NK and CTL Recognition of a Single Chain H-2Dd Molecule: Distinct Sites of H-2Dd Interact with NK and TCR

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*J Immunol* 1999; 163:3699-3708; 
http://www.jimmunol.org/content/163/7/3699

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NK and CTL Recognition of a Single Chain H-2D\textsuperscript{d} Molecule: Distinct Sites of H-2D\textsuperscript{d} Interact with NK and TCR

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We generated transgenic mice expressing a single-chain $\beta_2$-microglobulin ($\beta_2$m)-H-2D\textsuperscript{d}. The cell-surface $\beta_2$m-H-2D\textsuperscript{d} molecule was expressed on a $\beta_2$m-deficient background and reacted with appropriate mAbs. It was of the expected m.w. and directed the normal development of CD8$^+$ T cells in the thymus of a broad TCR repertoire. It also presented both exogenously provided and endogenous peptide Ags to effector CD8$^+$ T cells. In tests of NK cell education and function, it failed to reveal any interaction with NK cells, suggesting that the site of the interaction of NK receptors with H-2D\textsuperscript{d} was disrupted. Thus, the sites of TCR and NK receptor interaction with H-2D\textsuperscript{d} are distinct, an observation consistent with independent modes of TCR and NK receptor evolution and function.


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β₃m⁻⁻ mice, of a Sc H-2D⁺ (ScD⁺) molecule and examine its recognition in both education and effector phases by T and NK cells. These results lead to conclusions about the nature of the site of TCR and NKR interaction with H-2D⁺.

Materials and Methods

Expression of Sc H-2D⁺ in transgenic mice

To obtain a chimera gene encoding a Sc (β₃m-spacer-MHC-I H chain) H-2D⁺, in a form suitable for expression as a transgene, three different fragments from two plasmids were used: 1) a 5' fragment (XbaI-BamHI) from the pD⁺-1 plasmid (40, 41) containing promoter sequences from −400 bp upstream of the initiation ATG codon, modified in its BamHI site with Klenow polymerase and dGTP and dATP; 2) a 3' FspI fragment derived from a cDNA for a Sc H-2D⁺ protein that codes for β₃m, a peptide linker, and the mature H-2D⁺ H chain protein through to the end of the α₂ domain (encoded by exon 3), which had been modified at the SalI site with Klenow polymerase and dTTP and dCTP (32); and 3) an FspI-EcoRI fragment (which extends from the end of exon 3 to the 3' untranslated region) from pD⁺-1. These were ligated in two sequential steps into the pBluescript-IIKS (+) vector.

The DNA construct encoding the Sc H-2D⁺ molecule was injected into fertilized eggs of homozygous C57BL/6NCr mice to generate transgenic mice. Two transgenic founder mice were backcrossed to B6 mice that contained an induced defect in the expression of β₃m (C57BL/6Giphac-Ko)B2m N5, β₃m knockout (β₃m⁻⁻) mice (3), maintained and bred at Taconic, Germantown, NY) to generate transgenic mice expressing cell-surface Sc H-2D⁺ in the absence of other β₃m-dependent molecules. (The formal names for these transgenic strains are C57BL/6N/CrSc(β2m⁻⁻)1 and ScD⁺-2, β₃m⁻⁻ and -2, respectively, to refer to them as ScD⁺-1 and ScD⁺-2, respectively. When bred onto the H-2K bm3 mutations of H-2K b mice, were purchased from The Jackson Laboratory (Bar Harbor, ME) and are referred to here as B6.bm1 and B6.C-B2m⁻⁻ (B6.Wy) and C57BL6/J-By and C57BL6/J-H2bm1 mice (9) were obtained from The Jackson Laboratory (Lincoln Park, NJ) at 2 × 10⁶ cells/ml with 2 μg/ml Con A (Sigma). One day before injection, mice were injected i.p. with 2 μg/ml Con A (Sigma). On the day of assay, one-tenth volume of 1 M methyl-α-D-mannopyranoside (Sigma) in RPMI 1640 or PBS was added to target cells to block Con A sites before labeling for 1–2 h in 100 μl of 10 mM iodoacetamide, 1 mM sodium orthovanadate, and Complete protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN). Nuclei were removed by centrifugation, and lysates from 5 × 10⁷ cells were precleared with protein G-Sepharose and then subjected to immunoprecipitation with 10 μg of 34-2-12 and 150 μg of a 10% slurry of protein G-Sepharose beads (Pharmacia Biotech, Upplands, Sweden). Beads were washed and boiled in 1% SDS and 10 mM iodoacetamide for 5 min, and eluted proteins were then separated on a 14% SDS-polyacrylamide gel and transferred to an Immobilon P membrane (Millipore, Bedford, MA). Biotinylated proteins were visualized with streptavidin-HRP (Zymed, South San Francisco, CA) and enhanced chemiluminescence (Amersham, Arlington Heights, IL). Immunization with vaccinia virus and CTL Assay

