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Human MHC Class I Transgenic Mice Deficient for H2 Class I Expression Facilitate Identification and Characterization of New HLA Class I-Restricted Viral T Cell Epitopes

Eve Cheuk,*† Celine D’Souza,* Ningjie Hu,* Youan Liu,* Haili Lang,* and John W. Chamberlain2*‡

Although mice transgenic (Tg) for human MHC (HLA) class I alleles could provide an important model for characterizing HLA-restricted viral and tumor Ag CTL epitopes, the extent to which Tg mouse T cells become HLA restricted in the presence of endogenous H2 class I and recognize the same peptides as in HLA allele-matched humans is not clear. We previously described Tg mice carrying the HLA-B27, HLA-B7, or HLA-A2 alleles expressed as fully native (HLAnat) and as hybrid human/mouse (HLAhyb) molecules on the H2b background. To eliminate the influence of H2b class I, each HLA Tg was bred with a H2-Kb/H2-Dd-double knockout (DKO) strain to generate mice in which the only classical class I expression was the human molecule. Expression of each HLAhyb molecule and HLA-B27nat/human β2-microglobulin led to peripheral CD8+ T cell levels comparable with that for mice expressing a single H2-Kb or H2-Dd gene. Influenza A infection of Tg HLA-B27h/b/ DKO generated a strong CD8+ T cell response directed at the same peptide (flu nucleoprotein NP383–391) recognized by CTLs from flu-infected B27-humans. As HLA-B7/flu epitopes were not known from human studies, we used flu-infected Tg HLA-B7h/b/DKO mice to examine the CTL response to candidate peptides identified based on the B7 binding motif. We have identified flu NP418–426 as a major HLA-B7-restricted flu CTL epitope. In summary, the HLA class I Tg/H2-K/H2-D DKO mouse model described in this study provides a sensitive and specific approach for identifying and characterizing HLA-restricted CTL epitopes for a variety of human disease-associated Ags. The Journal of Immunology, 2002, 169: 5571–5580.

The ability of the immune system to detect and eliminate viral infection depends on recognition by CD8+ αβ-TCR CTLs of viral Ags in the form of peptides presented by self MHC class I molecules at the cell surface (1). In general, Ags present in the cytosol of infected cells are processed to peptides by the proteasome and transported into the endoplasmic reticulum by TAP, where they interact with MHC class I molecules (HLA-A, HLA-B, and HLA-C in humans; H2-K, H2-D, and H2-L in mouse) (2, 3). Effective binding leads to transport and expression of the MHC/peptide complex at the cell surface for survey by CTLs. A number of studies have shown that class I-associated peptides range from 8 to 11 aa in length, and that those able to bind a particular allele share the same or related amino acid residues at conserved positions (4–6). The existence of these allelic binding motifs demonstrates the importance of complementarity between the sequence of the presented peptide and the unique structure of the cleft of distinct alleles (4–6). Furthermore, given that only a limited number of viral peptide T cell epitopes restricted by specific human or mouse MHC alleles have been identified to date, the uncovering of such conserved motifs provides a reverse-genetics approach for initial identification of candidate MHC-binding peptides by scanning of amino acid sequences of Ags of interest (4–6, 7).

In addition to the multiplicity of MHC molecules encoded by distinct gene loci in both mice and humans, these proteins are also the most polymorphic molecules in both species, with many alleles identified for most loci (8). Despite this, the T cell response to infection is often observed to become focused in two ways. First, for a given infection, there tends to be a hierarchy or dominance within the responding CTL population with respect to usage of the available MHC class I alleles as restricting elements. Second, despite potential generation of up to hundreds of different peptides for a typical virus for recognition by T cells, a substantial proportion of the antiviral response comes to be directed at a limited number. This phenomenon is called immunodominance, with the preferred MHC/peptide complex(es) called the immunodominant epitope(s) and less favored ones referred to as subdominant epitopes (9, 10).

A detailed understanding of immunodominance is critical for delineating the determinants of effective antiviral immunity and developing vaccines for infections such as HIV-1. Although the inbred mouse is experimentally suited to studies of immunodominance as well as epitope identification and characterization, the results have little direct bearing on Ag recognition in the context of the human MHC molecules. However, performing such investigations for the human directly is complicated for several reasons. Aside from practical issues, MHC polymorphism results in most individuals being heterozygous and expressing up to six different class I alleles and an even greater number of class II alleles. This multiplicity of MHC allele expression, together with additional genetic influences on immune responsiveness, complicate analyses.
of the functional activities of individual HLA products in the outbred human population.

To attempt to reduce this complexity with respect to HLA function and recognition, we and others have explored the possibility that HLA class I molecules expressed in transgenic (Tg) mice might provide a useful model for studying HLA-specific T cell recognition in vivo (11–16). In particular, if human MHC class I alleles bind and present the same Ag-derived peptides to Tg T cells as they do in allele-matched humans, then a panel of Tg mice expressing different HLA alleles should facilitate studies in the context of the human Ag-presenting molecules of immunodominance. Furthermore, this will also have direct application in the identification of new viral and tumor Ag T cell epitopes, as well as developing vaccine immunization strategies. However, despite some efforts along these lines, the extent to which human MHC molecules function efficiently and with the identical specificity as in humans is not clear. Although some studies have suggested that fully native Tg HLA class I molecules are recognized poorly as restriction elements by Tg mouse T cells (11, 12), other reports using apparently similar strains or additional engineered versions of this model suggest that Tg HLA molecules can be recognized by the mouse immune system essentially as alternate mouse H2 class I alleles (13–16). It is possible that at least some of these discrepant results are due to allele-specific differences in the function of human MHC molecules in the mouse background. Furthermore, competition between Tg HLA and endogenous H2 could also limit HLA-dependent thymic selection and T cell recognition in a background coexpressing the normal array of H2 class I molecules.

