Cumulative Inhibition of NK Cells and T Cells Resulting from Engagement of Multiple Inhibitory Ly49 Receptors[1]

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Inhibitory receptors specific for MHC class I molecules are expressed on partially overlapping subpopulations of NK cells and memory T cells. A central question pertinent to NK cell development and function is how the combinatorial expression of different receptors with distinct class I specificities affects functional recognition. We therefore studied the quantitative effects resulting from class I engagement of multiple inhibitory Ly49 receptors. We used a transgenic mouse model in which all NK cells and T cells express two different Ly49 receptors with shared class I specificity. Comparisons of cells from these mice with cells from single transgenic mice and wild-type mice revealed that Ly49 receptors cumulatively inhibit lymphocyte effector functions. Multiple Ly49 interactions also had a cumulative impact on NK cell development. The findings suggest that the interactions of inhibitory receptors with class I are interpreted quantitatively rather than as on/off switches. They have intriguing implications concerning NK cell tolerance and reactivity toward cells with extinguished expression of a limited number of class I molecules. ***The Journal of Immunology,*** **2001, 166: 3002–3007.**

The cytolytic activity of NK cells is tightly regulated by the recognition of class I MHC molecules. Three families of NK cell surface receptors have been implicated in class I recognition: lectin-like CD94/NKG2 heterodimers interact with $Qa1^b$ (in mice) and HLA-E (in humans) class Ib molecules, KIR receptors belonging to the IgSF (in humans) interact with class Ia MHC molecules, and homodimeric Ly49 receptors belonging to the lectin family (in mice) also interact with class Ia MHC Ags (1, 2). All known class I-specific NK cell receptors in humans and mice share at least four characteristics: 1) they are members of receptor families, 2) they distinguish different class I alleles, 3) they contain activating as well as inhibitory family members, and 4) they are expressed on partially overlapping subpopulations of NK cells. When inhibitory class I-specific receptors expressed by NK cells engage target cell class I molecules, stimulatory signals are overridden, resulting in reduced cytolysis and cytokine release. In addition, during NK cell development, Ly49-class I interactions impact the expression of other inhibitory receptors expressed by NK cells, the effect being to minimize the coexpression of other Ly49 receptors by individual NK cells (3–5).

In addition to NK cells, inhibitory Ly49 receptors are expressed on some NK1 T cells and a small subpopulation of $CD8^+$ $\alpha\beta$-T cells, predominantly of the memory phenotype (6, 7). Ly49A engagement inhibits the activation and cytotoxicity of virus-specific $CD8^+$ T cells stimulated via their TCRs (8), and engagement of class I ligands by either Ly49A or Ly49G2 inhibits T cell proliferation in response to alloantigens (9, 10).

The specificity of interaction of class Ia molecules with Ly49 family members has been extensively studied (11, 12). Five of at least eight inhibitory Ly49 molecules expressed by NK cells from B6 mice have been shown to interact with class Ia molecules using cell-cell adhesion assays or class I tetramer binding studies: Ly49A binds to D^d, D^k, and MHC Ags of the $H\text{-}2^{f,q,r,s \text{ and } v}$ haplotypes, but not to K^d, L^d, K^k, or $H\text{-}2^b$ molecules (10, 12–15). Ly49C binds to K^d, D^d, D^k, K^b, and $H\text{-}2^{f,q,r,s \text{ and } v}$ Ags (10, 12, 16, 17); Ly49F binds weakly to $H\text{-}2^d$-encoded Ags (10); Ly49G2 binds to D^d, may interact weakly with L^d, and does not bind detectably to $H\text{-}2^{b,f,q,r,s \text{ and } v}$ Ags (10, 18); and Ly49I interacts with K^b and $H\text{-}2^{q,r,s \text{ and } v}$ molecules (10). The MHC reactivity of Ly49B, -E, and -J has not been reported.

Of the Ly49-MHC combinations tested in cell-cell adhesion assays, the Ly49A-D^d interaction is the strongest. Recent surface plasmon resonance analysis indicates a K_d of $\sim$10 μM for this interaction (19). Other Ly49-MHC interactions range widely in binding strength, based on the cell-cell adhesion assay results (10, 16). The strength of Ly49A or Ly49G2 binding to different class I alleles correlates with the degree of functional inhibition mediated by these receptors (10). These findings suggest that the inhibitory receptor interactions are interpreted quantitatively rather than as on/off switches.

Since Ly49 and other NK receptors are expressed on overlapping subsets of NK cells or memory $CD8^+$ T cells, individual cells typically express a combination of receptors (4, 20, 21). Thus, a central question pertinent to NK cell development and function is how combinatorial expression of different class I-specific receptors by individual NK cells affects functional and developmental outcomes. Previous studies demonstrate that NK cells expressing two different inhibitory Ly49 receptors can be inhibited through either of them (22). There is little information, however, about whether engagement of two receptors on an NK cell imparts greater inhibition than engagement of only one. This question is important because many receptor interactions with class I molecules are weak and might only become functionally relevant if additional receptor-class I interactions occurred in concert. To address this question, we have generated transgenic mice in which all NK cells and T cells express one or both of two different Ly49 receptors.