ScD⁺ β₃m⁻⁻ and D8 mice were immunized by i.p. injection of 2 × 10⁷ pfu of vaccinia virus expressing HIV-1 envelope glycoprotein gp160IBB (vPE16, the gift of P. Earl and B. Moss) (44). Three weeks later, splenocytes were cultured at 5 × 10⁶/ml in 24-well culture plates in complete T-cell medium (RPMI 1640 containing 10% FBS, 2 mM l-glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml), and 50 μg/ml gentamicin. Four days later, the cultures were supplemented with one-tenth volume of T-Stim (Collaborative Biomedical Products, Bedford, MA) as a source of IL-2. Splenocytes were cultured in vitro for 7 days with 1 μM P815BB H10 (RPGGRAFVY) peptide together with 4 × 10⁶ irradiated (3300 rad) syngeneic spleen cells as APC. Cytolytic activity of CTL lines was measured by a standard 4-h ³²Cr-release assay (45). SEs of the mean of triplicate cultures were all <5% of the mean. Generation of MLR and NK cell effector population

Primary mixed lymphocyte cultures were established essentially as described previously (46). Briefly, 2.5 × 10⁴ responder splenocytes and 2.5 × 10⁵ irradiated stimulator cells (3000 rad from a 137Cs source) were cultured together in 20 ml of complete T media with 5% FCS in upright T-25 flasks (Corning Glass Works, Corning, NY) for 5 days. Fresh NK effector cells were depleted of RBC, and nylon wool nonadherent splenocytes were passed over nylon wool columns to remove T cells. Before use, 150 μg poly I:C (Sigma, St. Louis, MO), an NK stimulator. Four-day lymphokine-activated killer (LAK) NK effector cells were prepared by a procedure based upon that of Chadwick and Miller (47). Briefly, splenocytes from cultures of RBC by hypotonic lysis and passed over nylon wool. Nylon wool nonadherent cells were cultured for 4 days in RPMI 1640 plus 10% FCS, supplements (including 50 μg/ml-2-ME, and 400 ng/ml recombinant human IL-2 (Chiron, Emeryville, CA). Adherent LAK (A-LAK) cells were prepared as described (15).

Lysis assays for NK cells and allogeneic CTL (MLR)

For target cell preparation, splenocytes were cultured in complete T medium containing 5% FCS for 24–24 h in 24-well plates (Falcon Plastics, Lincoln Park, NJ) at 2 × 10⁶ cells/ml with Con A (Sigma). On the day of assay, one-tenth volume of 1 M methyl-α-D-mannopyranoside (Sigma) in RPMI 1640 or PBS was added to target cells to block Con A sites before labeling for 1–2 h in 100 μl of 10 mM iodoacetamide, 1 mM sodium orthovanadate, and Complete protease (Biofluids, Rockville, MD), passed over nylon wool columns to remove T cells, and stained with PE-conjugated anti-NK1.1 and FITC-conjugated anti-Ly49A, C1, or G2 mAbs.

Cell-surface biotinylation and immunoprecipitation

Cells were surface-biotinylated using NHS-LC-biotin (Pierce, Rockford, IL) as previously described (43). The cells were then solubilized with 1% Nonidet P-40 in 10 mM Tris·HCl, pH 7.2, 140 mM NaCl, 1 mM PMSF, 5 mM iodoacetamide, 1 mM sodium orthovanadate, and Complete protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN). Nuclear proteins were then separated on a 14% SDS-polyacrylamide gel and transferred to an Immobilon P membrane (Millipore, Bedford, MA). Biotinylated proteins were visualized with streptavidin-HRP (Zymed, South San Francisco, CA) and enhanced chemiluminescence (Amersham, Chicago, IL).
Lymph node cells from ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\) mice were incubated with biotinylated 28-14-8 (anti-\(\alpha_1\) of H-2D\textsuperscript{d}) and stained with PE-streptavidin. In contrast, mice homozygous for the ScD\textsuperscript{d} transgene, Spleen cells from ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\), BALB/c, and B6 mice were stained with FITC-conjugated-mAbs against H-2D\textsuperscript{d}, D\textsuperscript{b}, and K\textsuperscript{b} molecules and PE-conjugated-CD4 mAb. Analysis for the expression of MHC-I molecules was performed on the gated CD4-positive cells. Lymph node cells from C3H/HeJ, ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\)/C\textsuperscript{57}Bl/6, B6, and SCID mice were stained for flow cytometry analysis using the biotinylated Ly49A and biotinylated proteins were visualized with strepavidin-HRP and enhanced chemiluminescent reaction; lane 1, BALB/c; lane 2, ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\); lane 3, B6. Spleen cells from ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\), B6, and BALB/c mice were surface-biotinylated and lysed. The lysates were precipitated with 34-2-12 (anti-\(\alpha_1\) of H-2D\textsuperscript{d}) and protein-G Sepharose beads, washed, separated on 14\% SDS polyacrylamide gel, and transferred to Immobilon P membrane. The biotinylated proteins were visualized with strepavidin-HRP and enhanced chemiluminescent reaction; lane 1, BALB/c; lane 2, ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\); lane 3, B6.

Biotinylation of Ly49A protein

The extracellular portion of Ly49A (amino acids 67 to 262) was expressed in bacteria as inclusion bodies, solubilized, refolded, and purified as described in detail elsewhere.\textsuperscript{5} The purified protein was chemically biotinylated with NHS-LC-biotin (Pierce) and further purified. Lymph node cells were stained for flow cytometry analysis using the biotinylated Ly49A and PE-streptavidin.