We previously described a panel of HLA Tg mice on a wild-type (WT) C57BL/6 background that express the class I alleles HLA-A2, HLA-B7, or HLA-B27 as fully native molecules in association with human β2-microglobulin (β2-m) or as human/mouse hybrid molecules consisting of the HLA α1α2 Ag-binding domains linked to the mouse α3, transmembrane, and cytoplasmic domains (16). Although such mice are useful for certain applications, coexpression of endogenous H2 class I may reduce the efficiency of detection of HLA-restricted Ag-specific responses. The possibility of a TCR repertoire bias inherently favoring mouse over human MHC recognition could also influence detection of HLA-restricted responses. Finally, related peptide-binding specificities of certain human and mouse alleles (i.e., HLA-B7 and H2-Lq) would also complicate analyses in HLA Tg mice coexpressing these mouse class I molecules. With these concerns in mind, we have transferred each of the above HLA class I Tgs onto a background naturally deficient for H2-L and double knockout (DKO) for H2-Kb and H2-Dq expression (17). The absence of H2 class I expression eliminates possible competition with Tg HLA, and thus the majority of CD8+ T cells in the periphery of these Tg HLA/H2 DKO mice are specific for the human allele. In this study, we compare each of the HLA\(^{\text{hybrid (hyb)}}\)/DKO and HLA\(^{\text{native (nat)}}\)/DKO strains with respect to the level of peripheral CD8+ T cells as well as antiviral CTL responses. Based on recognition of the same influenza A peptide in allele-matched HLA Tg mice and humans, we go on to show that this model provides a powerful approach for identifying new viral CTL epitopes recognized in the context of human MHC class I alleles.

### Materials and Methods

**HLA class I Tg/H2-K\(^{-/-}\)/D\(^{-/-}\) DKO mice**

The HLA\(^{\text{Aa}}\) and HLA\(^{\text{Am}}\) class I Tg mouse strains, originally generated in (B6/SILF1\(^{a}\)) backcrossed a minimum of 10 generations onto C57BL/6 (referred to as Tg HLA\(^{\text{nat}}\)/B6 and HLA\(^{\text{Aa}}\)/B6) and described in detail previously (15, 16). C57BL/6 J mice for precluding or experiments were from The Jackson Laboratory (Bar Harbor, ME). Tg offspring were identified by tail DNA hybridization or by flow cytometry of PBLs (15, 16). Although all Tg HLA class I molecules reacted with mAb B9.12.1, Tg HLA-B27\(^{\text{am}}\), HLA-B27\(^{\text{nat}}\), and HLA-B7\(^{\text{am}}\) and HLA-B7\(^{\text{nat}}\) could also be detected with mAb ME1, and Tg HLA-A2\(^{\text{hyb}}\) and A2\(^{\text{nat}}\) with mAb MA2.1 (15, 16).

Each Tg HLA class I line carries the cloned genomic gene encoding the fully native class I allele (15, 16). The HLA-B27\(^{\text{am}}\) (B*270252) and A2\(^{\text{nat}}\) (A*020111) were generated by coinjection of the HLA and β2-m genes and are referred to as Tg HLA-B27\(^{\text{am}}\)/β2-m and Tg HLA-A2\(^{\text{nat}}\)/β2-m, respectively (16). The HLA-B7\(^{\text{nat}}\)/(B*07021)/β2-m Tg mice were bred by deriving HLA-B7\(^{\text{nat}}\)/β2-m Tg mice with Tg for the β2-m gene, as described (15, 16). Each HLA\(^{\text{Aa}}\)/DKO line carries a cloned hybrid genomic gene containing the human exons for the B*270252, A*020111, or B*07021 a1 and a2 domains linked to the mouse exons for the α3, transmembrane, and cytoplasmic domains of H2-K\(^{\text{i}}\) (16). Although efficient surface expression of class I Tg HLA\(^{\text{Aa}}\) molecules requires coexpression of β2-m, Tg HLA\(^{\text{Aa}}\)/DKO class I molecules are expressed efficiently at the cell surface in the absence of β2-m due to association with endogenous murine β2-m (β2-m) (15, 16). For each HLA\(^{\text{Aa}}\)/DKO Tg construct, multiple lines were originally established and characterized with respect to Tg expression by tissue RNA blot hybridization and flow cytometry (15, 16). Individual Tg constructs were described in detail previously (15, 16). A representative study on the basis of a normal breeding and Tg transmission rate, an appropriate tissue distribution of Tg RNA, and cell surface expression at a level similar to each other and to endogenous H2 class I (16). These Tg HLA class I lines on the B6 (H2\(^{b}\) ) background are formally referred to as follows: Tg(B*270252/β2-m)/H2\(^{b}\) chamber; Tg(B*270252/β2-m)/H2\(^{b}\) chamber; Tg(A*020111/β2-m)/H2\(^{b}\) chamber; Tg(A*020111/β2-m)/H2\(^{b}\) chamber; Tg(B*07021/β2-m)/H2\(^{b}\) chamber, and Tg(B*07021/β2-m)/H2\(^{b}\) chamber.

The HLA\(^{\text{Am}}\) and HLA\(^{\text{Aa}}\) Tg mice on the B6 background described above were subsequently established on a background deficient for expression of endogenous H2 class I (i.e., H2-K\(^{-/-}\) and H2-D\(^{-/-}\) ) by backcrossing each Tg HLA mouse at least six times with H2-K\(^{-/-}\)/H2-D\(^{-/-}\) DKO mice previously backcrossed onto B6 (17). These HLA\(^{\text{Am}}\)/DKO and HLA\(^{\text{Aa}}\)/DKO strains are formally referred to as follows: Tg(B*270252/β2-m)/DKO chamber; Tg(B*270252/β2-m)/DKO chamber; Tg(A*020111/β2-m)/DKO chamber; Tg(A*020111/β2-m)/DKO chamber; Tg(B*07021/β2-m)/DKO chamber; and Tg(B*07021/β2-m)/DKO chamber.

Mice deficient for H2 (i.e., H2-K\(^{-/-}\)/D\(^{-/-}\)) (H2-K\(^{2-}\)/D\(^{-/-}\) (i.e., H2-D single KO) expression only (18) were also used for specific experiments as well as for breeding with DKO mice to generate offspring having only one H2-D gene (H2-K\(^{-/-}\)/D\(^{-/-}\) or one H2-K gene (H2-K\(^{2-}\)/D\(^{-/-}\)). Single KO and DKO parental strains were kindly provided by F. Lemonnier (Institut Pasteur, Paris, France). All mice were housed in a pathogen-free animal facility at The Hospital For Sick Children according to the guidelines of the Canadian Council of Animal Care.

