Human CD8 Transgene Regulation of HLA Recognition by Murine T Cells

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Summary

A series of human CD8 transgenic (hCD8 Tg) mice with differential expression in the thymus and periphery were produced to investigate CD8 coreceptor regulation of repertoire selection and T cell responses. Expression of hCD8 markedly enhanced responses to both HLA class I molecules and hybrid A2/K^b molecules providing functional evidence for a second interaction site, outside of the α3 domain, which is essential for optimal coreceptor function. Peripheral T cell expression of hCD8 was sufficient to augment responsiveness to HLA class I, as hCD8 Tg mice which lacked thymic expression responded as well as mice expressing hCD8 in the thymus and periphery. Both murine $CD8^+$ and $CD4^+$ T cells expressing hCD8 transgenes exhibited markedly enhanced responses to foreign HLA class I, revealing the ability of T cell receptor repertoires selected on either murine class I or class II to recognize human class I major histocompatibility complex (MHC). In contrast to recognition of foreign class I, thymic expression of hCD8 transgenes was absolutely required to enhance recognition of antigenic peptide restricted by self-HLA class I. Thus, our studies revealed disparate requirements for CD8 coreceptor expression in the thymus for selection of a T cell repertoire responsive to foreign MHC and to antigenic peptides bound to self-MHC, providing a novel demonstration of positive selection that is dependent on human CD8.

T cells have evolved to recognize foreign antigens displayed on the cell surface in association with class I or class II MHC proteins. The T cell receptor ligand being recognized is a binary MHC–peptide complex. However, TCR binding to MHC peptide is generally not sufficient for activation of T cells. Functional recognition usually requires concurrent binding of class I or class II MHC–peptide complexes by TCR and CD8 or CD4 coreceptors, respectively (1–4). Whereas the molecular basis for TCR binding to the class I MHC-peptide complexes involves specific interactions with peptide bound by the α_1, α_2 domains of MHC molecules (5, 6), CD8 coreceptors interact with a nonpolymorphic region within the α_3 domain of class I MHC proteins (7, 8). As a result of this requirement, T cells that recognize antigenic peptide in association with class I MHC proteins generally express CD8 coreceptors, whereas T cells that express CD4 coreceptors generally recognize antigenic peptide presented by class II MHC proteins (reviewed in [9]).

The interaction of TCR and coreceptors with MHC–peptide ligands is essential for the initiation of thymic selection, alloreactivity, and recognition of antigenic peptide presented by self-MHC–restricting elements. Furthermore, development of immune cells capable of mediating these functional responses requires expression of both TCR (10, 11) and coreceptors (12, 13). However, it has been difficult to assess the relative contribution of the TCR and coreceptors in directing the specificity of these functional responses. We generated human CD8 transgenic (hCD8 Tg)[1] mice to determine the specific affects of CD8 coreceptor binding to MHC class I on positive selection and peripheral T cell responses. The functional consequence of hCD8 coreceptor expression on T cell recognition of human class I molecules could readily be distinguished from endoge-

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[1] *Abbreviations used in this paper:* hCD8, human CD8; mCD8, murine CD8; pCTL, precursor CTL, Tg, transgenic.

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nous murine coreceptor function since mCD8 cannot efficiently bind with HLA class I (14–16). Additionally, we used hCD8 Tg mice with differential expression in the thymus and periphery to directly assess the functional role of CD8 coreceptors during T cell ontogeny for repertoire selection.

Materials and Methods

Mice. hCD8 Tg mice were generated using hCD8α or hCD8β full-length cDNA sequences subcloned into the p1013 transgene expression vector (17) to which a human β-globin intron sequence was added to stabilize expression. HLA-B7 Tg mice were generated using a full-length genomic clone containing *cis*-acting regulatory sequences to drive appropriate tissue-specific expression relative to endogenous *H-2* genes (18). All transgenic mice were produced in the Scripps core facility by microinjection of fertilized mouse embryos and implantation into pseudopregnant foster mothers according to the procedures of Hogen et al. (19). HLA-A2.1 Tg mice and HLA-A2/K^b Tg mice, expressing hybrid molecules bearing the α1and α2 domains of HLA-A2.1 fused to the α3 domain of H-$2K^b$, were generated as previously described (20, 21). Tg mice, C57BL/6, and B10.A-(18R) mice were maintained under specific pathogen–free conditions in the Scripps Animal Resources vivarium.

Flow Cytometry. FITC-conjugated anti–human CD8α (Becton Dickinson and Co., Cockeysville, MD) PE-conjugated anti–murine CD4 (Becton Dickinson and Co.), and red 613–conjugated anti–murine CD8α (GIBCO BRL, Gaithersburg, MD) antibodies were used to stain cell suspensions from thymus and spleen. For comparison of relative hCD8α and hCD8β expression, cells were stained with PE-conjugated anti–human CD8α (Becton Dickinson and Co.) and 5F2 (anti-hCD8β) Ab followed by addition of FITC-conjugated goat anti–mouse $F(Ab)_2$-anti-Fc Ab (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Compensation was done according to manufacturer's recommendations. Cells were analyzed with a FACScan® (Becton Dickinson and Co.) instrument (Lysis II software) on total cell (ungated) populations or cells gated for positive staining with anti–human CD8α Ab as indicated in the figures.

Autophosphorylation Assay. 10 million LN cells were lysed in 1.2 ml of 3% NP-40, 20 mM Tris-HCl (pH 8.4), 150 mM NaCl, 1 mM PMSF, 2 mM EDTA, 200 μM Na_3VO_4, and 50 mM NaF. Lysates were immunoprecipitated with Ab specific for hCD8 (OKT8) or antisera specific for $p56^{lck}$ (22) using protein A–Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Immunoprecipitates were then washed three times in lysis buffer and one time in 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, resuspended in 20 μl kinase buffer (40 mM Pipes, pH 7.1, 10 mM $MnCl_2$), and incubated at 30°C for 10 min in the presence of 10 μCi [γ-^{32}P]ATP (ICN K & K Laboratories, Inc., Plainview, NY). The reaction was stopped with 20 μl 2 × SDS sample buffer and analyzed by SDS-PAGE.

CTL Responses. Specific CTL activity was assessed in 4-h ^{51}Cr-release assays. Primary stimulation of allogeneic or xenogeneic effector cells was achieved by culturing unprimed responder spleen cells with irradiated (3,000 rad) stimulator spleen cells at a ratio of 7×10^6:6×10^6 in 24-well plates (RPMI 10, 37°C, 5% CO_2), which were pooled on day 6. Varying dilutions of effector cells were assayed for lysis of target cells including: P815 (H-2^d), 310 (HLA-A2.1, -B27), Jurkat (HLA-A3, -B7) or Jurkat transfectant cells expressing HLA-A2.1 (Jurkat/A2) or A2/K^b hybrid molecules (Jurkat/A2/K^b).

CTL cells specific for influenza virus were generated as described (21). Briefly, mice were primed intraperitoneally with 300 hemagglutinating U of A/PR/8/34 influenza virus (PR8) for 3 wk and then cultured with irradiated (3,000 rad) PR8-infected stimulator spleen cells. After 6 d, effector cells were assayed for cytotoxicity of ^{51}Cr-labeled target cells pulsed with 10 mM matrix peptide (GILGFVFTL; restricted by HLA-A2.1) or NP peptide (ASNENMETM; restricted by H-$2D^b$). Peptide pulsing was done at the same time as ^{51}Cr labeling.

