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

Doo Hyun Chung, Jeffrey Dorfman, Daniel Plaksin, Kannan Natarajan, Igor M. Belyakov, Rosemarie Hunziker, Jay A. Berzofsky, Wayne M. Yokoyama, Michael G. Mage and David H. Margulies

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

References
This article cites 74 articles, 41 of which you can access for free at: http://www.jimmunol.org/content/163/7/3699.full#ref-list-1

Subscription
Information about subscribing to J Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
NK and CTL Recognition of a Single Chain H-2D<sup>d</sup> Molecule: Distinct Sites of H-2D<sup>d</sup> Interact with NK and TCR

Doo Hyun Chung,* Jeffrey Dorfman,† Daniel Plaksin,1,* Kannan Natarajan,* Igor M. Belyakov,‡ Rosemarie Hunziker,2* Jay A. Berzofsky, ‡ Wayne M. Yokoyama,¶ Michael G. Mage,§ and David H. Margulies*‡

We generated transgenic mice expressing a single-chain β<sub>2</sub>-microglobulin (β<sub>2</sub>m)-H-2D<sup>d</sup>. The cell-surface β<sub>2</sub>m-H-2D<sup>d</sup> molecule was expressed on a β<sub>2</sub>m-deficient background and reacted with appropriate mAbs. It was of the expected m.w. and directed the normal development of CD8<sup>+</sup> T cells in the thymus of a broad TCR repertoire. It also presented both exogenously provided and endogenous peptide Ags to effector CD8<sup>+</sup> 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<sup>d</sup> was disrupted. Thus, the sites of TCR and NK receptor interaction with H-2D<sup>d</sup> are distinct, an observation consistent with independent modes of TCR and NK receptor evolution and function. The Journal of Immunology, 1999, 163: 3699–3708.

The proper expression of MHC class I molecules (MHC-I)<sup>4</sup> is critical for the development and function of NK cells (1, 2) and CD8<sup>+</sup> T lymphocytes (3, 4). Although both CD8<sup>+</sup> T cells and NK cells can recognize the same MHC-I on target cells, the precise requirements are quite different (5). CD8<sup>+</sup> T cells use their clonally expressed and somatically rearranged TCR to recognize peptide fragments presented on APC in complex with MHC-I (6–8). In contrast, the specificity of some NK receptors (NKR) is predominantly influenced by MHC-I itself and is unaffected by the particular peptide bound (9–11), while some other NKR exhibit a peptide preference (12, 13). NK cells employ a number of different receptors, both activating and inhibitory. Among the best understood NKR are the inhibitory receptors of the Ly49 family in the mouse (14). These NKR transmit inhibitory signals delivered upon engagement of MHC-I on target cells (15–17). The activation of the mature NK cell is modulated by the interaction of its inhibitory receptor(s) with appropriately conformed MHC-I on target cells. In addition, MHC-I expression of the host controls the proportion of NK cells expressing particular Ly49 receptors, and MHC-I expression tunes the functional potential of the NK cells in a developmental manner (18–21). Thus, the NK cell exploits the engagement of MHC-I in both education and effector phases.

Crystallographic structures of several TCR/MHC-I complexes indicate a common orientation of the αβ TCR in interacting with amino acid side chains of both the MHC-I molecule and the bound peptide (22–26). In contrast, relatively little is known about the structure of Ly49 receptors. Ly49A is a member of a family of related proteins encoded by closely linked genes and by amino acid sequence is distantly related to members of the C-type lectin family (14, 27). Ab blocking studies and transfection of target cells with MHC-I-encoding genes indicate that Ly49A is an inhibitory receptor for the H-2D<sup>d</sup> and H-2D<sup>b</sup> molecules (15, 28) and that this inhibitory effect for H-2D<sup>d</sup> results from interactions with the α<sub>1</sub>α<sub>3</sub> domains (11, 15). Other studies indicate that Ly49A binds H-2D<sup>d</sup> directly, although the precise location of the Ly49A interaction with the MHC-I molecule is unclear (29–31).

