CD8 Controls T Cell Cross-Reactivity

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Estimates of human αβ TCR diversity suggest that there are $<10^8$ different Ag receptors in the naive T cell pool, a number that is dwarfed by the potential number of different antigenic peptide-MHC (pMHC) molecules that could be encountered. Consequently, an extremely high degree of cross-reactivity is essential for effective T cell immunity. Ag recognition by T cells is unique in that it involves a coreceptor that binds at a site distinct from the TCR to facilitate productive engagement of the pMHC. In this study, we show that the CD8 coreceptor controls T cell cross-reactivity for pMHCI Ags, thereby ensuring that the peripheral T cell repertoire is optimally poised to negotiate the competing demands of responsiveness in the face of danger and quiescence in the presence of self.   *The Journal of Immunology*, 2010, 185: 4625–4632.

CD8^{+} CTLs are key determinants of immunity to intracellular pathogens and cancer cells. CTLs recognize protein Ags in the form of short peptides (8–13 aa), presented in association with MHC class I (MHCI) molecules on the surface of target cells. Almost all nucleated cells express MHCI, thereby enabling the immune system to scan the cell surface to detect internal anomalies. The Ag specificity of CTLs is conferred by the highly variable CDRs of the TCR that interact with the peptide-binding platform of the MHC (1–3). Adaptive T cell immunity requires that naive T cells from a limited precursor pool, which has been estimated at $<10^8$ different AgR clonotypes (4), respond effectively to a multitude of potential peptide-MHC (pMHC) Ags associated with cellular abnormalities; this discrepancy is resolved, at least in part, by the phenomenon of T cell cross-reactivity (5). To satisfy the conditions required for effective immune coverage, both theoretical considerations (5) and experimental evidence (6) suggest that a single TCR must recognize over one million different peptides in the context of a single MHCI molecule. However, despite the huge importance of T cell cross-reactivity, little is known about the factors that control this phenomenon.

T cell recognition of pMHCI Ag involves the binding of two receptors (TCR and CD8) to a single ligand (pMHCI) (7, 8). Indeed, this mode of Ag engagement is unique to T cell biology. The αβ heterodimeric CD8 coreceptor has multiple enhancing effects on early activation events (9, 10), which are mediated by the following: 1) localization of the TCR to specific membrane domains believed to be privileged sites for TCR-mediated signal transduction (11); 2) recruitment of essential signaling molecules to the intracellular side of the TCR/CD3/ζ complex (12, 13); and 3) stabilization of the TCR–pMHCI interaction at the cell surface (14, 15). Furthermore, a number of studies have demonstrated that both the on-rate (k_{on}) and off-rate (k_{off}) of the TCR–pMHCI interaction determine the consequences of Ag engagement at the functional level (16–19). CD8 has the ability to influence both of these kinetic parameters (15, 20–22) and, therefore, may play an important role in tuning the duration of short-lived TCR–pMHCI interactions to mediate distinct biological outcomes (23–25). In this study, we extend these observations to investigate the role of CD8 in the phenomenon of T cell cross-reactivity.

Materials and Methods

Cells

The ILA1 CTL clone was generated and restimulated as described previously (20). CTLs were maintained in RPMI 1640 (Life Technologies, Paisley, U.K.) containing 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies), and 10% heat inactivated FCS (Life Technologies) supplemented with 2.5% Cellkines (Helvetica Healthcare, Geneva, Switzerland), 200 IU/ml IL-2 (PeproTech EC, London, U.K.), and 25 ng/ml IL-15 (PeproTech). HLA A*0201 (HLA A2 from hereon) and mutants thereof were expressed as full-length molecules in pCDNA3.1 (Invitrogen, Carlsbad, CA) under G418 (Neo) selection (26). Cells were transfected with D227K/T228A HLA A2, A245V HLA A2, wild-type HLA A2, Q115E HLA A2, or A2/K[b] HLA A2 (15); these molecules exhibit abrogated, weak, normal, enhanced, or superenhanced interactions with CD8, respectively, yet retain TCR-binding integrity (15). C1R B cell transfectants were established from single cells and clones with similar expression levels of HLA A2, as determined by flow cytometry with the HLA A2-specific Ab BB7.2 (FITC conjugated; Serotec, Oxford, United Kingdom), were selected. C1R transfectants were maintained in RPMI 1640 (Life Technologies) containing 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies), and 20% heat-inactivated FCS (Life Technologies).