Results

Expression of transgenic and native MHC-I in ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\) knockout mice

To explore the expression of the ScD\textsuperscript{d} and other MHC-I molecules in \(\beta_m^{m^{-/-}}\) transgenic mice, lymph node cells from ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\), BALB/c, and B6 mice were stained with mAb against H-2D\textsuperscript{d}, H-2D\textsuperscript{b}, and H-2K\textsuperscript{b}. As expected, lymph node cells of the \(\beta_m^{m^{-/-}}\) transgenic mice expressed no detectable H-2K\textsuperscript{b} or H-2D\textsuperscript{b} above the level observed in \(\beta_m^{m^{-/-}}\) nontransgenic cells (Fig. 1A). In contrast, mice homozygous for the ScD\textsuperscript{d} transgene, expressed in the \(\beta_m^{m^{-/-}}\) background, revealed apparently normal levels of H-2D\textsuperscript{b} epitopes as indicated by reactivity with Abs 34-2-12 (\(\alpha_3\) domain), and 3-25-4 (\(\alpha_1\alpha_3\) domain) (Fig. 1A) as well as with 34-5-8 (\(\alpha_1\alpha_2\) domain) and 34-4-20 (data not shown).

Although \(\beta_m^{m^{-/-}}\) cell lines and mice are profoundly deficient in expression of MHC-I molecules, it is well known that low levels of H-2D\textsuperscript{b} are detectable by flow cytometry or by sensitivity to lysis by H-2D\textsuperscript{b}-specific alloreactive CTL (48–50). As shown in Fig. 1B, we detected low levels of H-2D\textsuperscript{b} in both \(\beta_m^{m^{-/-}}\) and in ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\) mice expressing the ScD\textsuperscript{d} transgene. Presence of the ScD\textsuperscript{d} transgene did not perturb the expression of H-2D\textsuperscript{b} in \(\beta_m^{m^{-/-}}\) mice (Fig. 1B).

With biochemical techniques we examined the Sc molecules made in these transgenics. Although we expected that the Sc H-2D\textsuperscript{d} molecules expressed at the cell surface were the basis of the serological reactivity, it was possible that the Sc molecules were proteolytically cleaved in the spacer region, leading to cell surface H-2D\textsuperscript{b}/\(\beta_m^{m^{-/-}}\) heterodimers. H-2D\textsuperscript{b} molecules expressed on the surface of cells of the transgenic mice were exclusively detected as molecules with a molecular mass of 60–65 kDa as determined by immunoprecipitation from splenocytes using 34-2-12 mAb (anti-\(\alpha_3\) domain of H-2D\textsuperscript{b}) (Fig. 1C). In contrast, the normal MHC-I H-2D\textsuperscript{d} H chain on the spleen cells from BALB/c was detected as a 50-kDa protein. Thus, the H chain of transgenic ScD\textsuperscript{d} is expressed as a molecular species that is covalently linked with \(\beta_m\) on the cell surface, and there is no evidence for proteolytic cleavage of the Sc molecule. (Because the 34-2-12 Ab used for the immunoprecipitation from splenocytes used 34-2-12 mAb (anti-\(\alpha_3\) domain) (51), this Ab would have detected membrane molecules truncated in the \(\beta_m\) spacer, \(\alpha_1\), or \(\alpha_2\) regions.)

ScD\textsuperscript{d} educates a diverse repertoire of CD8\textsuperscript{+} T cells, similar to that induced by native H-2D\textsuperscript{d}

It is well known that normal expression of MHC-I molecules in the thymus is necessary for the normal maturation of CD8\textsuperscript{+} T cells, that is, the progression of immature CD4\textsuperscript{+}CD8\textsuperscript{+} cells to mature single positive cells. Animals defective in \(\beta_m\) expression, and as a result lacking normal cell-surface MHC-I expression, show a profound decrease in the number of CD8\textsuperscript{+} (single positive) cells in the thymus as well as in lymph node and spleen (3, 4). In addition, animals defective in TAP expression, and thus deficient in the delivery of self-peptides to MHC-I, show similarly abnormal CD8\textsuperscript{+} T cell development (52). To assess the selection of CD8\textsuperscript{+} T cells by ScD\textsuperscript{d} in the absence of normal expression of other MHC-I molecules, we analyzed thymocytes, lymph node cells, and splenocytes from B6, B6 \(\beta_m^{m^{-/-}}\), and ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\) mice for the presence of mature CD8\textsuperscript{+} T cells by flow cytometry (Fig. 2). Unlike nontransgenic \(\beta_m^{m^{-/-}}\) thymocytes, those from ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\) mice contained mature CD8\textsuperscript{+} cells in numbers similar to those seen in B6 thymuses (Fig. 2). As reported by others, normal B6 animals showed a significant proportion of CD4 and CD8 single positive cells in the thymus (10.65 and 1.70\%, respectively), lymph node (62.39 and 36.32\% of TCR C\textsuperscript{d} \(\beta_m^{m^{-/-}}\) cells), and spleen (65.39 and 32.29\% of TCR C\textsuperscript{b} \(\beta_m^{m^{-/-}}\) cells). In contrast, thymocytes from \(\beta_m^{m^{-/-}}\) animals showed a profound decrease in the proportion of CD8 single positive cells in each tissue (0.01, 0.21, and 0.14\% in thymus, lymph node, and spleen, respectively) and a compensatory increase in the proportion of CD4 single positive cells in the peripheral lymph nodes and spleen. There was no apparent change in total number of CD4\textsuperscript{+} cells. Despite the lack of proper expression of MHC-I molecules other than the ScD\textsuperscript{d} in the transgenic \(\beta_m^{m^{-/-}}\) animals, substantial numbers of CD8\textsuperscript{+} T cells were detected in the thymus, lymph nodes, and spleen of ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\) mice (1.70, 33.69, and 31.63\% of total, respectively), indicating their normal migration to peripheral lymphoid organs (Fig. 2).

