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Cutting Edge: Inhibitory Effects of CD4 and CD8 on T Cell Activation Induced by High-Affinity Noncognate Ligands

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It has been proposed that MHC restriction during thymocyte selection is controlled by coreceptor (CD4 or CD8) sequestration of the signaling molecule Lck. We explored this model as a mechanism for preventing peripheral T cell activation due to non-MHC ligand cross-reactivities of TCRs. TCRs that have a range of affinities for a class I MHC ligand were transduced into a T cell hybridoma in the absence or presence of coreceptors. High and intermediate affinity TCRs ($K_D = 17$ and $540$ nM) did not require CD8 for T cell activity, but CD4 acted as a potent inhibitor of the intermediate affinity TCR. These and other findings support the view that even high-affinity TCR:ligand interactions can be influenced by coreceptor sequestration of Lck. Thus, CD4 and CD8 act as “coreceptor inhibitors” to maintain appropriate TCR-mediated MHC restriction in peripheral T cell activity. The Journal of Immunology, 2009, 183: 7639–7643.

Affinities of normal TCR:peptide-MHC interactions are relatively weak ($K_D = 1 - 100$ μM) (1), requiring the binding and synergies of coreceptors (CD8 on cytolytic T cells or CD4 on helper T cells) to initiate proximal signaling, ultimately leading to activation of the T cell (2, 3). These binding events facilitate colocalization of the coreceptor and the TCR, bringing the Src-family tyrosine kinase Lck, which is associated with the cytoplasmic tail of the coreceptor, into close proximity of the ITAMs of the CD3 subunits of the TCR complex to initiate the early signaling machinery of the T cell (4, 5).

The role of Lck in the development of thymocytes has been studied extensively (6). Recently, Singer and colleagues have proposed an Lck sequestration model that might drive positive selection, ensuring that TCRs are selected only by binding to an MHC ligand (7). They concluded that Lck sequestration by CD4 and CD8 could prevent TCR-mediated signaling in thymocytes that were not restricted by MHC. However, even the thymically selected peripheral repertoire of TCRs is diverse, arguably as diverse as Abs, and one might expect there to be frequent reactivity with noncognate ligands. Because it is clear that an Ab repertoire with diversity restricted only to CDR3 regions can bind to virtually any ligand (8), one would expect these TCRs to have the potential to bind to various ligands in the periphery, including cell surface proteins that have not been expressed in the thymus. As the coreceptor independent affinity threshold of TCRs appears to be $\sim 1$ μM $K_D$ (9), these types of cross-reactive affinities are in the same range as Abs from a primary response.

If the Lck sequestration model of Singer and colleagues is correct (6), then forced expression of the coreceptors in T cells that are restricted by the noncognate MHC should lead to reduced activity mediated by the TCR. Indeed, it has been shown previously that signals leading to activation can be inhibited by preventing the association of the CD4 coreceptor with the TCR-CD3 complex (10). In this study we were interested in examining which affinity of the TCR might be impacted by the Lck sequestration property of coreceptors. We used three TCRs engineered for a wide range of affinities ($K_D$ values of $30$ μM, $540$ nM, and $17$ nM) to the same peptide/class I MHC (SIY/Kb) to explore these effects. We show that introduction of the noncognate coreceptor (CD4) into coreceptor-negative T cells inhibited the activity mediated by the TCR with intermediate affinity for a class I MHC ligand, presumably through the sequestration of Lck. CD4 had little effect on the activity of the high-affinity TCR, consistent with the notion that it may require less noncoreceptor-associated Lck (i.e., free Lck) to mediate T cell activity. Surprisingly, T cells that overexpressed a high level of CD8 could be completely inhibited by Abs to CD8 (i.e., even though the TCRs bound strongly to the class I MHC without CD8). In addition, activation of CD8$^+$ T cells that expressed high and intermediate affinity TCRs ($K_D = 14$ nM and $1.5$ μM, respectively) for a class I MHC (L$\alpha$) that lacked the CD8 binding domain was greatly reduced compared with their CD8-negative counterparts. The mechanism of CD8 inhibition is likely due to the sequestration of Lck such that even though the TCR has high-affinity for the class I MHC, engagement of it alone without colocalization...
of CD8 and associated Lck leads to the impairment of activity.

The results support a key role for coreceptor-Lck sequestration in the activity of mature T cells as a mechanism for maintaining MHC restriction in the periphery. The process is influenced by both the affinity of the TCR for the ligand and the level of the coreceptor, providing a system for controlling the specificity of MHC restriction by T cells and possibly providing yet another mechanism for peripheral tolerance to potential cross-reactive Ags that are not appropriately presented to the T cell.