Nonamer combinatorial peptide library and MIP1β ELISA

The nonamer combinatorial peptide library (CPL) contains a total of 4.8 × 10^{11} [$(9 + 19) \times 19^8$] different nonamer peptides (Pepscan, Lelystad, The Netherlands) (6, 27) and is divided into 180 different peptide mixtures (Fig. 1). In every peptide mixture, one position has a fixed L-amino acid residue, but all other positions are degenerate, with the possibility of any 1 of 19 natural L-amino acids being incorporated in each individual position (cysteine is excluded in nonfixed positions). Each library mixture consists of 1.7×10^{10} (19^8) different nonamer peptides in approximately equimolar concentrations. For CPL screening, ILA1 CTLs were washed and rested overnight in RPMI 1640 containing 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 2 mM L-glutamine
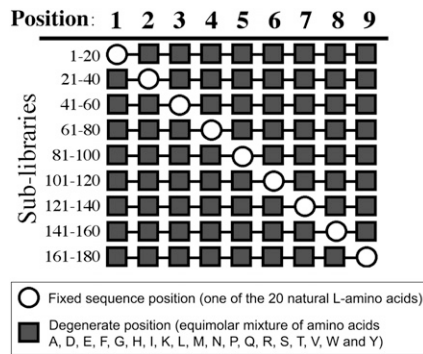
FIGURE 1. Schematic representation of a nonamer CPL. The nonamer CPL contains a total number of 4.8×10^{11} [$(9 + 19) \times 19^8$] different nonamer peptides and is divided into 180 different peptide mixtures (or sublibraries), as indicated. In every peptide mixture, 1 of the 20 natural proteogenic L-amino acids is fixed at one position (circles), but all other positions are degenerate (squares), with the possibility of any 1 of 19 natural L-amino acids being incorporated at the degenerate positions (cysteine is excluded at the degenerate positions). Thus, each library mixture consists of 1.7×10^{10} (19^8) different nonamer peptides in approximately equimolar concentrations. The first mixture has alanine fixed at position 1 with all other positions being degenerate; the second mixture has cysteine fixed at position 1 with all other positions being degenerate. This occurs for all 20 L-amino acids at position 1 and then is repeated for position 2 and so on.

(Life Technologies), and 2% heat-inactivated FCS (Life Technologies). In 96-well U-bottom plates, 6×10^4 C1R B cells were pulsed with various library mixtures at a concentration of 100 μg/ml for 2 h at 37°C. After peptide pulsing, 3×10^4 ILA1 CTLs were added, and the assay was incubated overnight at 37°C. Subsequently, the supernatant was harvested and assayed for MIP1β by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

IFN-γ ELISPOT assay

C1R B cells were used as APCs in the presence of 100 CTLs and 10^{-7} M peptide. The assay was applied to duplicate wells of polyvinylidene difluoride-backed plates (Millipore, Bedford, MA) precoated with IFN-γ

capture Ab 1-DIK (Mabtech, Nacka, Sweden) and incubated for 4 h at 37°C. Plates were developed according to the manufacturer's instructions (Mabtech), and spots were counted using an automated ELISPOT Reader System ELR02 (Autoimmun Diagnostika, Strassberg, Germany).

Degranulation assay

Surface CD107a mobilization was used to assess degranulation, as described previously (28). Briefly, 10^4 ILA1 CTLs were incubated at 37°C with 5×10^4 peptide-pulsed C1R B cells in the presence of FITC-conjugated anti-CD107a (clone H4A3; BD Biosciences, San Jose, CA) and 0.7 μl/ml monensin (GolgiStop; BD Biosciences). After incubation, the cells were washed twice and resuspended in PBS. Data were acquired using a FACSCalibur flow cytometer and analyzed with CellQuest software.

Peptide-binding assays

T2 cells were incubated overnight in the presence of the indicated peptides at a concentration of 10^{-4} M, and then stained with fluorochrome-labeled W6/32 Ab (Dako, Glostrup, Denmark) for 40 min at 4°C. Data were acquired using a FACSCalibur flow cytometer and analyzed with CellQuest software.

