) blasts, which have high H-2D^d expression, it was capable of killing SP2/0 tumor cells, which have low H-2D^d expression. Ly-49$^+$ NK cells (Ly-49hi) from nontransgenic mice failed to kill both of these H-2D^d-expressing target cells. (*iii*) In the transgenic mice, the Ly-49$^+$ subset acquired the ability to kill C57BL/6 Con A blasts, in contrast to the Ly-49$^+$ NK cells of C57BL/6 mice. We propose a "receptor-calibration" hypothesis, where low receptor density on the effector cells imposed by selection or adaptation to the environment allows higher sensitivity for detection of reduced self-MHC ligands on potential target cells.

Natural killer (NK) cells have been shown to reject bone marrow cells and kill virally infected or various tumor cells in what has been considered a non-major histocompatibility complex (MHC) restricted manner. However, accumulating data indicate that the functions of NK cells can be influenced by the expression of MHC class I molecules (1, 2). Unlike T lymphocytes, NK cells seem to be inhibited by the presence, and presumed recognition, of MHC class I molecules. There is further evidence that the presence of self-MHC class I molecules is necessary for the complete inhibition of NK cell responses *in vivo* (3, 4). RBL-5 lymphoma cells (H-2^b) grow in syngeneic C57BL/6 mice but not in D8 mice (H-2D^d transgene in C57BL/6 mice) and the resistance to tumor cells by D8 mice is dependent upon NK1.1$^+$ cells. However, transfection of RBL-5 cells with the H-2D^d gene allows these lymphoma cells to grow in D8 mice. These data and data from β_2-microglobulin deficient (β_2m −/−) mice indicate that NK cells somehow "learn" the nature of self-MHC and do not react against it (5, 6). It is therefore of interest to identify how NK cell surface receptor phenotypes change in response to defined alterations in "self"—i.e., MHC class I molecules. Such studies would allow insights similar to those obtained in T-cell biology, made possible by the use of anti-clonotypic as well as anti-T-cell receptor/Vβ antibodies in combination with mouse genetics, which enabled experimental control of receptors and their ligands during development.

According to the effector inhibition model for missing self recognition, an NK cell will lyse a target cell unless the NK cell receives an inhibitory signal (7, 8). This inhibitory signal would occur through a cell surface receptor on the NK cell that interacts with a self-MHC class I molecule. The Ly-49 molecule is a good candidate for such an MHC class I receptor. The Ly-49 molecule is a type II integral membrane protein with a lectin-like domain, and there is evidence from antibody blocking and physical binding assays that Ly-49 directly interacts with H-2D^d (9–11). It is a member of a multigene family on murine chromosome 6 (the NK gene complex) coding for a set of related proteins (12). Another NK-specific molecule, 5E6, is expressed on a subset of murine NK cells and has a homologous DNA sequence to Ly-49 (13, 14). The Ly-49 antigen is expressed by a subset of NK cells, a small population of CD3$^+$ thymocytes, and a subset of CD3$^+$ intestinal intra-epithelial lymphocytes but not by other lymphoid cells (15). The ability of C57BL/6 Ly-49$^+$ NK cells to kill was inhibited by the expression of H-2D^d, but not H-2K^d or H-2L^d, molecules on transfectants of an H-2^b tumor (9). However, the Ly-49$^+$ NK cells were from H-2^b mice and therefore were inhibited not by interaction with self-MHC but rather by interaction with non-self-MHC. Furthermore, the Ly-49$^+$ NK cell subset was not found in mice that expressed H-2D^d, and the absence of the Ly-49$^+$ NK cells was found in bone marrow chimeric studies to be determined primarily by the expression of H-2D^d on bone marrow-derived cells (16, 17).