### Influenza A virus, peptides, and cytotoxicity assays

Mice were infected by i.p. injection of 300 hemagglutinating units of influenza A virus X31 (SPAFAS, North Franklin, CT) in PBS. In H2\(^{a}\) mice, the anti-flu CTL response has been shown to recognize nucleoprotein (NP) peptide aa 366–374 (NP366–374; ASNENMETM) in association with H2-D\(^{a}\) in association with H2-D\(^{a}\) single KO) expression only (19). In HLA-B27\(^{\text{am}}\) humans, B27-restricted CTLs have been shown to recognize flu peptide NP383–391 (SRYWAIRTR) (20). These and all other peptides used in this study were from Research Genetics (Huntsville, AL).

Spleen cells from mice infected 3 wk earlier were restimulated in vitro for two 6- to 7-day periods with the indicated peptide in αMEM (Life Technologies, Grand Island, NY) containing 10% FCS (Sigma-Aldrich, St. Louis, MO), 10 mM HEPEs, 5 x 10^-5 M 2-ME, penicillin/streptomycin (Life Technologies), and 0.5 U/ml of mouse IL-2 (21). Autologous peptide-pulsed spleen cells were the source of APCs and T cells for these methods. For the second period, viable cells were harvested and restimulated with peptide-pulsed irradiated (200 rad) strain-matched spleen cells that served as APCs. Cell aliquots were stained on the indicated day with anti-CD8 and anti-CD4 mAbs to monitor the CD8+ and CD4+ populations. On the day of 51Cr release assay, target cells were labeled with Na2 51CrO4 (16) and then pulsed with the indicated peptide. Targets were Con A-stimulated (2 days with 2 μg/ml) strain-matched spleen cells. After 4 h of incubation of

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3 Abbreviations used in this paper: Tg, transgenic; β2-m, β2-microglobulin; DKO, double knockout; β2-m, human β2-m; HA, hemagglutinin; hyb, hybrid; KO, knockout; LN, lymph node; mfi, mean fluorescence; nat, native; NP, nucleoprotein; WT, wild type.
effects with targets at various E:T ratios, supernatants were harvested and counted. Specific lysis was calculated as (experimental — spontaneous release)/(maximal — spontaneous release) × 100% (16, 21).

Flow cytometry

The mAbs and detection reagents used for flow cytometry and their specificities and sources are as follows: CD3 PerCP, CD4 PE, CD8a FITC, CD8α PerCP, TCR Vβ8.3 FITC, H2-K b biotin, and SA-PE were from BD Pharmingen (San Diego, CA); ME1 (specific for HLA-B7, HLA-B27, and HLA-Bw22), MA2.1 (specific for HLA-A2), and 28-14-85 (specific for H2-D b, H2-L b, and H2-Dq) were from American Type Culture Collection (Manassas, VA). FITC-conjugated F(ab̲)₂ goat anti-mouse IgG (Fc specific) and FITC-conjugated F(ab̲)₂ goat anti-rat IgG (Fc specific) were from Accurate Chemical and Scientific (Westbury, NY). The anti-TCR Vβ mAbs were obtained from J. Penninger (Amgen Institute, Toronto, Canada), and were specific for Vβ2 (B20.6), Vβ6 (44.22.1), Vβ7 (TR310), Vβ8.2 (F23.2), Vβ8.1/8.2 (K116), Vβ11 (KT11), Vβ12 (MR11-1), and Vβ14 (14.2) (22). Following two- or three-color staining, cells were washed, fixed in 1% paraformaldehyde in PBS, and then analyzed on a BD Biosciences FACScan flow cytometer (Mountain View, CA) (16, 21). Data analysis was performed using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

Results

Expression of Tg HLA class I leads to an increased population of peripheral CD8⁺ T cells in H2 class I-deficient mice

To facilitate studies of allele-specific human MHC class I function in vivo, a series of HLA class I transgenic (HLA class I Tg) mice that express the HLA-B27, HLA-B7, or HLA-A2 alleles as either fully human native (HLA nat ) molecules or as human (αα/αβ) mouse (α3, transmembrane, cytoplasmic domains) hybrid (HLA hyb ) molecules were established on the C57BL/6 (B6; H2 b ) background and described previously (16). Individual representative lines for each Tg were selected for further studies based on expression of the human molecules at close to physiological levels that were similar to each other and to endogenous H2 class I (16, 23). Tg B27 nat and A2 nat were coexpressed with hβm (B27 nat/hβm, A2 nat/hβm), while B7 nat was expressed in the presence of B7 nat/hβm or absence (B7 mut ) of human βm (15, 16, 23). Compared with the Tg HLA nat molecules, the B27 hyb, B7 hyb, and A2 hyb molecules associated with mβm and were efficiently expressed at the surface of Tg cells in the absence of human βm (15, 16).

As H2-K b and H2-D b were both expressed in these original Tg mice on the H2 b B6 background, the proportion of the peripheral T cell repertoire that was specific for Tg HLA vs endogenous H2 class I was unknown. Furthermore, given some apparently inconsistent results from different groups using various HLA Tg lines (11, 18.7%; 12.5-fold increase), and HLA-B27 nat /h2 mut (Fig. 2i; 11%; 7.3-fold increase), HLA-B7 hyb (Fig. 2i; 13.9%; 9.3-fold increase), HLA-A2 hyb (Fig. 2i; 18.7%; 12.5-fold increase), and HLA-B27 nat /hβm (Fig. 2i; 7.9%; 5.3-fold increase). Thymocytes also contained a slightly increased percentage of single-positive CD8⁺ thymocytes for each strain compared with the DKO (not shown). In contrast to the above results, the percentage of CD8⁺ T cells for LN (and spleen and thymus (not shown)) in HLA-B7 hyb/DKO mice with (Fig. 2ii) or without hβm (not shown) and HLA-A2 hyb/hβm/DKO (Fig. 2viii) mice was only slightly greater than the level in the non-Tg DKO strain (i.e., less than 2-fold).