Ab blocking of coreceptors was achieved by adding 2× mAb (titrated for efficient blocking) to the effector cells 30 min before addition of target cells. The blocking Ab included: 3.168 (anti–murine CD8) and OKT8 (anti–human CD8). Depletion of effector cells with Ab + C′ was done just before addition of target cells using the following mAb: 3.168 (anti–murine CD8), Leu2b (anti–human CD8), and RL172 (anti–murine CD4). Ab was added to effector cells along with guinea pig complement (Pel-Freez Biologicals, Rogers, AR), incubated 1.5 h at 37°C, and washed three times. The efficacy of cell depletion was assessed by FACS® analysis to confirm that the appropriate subpopulations were eliminated. Relative cytotoxic activity was assessed as: 100 × (sample release − spontaneous release)/(maximum release − spontaneous release).

Limiting dilution analysis was done using varying numbers of unprimed spleen cells cultured at limiting dilution ($n = 24$) with 2.5×10^5 irradiated (3,000 rad) stimulator spleen cells at 37°C in 0.2 ml RPMI 10 medium supplemented with 2% T cell growth factor for 7 d. The contents of each well were then divided into two aliquots and tested for CTL activity against ^{51}Cr-labeled target cells (10^4) in a 6-h assay. Significant lysis was defined as being at least three times the standard deviation above the mean spontaneous release value from wells containing syngeneic target cells. The frequency of pCTL was determined according to the Poisson probability distribution (23).

Proliferation Assays. Responder cells (enriched for $mCD4^+$ T cells) prepared from single-cell suspensions of pooled lymph nodes were passed over nylon wool and then incubated with 3.168 (anti-mCD8) and J11d (anti–B cell) Ab + C′ (Pel-Freez Biologicals) for 1.5 h at 37°C followed by three washes. Stimulator cells were prepared from irradiated (2,000 rad) single-cell suspensions of splenocytes. 3×10^5 responder and 5×10^5 stimulator cells were incubated in 0.25 ml RPMI 10 medium at 37°C, 5% CO_2. Each well was pulsed with 1 μCi [^{3}H]TdR and harvested on days 2.5, 3.5, and 4.5.

Results

Human CD8 Transgenic Mice with Differential Thymic and Peripheral Expression. T cell–specific expression of hCD8 molecules was obtained using a transgene expression vector bearing the murine $p56^{lck}$ proximal promoter (17) (Fig. 1). The hCD8α and hCD8β transgene constructs were microinjected separately or coinjected into $(C57BL/6 \times SJL)_{F2}$ embryos to generate lines with differential expression. Transgenic founder mice were backcrossed to C57BL/6 mice. All mice used for these studies were backcrossed a minimum of three times and were homozygous for the H-2^b haplotype. Five lines (hCD8α.18, hCD8α.40, hCD8β.10, hCD8α+β.42, and hCD8α+β.57) that demonstrated stable transgene expression were selected for further analysis.

Cell surface expression of transgenic hCD8 on thymocytes

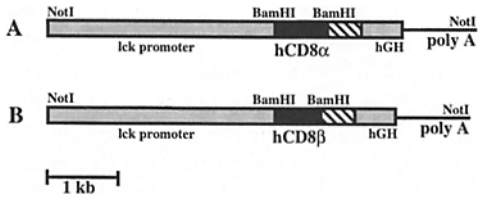

Figure 1. DNA constructs used to generate human CD8 transgenic mice. The cDNA constructs used to generate hCD8α and hCD8β transgenic mice were under the control of the murine $p56^{lck}$ proximal promoter. The shaded areas represent the p1013 transgene expression vector, black areas represent the hCD8α and hCD8β cDNA sequences, and striped areas represent the human β-globin intron sequence added to stabilize expression.

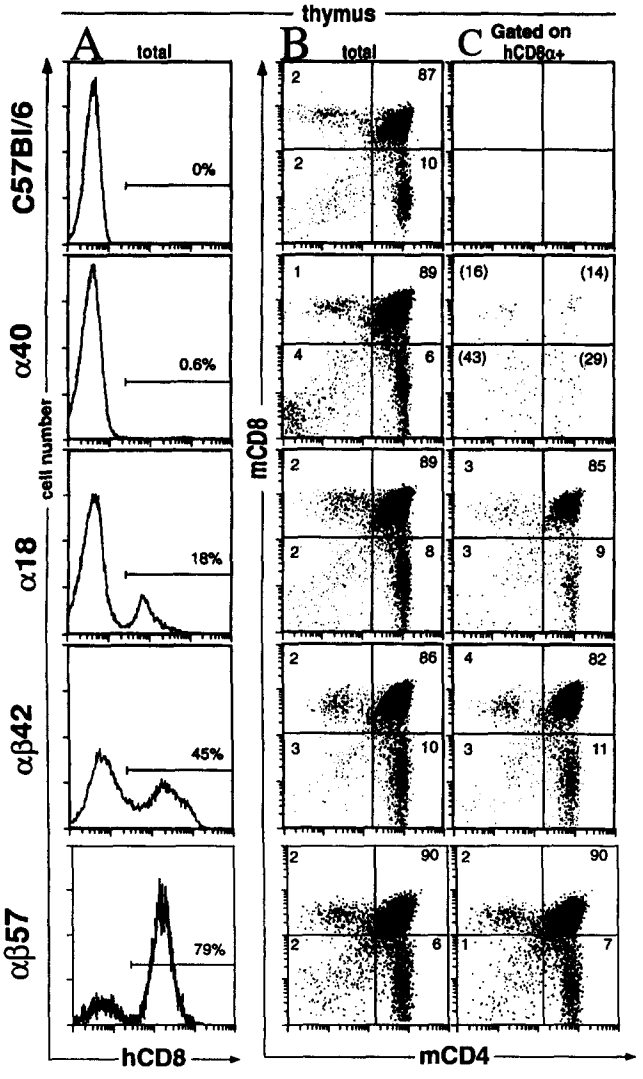

Figure 2. Thymic coreceptor expression in hCD8 transgenic mice. Thymocytes from nontransgenic C57BL/6, hCD8α.40, α.18, α+β.42, and α+β.57 Tg mice were analyzed for expression of hCD8α (column *A*), and for the relative expression of endogenous mCD4 and mCD8 on total thymocytes (column *B*), and on thymocytes gated for expression of hCD8 (column *C*). Percentages of cell populations from a total of 10,000 events are indicated in the relevant quadrants. Staining and analysis were run in parallel for direct comparison with the exception of hCD8α+β.57 thymocytes which were from a comparable experimental set.

and splenocytes was assessed by three-color FACS® analysis. Expression of hCD8α on thymocytes (Fig. 2 *A*) of three transgenic lines (hCD8α.18, hCD8α+β.42, and hCD8α+β.57) was observed on immature $mCD4^-8^-$ (DN), $mCD4^+8^+$ (DP) cells, and on mature single positive (SP) thymocytes from both the mCD8 and mCD4 lineages (Fig. 2 *C*). The normal ratio for DN, DP, mCD4 SP, and mCD8 SP thymocytes was observed in all three transgenic lines (Fig. 2 *B*). Furthermore, the mCD4/mCD8 ratio among $hCD8^+$ gated thymocytes was normal, as compared to total thymocyte populations. The relative expression levels of mCD4, mCD8, and transgenic hCD8 coreceptors were equivalent. Thus, the expression of hCD8 in these transgenic mice did not appear to significantly alter the normal ratio or the level of expression of endogenous coreceptors in the thymus.