These data provide strong evidence that an NK cell surface molecule can receive an inhibitory signal from an MHC class I antigen. They also present a paradox (18, 19). How might NK cells receive inhibitory signals following interaction with self-MHC molecules, when the NK cells that express the receptor (i.e., Ly-49) for a particular self-MHC molecule (i.e., H-2D^d) are absent in animals that express that MHC molecule? We have investigated this issue by analyzing the expression and function of Ly-49$^+$ NK cells from mice that express H-2D^d. Our data indicate that an Ly-49$^+$ NK cell subset of similar size is indeed present in mice that express H-2D^d, but the expression of this receptor is reduced by almost half compared with Ly-49$^+$ NK cells from mice that do not express H-2D^d. Analysis of D8 Ly-49$^+$ cells (Ly-49lo) demonstrated that they act functionally different compared to Ly-49$^+$ cells (Ly-49hi) from C57BL/6 mice. We propose a "receptor-calibration" model to explain the functional consequences of low-level Ly-49 expression on NK cells that reside in an H-2D^d environment.

MATERIALS AND METHODS

Animals and Cell Lines. DBA/2 and (BALB/c × C57BL/6) F$_1$ mice were purchased from Bomholtgård Breeding and Research Center (Ry, Denmark). C57BL/6 mice were purchased from B & K Universal (Sollentuna, Sweden). All other

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Abbreviations: β_2m −/−, β_2-microglobulin deficient; NWNA, nylon wool nonadherent; Con A, concanavalin A; NK, natural killer; MHC, major histocompatibility complex; MFI, mean fluorescence intensity; FITC, fluorescein isothiocyanate.

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mice were bred and maintained at the Department of Tumor Biology, Karolinska Institutet (Stockholm). C57BL/6 β_2m −/− mice were generated by backcrossing β_2m −/− mice to C57BL/6 mice. SP2/0 (H-2^d) is a myeloma of BALB/c origin.

Enrichment of NK Cells. To enrich for NK cells, spleens were aseptically isolated, and the cells were gently released in phosphate-buffered saline by disruption with a syringe. After removal of the erythrocytes by hypotonic shock, the spleen cells were loaded onto nylon wool (Polyscience) columns as described (20). The nonadherent (NWNA) cells were either analyzed immediately by flow cytometry or cultured in complete medium (RPMI 1640 medium/0.05 mM 2-mercaptoethanol/penicillin/streptomycin/1 mM sodium pyruvate/minimum essential medium nonessential amino acids/10% fetal calf serum) with 1500 units of recombinant interleukin 2 (rIL-2) per ml. After 3 days in culture, the nonadherent cells are removed, and the adherent cells were cultured with rIL-2 medium for 1 additional day.

Antibodies and Flow Cytometry. Fresh NWNA spleen cells (10^6) were labeled and analyzed on a FACScan flow cytometer (Becton Dickinson) as described (19). Phycoerythrin (PE)-conjugated anti-NK1.1 and anti-CD3, biotin-conjugated anti-5E6 (PharMingen), fluorescein isothiocyanate (FITC)-conjugated goat F(ab′)$_2$ anti-mouse IgG (H + L) sera (Caltag, South San Francisco, CA), and FITC-conjugated streptavidin (Dakopatts, Glostrup, Denmark) were used at 0.5–1.0 μg per 10^6 cells. Biotinylated JR9-318 (anti-Ly-49) was generously provided by Jacques Roland (Department d'Immunologie, Institut Pasteur, Paris). The A1 hybridoma was generously provided by Osami Kanagawa (Department of Pathology, Washington University Medical School, St. Louis). A1 ascites (anti-Ly-49) was generously provided by Wayne Yokoyama (Mt. Sinai Medical School, New York).

Isolation of Ly-49$^+$ Cells. Four-day cultured NWNA spleen cells were incubated at 4°C with anti-Ly-49 antibody (A1 monoclonal antibody) followed by FITC-goat anti-mouse IgG (H + L) antibody and PE-conjugated anti-NK1.1 antibody. The antibody-bound cells were subsequently sorted on a FACS IV flow cytometer (Becton Dickinson). Ly-49$^+$/NK1.1$^+$ and Ly-49$^-$/NK1.1$^+$ cells were isolated and cultured in complete medium with 1500 units of rIL-2 per ml for 1 day to dissociate the antibodies used for sorting.

