Brief Definitive Report

Transgenic Expression of the Ly49A Natural Killer Cell Receptor Confers Class I Major Histocompatibility Complex (MHC)-specific Inhibition and Prevents Bone Marrow Allograft Rejection

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Summary

Natural killer (NK) cells and some T cells are endowed with receptors specific for class I major histocompatibility complex (MHC) molecules that can inhibit cellular effector functions. The function of the Ly49 receptor family has been studied in vitro, but no gene transfer experiments have directly established the role of these receptors in NK cell functions. We show here that transgenic expression of the H-2D^d-specific Ly49A receptor in all NK cells and T cells conferred class I–specific inhibition of NK cell–mediated target cell lysis as well as of T cell proliferation. Furthermore, transgene expression prevented NK cell–mediated rejection of allogeneic H-2^d bone marrow grafts by irradiated mice. These results demonstrate the function and specificity of Ly49 receptors in vivo, and establish that their subset-specific expression is necessary for the discrimination of MHC-different cells by NK cells in unmanipulated mice.

Two families of NK cell receptors with specificity for MHC class I molecules have been described. Immunoglobulin-like killer inhibitory receptors (KIRs) were identified on human NK cells, whereas the structurally unrelated Ly49 family of receptors, homologous to C-type lectins, were identified in the mouse (1, 2). The Ly49 receptor family comprises eight closely related genes (Ly49A–H) on mouse chromosome 6 (2). Expression of Ly49 receptors is largely restricted to NK cells and a small fraction of T cells (3–5). The Ly49A, Ly49C, and Ly49G2 receptors are each expressed by 20–50% of NK cells, with some cells expressing two or even all three of the receptors (6–10). Mouse NK cells expressing the Ly49A receptor are unable to kill H-2D^d- and H-2D^k-expressing tumor cell or lymphoblast target cells (3, 11). Evidence that Ly49A binds to D^d, and that anti-Ly49A antibodies allow Ly49A$^-$ NK cells to kill D^d-expressing target cells, suggested that Ly49A is a D^d-specific receptor that delivers an inhibitory signal to NK cells (3, 12, 13). NK cell subsets expressing the Ly49C or Ly49G2 receptors are inhibited by distinct MHC class I molecules, K^b for the B6 allele of Ly49C and D^d or L^d for Ly49G2 (7, 9).

To date, there is no evidence that genetic transfer of Ly49 receptors confers class I–specific inhibition. Therefore, the role of Ly49 receptors in regulating the various effector functions ascribed to NK cells remains unclear. This is particularly true for in vivo effector functions such as the NK cell–mediated rejection of bone marrow cells. Furthermore, the possibility has been raised that Ly49 receptors play a role as accessory receptors in class I–specific inhibition, rather than as the primary determinants of specificity (14). These concerns are enhanced by the failure to date to isolate murine homologues of the human KIR family of class I–specific inhibitory receptors. It remains possible that the two receptor families coexist in mice and mediate distinct functions. To address directly the role of Ly49A in class I-specific inhibition of NK and T cell functions, we have generated transgenic mice in which the Ly49A cDNA is expressed by all NK cells and T cells. Transgenic expression of Ly49A in all NK cells from H-2^b mice prevented these mice from rejecting an H-2^d bone marrow allograft, and resulted in impaired in vitro cytolysis of tumor target cells expressing the D^d Ly49A ligand. Furthermore, expression of Ly49A in T cells specifically inhibited their capacity to mount a proliferative response to stimulator cells that express class I ligands of Ly49A.