The transgenic line designated hCD8α.40 showed almost no detectable levels of hCD8α expression in the thymus (Fig. 2, *α40*). Transgene expression was detected on <1% of thymocytes and attempts to sort $hCD8^+$ cells for enrichment failed to increase the relative percentage of cells expressing hCD8 coreceptors. However, hCD8α transgene expression on mature peripheral T cells of hCD8α.40 Tg mice was very similar to that of the hCD8α.18 line (see below). The same transgene construct was used to generate the hCD8α.40 and hCD8α.18 lines and the founder mice came from the same set of injections, indicating that the lack of thymic expression may be due to the site of transgene integration into the genome.

Expression of hCD8α coreceptors on splenocytes was observed on mature T cells from both the mCD8 and mCD4 lineages (Fig. 3). Transgene expression in the periphery was T cell specific and was stable throughout the life of the mice (data not shown). The levels of mCD4 and mCD8 expression on transgenic lymphocytes were about the same, or only slightly lower, as that on non-transgenic C57BL/6 lymphocytes. The expression levels of transgenic hCD8 coreceptors were comparable to CD8 expression on human PBL (data not shown). The ratio of mature mCD4:mCD8 cells was not altered. Thus, hCD8 transgene expression did not impair endogenous murine coreceptor expression and was comparable to hCD8 expression on human PBL.

The relative cell surface expression of hCD8α and hCD8β on thymocytes and spleen cells in hCD8α+β.42 and hCD8α+β.57 Tg mice is shown in Fig. 3 *C*. There was a relatively large proportion of thymocytes expressing hCD8α homodimers as well as hCD8α/β heterodimers in the thymus. In contrast, mature T cells from transgenic spleens expressed primarily hCD8α/β heterodimers. The ratio of cells expressing hCD8α homodimers to hCD8α/β heterodimers was decreased dramatically in the periphery compared to thymocyte expression (see ratios in parentheses, Fig. 3 *C*). This differential expression pattern of hCD8α and hCD8β was particularly striking in the hCD8α+β.57 Tg strain. These data suggest a preferred survival during thymic selection of T cells coexpressing hCD8α and hCD8β in coinjected hCD8 Tg mice. How-

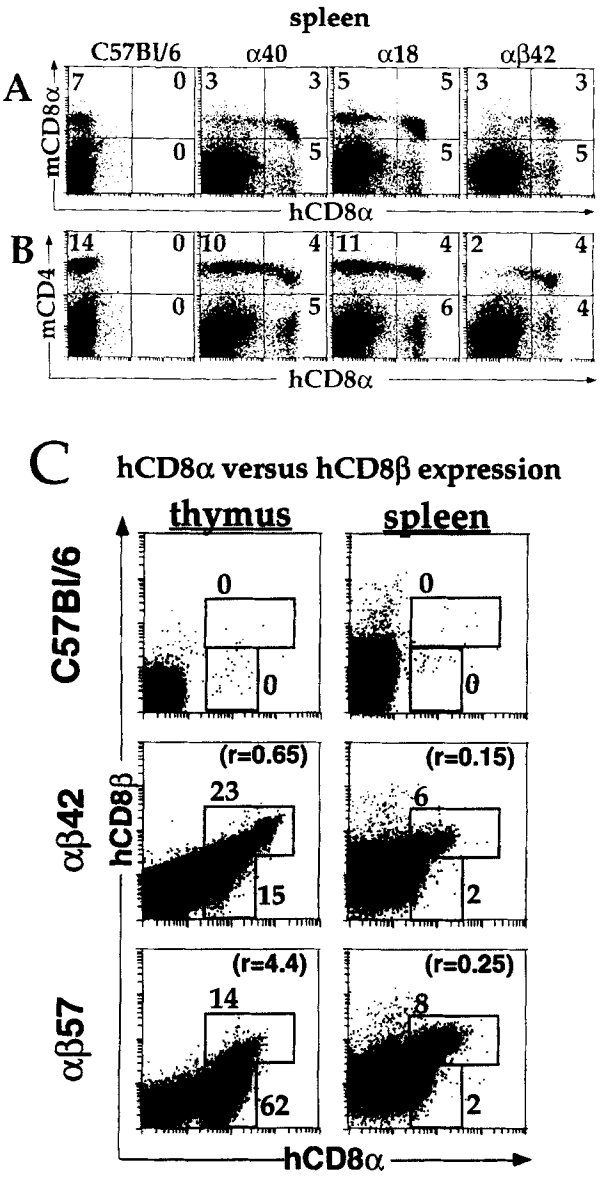

Figure 3. Peripheral T cell expression of coreceptors in hCD8 Tg mice and the relative expression of hCD8α and β chains. (*A* and *B*) Splenocytes from nontransgenic C57BL/6, hCD8α.40, α.18, and α+β.42 Tg mice were analyzed for staining with Ab to hCD8 and mCD8 (*A*) or for staining with Ab to hCD8 and mCD4 (*B*). The percentages of total splenocytes frmo 10,000 events collected are indicated in the relevant quadrants. (*C*) The relative cell surface expression of hCD8α and hCD8β on thymocytes and splenocytes in hCD8α+β.42 and hCD8α+β.57 Tg was assessed by FACS® analysis. The percentages of cell populations expressing hCD8α only and cells expressing both hCD8α and hCD8β are indicated next to the relevant boxes. The ratio of cells expressing hCD8α: hCD8α/β is shown in parentheses for each panel.

ever, the lack of hCD8β expression in hCD8α.18 Tg mice did not inhibit the development of a significant population of mature T cells expressing only hCD8α (Fig. 3, *A* and *B*).

Transgenic hCD8 Proteins Fully Integrate into the Murine Coreceptor Signal Transduction Pathway. Functional competence of coreceptors requires an interaction with the lymphocyte-specific protein tyrosine kinase $p56^{lck}$ through which signal transductions are mediated (24). A conserved 12–amino acid motif in the cytoplasmic domain of both murine and human CD8α chains has been shown to noncovalently interact with murine $p56^{lck}$ (25). Cell lysates from LN cells of hCD8 Tg mice were subjected to immunoprecipitation with hCD8α-specific Ab (OKT8) or $p56^{lck}$-specific antiserum (22) to analyze the physical association of transgenic hCD8 with murine $p56^{lck}$. An in vitro protein kinase assay for autophosphorylated $p56^{lck}$ was performed on the immunoprecipitates from C57BL/6, hCD8α.18, and hCD8α+β.42 Tg mice. As demonstrated in Fig. 4, murine $p56^{lck}$ coprecipitated with hCD8 molecules in OKT8 immunoprecipitated lysates from hCD8 Tg mice but not C57BL/6 mice. This suggested that the transgenic hCD8 proteins were appropriately integrated into the murine coreceptor signal transduction system.