We previously described the expression and function in transfected cells of single-chain (Sc) forms of the MHC-I molecules, H-2D<sup>d</sup> and HLA-A2, which consist of the MHC-I L chain, β<sub>2</sub>-microglobulin (β<sub>2</sub>m), covalently linked to the MHC-I H chain through a peptide spacer (32–34). Others have reported similar Sc constructs of H-2K<sup>d</sup> (35) and HLA-A2 (36). Sc H-2D<sup>d</sup> molecules were expressed on the surface of transfected cells as detected by flow cytometry and could stimulate T hybridoma cells (B4.2.3 specific for H-2D<sup>d</sup> complexed with P18-I10, a decapeptide from the HIV-1 envelope glycoprotein V3 loop (37, 38) in vitro when loaded with peptide. Analysis of the fine specificity of Ag presentation by the Sc molecules revealed only subtle quantitative differences when a panel of synthetic substituted peptide Ags was employed, suggesting that there are no major differences in the way the Sc molecules bind peptide or in the conformation of the resulting MHC/peptide complex (39). These data indicated that the overall conformation of the Sc H-2D<sup>d</sup> molecule was adequate for binding peptides and for stimulating H-2D<sup>d</sup>-restricted T cells. Because these earlier experiments addressed issues of mature T cell recognition of MHC/peptide complexes, we expected that transgenic expression of the Sc MHC-I molecules would allow questions of T cell and NK cell development and education to be explored. Here, we examine the expression, both in B6 and in B6...
Materials and Methods

Expression of Sc H-2D<sup>d</sup> in transgenic mice

To obtain a chimeric gene encoding a Sc (β<sub>m</sub>-spacer-MHC-I H chain) H-2D<sup>d</sup>, in a form suitable for expression as a transgene, a different fragment from two plasmids were used: (1) a 5′ fragment (XbaI-BanHII) from the pD1-1 plasmid (40, 41) containing promoter sequences from −400 bp upstream of the initiation ATG codon, modified in its BanHII site with Klenow polymerase and dGTP and dATP; 2) a 3′- DNA fragment derived from a cDNA for a Sc H-2D<sup>d</sup> construct that codes for β<sub>m</sub>, a peptide linker, and the mature H-2D<sup>d</sup> H chain protein through to the end of the α<sub>β</sub> domain (encoded by exon 3), which had been modified at the SalI site with Klenow polymerase and dTTP and dCTP (32); and 3) an Fspl- EcoRI fragment (which extends from the end of exon 3 to the 3′ untranslated region) from pD1-1. These were ligated in two sequential steps into the pBluescript-IKS (+) vector.

The DNA construct encoding the Sc H-2D<sup>d</sup> 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 β<sub>m</sub> (C57BL/6GphTac-Ko) B2m N5, β<sub>m</sub> knockout (β<sub>m</sub>−/−) mice (3), maintained and bred at Taconic, Germantown, NY) to generate transgenic mice expressing cell-surface Sc H-2D<sup>d</sup> in the absence of other β<sub>m</sub>-dependent molecules. (The formal names for the transgenic strains are C57BL/6Ncr (Sc<sub>β</sub>2m<sup>-</sup>C1) and Sc<sub>β</sub>2m<sup>-2</sup> β<sub>m</sub>−/− as well.) Offspring were screened for H-2 expression with Abs specific for H-2D<sup>d</sup> (34-2-12, 12-H-2D<sup>d</sup> (28-14-8), and H-2K<sup>b</sup> (AF6-88.5) by flow cytometry. Most of the experiments reported here have been performed with Sc<sub>β</sub>2m<sup>-1</sup>, and some have been performed with Sc<sub>β</sub>2m<sup>-2</sup>. Surface expression of the transgene-encoded molecule and functional behavior were indistinguishable for the two strains.

