CD8+ T Cells Are Necessary for Recognition of Allelic, But Not Locus-Mismatched or Xeno-, HLA Class I Transplantation Antigens

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CD8+ T Cells Are Necessary for Recognition of Allelic, But Not Locus-Mismatched or Xeno-, HLA Class I Transplantation Antigens

Steven H. Borenstein,* Jeremy Graham, Jeremy Graham, Xiao-Li Zhang,‡ and John W. Chamberlain*‡‡

Although HLA transgenic mice (HLA TgM) could provide a powerful approach to investigate human MHC-specific T cell responsiveness, the extent to which these molecules are recognized by the mouse immune system remains unclear. We established TgM expressing HLA class I alleles A2, B7, or B27 in their fully native form (HLA\textsuperscript{nat}) or as hybrid molecules (HLA\textsuperscript{hyb}) of the HLA α1/α2 domains linked to the H-2K\textsuperscript{*} α3, transmembrane, and cytoplasmic domains (i.e., to maintain possible species-specific interactions). Comparison of each as xeno- (i.e., by non-TgM) vs allo- (i.e., by TgM carrying an alternate HLA allele) transplantation Ags revealed the following: 1) Although HLA\textsuperscript{hyb} molecules induced stronger xeno-CD8\textsuperscript{+} T cell responses in vitro, additional effector mechanisms must be active in vivo because HLA\textsuperscript{nat} skin grafts were rejected faster by non-TgM; 2) gene knockout recipients showed that xenorejection of HLA\textsuperscript{nat} and, unexpectedly, HLA\textsuperscript{hyb} grafts doesn’t depend on CD8\textsuperscript{+} or CD4\textsuperscript{+} T cells or B cells; 3) each HLA\textsuperscript{hyb} strain developed tolerance to “self” but rejected allele- (-B27 vs -B7) and locus- (-B vs -A) mismatched grafts, the former requiring CD8\textsuperscript{+} T cells, the latter by CD8\textsuperscript{+} T cell-independent mechanisms. The finding that recognition of xeno-HLA\textsuperscript{hyb} does not require CD8\textsuperscript{+} T cells while recognition of the identical molecule in a strictly allo context does, demonstrates an α1/α2 domain-dependent difference in effector mechanism(s). Furthermore, the CD8\textsuperscript{+} T cell-independence of locus-mismatched rejection suggests the degree of similarity between self and non-self α1/α2 determines the effector mechanism(s) activated. The HLA Tg model provides a unique approach to characterize these mechanisms and develop tolerance protocols in the context of human transplantation Ags. The Journal of Immunology, 2000, 165: 2341–2353.

The MHC class I and II molecules are highly polymorphic cell surface glycoproteins centrally involved in development and responsiveness of the immune system through their capacity to present self and foreign Ag-derived peptides to T lymphocytes (1, 2). MHC class I molecules (HLA-A, -B, and -C in humans; H-2K, -D, and -L in mice) present peptides to CD8\textsuperscript{+} (cytotoxic) T cells, whereas MHC class II molecules (HLA-DR, -DP, and -DQ in humans; I-A and I-E in mice) present peptides to CD4\textsuperscript{+} (helper) T cells (1, 3, 4). Class I molecules are expressed in association with a smaller nonpolymorphic chain called β2-microglobulin (β2m)\textsuperscript{3} on most somatic cells at levels that vary significantly between tissues and cell types (5, 6). Class II molecules are found mainly on B cells, macrophages, and other APCs as well as on thymic stromal cells.

Although conventional T cell recognition of foreign (i.e., viral) Ag is self-MHC- restricted (7), CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells also respond vigorously when confronted with allogeneic cells expressing non-self class I and II molecules, respectively. This potent cell-mediated immune response is believed to be the primary event involved in allograft rejection, and results from the high frequency of T cells that are alloreactive (i.e., 1–10% of the T cell repertoire) (8, 9). Although it was long believed that such cells directly recognized polymorphic differences between MHC alleles, now known to reside largely within the Ag-binding cleft (10–12), more recent evidence also suggests an important role for indirect recognition of donor MHC (or non-MHC) Ag-derived peptides in association with recipient MHC (13–15). In addition, the finding that different MHC alleles have different peptide-binding specificities (16) suggests that some alloreactive T cells may be influenced by the distinct array of self-peptides presented by foreign MHC molecules at the surface of transplanted tissues (9). Although both the direct and indirect pathways are now generally believed to be important, the relative contribution of each to allograft rejection in vivo is not clear.

In contrast to allografts, the role of the T cell response in rejection of grafts between species (i.e., xenografts) is much less characterized, in part because it occurs only after two earlier stages of rejection, namely, hyperacute rejection and delayed acute vascular rejection (17–22). Studies conducted in vitro circumvent these earlier events and have generally detected a very low T cell response to xenogeneic cells (21, 23). This could be interpreted as resulting from either a low frequency of T cells bearing TCRs able to interact with the polymorphic domains of xenogeneic MHC and/or inefficient cross-species interactions involving coreceptor, costimulatory or accessory molecules, or other incompatibilities between species that influence T cell responsiveness. Given the multiple molecular interactions necessary...
for T cell activation to foreign Ag, it has been difficult to distinguish which of these is correct. This is important to resolve because, although progress has been made toward eliminating the hyperacute response (22), successful in vivo xenotransplantation protocols will require overcoming all three barriers.

Most investigations of the mechanisms of allograft rejection have been conducted with mouse strains differing at multiple major (class I and II) and/or minor MHC loci. With the exception of the nonallelic (i.e., mutant) class I (e.g., K\textsuperscript{bm1} or class II molecules (24) and a limited number of H-2 transgenes (25, 26), few studies have addressed the influence of individual natural class I or II alleles on their own to mediate rejection. It is also unclear whether individual mouse or human alleles are functionally equivalent as transplantation Ags or whether a hierarchy exists. There are also a number of questions that need to be clarified about the cellular response to xenografts, including how complex the xenoreactive T cell population is and whether anti-xeno-MHC T cells respond to the same types of allelic polymorphisms seen by alloreactive T cells or whether there are other species-specific nonallelic differences that dominate. It will also be important to determine whether xeno-MHC molecules induce additional non-T cell effector mechanisms that contribute to rejection.

With these issues in mind, and because current mouse models are limited in what they can reveal about T cell recognition in the context of the human MHC molecules, we and others have explored the possibility that HLA class I and II molecules expressed in transgenic (Tg) mice might provide a useful model for studying HLA-dependent immune function in vivo (5, 27–33). For instance, characterization of the non-Tg mouse T cell response to human MHC expressed in tissues of otherwise genetically identical HLA Tg mice (TgM) should make it possible to identify the specific immune mechanisms involved in xeno-MHC recognition and rejection as well as the importance of MHC-dependent vs -independent interactions in the apparent reduced xenogeneic cellular response detected in vitro. Furthermore, comparison of the non-Tg (i.e., xeno-) and HLA Tg (i.e., allo-) T cell responses to alternate (non-self) HLA Tg alleles could provide a unique approach to compare the structures recognized by T cells that respond to xeno- vs allo-MHC. However, despite some efforts in these directions, the extent to which Tg HLA molecules are functionally recognized by non-TgM or TgM T cells remains relatively unclear. Some reports suggest that fully human (native) HLA class I molecules are recognized only inefficiently at best by the mouse immune system as either xeno- or allotransplanted, or as restriction elements (30, 31, 34–39). In contrast, other reports, often using apparently similar alleles and strains of mice, suggest that Tg HLA class I molecules are recognized by the mouse immune system and T cells much the same as alternate mouse class I alleles (28, 29, 32, 33, 40–48).