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The results reveal that multiple Ly49 receptor interactions cumulatively impact the elaboration of effector activity as well as NK cell development.

Materials and Methods

Animals and cell lines

C57BL/6J (B6) mice were bred at the animal facilities of the University of California (Berkeley, CA) or at the animal facilities of the Institute for Virology and Immunobiology, University of Wurzburg (Wurzburg, Germany). B10.D2/nSnJ mice for breeding Ly49A and Ly49G2 transgenic H-$2^{b/d}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.D2/nOlaHsd (H-2^{d}, B10.D2), B10.BR/OlaHsd (H-2^{k}, B10.BR), B10.M/OlaHsd (H-2^{f}, B10.M), B10.RIII(71NS)/OlaHsd (H-2^{r}, B10.RIII), and B10.S/OlaHsd (H-2^{s}, B10.S) mice were purchased from Harlan Winkelmann (Borchen, Germany). Ly49A-transgenic mice (line 2) and Ly49G2-transgenic mice (line 5) have been described previously (9, 10). Both transgenic lines were backcrossed at least four times to the B6 background before crossing with B10.D2 mice and/or intercrossing the two different lines to generate double-transgenic animals. All mice expressed MHC and NK cell complex genes of the B6 haplotype by serological analysis. Conventionally housed mice were monitored continuously for health conditions. In the case of Ly49A and/or Ly49G2 transgenic H-$2^{b/d}$ mice, moribund (wasted, hunched, and/or sessile) animals were sacrificed. The following cell lines were used: EL-4 (provided by T. Potter, University of Colorado, Denver, CO), EL-4 cells transfected with D^{d} (23), P815 (American Type Culture Collection, Manassas, VA; TIB 64), RMA (provided by M. Bevan, University of Washington, Seattle, WA), and RMA transfected with D^{d} (23).

Antibodies

The following mAbs were used: 4D11 (anti-Ly49G2) (24), JR9-318 (anti-Ly49A, provided by J. Roland, Institut Pasteur, Paris, France), SW-5E6 (anti-Ly49C/I, provided by V. Kumar and M. Bennett, University of Texas, Dallas, TX), 14B11 (anti-Ly49C/I/F/H) (25), 34-5-8S (anti-$D^{d}\alpha1/\alpha2$, American Type Culture Collection HB-102), and 2.4G2 (anti-FcγRII/III) (26). The mAbs were purified on protein A- or protein G-Sepharose columns, and Ig fractions were conjugated to biotin or FITC using standard techniques. Anti-NK1.1-biotin, anti-NK1.1-PE, and anti-CD3-PE were purchased from PharMingen (San Diego, CA), and anti-CD3-Tricolor was obtained from Caltag (South San Francisco, CA).

Cell surface staining and flow cytometry

Cell staining was performed on ice in PBS/3% FCS/0.02% sodium azide with saturating amounts of staining reagents. The cells were washed two or three times between steps or before analysis. Cells ($0.5–1.5 \times 10^{6}$) were treated with unconjugated mAb 2.4G2 to block FcγRII/III. Two staining procedures were used as indicated: 1) 4D11-biotin/streptavidin-Cychrome (PharMingen), JR9-318-FITC, anti-NK1.1-PE (see Fig. 1); or 2) anti-NK1.1-biotin/streptavidin-Tricolor (Caltag), anti-CD3-PE, JR9-318-FITC, 4D11-FITC, 14B11-FITC, or 5E6-FITC (see Figs. 5 and 6). Samples were analyzed on an Epics XL (Coulter, Hialeah, FL) or a FACScan (Becton Dickinson, San Jose, CA) flow cytometer, gating on live cells based on the forward and side light scatter profile. WinMDI and CellQuest software (Becton Dickinson) were used for data display. Representative dot plots are shown as $\log_{10}$ fluorescence intensities on a four-decade scale.

Lymphocyte-activated killer (LAK)[3] cell culture and cytotoxicity assay

RBC-depleted spleen cells were cultured at 2×10^{6} cells/ml in RPMI 1640/5% FCS medium (Life Technologies, Gaithersburg, MD) containing 4500 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA). After 4 days, the IL-2 was washed away, and the cells were cocultured in round-bottom 96-well plates at the indicated E:T cell ratios with 1×10^{4} target cells that had been labeled with 1 mCi of ^{51}Cr (Amersham, Arlington Heights, IL) for 1 h and washed extensively. After 4 h, radioactivity released into the supernatant was determined by gamma counting. All determinations were performed in triplicate.