Results

T cell recognition of pMHCI Ag is highly cross-reactive

We examined the degree of cross-reactivity exhibited by the CTL clone ILA1, which recognizes residues 540–548 (ILAKFLHWL) of the ubiquitous tumor Ag human telomerase reverse transcriptase bound by HLA A2 (20, 29). For this purpose, we used CPLs, which represent the entire peptide universe of a given length (4.8×10^{11} peptides for a nonamer library; Fig. 1) (6). Fig. 2 shows a CPL scan conducted with the ILA1 CTL clone (summarized in Fig. 3A). The number of amino acid combinations that were recognized by the ILA1 TCR was restricted in the central region of the peptide (residues 3–5), thereby suggesting that this TCR makes the majority of its peptide contacts in this region. This is supported by the observation that alanine substitutions in the central region of the peptide were highly deleterious to Ag recognition (Fig. 3B). In contrast, recognition was highly degenerate at the remaining positions. This degeneracy was confirmed by the
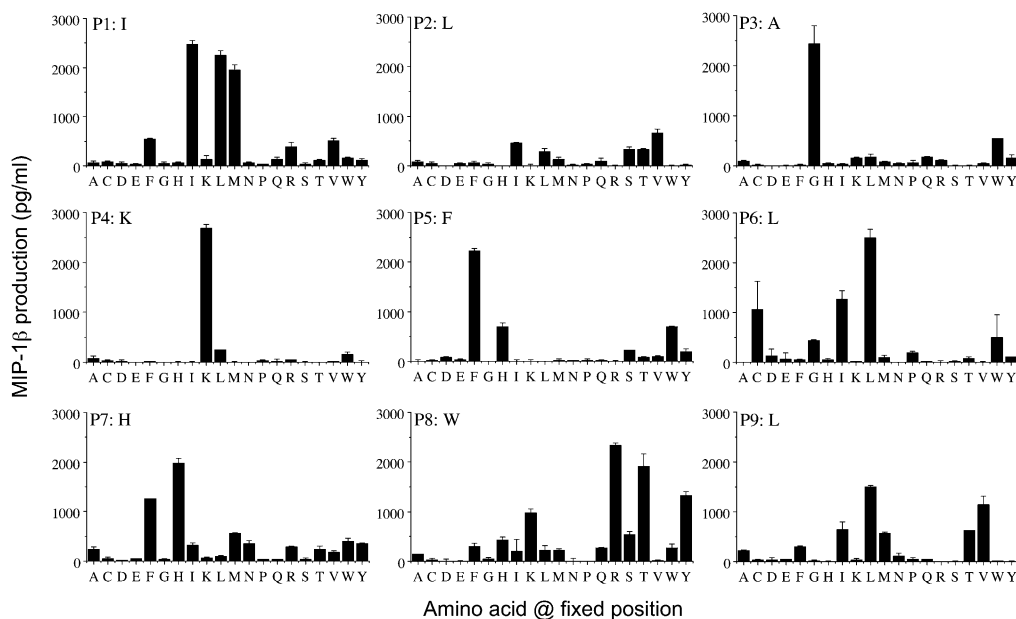