Materials and Methods

Generation of Ly49A Transgenic Mice. A Ly49A cDNA clone was isolated from the C57BL/6 (B6)-derived thymoma EL-4 using reverse transcriptase and the polymerase chain reaction (RT–PCR) with Ly49A-specific primers containing an EcoRI site (8). Compared with the published Ly49A sequence (2), our cDNA contained two silent base pair changes, and one change that resulted in a substitution at position 106 of Met to Ile. This amino acid substitution, however, was also found in Ly49A cDNAs we isolated from B6 A-LAK cells. The Ly49A cDNA clone was subcloned into the class I promoter expression cassette (15) and injected into fertilized (B6 × CBA/J)F_2 eggs. Transgenic founder mice were identified by Southern blotting with an Ly49A cDNA probe and by staining peripheral blood lymphocytes with FITC-labeled anti-Ly49A mAbs A1 or JR9-318. B6 backcross mice

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were typed for H-2 expression with antibodies specific for H-2D^b (28-14-8s) or H-2K^k (15-5-5, PharMingen, San Diego, CA). The allotype of the NK complex on chromosome 6 was monitored with the B6 allele-specific mAb PK136 (anti-NK1.1). The mice used had been backcrossed two or three times to B6 mice, were homozygous H-2$^{b/b}$, and were homozygous for the B6 NK gene complex.

Flow Cytometry. 5 × 10^5 nylon wool nonadherent spleen cells from individual mice were stained with PE-labeled goat anti-mouse IgG (Southern Biotechnology, Birmingham AL). Free binding sites were saturated with mouse IgG before addition of PE-conjugated anti-CD3 (PharMingen) and biotinylated PK136 (anti-NK1.1). The washed cells were incubated with streptavidin-Tricolor (Caltag Laboratories, South San Francisco, CA) plus FITC-labeled JR9-318 (anti-Ly49A) (4). Lymph node cells were stained with PE-conjugated anti-CD4 (PharMingen) plus FITC labeled anti-CD8 (Caltag). Alternatively, lymph node cells were stained with PE-conjugated anti-CD4, anti-CD8, or anti-Ig followed by FITC-labeled JR9-318. 5 × 10^4 to 10^5 events were analyzed on an EPICS XL (Coulter, Hialeah, FL) flow cytometer. Expression of Ly49A on NK cells was assessed by gating on NK1.1$^+$ CD3$^-$ Ig$^-$ cells. Graphics were generated using the WindMi software (John Trotter, Salk Institute, La Jolla, CA).

Killer Assay. Spleen cells were passed over nylon wool columns and nonadherent cells were cultured in RPMI-1640 (GIBCO BRL, Gaithersburg, MD) containing 250 ng/ml recombinant interleukin-2. LAK cells were harvested at day 3, and incubated in duplicate for 4 h with 1 × 10^4 ^{51}Cr-labeled target cells, either R8.15 tumor cells, which express only the D^b class I molecule, or D^d-transfected R8.15 cells (16).

Mixed Lymphocyte Reactions. Nylon wool nonadherent lymph node cells were enriched for CD4$^+$ T cells by treating the cells with AD4(15) (anti-CD8) ascites fluid and rabbit complement, followed by removal of dead cells over a ficoll gradient. 1 × 10^5 Ly49A-expressing CD4$^+$ T cells (82% pure) or control CD4$^+$ T cells (87% pure) were stimulated with 2 × 10^5 irradiated (2,000 rads) spleen cells from which T cells had been depleted with mAb J1J (anti-Thy 1) and rabbit complement. No cytokines were added. After 4 d, the cultures were pulsed overnight with 0.5 μCi [^{3}H]thymidine/well.

Bone Marrow Graft Rejection. Recipient B10.D2 and 8- to 12-week-old Ly49A transgenic mice or control littermates, backcrossed three times to B6 mice, were irradiated (950 rads from a ^{137}Cs source) and inoculated intravenously with 5 × 10^6 bone marrow cells from B6 or B10.D2 mice, or from β2m$^{-/-}$ B6. Recipient mice were injected intraperitoneally five days later with 3 μCi of ^{125}I-labeled 5-iodo-2′ deoxyuridine (^{125}I-UdR). Isotope incorporation in the recipient spleens was determined 24 h later, after rinsing the spleens with PBS. For NK cell depletion, recipient mice were injected intraperitoneally 24 h prior to irradiation and bone marrow transfer with either 200 μg of mAb PK136 (anti-NK1.1, mouse IgG2a) or the isotype matched control mAb 14-4-4 (anti-MHC class II E^k, mouse IgG2a).