The finding of a higher level of peripheral CD8⁺ T cells for mice Tg for each HLA hyb allele vs the corresponding HLA nat allele (i.e., HLA-B27, 11.0 vs 7.9%; HLA-B7, 13.9 vs 2.8%; HLA-A2, 18.7 vs 2.9%) is presumably due to the influence of improved intermolecular interactions during selection and in the periphery dependent on the α3, transmembrane, and/or cytoplasmic domains of the hybrid vs native molecules in the mouse background (14, 16). Interestingly, unlike HLA-A2 nat and HLA-B7 nat mice whose peripheral CD8⁺ T cell level was only slightly above that in non-Tg DKO mice, HLA-B27 nat lymphoid tissues contained a significantly higher proportion of CD8⁺ T cells (p < 0.002). Allelic differences in the level of CD8⁺ T cells also exist for the HLA hyb molecules because expression of HLA-A2 hyb consistently gave rise to the highest level of peripheral CD8⁺ T cells, followed by HLA-B7 hyb and HLA-B27 hyb. Quantitation of Tg HLA expression at the cell surface for each native and hybrid molecule indicates that these allelic differences in peripheral CD8⁺ T cell levels are not simply due to differences in expression level of Tg HLA in the various Tg lines (16, 23) (Fig. 1).

Although the level of peripheral CD8⁺ T cells for several of the Tg HLA lines was significantly greater than in the DKO strain, it was still less than that for WT non-Tg H2 b mice (Fig. 2). This lower level could be due to quantitative effects of MHC expression
level because there are four expressed H2b gene loci in C57BL/6 mice, while there is only one HLA gene locus in the HLA Tg DKO mice. To investigate this possibility, we first examined the influence of the number of expressed H2 class I genes on the size of the peripheral CD8 T cell population. For this purpose, mice having only one H2-D (K/−/−D+/+) or one H2-K (K+/−/−D−/−) gene were generated by appropriate breedings (see Materials and Methods). LN and spleen cells from each strain were analyzed by flow cytometry to determine the level of CD8 and CD4 cells (Fig. 3A). Compared with the WT (K+/+D+/+), mice with a single expressed H2-D or H2-K gene have a reduced level of CD8 T cells in the periphery (Fig. 3Ai; WT, 24.2%; iii, K+/−/−D−/−, 8.6%; iv, K+/−/−D−/−, 16.3%). Thus, mice with a greater number of H2b class I genes (e.g., four H2b genes in WT C57BL/6) have a higher percentage of peripheral CD8 T cells than mice having a lower number of H2b genes. This relationship is particularly evident when the results are displayed graphically, as in Fig. 3B. Interestingly, these studies also reveal a locus-specific effect, as mice with only one H2-K gene have a higher percentage of CD8 T cells than H2-D counterparts (Fig. 3).

The finding that the levels of peripheral CD8 T cells for all three HLAαβb class I Tg/DKO strains carrying a single human allele (B7αβb, B27αβb, or A2αβb) are in the same range as for mice having only one H2-K or H2-D gene (Fig. 3B) implies that there is no significant limitation in HLA class I-dependent selection of CD8 T cells in this Tg HLA/DKO model. Furthermore, the allele- and locus-specific differences between the HLA Tg lines may reflect similar effects as for endogenous H2 class I molecules.

Peripheral CD8 T cells in Tg HLA/H2-K/−/−D−/− DKO mice display a similar spectrum of TCR Vβ chain usage compared with non-Tg WT

Although expression of all Tg HLAαβb, and to a lesser degree HLAααb, molecules leads to the appearance of CD8+ T cells in the periphery, it was unknown whether the naive TCR repertoire was similar or deviated compared with the non-Tg H2b WT mouse. To examine this, three-color flow cytometry was used to identify CD3+CD8+ LN T cells for subsequent analysis of the relative abundance of cells expressing various TCR Vβ-chains (Vβ2, 6, 7, 8.1, 8.2, 8.3, 11, 12, and 14) that are useful for distinguishing subfamilies of a portion of the T cell repertoire (22). Fig. 4 shows the percentage of CD3+CD8+ T cells that express each indicated TCR Vβ for the various mice (i, non-Tg WT (K+/+D+/+); ii, DKO (K/−/−D−/−); iii, B27αβb/DKO; iv, B27αβb/DKO; v, B7αβb/DKO; and vii, A2αβb/DKO). Although there was some variation for mice of a given non-Tg or HLA Tg genotype, the trend is readily apparent. Each TCR Vβ subpopulation that is present at a higher level in CD8+ cells of H2b WT mice is also more abundant in CD8+ T cells of each of the HLAαβb and HLAααb Tg strains examined. Similarly, those that are less abundant in WT mice are also less abundant in each HLA Tg strain. Thus, not only does expression of Tg HLA class I lead to a significant level of peripheral CD8+ T cells in the absence of H2-K and H2-D expression, these cells display an overall similar distribution of TCR Vβ-chains, signifying a grossly similar TCR repertoire compared with the non-Tg WT.

Recognition of the same immunodominant influenza A NP peptide by Tg HLA-B27αβb and B27ααb CD8+ T cells as in B27+ humans