Mixed leukocyte reactions

MLRs were performed in RPMI 1640/FCS medium in 96-well round-bottom plates in triplicate. As responder cells, lymph node cells (>85% CD3^{+}) passed through nylon wool were used at 1×10^{5} cells/well. For preparation of stimulator cells, RBC-depleted spleen cell suspensions were irradiated with 25 Gy, washed three times, and cultured at 5×10^{5} cells/well. Cultures were pulsed on day 3 with 12.5 μCi of [^{3}H]thymidine (Amersham) and harvested 20 h later. Radioactive incorporation was determined by beta counting. Stimulation indexes were determined as cpm obtained with allogeneic stimulator cells divided by mean cpm obtained with syngeneic stimulator cells.

Statistics

Tests for statistical differences of indicated pairwise comparison groups were conducted using Student's two-tailed *t* test.

Results

Transgenic coexpression of Ly49A and Ly49G2

To determine whether MHC engagement by two inhibitory Ly49 receptors with shared class I MHC specificity cumulatively inhibits lymphocyte function, we generated Ly49A/Ly49G2 double-transgenic mice by intercrossing mouse lines that transgenically express either receptor (9, 10) (Fig. 1). Cell surface coexpression

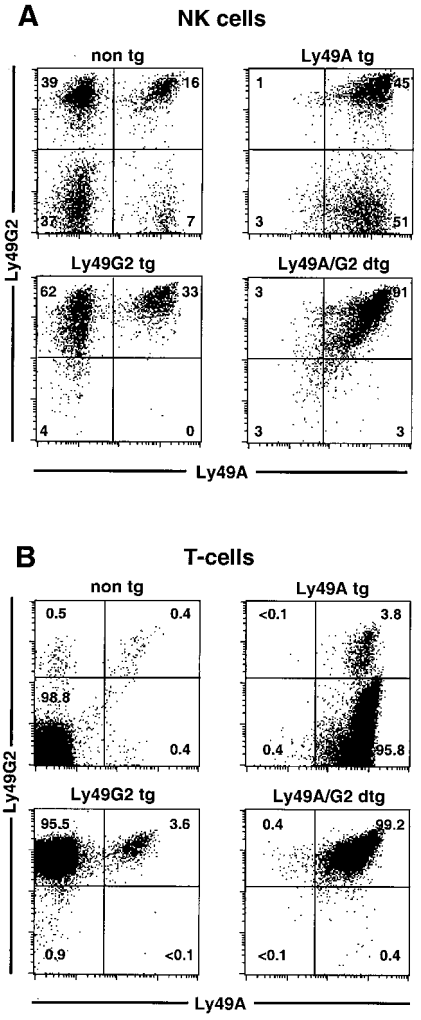

FIGURE 1. Ly49A and Ly49G2 are coexpressed on Ly49A/G2 double-transgenic LAK cells and T cells. LAK cells (*A*) or freshly isolated T cells (*B*) from nontransgenic (non tg) littermates or the indicated transgenic (tg) lines of the B6 (H-2^{b}) background were passed through nylon wool and stained with mAbs specific for Ly49A (JR9-318) and Ly49G2 (4D11). *A*, Gated NK1.1^{high} cells are shown; *B*, gated CD3^{+} cells are shown. Results similar to those in *A* were obtained with freshly isolated NK cells. Numbers represent percentages of cells per quadrant.

[3] Abbreviation used in this paper: LAK, lymphocyte-activated killer.

of Ly49A and/or Ly49G2 transgenes on short-term IL-2-activated NK cells (LAK cells) and T cells was determined by staining with Ly49A-specific mAb JR9-318 and Ly49G2-specific mAb 4D11. As expected, nontransgenic littermates expressed Ly49A and Ly49G2 on partially overlapping subpopulations of NK cells and a small number of T cells, while Ly49A and/or Ly49G2 transgenic mice efficiently expressed the respective transgene(s) on virtually all NK1.1^{high} LAK cells and on all ex vivo isolated T cells (Fig. 1). Transgene expression on freshly isolated NK cells was similar to expression on NK1.1^{high} LAK cells (data not shown). The cell surface expression level of Ly49A directed by the transgene was essentially identical with that of endogenously expressed Ly49A on both NK cells and T cells (Fig. 1) (9). The Ly49G2 transgene was expressed at marginally lower levels than endogenous Ly49G2 on NK cells and at levels equivalent to wild-type Ly49G2 on T cells (Fig. 1 and data not shown).