Results and Discussion

A mouse Ly49A cDNA clone was isolated from the C57BL/6 (B6) thymoma EL-4 and inserted into the class I promoter/immunoglobulin enhancer expression cassette (15) to generate transgenic mice. A transgenic (B6 × CBA/J)F$_2$ founder, with a high (>15) transgene copy number, was back-crossed three times to B6 (H-2^b) mice to establish a transgenic line (line No. 2). The transgenic mice and nontransgenic littermates contained a comparable frequency (Fig. 1 *A*) and number (1.0 ± 0.4 × 10^6, n = 6 vs. 0.9 ± 0.3 × 10^6, n = 4, respectively) of NK cells, indicating that transgene expression did not prevent NK cell development. Approximately 20% of NK cells in nontransgenic littermates expressed Ly49A (Fig. 1 *A*), similar to the proportion in B6 mice (3). In contrast, virtually all NK cells (>98.0%) in transgenic mice expressed Ly49A on the cell surface (Fig. 1 *A*). Importantly, the cell surface expression levels of Ly49A on NK cells derived from transgenic mice and nontransgenic littermates were not significantly different, as determined by staining with the anti-Ly49A mAbs JR9-318 (mean fluorescence intensity (MFI) 94 ± 12 vs. 105 ± 7, respectively) or A1 (data not shown).

The Ly49A transgene was expressed not only by NK cells, but also on the surface of CD4$^+$ and CD8$^+$ T cells (Fig. 1 *B*). The peripheral (Fig. 1 *B*) and thymic (data not shown) T cell compartments appeared normal, suggesting that the transgene did not alter T cell development in H-2^b mice. Furthermore, transgene expression was detected on TCRγδ$^+$ and NK1.1$^+$ T cells (data not shown) and at variable levels on B cells (Fig. 1 *B*). Transgenic macrophages expressed little or no Ly49A on the cell surface (data not shown). These results extend a previous report that non-NK1.1$^+$ cells can support cell surface expression of Ly49 receptors (6, 13).

The specificity and function of the Ly49A transgene in NK cells was established by its ability to confer H-2D^d–

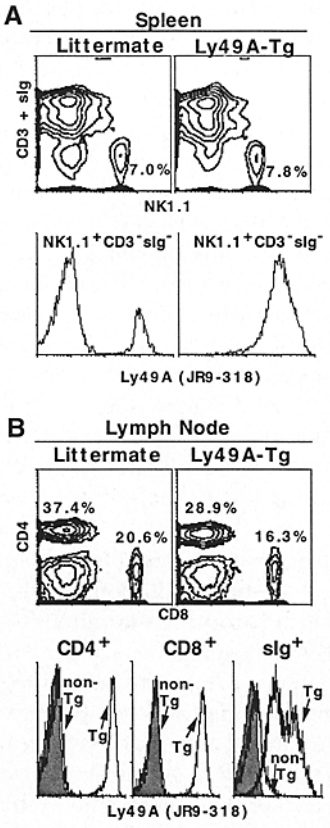

Figure 1. Analysis of Ly49A transgenic mice. (*A*) Nylon wool nonadherent spleen cells from Ly49A transgenic mice and nontransgenic littermates contained a similar frequency of NK cells. Ly49A expression defines a subset of ~20% of NK cells in nontransgenic littermates, whereas virtually all transgenic NK cells expressed Ly49A. (*B*) Comparable frequencies of CD4$^+$ and CD8$^+$ T cells were observed in lymph nodes from transgenic mice and nontransgenic littermates. Compared with unstained lymph node cells (*filled histograms*), nontransgenic CD4$^+$ and CD8$^+$ T cells did not express Ly49A, whereas all CD4$^+$ and CD8$^+$ T cells from transgenic mice expressed Ly49A. Ly49A levels on surface immunoglobulin-positive (sIg$^+$) B cells were variable.