To determine whether the CD8+ T cells in the periphery of the Tg HLA/H2-K, H2-D DKO mice are functionally restricted by the human class I allele, the CD8+ CTL response to influenza A infection was examined. In B27+ humans, a major portion of the B27-restricted flu-specific CTL response is directed at flu NP peptide 383–391 (NP383–391), with most of these T cells expressing the TCR Vβ7 chain (20, 25). In H2b mice, the anti-flu CTL response is directed mainly at NP366–374, with the TCR Vβ8.3 chain dominating this response (19, 26). To test for these responses, splenic T cells from influenza A-infected Tg HLA-B27αβb/DKO, Tg HLA-B27ααb/hβ2m/DKO, non-Tg/DKO, and non-Tg/WT mice were restimulated in vitro for two to seven days, as described in Materials and Methods, with NP383–391 (B27-restricted), NP366–374 (H2-Dβ-restricted), control peptide, or no peptide. Fig. 5 shows the results of flow cytometric analyses performed at 0, 6, and 13 days of stimulation to monitor changes in the CD8+ and CD4+ populations. Incubation of splenic cells from infected Tg B27αβb/DKO mice with NP383–391 resulted in a strong induction of CD8+ cells from 6.7% on day 0 to 66.0% on day 6 to 97.5% on day 13 (Fig. 5c, ii and iii). This expansion was similar to that for cells from infected non-Tg WT H2b mice stimulated with H2-Dβ-restricted NP366–374 (Fig. 5a, i–iii; 12.5% on day 0 to 63.3% on day 6 to 96.7% on day 13). There was no significant expansion of CD8+ cells for infected DKO mice incubated with either NP366–374 (Fig. 5b, i–iii) or NP383–391 (not shown), or for cells from infected non-Tg WT H2b mice incubated...
with NP383–391 (not shown). There was also a significant CD8+ cell expansion for the Tg B27

with NP383–391 peptide, although the increase was slightly less than that for B27

vi. For these reasons, we concluded that the CD8+T cell expansion for Tg B27

and B27 nat/h cells was due to the induction of a CD8+ T cell response to NP383

H2-D\(^d\)/NP366–374-specific responders did not kill H2b targets pulsed with NP383–391 (Fig. 6Ai, line B) or B27

in HLA-B27 hyb/DKO and HLA-B27 nat/h mice was 80.0% on day 13; Fig. 5d, i–ii). Thus, peripheral CD8+ T cells detected in LN cells for each of the strains shown in A (i.e., i–iv), as well as for several of the HLA Tg strains shown in Fig. 1 (i.e., B27

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with NP383–391 pulse, stimulating cells were established as above and tested with

infection of B27

374 (Fig. 6Ai, line A, and not shown). Thus, CD8+ T cells in the periphery of Tg B27

and B27 nat/h mice were B27 restricted and respond to the same viral peptide recognized in the context of HLA-B27 in humans.

CTL assays were performed to test for HLA-B27-restricted killing using cultures stimulated with peptide for 13 days as above. Strong lysis was observed for NP383–391-stimulated responders from infected Tg B27

and B27 nat/h mice when tested on NP383–391-pulsed B27

and B27nat/h\(\beta_2m\)/DKO Con A-stimulated target cells (lines E and G in Fig. 6A, ii and iii). These same NP383–391-stimulated responders did not give killing above the background levels observed for B27

and B27nat/h\(\beta_2m\)/DKO targets pulsed with NP366–374 (lines F and H in Fig. 6A, ii and iii) or no peptide (not shown), or non-Tg H2b targets pulsed with NP383–391 (line I, Fig. 6A, ii and iii). The level of B27/NP383–391-specific killing by B27

and B27nat/h\(\beta_2m\)/DKO responders was only slightly higher than for B27

and B27nat/h\(\beta_2m\)/DKO responders and comparable with that for responders from infected non-Tg H2b mice stimulated and tested on H2b cells pulsed with NP366–374 (Fig. 6Ai, line A). These

H2-D\(^d\)/NP366–374-specific responders did not kill H2b targets pulsed with NP383–391 (Fig. 6Ai, line B) or B27

and B27nat/h\(\beta_2m\)/DKO targets pulsed with NP366–374 (Fig. 6Ai, lines C and D) or NP383–391 (not shown). No killing was observed for cultures from infected DKO mice stimulated and tested with NP366–374 or NP383–391 on H2b or B27 target cells (Fig. 6Aii, line M, and not shown). Thus, CD8+ T cells in the periphery of Tg B27

and B27nat/h\(\beta_2m\)/DKO mice are B27 restricted and respond to the same viral peptide seen in natural influenza A infection of B27+ humans as immunodominant.

To examine the complexity of the TCR V\(\beta\) repertoire of infected Tg HLA-B27/DKO mice following in vitro stimulation with NP383–391 peptide, 13-day cultures established as above were stained for expression of CD4 and CD8 in conjunction with the TCR V\(\beta\) chain expression by

and B27 nat/h mice (i.e., i–iv) as well as for several of the HLA Tg strains shown in Fig. 1 (i.e., B27

and DKO; B27

and A2 hyb/DKO) are represented as bar graphs. The number of expressed MHC class I gene loci is indicated below each mouse genotype.

FIGURE 3. The influence of the number of H2b class I gene loci on the level of CD8+ T cells detected in peripheral lymphoid tissues. A, LN cells from i, non-Tg H2b WT (WT, K\(^{\text{\text{ii}}}/D\(^{\text{\text{ii}}}\)); ii, K\(^{\text{\text{ii}}}/D\(^{\text{\text{ii}}}\)/DKO; iii, K\(^{\text{\text{ii}}}/D\(^{\text{\text{ii}}}\); and iv, K\(^{\text{\text{ii}}}/D\(^{\text{\text{ii}}}\) mice were stained for expression of CD4 and CD8. The numbers in the lower right quadrants represent the percentage of CD8+ cells in a lymphocyte gate. B, The percentages of CD8+ T cells detected in LN cells for each of the strains shown in A (i.e., i–iv), as well as for several of the HLA Tg strains shown in Fig. 1 (i.e., B27

and A2 hyb/DKO mice. LN cells from each mouse type were stained for expression of CD3, CD8, and B27 in humans. sponsive to the same

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on day 6 to 80.0% on day 13; Fig. 5d, i–iii). Thus, peripheral CD8+ T cells in HLA-B27

and HLA-B27

and B27 nat/h cells was due to the induction of a CD8+ T cell response to NP383

FIGURE 4. Three-color flow cytometric analysis of the repertoire of TCR V\(\beta\)-chain expression by i, non-Tg WT (WT, K\(^{\text{\text{ii}}}/D\(^{\text{\text{ii}}}\)); ii, DKO (K\(^{\text{\text{ii}}}/D\(^{\text{\text{ii}}}\)); iii, B27

and B27 nat/h targets pulsed with NP366–374 (Fig. 6Ai, lines C and D) or NP383–391 (not shown). No killing was observed for cultures from infected DKO mice stimulated and tested with NP366–374 or NP383–391 on H2b or B27 target cells (Fig. 6Aii, line M, and not shown). Thus, CD8+ T cells in the periphery of Tg B27

and B27nat/h\(\beta_2m\)/DKO mice are B27 restricted and respond to the same viral peptide seen in natural influenza A infection of B27+ humans as immunodominant.