Cytotoxicity Assay. Sorted Ly-49$^+$ and Ly-49$^-$ cells were used as effectors in a ^{51}Cr-release assay (5 h) with concanavalin A (Con A)-stimulated splenocytes or SP2/0 tumor target cells as described (20).

RESULTS

Decreased Expression of Ly-49 in CD3$^-$ NWNA Spleen Cells from H-2^d and H-2^k Mice. The expression of Ly-49 on fresh NK cells was examined in C57BL/6 (H-$2^{b/b}$), BALB/c (H-$2^{d/d}$), DBA/2 (H-$2^{d/d}$), AKR (H-$2^{k/k}$), and (BALB/c × C57BL/6) F_1 mice (H-$2^{d/b}$). It has been reported that no Ly-49$^+$ NK cells exist in mice of the H-2^d or H-2^k haplotypes. To enrich for NK cells, NWNA spleen cells were used for two-color flow cytometric analysis of CD3 and Ly-49 expression. Since most of these strains do not express the pan-NK-specific antigen NK1.1, the expression of Ly-49 on CD3$^-$ NWNA spleen cells was determined. As shown in Fig. 1*A*, we detected expression of Ly-49$^+$ cells in BALB/c and C57BL/6 mice, and the total percentage of splenic CD3$^-$, Ly-49$^+$ cells was similar for all strains tested. However, we observed that the mean fluorescence level of Ly-49 was consistently less in BALB/c (66%), DBA/2 (64%), and AKR (54%) mouse strains than in C57BL/6 mice (Table 1). The decreased expression of Ly-49 on these cells was a dominant trait since the level of Ly-49 expression in (BALB/c × C57BL/6)F_1 mice was virtually identical to that seen in BALB/c mice. In contrast to the expression of Ly-49, the expression of the 5E6 antigen was similar between the different mouse strains indicating that there was not a general decrease in the expression of Ly-49-like molecules on NK cells in these mice (data not shown).

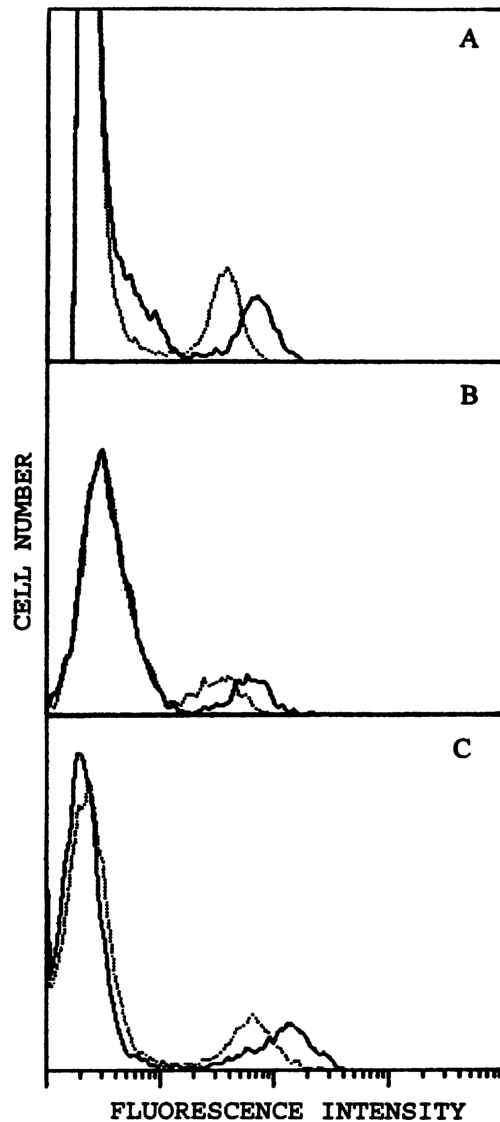

FIG. 1. Expression of Ly-49 on NWNA spleen cells. (*A*) Histogram of JR9-318 expression on CD3$^-$ gated NWNA spleen cells from C57BL/6 (solid line) and BALB/c (dotted line) mice. Mean fluorescence intensity (MFI, percentage of C57BL/6): BALB/c, 52%. (*B*) Histogram of JR9-318 expression on NK1.1$^+$ gated NWNA spleen cells from C57BL/6 (solid line) and D8 (dotted line) mice. MFI: D8, 59%. (*C*) Histogram of A1 expression on NK1.1$^+$ gated NWNA spleen cells from C57BL/6 (solid line) and D8 (dotted line) mice. MFI: D8, 55%.