Abs and mice

The following Abs, purchased from PharMingen, San Diego, CA, were used: anti-CD16/CD32 (2.4G2); FITC-conjugated anti-H-2<sup>d</sup>-2m (34-5-8, α<sub>A</sub> domain specific), (34-2-12, which binds the α<sub>A</sub> domain), (34-4-20, 25-34.5), anti-H-2<sup>k</sup>-2m (AF6-88.5); anti-H-2<sup>d</sup>-2m (KH95); anti-CD4 (14-4-11); anti-CD8 (53-6.7); and anti-Ly49A (A1). PE-conjugated CD4 (H129.19); anti-TCR (TCR) V<sub>α</sub>2C11; anti-CD8 (53-6.7); anti-Ly49A (A1); PE-conjugated CD4 (H129.19); anti-TCR (TCR) V<sub>α</sub>2C11; anti-CD8 (53-6.7); anti-Ly49A (A1); PE-conjugated F(ab′)<sub>2</sub>-goat anti-mouse IgG mAb was purchased from Jackson ImmunoResearch (West Grove, PA). A1 (anti-Ly-49A), SW5E6 (anti-Ly49C/I), and 4D11 (anti-Ly49G2) were also used. 34-5-8S (anti-H-2D<sup>d</sup>) and 34-4-20S (anti-H-2D<sup>d</sup>) were obtained from American Type Culture Collection (Villignton, VA). Anti-Ly49A, C/I, or G2 mAbs. 34-5-8S (anti-H-2D<sup>d</sup>) and 34-4-20S (anti-H-2D<sup>d</sup>) were obtained from American Type Culture Collection (Villignton, VA). Anti-Ly49A, C/I, 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 mMD-sorbitol, 0.6 M NaCl, 1 mM PMSF, 5 mM iodoacetamide, 1 mM sodium orthovanadate, and Complete protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN). Nuclei were removed by centrifugation, and lysates from 5× 10<sup>7</sup> cells were precleared with protein G-Sepharose and then subjected to immunoprecipitation with 10 μg of 34-2-12 and 150 μl of a 10% slurry of protein G-Sepharose beads (Pharmacia Biotech, Uppsala, 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, Chicago, IL).

Immunization with vaccinia virus and CTL Assay

Sc<sub>D</sub><sup>d</sup> β<sub>m</sub>−/− and D8 mice were immunized by i.p. injection of 2× 10<sup>6</sup> plaque-forming units of vaccinia virus expressing HIV-1 envelope glycoprotein gp160BIB (vPE16, the gift of P. Earl and B. Moss) (44). Three weeks later, splenocytes were cultured at 5× 10<sup>7</sup>/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 μg/ml), and 50 μM 2-ME). Three 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 at 2× 10<sup>6</sup> cells/ml and 1 μM P188B110 (RGPGRAFVTL) peptide together with 4× 10<sup>6</sup> irradiated (3300 rad) syngeneic spleen cells as APC. Cytolytic activity of CTL lines was measured by a standard 4-h 51Cr-release assay (56). 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× 10<sup>5</sup> responder splenocytes and 2.5× 10<sup>6</sup> 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 (Conning Glass Works, Conning, NY) for 5 days. Fresh NK effector cells were depleted of RBC, and nylon wool nonadherent splenocytes were taken from groups of mice that had been treated 20–24 h previously with 150 μg poly IC (Sigma, St. Louis, MO), an NK stimulator. Four-day lymphokine-activated killer (LAK) NK cell effector cells were prepared by a procedure based upon that of Chadwick and Miller (47). Briefly, splenocytes were cultured in absence 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 recombinant human IL-2) (Chiron, Emeryville, CA). Adherent LAK (A-LAK) cells were prepared as described (15).

Lysis assays for NK cell and allogeneic CTL (MLR)

For target cell preparation, splenocytes were cultured in complete T medium containing 5% FCS for 24–36 h in 24-well plates (Falcon Plastics, Lincoln Park, NJ) at 2× 10<sup>5</sup> cells/ml with 2 μg/ml Con A (Sigma). On the day of assay, one-tenth volume of 1 M methyl-x-o- naphthoysansoide (Sigma) in RPMI 1640 or PBS was added to target cell cultures to block Con A sites before labeling for 1–2 h in 10 μl of 10 mCi/ml 51CrNa2CrO4 (Amersham, Arlington Heights, IL) in PBS. All points were determined in triplicate using 5× 10<sup>3</sup> to 1× 10<sup>6</sup> target cells per well.