We considered the possibility that relatively few CD8\textsuperscript{+} clones might mature in ScD\textsuperscript{d} \(\beta_m^{m^{-/-}}\) mice and expand to fill the available space. If such were the case, the diversity of the expressed...
TCR would be expected to be low. We examined the Vα and Vβ TCR repertoires of CD8^+ T cells using available mAbs. CD8^+ T cells in lymph nodes from ScD^d β_m~m~ mice exhibited a diverse repertoire of TCR, indicating that these CD8^+ T cells do not represent expansion of a small, oligoclonal population (Fig. 3A). Quantitative differences in the level of expression of different TCR V regions might reveal subtle differences in the TCR repertoire selected by the ScD^d on the β_m~m~ background as compared with those repertoires selected by either B6 or B10.D2.

We also wished to explore the extent of overlap in the TCR repertoires selected by the ScD^d molecules and normal H-2D^d. To do this, we stimulated D8 (B6, native H-2D^d transgenic) splenocytes with ScD^d B6 (B6, ScD^d transgenic) splenocytes and vice versa in MLR (Fig. 3B). T cells that develop in an environment in which their TCR are selected by the ScD^d molecule but retain discriminating activity against the native two-chain H-2D^d molecule would be expected to be present and be stimulated in the ScD^d anti-D8 MLR and should be capable of killing D8 targets. Similarly, T cells expressing TCR that are selected on H-2D^d yet remain reactive against unique epitopes of ScD^d would be expected to be stimulated in the D8 anti-ScD^d B6 MLR and show cytolysis against ScD^d B6 targets. A low level of lysis of target cells of the stimulator mouse strain indicates that few such cells are present and/or they are not particularly reactive. CTL stimulated in both the D8 anti-ScD^d B6 MLR and the ScD^d B6 anti-D8 MLR exhibited very low cytolytic activity against target cells of the stimulator type (Fig. 3B). This was not due to a general inability of these mice to respond to any MHC-I difference, as B6.bm3 stimulators elicited substantial activity from the same responders against H-2 bm3 target cells (Fig. 3B). H-2bm3 stimulators also elicited substantial activity from the same responders (data not shown). This also was not due to the inability of H-2D^d or ScD^d proteins to stimulate responses generally, as B6 cells transgenic for either the native two chain or the ScD^d elicited substantial CTL activity from nontransgenic B6 splenocytes (Fig. 3C). Furthermore, the anti-H-2D^d alloreactive CTL raised against either the Sc or the native molecule do not effectively distinguish native H-2D^d from the Sc. Taken together, the serological and functional data indicate that ScD^d participates in positive selection of CD8^+ T cells with a broad repertoire and that this repertoire is functionally similar to that induced by the native H-2D^d.

ScH-2D^d mice can induce a virus-specific H-2D^d-restricted CD8^+ CTL response

The generation of a diverse repertoire of CD8^+ T cells indicated that the ScD^d molecule was effective in the presentation of self-Ag (presumably as peptides) to developing thymic cells. In addition, the ability of cells expressing ScD^d to elicit allospecific CTL from B6 T cells indicated that the molecule is effective in Ag presentation to mature T cells. We also wished to evaluate the ability of the ScD^d molecules to effectively participate in an immune response by presenting foreign Ags, such as those generated by a viral infection, to specific CD8^+ T cells. ScD^d β_m~m~ and D8 mice were infected with recombinant vaccinia virus directing the expression of the HIV-1 gp160 envelope glycoprotein (vPE16). Three weeks following infection, spleen cells were restimulated in vitro by syngeneic spleen cells loaded with gp160-derived H-2D^d-restricted peptide, P18-I10 (37), and then were tested for effector function in a 51Cr-release assay. CTL induced in ScD^d β_m~m~ mice could kill P18-I10-loaded P815 cells expressing wild-type H-2D^d on the cell surface, whereas these cells could not lyse P815 target cells that had not received the sensitizing peptide (Fig. 3D). ScD^d β_m~m~ mice developed a P18-I10 specific, H-2D^d-restricted CD8^+ CTL response that was comparable to that of D8 mice in extent of cytolysis. Furthermore, these cells were able to recognize peptide-loaded native H-2D^d efficiently. Such CD8^+ CTL had been generated in vivo with the peptide produced endogenously from the full-length gp160, and these T cells had not been exposed to native H-2D^d-expressing APC before the cytolytic assay. These findings clearly demonstrate that CD8^+ T cells selected by peptide in ScD^d β_m~m~ mice are capable of mounting an effective peptide-specific anti-viral cytotoxic response. Most importantly, CTL induced by ScD^d expressed on APC of the transgenic mice interact effectively with native H-2D^d/P18-I10 complexes, that is, they are unable to distinguish native H-2D^d from ScD^d.
ScDd is defective in the education of NK cells