Human CD8 Transgene Expression Enhances HLA Recognition by Murine T Cells. It has been demonstrated that the main barrier to recognition of human class I MHC by murine T cells is poor interaction between mCD8 and HLA class I molecules (14–16). To characterize the function of $hCD8^+$ T cells, we tested their ability to mount a cytolytic response to HLA-A2.1 molecules after primary in vitro stimulation with splenocytes from HLA-A2.1^+ Tg mice. Unprimed responder splenocytes from hCD8α.18, hCD8β.10, and hCD8α+β.42 Tg mice were directly compared to assess the relative contribution of hCD8 subunits to enhance HLA-A2.1 recognition and CTL reactivity (Fig. 5). Effector cells from hCD8α.18 or hCD8α+β.42 Tg mice showed markedly enhanced CTL responses to HLA-A2.1 transfected Jurkat (Jurkat/A2), and 310 target cells (HLA-A2, B27) as compared to nontransgenic C57BL/6 or hCD8β.10 Tg effector cells (Fig. 5, *A* and *C*). The enhanced xenogeneic reactivity by hCD8α.18 and hCD8α+β.42 transgenic effector CTL was comparable to the allogeneic B10.A(18R) stimulated responses to P815 ($H\text{-}2D^d$) target cells (Fig. 5 *D*). The low cytolytic response to nontransfected Jurkat cells (HLA-A3/B7) verified that the CTL responses were specific for HLA-A2.1 (Fig. 5 *B*). Responder cells from hCD8 Tg mice cocultured with allogeneic B10.A(18R) stimulator cells showed a strong response to $H\text{-}2D^d$ (Fig. 5 *D*) indicating that the expression

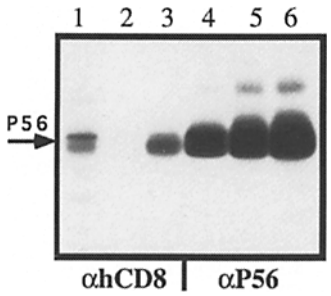

Figure 4. Analysis of hCD8 coreceptor association with murine $p56^{lck}$. Lysates were prepared from LN cell suspensions and immunoprecipitated with anti-human CD8 Ab (*αhCD8*) (lanes *1–3*) or anti-$p56^{lck}$ antisera (*αP56*) (lanes *4–6*) and then assessed for autophosphorylation of $p56^{lck}$ using an in vitro kinase assay. LN cell suspensions from hCD8α.18 Tg mice (lanes *1* and *4*), hCD8α+β.42 Tg mice (lanes *3* and *6*) or nontransgenic C57BL/6 mice (lanes *2* and *5*) were tested.

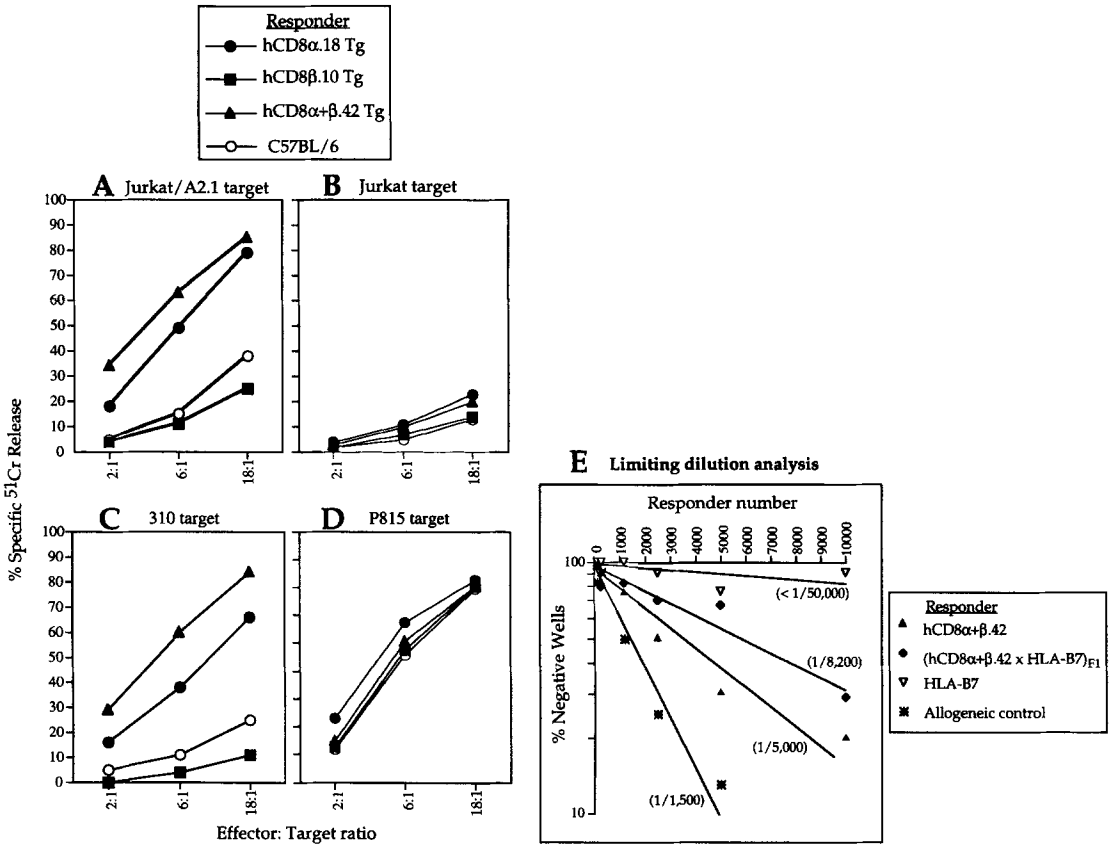

Figure 5. Primary CTL activity to foreign MHC in transgenic mice expressing hCD8. (*A–D*) Unprimed splenocytes from hCD8α.18 Tg (*closed circles*), hCD8β.10 Tg (*closed squares*), hCD8α+β.42 Tg (*closed triangles*), and nontransgenic C57BL/6 mice (*open circles*) were stimulated with HLA-A2.1 Tg splenocytes (*A–C*) or B10.A(18R) splenocytes (*D*). The resulting effector cells were assessed for CTL activity against the following target cells: Jurkat/A2.1 transfectants (*A*), Jurkat (*B*), 310 (*C*), and P815 (*D*). (*E*) The relative frequency of pCTL specific for HLA-A2.1 was assessed for hCD8α+β.42 Tg (*closed triangle*), (hCD8α+β.42 × HLA-B7)F1 Tg (*closed diamond*), and HLA-B7 Tg (*inverted, open triangle*) mice using Jurkat/A2.1 target cells. For comparison, limiting dilution analysis of allogeneic responses by hCD8α+β.42 Tg responders to B10.A(18R) ($H\text{-}2D^d$) were assessed (*) using P815 target cells. The relative pCTL frequencies were determined from the number of responder cells associated with 37% negative wells and are presented in parentheses.

of hCD8 transgenes did not inhibit the recognition of allogeneic murine class I determinants. Thus, the expression of hCD8α or hCD8α+β transgenes greatly enhanced the capacity of murine T cells to recognize human class I molecules without interfering with the normal recognition of allogeneic murine class I. Interestingly, expression of hCD8β protein alone, by effector cells from hCD8β.10 Tg mice, was not sufficient to enhance HLA class I recognition (Fig. 5, *A* and *C*). Cell surface expression was confirmed by FACS® analysis with the monoclonal antibody 5F2, which can recognize hCD8β associated with either hCD8α or mCD8α (26). Moreover, coexpression of hCD8β with hCD8α in $(hCD8\beta.10 \times hCD8\alpha.18)_{F1}$ Tg mice did not increase the relative cytolytic reactivity to HLA-A2.1 compared to parental hCD8α.18 Tg mice (data not shown). These results extend previous studies indicating that the recognition function of hCD8 for HLA class I appears to be primarily a function of the hCD8α subunit (8, 27).