It is unclear whether these inconsistencies are actually due to differential function of distinct HLA alleles in the mouse background, as opposed to other quantitative or qualitative aspects of expression, or possibly differences in specific functional assays. To distinguish between these possibilities, with the longer-term objective being development of this model as an accurate reflection of HLA function in humans, we have established a panel of TgM that express the class I alleles HLA-A2, -B7, or -B27. One set of mice expresses the fully human native heavy chain in conjunction with human \(\beta_2\)m (h\(\beta_2\)m), whereas the other set expresses a hybrid form of each allele, consisting of the exons encoding the human \(\alpha_1\) and \(\alpha_2\) polymorphic domains (i.e., the peptide binding cleft) linked to the \(\alpha_3\), transmembrane, and cytoplasmic domains of the mouse H-2\(K^b\) protein. Development of the HLA/H-2\(K^b\) hybrid TgM was based on results of our own and others, which suggested the possibility that species-specific molecular interactions may influence how efficiently a human class I molecule expressed in Tg cells is able to undergo intracellular interactions (i.e., with \(\beta_2\)m, chaperones) and transport, have access to a suitable array of self and foreign peptides, or interact with the T cell co-activator CD8 at the cell surface (6, 27, 30, 36, 37, 49, 50). At least some of these interactions are known to depend partly or completely on class I domains outside the Ag-binding \(\alpha_1/\alpha_2\) domains (i.e., \(\beta_2m-a3\); CD8-a3), the rationale for the HLA/H-2 hybrid construct was that the encoded molecule should retain the peptide-binding specificity of the human allele and be able to efficiently undergo these other interactions when expressed in a mouse background.

The studies in this paper investigate the immune mechanisms activated in vitro and in vivo by non-TgM and TgM in response to these three well-characterized human MHC class I alleles expressed as fully native HLA (HLA\textsuperscript{nat}) vs HLA/H-2 hybrid (HLA\textsuperscript{hyb}) Tg xenotransplantation Ags. The results demonstrate that distinct effector mechanisms are involved in recognition of, first, the identical HLA allele as a xeno- vs an allo-MHC molecule, and second, different locus-matched alleles (HLA-B7 vs -B27) vs locus-mismatched products (HLA-B vs HLA-A).

### Materials and Methods

**Inbred, knockout (KO), and HLA Tg Mice**

(B6/SJL/J, H-2\(^s\)), C57BL/6J (BL6) (H-2\(^b\)), DBA/2J (H-2\(^d\)), B6.C-H-2bm1 (H-2\(^K^b\)\textsuperscript{bm1}) (24), and IgM\textsuperscript{K \textsuperscript{-}} (H-2\(^d\)) (51) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), CD8\textsuperscript{-} (52), CD4\textsuperscript{-} (53), and CD4\textsuperscript{+}CD8\textsuperscript{-} (54) mice were obtained from Dr. Tak Mak (Agen Institute and Ontario Cancer Institute, Toronto, Ontario, Canada).

The DNA constructs used to generate the HLA TgM are shown in Fig. 1. For each construct, multiple founder mice were generated by microinjection of (B6/SJL/J)\textsubscript{2} embryos (5, 55, 56), bred with non-Tg C57BL/6J mates to establish lines, and characterized with respect to Tg expression by RNA blot hybridization of tissue RNAs and by flow cytometry (5, 56). The HLA Tg lines used for these studies were selected on the basis of a normal breeding and transgene transmission rate, an appropriate tissue distribution of Tg HLA RNA (5, 56), and cell surface expression at a level similar to each other and to endogenous H-2 class I (5, 39). All constructs were genomic clones containing MHC class I exons, introns, and 5' and 3' flanking DNA in the genomic configuration. Each construct contained several hundred base pairs of HLA gene 5' flanking DNA, which we and others have shown previously to include all MHC class I cis-active transcriptional regulatory information sufficient to direct appropriate HLA class I Tg expression in TgM (55, 56). The HLA-B\textsuperscript{-} (B27\textsuperscript{-}) mice carried a 6.0-kb EcoRI RI fragment containing the fully human HLA-B7 molecule (5). The HLA-B7\textsuperscript{hyb} (B7\textsuperscript{hyb}) double TgM were derived by breeding the B7\textsuperscript{-} mice above with TgM carrying a 14-kb PvuII Sall fragment encoding h\(\beta_2\)m as described (5). The HLA-B27\textsuperscript{hyb} (B27\textsuperscript{hyb}) and HLA-A2\textsuperscript{hyb} (A2\textsuperscript{hyb}) mice were derived by co-injecting the human \(\beta_2\)m gene together with either a 6.5-kb EcoRI fragment encoding HLA-B27 (57) or a 6-kb EcoRI fragment encoding HLA-A2 (58). For both the B27\textsuperscript{hyb} and A2\textsuperscript{hyb} Tg lines used here, the h\(\beta_2\)m and HLA genes were cotransfected to offspring, indicating that both genes were cotransolated at a single chromosomal site.

The hybrid HLA-B7/H-2K\(^b\) (B7\textsuperscript{K} or B7\textsuperscript{hyb}), HLA-B27/H-2K\(^b\) (B27\textsuperscript{K} or B27\textsuperscript{hyb}), and HLA-A2/H-2K\(^b\) (A2\textsuperscript{K} or A2\textsuperscript{hyb}) TgM were generated by microinjection of a hybrid genomic clone containing the human \(\alpha_1\) and \(\alpha_2\) domains of H-2\(K^b\) B, B27, or A2, respectively, and the mouse exons for the \(\alpha_3\), transmembrane, and cytoplasmic domains of H-2\(K^b\). Each hybrid construct was made by linking a 2.35-kb 3' fragment of the genomic H-2\(K^b\) gene (beginning at a midpoint in intron 3 and containing exons 4–8 and 3' flanking DNA) to a 5' fragment of the specific HLA gene containing several hundred base pairs of 5' flanking DNA and exons 1–3 up to a midpoint in intron 3.

All Tg strains were maintained by backcrossing to C57BL/6J (H-2\(^b\)) mates. The strains used in the experiments described here had been backcrossed at least 8–10 generations. The HLA-B7\textsuperscript{hyb}/CD8\textsuperscript{-} and HLA-B27\textsuperscript{hyb}/CD8\textsuperscript{-} TgM were generated by breeding the corresponding HLA\textsuperscript{hyb}/CD8\textsuperscript{-} line (B7\textsuperscript{-} or B27\textsuperscript{-}) with CD8\textsuperscript{-} (H-2\(^b\)) (52) mice and subsequently breeding the HLA\textsuperscript{hyb}/CD8\textsuperscript{-} heterozygous offspring with CD8\textsuperscript{-} mice. All inbred, KO, and Tg mice were housed in a pathogen-free animal facility at The Hospital For Sick Children in accordance with the current regulations and standards of the Canadian Council of Animal Care.
**Transfection of P815 cells**

The DBA/2 (H-2^d^)-derived mastocytoma cell line P815 (59) was cotransfected by electroperoration of the bacterial neomycin (G418) resistance gene pμHμp-Neo (60) with individual HLA^{nat} or HLA^{hyb} gene constructs using a Bio-Rad gene pulser (Bio-Rad, Richmond, CA). Electroporated cells were cultured and selected in ñMEM supplemented with 5% newborn calf serum (St. Louis, MO) and 200 μg/ml G418 (Life Technologies, Rockville, MD) at 37°C and 5% CO_2_, Clones expressing high comparable levels of cell surface HLA for each transfected population were isolated by limiting dilution and characterized by flow cytometry.

**Flow cytometry**

The mAbs used, their specificities, and their sources were as follows: ME-1 (specific for HLA-B7, -B27, and -Bw2) was obtained from American Type Culture Collection (ATCC, Manassas, VA) (5, 61); MA2.1 (specific for HLA-A2 and -B17) was purchased from ATCC (62); B9.12.1 (pan-HLA class I-specific) was a generous gift of F. Lemmonier (Pasteur Institute, Paris, France; Ref. 63); and 2B.11.4S (specific for DR^d^, L^d^, and D^d^) (64) was obtained from ATCC. For flow cytometry, single cell suspensions from lymph nodes were prepared, and 1 × 10^6_ cells were stained with the primary mAb for 45 min at 4°C in 75 μl of PBS (specific for HLA-B7, -B27, and -Bw2) (Accurate Chemical and Scientific, Westbury, NY). Cells were then analyzed on a Becton Dickinson FACScan flow cytometer (Fc-specific) (Accurate Chemical and Scientific, Westbury, NY). Cells were cultured and selected in ñMEM containing 5% BSAs and 0.1% sodium azide. Then, cells were washed and incubated for 30 min with FITC-conjugated F(ab\')_2 goat anti-mouse IgG (Fc-specific) (Accurate Chemical and Scientific, Westbury, NY). Cells were then analyzed on a Becton Dickinson FACScan flow cytometer (Mountain View, CA).