Table 1. Expression of Ly-49 on NWNA spleen cells from inbred and F_1 hybrid mice

Strain	H-2	MFI JR9-318,* % of C57BL/6
C57BL/6	b/b	100
BALB/c	d/d	66 (5)
DBA/2	d/d	64 (4)
AKR	k/k	54 (4)
C57BL/6 × BALB/c)F_1	b/d	58 (3)

*MFI of CD3$^-$ NWNA spleen cells. In each experiment the expression on C57BL/6 NWNA spleen cells was used as 100% expression. *n* values are given in parentheses.

Decreased Expression of Ly-49 in Transgenic and Congenic Mice That Express H-2D^d. To determine whether the decrease in the level of Ly-49 expression was due to the expression of H-2D^d, fresh NK cells from C57BL/6 (H-2$^{b/b}$), congenic B10.D2 (H-2$^{d/d}$), D8 (H-2$^{b/b}$, D$^{d/d}$), (D8 × C57BL/6)F_1 (H-2$^{b/b}$, D^d), and C57BL/6 β_2m −/− (H-2$^{b/b}$) were analyzed for the expression of Ly-49. Since all mice of the C57BL background express the NK-specific marker NK1.1, NK1.1 and Ly-49 expression on NWNA spleen cells derived from these strains was analyzed by two-color flow cytometry. As shown in Fig. 1*B*, there was a population of NK1.1$^+$, Ly-49$^+$ cells in the NWNA spleen cells from both C57BL/6 and D8 mice. The percentage of NK1.1$^+$ cells that expressed Ly-49 was similar between D8 and C57BL/6 mice (13–18%). Similar to the data observed from the BALB/c mice, there was decreased expression of Ly-49 on D8 NK1.1$^+$ cells. All of the strains tested that express H-2D^d had lower expression of Ly-49 on their NK1.1$^+$ cells (52–64%) compared to C57BL/6 mice (Table 2).

Since our data using the JR9-318 anti-Ly-49 antibody to analyze NK cells from H-2D^d-expressing mice was different from those of previous studies that used the A1 antibody (16, 17), we also analyzed NWNA spleen cells for Ly-49 expression using the same anti-Ly-49 antibody (A1). A1 and JR9-318 monoclonal antibodies recognize different epitopes on the Ly-49 molecule (15). There was a significant population of NK1.1$^+$ cells that expressed the A1 epitope (Fig. 1*C*); however, the expression of the A1 epitope of Ly-49 was also decreased in D8 (66%), (D8 × C57BL/6)F_1 (66%), and B10.D2 (82%) mice, all of which express H-2D^d (Table 2). A single copy of the H-2D^d gene was sufficient to induce this decreased expression of Ly-49 since (D8 × C57BL/6)F_1 mice had a decrease in Ly-49 expression similar to that of D8 and B10.D2 mice. Although the level of Ly-49 in these different mouse strains was dependent on the expression of H-2D^d, the expression of 5E6 was similar for all strains tested (data not shown).