Bone marrow transplantation

Bone marrow cells were obtained by flushing the lumina of the tibiae and femora of donor mice with HBSS under aseptic conditions. Suspensions of 3× 10<sup>6</sup> cells were injected i.v. (subocular under anesthesia or by tail vein) into groups of six (or occasionally five) irradiated (900 rad from a 137Cs source) hosts. Five days after inoculation with bone marrow cells, mice were injected i.p. with 2× 10<sup>12</sup> cpm of [14C]iodoateoxuridine (125IUDR; Amersham). Then, 20–24 h after injection of radiolabel, the mice were sacrificed and incorporation of radioactivity into the spleens was determined by gamma spectrometry.
Lymph node cells from ScDdβm−/− mice were incubated with biotinylated 28-14-8 (anti-H-2Dd) as indicated by reactivity with Abs 34-2-12 (Fig. 1B). In contrast, mice homozygous for the ScDd transgene, ScDdβm+/+ BALB/c, were stained with FITC-conjugated-mAbs against H-2Dd, Db, and Kb 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, ScDdβm−/−, BALB/c, and B6 mice were stained with FITC-conjugated-mAbs against H-2Dd, Dd, and Kb molecules. Spleen cells from ScDdβm−/−, B6, and ScDdβm+/+ mice were surface-biotinylated and lysed. The lysates were precipitated with 34-2-12 (anti-H-2Dd) mAb and protein-G Sepharose beads, washed, separated on 14% SDS polyacrylamide gel, and transferred to Immobilon P membrane. The biotinylated proteins were visualized with streptavidin-HRP and enhanced chemiluminescent reaction.

Immunoprecipitation from splenocytes using 34-2-12 mAb (anti-H-2Dd) and protein-G made in these transgenics. Although we expected that the ScH-2Dd H chain on the spleen cells from BALB/c was detected as a 50-kDa protein. Thus, the H chain of transgenic ScDd is expressed as a molecular species that is covalently linked with βm 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 is α3 domain-specific and reacts with the isolated H-2Dd α3 domain (51), this Ab would have detected membrane molecules truncated in the βm, spacer, α1, or α2 regions.)

Although βm−/− cell lines and mice are profoundly deficient in expression of MHC-I molecules, it is well known that low levels of H-2Dd are detectable by flow cytometry or by reactivity with lysis by H-2Dd-specific alloreactive CTL (48–50). As shown in Fig. 1B, we detected low levels of H-2Dd both in βm−/− and in ScDdβm−/− mice expressing the ScDd transgene. Presence of the ScDd transgene did not perturb the expression of H-2Dd in βm−/− mice (Fig. 1B).

With biochemical techniques we examined the Sc molecules made in these transgenics. Although we expected that the ScH-2Dd 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-2Dd/βm heterodimers. H-2Dd 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-α3 domain of H-2Dd (Fig. 1C)). In contrast, the normal MHC-I H-2Dd H chain on the spleen cells from BALB/c was detected as a 50-kDa protein. Thus, the H chain of transgenic ScDd is expressed as a molecular species that is covalently linked with βm 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 is α3 domain-specific and reacts with the isolated H-2Dd α3 domain (51), this Ab would have detected membrane molecules truncated in the βm, spacer, α1, or α2 regions.)

ScDd educates a diverse repertoire of CD8+ T cells, similar to that induced by native H-2Dd

It is well known that normal expression of MHC-I molecules in the thymus is necessary for the normal maturation of CD8+ T cells, that is, the progression of immature CD4−CD8+ T cells to mature single positive cells. Animals defective in βm expression, and as a result lacking normal cell-surface MHC-I expression, show a profound decrease in the number of CD8+ (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+ T cell development (52). To assess the selection of CD8+ T cells by ScDd in the absence of normal expression of other MHC-I molecules, we analyzed thymocytes, lymph node cells, and splenocytes from B6, B6 βm−/−, and ScDdβm−/− mice for the presence of mature CD8+ T cells by flow cytometry (Fig. 2). Unlike nontransgenic βm−/− thymocytes, those from ScDdβm−/− mice contained mature CD8+ 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 β− cells), and spleen (65.39 and 32.29% of TCR β− cells). In contrast, thymocytes from βm−/− 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+ cells. Despite the lack of proper expression of MHC-I molecules other than the ScDd transgenic βm−/− animals, substantial numbers of CD8+ T cells were detected in the thymus, lymph nodes, and spleen of ScDdβm−/− 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+ clones might mature in ScDdβm−/− mice and expand to fill the available space. If such were the case, the diversity of the expressed