H-2^d-specific cumulative inhibition of NK cell cytotoxicity through Ly49A and Ly49G2

LAK cells from wild-type or Ly49A, Ly49G2, or double-transgenic were nearly equal in their capacity to kill H-2^b EL-4 target cells (Fig. 2*A*). In contrast, while nontransgenic effector cells killed H-2^d P815 tumor cells efficiently, both Ly49G2 and Ly49A transgenic LAK cells lysed P815 cells less well, indicating partial inhibition (Fig. 2*B*). Significantly, in each of three experiments Ly49A/G2 double-transgenic LAK cells killed P815 cells less well than did either of the single-transgenic populations (Fig. 2*B* and data not shown), indicating a cumulative effect when two Ly49 receptors are engaged.

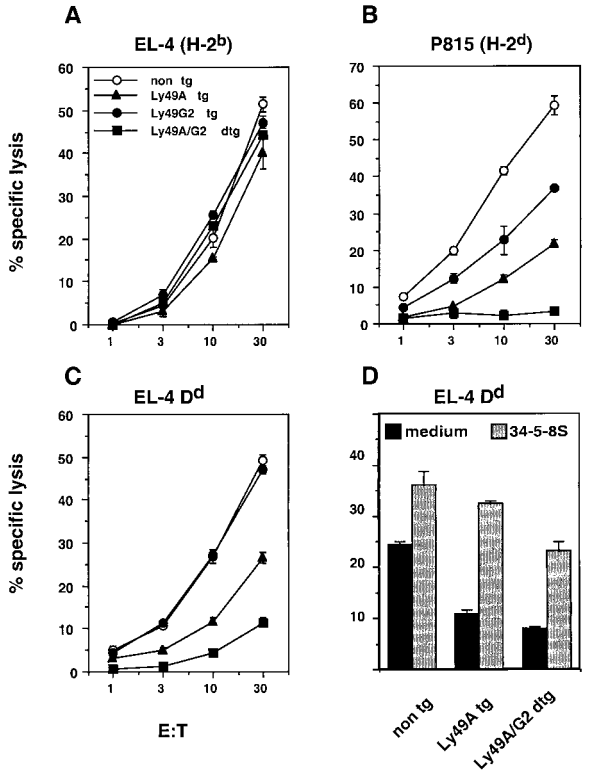

FIGURE 2. Cumulative inhibition of NK cytolytic activity resulting from multiple Ly49 interactions. *A—C*, Unseparated LAK cells from the indicated strains (all H-2^b) were used as effector cells against EL4 (H-2^b), P815 (H-2^d), or D^d-transfected EL-4 target cells as indicated. Comparable results were obtained in two repetitions of the experiment. *D*, 34-5-8S mAb specific for the D^d α1/α2 domain reverses most inhibition attributable to the transgenically encoded receptors. The mAb was added at a 10 μg/ml final concentration to the cytotoxicity assay (E:T cell ratio, 10:1). Addition of D^d α3 domain-specific mAb 34-2-12S had no effect (data not shown). Representative data from one experiment of two with similar results are shown. *A—D*, Data are shown as the mean ± SD of triplicate determinations.

To corroborate this finding and determine whether inhibition could be assigned to the D^d molecule, we used D^d-transfected EL-4 cells as targets. As shown in Fig. 2*C*, Ly49A-transgenic effector cells were less potent than wild-type effector cells in killing the D^d transfectants. Unexpectedly, Ly49G2-transgenic effector cells were not inhibited by the D^d-transfected EL-4 cells (Fig. 2*C*), possibly due to the slightly lower D^d levels on these cells compared with P815 cells (data not shown). Alternatively, D^d alone may not be sufficient to inhibit through Ly49G2 (see *Discussion*). Most significantly, the Ly49G2 transgene functionally synergized with the Ly49A transgene, resulting in substantially lower lysis of D^d-transfected target cells with double-transgenic effector cells compared with Ly49A-transgenic effector cells (Fig. 2*C*). Similar results were obtained in two other experiments. Addition of D^d α1/α2 domain-specific mAb 34-5-8S, but not of the α3 domain-specific mAb 34-2-12S, to EL-4/D^d targets substantially reversed the inhibition of Ly49A transgenic and Ly49A/G2 double-transgenic cells (Fig. 2*D* and data not shown), indicating that D^d is directly recognized by the NK cells. Together, the data show that D^d recognition by Ly49A and Ly49G2 cumulatively inhibits cytolytic NK cell activity toward tumor cells that express corresponding MHC ligands.

Cumulative inhibition of T cell proliferation through Ly49A and Ly49G2

To test whether the proliferative T cell response to alloantigens is also inhibited cumulatively by Ly49A and Ly49G2, we performed MLRs with T cells from nontransgenic and Ly49A- and/or Ly49G2-transgenic mice. As stimulator cells, we used spleen cells from five different MHC congenic B10 strains (H-2^{d,k,f,r,s}; Fig. 3*A*). To determine whether inhibition in the case of H-2^d stimulator cells was due to engagement of class I molecules, MLRs were performed with stimulator cells from class I-deficient (β_2-microglobulin$^{-/-}$) B6-H-2$^{b/b}$ or B6-H-2$^{d/d}$ spleen cells (Fig. 3*B*). The latter cells can stimulate MLRs by virtue of the allogeneic class II molecules they express. Responses are shown as stimulation indexes relative to the responses to syngeneic B6 stimulator cells.