The effect of transgene expression on the frequency of CTL precursors (pCTL) capable of recognizing xenogeneic HLA class I molecules was determined by limiting dilution analysis of primary in vitro stimulated spleen cells (Fig. 5 *E*). The frequency of pCTL from HLA-B7 Tg mice specific for HLA-A2.1 was very low (<1:50,000) and was essentially the same as from nontransgenic C57BL/6 mice (data not shown). These results are in accord with a previous report demonstrating that expression of HLA class I transgenes did not increase the frequency of pCTL capable of responding to foreign HLA class I determinants (28). In contrast, the expression of the hCD8 coreceptor in hCD8α+β.42 Tg mice markedly increased the frequency of HLA-A2.1-specific pCTL (1:5,000) to a level comparable with allogeneic responses to B10.A(18R) spleen cells (1:1,500). Coexpression of HLA-B7, by $(hCD8\alpha+\beta.42 \times HLA\text{-}B7)_{F1}$ Tg mice, did not increase the frequency of HLA-A2.1-specific pCTL compared to hCD8α+β.42 Tg mice expressing only hCD8. Thus, as revealed by the presence of hCD8, a significant proportion of the conventional murine T cell repertoire bears TCR with potential specificity for foreign HLA class I molecules.

Coreceptor Expression in CTL Reactive to HLA Class I Molecules. Antibody blocking studies with CD8 specific Ab were performed, to assess the relative contribution of transgenic hCD8 or endogenous mCD8 proteins in medi-

ating the observed increase in reactivity to HLA-A2.1 (Fig. 6, *A* and *B*). After in vitro stimulation, effector cells were treated with Ab specific for hCD8 or mCD8 just before the addition of target cells for the CTL assay. Blocking with Ab specific for hCD8α (OKT8) markedly decreased the ability of the hCD8α+β.42 Tg–derived CTL to kill Jurkat/A2 target cells, to a level similar to CTL from nontransgenic C57BL/6 mice (Fig. 6 *A*). However, blocking with Ab specific for mCD8α (3.168) did not effect the ability of hCD8α+β.42 Tg derived CTL to specifically kill Jurkat/A2 target cells. The mCD8α specific Ab was shown to be an efficient blocking reagent as it completely inhibited the lysis of P815 target cells by C57BL/6 CTL stimulated with irradiated B10.A(18R) spleen cells (Fig. 6 *B*). These results indicate that the hCD8 transgenic molecules augmented responses to HLA-A2.1 and that the murine CD8 molecules were not required, and are thus consistent with the coreceptor model for CD8 function (4).

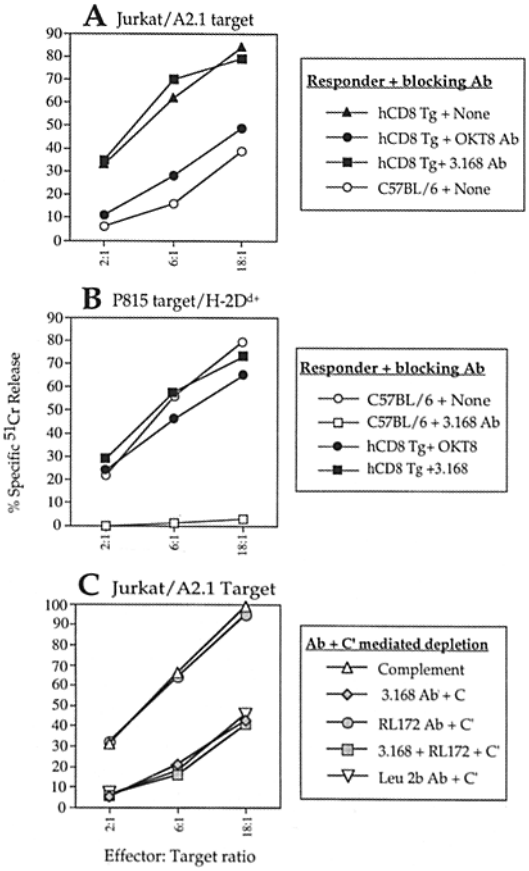

Figure 6. CTL coreceptor utilization. Blocking antibodies specific for endogenous mCD8 (3.168) or transgenic hCD8 (OKT8) coreceptors were added to the indicated hCD8α+β.42 Tg or nontransgenic C57BL/6 CTL effector cells 30 min before adding the Jurkat/A2.1 (*A*), or P815 (*B*) target cells to assess coreceptor usage in xenogeneic responses to HLA-A2.1 or allogeneic responses to H-2D^d, respectively. (*C*) Ab + C'–mediated depletion of potential CTL effector cells, from hCD8α+β.42 Tg mice was done to assess which effector cells were cytolytic for Jurkat/A2.1 target cells. Effector cell populations were depleted of mCD8$^+$ cells (*shaded diamonds*), mCD4$^+$ cells (*shaded circles*), mCD4$^+$ and mCD8$^+$ cells (*shaded squares*), or hCD8$^+$ cells (*inverted, open triangles*). Nondepleted CTL populations were treated with complement alone (*open triangles*).

Although lysis of P815 target cells by C57BL/6 CTL specific for allogeneic H-2D^d determinants was completely inhibited by mCD8 specific Ab (3.168), lysis by H-2D^d-specific CTL cells derived from hCD8α+β.42 Tg mice was not inhibited (Fig. 6 *B*). This suggests that CTL from hCD8 Tg responder mice were capable of using transgenic hCD8 coreceptors as well as the endogenous mCD8 coreceptors in responses to allogeneic H-2D^d determinants. This was confirmed by the complete inhibition of responses by hCD8α+β.42 Tg effectors to allogeneic murine class I by the addition of Ab specific for both mCD8 and hCD8 (see below, Fig. 7 *C*). Thus, whereas mCD8 interacts inefficiently with human class I, hCD8 can associate with murine class I and function as a coreceptor. These results extend previous studies indicating that hCD8 could functionally interact with murine class I MHC (27).

Coreceptor expression by HLA-A2.1–specific CTL from hCD8 Tg mice was determined by depletion of specific effector populations using Ab + C'–mediated cytotoxicity just before the addition of labeled target cells (Fig. 6 *C*). Specific lysis of Jurkat/A2 target cells was markedly reduced when hCD8α+β.42 Tg effector cells expressing hCD8 or mCD8 were depleted (Fig. 6 *C*). Depletion of effector cells expressing mCD4 did not effect the ability to lyse specific targets suggesting that the predominant T cell phenotype was associated primarily with endogenous coreceptor expression. These results are in accord with a recent study of rescued class I specific CD4 cells in (mCD8 Tg × class II$^{-/-}$) mice which indicated that the expression of helper versus CTL effector phenotypes appeared to be independent of MHC recognition and correlated with endogenous coreceptor gene expression (29). A residual CTL response was observed when effector cells expressing mCD8 and mCD4 were both depleted. However, the hCD8$^+$/mCD4$^-$8$^-$ effector cells accounted for only 10% of the anti–HLA-A2.1 CTL activity (data not shown). Thus, the majority of HLA-A2.1–specific CTL effector cells from hCD8α+β.42 Tg mice were of the hCD8$^+$, mCD8$^+$ phenotype.