FIGURE 2. CPL scan of ILA1 CTLs. In each well of a 96-well U-bottom plate, 6×10^4 C1R A2 cells were pulsed in duplicate with 1 of the 180 mixtures from a nonamer CPL library (100 μg/ml) at 37°C. After 2 h, 3×10^4 ILA1 CTLs were added to each well and incubated overnight at 37°C. Supernatant was then removed and assayed for MIP1β by ELISA. Results are displayed as histogram plots and are representative of five independent experiments. SD from the mean of two replicates is shown. Of note, isoleucine and valine were recognized more prominently at position 2 in the CPL scan compared with leucine, which is the preferred HLA A2 anchor residue. These data suggest that ILA1 CTLs recognize a much greater number of nonamer peptides with either isoleucine or valine at this position.
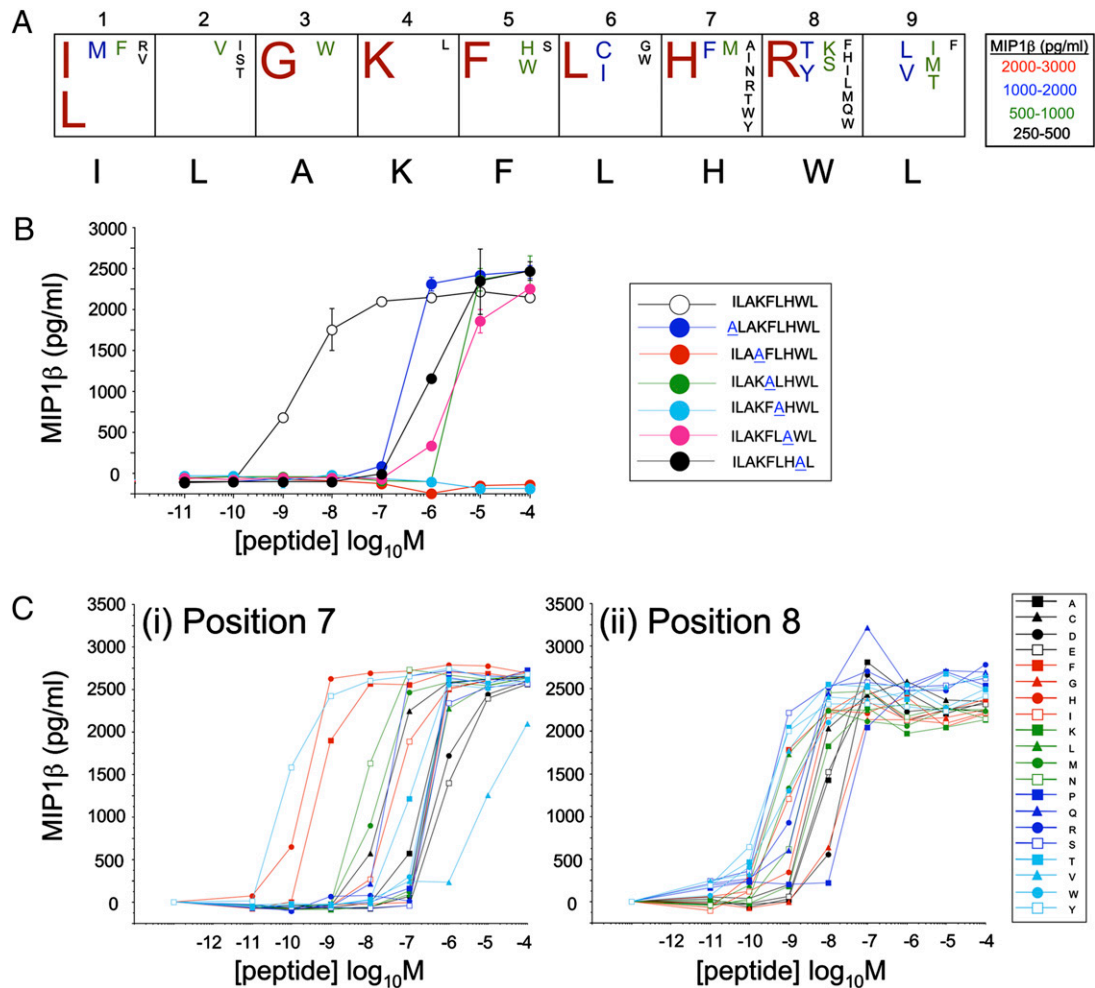
FIGURE 3. *A*, Box plot summary of CPL scan (shown in Fig. 2). *B*, Index peptide alanine scan of ILA1 CTLs. A total of 6×10^4 C1R A2 cells was pulsed in duplicate with various concentrations of the indicated peptides at 37°C. After 1 h, 3×10^4 ILA1 CTLs were added and incubated overnight at 37°C. Supernatant was then harvested and assayed for MIP1β by ELISA. SD from the mean of two replicates is shown. *C*, Recognition of fixed position variants by ILA1 CTL. MIP1β activation data for a set of peptides with the sequence 1) ILGKFLxWL or 2) ILGKFLHxL, where x is 1 of the 20 natural proteogenic ʟ-amino acids. Experimental details as for *B*.
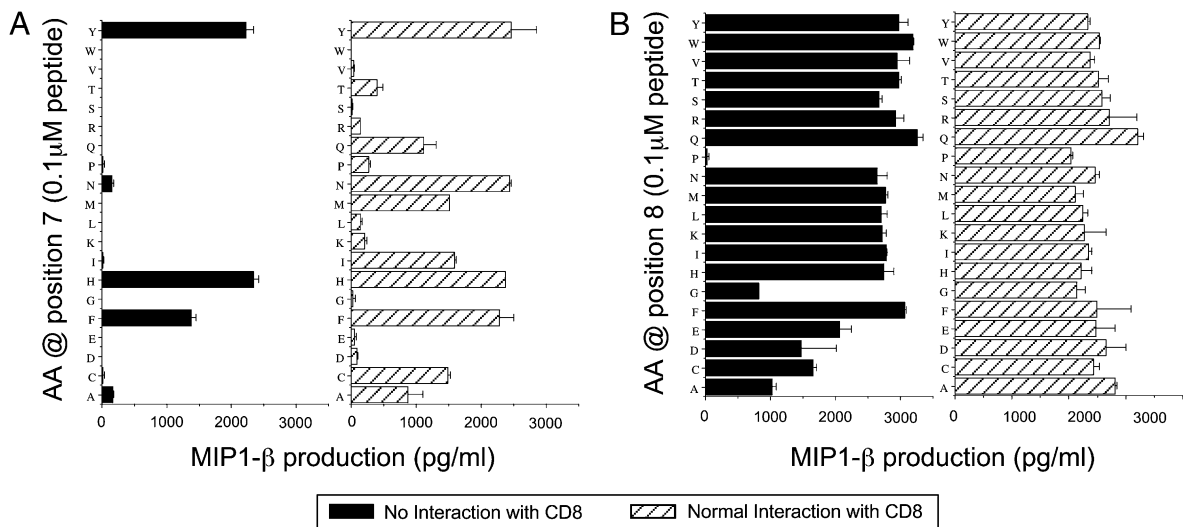
FIGURE 4. CD8 extends the range of pMHCI ligands recognized by the TCR. MIP1β activation data for a set of peptides with the sequence ILGKFLxWL (*A*) or ILGKFLHxL (*B*), where x is 1 of the 20 natural proteogenic ʟ-amino acids, presented in the context of a normal (A2) or abrogated (A2 D227K/T228A) interaction with CD8. Experimental details as for Fig. 3*B*. SD from the mean of two replicates is shown. Lack of CD8 engagement affected recognition of the majority of ILGKFLxWL peptides. However, recognition of only two ILGKFLHxL peptides (glycine and proline) was similarly affected; this may be due to effects on the peptide backbone mediated by these amino acids.