To examine the complexity of the TCR V\(\beta\) repertoire of infected Tg HLA-B27/DKO mice following in vitro stimulation with NP383–391 peptide, 13-day cultures established as above were stained for expression of CD4 and CD8 in conjunction with the same panel of TCR V\(\beta\)-chains tested previously. Consistent with a previous report (26), CD8+ T cells expressing TCR V\(\beta\)8.3 dominated the CD8+ T cell response of flu-infected non-Tg H2b WT mice following 13 days of stimulation with NP366–374 (Fig. 6Bi). In contrast, the anti-NP383–391 CD8+ CTL response of Tg B27

and B27nat/h\(\beta_2m\)/DKO mice consisted of >40% V\(\beta\)8.1+ cells at day 13 (Fig. 6Bii). Interestingly, although NP383–391 also stimulated a strong CD8+ effector population for Tg B27

and B27 nat/h mice (Figs. 5d and 6Aii), none of the TCR V\(\beta\)-chains tested, including
Vβ8.1. was found to consistently dominate this population (not shown). This lack of expansion of Vβ8.1+ cells for the B27αβm/DKO group is not because this population is absent from the naive repertoire of these mice (Fig. 4iv). Thus, although CD8+ T cells from both B27αβm/DKO and B27αβm/DKO mice are able to respond to a specific flu peptide known to be restricted by B27 in humans, different TCR Vβ chains are used.

**Identification of candidate flu epitopes restricted by HLA-B7**

The above findings showing that the same peptide is recognized in response to flu infection of B27/DKO Tg mice as in B27+ humans suggest that this model should be useful for identifying new HLA class I-restricted viral T cell epitopes in vivo. Although flu peptides recognized in association with HLA-B27 and HLA-A2 are known (20, 27), flu peptides restricted by HLA-B7 have not been reported. To identify such B7-restricted flu peptides, splenocytes from flu-infected HLA-B7αβm/DKO mice were stimulated as above with individual candidate peptides (see below) and then analyzed by flow cytometry to test for an increased relative abundance of CD8+ cells and by cytotoxicity for B7-restricted killing. Candidate peptides were first identified by using the SYFPEITHI program from Rammensee et al. (Ref. 28; www.syfpeithi.de/) to screen influenza A X31 protein sequences for the HLA-B7 binding motif (29, 30). Based on this analysis, two high ranking peptides were identified (NP418–426 and hemagglutinin (HA)339–347) and synthesized for the studies in this work. HA339–347 ranked highest of all HA peptides and was assigned a score of 22 of 30 by the program (28). NP418–426 ranked second of all X31 NP peptides, receiving a program score of 19 of 30. When this type of analysis was applied to HLA-A2, the program successfully identifies a flu peptide known to be functional as a CTL epitope (M58–66) at the top of the list of candidates. Interestingly, this was not the case for B27, as the program identified and ranked a number of candidate peptides in multiple flu X31 proteins above NP383–391, the only functional B27 CTL epitope known to date.

In the case of the HLA-B7 peptides, while NP418–426 from influenza A PR8 strain has been shown to bind HLA-B7 in vitro, HA339–347 PR8 did not perform as well in this assay (31). Although NP418–426 is conserved between both X31 and PR8 strains (LPPDRTTVM), HA339–347 is not (VPEKQTRGL in X31; IPSIQSRGL in PR8) (32). Fig. 7A shows that incubation of splenocytes from flu X31-infected Tg HLA-B7αβm/DKO mice with NP418–426 leads to strong induction of a CD8+ population by day 14 (96.6% CD8+ on day 14 (ii) vs 11.6% on day 0 (i). The cytotoxicity assays performed with day 14 cultures (Fig. 7Bii) showed that this population is able to lyse B7αβm/DKO targets pulsed with NP418–426, but not pulsed with an irrelevant peptide (NP383–391) or no peptide. These responders also did not kill non-Tg DKO cells or B27αβm/DKO cells pulsed with or without NP418–426 (not shown). Analysis of the TCR Vβ repertoire of the 14-day NP418–426-stimulated Tg HLA-B7αβm/DKO culture showed that cells expressing the Vβ8 chain were a dominant population (Fig. 7Bii). In contrast to the results of Fig. 7 for peptide NP418–426, similar analyses conducted for peptide candidate HA339–347 failed to detect any significant induction of CD8+ CTLs from flu-infected Tg HLA-B7αβm/DKO mice (results not shown).

**Discussion**

Tg mice expressing individual HLA alleles have significant potential as a model for studying T cell responsiveness to infection- or tumor-associated Ags in the context of the human MHC molecules. However, due in part to coexpression of endogenous mouse H2 class I molecules, it was not clear from previous studies the extent to which Tg mouse T cells were restricted by the human allele and recognized the same peptides as in allele-matched
humans. For instance, at several levels in MHC class I biogenesis in thymic development and peripheral function of CD8$^+$ T cells, it was possible that competition with endogenous H2 class I limited the ability of Tg HLA class I molecules to achieve optimal function despite expression as self. Attempts to overcome some of these effects include coexpression of hβ2m to overcome inefficient...
FIGURE 7. Analysis of anti-influenza A peptide-specific CD8+ T cell response in Tg HLA-B7hyb/DKO mice. A. CD4 vs CD8 flow cytometric profiles following 0 (i) and 14 (ii) days of stimulation of splenocytes from influenza A, splenocytes from Tg HLA-B7hyb/DKO mice were stimulated in vitro as above for 14 days with flu peptide NP418–426. B. Analysis of cytotoxicity of peptide-induced CD8+ T cells of Tg HLA-B7hyb/DKO mice. Three weeks postinfection with influenza A, spleen cells from Tg HLA-B7hyb/DKO mice were stimulated in vitro as above with peptide NP418–426. On days 0 and 14, cells were stained for expression of CD4, CD8, and various TCR Vβ-chains. By first gating on the CD8+ population, the percentage of CD8+ T cells expressing each of the tested TCR Vβ chains was determined. The values shown are corrected for any differences in the extent of CD8+ T cell induction in each mouse on the day of the experiment, in which the CD8+ T cells in influenza A-infected non-Tg H2b WT mice stimulated with NP366–374 (not shown) are taken as 100%. The anti-NP418–426/B7hyb response is dominated by Vβ6+ cells. association of fully native HLA class I H chains with mβ2m (15), replacement of the human class I α3 domain with mouse α3 to overcome inefficient interactions with mβ2m and mouse CD8 (14, 16), breeding of the HLA Tg onto apparently more favorable mouse H2 backgrounds (33), introduction of a Tg construct encoding linked β2m and HLA onto a mβ2m-deficient background to reduce surface expression of H2 class I (34), and coexpression of a human CD8 Tg along with Tg HLA to facilitate species-specific CD8 interactions (35). Although these efforts have been instructive, questions still remain about what form this type of model should take to optimize efficient and specific function of the human MHC molecules.