**Cell-mediated lympholysis (CML) assay**

CML assays were performed essentially as described (65). Mouse responder lymph node cells (LNCs) (1 × 10^6_ to 3 × 10^6_ cells/well) were cultured in 96-well round-bottom microtiter plates (Nunc, Naperville, IL) with 3 × 10^5_ irradiated (2000 rad) stimulator cells for 5 days at 37°C and 5% CO_2_ in ñMEM supplemented with 10% FCS (Sigma), 10 mM HEPES, 5 × 10^-6_ M 2-ME, and penicillin/streptomycin (Life Technologies). Stimulator cells were obtained from the spleens of (B6/SJL)F_1_ at 37°C, 5% CO_2_ for 1.5 h. Subsequently, 100 μl of media containing stimulator cells at the ratios indicated in a 200-μl final volume for 4 days at 37°C and 5% CO_2_. CML assays were performed essentially as described (65). Mouse responder lymph node cells (LNCs) (1 × 10^6_ to 3 × 10^6_ cells/well) were cultured in 96-well round-bottom microtiter plates (Nunc, Naperville, IL) with 3 × 10^5_ irradiated (2000 rad) stimulator cells for 5 days at 37°C and 5% CO_2_ in ñMEM supplemented with 10% FCS (Sigma), 10 mM HEPES, 5 × 10^-6_ M 2-ME, and penicillin/streptomycin (Life Technologies). Stimulator cells were obtained from the spleens of (B6/SJL)F_1_ (H-2^b^) C57BL/6 (H-2^b^), H-2^K^\_I, and DBA/2 (H-2^d^) mice or HLA^{nat} or HLA^{hyb} class I TgM. Targets were either spleen cells that had been stimulated for 3 days with Con A (see below) or HLA-transfected P815 cells. After 5 days of in vitro stimulation, 100 μl of the culture supernatant was removed from each well and 51Cr-labeled targets (3 × 10^5_ ) were added to the effector cells at the ratios indicated in a 200-μl final volume for 4 h at 37°C. Subsequently, 100 μl of supernatant was removed from each well and counted on a Wallac gamma counter (Gaithersburg, MD). Specific lysis was calculated as \[
\text{specific lysis} = \frac{(\text{experimental count} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})} \times 100%
\]

Statistical significance was determined with unpaired Student’s t test for the comparison of means with unequal variances (Microsoft Excel software; Redmond, WA). Differences between groups were considered to be significant if p < 0.05.

**Results**

HLA^{nat} and HLA^{hyb} -A2, -B7, and -B27 TgM

To further characterize and develop the HLA Tg model for studies of human MHC-dependent T cell recognition and responsiveness, we established a panel of TgM that expresses the HLA class I alleles HLA-A2, -B7, or -B27 in either the fully native form (i.e.,

![FIGURE 1. Schematic representation of HLA class I native (HLA^{nat}) and hybrid (HLA^{hyb}) transgene constructs. The HLA^{nat} class I -A2, -B7, and -B27 TgM were generated by pronuclear microinjection of (B6/SJL)F_2_ embryos with DNA constructs as represented in A encoding HLA-A2, -B7, or -B27, respectively. Each construct was fully human containing all exons, introns, 5' and 3' flanking DNA, and 5' transcriptional control sequences in their native genomic configuration. Aside from their natural allelic differences, the three constructs differed from each other only slightly in the amount of 5' and 3' flanking DNA. The HLA-A2^{nat} construct was a 6.0-kb EcoRI fragment, whereas the B7^{nat} construct was a 6.0-kb EcoRI-BamHI fragment and the B27^{nat} construct was a 6.5-kb EcoRI fragment. Each of these HLA^{nat} fragments was introduced into TgM along with a cloned genomic fragment encoding h6-Thy (as described (see Materials and Methods for details)) (5). The HLA^{hyb} class I -A2-H2-K^d_ and -B7-H2-K^d_ and -B27-H2-K^d_ (or -B27^{nat}) TgM were generated by pronuclear microinjection of (B6/SJL)F_2_ embryos with DNA constructs, as represented in B, encoding the hybrid proteins HLA-A2-H2-K^d_ (A^{nat}B^{hyb}), B7/H2-K^d_ (B^{nat}B^{hyb}), or B27/H2-K^d_ (B^{nat}B^{hyb}), respectively. The A^{hyb} construct contained HLA-A2 5' flanking DNA and -A2 exons 1–3 on a 2.68-kb fragment linked in intron 3 to a 2.35-kb fragment containing exons 4–8 and 3' flanking DNA of the self H-2^K^ gene (the approximate position of the HLA/A-B2-K^d_ junction is indicated by a small vertical arrowhead). The B7^{hyb} construct contained HLA-B7 5' flanking DNA and B7 exons 1–3 on a 1.9-kb fragment linked in intron 3 to the 2.35-kb exon 4–8 H-2^K^ fragment. The B27^{hyb} construct contained HLA-B27 5' flanking DNA and B27 exons 1–3 on a 1.7-kb fragment linked in intron 3 to the 2.35-kb exon 4–8 H-2^K^ fragment. For both the native (A) and hybrid (B) constructs, the human exons are depicted as II, the mouse H-2^{K} exons as II, and 5' and 3' flanking DNA and introns as a thin line. The exons 1–8 are numbered below the map together with the corresponding protein domains (α1, α2, α3, membrane (Tm), and cytoplasmic (Cyt)). The scale is approximate.
Expression of HLA<sup>nat</sup> and HLA<sup>hyb</sup> transgenes in TgM

Cell surface expression of Tg HLA<sup>nat</sup> and HLA<sup>hyb</sup> class I was analyzed for LNCs by flow cytometry with several anti-HLA class I mAbs, including B9.12.1 (reacts with all HLA class I alleles) (63), ME-1 (reacts with HLA-B7, -B27, and -Bw22) (61), and MA2.1 (reacts with HLA-A2 and -B17) (62). Endogenous H-2 class I expression was examined with the H-2D<sup>b</sup>-reactive mAb 28-14-8S (64). Following analysis of all Tg lines, single representative lines for each construct were selected for the studies described in this article on the basis of the most similar levels of Tg HLA expression to each other and to endogenous H-2 class I. As already described (5), surface expression of HLA-B7<sup>nat</sup> increased ~10-fold in LNCs of mice coexpressing hβ<sub>2m</sub> (B<sup>7азветалик м</sup>), indicating suboptimal interaction of the human heavy chain with mouse β<sub>2m</sub> in mice Tg for the HLA<sup>nat</sup> class I gene alone. As a result, and to ensure the appropriate conformation of the Tg HLA<sup>nat</sup> heavy chains, the HLA-A2<sup>nat</sup> and HLA-B27<sup>nat</sup> TgM were derived by coinjection of fertilized eggs of the heavy chain gene along with the hβ<sub>2m</sub> gene. Fluorescent in situ hybridization analyses (67), as well as cotransmission of both the coinjected HLA and hβ<sub>2m</sub> genes to offspring, demonstrated that both genes were cotegrated at a single chromosomal site (results not shown). The HLA-B7<sup>nat</sup>/hβ<sub>2m</sub> mice were derived from the breeding of singly Tg parental HLA-B7<sup>nat</sup> and hβ<sub>2m</sub> lines (5). The HLA<sup>hyb</sup> gene constructs (Fig. 1B) were designed such that the encoded molecules would associate much more efficiently than the HLA<sup>nat</sup> class I proteins with endogenous mouse β<sub>2m</sub>s, thereby obviating the need for coexpression of hβ<sub>2m</sub>.