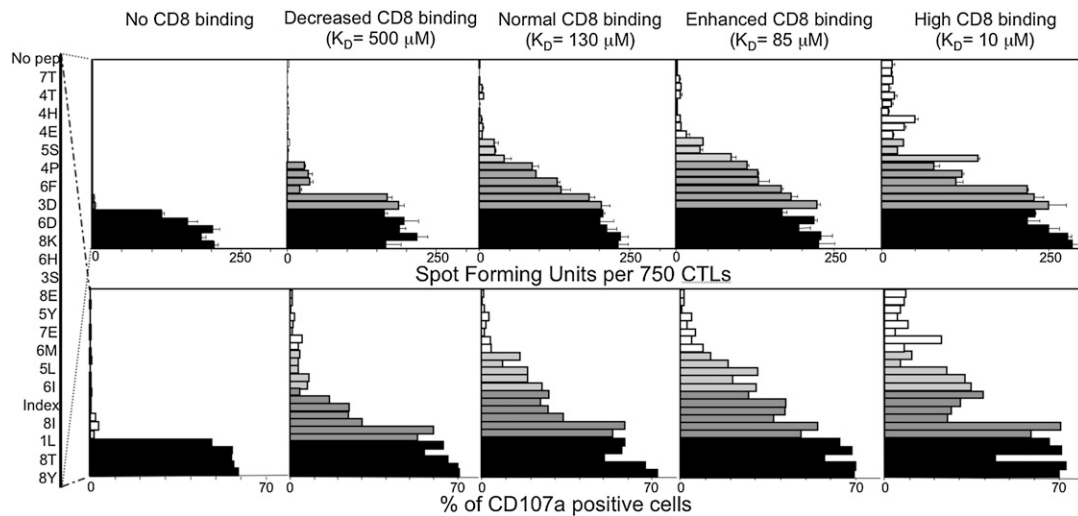**FIGURE 5.** Alterations in pMHCI–CD8 binding affect CTL specificity. The equilibrium CD8αα-binding affinity for each of the HLA A2 mutants is indicated above the respective bar charts. Twenty-three peptides, selected on the basis of robust binding to HLA A2 (Supplemental Fig. 2), were used at a final concentration of 10^{-7} M in IFN-γ ELISPOT (*upper panels*) and degranulation assays (*lower panels*) with ILA1 CTLs and C1R B cell clones expressing D227K/T228A, A245V, wild-type, Q115E, or A2/K^b versions of HLA A2 (15, 26, 33).

ability of ILA1 CTLs to recognize robustly a panel of peptides with any of the 20 natural proteogenic L-amino acids at either peptide position 7 or 8 (Fig. 3C). Thus, positional peptide degeneracy is restricted in the TCR contact region, but extreme at other peptide residue positions, allowing a single TCR to recognize huge numbers of amino acid combinations.

CD8 extends the range of pMHCI ligands that can be recognized

Next, we examined the influence of CD8 binding on the recognition of altered peptide ligands (APLs) with all 20 natural proteogenic L-amino acids at position 7. The number of peptides recognized at

a concentration of 10^{-7} M was substantially reduced on abrogation of the pMHCI–CD8 interaction (Fig. 4A). The effect of CD8 binding was less apparent overall, although essential in some cases, for APLs with substitutions at position 8 (Fig. 4B). Thus, position 8 of the peptide is more degenerate than position 7 and, as a result, less dependent on CD8 engagement. CD8 coreceptor engagement was also important for the recognition of peptides with an xLxKFLxxL motif (Supplemental Fig. 1) and for the recognition of APLs with single amino acid substitutions at each of the non-MHCI anchor positions (Supplemental Fig. 2). Taken together, these results indicate that CD8 binding extends the range of pMHCI
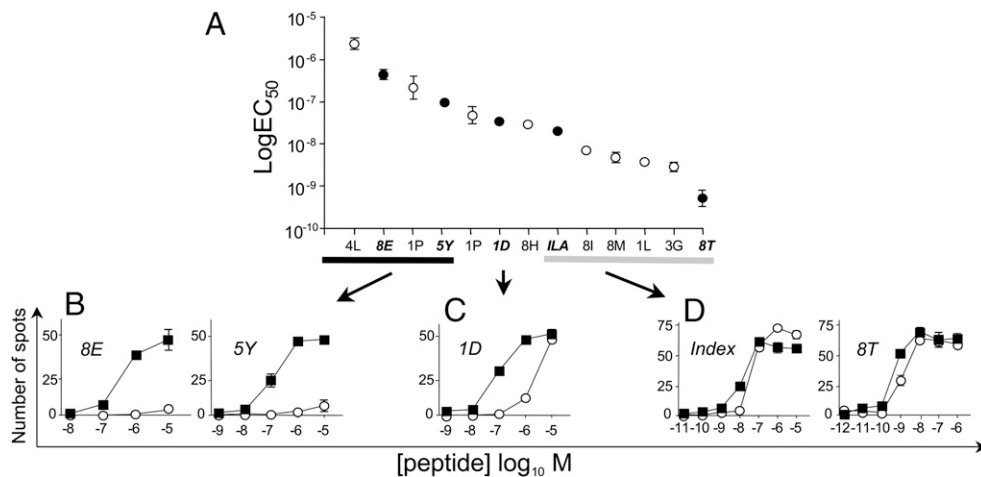