We previously described a panel of HLA class I Tg mice on the WT H2b background that express the HLA-B27, HLA-B7, or HLA-A2 alleles as fully human (HLAnat) molecules or as hybrid human/mouse (HLAhyb) molecules (16). To eliminate the influence of H2b class I expression on Tg HLA function, each strain was bred with a H2-Kb/H2-Dd doubly-deficient (DKO) strain (17) to generate mice in which the only classical class I expression is the human molecule. A similar approach has been taken by another group for a HLA-A2 Tg analogous to the A2hyb Tg studied in this work (24). Relative to the greatly reduced number of CD8+ T cells in lymphoid tissues of the parental DKO strain, expression of each of the three Tg HLAhyb alleles, as well as Tg HLA-B27nat/hβ2m, led to development of a significant peripheral CD8+ T cell population (Fig. 2). As the only classical class I expression in these mice is the human allele, these cells are assumed to be restricted and tolerant to the Tg HLA molecule.

Compared with previous studies of HLA Tg mice on a WT background (11–16), the absence of endogenous H2 class I-specific CD8+ T cells in the mice described in this work allows a more direct comparison of the ability of each Tg HLA class I allele to mediate thymic development and peripheral function. Our finding on the DKO background of a higher level of peripheral CD8+ T cells for all three HLAhyb class I Tg strains compared with the corresponding HLANat strains implicates the mouse class I α3, transmembrane, and/or cytoplasmic domains in the HLAhyb molecules as mediating this effect. Although intermolecular species-specific interactions involving any of these three domains may be improved, it is likely that a major effect is on interactions of mouse CD8 on developing and mature T cells with the Tg HLABhyb molecule. These increased interactions would be expected to facilitate improved positive selection in the thymus, with the result being a greater number of CD8+ T cells exiting the thymus and populating the periphery. Such improved interactions may also facilitate the response of peripheral CTLs to Ag, as suggested by previous results from ourselves and others (14, 16).

For HLA-B7nat/hβ2m/DKO and A2nat/hβ2m/DKO Tg mice, the level of peripheral CD8+ T cells is only slightly above the background level in the parental DKO mice. These results suggest that positive selection on Tg HLA-A2 and HLA-B7 class I molecules...
in this model is strongly dependent on interactions with CD8 during T cell development. A possible explanation for the cells that do appear in the periphery is that the B*705 and A2*0401 molecules may be able to weakly interact with mouse CD8, thus possibly allowing positive selection of T cells with TCRs with increased affinity able to compensate for the reduced contribution from CD8 (14). Alternatively, if these two HLA*0101 molecules are unable to functionally interact with mouse CD8 at all during selection, then this peripheral population may express even higher affinity TCRs and be completely CD8 independent. Despite the low level of these cells in the A2*0401/h-2 m/DKO and B7*0501/h-2 m/DKO mice, it is interesting that the level of CD8 expression appears to be in the normal range (Fig. 2). It will be important to distinguish between the above possibilities.

In contrast to the Tg A2*0401/h-2 m/DKO or B7*0501/h-2 m/DKO mice, there was a significant peripheral CD8+ population in the Tg HLA-B27*0501/h-2 m/DKO mice. This was not due simply to differing levels of Tg HLA expression, as the level of B7*0501 and B7*0501 at the cell surface was similar to each other and only slightly greater than A2*0401 (16, 23, and not shown). Therefore, some aspect of the structure of the HLA-B27*0501 molecule permits development of CD8+ T cells in the DKO background more efficiently than the other two HLA+ alleles. Presumably, this is due to one of the following reasons: either 1) B7*0501 interacts suboptimally with mouse CD8 to the same extent as A2*0401 and B7*0501, but due to some feature of the a1/a2 cleft structure, is inherently capable of interacting with and selecting a broader array of TCRs; or 2) B7*0501 interacts more effectively with mouse CD8 than the other two HLA+ alleles, and as a result, selects a greater number of developing T cells to the CD8+ lineage. Whichever is correct, the CD8+ T cells in the Tg B27*0501/h-2 m/DKO mice do not appear to be an unusual subpopulation, as the TCR Vß repertoire was broad and grossly similar to both WT H2b and Tg HLA-B27*0501/DKO mice (Fig. 4). In addition, at least a portion of this population is restricted to B27 and responsive to infection and stimulation with the same flu NP peptide (383–391) restricted by this allele in Tg HLA-B27*0501 mice and B27+ humans (Figs. 5d and 6a).

Although the level of CD8+ T cells for all three HLA*0101 alleles was greater than for the corresponding HLA+ alleles, it appeared initially that this was still less than for WT H2b mice. However, more detailed analyses showed that the level of CD8+ T cells for each Tg HLA*0101 class I molecule was in the same range as for non-Tg mice carrying a single expressed H2-Kb or H2-Dd gene (Fig. 3). Interestingly, these studies also revealed that expression of a single H2-Kb gene led to a higher level of CD8+ T cells than for a single H2-Dd gene. Based on these differences in efficiency in positive selection for H2 class I molecules, the differences in the CD8+ T cell levels for the three HLA*0101 strains may result from similar locus- or allele-specific effects. Taken together, these analyses lead us to conclude that development of the CD8+ lineage in Tg HLA*0101/DKO mice occurs efficiently and that there are no major limitations compared with H2 class I-dependent CD8+ T cell development.