Native H-2Dd interacts with some of the Ly49 receptors on NK cells, in particular Ly49A (15) and Ly49G2 (16), and plays a critical role in the function and development of NK cells as well as CD8+ T cells (14). To explore the effects of the ScDd transgene in the development of NK cells, we tested whether the presence of ScDd could alter NK cell development in transgenic mice. Expression of native H-2Dd is sufficient to alter the NK cell specificity: H-2Dd, when expressed as a transgene in B6 mice, confers upon the NK cells in these mice the ability to reject B6 bone marrow grafts in vivo and the ability to lyse B6 target cells in vitro (53, 54). Additionally, expression of MHC-I molecules in normal mice is sufficient to permit development of NK cells capable of rejecting β2m-/- bone marrow in vivo and killing β2m-/- target cells in vitro (2, 55).

Surprisingly, we found that expression of the ScDd transgene in β2m-/- mice did not confer any similar function upon the NK cells in these mice. Poly I:C-stimulated NK cells from ScDd β2m-/- mice could not kill β2m-/- Con A blasts as measured in cytotoxicity assays (Fig. 4A), and ScDd β2m-/- mice do not reject bone marrow cells from β2m-/- (B), nor do ScDd B6 animals reject grafts from B6 (C). Each group of recipients contains five or six mice in the bone marrow transplantation. The results in B and C each show two independent bone marrow transplantation experiments.

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FIGURE 3. Sc H-2Dd functions normally in thymic selection and in allo- and Ag-specific recognition. A, CD8+ T cells were gated and analyzed for expression of various Vα and Vβ segments of TCRs by flow cytometry. The percentage of CD8+ T cells expressing TCRs were determined in three mice analyzed independently and the mean and SE are plotted. 

B and C. The indicated cells from primary mixed lymphocyte cultures were used for a cytolyis assay using Con A-activated spleen cells from the indicated strains as targets. In B, triangles represent effector cells of ScDd B6 raised against bm3, and circles are for D8 effector cells raised against bm3. Filled circles and triangles are for bm3 targets, and open circles and triangles are for self-targets.

D, ScDd β2m-/- (circles) and D8 (squares) mice were immunized by i.p. injection with vaccinia virus expressing gp160IIIB (vPE16) as described in Materials and Methods. Three weeks later, spleen cells from immunized mice were stimulated in vitro with P18-I10 peptide-pulsed irradiated syngeneic spleen cells for 7 days as described. Cytolytic activity of CTL was measured in a 4-h assay using 51Cr-labeled P815 target cells pulsed with P18-I10 (solid markers) for 2 h or without peptide (open markers).

FIGURE 4. ScDd is defective in promoting the education of NK cells. Fresh poly I:C-stimulated NK cells from B6, ScDd β2m-/-, and β2m-/- mice were generated as described in Materials and Methods and tested on target Con A-activated lymphoblasts from the indicated strains A. Bone marrow grafts were performed in the indicated donor → host combinations as described in Materials and Methods (B and C). ScDd β2m-/- mice do not reject bone marrow cells from β2m-/- (B), nor do ScDd B6 animals reject grafts from B6 (C). Each group of recipients contains five or six mice in the bone marrow transplantation. The results in B and C each show two independent bone marrow transplantation experiments.

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on NK cells from ScD\(^d\) \(\beta_m^{-/-}\) mice were indistinguishable from those of NK cells from \(\beta_m^{-/-}\) mice (Table I). This contrasts with the surface expression of NKR of D8 mice that have significantly fewer Ly49A- and Ly49G2-expressing NK cells. These cells also exhibit a lower cell surface density of Ly49A when compared with B6 animals. Similarly, ScD\(^d\) B6 mice are not significantly different from the B6 parental line. These results further support the conclusion that NK cells of ScD\(^d\) \(\beta_m^{-/-}\) and of ScD\(^d\) B6 mice could not be properly educated by ScD\(^d\) in vivo during their development.

NK cells might require interactions with other molecules whose expression is dependent on \(\beta_m^{-/-}\), such as MHC-Ib molecules, to be properly educated and active in killing target cells. It is also possible that only a single MHC-I molecule expressed at proper levels is not sufficient to drive NK development. To investigate these possibilities, we employed ScD\(^d\) B6 mice. These mice are suited to test the above possibilities because they express normal levels of \(\beta_m\) and thus normal levels of all MHC-Ia and MHC-Ib molecules of B6 mice. NK cells from B6 mice transgenic for native H-2D\(^d\) (D8) reject B6 bone marrow in vivo and lyse B6 target cells in vitro (53). In addition, cells from D8 mice could kill both B6 and ScD\(^d\) B6 ConA blasts in the cytotoxicity assay (Fig. 5A). Furthermore, ScD\(^d\) B6 hosts could not reject B6 bone marrow grafts (Fig. 4C). This result is particularly striking because animals expressing a native H-2D\(^d\) transgene are capable of rejecting B6 bone marrow grafts. Thus, the ScD\(^d\) transgene is unable to function like native H-2D\(^d\) in the education of NK cells.