Fig. 2A shows flow cytometry staining results for LNCs from HLA-A2<sup>nat/hβ2m</sup> and HLA-A2<sup>hyb</sup> TgM, whereas Fig. 2B shows similar analyses for HLA-B7<sup>nat</sup>-B7<sup>hyb</sup>, -B7<sup>hyb</sup>-B27<sup>nat/hβ2m</sup>, and -B27<sup>hyb</sup> TgM. Relative to the background levels of fluorescence observed for non-Tg LNCs stained with the same anti-HLA-A2.1 (Fig. 2A), anti-HLA-B7/B27 (Fig. 2B), or anti-pan-HLA class I mAbs, significant surface expression of both the native and hybrid molecules for each allele was detected on Tg cells (Fig. 2). A high level of hβ<sub>2m</sub> was also detected at the surface of cells from HLA-A2<sup>nat/hβ2m</sup>, -B7<sup>nat/hβ2m</sup>, and -B27<sup>nat/hβ2m</sup> TgM (not shown). The level of endogenous H-2D<sup>b</sup> (and K<sup>b</sup>, not shown) class I expression was similar in all HLA Tg strains compared with non-TgM (Fig. 2A, and not shown).

A more quantitative estimate of the level of Tg HLA and H-2K<sup>D</sup>D<sup>b</sup> expression was previously determined for splenocytes for some of the mice shown in Fig. 2B using Quantum Simply Cellular Microbeads (xxiv) (Sigma) to measure the number of binding sites with anti-HLA-B7/B27 mAb ME-1 and anti-H-2K<sup>D</sup>D<sup>b</sup> mAb HB-51 (39). These analyses showed that relative to non-Tg C57BL/6 (H-2<sup>D</sup>D<sup>b</sup>) and (B6/SJL)F<sub>1</sub> (H-2<sup>D</sup>D<sup>b</sup>) mice, which carry ~4 × 10<sup>5</sup> and 2 × 10<sup>6</sup> K<sup>D</sup>D<sup>b</sup> binding sites per cell, respectively, the levels of HLA-B7<sup>nat</sup>-B7<sup>hyb</sup>, and -B27<sup>nat/hβ2m</sup> expression in the lines shown in Fig. 2B were comparable, ranging from 1 to 3 × 10<sup>5</sup> binding sites per cell (39). As an alternate means of estimating the relative level of expression of each of the Tg...
**Enhanced recognition of Tg HLA^{hyb} class I molecules in vitro**

To evaluate the ability of non-Tg H-2-matched T cells to recognize and respond to Tg HLA^{nat} and HLA^{hyb} class I molecules as transplantation Ags in vitro, CML assays were performed. As the immune system of the responder strain used in these initial assays had not previously been exposed to the HLA molecule during immune development (i.e., in vivo), we consider this situation to represent a mouse anti-human MHC class I xenoresponse. Following a 5-day primary in vitro stimulation of non-Tg LNCs from (B6/SJL)F_{1} mice with spleen cells from each of the three HLA^{hyb} or three HLA^{nat/h2m} TgM, {^{51}}Cr release assays were performed using Con A-stimulated spleen cells from various non-TgM and TgM strains as targets. Fig. 3, A and D, shows the results obtained for the anti-HLA^{nat} and anti-HLA^{hyb} responses, respectively. For all allelics, the hybrid molecule (Fig. 3D) induced a higher level of killing than its native counterpart (Fig. 3A). This increase was most apparent for B7 and B27 because the native forms of these induced only very weak responses (Fig. 3A). The elevated anti-HLA^{hyb} response was due not simply to higher cell surface expression because flow cytometry (Fig. 2), quantitative measurements (39), and RNA analyses (not shown) showed that each of the HLA^nat molecules in HLA^{nat/h2m} TgM was expressed at similar or greater levels than the corresponding HLA^{hyb} molecule (Fig. 2, A and B). In contrast to the very low level of killing detected for HLA-B^{7nat} or -B^{27nat}, the A^{2nat} molecule induced a stronger response, which was further increased when A2 was expressed as a hybrid molecule (Fig. 3, A and D). The higher response to HLA-A^{2nat} compared with -B^{7nat} or -B^{27nat} was not due to differences in expression level because B^{7nat/h2m} was expressed at a higher level on the cell surface than A^{2nat/h2m}, and B^{27nat/h2m} expression was only slightly less (Fig. 2, A and B, and not shown). The magnitude of the anti-HLA^{hyb} response for all alleles was close to that generated against the strong mouse class I allotransplantation Ag H-2K^{m1} (68, 69), whereas the response to HLA-A^{2nat} was only slightly less (Fig. 3, A and D).

As the above studies were performed using HLA Tg spleen-derived Con A-stimulated target cells sharing the same H-2^{h} haplotypic as the responding/effector cells (i.e., H-2^{h}-matched), it was not possible to distinguish the extent to which the observed killing was due to recognition of intact HLA molecules directly as opposed to recognition of processed HLA-derived peptides presented by mouse H-2^{h} MHC molecules. Therefore, this type of experiment was repeated using H-2 mismatched HLA class I gene-transfected mouse P815 (H-2^{k}) cells as targets. Any killing observed for these targets must be due to direct recognition of intact HLA molecules. As shown in Fig. 3E, non-Tg LNCs stimulated with HLA-A^{2hyb}, -B^{7hyb}, or -B^{27hyb} spleen cells gave significant lysis of HLA-A^{2hyb}, -B^{7hyb}, or -B^{27hyb}-expressing P815 cells, comparable to that observed for the Con A-stimulated targets (Fig. 3D). Parental nontransfected P815 cells were not killed. Fig. 3B shows that LNCs stimulated with Tg HLA-A^{2nat} cells gave a modest but significant level of lysis of HLA-A^{2nat}-P815 targets, whereas killing of HLA-B^{7nat/-}P815 cells by Tg B^{7nat/-}-stimulated responders was only slightly above background and of HLA-B^{27nat/-}P815 targets by Tg B^{27nat/-}-stimulated responders was undetectable. Therefore, the results of Fig. 3 demonstrate that, even when presented as xeno-Ags, all three HLA^{hyb} alleles, and at least the HLA-A^{2nat} molecule, are recognized largely, if not entirely, as intact MHC class I molecules by mouse T cells. This xeno-MHC recognition is also HLA allele-specific as the responding culture generated against one HLA^{hyb} (or HLA^{nat}) allele did not lyse targets expressing an alternate HLA^{hyb} (or HLA^{nat}) allele (not shown). Thus these xenoresponses are directed toward allele-specific polymorphisms as opposed to human-specific monomorphic determinants.

To investigate the basis of the apparent weak anti-HLA^{nat} response further (Fig. 3, A and B), cultures derived from stimulating non-Tg (B6/SJL)F_{1} LNCs with HLA-B^{7nat} Tg spleen cells were tested on HLA-B^{7hyb} vs HLA-B^{7nat}-expressing P815 target cells (Fig. 3C). As in Fig. 3B, the killing of B^{7nat/-}P815 was very low and only slightly above the background level of lysis observed for parental P815 (not shown). In contrast, these same HLA-B^{7nat}-stimulated cultures gave a much higher level of killing of HLA-B^{7nat}-P815 cells (Fig. 3C). Similar experiments conducted with cultures derived from stimulating (B6/SJL)F_{1} LNCs with HLA-A^{2nat} or -B^{27nat} Tg spleen cells also showed a significantly increased level of killing of allele-matched HLA-A^{2nat/-}P815 compared with HLA-A^{2nat/-}P815 targets (i.e., specific lysis increased from 34.6% for HLA-A^{2nat/-}P815 to 77.3% for A^{2nat}-P815 at an E:T ratio of 100:1 (p = 0.03), and from <10% for HLA-B^{27nat/-}P815 to 71.3% for B^{27nat/-}P815 at an E:T ratio of 100:1 (p = 0.001)). This increased killing of allele-matched HLA^{hyb}-expressing targets by HLA^{nat}-stimulated (B6/SJL)F_{1} LNCs indicates that the low level of killing of HLA^{nat}-expressing targets is not due to the absence of xenoreactive T cells in the normal non-Tg mouse repertoire able to recognize and be stimulated by the HLA^{nat} molecule. Rather, the results suggest that some aspect of the structure of each HLA^{nat} molecule on target cells important for mediating lysis is suboptimal compared with the HLA^{hyb} molecule. This observation implies differential HLA domain-dependent interactions occurring at the induction vs effector phases of these in vitro CML assays.