An important application for the HLA Tg model is in identifying and characterizing T cell epitopes recognized in the context of particular HLA alleles for specific disease- or infection-associated Ags. To become useful in this way, it is essential that the Tg HLA alleles act as restriction elements in the mice with an Ag-presentation specificity similar to that in allele-matched humans. To date, only few studies have been conducted to address this issue, and these have been mainly for HLA-A2 (14, 24, 36). Our results demonstrate that CD8+ T cells in both the Tg HLA-B27*0501/DKO and HLA-B27*0501/h-2 m/DKO mice are functional and B27-restricted for recognition of influenza A Ags following infection. Furthermore, both B27*0501 and B27*01 mediate strong CTL responses against flu NP383–391 known to be immunodominant in the B27-restricted response against flu in B27+ humans (Figs. 5 and 6). Although we have not shown this peptide to be immunodominant in the B27 Tg mice studied in this work, it is clear that this is a major response against flu infection in these animals. Additional experiments are in progress to address this point directly.

Although the in vitro stimulated B27*0501-restricted anti-NP383–391 CD8+ T cell response occurred with a similar kinetics and reached a similar level compared with the H2-Dd-restricted anti-NP366–374 response of non-Tg WT mice (Fig. 5, c and a), the B27*0501/NP383–391-specific response appeared to reach a lower maximal level of CD8+ cells (i.e., ~80% for B27*0501/DKO vs 97.5% for B27*0501/DKO and 96.7% for H2b WT by day 13; see Fig. 5). We are uncertain whether this reflects a lower Ag-specific frequency in the naive repertoire or reduced proliferative expansion of Ag-specific cells in the B27*0501/h-2 m/DKO mice, but are examining this issue. If the latter, then it will suggest a role for optimal CD8+ class I interactions in mediating this effect.

Another feature of the Ag-specific response in Tg B27*0501/DKO mice that was similar to the H2-Dd-restricted response was that the induced CD8+ populations were dominated by expression of a single TCR Vß chain (Vß8.1 for B27*0501/NP383–391 and Vß8.3 for Dd/NP366–374; Fig. 6). This finding further demonstrates that HLA-restricted Ag recognition in these mice occurs in an overall similar fashion to that restricted by endogenous H2 class I. However, despite recognition of the same flu peptide (NP383–391) in the context of B27, the response in B27*0501/h-2 m/DKO mice is not mediated by cells expressing TCR Vß8.1 or any other dominant Vß-chain tested. This was not because Vß8.1+ cells were absent from the naive repertoire of B27*0501/h-2 m/DKO mice (Fig. 4), and therefore is most likely due to one of two main reasons. First, it is possible that this induced population is dominated by cells expressing a specific TCR Vß chain different from any tested for with available anti-TCR Abs. Alternatively, it may be that no single TCR Vß chain comes to dominate this Ag-stimulated population. If the naive repertoire in B27*0501/h-2 m/DKO mice is in fact less CD8 dependent, then this latter possibility would suggest that one effect of optimal CD8 interactions is to facilitate efficient stimulation and expansion of specific CD8+ T cell subpopulations following exposure to Ag, with the outcome being oligo- or monoclonal dominance recognizable by a limited distribution of TCR Vß chain expression.

Despite the above, CD8+ T cells from B27*0501 mice stimulated with NP383–391 lyse both peptide-pulsed B27*0501 and B27*01 target cells (Fig. 6a). Similarly, CD8+ T cells from B27*0501 mice stimulated with this peptide kill both NP383–391-pulsed B27*0501 and B27*01 targets. Together, these findings indicate that although B27*0501 appears to function more effectively than B27*01 in mediating development of the CD8+ T cell sublineage in Tg mice, both molecules are able to stimulate strong responses for autologous T cells and can be recognized at the effector stage by induced T cells both from self as well as the alternate strain. It will be of interest to determine the extent to which the naive T cell repertoires of these two strains differ.

An important aspect of the above studies is that by removing the background of H2-Kb- and H2-Dd-restricted CD8+ T cells, any changes detected in the size or repertoire of the CD8+ T cell population following Ag exposure in vivo or in vitro must be due to recognition events in the context of Tg HLA. This was not the case in prior versions of this model in which the Tg HLA was expressed on the WT H2 class I+ background. This complicated previous attempts to use this model to identify new HLA class I-restricted T cell epitopes for a given Ag or to characterize the responding
CD8+ population because one had to contend with and control for a background of H2 class I-restricted Ag-specific responses. Our ability to identify a new HLA-B7-restricted CTL epitope for influenza A by making use of the Tg HLA-B7/b/b/DKO mice (Fig. 7) demonstrates that this model and the approach taken provide an effective strategy and solution to this problem. Although we do not know that the identified peptide (NP418–426) is immunodominant in this anti-flu A/B7/b/b-restricted CTL response, the high level of killing observed implies that this is a major epitope recognized in the context of this allele. We are attempting to confirm this finding by testing for recognition of this peptide by CTLs from HLA-B7+ humans previously exposed to influenza A.

Although the Tg B27/hyb-restricted anti-flu NP383–391 response was dominated by CD8+ T cells that expressed the TCR Vβ8.1 chain, the Tg HLA-B7/b/b/flu NP418–426 response was found to be dominated by Vβ6+ CD8+ T cells (Fig. 7). In humans, the HLA-B27-restricted response against flu NP383–391 has been reported to be dominated by human CTLs that express Vβ7 with Vα12.1, 14.1, or 22 (20, 25), while the HLA-A2-restricted response against flu matrix M58–66 is dominated by Vβ17+ CTLs (27, 37). To further characterize the degree to which HLA-dependent recognition is conserved in HLA allele-matched Tg HLA/DKO mice and humans, it will be important to examine the specific TCR Vβ/ve-chain combinations that are preferentially used in both species.

References