Ly-49lo NK Cells But Not Ly-49hi NK Cells Kill SP2/0 Tumor Cells and C57BL/6 Con A Blasts. To investigate whether H-2D^d expression influenced cytotoxic functions of the Ly-49$^+$ subset, flow cytometer-sorted Ly-49$^+$ and Ly-49$^-$ NK cells from D8 and C57BL/6 were used as effectors against BALB/c (H-2^d) Con A lymphoblasts and SP2/0 (H-2^d) tumor cell targets. BALB/c Con A lymphoblasts express high levels of H-2D^d on the cell surface, whereas SP2/0 cells express lower levels of H-2D^d, as shown in Fig. 2. Ly-49$^+$ NK cells from C57BL/6 and D8 failed to kill BALB/c Con A targets (Fig. 3 *A* and *B*), while the Ly-49$^-$ cells from both types of mice did kill the BALB/c target (Fig. 3 *A* and *B*). There was a greatly reduced ability of C57BL/6 Ly-49hi cells to kill SP2/0 tumor cells compared to C57BL/6 Ly-49$^-$ cells (Fig. 3 *C*). In contrast, Ly-49lo and Ly-49$^-$ cells from the H-2D^d transgenic mice (D8 mice) had similar ability to kill SP2/0 tumor cells (Fig. 3*D*). We also tested C57BL/6 Con A blasts as targets, which would represent complete "self" to C57BL/6 NK cells but not to D8 NK cells, since these Con A blasts do not express H-2D^d. Ly-49$^+$ NK cells from D8 mice killed C57BL/6 blasts (Fig. 3*F*), but Ly-49$^-$ cells did not. Both NK cell subsets from C57BL/6 mice failed to kill the syngenic blasts (Fig. 3*E*). It was sufficient to introduce the H-2D^d gene in the target cell to achieve protection from the Ly-49lo NK cells of D8 mice. Neither the NK cells (Ly-49$^+$ or Ly-49$^-$) from C57BL/6 nor the NK cells from D8 were able to kill D8 Con A blasts (Fig. 3 *G* and *H*). D8 Con A blasts express all of the self class I alleles of C57BL/6 and D8 mice.

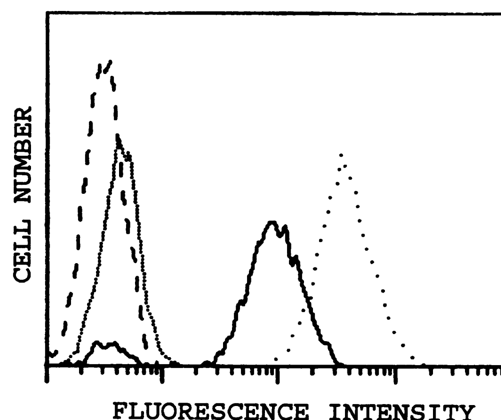

FIG. 2. Expression of H-2D^d on BALB/c Con A blasts and SP2/0 tumor cells. Histogram of H-2D^d expression on BALB/c Con A blasts (dotted line) and SP2/0 tumor cells (solid line). BALB/c Con A blasts with FITC-goat anti-mouse serum only (small dotted line); SP2/0 tumor cells with FITC-goat anti-mouse serum only (dashed line).

DISCUSSION

Our data indicate that the number of Ly-49$^+$ NK cells is similar regardless of H-2 haplotype of the host. However, the expression of Ly-49 in transgenic or inbred strains expressing H-2D^d is reduced to 60% (Ly-49lo NK cells) of that in C57BL/6 mice (Ly-49hi NK cells). The reason that we can detect these cells is likely due to the increased sensitivity of our analysis since we employ a two-step system to detect the Ly-49 antigen. Our data are thus not irreconcilable with previous reports in which there was no detection of Ly-49$^+$ NK cells in H-2^k or H-2^d expressing mice using a single-step method with FITC-conjugated A1 antibody (16). Upon further purification of NK cells, it was indeed possible to detect a distinct population of Ly-49$^+$, NK1.1$^+$ cells in mice that expressed H-2D^d (21). The most likely interpretation of our data is that the number of Ly-49 molecules on D8 NK cells is reduced, and analysis of Ly-49lo and Ly-49hi cells demonstrated that they act differently upon encountering a given target cell. Ly-49lo NK cells lysed SP2/0 tumor cells, which express low levels of H-2D^d, but Ly-49hi NK cells did not. Target cells expressing normal levels of H-2D^d (BALB/c or D8 lymphoblasts) were not killed by either Ly-49hi NK cells or Ly-49lo NK cells.