**Rapid xenorejection of both HLA^{hyb} and HLA^{nat} class I Tg skin grafts**

To extend assessment of the relative immunogenicity of each of the HLA^{hyb} and HLA^{nat} class I alleles in vivo, tail skin from each Tg strain was grafted onto non-Tg H-2-matched ((B6/SJL)F_{1}; H-2^{k}) recipients, and the mean survival time (MST) was determined (Fig. 4). Although grafts from HLA^{hyb}- or HLA^{nat}-Tg-negative offspring (HLA Tg neg) were indefinitely accepted (Fig. 4A and Table I), grafts from HLA-A^{2hyb}, -B^{7hyb}, or -B^{27hyb} TgM were rejected after 11.5 ± 2.4, 16.4 ± 1.8, and 17.2 ± 1.3 days, respectively (Fig. 4B and Table I). These rates of rejection were similar to that for skin mice carrying the known strong mouse class I allotyping H-2K^{m1} (13.8 ± 1.5 days, Fig. 4A), whereas a full MHC class I/class II strain mismatch (DBA/2; H-2^{k}) was rejected slightly faster at about 9.4 ± 0.5 days (Fig. 4A).

Based on the low level of response detected after in vitro stimulation with each of the HLA^{nat} Tg class I molecules (Fig. 3, A and B), we expected skin grafts from these mice to be rejected slowly, if at all, by non-Tg recipients. However, the MST of grafts expressing HLA-B^{7nat} with or without h^{2m} on non-Tg recipients was found to be 11.6 ± 0.5 and 12.2 ± 1.8 days and for HLA-B^{27nat/-}h^{2m} or HLA-A^{2nat/-}h^{2m} grafts was 10.5 ± 2.0 and 11.5 ± 1.3 days (Fig. 4C and Table I). For both B7 and B27, the rate of rejection of grafts expressing the HLA^{nat} allele was significantly faster than grafts expressing the corresponding HLA^{hyb} allele (p < 0.001). HLA-A^{2nat} and -A^{2hyb}-expressing grafts were rejected equally fast (MST of 11.5 days; see Fig. 4, B and C, and Table I).
In the case of the HLA hyb alleles, these results demonstrate that the immune system of non-TgM is able to recognize and respond to the xenogeneic human α1/α2 polymorphic domains of all three alleles such that graft rejection occurs at a similar rapid rate as for the strong allotransplantation Ag H-2K bm1. In the case of the HLA nat alleles, despite only being recognized weakly in cytotoxicity assays in vitro, these molecules are equal or more potent than the corresponding HLA hyb alleles as xenotransplantation Ags in vivo.

FIGURE 3. Comparison of primary in vitro-stimulated CML response of non-Tg LNCs against Tg cells expressing xenogeneic HLA nat vs HLA hyb class I molecules. Responder LNCs from non-Tg (B6/SJL)F1 (H-2 b/s) mice were stimulated in vitro with irradiated spleen cells from HLA nat class I (HLA nat/h h b m) (A, B, and C) or HLA hyb class I TgM (HLA hyb/h h b m) (D and E). The response against cells from mice carrying the H-2K bm1 class I alloantigen was included for comparison in all experiments and is shown in (A). After 5 days of stimulation, the responding cultures were tested for specific cytotoxicity on target cells in 4-h 51Cr-release assays (65). A, (B6/SJL)F1 (H-2 b/s) responders stimulated by HLA-A2 nat, HLA-B7 nat, or HLA-B27 nat class I-expressing Tg cells (on a H-2 b/matched background) were tested on Con A-stimulated mouse lymphoblast targets from HLA-A2 nat, HLA-B7 nat, or HLA-B27 nat TgM (H-2 b/matched), respectively. All anti-HLA nat responders were tested on non-Tg targets and are shown for anti-HLA-A2 nat responders. B, all three anti-HLA nat responding cultures were tested on HLA-A2 nat-, HLA-B7 nat-, or HLA-B27 nat-expressing H-2 m/mismatched P815 (H-2 d) cells respectively. Analogous experiments are shown in (D) and (E) for the anti-HLA hyb class I responders tested on HLA hyb/matched Con A-stimulated (D; H-2 b/matched) or P815 (E; H-2 d/mismatched) targets. C, responders stimulated by HLA-B7 nat were tested on HLA-B7 nat- and HLA-B7 hyb-expressing P815 cells. The E:T ratio and the percentage of specific lysis are indicated on the x- and y-axes, respectively. The data represent the means of three individual experiments.
Non-Tg Recipients

A) Control Donor Grafts

B) HLA hyb Donor Grafts

C) HLA nat Donor Grafts

Days Post Transplantation

FIGURE 4. Rejection of Tg HLA hyb and HLA nat class I tail-skin xenografts by H-2b-matched non-Tg recipients. Groups of non-Tg (B6/SJL)F1 recipient mice (see Table I for number of mice per group) were grafted with full-thickness skin from the following donor mice: HLA hyb or HLA nat transgene-negative [Tg neg] offspring from backcrosses of heterozygous TgM (h 2 m, -B7 nat , -B7 nat /h 2 m TgM (h 2 m)), from DBA/2 (Fig. 5A). Similarly, the allele-specific rejection rates of all HLA nat alleles as well as two of the three HLA hyb alleles (A2 hyb and B7 hyb ) were rejected at rates (Fig. 5B) very similar to those observed for CD8 KO recipients of bm 1 grafts, five of seven mice did not reject grafts by 200 days, whereas one rejected at 53 days and another at 57 days. As transplantation Ags, we wished to examine the role of conventional cellular and humoral immune effector mechanisms in HLA Tg skin graft rejection. Therefore, HLA hyb or HLA nat class I Tg skin for each allele was grafted onto H-2b-matched gene-KO recipient mice that were deficient for either CD8 T cells (CD8 KO) (53) (Fig. 5D), CD4 T cells (CD4 KO) (53) (Fig. 5B), CD4 + and CD8 T cells (CD4 KO/CD8 KO) (54) (Fig. 5C), or B cells (IgM KO) (51) (Fig. 5D). Although five of seven CD8 KO recipients retained mouse class I disparate H-2K bm1 allografts for longer than 200 days (Fig. 5A), grafts from TgM for all three HLA nat alleles as well as two of the three HLA hyb alleles (A2 hyb and B7 hyb ) were rejected at rates (Fig. 5A) very similar to those observed for the non-KO recipients (i.e., as in Fig. 4; see Table I). The only exception was that rejection of B27 hyb skin was somewhat delayed to 29.9 ± 8.1 days compared with wild-type recipients (p = 0.01) (Fig. 5A). Similarly, the allele-specific rejection rates of all HLA nat and HLA hyb grafts on CD4 KO and IgM KO recipients (Fig. 5, B and D; Table I) were virtually identical with those observed in the non-KO recipients. Grafts from TgM for all three HLA hyb alleles as well as HLA-A2 nat /h 2 m were rejected by CD4 KO/CD8 KO double

Rejection of HLA hyb or HLA nat class I Tg xenoskin grafts does not depend on CD8 T cells, CD4 + T cells, or B cells

Given the apparent discrepancy between the in vitro and in vivo results on recognition of Tg HLA nat vs HLA hyb class I molecules as transplantation Ags, we wished to examine the role of conventional cellular and humoral immune effector mechanisms in HLA Tg skin graft rejection. Therefore, HLA hyb or HLA nat class I Tg skin for each allele was grafted onto H-2b-matched gene-KO recipient mice that were deficient for either CD8 T cells (CD8 KO) (52) (Fig. 5A), CD4 T cells (CD4 KO) (53) (Fig. 5B), CD4 + and CD8 T cells (CD4 KO/CD8 KO) (54) (Fig. 5C), or B cells (IgM KO) (51) (Fig. 5D). Although five of seven CD8 KO recipients retained mouse class I disparate H-2K bm1 allografts for longer than 200 days (Fig. 5A), grafts from TgM for all three HLA nat alleles as well as two of the three HLA hyb alleles (A2 hyb and B7 hyb ) were rejected at rates (Fig. 5A) very similar to those observed for the non-KO recipients (i.e., as in Fig. 4; see Table I). The only exception was that rejection of B27 hyb skin was somewhat delayed to 29.9 ± 8.1 days compared with wild-type recipients (p = 0.01) (Fig. 5A). Similarly, the allele-specific rejection rates of all HLA nat and HLA hyb grafts on CD4 KO and IgM KO recipients (Fig. 5, B and D; Table I) were virtually identical with those observed in the non-KO recipients. Grafts from TgM for all three HLA hyb alleles as well as HLA-A2 nat /h 2 m were rejected by CD4 KO/CD8 KO double
KO recipients at rates similar to non-KO mice, whereas rejection of HLA-B7\(^{\text{a}}\)/\(\beta_2\)m and -B27\(^{\text{a}}\)/\(\beta_2\)m grafts were slightly delayed to 27.2 ± 12.5 days (\(p = 0.02\)) and 22.0 ± 5.2 days (\(p < 0.001\)) (Fig. 5C and Table I). Taken together, these data suggest that although the relative contribution of CD8- and CD4-dependent T cell mechanisms to HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\) class I Tg skin graft rejection demonstrate some variability depending on the allele, neither CD8\(^{+}\) or CD4\(^{+}\) T cells are absolutely required for graft rejection. This lack of dependence on CD8\(^{+}\) T cells for rejection of HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\) grafts was particularly surprising in view of the in vitro CML results above as well as previous results of others (30, 36, 37).