FIGURE 6. Differential effect of the CD8 coreceptor on CTL recognition of Ag variants. The response of ILA1 CTLs to varying concentrations of 13 different agonist ligands was determined by staining for the degranulation marker CD107a. *A*, The concentration of peptide that elicited half-maximum activation (logEC$_{50}$) of ILA1 CTLs in dose/response experiments using C1R B cells expressing wild-type HLA A2 molecules was used to rank the peptides according to their potency. ●, Used to indicate the peptides shown in *B–D*. *B–D*, Each agonist peptide was subsequently presented by C1R B cells expressing equivalent surface densities of either D227K/T228A (○) or wild-type (■) HLA A2 molecules. The ligands tested fell into three different categories with respect to the degree of CD8 dependency that they exhibited in IFN-γ ELISPOT dose/response experiments. *B*, As represented, recognition of the low potency peptides was strictly dependent on the engagement of CD8 regardless of their concentration. *C*, Agonist peptides of intermediate potency showed partial CD8 dependency, such that high concentrations were required to elicit CTL activation in the absence of a pMHCI/CD8 interaction. *D*, In contrast, potent agonist ligands exhibited nearly identical dose/response activation patterns when presented by either D227K/T228A or wild-type HLA A2 molecules, and were thus CD8-independent. Recognition of the following APLs is represented from *left* to *right*: *B*, 8E and 5Y; *C*, 1D; and *D*, index and 8T. SD from the mean of two replicates is shown. Data are representative of at least two independent experiments.
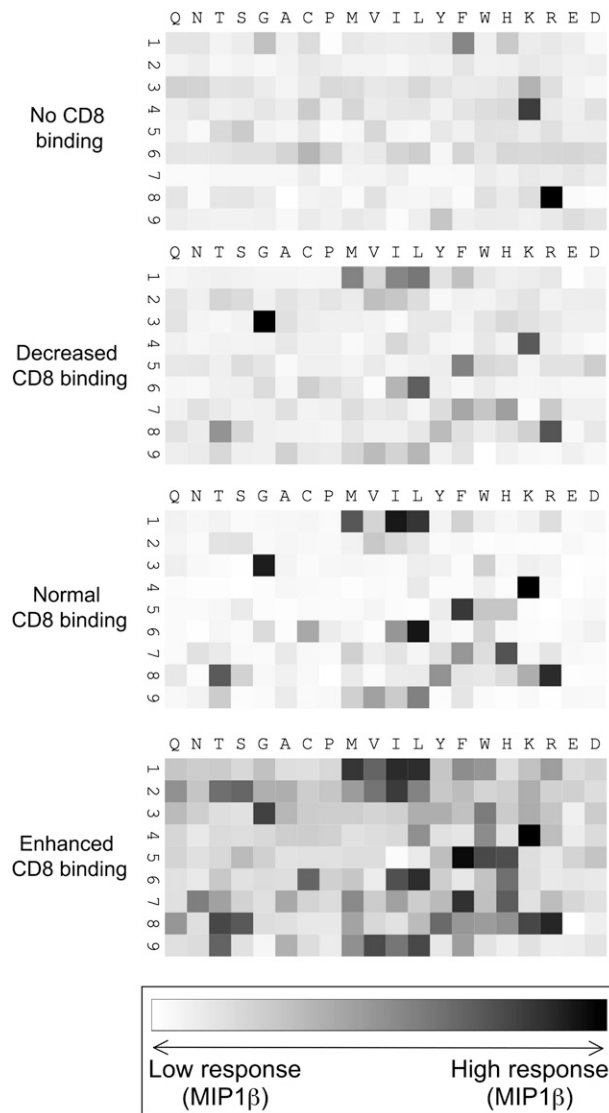
FIGURE 7. Influence of CD8 on CTL visualization of the nonamer peptide universe: heat map representations. CPL scans of ILA1 CTLs using C1R B cell clones expressing equivalent surface densities of D227K/T228A, A245V, wild-type, or Q115E versions of HLA A2 to present peptide mixtures (see Fig. 8, Supplemental Fig. 3 for summary of the raw data used). Effector function was assayed by MIP1β ELISA. Results are presented as greyscale plots, scaled such that the lowest result among the 180 mixtures is assigned white and the highest result is assigned black (see key). Vertical axis (numbers) indicates the position of the fixed amino acid residue in the incubation mixture; horizontal axis (single-letter amino acid code) indicates the residue that was fixed in the mixture. Amino acids are grouped according to their physicochemical properties, as follows: polar, uncharged amines: Q, N; polar, uncharged alcohols: T, S; small: G, A, C; hydrophobic: A–K; aliphatic: V, I, L; aromatic: Y, F, W, H; large: F, W; charged basic: H, K, R; and charged acidic: E, D.

ligands that can be recognized by a single cell surface-bound TCR. This is consistent with previous murine studies, which show that coreceptor recruitment can influence the outcome of TCR–pMHCI engagement (30–32).