We propose that the level of inhibitory signals received upon encountering MHC class I is dependent upon the number of

Table 2. Expression of Ly-49 on NWNA spleen cells from C57BL/6 derived transgenic, congenic, and knockout mice

Strain	H-2	MFI A1,* % of C57BL/6	MFI JR9-318,* % of C57BL/6	Ly-49$^+$ cells, % of NK1.1$^+$ cells†
C57BL/6	b/b	100	100	17.5 ± 1.4
D8	b/b, D^d/D^d	66 (5)	64 (5)	12.6 ± 6.5
(C57BL/6 × D8)F_1	b/b, D^d	66 (3)	54 (4)	16.1 ± 2.7
B10.D2	d/d	82 (4)	52 (4)	15.4 ± 2.2
C57BL/6 β_2m −/−	b/b	99 (3)	107 (4)	16.5 ± 2.3

*MFI of NK1.1$^+$ NWNA spleen cells. In each experiment the expression on C57BL/6 NWNA spleen cells was used as 100% expression. *n* values are given in parentheses.
†Mean ± SD.

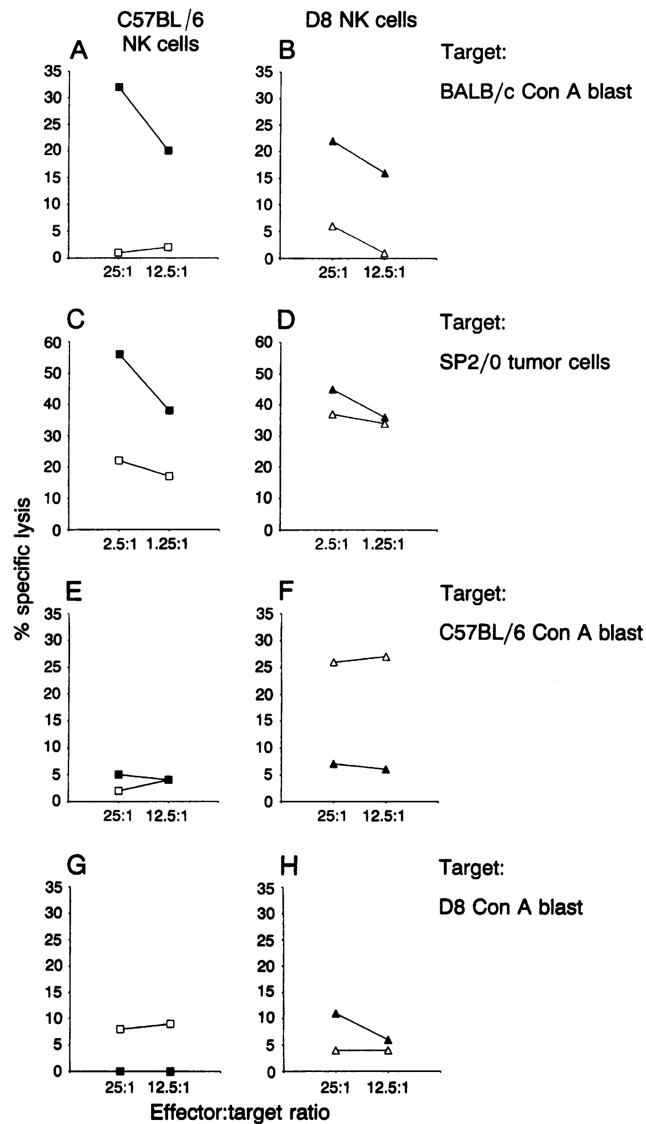

FIG. 3. Cytolysis of Con A blasts and tumor cells by Ly-49+ and Ly-49− NK cells from C57BL/6 (*A, C, E,* and *G*) and D8 (*B, D, F,* and *H*) mice. Sorted effector cells: Ly-49+ NK cells (open symbols); Ly-49− NK cells (solid symbols). Targets: BALB/c Con A lymphoblasts (*A* and *B*), SP2/0 tumor cells (*C* and *D*), C57BL/6 Con A lymphoblasts (*E* and *F*), D8 Con A lymphoblasts (*G* and *H*). Lysis of target cells was determined in triplicate at the indicated effector:target ratios. Ly-49 cells were sorted with A1 monoclonal antibody.