Optimal Responsiveness to HLA Class I Involves Two Functional Interaction Sites. The results presented above clearly indicated that the expression of hCD8 transgenes markedly enhanced the magnitude of reactivity and the precursor frequency of murine T cells capable of responding to HLA class I molecules. These results were consistent with reports of enhanced reactivity of murine T cells to hybrid HLA/H-2 molecules bearing the α3 domain from murine class I, which facilitated interaction with endogenous mCD8 coreceptors (14–16). However, a second interaction site that affects hCD8 coreceptor binding to HLA-A2.1 was recently revealed using site directed mutagenesis of hCD8α to determine which residues were essential for binding to HLA-A2.1 in cell–cell binding assays (30). The mutational studies of hCD8 along with topographical models of hCD8-HLA class I interactions, based on the crystal structures of hCD8 and HLA-A2.1, suggested that residues within the MHC class I α2 domain may be involved in CD8 coreceptor binding (30). Mutational analysis of HLA-A2.1 has now

been done to define this second interaction site (31). The hCD8 Tg model provided the means to determine the functional impact of a second interaction site, outside of the α3 domain, on the ability of hCD8 to augment responses to HLA class I molecules.

The observation that human CD8 coreceptors can functionally interact with murine class I (see Fig. 6 *B*) suggested that hCD8 coreceptors should interact with the A2/K^b hybrid molecules at both the α_3 and the putative α_2 domain binding sites. In contrast, it would be anticipated that mCD8 coreceptors would interact with only the α_3 domain of the hybrid A2/K^b molecules. If this were the case it would be predicted that hCD8 Tg mice would respond better than nontransgenic C57BL/6 mice to spleen cells from A2/K^b Tg mice. In accordance with this, primary in vitro responses to A2/K^b hybrid molecules by hCD8α+β.42 Tg mice were consistently fivefold higher than responses by nontransgenic C57BL/6 mice as measured in CTL assays using Jurkat-A2/K^b targets (Fig. 7 *A*). Furthermore, Ab blocking of hCD8α×β.42 Tg CTL with anti-hCD8 Ab (OKT8) reduced the cytolytic response to Jurkat-A2/K^b target cells, to a level just below that of nontransgenic C57BL/6 mice (Fig. 7 *B*). Blocking of C57BL/6-derived CTL with anti-mCD8 antibody (3.168) completely abrogated the response (Fig. 7 *B*).

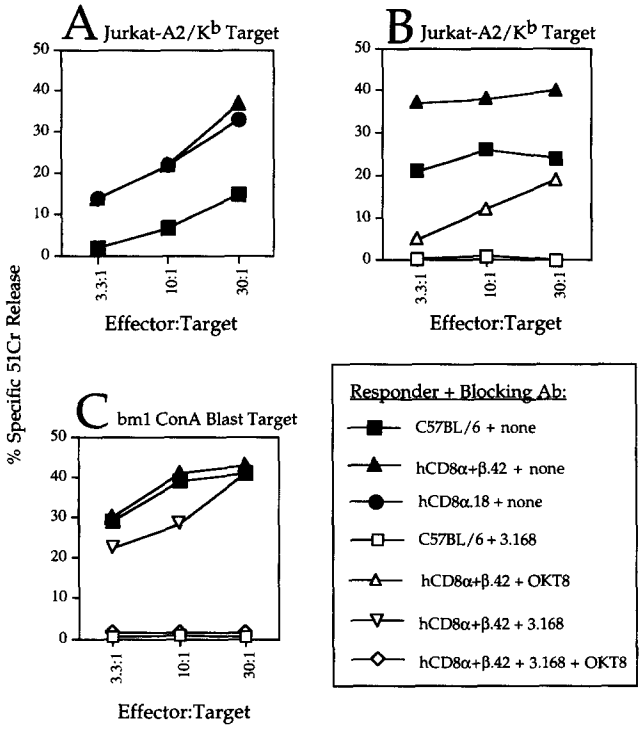

Figure 7. Human CD8 coreceptor binding involves two functional interaction sites with HLA class I MHC. Primary in vitro stimulated CTL cells from nontransgenic C57BL/6 mice (*closed squares*), hCD8α+β.42 Tg (*closed triangles*), or hCD8α.18 Tg (*closed circles*) were assessed for CTL activity against Jurkat-A2/K^b transfectant target cells (*A* and *B*) or bm1-derived Con A blast target cells (*C*). Ab blocking of CTL effector cells from C57BL/6 mice with mCD8-specific Ab (3.168) (*open squares*), and blocking of CTL from hCD8α+β.42 Tg mice with hCD8-specific Ab (OKT8) (*open triangles*), with mCD8-specific Ab (3.168) (*inverted, open triangles*), or both (OKT8+3.168) (*open diamonds*) was done to assess the CD8 coreceptor usage in responses to A2/K^b hybrid molecules (*B*) or to bm1 alloantigens (*C*).

The marked enhancement in responses to A2/K^b in hCD8 Tg mice could be explained in two ways: the hCD8 coreceptor may interact with α_3 domain of H-2K^b class I molecules more efficiently than mCD8 coreceptors or, alternatively, an interaction site within the first two domains of HLA-A2.1 was essential to optimize the functional interaction of CD8 coreceptors with the A2/K^b hybrid molecule. To determine if hCD8 interacted with the α_3 domain of H-2K^b more efficiently than mCD8 we compared the response to H-2K^{bm1} by C57BL/6 mice and hCD8α+β.42 Tg mice since the α_3 domain of H-2K^{bm1} is identical to that of the H-2K^b. The H-2K^{bm1} allele differs from the H-2K^b allele at residues 152, 155, and 156 within the α_2 domain and induces a very strong alloresponse by mice of the b-haplotype such as C57BL/6. Effector cells from C57BL/6 mice and hCD8α+β.42 Tg mice responded equivalently as determined by lysis of bm1 Con A blast target cells in a primary in vitro CTL assay (Fig. 7 *C*). The response to bm1 by nontransgenic C57BL/6-derived CTL was completely inhibited by the addition of Ab specific for mCD8 (3.168) whereas inhibition of hCD8α+β.42 Tg–derived CTL required the addition of Ab specific for both mCD8 and hCD8 (3.168 + OKT8). Together with the studies of hCD8 site-directed mutagenesis discussed above (30), these results suggested that hCD8 interactions with residues within the α_2 domain as well as the α_3 domain of HLA class I are required for optimal coreceptor function. Thus optimal recognition of HLA class I–restricting elements by murine T cells requires expression of human CD8 coreceptors.

Peripheral Expression of hCD8 Is Sufficient to Modulate Foreign MHC Class I Recognition. The lack of hCD8 transgene expression in the thymus of hCD8α.40 Tg mice (see above) allowed us to examine the ability of hCD8 to modulate recognition of MHC molecules by an established peripheral T cell repertoire which was conventionally selected on murine MHC. Thymic expression in hCD8α.18 Tg mice could have altered the development of the murine T cell repertoire as human CD8 is capable of functionally interacting with murine class I molecules (see Fig. 6 *B*). Hence, CTL responses to HLA-A2.1 by C57BL/6, hCD8α.18, and hCD8α.40 mice were directly compared to determine if hCD8 expression in the thymus was required for enhanced anti–HLA class I CTL responses (Fig. 8 *A*). Effector cells from hCD8α.18 and hCD8α.40 Tg showed equivalent enhancement of CTL responses to HLA-A2.1 compared to C57BL/6. Thus, peripheral expression of hCD8 coreceptors, in hCD8 Tg mice lacking thymic expression, was sufficient to enhance recognition of foreign HLA class I.