**Sc H-2D\(^d\) cannot deliver inhibitory or stimulatory signals for NK cell-mediated lysis**

The MHC-I-dependent resistance to NK lysis is mediated through inhibitory surface NKR that engage target cell MHC-I (57). The native H-2D\(^d\) molecule interacts with at least Ly49A and Ly49G2 receptors on NK cells and thus is capable of delivering inhibitory signals to NK cells, preventing or reducing lysis (15, 16, 28, 29). We wished to understand whether the expression of the ScD\(^d\) transgene-encoded molecules on the target cells resulted in the delivery of inhibitory signals to NK effector cells. In in vitro cytotoxicity assays, short-term cultured NK cells derived from B10.D2 (data not shown) and D8 mice killed ScD\(^d\) B6 and nontransgenic B6 target cells equivalently (Fig. 5A) and also lysed ScD\(^d\) \(\beta_m^{-/-}\) target cells and \(\beta_m^{-/-}\) target cells equivalently. Expression of ScD\(^d\) on donor bone marrow cells used in grafts was insufficient to prevent rejection of either \(\beta_m^{-/-}\) (data not shown) or B6 bone marrow by B10.D2 hosts (Fig. 5B). Although it is unclear whether expression of only native H-2D\(^d\) on \(\beta_m^{-/-}\) bone marrow grafts would be sufficient to prevent rejection by B10.D2 mice, expression of native H-2D\(^d\) as a transgene was sufficient to prevent rejection of B6 marrow by B10.D2 mice (Fig. 5B), as previously shown by Öhlén and colleagues (53). Taken together, these data indicate that the ScD\(^d\) transgene, in contrast to the H-2D\(^d\) transgene of D8 animals, has little or no function with respect to NK cell inhibition.

The rejection of D8 bone marrow by B6 is dependent on an NK1.1\(^+\) cell population, and is believed to be due to engagement of H-2D\(^d\)-specific activation receptors on B6 NK cells (53). Although the mechanism of this phenomenon is not completely clear, it may involve recognition by stimulatory receptors that lack cytoplasmic immunoreceptor tyrosine-based inhibitory motifs, such as Ly49D and Ly49H (58, 59). Here we show that B6 mice are unable to reject ScD\(^d\) B6 bone marrow grafts and confirm the ability of B6 mice to reject D8 grafts (Fig. 5C). (Because the rejection of bone marrow grafts in this experimental system is due to NK and not CTL activity, allospecific rejection is not observed.)

### Table I. Cell-surface expression of NKR in transgenic and knockout mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Ly49A (A1) Mean fluorescence intensity (MFI)</th>
<th>Ly49C/I (5E6) MFI</th>
<th>Ly49G2 (4D11) MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta_m^{-/-})</td>
<td>24.6 ± 2.5</td>
<td>65.7 ± 2.8</td>
<td>61.6 ± 2.0</td>
</tr>
<tr>
<td>ScD(^d) (\beta_m^{-/-})</td>
<td>23.6 ± 0.9</td>
<td>64.2 ± 3.9</td>
<td>64.5 ± 1.0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>19.5 ± 1.0</td>
<td>43.4 ± 2.2</td>
<td>47.1 ± 2.7</td>
</tr>
<tr>
<td>B6 tgD(^d) (D8)</td>
<td>16.2 ± 1.4</td>
<td>39.9 ± 2.9</td>
<td>40.5 ± 3.5</td>
</tr>
<tr>
<td>ScD(^d) B6</td>
<td>19.9 ± 2.3</td>
<td>40.0 ± 3.6</td>
<td>56.1 ± 6.6</td>
</tr>
</tbody>
</table>

* a The percentage of NK cells expressing the indicated NKR among the NK1.1\(^+\) splenocytes is indicated.

* b MFI, Mean fluorescence intensity. The mean ± SD of three different mice of each strain is indicated. MFI values are normalized to C57BL/6 = 100%.
deficiency of ScDd-expressing NK cells, we investigated the ability to explore the mechanism for the functional and developmental spectrum of CD8 that recognize H-2Dd. Unseparated A-LAK populations were effectively inhibited by D8 target cells due to their known lysis from alloreactive T cells selected by native H-2Dd. In contrast, the expressed products of CD8+ T cells restored by ScDd and Sc HLA-A2.1 molecules were quite different from each other, the strategy using transgenic Sc molecules was very effective for education of CD8+ T cells in both cases. These results confirm that both the peptide binding site and the region of the MHC-I needed for interaction with TCR are conserved even after the structural modification generating Sc molecules. The similarity of TCR repertoires generated by native and ScDd is demonstrated by the virtual lack of alloseactivity of T cells from D8 for stimulators from ScDd B6 and of T cells stimulated in the reverse MLR (Fig. 3).