MHC receptor–class I interactions. A critical threshold must be achieved to inhibit NK cell lysis. The likelihood of this threshold being achieved can be decreased by lowering the level of either the receptor or the class I ligand. This model then suggests that the Ly-49lo phenotype represent a calibration of receptor levels that allow NK cells to perform optimally in an environment in which normal cells express high levels of H-$2D^d$. As a consequence, there are functional differences between Ly-49lo and Ly-49hi NK cells. When the Ly-49hi NK cells encounter a target with low H-$2D^d$ density, they receive an inhibitory signal above a critical threshold, which turns off cytolysis. On the contrary, the Ly-49lo NK cells fail to receive an inhibitory signal that is above the threshold, so lysis is not prevented. Thus, by lowering the level of receptors to self-MHC, NK cells may be calibrated to detect small alterations in self-MHC. This type of receptor calibration to self-MHC allows protection of normal cells from lysis but would leave NK cells able to kill cells that express suboptimal levels or altered MHC, perhaps as a consequence of viral infection or transformation.

It is possible that reduced Ly-49 expression is simply the consequence of interactions between mature effector cells and autologous normal cells presenting H-$2D^d$, analogous to T-cell receptor or immunoglobulin down-regulation in mice that express that receptor ligand (22–24). Another explanation is that the lower Ly-49 expression is determined during development from immature NK cell precursors, as a means of adapting the NK cell repertoire to the H-$2D^d$-expressing "self" environment. Ly-49 NK cells developing in an H-$2D^d$ environment in which strong receptor–ligand interactions occur may thus receive signals that set the expression of the Ly-49 receptor at a low level.

A second functional effect of introducing the H-$2D^d$ molecule was that Ly-49lo NK cells acquired the ability to kill C57BL/6 lymphoblasts, in contrast to Ly-49hi NK cells of C57BL/6 mice. Neither the Ly-49^+ nor the Ly-49^- NK cells from C57BL/6 or D8 were able to kill D8 Con A blasts. Why do Ly-49lo cells of D8 but not Ly-49hi cells of C57BL/6 kill C57BL/6 blasts? This may not be explained by the different levels of Ly-49 alone. We favor a model based on a second receptor with affinity for H-2^b class I molecules, expressed on NK cells of both types of mice. In this model, occupation of the H-2^b receptor would be sufficient and necessary to generate a negative signal for Ly-49^- cells of C57BL/6 and D8 mice (explaining why they kill H-2^d but not H-2^b blasts). For Ly-49hi NK cells of C57BL/6 mice, occupation of the receptor for H-2^b or the H-$2D^d/D^k$ receptor (Ly-49) would generate a sufficient negative signal (explaining why they do not kill H-2^b or H-2^d blasts). For Ly-49lo NK cells from D8 mice, occupation of the H-$2D^d/D^k$ receptor (Ly-49) would be sufficient but also necessary to turn off killing (explaining why they kill H-2^b but not H-2^d blasts). The balance between signaling mediated by the NK H-$2D^d$ receptor and the postulated H-2^b receptor would thus be altered in these cells; alternatively, the H-2^b receptor may not be expressed at all.

Other models are possible, based, for example, on the idea that Ly-49 also has a weak affinity to H-2^b molecules. This would help to explain why Ly-49 is expressed at all on C57BL/6 NK cells. However, data obtained with a recently developed binding assay do not support a Ly-49–H-2^b interaction (10). One possibility is that receptors without affinity for self-MHC can get highly expressed by default. It is important to remember that in a system in which specific recognition turns off effector cells, non-self receptors are not useful, but they are not harmful either. They can be allowed to persist, as long as each NK cell expresses at least one receptor with affinity for self-MHC.

Determining the mechanism behind the ability of NK cells to "learn" the nature of self-MHC is a major challenge in understanding NK cell function. Future studies will be needed to determine if other NK cell receptors are calibrated to self-MHC class I molecules.

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