The $p56^{lck}$ promoter used to drive expression of the transgenes induced expression of hCD8 on both mCD8 and mCD4 T cells. This enabled us to assess the ability of hCD8 to modulate TCR recognition of MHC by mature

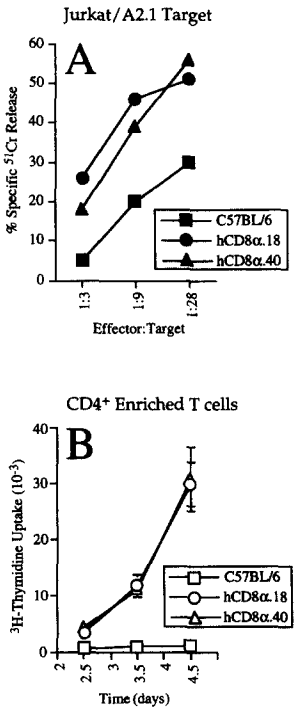

Figure 8. Requirement for hCD8 expression in the thymus for reactivity to foreign class I or class II MHC. (*A*) Cytolytic activities of hCD8α.18 Tg (*closed circle*), hCD8α.40 Tg (*closed triangle*) (no thymic expression), and nontransgenic C57BL/6 (*closed squares*) derived CTL were assessed against labeled Jurkat/A2.1 target cells. (*B*) Proliferative responses of mCD4+–enriched T cells from hCD8α.18 Tg (*open circle*), hCD8α.40 Tg (*open triangle*) (no thymic expression), and nontransgenic C57BL/6 mice (*open squares*) to splenocytes from HLA-A2.1 Tg mice were assessed by [^{3}H]TdR uptake at the indicated times after coculture.

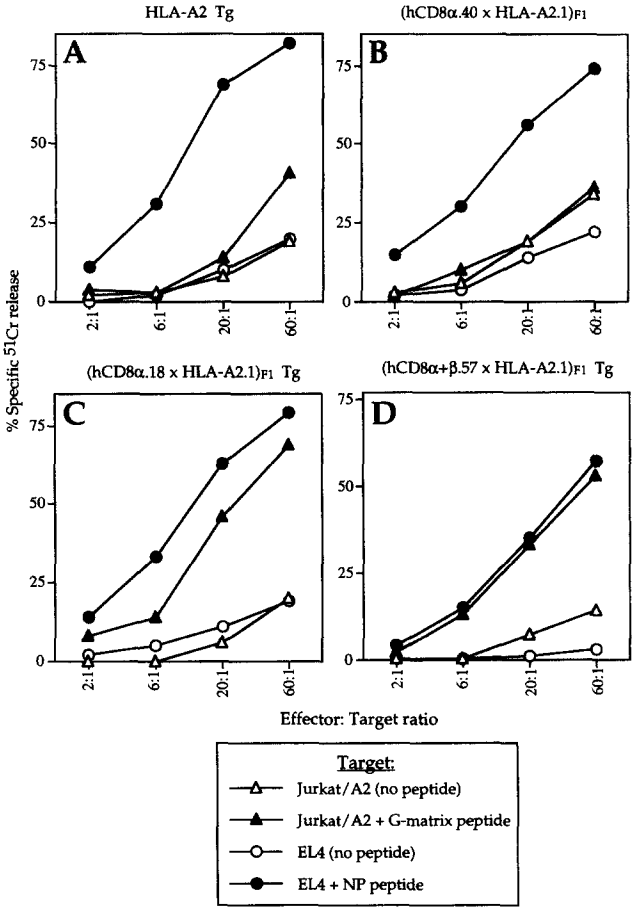

Figure 9. Requirement of hCD8 expression in the thymus for reactivity to antigenic viral peptides in the context of self-HLA class I MHC. CTL activity of effector cells against influenza virus peptides presented by HLA-A2.1 (*triangles*) or H-2D^{d} (*circles*) restricting elements were assessed in a 4-h ^{51}Cr-release assay. Effector cells from HLA-A2.1 Tg (*A*), (hCD8α.40 × HLA-A2.1)$_{F1}$ Tg (*B*), (hCD8α.18 × HLA-A2.1)$_{F1}$ Tg (*C*), and (hCD8α+β.57 × HLA-A2.1)$_{F1}$ Tg (*D*) mice were compared for lysis of Jurkat/A2.1 target cells (*triangles*) or EL4 target cells (*circles*) in the absence of antigen (*open symbols*) or prepulsed with the indicated peptides (*closed symbols*).

CD4+ cells that were educated on murine class II–restricting elements in the thymus of hCD8α.40 Tg mice (which lacked thymic expression of hCD8). Proliferative responses to HLA-A2.1, by mCD4+ purified T cells, were enhanced in mice expressing hCD8 in the thymus and periphery (hCD8α.18) and in mice expressing hCD8 in the periphery only (hCD8α.40) as compared to nontransgenic C57BL/6 mice (Fig. 8 *B*). Thus, mCD4+ T cells (educated on murine class II MHC) that expressed hCD8 after exiting the thymus were readily recruited for recognition of HLA class I in proliferative responses.

Thymic Expression of hCD8 Is Required for Self-HLA Class I–restricted Responses to Viral Peptide. Previous studies have shown that CD8 coreceptors are essential for the development of class I–restricted T cells (12, 13, 32) and for the enhanced positive selection of clonotypic T cells expressing TCR transgenes (33). The hCD8 transgenic model provided a means to directly assess the requirement for human CD8 coreceptor expression in the thymus for selection of T cells restricted by self-HLA class I molecules. We compared influenza virus primed effector cells from HLA-A2.1, (hCD8α.40 × HA-A2.1)$_{F1}$, (hCD8α.18 × HLA-A2.1)$_{F1}$, and (hCD8α+β.57 × HLA-A2.1)$_{F1}$ Tg mice for CTL reactivity to HLA-A2.1+ target cells pulsed with HLA-A2.1 restricted influenza matrix peptide (GILGFVFTL). CTL reactivity to H-2D^{b}–restricted NP peptide (ASNENMETM) was assessed simultaneously as an internal control for efficient priming after infection with influenza virus PR8. The results indicated that parental HLA-A2.1 Tg–derived CTL (Fig. 9 *A*) responded weakly to the HLA-A2.1–restricted matrix peptide. Coexpression of hCD8α in the periphery only, by (hCD8α.40 × HLA-A2.1)$_{F1}$ Tg mice, was not sufficient to augment the HLA-A2.1–restricted response to matrix peptide (Fig. 9 *B*). In contrast, coexpression of hCD8α (Fig. 9 *C*) or hCD8α+β (Fig. 9 *D*) in both the thymus and periphery markedly enhanced the HLA-A2.1–restricted response to matrix peptide. The weak responses by CTL from HLA-A2.1 and (hCD8α.40×HLA-A2.1)$_{F1}$ Tg mice were not due to an inefficient priming of the mice, as strong responses to H-2D^{b}–restricted NP peptide were observed (Fig. 9, *A* and *B*). Thus, thymic expression of hCD8 during positive selection was required for efficient development of an HLA-A2.1–restricted response to viral peptide. This requirement for CD8 coreceptor expression in the thymus was in marked contrast to the responses to alloantigens, which were mediated by a relatively large proportion of the T cell repertoire. These results indicate that an important functional consequence of hCD8 coreceptor expression in the thymus is an increased likelihood that individuals expressing a particular HLA class

I molecule will positively select a sufficient repertoire to insure development of strong immune responses to antigenic peptides presented by that particular restriction element.