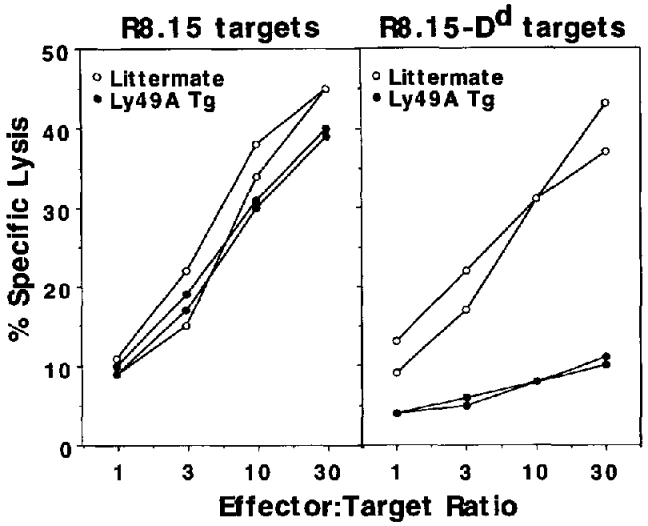

Figure 2. Ly49A transgene expression confers MHC class I D^d-specific inhibition to unfractionated NK cells. The results of two independent experiments are shown. Interleukin-2 activated killer cells from Ly49A transgenic and nontransgenic littermates efficiently lysed the NK cell–sensitive tumor cell line R8.15, which expresses only the D^b class I molecule. R8.15 cells transfected with the D^d class I molecule were resistant to lysis by killer cells from Ly49A-transgenic mice but were lysed by killer cells from nontransgenic littermates.

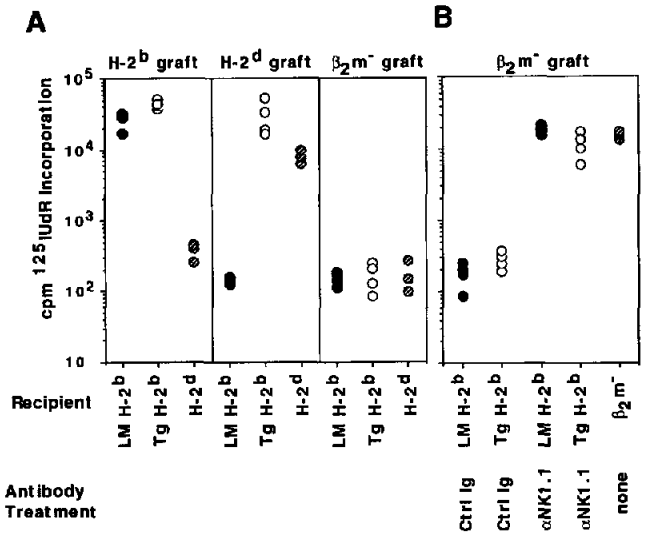

Figure 3. Ly49A transgenic H-2^b mice are unable to reject an H-2^d bone marrow allograft. (*A*) Rejection of bone marrow grafts by irradiated mice was assayed with the splenic ^{125}I-UdR incorporation assay, in which low levels of isotope incorporation reflect rejection of the graft, and high levels of incorporation reflect graft acceptance. Each symbol represents an individual recipient animal. Four recipient mice were used per experimental group in most cases. The transgenic mice, like the control mice, accepted H-2–identical (*H-2^b*) grafts. In contrast with nontransgenic littermates, Ly49A-transgenic H-2^b mice were unable to reject H-2^d bone marrow grafts. This was not due to a general inability to reject bone marrow cells, since grafts derived from β2m-deficient mice were readily rejected. (*B*) Bone marrow graft rejection in Ly49A transgenic mice is mediated by NK1.1$^+$ cells since depletion of NK1.1$^+$ cells results in the inability to reject grafts derived from β2m-deficient mice.

specific inhibition to unfractionated interleukin-2–activated NK cells. Whereas NK cells from Ly49A-transgenic mice (H-2^b) and control nontransgenic littermates (H-2^b) efficiently lysed the tumor target cell R8.15, the lytic activity of transgenic NK cells was specifically inhibited by D^d expression as shown by their failure to lyse D^d-transfected R8.15 cells (Fig. 2). Similar results were obtained with freshly isolated, poly I/C-activated NK cell preparations (data not shown). These results represent direct evidence that Ly49 molecules are sufficient to confer class I–specific inhibition of target cell lysis to NK cells. A previous study employing viral-mediated gene transduction reached a similar conclusion with respect to the structurally unrelated human KIR gene family (17).