Ly49A- or Ly49G2-transgenic T cells proliferated at least 8-fold less well than wild-type T cells in response to allogeneic H-2$^{d,k,f,r\ or\ s}$ (Fig. 3*A*). For the H-2$^{k,f,\ r\ and\ s}$ stimulator haplotypes, the proliferation of Ly49A/G2 double-transgenic T cells was significantly (2.5- to 6.7-fold; $p < 0.05$) lower than the proliferation of Ly49A or Ly49G2 single-transgenic T cells. The H-2^d stimulator cells caused the strongest inhibition of Ly49A- or Ly49G2-transgenic responders, with nearly complete inhibition occurring in the case of Ly49A-transgenic T cells (Fig. 3*A*) (10). Ly49A/G2 double-transgenic T cells proliferated even less well to H-2^d stimulator cells, although this effect was not statistically significant due to the limited possibility of additional inhibition with these stimulator cells. The inhibition in these experiments was mediated by class I molecules (in the case of H-2^d, presumably D^d), since no inhibition of any population was observed when class I-deficient allogeneic (H-2^d) cells were used as stimulator cells (Fig. 3*B*) (10). This control experiment demonstrates that Ly49 transgene coexpression by itself, without ligand engagement, does not cause inhibition. Together, the data show that Ly49A and Ly49G2 mediate dominant and cumulative inhibition of T cell alloresponses upon engagement of MHC molecules.

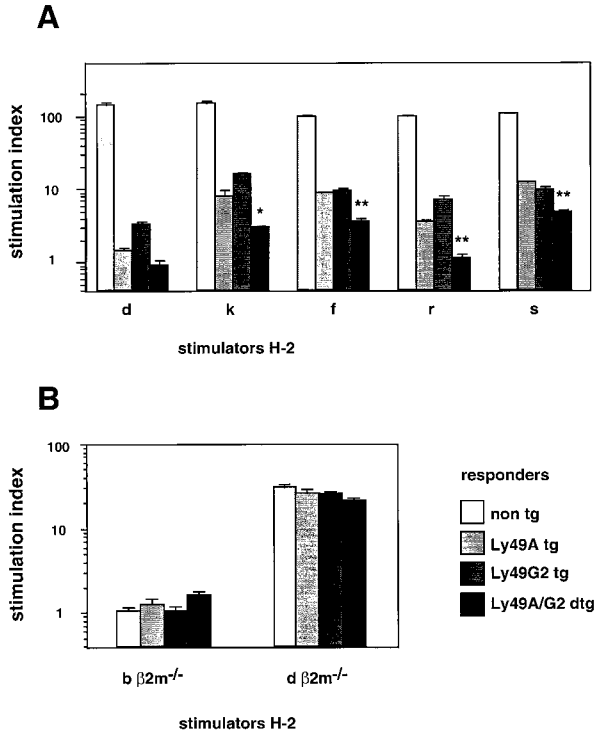

FIGURE 3. Coengagement of allogeneic MHC molecules by Ly49A and Ly49G2 cumulatively inhibits mixed lymphocyte proliferative responses by T cells. Lymph node cells from the indicated lines (all H-$2^{b/b}$, >85% TCR$\alpha\beta^+$, Ly49A and Ly49G2 expression as shown in Fig. 1) passed through nylon wool were used as responders in MLRs. *A*, Irradiated spleen stimulator cells from the indicated strains were used (H-2 haplotype in parentheses): B10. D2 (d), B10. BR (k), B10. M (f), B10. RIII (r), or B10. S (s) mice. *B*, Stimulator cells from H-$2^{b/b}$ or H-$2^{d/d}$ β_2-microglobulin-deficient mice of the B6/B10 background (b $\beta_2m^{-/-}$ or d $\beta_2m^{-/-}$, respectively) were used. Results are shown as the mean ± SD stimulation indexes (cpm obtained with allogeneic stimulators/mean cpm obtained with syngeneic stimulators) from one representative experiment of three (*A*) or two (*B*). For statistical significance determinations, we compared Ly49A/G2 dtg responders with Ly49A tg responders: *, $p < 0.05$; **, $p < 0.01$.