Discussion

The hCD8 Tg system used here provided a model to study CD8 coreceptor regulation of MHC–peptide complex recognition under conditions in which expression of the hCD8 transgene occurred in both thymocytes and peripheral T cells or in the periphery alone. The general effect of hCD8α or hCD8α+β transgene expression on T cell recognition was a greatly enhanced capacity of murine T cells to recognize HLA class I molecules. Interestingly, expression of hCD8β alone, by T cells from hCD8β.10 Tg mice, was not sufficient to enhance HLA class I recognition (Fig. 5). Thus, augmentation of HLA class I recognition was primarily a function of the hCD8α subunit.

The potential specificities of murine and human T cell repertoires for MHC–peptide complexes appear to overlap to a significant degree. This may reflect the strong homology between human and murine MHC molecules (34). Conventional murine T cell repertoires have previously been shown to contain T cells capable of strong reactivity to hybrid HLA class I molecules, bearing murine class I α_3 domains (14–16). However, the studies presented here demonstrated that optimal recruitment of murine T cells for responsiveness to HLA class I molecules required an additional interaction site for hCD8 coreceptor binding to HLA class I (Fig. 7). We have consistently observed that C57BL/6 responses to A2/K^b were enhanced compared to responses to HLA-A2.1 due to the enhanced interaction of the mCD8 coreceptors with the murine class I α_3 domain (21). However, the further enhanced responses to hybrid A2/K^b as well as HLA-A2.1 molecules induced by hCD8 transgene expression indicated that the second interaction site, shown to be within the α2 domain of HLA class I (31), is essential for optimal recognition of HLA class I restriction elements. Therefore, the hCD8 Tg model described here represents a significant advancement for the study of HLA class I–restricted responses in vivo using murine experimental models.

The results presented above demonstrated that peripheral expression of hCD8α, in hCD8α.40 Tg mice lacking thymic expression, proved sufficient to mediate augmented responses to foreign HLA class I by conventionally selected murine T cells (Fig. 8 *A*). The plasticity of the repertoire further extended to recognition of MHC class I by $mCD4^+$ T cells that had been selected by class II MHC (Fig. 8 *B*). These results were analogous to previous studies (35) in which expression of transgenic mCD4 on $mCD8^+$ T cells facilitated class II recognition by cells positively selected on class I MHC. Together, these results are consistent with models proposing that CD4 and CD8 T cells use largely overlapping TCR gene pools in their recognition of class II and class I MHC and underscore the significant role of coreceptors in determining the ability of a T cell to respond to MHC.

The hCD8-augmented responses to HLA class I–restricted epitopes could readily be distinguished from the endogenous mCD8-regulated responses since mCD8 could not effectively interact with HLA class I molecules. This provided a direct means to assess the requirement of hCD8 expression in the thymus for the development of responses to foreign antigen restricted by self-HLA class I. Although expression of hCD8α in the periphery only was sufficient to obtain strong xenogeneic responses against HLA-A2.1, both thymic and peripheral expression of hCD8 coreceptors was required for development of HLA-A2.1–restricted responses to antigenic peptide. There are several potential mechanisms that may explain this observation. First, the binding of hCD8 to HLA-A2.1 molecules could increase the overall avidity for self-HLA-A2.1–peptide complexes resulting in a significant increase in the number of thymocytes that could achieve positive selection. Previous results from cell–cell binding assays have demonstrated that hCD8 coreceptor expression enhanced cellular adhesion to cells expressing HLA-A2.1 (36, 37), and over-expression of mCD8 coreceptors has been shown to augment positive selection of T cells expressing anti-HY TCR transgenes (33). Of course, increasing relative avidity could also result in elimination of thymocytes expressing TCR with higher affinity for HLA-A2.1-peptide complexes that may have undergone positive selection in the absence of hCD8 coreceptors (20). Nevertheless, since it is likely there are fewer cells with this higher range of receptor affinity, the presence of hCD8 during thymic development would result in a net increase in the size of the HLA-A2.1–restricted repertoire.

Another potential mechanism for hCD8-dependent enhancement of HLA-A2.1-restricted responses would be the coreceptor function of hCD8 proteins, which would result in a more effective dual signal transduction for selection and activation. A recent study of "tailess CD8α" transgenic mice demonstrated that signal transduction through the CD8 coreceptor was essential for the development of most class I–restricted T cells (38). It would appear in the hCD8 Tg model presented here, that the hCD8α coreceptor protein is compatible with the murine cytoplasmic proteins required for signal transduction during thymic development.

The expression of hCD8β proteins was not essential to obtain strong reactivity to either foreign HLA class I or viral peptide restricted to self-HLA-A2.1. However, the relative expression of hCD8α and hCD8β in the thymus and periphery (Fig. 3 *C*) suggested there was a preferential selection of cells expressing hCD8α/β heterodimers. These results are particularly interesting in light of recent reports indicating that CD8β expression was essential for the development of a significant proportion of $CD8^+$ T cells (38a, 39, 40). However, the expression of only hCD8α was sufficient for the development of mature $hCD8^+$ T cells (Fig. 3) and for enhancing HLA class I–restricted responses to viral antigens (Fig. 9). Although there appeared to be a relatively small proportion of mCD8β associated with hCD8α compared to hCD8β or hCD8α (homodimers) (data not shown), it may have been sufficient to influence

development. Alternatively, the hCD8α^+ cells may have been a population of T cells that was not dependent on the expression of CD8β proteins for development (38a, 39, 40). Full assessment of the relative contribution of hCD8β for the development of T cells specific for HLA class I MHC–peptide complexes would require backcrossing to mutant mice with the mCD8β^{null} phenotype.

The results from this hCD8 Tg model demonstrated a marked enhancement of positive selection for HLA class I–restricted responses to antigenic peptide that was expressly dependent on the expression of hCD8 in the thymus. Previous studies indicating an essential role for CD8 coreceptor expression in the thymus for positive selection have shown that the elimination of CD8 expression abrogated the development of class I restricted cells (12, 13, 32) and that overexpression of endogenous mCD8 augmented the biased TCR expression by thymocytes generated by TCR cDNA transgenes (33). Here, we further demonstrate that a relatively small population of thymocytes specific for a single peptide epitope restricted by HLA-A2.1 required the expression of hCD8 in the thymus for the subsequent development of strong responses after antigenic stimulation. Moreover, the lack of hCD8 expression in the thymus precluded positive selection of HLA-restricted responses to peptide while leaving hCD8-dependent enhancement of alloreactive specificities intact. These disparate requirements for hCD8 coreceptor expression in the thymus indicate that neither class specific nor allele specific thymic selection is required to obtain recognition of foreign MHC molecules, yet allele-specific thymic selection is a requirement for self-MHC–restricted recognition of antigenic viral peptides. Finally, the ability of hCD8 to optimize utilization of HLA class I molecules as effective restricting elements should facilitate the study of HLA-restricted responses to peptide epitopes in vivo as the hCD8 Tg mice can readily be bred to any HLA class I transgenic strain.

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