It is valuable to think about our experiments along with those using transgenic mice that express MHC-II molecules covalently linked to a single peptide (60, 61) and those that, as a result of the MHC-II processing deficit caused by H-2M deficiency, express MHC-II molecules predominantly in complex with the class II-associated invariant chain peptide (62–64). These experiments suggest that the normal diversity of self-peptides is not critical for positive selection of a broad TCR repertoire. However, the H-2M-/- animals, which express normal levels of MHC-II, mount a response against cells bearing normal MHC-II, indicative of the differences between their repertoires. The single peptide MHC-II transgenic mice show a similar response to parental MHC-II, but disparities in the level of expression make these experiments more difficult to interpret. Thus, it appears that in the MHC-II-restricted examples discussed above, there are significant differences in the full MHC-II/peptide repertoire of the mutant animals as compared with the parental strains, and the resulting TCR repertoires are quite distinct. In our studies, the ScDd functions well to positively select a broad TCR repertoire, a repertoire that shows little reactivity against native H-2Dd. Thus, the ScDd animals select a repertoire very similar to that selected by the native molecule. We conclude that the ScDd functions properly with respect to presentation of most self-peptides presented by native H-2Dd, presumably because the conformation of the peptide groove of ScDd is well conserved and ScDd present a broad array and an appropriate distribution of MHC/peptide complexes to TCR of developing thymocytes. The ability of ScDd to present peptides derived from the endogenous processing pathway is consistent with our earlier studies examining the presentation of synthetic peptides by transfected cells expressing ScDd (33). In addition, we showed that the sequence motif of peptides eluted from ScDd molecules was the same as that of peptides derived from native H-2Dd (65).

In contrast to its ability to serve both in the education of and the target cell recognition by CD8+ T cells, the transgenic encoded ScDd did not function with respect to the education of NK cells or inhibition of NK-mediated cytotoxicity in a variety of assays. ScDd β2m-/- derived NK cells did not reject β2m-/- bone marrow like native H-2Dd in any NK assay we employed; it was unable to educate NK cells in the transgenic mice, and was also unable to affect NK cell function in vitro or in vivo or detectably to bind the recombinant Ly49A receptor.

A recent report described the expression of a similar Sc human HLA-A2.1 at normal levels on cells in a transgenic mouse (36). In those experiments, Sc constructs based on either murine or human β2m were expressed in β2m-/- or double β2m-/- H-2Dd-/- animals. This transgene also restored a sizable T cell population of functional CD8+ cells when expressed in such MHC-I-deficient mice. As shown in our experiments, transgenic ScDd restored a significant number of CD8+ T cells expressing a broad TCR repertoire in the absence of proper expression of other MHC-Ia and MHC-Ib molecules. Furthermore, these CD8+ T cells could mount a response against specific endogenously processed peptide/MHC complexes in the periphery and efficiently recognized specific peptide presented by native H-2Dd. Although the proportion of CD8+ T cells restored by ScDd and Sc HLA-A2.1 mice were quite different from each other, the strategy using transgenic Sc molecules was very effective for education of CD8+ T cells in both cases. These results confirm that both the peptide binding site and the region of the MHC-I needed for interaction with TCR are conserved even after the structural modification generating Sc molecules. The similarity of TCR repertoires generated by native and ScDd is demonstrated by the virtual lack of alloseactivity of T cells from D8 for stimulators from ScDd B6 and of T cells stimulated in the reverse MLR (Fig. 3).

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grafts in vivo, nor did they kill β2m−/− target cells in vitro (Figs. 4 and 5), indicating that the transgenic ScD Δ protein was unable to alter the MHC-I reactivity of NK cells (i.e., “educate” the NK cells). Consistent with the failure of ScD Δ to educate NK cells, the pattern of expression of Ly49 receptors on NK cells in ScD Δ ββ.m−/− mice was indistinguishable from that of ββ.m−/− mice (Table I). Furthermore, expression of the ScD Δ protein on either B6 or ββ.m−/− bone marrow grafts was unable to prevent their rejection from B10.D2 (H-2b) mice and unable to reverse their sensitivity to cytolysis in vitro. This contrasts strikingly with the function of the native H-2D d expressed as a transgene in B6 mice, which rescues the graft from bone marrow rejection.

Recently, a ligand on NK cells for nonclassical MHC-I molecules has been identified. A cell-surface heterodimer consisting of NKG2 and CD94 recognizes HLA-E in humans (66, 67) and Qa-1 in mice (68). Furthermore, a significant proportion of marine NK cells express a receptor that binds to soluble Qa-1 tetramers (69). Because ScD Δ ββ.m−/− mice cannot properly express MHC-Ib molecules, we examined whether ββ.m-dependent Hc-Ib molecules may be critical in education of NK cells in vivo. It was also possible that the expression of only one MHC-Ia molecule might not be sufficient to educate functional NK cells. Thus, additional expression of other classical MHC-I molecules may be required to restore NK cell function from the state found in ββ.m−/− mice. To explore these possibilities further, we performed bone marrow reversion assays and in vitro cytolytic assays using ScD Δ B6 mice expressing ββ.m normally on the cell surface. However, ScD Δ could not restore the education and function of NK cells even in a ββ.m−/− environment. This conclusion is strengthened by the observation that expression of native H-2D Δ as a transgene (in D8) is sufficient to induce all of the NK activities that we assayed and failed to find in ScD Δ B6 mice or cells. These results are all consistent with the view that ScD Δ molecules cannot interact properly with any H-2D Δ-specific inhibitory NKR and thus that the expression of this molecule is simply not sensed effectively by NK cells.