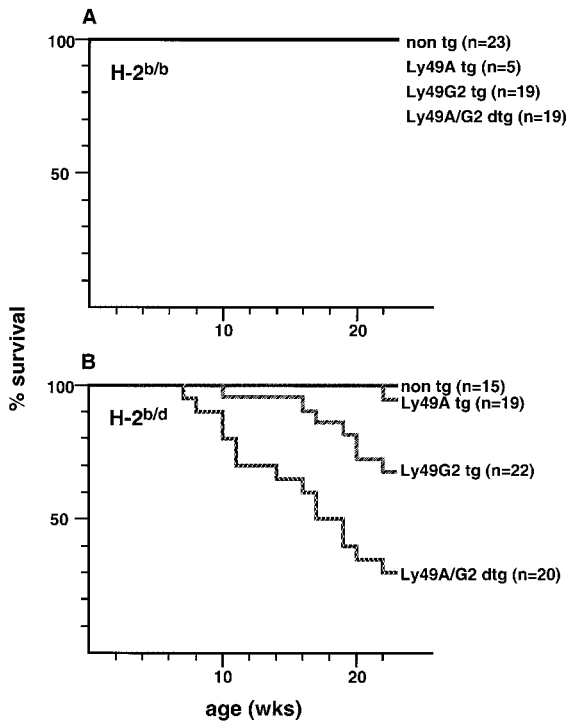

FIGURE 4. Reduced survival of Ly49A/G2 double-transgenic mice in H-$2^{b/d}$, but not H-$2^{b/b}$, mice. The health and survival of nontransgenic and Ly49A and/or Ly49G2 transgenic H-$2^{b/b}$ (*A*) or H-$2^{b/d}$ (*B*) littermate mice of the B6/B10 background were monitored continuously over a period of 5 mo.

Enhanced wasting disease in Ly49A/G2 double-transgenic H-$2^{b/d}$ mice

In two recent reports Ly49A-transgenic H-2^d mice exhibited a fatal chronic wasting disease (27, 28). The disease was dependent on both the Ly49A transgene and expression of the appropriate ligand (H-2^d), and is believed to result from impaired T cell tolerance induction as a result of Ly49 receptor engagement in the thymus. Our Ly49A-transgenic mice, in contrast, exhibit little or no fatal disease (Fig. 4 and data not shown), perhaps due to a lower (although physiological) level of transgene expression. The Ly49G2-transgenic mice, however, do exhibit a partially penetrant wasting disease, leading to the death of 32% of the mice by 22 wk (Fig. 4). The disease is evident in H-$2^{b/d}$ mice, but not in H-2^b mice. Strikingly, Ly49A/G2 double-transgenic mice exhibit a much more severe disease with earlier onset, leading to the death of 70% of the mice after 22 wk. Again, disease was observed in H-$2^{b/d}$ mice, but not in H-2^b mice. The differences in disease rates were not due to variations in housing or inadvertent genetic differences between the mice tested, since they were littermates and were housed together. These data suggest that Ly49A and Ly49G2 transgenes exhibit synergistic effects in the initiation and/or progression of fatal disease.

Enhanced alterations in the repertoire of NK cell receptors resulting from coexpression of Ly49A and Ly49G2

Previous studies demonstrated that transgenic expression of Ly49A in all developing NK cells results in an altered repertoire of endogenous Ly49 expression. In Ly49A-transgenic H-$2^{b/d}$ mice, the Ly49G2$^+$ NK cell subset was 2- to 3-fold smaller than that in Ly49A-transgenic H-$2^{b/b}$ mice (3) (Fig. 5*A*).We therefore tested whether the Ly49G2 transgene reciprocally impairs the expression of Ly49A. Indeed, the frequency of Ly49A$^+$ NK cells was significantly and substantially (~2.5-fold) lower in Ly49G2-transgenic

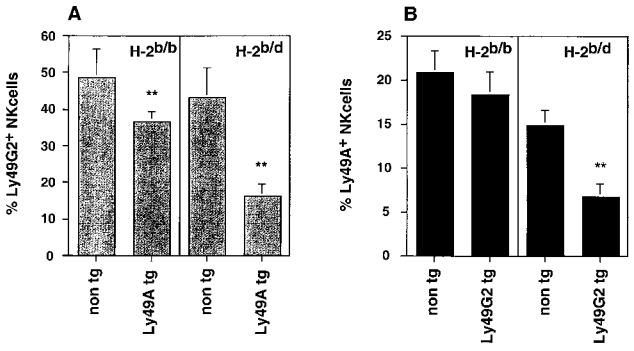

FIGURE 5. MHC recognition by transgenic Ly49A and Ly49G2 reciprocally reduces expression of endogenous Ly49G2 and Ly49A, respectively. The frequency of NK1.1$^+$ CD3$^-$ splenocytes of the indicated H-2 haplotypes that express Ly49G2 (*A*) or Ly49A (*B*) was determined by staining spleen cells passed through nylon wool with mAbs JR9-318 or 4D11 (see *Materials and Methods*). Values represent the mean ± SD from three to nine individual mice. **, $p < 0.01$ Ly49, transgenic vs nontransgenic mice of the same MHC type.