The role of CD8 binding on CTL cross-reactivity was explored in more detail using a series of point-mutated cell surface-expressed HLA A2 molecules that exhibit a range of pMHCI–CD8-binding affinities spanning >3 orders of magnitude (K_D 10–10,000 μM) (15). Recognition of APLs by the ILA1 CTL clone became more stringent in the absence of a CD8 interaction. At a concentration

of 10^{-7} M, only 5 peptides (index, 8I, 1L, 8T, and 8Y) in a series of 23 selected for optimal HLA A2 binding were able to elicit a response in the absence of a pMHCI–CD8 interaction (Fig. 5). Importantly, increasing the concentration of peptides with agonist properties that were strictly dependent on CD8 binding could not compensate for the lack of coreceptor engagement (Fig. 6). In contrast, even an extremely weak pMHCI–CD8 interaction ($K_D \sim$ 500 μM) (15) enabled the efficient recognition of 6 additional APLs; an additional 3 APLs were recognized in the context of the wild-type pMHCI–CD8 interaction ($K_D \sim$ 130 μM) (Fig. 5). The pattern of ligand recognition in the context of cell surface Q115E-mutated HLA A2, which binds CD8 with enhanced affinity ($K_D \sim$ 85 μM) (15), remained unchanged for this set of APLs (Fig. 5). However, this mutation did increase the response magnitude to most agonist ligands in activation assays (data not shown). In contrast, the A2/K^b hybrid, which exhibits >10-fold increased CD8 binding (15, 33), allowed the recognition of all APLs. In fact, CTL activation was observed in the absence of pulsed peptide when the pMHCI–CD8 interaction was superenhanced to this extent (Fig. 5).

CD8 controls levels of T cell cross-reactivity

We also examined the effect of CD8 binding on TCR visualization of the nonamer peptide universe using CPL scan technology (Figs. 7, 8, Supplemental Fig. 3). Very few sublibraries activated ILA1 CTLs in the absence of a pMHCI/CD8 interaction. Increasing the strength of the pMHCI/CD8 interaction to just $K_D \sim$ 500 μM resulted in a substantial increase in the number of recognized peptide mixtures, thereby confirming that an interaction in this affinity range can have profound biological consequences (34). The number of agonist sublibraries was increased further in the presence of a wild-type pMHCI/CD8 interaction. Strikingly, a 50% increase in the strength of the pMHCI/CD8 interaction resulted in the recognition of a large number of sublibraries with additional amino acids recognized at every position of the peptide (Figs. 7, 8, Supplemental Fig. 3). Thus, the engagement of CD8 with the invariant domain of MHCI can act to control levels of T cell cross-reactivity.

Discussion

The evolution of T cell immunity presented a huge challenge because individual TCRs were expected to recognize millions of different peptide Ags that they had never encountered before, and were unable to adapt to, without reacting to structurally similar self-derived molecules. However, the unique character of T cell ligands, which comprise a conserved presenting molecule and a variable antigenic component, allowed for the evolution of a different receptor for each component. The coreceptor acts to direct T cells toward pMHC molecules by preventing thymocytes from being signaled by non-MHC ligands (35). Our findings show that CD8 extends the range of pMHCI ligands that can be seen by an individual cell surface-bound TCR, a feature that is essential for effective immune coverage. On this basis, we propose that the bipartite receptor/coreceptor recognition system has evolved to provide an unparalleled solution to the unique challenges of effective T cell immunity and is necessary to regulate the balance between optimal cross-reactivity and cognate Ag specificity.