The failure of biotinylated Ly49A protein to stain cells from the ScD Δ transgenic animals (Fig. 6) as well as the failure of ScD Δ on targets to inhibit killing by sorted-Ly49A+G2− NK cells (data not shown), indicates that structural alterations of the ScD Δ protein prevent it from interacting effectively with NK inhibitory receptors. Using chimeric H-2K Δ/H-2D Δ molecules, Matsumoto et al. showed that residues 53–65 of the α1 domain and 90–107 in the N-terminal part of the α2 domain of H-2D Δ contributed to Ly49A recognition (70). It is possible that these differences indirectly affect NKR binding by influencing the conformation or selection of bound peptides. The specificity of Ly49A for different MHC-I molecules may result from polymorphic residues between reactive and nonreactive MHC-I alleles and/or nonpolymorphic residues having different side chain conformations in different MHC-I molecules (14, 71). Other mutagenesis studies using cultured cell lines transfected with in vitro mutated H-2D Δ molecules suggest a role of specific residues in the α1 and α2 domains in Ly49A recognition (72).

Unlike the Ly49A/H-2D Δ interaction, very little is known about the interaction of H-2D Δ with Ly49G2. The expression level of Ly49G2 on NK cells is not perturbed by expression of the ScD Δ transgene. This result stands in contrast to the observed changes in the expression of the Ly49G2 receptor among NK cells when expressed in the presence of native H-2D Δ (19, 21). Although this is not a direct indication, the fact that we fail to observe a change in Ly49G2 expression in the ScD Δ transgenic mouse suggests that Ly49G2 also fails to effectively interact with the ScD Δ molecule. Furthermore, Ly49A+G2− A-LAK cells were not inhibited in their cytolyis of ScD Δ expressing target cells. Finally, Ly49G2 is expressed on approximately half of NK cells in various mouse strains, and an effective inhibitory interaction of the ScD Δ protein with Ly49G2 could be expected to alter NK function in bulk NK populations, which was not observed. Thus, we conclude that Ly49G2 also cannot interact effectively with the ScD Δ protein.

Although the mechanism of the failure in physical interaction between Ly49A or Ly49G2 and ScD Δ molecules is unclear, this may result from: 1) direct blocking of Ly49A binding by the cognate peptide spacer linking the C terminus of ββ.m and the N terminus of the H-2D Δ H chain in this construct; 2) a conformational change of the Ly49A binding site induced by this cognate link; or 3) the formation of an obligate ScD Δ/ScD Δ noncovalent dimer on the cell surface due to “domain swapping” of the tethered ββ.m whereby ββ.m covalently linked to one molecule binds to the other, resulting in the sequestration of the Ly49A binding site in the interface between the two heterodimers. (Sc Ab Fv are known to form either dimers (“diabodies”) (73, 74) or trimers depending on the length of the peptide spacer that joins V h and V l (75).) Because our data show no evidence of function with respect to the entire population of NK cells of the mouse, we conclude that this lack of interaction must apply to most or all of the H-2D Δ-specific inhibitory NKR, including Ly49A and Ly49G2. In addition, we provide evidence that the lack of interaction extends to H-2D Δ-specific stimulatory receptors as well (see Fig. 5C). We thus conclude that some structure that is necessary for interaction with most or all H-2D Δ-specific NK activating and inhibitory receptors is not present in the ScD Δ protein, while the structures required for effective interaction with a broad range of H-2D Δ-specific TCR remain intact. Because the site of interaction of MHC-I/peptide complexes with TCR has been shown to consist of the α1 and α2 helices of the MHC and of exposed side chains of the bound peptide, (22–25) it seems likely that this structural surface is not significantly distorted in the ScD Δ molecule. Clearly, the general lack of peptide specificity of Ly49A interaction with H-2D Δ as well as the profound difference in the reactivity of ScD Δ with Ly49A both functionally and in a direct binding assay using the recombinant ScD Δ indicate that the TCR and the NKR interact with distinct sites on H-2D Δ. In addition, these results support the view that several different activating and inhibitory receptors of the same structural family employ a structurally conserved surface of the molecule to interact with H-2D Δ.

In conclusion, transgenic Sc H-2D Δ can induce development and function of a large number of mature functional CD8+ T cells expressing a broad TCR repertoire highly similar to the repertoire selected by native H-2D Δ but cannot function in the education of NK cells or interact with H-2D Δ-specific inhibitory receptors expressed by NK cells. Thus, we demonstrate that TCRs bind a part of the MHC/peptide complex distinct from the site where Ly49A and Ly49G2 bind.

Acknowledgments

We thank the staff of the National Institute of Allergy and Infectious Diseases Transgenic Mouse Facility; also, Howard Adams, Jose Austin, Kim Beck, and Michelle Klein for technical help, Drs. S. Pack and K. Polakova for assistance, Drs. P. Earl and B. Moss for recombinant vaccinia virus vPE16, Dr. E. Shevach for comments on the manuscript, and Dr. R. Germain for helpful discussions during the course of this work.

References


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