**Allo-, but not xeno-, recognition of HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\) class I Tg molecules is CD8 dependent**

The above results show that rejection of HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\)-expressing grafts in vivo does not depend on CD8\(^{+}\) T cells. This contrasts to the clear CD8 dependence of rejection of grafts expressing a single mouse class I alloantigen (i.e., H-2\(^{\text{bm1}}\), Fig. 5A). These observations may indicate that even when the “xeno” component of the foreign MHC molecule is limited to only the \(\alpha 1/\alpha 2\) domains as in the HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\) molecules, there is still an inherent difference between the immune effector mechanisms that respond to this molecule compared with those that respond to an allo-MHC class I molecule. The HLA Tg model provides a unique opportunity to investigate this issue directly because it is possible to compare recognition of a given HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\) class I allele as a xenotransplantation Ag (i.e., by the immune system of non-TgM) with recognition of the identical molecule as an allotransplantation Ag (i.e., by the immune system of mice that are Tg for an alternate HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\) class I allele and thus developed in this human MHC-expressing environment). To this end, in vitro CML and in vivo graft rejection studies similar to the above were conducted, but rather than the responder LNCs and recipient mice being non-Tg, responder LNCs and graft recipients that expressed an alternate HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\) class I Tg allele relative to the stimulator and donor strain were used.

Following primary in vitro stimulation with HLA-B27\(^{\text{b}}\)/CD8 Tg spleen (Spl) cells, LNCs from HLA-matched B7\(^{\text{b}}\)/CD8 TgM did not lyse HLA-B7\(^{\text{b}}\)/P815 targets above the background levels observed for P815 parental cells (Fig. 6A). Compared with the strong xenogeneic anti-HLA-B7\(^{\text{b}}\) response generated by non-Tg LNCs (Fig. 3, D and E), this lack of killing by B7\(^{\text{b}}\)/CD8 Tg LNCs indicates that CTL in these mice are tolerant to the HLA-B7\(^{\text{b}}\) molecule. When LNCs from these same mice were stimulated with spleen cells from H-2\(^{\text{bm1}}\) mice and assayed on H-2\(^{\text{bm1}}\) targets, a high level of lysis was observed (Fig. 6A) similar to that seen with non-Tg H-2\(^{\text{b}}\) responders (Fig. 3A). Following stimulation with HLA-B27\(^{\text{b}}\)/CD8 Tg spleen cells, LNCs from HLA-B7\(^{\text{b}}\)/CD8 TgM gave a significant level of lysis of both B27\(^{\text{b}}\)/CD8 Tg spleen Con A blast targets (B27\(^{\text{b}}\)/CD8 Tg Spl (H-2\(^{\text{b}}\))) and B27\(^{\text{b}}\)/CD8-P815 targets (Fig. 6B). This lysis appeared to be allele-specific as these same B27\(^{\text{b}}\)/CD8 responder LNCs did not lyse HLA-A2\(^{\text{b}}\)/CD8 expressing P815 cells (or A2\(^{\text{b}}\)/CD8 Con A blasts; not shown) above background levels observed for parental P815 or C57BL/6J Con A blast targets (non Tg Spl (H-2\(^{\text{b}}\)); Fig. 6B). These results demonstrate that the peripheral T cell repertoire of HLA-B27\(^{\text{b}}\)/CD8 TgM has become tolerant to the self-B7\(^{\text{b}}\) allele but is alloreactive to alternate related human alleles such as HLA-B27\(^{\text{b}}\). Additional experiments of the peripheral repertoire of HLA-B27\(^{\text{b}}\)/CD8 TgM yielded similar results, demonstrating tolerance to the self-HLA-B27\(^{\text{b}}\) allele and allele-specific alloreactivity to related non-self human alleles (i.e., B7\(^{\text{b}}\); not shown).

Although CD8\(^{+}\) T cells are the main effectors responsible for rejection of H-2 class I disparate skin allografts (Fig. 5A), rejection of xeno-MHC class I grafts from HLA\(^{\text{b}}\)/HLA\(^{\text{a}}\)TgM does not depend on either CD8\(^{+}\) or CD4\(^{+}\) T cells (Fig. 5, A and B). This was surprising given the high degree of amino acid and structural similarity of human and mouse MHC class I molecules (70, 71). It was unclear whether these results were due to an inherent difference in immune recognition mechanisms of xeno- vs allo-MHC in vivo even when the difference is limited solely to the \(\alpha 1/\alpha 2\) domains as in the HLA\(^{\text{a}}\)/HLA\(^{\text{b}}\) molecules, or rather reflects certain allele-specific and/or species-specific functional differences, or possibly other limitations of the HLA Tg model. To examine the role of CD8\(^{+}\) T cells in recognition of HLA\(^{\text{b}}\) class I molecules as allo- vs xenotransplantation Ags, primary in vitro CML experiments were conducted as before but with responder LNCs from HLA-B7\(^{\text{b}}\)/CD8 TgM bred to homozygosity for the CD8\(^{a}\) KO mutation and thus deficient for CD8\(^{+}\) T lymphocytes. Compared with the strong response of B7\(^{\text{b}}\)/CD8 TNCs (Fig. 6, upper panels) against both H-2\(^{\text{bm1}}\) (Fig. 6A) and HLA-B27\(^{\text{b}}\)/CD8 (Fig. 6B) expressing stimulators and targets, LNCs from HLA-B7\(^{\text{b}}\)/CD8 KO mice (Fig. 6, lower panels) were unable to mount cytotoxic responses to either of these mouse (bm1) (Fig. 6D) or human (B27\(^{\text{b}}\)) (Fig. 6C) alloantigens above background. CD8\(^{-/}\) KO mice that did not
carry the HLA transgene were also unable to respond to H-2K\textsuperscript{bm1} (Fig. 6D). Additional studies using LNCs from HLA-B\textsuperscript{B7hyb} CD8^{-/-} KO mice (instead of HLA-B\textsuperscript{B7hyb} CD8^{-/-} KO) as a source of responders gave analogous results (not shown). Therefore, similar to the in vitro response to mouse class I alloantigens such as H-2K\textsuperscript{bm1}, CD8\textsuperscript{1} T cells that develop in HLA hyb TgM are able to recognize and respond to alternate human MHC HLA hyb class I alleles as alloantigens.