The CD8 effect (Fig. 9) can be controlled to optimize the degree of cross-reactivity and Ag sensitivity of CD8 T cells at various stages of their development. Mechanisms that regulate the role of CD8 as a coreceptor both during thymic education and in the periphery include transcriptional inhibition of CD8 expression in double-positive thymocytes (36), selective coreceptor internalization following antigenic stimulation (37), switching to the expression of
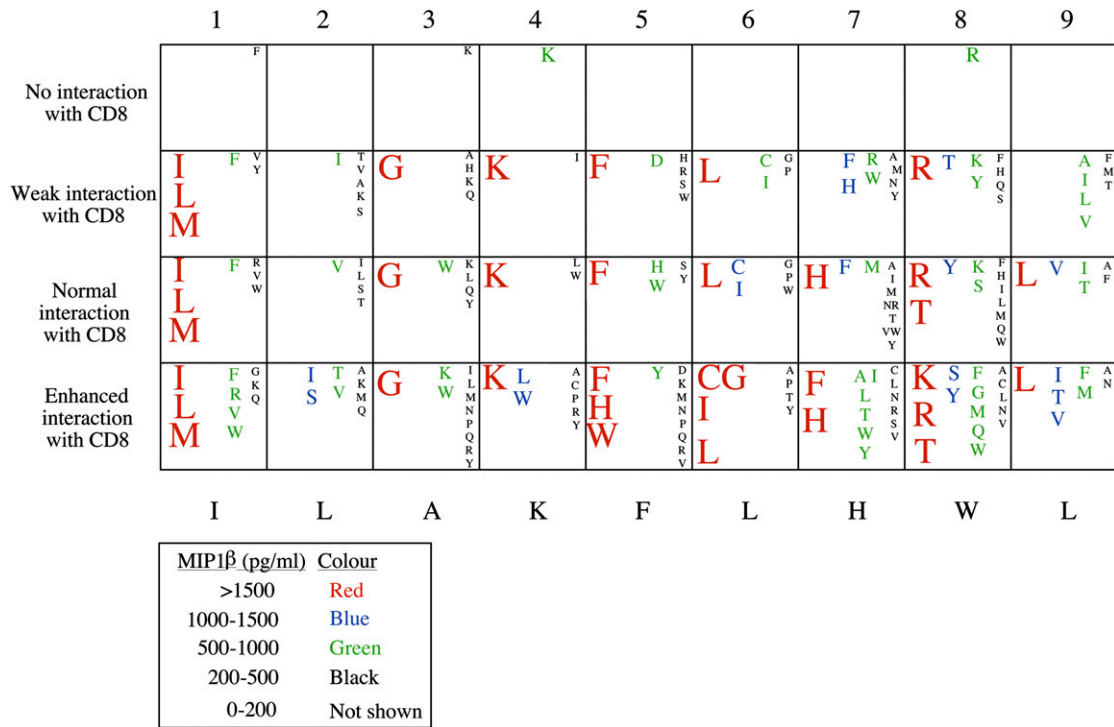
FIGURE 8. Influence of CD8 on CTL visualization of the nonamer peptide universe: summary of raw data. The results shown in Fig. 7 and Supplemental Fig. 3 are summarized as a degeneracy box diagram.
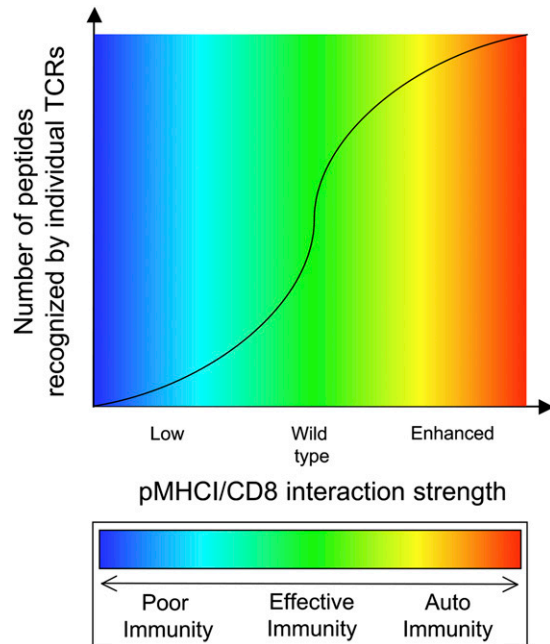

FIGURE 9. CD8 controls optimal levels of T cell cross-reactivity. Proposed model demonstrating how CD8 might function to control T cell cross-reactivity in vivo. The TCR constantly scans pMHCI complexes on the surface of target cells. The vast majority of these interactions have very low affinity and are too short-lived to trigger T cell activation. CD8 tunes the range of ligands that the TCR can productively engage, and thereby controls T cell cross-reactivity. When the pMHCI/CD8 interaction is weak, very few ligands can be recognized. The substantially lower level of peptide cross-reactivity observed in the absence of CD8 binding is likely to result in poor immunity due to a failure of the peripheral CTL repertoire to recognize all potential foreign peptides. When CD8 binding is enhanced, cross-reactivity increases to the point in which mature CTLs can recognize large numbers of peptide sequences, which increases the possibility of self-pMHCI complex recognition and consequent autoimmunity. The effect of CD8 can be regulated at different stages of T cell development.

the CD8αα isoform (reviewed in Ref. 38), changes in the pattern of glycosylation (39–41), and cytokine signals that transcriptionally tailor CD8 coreceptor expression (42). These mechanisms work together to fine-tune the degree of functional cross-reactivity at particular stages of development, facilitating selection of the TCR repertoire in the thymus while restraining deleterious activation in the periphery.

Interestingly, pMHCI/CD8 interactions are stronger in mice than in humans; this is likely to generate a greater degree of cross-reactivity. Indeed, an enhanced level of T cell cross-reactivity in the mouse was predicted on theoretical grounds (5), because a more limited pool of T cells in small animals is still required to recognize a similar number of potential foreign peptides. Although further studies are needed to test this, we predict that phylogenetic differences in coreceptor function will correlate inversely with the size of the naive TCR pool. In summary, our findings underline the assertion that T cell recognition is one of the extremely rare special cases in evolution and suggest that, when both specificity and cross-reactivity are required, two receptors are better than one.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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