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. 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 ScDdβm−/− knockout mice

To explore the expression of the ScDd and other MHC-I molecules in βm−/− transgenic mice, lymph node cells from ScDdβm−/−, βm−/−, BALB/c, and B6 mice were stained with mAb against H-2Dd, H-2Db, and H-2Kk. As expected, lymph node cells of the βm−/− transgenic mice expressed no detectable H-2Kk or H-2Db above the level observed in βm−/− nontransgenic cells (Fig. 1A). In contrast, mice homozygous for the ScDd transgene, expressed in the βm−/− background, revealed apparently normal levels of H-2Dd epitopes as indicated by reactivity with Abs 34-2-12 (α3 domain), and 2-35.4 (αα domain) (Fig. 1A) as well as with 34-5-8 (ααβα domain) and 34-4-20 (data not shown).

Natarajan et al. Submitted for publication.

Submitted for publication.

Image 57x477 to 271x734

FIGURE 1. Expression of ScDd in the absence of normal βm.

A, Lymph node cells from ScDdβm−/−, C57BL/6, and ScDdβm+/+ mice were stained with FITC-conjugated-mAbs against H-2Dd, Dd, and Kb molecules and PE-conjugated-CD4 mAb. Analysis for the expression of MHC-I molecules was performed on the gated CD4-positive cells. B, Lymph node cells from C3H/HeJ, ScDdβm−/−, BALB/c, and B6 mice were incubated with biotinylated 28-14-8 (anti-α3 of H-2Db) and stained with PE-streptavidin. C, Immunoprecipitation of transgenic Sc H-2Dd. Spleen cells from ScDdβm−/−, B6, and BALB/c mice were surface-biotinylated and lysed. The lysates were precipitated with 34-2-12 (anti-α3 of H-2Db) and protein-G Sepharose beads, washed, separated on 14% SDS polyacrylamide gel, and transferred to Immobilon P membrane. The biotinylated proteins were visualized with streptavidin-HRP and enhanced chemiluminescent reaction; lane 1, BALB/c; lane 2, ScDdβm−/−; lane 3, B6.
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 ScDd β₂m⁻/⁻ 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 region genes might reveal subtle differences in the TCR repertoire selected by the ScDd on the β₂m⁻/⁻ 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 ScDd molecules and normal H-2Dd. To do this, we stimulated D8 (B6, native H-2Dd transgenic) splenocytes with ScDd B6 (B6, ScDd transgenic) splenocytes and vice versa in MLR (Fig. 3B). T cells that develop in an environment in which their TCR are selected by the ScDd molecule but retain discriminating activity against the native two-chain H-2Dd molecule would be expected to be present and be stimulated in the ScDd anti-D8 MLR and should be capable of killing D8 targets. Similarly, T cells expressing TCR that are selected on H-2Dd yet remain reactive against unique epitopes of ScDd would be expected to be stimulated in the D8 anti-ScDd B6 MLR and show cytolyis against ScDd 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-ScDd B6 MLR and the ScDd B6 anti-D8 MLR exhibited very low cytoytic 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-2bm3 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-2Dd or ScDd proteins to stimulate responses generally, as B6 cells transgenic for either the native two chain or the ScDd elicited substantial CTL activity from nontransgenic B6 splenocytes (Fig. 3C). Furthermore, the anti-H-2Dd alloreactive CTL raised against either the Sc or the native molecule do not effectively distinguish native H-2Dd from the Sc. Taken together, the serological and functional data indicate that ScDd 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-2Dd.