Although CD8\textsuperscript{+} T cells from non-Tg (xeno) and HLA\textsuperscript{hyb} Tg (allo) mice are able to respond in vitro to Tg cells expressing a foreign HLA\textsuperscript{hyb} allele, CD8\textsuperscript{+} cells are not required for the rapid xenorejection by non-TgM of grafts from HLA\textsuperscript{hyb} TgM in vivo. To examine whether expression of the HLA class I molecule as a self-MHC allele influences the mechanisms by which the immune system responds in vivo to grafts from mice expressing alternate HLA class I alleles (i.e., HLA\textsuperscript{A2hyb}), a series of skin-grafting experiments were conducted (Fig. 7). Compared with non-Tg recipients (i.e., 17.2 ± 4.0 and 11.7 ± 2.9 days for HLA-B\textsuperscript{B7hyb} recipients (Fig. 7A, Table II) vs 16.4 ± 1.8 and 11.5 ± 2.4 days for non-Tg recipients (Table I)), a similar pattern was seen for HLA-B\textsuperscript{B7hyb} TgM that, compared with non-TgM, which rejected B7 hyb grafts at 16.4 ± 1.8 days (Table I), B7 hyb recipients indefinitely accepted these grafts (Table II) but rejected HLA-B27 hyb and -A2 hyb Tg skin after 17.3 ± 3.4 and 13.3 ± 2.9 days (Fig. 7B, Table II) (compared with 17.2 ± 1.3 and 11.5 ± 2.4 days, respectively, for non-Tg recipients).

The above results demonstrate that HLA-B\textsuperscript{B7hyb} and HLA-B\textsuperscript{B27hyb} class I TgM become tolerant to the α1 and α2 domains of the self-Tg HLA allele but are reactive to the corresponding domains in alternate class I HLA\textsuperscript{hyb} alleles as well as in alternate locus products (i.e., HLA-A\textsuperscript{A2hyb}). To evaluate the requirement for CD8\textsuperscript{+} T cells in rejection of these HLA\textsuperscript{hyb}-expressing Tg allografts, HLA-B\textsuperscript{B27hyb} or -B7 hyb mice that were bred to be deficient for CD8 expression were transplanted with HLA-B\textsuperscript{B27hyb}, B\textsuperscript{hyb}, or -A\textsuperscript{hyb} Tg skin. HLA-B\textsuperscript{B27hyb}/CD8^{-/-} recipients did not reject allele-matched (B\textsuperscript{27hyb}) or allele-mismatched (B\textsuperscript{hyb}) grafts but did reject locus-mismatched (A\textsuperscript{hyb}) grafts at a rate similar to that for B\textsuperscript{27hyb}/CD8^{-/-} TgM and non-TgM (i.e., 12.4 ± 1.7 vs 11.7 ± 2.9 vs 11.5 ± 2.4 days). Similarly,

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**FIGURE 6.** Comparison of primary in vitro-stimulated CMR response of HLA\textsuperscript{hyb}/CD8\textsuperscript{-/-} and HLA\textsuperscript{hyb}/CD8\textsuperscript{-/-} KO LNCs against H-2-matched Tg cells expressing alternate HLA\textsuperscript{hyb} class I alloantigens. Responder LNCs from HLA-B\textsuperscript{B7hyb} CD8\textsuperscript{-/-} KO LNCs against H-2-matched Tg cells expressing alternate HLA\textsuperscript{hyb} class I alloantigens. Responder LNCs from HLA-B\textsuperscript{B7hyb} and HLA-B\textsuperscript{B27hyb} expressing P815 target cells. Con A-stimulated blasts from C57BL/6J or bm1 mice were included as control targets. C, Responder LNCs from HLA-B\textsuperscript{B7hyb} CD8\textsuperscript{-/-} KO mice were stimulated with HLA-B\textsuperscript{B7hyb} spleen cells and assayed on HLA\textsuperscript{hyb}-mismatched targets. D, Responder LNCs from non-Tg CD8\textsuperscript{-/-} KO or HLA-B\textsuperscript{B7hyb} CD8\textsuperscript{-/-} KO mice were stimulated with irradiated H-2K\textsuperscript{bm1} or HLA-B\textsuperscript{B7hyb} Tg spleen cells and assayed on bm1 Con-A blast targets or HLA-B\textsuperscript{B7hyb} expressing P815 cells. The E:T ratios and the percentage of specific lysis are indicated on the x- and y-axes, respectively.
HLA-B7\textsubscript{hyb}/CD8\textsuperscript{+/+} recipients did not reject skin expressing either self (i.e., B7\textsubscript{hyb}) or non-self (B27\textsubscript{hyb}) HLA-B Tg alleles but did reject skin from HLA-A2\textsubscript{hyb} TgM (Fig. 7, C and D; Table II). These results demonstrate that rejection of Tg skin expressing alternate HLA class I B alleles depends exclusively on CD8\textsuperscript{+} T cells, whereas rejection of locus-mismatched HLA Tg skin depends on other CD8 T cell-independent mechanisms. Rejection of Tg skin expressing any of the three HLA alleles by non-TgM (i.e., as xenotransplantation Ags) was also found to occur through a CD8 T cell-independent route.

**Discussion**

Although HLA TgM have the potential to provide a useful model for studies of human MHC-dependent immune function in vivo, the extent to which mouse T cells are able to functionally respond to human class I or II alleles in this system was not clear (27–48). We established a panel of TgM carrying different HLA alleles in both the fully human (HLAnat) and hybrid human/mouse (HLA\textsubscript{hyb}) forms to address several aspects of immune recognition to clarify this issue. Here we have examined whether the same in vitro and in vivo immune mechanisms are responsible for recognition of a given MHC class I allele as a xeno- as opposed to an allotransplantation Ag. To minimize any influence of quantitative differences in expression level on differential function of the three HLA alleles in either the native or hybrid form, we selected single lines for each that expressed the most similar cell surface levels of each Tg product to each other and to endogenous mouse H-2 class I (Fig. 2). The close to physiological expression levels of all Tg HLA\textsubscript{hyb} and HLA\textsubscript{nat} molecules, together with reactivity with all anti-HLA class I mAbs specific for polymorphic or monomorphic determinants tested, implies that each Tg HLA\textsubscript{hyb} and HLA\textsubscript{nat} (in the presence of h\textsubscript{b}2 m) molecule is able to efficiently undergo intracellular trafficking and association with endogenous peptides and to adopt an appropriate conformation at the cell surface.

**Table II.** MST of HLA class I Tg skin grafts on HLA-CD8\textsuperscript{+/+} and HLA-CD8\textsuperscript{+/+} recipient mice

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<th>Donor</th>
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<td>B27/K\textsuperscript{K}-CD8\textsuperscript{+/+}</td>
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<td>9</td>
<td>&gt;200</td>
</tr>
<tr>
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<td>11.7 ± 2.9</td>
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</tr>
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<td>A2\textsubscript{hyb}</td>
<td>6</td>
<td>20.8 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>B27\textsubscript{hyb}</td>
<td>7</td>
<td>&gt;200</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) No. of recipient mice receiving the indicated skin graft.
\(^b\) MST (in days) ± SD. In cases where all recipients failed to reject grafts from a certain donor strain, the MST is considered as greater than the number of days the mice were maintained (i.e., 200, 300, 400 days) until the experiment was terminated.
Despite physiological surface levels of Tg HLA\textsuperscript{hyb} class I, the in vitro cytotoxicity studies in Fig. 3, A and B, together with previous results of our own and others, implied a limitation at some level in the efficiency of recognition of fully human MHC class I molecules by non-Tg cytotoxic T cells. To investigate this issue, a number of groups, including ours, have studied TgM expressing HLA\textsuperscript{hyb}Tg constructs to distinguish whether inefficient xenorecognition of HLA\textsuperscript{nat} class I was due to the low frequency of mouse TCRs able to interact with the HLA class I α1/α2 domains (i.e., “holes” in the repertoire) as opposed to other species-specific molecular incompatibilities involving interactions outside the peptide-binding α1/α2 domains. The results in Fig. 3, D and E, demonstrate that the in vitro primary xenoreaction of non-TgM T cells to HLA class I molecules is enhanced for all three alleles when cells from HLA\textsuperscript{hyb} rather than HLA\textsuperscript{nat} TgM are used as stimulators and targets. In the HLA\textsuperscript{hyb} form, all alleles induced levels of killing that were close to that against the strong mouse class I transplantation Ag H-2K\textsuperscript{bm1} (68, 69). In the HLA\textsuperscript{nat} form, only HLA-A2 induced a reproducibly significant level of killing. It is unclear why this HLA\textsuperscript{nat} allele induces a stronger response than the others but, given the similar cell surface expression levels, it must be due to structural polymorphic differences. This type of HLA\textsuperscript{nat} allelic difference may explain some of the differing results reported by groups working with various HLA\textsuperscript{nat} class I Tg strains.