Previous studies have demonstrated that NK cells mediate the rejection by irradiated mice of allogeneic or class I–deficient bone marrow cells, and of parental-type marrow grafts by F_1 hybrids of H-2 disparate mouse strains (18). To establish the role of Ly49 receptors in NK cell specificity and function in vivo, we determined the effects of Ly49A transgene expression on the capacity of irradiated H-2^b mice to reject an allogeneic H-2^d bone marrow graft, a reaction that is mediated by NK cells (19). Marrow engraftment was monitored by the incorporation of 5-[^{125}I]iodo-2′-deoxyuridine in spleen cells, indicating donor cell proliferation (18). Whereas nontransgenic H-2^b mice rejected the allogeneic H-2^d (B10.D2) bone marrow grafts, Ly49A-transgenic H-2^b mice completely accepted these grafts, as demonstrated by the high level of isotope incorporation in their spleens (Fig. 3 *A*). As expected, both Ly49A transgenic and nontransgenic mice accepted H-2–identical B6 (H-2^b) bone marrow grafts. The Ly49A-transgenic mice retained the capacity to reject bone marrow grafts from class I–deficient (i.e., β2m$^-$) mice (20) (Fig. 3 *A*), a reaction that was prevented by pretreating the mice with anti-NK1.1 antibody, but not with irrelevant (anti-MHC class II I-E) antibody (Fig. 3 *B*). Hence, the transgene specifically prevented rejection of H-2^d bone marrow grafts, and did not affect NK-mediated rejection of β2m$^-$ bone marrow grafts. This experiment demonstrates that the capacity to reject H-2^d grafts is prevented when Ly49A is expressed on all NK cells, as opposed to on a subset of NK cells as observed in normal mice. Therefore, a significant conclusion of our results is that the alloreactivity observed in the NK cell compartment is due to the failure of many NK cells to express a given class I–specific inhibitory receptor, i.e., to the fact that Ly49 receptor expression is normally restricted to NK cell subsets.

The demonstration that the Ly49A transgene prevents rejection of H-2^d bone marrow cell grafts provides direct evidence of a role for Ly49 receptors in vivo. A role for Ly49 receptors in bone marrow cell rejection was previously inferred from in vitro cytolysis experiments that mirror the specificity of bone marrow cell rejection (9). Some caution is warranted in equating the cytolysis experiments with bone marrow cell rejection; however, when it is considered that marrow rejection remains largely intact in mice