**ScH-2Dd mice can induce a virus-specific H-2Dd-restricted CD8⁺ CTL response**

The generation of a diverse repertoire of CD8⁺ T cells indicated that the ScDd molecule was effective in the presentation of self-Ag (presumably as peptides) to developing thymic cells. In addition, the ability of cells expressing ScDd 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 ScDd 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. ScDd β₂m⁻/⁻ 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-2Dd-restricted peptide, P18-110 (37), and then were tested for effector function in a ⁵¹Cr-release assay. CTL induced in ScDd β₂m⁻/⁻ mice could kill P18-110-loaded P815 cells expressing wild-type H-2Dd on the cell surface, whereas these cells could not lyse P815 target cells that had not received the sensitizing peptide (Fig. 3D). ScDd β₂m⁻/⁻ mice developed a P18-110 specific, H-2Dd-restricted CD8⁺ CTL response that was comparable to that of D8 mice in extent of cytolyis. Furthermore, these cells were able to recognize peptide-loaded native H-2Dd 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-2Dd-expressing APC before the cytolyis assay. These findings clearly demonstrate that CD8⁺ T cells selected by peptide in ScDd β₂m⁻/⁻ mice are capable of mounting an effective peptide-specific anti-viral cytotoxic response. Most importantly, CTL induced by ScDd expressed on APC of the transgenic mice interact effectively with native H-2Dd/P18-110 complexes, that is, they are unable to distinguish native H-2Dd from ScDd.
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.

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 cytolysis assay using Con A-stimulated 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).

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 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.
on NK cells from ScDd $\beta_m^{-/-}$ 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, ScDd B6 mice are not significantly different from the B6 parental line. These results further support the conclusion that NK cells of ScDd $\beta_m^{-/-}$ and of ScDd B6 mice could not be properly educated by ScDd 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 ScDd 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-2Dd (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 ScDd B6 ConA blasts in the cytotoxicity assay (Fig. 5A). Furthermore, ScDd B6 hosts could not reject B6 bone marrow grafts (Fig. 4C). This result is particularly striking because animals expressing a native H-2Dd transgene are capable of rejecting B6 bone marrow grafts. Thus, the ScDd transgene is unable to function like native H-2Dd in the education of NK cells.

**Sc H-2Dd cannot deliver inhibitory or stimulatory signals for NK cell-mediated lysis**

The MHC-I-dependent resistance to NK lysis is mediated by inhibitory surface NKR that engage target cell MHC-I (57). The native H-2Dd 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 ScDd transgene-encoded molecules on the target cells resulted in the delivery of inhibitory signals to NK effector cells. In vitro cytotoxicity assays, short-term cultured NK cells derived from B10.D2 (data not shown) and D8 mice killed ScDd B6 and nontransgenic B6 target cells equivalently (Fig. 5A) and also lysed ScDd $\beta_m^{-/-}$ target cells and $\beta_m^{-/-}$ target cells equivalently. Expression of ScDd 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-2Dd on $\beta_m^{-/-}$ bone marrow grafts would be sufficient to prevent rejection by B10.D2 mice, expression of native H-2Dd as a transgene was sufficient to prevent rejection of B6 marrow by B10.D2 mice (Fig. 5B), as previously shown by Öhle and colleagues (53). Taken together, these data indicate that the ScDd transgene, in contrast to the H-2Dd 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-2Dd-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 ScDd 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.) If

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

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Ly49A (A1)</th>
<th>Ly49C/I (5E6)</th>
<th>Ly49G2 (4D11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_m^{-/-}$</td>
<td>24.6 ± 2.5</td>
<td>117.3 ± 25.4</td>
<td>65.7 ± 2.8</td>
</tr>
<tr>
<td>ScDd $\beta_m^{-/-}$</td>
<td>23.6 ± 0.9</td>
<td>107.0 ± 16.6</td>
<td>64.2 ± 3.9</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>19.5 ± 1.0</td>
<td>100 ± 41.5</td>
<td>43.4 ± 1.2</td>
</tr>
<tr>
<td>B6 tgDd (D8)</td>
<td>16.2 ± 1.4</td>
<td>26.5 ± 7.2</td>
<td>39.9 ± 2.9</td>
</tr>
<tr>
<td>ScDd B6</td>
<td>19.9 ± 2.3</td>
<td>65.0 ± 11.7</td>
<td>40.0 ± 3.6</td>
</tr>
</tbody>
</table>

* The percentage of NK cells expressing the indicated NKR among the NK1.1+ splenocytes is indicated.