H-$2^{b/d}$ mice compared with that in nontransgenic H-$2^{b/d}$ mice (Fig. 5*B*). In H-$2^{b/b}$ mice the Ly49G2 transgene had no significant effect on the size of the Ly49A$^+$ NK cell subset. Thus, transgenic Ly49A and Ly49G2 reciprocally and equivalently inhibited expression of the other (endogenous) receptor in a ligand-dependent manner.

To test whether Ly49A- and Ly49G2-mediated signals cumulatively shape the Ly49 repertoire, we determined the effects of Ly49 transgene expression on third-party inhibitory receptors. The frequency of NK cells expressing Ly49C/I, as detected with the SW-5E6 Ab, was significantly lower in Ly49A or Ly49G2 single-transgenic mice than in nontransgenic mice (Fig. 6), but the reduction was quite modest compared with the previously observed reductions in Ly49G2$^+$ or Ly49A$^+$ NK cells (Fig. 5). In H-$2^{b/d}$ Ly49A/G2 double-transgenic mice, however, the reduction was significantly larger, resulting in a >2-fold overall reduction. No effect was observed in H-2^b mice. A similar effect was observed when NK cells were stained with the 14B11 Ab (25), which detects Ly49C/I/F/H (Fig. 6). Therefore, engagement of both transgenic Ly49 receptors by developing NK cells has a greater effect on expression of additional Ly49 receptors than does engagement of only one.

Discussion

The studies herein demonstrate that engagement of two Ly49 receptors expressed by the same cell can lead to cumulative effects on lymphocyte function and development. At the level of NK cell function, the data indicate that D^d corecognition by Ly49A and Ly49G2 inhibits NK cell cytotoxicity more strongly than recognition by either Ly49A or Ly49G2 alone. This finding has intriguing implications concerning NK cell tolerance to normal self cells. It raises the possibility that inhibition of NK cells by self cells is not necessarily attributable to any single receptor-ligand interaction. Thus, it is plausible that many NK cells may be prevented from attacking self cells as a result of cumulative signaling through several receptors, each with weak reactivity toward self class I molecules. Therefore, in assessing self tolerance of NK cells, it may be necessary to consider the cumulative affects of several interactions of varying strength, accounting for all the receptors expressed by each NK cell.

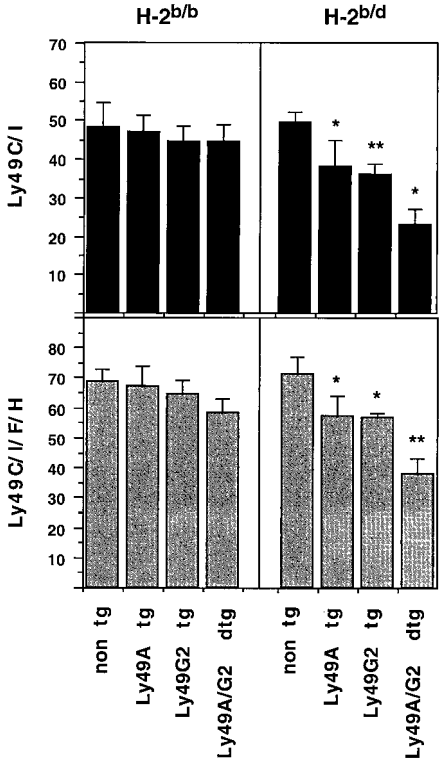

FIGURE 6. Ly49 repertoire in Ly49A- and/or Ly49G2-transgenic mice. The frequency of NK1.1$^+$ CD3$^-$ cells of the indicated H-2 haplotypes that express Ly49C/I or Ly49C/I/F/H was determined by staining splenocytes passed through nylon wool with mAbs 5E6 or 14B11 mAbs, respectively (see *Materials and Methods*). Values represent the mean ± SD from four to nine individual mice. For statistical significance determinations, we compared single-transgenic mice with nontransgenic mice, or double-transgenic mice with Ly49G2-transgenic mice. *, $p < 0.05$; **, $p < 0.01$.

An extension of this thinking concerns the scenario where NK cells express two (or more) receptors with weak reactivity for different self class I molecules. Such NK cells would be expected to attack self cells in which class I molecules are globally down-regulated. The present results raise the possibility that such cells might also be capable of attacking self cells in which one or the other of the cognate class I molecules is individually down-regulated, since the weak engagement of the second receptor would be insufficient to fully inhibit the NK cell. Recent studies suggest that selective class I down-regulation is common in both tumor cells and virus-infected cells (29, 30). It has been speculated elsewhere that the overlapping expression pattern of inhibitory MHC-specific receptors may have arisen by natural selection to equip the NK cell population with the capacity to attack cells in which some, but not all, class I molecules have been extinguished (4).