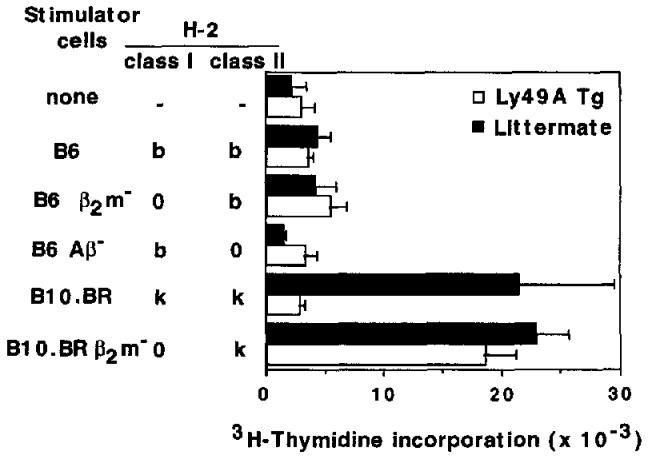

Figure 4. Ly49A transgene expression in $CD4^+$ T cells prevents the allogeneic mixed lymphocyte reaction. $CD4^+$ responder T cells from B6 backcross ($H\text{-}2^b$) Ly49A transgenic mice proliferated in response to β2m-deficient B10.BR stimulator spleen cells. In contrast, the response to $\beta 2m^+$ B10.BR stimulators, which express the D^k Ly49A ligand, was strongly inhibited. $CD4^-$ T cells from nontransgenic littermates proliferated in response to β2m as well as $\beta 2m^+$ B10.BR stimulator cell populations. Data represent mean values ± standard deviation of quadruplicate determinations based on two experiments.

that lack perforin- or Fas-mediated cytotoxic function (21). Furthermore, the possibility that MHC recognition by NK cells can in some cases induce, rather than inhibit bone marrow rejection is not reflected by the in vitro assays (22, 23). A role for Ly49 receptors in bone marrow cell rejection was also previously suggested by correlative evidence that $Ly49C^+$ NK cells are essential for rejection of $H\text{-}2^d$ bone marrow grafts by $H\text{-}2^b$ mice (24). Since cells expressing Ly49C are present in normal numbers in Ly49A transgenic mice (data not shown), a likely explanation of our results is that Ly49A transgene expression confers D^d-specific inhibition to all $Ly49C^+$ NK cells, thus preventing these cells from rejecting the $H\text{-}2^d$ bone marrow cell allograft.

To address whether Ly49 receptors can confer class I–specific inhibition to lymphocytes other than NK cells, we addressed the effects of Ly49A transgene expression on the proliferative response of conventional $CD4^+$ T cells, most of which are normally $Ly49A^-$ (Fig. 1 *B*), to allogeneic class II molecules. $CD4^+$ T cell–enriched responder cells from transgenic and nontransgenic mice responded equivalently to stimulation with β_2m-deficient B10.BR spleen cells (Fig. 4). These stimulator cells present the allogeneic A^k and E^k class II molecules, but do not express class I molecules. Stimulator cells from class I^+ B10.BR mice, which also express A^k and E^k but express in addition the Ly49A-specific D^k class I molecule (11), elicited a good response from nontransgenic $CD4^+$ T cells, but the response of the Ly49A-transgenic $CD4^+$ T cells was completely inhibited. Thus, the Ly49A–class I interaction can even inhibit T cell receptor–mediated responses to allogeneic class II molecules. Similar results were obtained when we tested the $CD8^+$ T cell proliferative response to allogeneic MHC molecules (data not shown).

The finding that Ly49A functions in T cells indicates that no additional NK cell–specific molecules are required for Ly49 receptor activity, and suggests that the relevant inhibitory signaling pathways are conserved in NK and T cells and perhaps all lymphoid cells. In line with this observation, previous studies in the human system demonstrated KIR-mediated, class I–specific inhibition of cytolysis by certain T cell clones (25). The recent implication of the tyrosine phosphatases PTP1C and/or PTP1D in the down-modulation or prevention of immune responses by B cells, T cells, and NK cells lends further support for this notion (26–28). With regard to Ly49A, the retention of the signaling pathway in T cells is of particular interest because a small fraction of T cells express Ly49 receptors on their surface (4, 5). Therefore, besides regulating NK cell function, Ly49A–class I interactions may be important in normal mice for the regulation of some T cells (25).

In conclusion, we have demonstrated that the Ly49A molecule is the only NK cell–specific receptor required to confer inhibitory class I specificity to NK cells and T cells, and most significantly, that these receptors regulate NK cell function in vivo. The results suggest that Ly49 receptors regulate multiple cellular effector functions, and do not represent accessory receptors for class I recognition. Furthermore, the data indicate that the expression of Ly49 receptors by NK cell subsets in normal mice is essential for the discrimination of class I–different cells. This property is probably necessary to allow NK cells to attack variant cells in which the expression of some but not all host class I molecules are repressed due to infection, mutation, or transformation (29).

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