* MFI, Mean fluorescence intensity. The mean ± SD of three different mice of each strain is indicated. MFI values are normalized to C57BL/6 = 100%.

**FIGURE 5.** Unlike native H-2Dd, ScDd molecules cannot deliver inhibitory or stimulatory signals for NK cell-mediated lysis. IL-2-activated NK cells from D8 mice kill ScDd transgenic target cells and nontransgenic target cells equivalently (A). In bone marrow assays, bone marrow cells from ScDd B6 mice were rejected by B10. D2 mouse recipients (B) and accepted by B6 recipients (C). Each group of recipients contains six mice (or occasionally five) except for one group (n = 3). These results are of two independent bone marrow transplantation experiments. Recipient (B6 + PK136) mice were pretreated with anti-NK1.1 Abs.
To explore the mechanism for the functional and developmental

trum of CD8 specificity for H-2Dd. Unseparated A-LAK populations were un-
cellular cytotoxicity (data not shown). However, both populations
lysed them all equivalently (data not shown).

able to distinguish any of the three target cell populations and
T cells selected by native H-2Dd. In contrast, the expressed prod-
bred (Fig. 5).

These data were analyzed among the TCR Cβ-positive cells of lymph node.

\( Sc \) H-2Dd are defective in binding Ly49 receptors on NK cells

To explore the mechanism for the functional and developmental
diciency of ScDd-expressing NK cells, we investigated the abili-
ity of ScDd to interact directly with Ly49 receptors known to bind
native H-2Dd. A soluble recombiant form of the
Ly49A extracellular domain was employed to probe for binding to
lymph node cells expressing various types of MHC-I, and binding
was detected by flow cytometry (Fig. 6). Biotinylated Ly49A
stained lymph node T cells expressing native H-2Dd (BALB/c, B10.D2, and D8) but did not stain lymph node T cells from B6 or
\( \beta_2m^- \) and ScDd \( \beta_2m^- \) mice. This reagent also failed to stain
cells from ScDd transgenic mice on either the B6 or
back-

\( Sc \)Dd is demonstrated by the virtual lack of alloreactivity 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 defect 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 repertoirees. 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
elements 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 repertoirees are
quite distinct. In our studies, the ScDd functions well to positively
select a broad TCR repertoire, a repertoire that shows little reac-
tivity against native H-2Dd. Thus, the ScDd animals select a rep-
ertoire 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 thy-
ocytes. The ability of ScDd to present peptides derived from the
endogeneous processing pathway is consistent with our earlier
studies examining the presentation of synthetic peptides by trans-
fected 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
\( \beta_2m^- \)-derived NK cells did not reject \( \beta_2m^- \) bone marrow

Discussion

In this paper, we describe transgenic mice expressing a ScDd mol-
cule on somatic cells at densities similar to those of the native
H-2Dd molecule in other mouse strains. We have demonstrated that
the transgenic ScDd can induce development of a broad spec-
trum of CD8\(^{+}\) T cells whose TCR repertoire resembles that of the
T cells selected by native H-2Dd. In contrast, the expressed pro-
duct of this transgene is essentially or completely inert to NK cells
and their receptors that normally interact with native H-2Dd. The
cell surface molecule encoded by the Sc construct did not function
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 on normal levels on cells in a transgenic mouse (36). In
those experiments, Sc constructs based on either murine or human
\( \beta_2m \) were expressed in \( \beta_2m^- \) or double \( \beta_2m^- / H-2Dd^- \) an-
imals. 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 rep-
ertoire 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 pep-
tide presented by native H-2Dd. Although the proportion of CD8\(^{+}\)
T cells restored by ScDd and Sc HLA-A2.1 molecules were quite
different from each other, the strategy using transgenic Sc mole-
cules 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 con-
served even after the structural modification generating Sc mole-
cules. The similarity of TCR repertoirees generated by native and
ScDd is demonstrated by the virtual lack of alloreactivity of T cells
from D8 for stimulators from ScDd B6 and of T cells stimulated in
the reverse MLR (Fig. 3).
 NK AND CTL RECOGNITION OF SINGLE CHAIN H-2D^d