With regard to the non-Tg response against HLA\textsuperscript{hyb} class I, it is important to recognize that the strong lysis of H-2-mismatched HLA\textsuperscript{hyb}-transfected P815 targets indicates that killing is due largely to direct recognition of intact MHC molecules. The induced responses were also allele-specific, indicating that they were directed primarily at the polymorphic regions as opposed to shared human-specific determinants. Together, these results imply that the weak anti-HLA\textsuperscript{nat} response detected in vitro is not due to a low frequency of xeno-MHC reactive mouse T cells in the non-Tg repertoire, but rather mainly to species-specific interactions outside of the Ag-binding cleft. A similar conclusion was reached previously by others studying HLA-A2 (30, 37) and -B27 (36), but not for -B7 (41).

Although HLA\textsuperscript{nat}-stimulated non-Tg responders were unable to give significant levels of killing of HLA\textsuperscript{nat} allele-matched targets, these same responders gave much higher levels of killing of HLA\textsuperscript{hyb} allele-matched targets for HLA-B7 (Fig. 3C), -A2, and -B27 (not shown). These results suggest that the low killing observed in the experiments of Fig. 3, A and B, is not necessarily because the xenogenic HLA\textsuperscript{nat} molecules are unable to induce a cellular response but rather may be due in part to a suboptimal ability of the induced cells to recognize the HLA\textsuperscript{nat} molecule on target cells. In studies of the anti-influenza T cell response in analogous strains of HLA\textsuperscript{nat} class I disparate graft rejection in vivo (72–75). Based on this, it would be expected that if recognition of HLA Tg class I molecules as xenotransplantation Ags by the non-TgM immune system is similar to that of mouse H-2 class I molecules, then the mechanisms mediating HLA Tg and H-2 class I disparate graft rejection should be similar and be reflected by these assays. However, based on our skin graft rejection studies, we believe that some of the previous inconsistencies among studies using this type of model result from the apparent breakdown of this correlation. For example, given the very low level of killing observed following stimulation with each Tg HLA\textsuperscript{nat} product (Fig. 3), the rapid rejection of skin grafts from these same HLA\textsuperscript{nat} TgM was not expected (Fig. 4C). However, although Van Twueyer et al. (76) also reported rapid rejection of skin grafts from mice Tg for HLA-B27\textsuperscript{nat}, others appear to have limited their analyses to in vitro primary CML assays with the assumption that the in vitro results reflected in vivo graft recognition and rejection (36).

The mechanisms underlying the very rapid rejection of HLA\textsuperscript{nat} Tg grafts are not obvious. Clearly, it is not due to the influence that coexpressing hβ\textsubscript{2m} has on either the quantitative level of expression or the conformation of the HLA\textsuperscript{nat} class I heavy chain as skin grafts from singly Tg HLA-B7\textsuperscript{nat} mice (i.e., hβ\textsubscript{2m}-negative) are also rapidly rejected (Fig. 4C) and those from singly Tg hβ\textsubscript{2m} mice (i.e., HLA-negative/hβ\textsubscript{2m}-positive) are independently affected (Fig. 4A). Also, the use of gene KO graft recipient mice deficient for either CD8\textsuperscript{+} or CD4\textsuperscript{+} T cells or B cells showed that these populations on their own were not responsible for rejection of Tg HLA\textsuperscript{nat} grafts. Either other effector mechanisms (i.e., NK cells), or multiple mechanisms as suggested by the somewhat prolonged survival of grafts from two of the three HLA\textsuperscript{nat} Tg strains in CD4\textsuperscript{−}/CD8\textsuperscript{−} double KO recipients, are operative in the rapid rejection of these grafts. Studies to investigate these possibilities are in progress.

The second instance in which there is a discrepancy between results obtained from in vitro CML vs in vivo graft rejection assays was with xenorecognition of the HLA\textsuperscript{hyb} class I molecules. In the in vitro CML assays shown here, together with results from others (36), demonstrate that each HLA\textsuperscript{hyb} class I allele is recognized as efficiently as the mouse class I alloantigen bm1, presumably due to improved interaction of the HLA\textsuperscript{hyb}, compared with the HLA\textsuperscript{nat} class I molecule, with mouse CD8 as a result of including the mouse α3 domain (36, 37). However, our results showed that when CD8\textsuperscript{−}/CD4\textsuperscript{−} KO mice were engrafted with donor HLA\textsuperscript{hyb} Tg skin, the allele-specific rejection rates (Fig. 5A) were very similar to those observed for wild-type (CD8\textsuperscript{+}/CD4\textsuperscript{+}) recipients. Thus, despite the differences detected in vitro for the HLA\textsuperscript{nat} and HLA\textsuperscript{hyb} xeno-MHC molecules, these effects are of no significance in vivo at least two respects: first, despite poor recognition in vitro, HLA\textsuperscript{nat} grafts are rejected very rapidly in vivo at rates that are equal to or faster than those for the corresponding HLA\textsuperscript{hyb} grafts or even bm1 grafts; and second, despite improved recognition in vitro of HLA\textsuperscript{hyb} class I molecules by non-TgM CD8\textsuperscript{+} T cells, this effect is completely irrelevant to rejection of HLA\textsuperscript{hyb} grafts in vivo. In contrast, the absence of CD8\textsuperscript{+} T cells had a significant effect on the survival of H-2K\textsuperscript{bm1} skin grafts. This finding of prolonged survival of mouse class I allogeneic, but not HLA\textsuperscript{hyb} class I Tg, skin grafts in CD8\textsuperscript{−}/CD4\textsuperscript{−} KO recipients argues that CD8\textsuperscript{+} T cells play an important role in the rejection of murine allografts disparate at a single MHC class I molecule but not in class I disparate xenografts. A similar independence of HLA\textsuperscript{hyb} Tg graft rejection on CD4\textsuperscript{+} T cells was revealed when CD8\textsuperscript{+}/KO recipients were grafted with HLA Tg skin (Fig. 5B). Thus, similar to HLA\textsuperscript{nat} grafts, data indicate that neither CD8\textsuperscript{+} nor CD4\textsuperscript{+} T cells on their own are necessary for rejection of xeno-HLA\textsuperscript{hyb} Tg grafts and that either other or multiple mechanisms must be involved. As the only difference between the HLA\textsuperscript{hyb} molecules and the “self” H-2K\textsuperscript{bm1} class I molecule is in the α1/α2 domains, our results show that although the human (xeno) α1/α2 domains are sufficient for recognition as a very strong major histocompatibility transplantation Ag, the immune mechanisms induced by this
xeno-MHC class I molecules are distinct from those induced by an allo-MHC (mouse) class I molecule.

The strong non-Tg anti-HLA<sub>B</sub> xenoresponses detected in vitro and in vivo were not detected when immune cells from HLA<sub>B</sub> TgM were stimulated with HLA<sub>B</sub>-allele-matched cells (Fig. 6A) or grafts (Fig. 7, A and B). In contrast, stimulation of HLA<sub>B</sub>-Tg cells with HLA<sub>B</sub>-allele-mismatched (i.e., allogeneic) Tg cells led to strong lysis of target cells expressing the mismatched HLA<sub>B</sub>-allele but not the HLA<sub>B</sub>-self allele or an alternate third party allele (Fig. 6, A and B). Similarly, although HLA<sub>B</sub>-TgM were tolerant to grafts expressing their self-HLA<sub>B</sub>-allele, they rapidly rejected grafts from mice expressing a HLA<sub>B</sub>-allele-mismatched (rejection of B<sup>B7</sup>-grafts by B<sup>B7</sup>-TgM, Fig. 7A) as well as locus-mismatched grafts (rejection of HLA-A2<sub>B</sub>-grafts by B<sup>B7</sup>-TgM, Fig. 7A) and by B<sup>B7</sup>-TgM, Fig. 7B). Thus, given that the non-Tg response to HLA<sub>B</sub>-class I molecules as xenogeneic HLA class I alleles develop within the hosts, allele-specific tolerance in each of these compartments develops to the xenogeneic HLA class I molecules as xenogeneic HLA class I alleles develop within the hosts, allele-specific tolerance in each of these compartments develops to the xenogeneic HLA class I alleles.

References