Functional inhibitory interactions of D^d with endogenous (31) and transgenic (9) Ly49A are well documented. Using P815 (H-2^d) cells as targets, we observed partial inhibition of both Ly49A-transgenic and Ly49G2-transgenic LAK effector cells. Inhibition of Ly49G2-transgenic LAK cells by P815 targets was less efficient than inhibition of Ly49A-transgenic cells, in line with a previous report using sorted LAK cells (18). This functional difference between Ly49A and Ly49G2 is likely to reflect differences in D^d binding, because in both cell-cell adhesion and tetramer binding assays D^d interacts more strongly with Ly49A than with Ly49G2 (10).

Surprisingly, our D^d-transfected EL-4 cells did not inhibit cytolysis by Ly49G2-transgenic effector cells (Fig. 2). The larger effects observed with P815 cells may reflect higher levels of D^d expression (data not shown) and/or the participation of L^d as a functional ligand for Ly49G2 (18). P815 cells, but not the transfected cells, express L^d. Although little, if any, specific binding between Ly49G2 and L^d was observed in adhesion assays (10) or functional assays with recombinant L^d (32), it is possible that a weak interaction is sufficient to cooperate with other interactions, such as the stronger D^d-Ly49G2 interaction. The key finding was that although the Ly49G2-transgenic effector cells were not inhibited by D^d transfectants, the combination of Ly49A and Ly49G2 resulted in synergistic inhibition. The explanation for receptor synergy remains to be established. One possibility is that inhibition exhibits a cooperative effect with respect to the number of engaged Ly49 receptors.

Analysis of the effects of the Ly49A and Ly49G2 transgenes on the expression of endogenous Ly49 receptors indicates that cumulative receptor interactions impact formation of the NK cell repertoire as well. Coexpression of both self-specific receptor transgenes clearly caused a greater reduction in the expression of Ly49C/I receptors than either receptor transgene alone. Previous studies suggest that Ly49 receptor expression in ontogeny occurs in a sequential fashion (5, 33). Several features of the repertoire led to the proposal that the sequential accumulation of Ly49 receptors is inhibited as NK cells accumulate sufficient self class I-specific receptors (3–5). It appears, however, that any one receptor may

only partially inhibit expression of other receptors, as we observe in the case of both Ly49A and Ly49G2 single-transgenic mice. A possible explanation for this phenomenon is that individual receptors are in many cases insufficiently inhibitory to reliably achieve self tolerance of NK cells, necessitating the expression of additional receptors. Hence, the termination of new receptor expression by developing NK cells might require that the NK cell expresses two or even more self-specific receptors. The possibility that multiple self-specific receptors might be necessary to achieve full nonresponsiveness to self cells was previously addressed in mathematical models of the formation of the NK cell repertoire (34).

It is notable also that while the frequencies of cells expressing endogenous Ly49A or Ly49G2 were substantially reduced by expression of a Ly49G2 or Ly49A transgene, respectively, both transgenes by themselves had a minimal effect on the frequency of Ly49C/I$^+$ cells. This was previously reported in the case of Ly49A transgenic mice (3). Only with both Ly49A and Ly49G2 transgenes did we observe a substantial reduction in Ly49C/I$^+$ cells. One possibility is that the signals that control de novo expression of Ly49C and -I differ in some respects from those that control de novo expression of Ly49A and Ly49G2.

As was the case for NK cells, coengagement of two specific receptors in the double-transgenic mice had a greater impact on T cell activation than engagement of either alone. These data serve as independent evidence that inhibitory signaling by multiple Ly49 receptors is cumulative, in this case during the activation phase of T cells. It is somewhat difficult to gauge the possible significance of the results for T cells, since there is little understanding of the biological role of Ly49 receptors on the T cells that express them in normal mice. Nevertheless, it is significant that the T cells that express Ly49 receptors, particularly the CD8$^+$CD44$^+$ memory subset, exhibits a highly overlapping pattern of receptor expression (7).

Similarly, the development of a wasting disease thought to result from dysregulation of TCR stimulation during development (27, 28) was also more severe in double-transgenic than in single-transgenic mice. Disease only occurred H-2$^{b/d}$ mice, not in H-2^b mice (Fig. 4). Although the cause and pathologic manifestations of the observed fatal disease are not yet fully resolved, two possibilities may be considered: 1) H-2^d engagement by the transgenic receptors impairs the establishment of tolerance resulting in the appearance of autoimmune T cells; or 2) H-2^d engagement by the transgenic receptors inhibits lymphocyte activation, resulting in immunodeficiency and consequent infection. Regardless of the underlying cause of fatal disease, the data clearly provide another example of an H-2^d-specific and synergistic effect of the two transgenes.

In future studies it will be important to address whether cumulative class I-specific effects as observed for the Ly49A and Ly49G2 receptor combination apply equally to various other receptor combinations and to ascertain how these combinatorial interactions affect NK cell self tolerance and functional inhibition.

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