grants in vivo, nor did they kill β_m^-/- target cells in vitro (Figs. 4 and 5), indicating that the transgenic ScD^d protein was unable to alter the MHC-I reactivity of NK cells (i.e., “educate” the NK cells). Consistent with the failure of ScD^d to educate NK cells, the pattern of expression of Ly49 receptors on NK cells in ScD^d β_m^-/- mice was indistinguishable from that of β_m^-/- mice (Table I). Furthermore, expression of the ScD^d protein on either B6 or β_m^-/- bone marrow grafts was unable to prevent their rejection from B10.D2 (H-2^d) 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 murine NK cells express a receptor that binds to soluble Qa-1 tetramers (69). Because ScD^d β_m^-/- mice cannot properly express MHC-Ib molecules, we examined whether β_m-dependent MHC-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 β_m^-/- mice. To explore these possibilities further, we performed bone marrow retransplantation assays and in vitro cytolytic assays using ScD^d B6 mice expressing β_m normally on the cell surface. However, ScD^d could not restore the education and function of NK cells even in a β_m^-/^- environment. This conclusion is strengthened by the observation that expression of native H-2D^d as a transgene (in D8) is sufficient to induce all of the NK activities that we assayed and failed to find in ScD^d B6 mice or cells. These results are all consistent with the view that ScD^d molecules cannot interact properly with any H-2D^d-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^d transgenic animals (Fig. 6) as well as the failure of ScD^d on targets to inhibit killing by sorted-Ly49A^-G2^- NK cells (data not shown), indicates that structural alterations of the ScD^d protein prevent it from interacting effectively with NK inhibitory receptors. Using chimeric H-2^K/2^H-J^d^ molecules, Matsumoto et al. showed that residues 53–65 of the α_d domain and 90–107 in the N-terminal part of the α_d domain of H-2^d^ 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^d^ molecules suggest a role of specific residues in the α_d and α_s domains in Ly49A recognition (72).

Unlike the Ly49A/H-2D^d^ interaction, very little is known about the interaction of H-2D^d^ with Ly49G2. The expression level of Ly49G2 on NK cells is not perturbed by expression of the ScD^d^ 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^d^ (19, 21). Although this is not a direct indication, the fact that we fail to observe a change in Ly49G2 expression in the ScD^d^ transgenic mouse suggests that Ly49G2 also fails to effectively interact with the ScD^d^ molecule. Furthermore, Ly49A^-G2^-A-LAK cells were not inhibited in their cytolysis of ScD^d^ 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^d^ 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^d^ protein.

Although the mechanism of the failure in physical interaction between Ly49A or Ly49G2 and ScD^d^ molecules is unclear, this may result from: 1) direct blocking of Ly49A binding by the covalent peptide spacer linking the C terminus of β_m and the N terminus of the H-2D^d^ H chain in this construct; 2) a conformational change of the Ly49A binding site induced by this covalent link; or 3) the formation of an obligate ScD^d^/ScD^d^ noncovalent dimer on the cell surface due to “domain swapping” of the tethered β_m whereby β_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^d^-specific inhibitory NKR, including Ly49A and Ly49G2. In addition, we provide evidence that the lack of interaction extends to H-2^d^ 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^d^-specific NK activating and inhibitory receptors is not present in the ScD^d^ protein, while the structures required for effective interaction with a broad range of H-2D^d^-specific TCR remain intact. Because the site of interaction of MHC-I/peptide complexes with TCR has been shown to consist of the α_d and α_s 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^d^ molecule. Clearly, the general lack of peptide specificity of Ly49A interaction with H-2D^d^ as well as the profound difference in the reactivity of ScD^d^ with Ly49A both functionally and in a direct binding assay using the recombinant Ly49A indicate that the TCR and the NKR interact with distinct sites on H-2D^d^.

In conclusion, transgenic Sc H-2D^d^ can induce development and function of a large number of mature functional CD^1^ T cells expressing a broad TCR repertoire highly similar to the repertoire selected by native H-2D^d^ but cannot function in the education of NK cells or interact with H-2D^d^-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