Materials and Methods

Peptides, Abs, and cell lines

SIYRYYGL (SIY), SIINFEKL (OVA), and QLSPFPFDL (QL9) peptides were synthesized by the Macromolecular Core Facility at Penn State University (Hershey, PA). T cell hybridomas (58–/– line) used in SIY/Kb experiments (2C\textsubscript{low}, 2C\textsubscript{int}, 2C\textsubscript{high}; where int is intermediate) were transduced with Moloney sarcoma virus retroviral vectors that contained each TCR; 2C\textsubscript{low} refers to the wild-type 2C TCR, 2C\textsubscript{int} refers to the S51/Y48 mutant of the m33 TCR; and 2C\textsubscript{high} refers to the Y26 mutant of the m33 TCR (9). Independently, 58–/– T cells were transfected with plasmids that contained CD8α and β, as described (11). The 58–/– T cells with 2C\textsubscript{low} and 2C\textsubscript{high} TCRs were transduced with a retroviral vector that contains CD8α and β, as described (11).

The following Abs were used: PE-labeled H57–597 anti-mouse TCR Cβ (BD Pharmingen); PE-labeled 53–6.7 anti-mouse CD8α (BD Pharmingen); CT-CD8α (Cedarlane); PE-labeled 53–8 anti-mouse CD8β (BD Pharmingen); and allophycocyanin-labeled GK1.5 anti-mouse CD4 (eBioscience). For cell surface staining, cells were analyzed with an Accuri C6 flow cytometer (Accuri Cytometers).

Expression and purification of soluble Ld

The m31-Ld\textsuperscript{Δ} protein was expressed and purified as described (12). Protein was biotinylated via a C-terminal biotinylation signal peptide sequence using a BirA ligase (Avida). A QL9/Ld\textsuperscript{Δ} Ig dimer (BD Biosciences) was generated by incubation of the BD DimerX Ld IgG1 dimer (BD Biosciences) with a 40-fold molar excess QL9 peptide.

T cell activation assays

T cells (10\textsuperscript{5}) and T2-Kb cells (10\textsuperscript{5}) were incubated with various concentrations of the SIY peptide for 24 h. Supernatants (50 μl) were collected and assayed for IL-2 using an IL-2 capture assay (BD Pharmingen). For inhibition with anti-CD8 Abs, the Ab CT-CD8α (Cedarlane Labs) was preincubated at 10 μg/ml with T cells for 20 min before the addition of T2-Kb cells and peptides. For activation assays involving immobilized peptide-Ld, QL9/Ld\textsuperscript{Δ} Ig dimers containing 10 μM excess QL9 peptide were immobilized at various concentrations to 96-well plates (Corning); PE-labeled m31-Ld\textsuperscript{Δ} Ig dimer (BD Biosciences) was generated by incubation of the BD DimexLd\textsuperscript{Δ} IgG1 dimer (BD Biosciences) with a 40-fold molar excess QL9 peptide.

Results and Discussion

CD4 modulates activity of T cells that express an intermediate affinity, class-I restricted TCR

Three TCRs derived from the 2C TCR that binds to the class I MHC K\textsuperscript{b} associated with peptide SIY were used in the present study. These TCRs have widely different affinities for SIY/Kb. We refer to them here as low affinity (2C\textsubscript{low} TCR, K\textsubscript{D} = 30 μM; the wild-type 2C TCR), intermediate affinity (2C\textsubscript{int}, K\textsubscript{D} = 540 nM), and high affinity (2C\textsubscript{high}, K\textsubscript{D} = 17 nM), as measured by surface plasmon resonance using soluble TCR and immobilized SIY/Kb (9). The 2C\textsubscript{low} TCR has been shown previously to mediate activity in 58–/– T cell hybridoma only in the presence of CD8, whereas 2C\textsubscript{int} and 2C\textsubscript{high} TCRs can mediate activity in T cells that lack CD8 (9, 11). To examine the possible impact that CD4 would have on activity mediated by these two CD8-independent TCRs, CD4 was introduced, along with the TCRs, into the TCR-negative, CD8-negative T cell hybridoma 58–/– (13). Independently, these TCRs were also introduced into a CD8αβ\textsuperscript{+} 58–/– cell line (11). The six cell lines (coreceptor negative, CD4\textsuperscript{−}, and CD8\textsuperscript{−} for each of the TCRs) were sorted for TCR expression with anti-CD3 Ab, and the levels of TCR were shown to be approximately the same among the different cell lines (supplemental Fig. 1, A and B, top panel).

The CD4\textsuperscript{+} and CD8\textsuperscript{−} cell lines (2C\textsuperscript{low} and 2C\textsuperscript{high}) expressed approximately the same levels of CD4 and CD8 (supplemental Fig. 1, A and B). As expected, expression of the CD4 coreceptor did not interfere with binding of the class I ligand to either 2C\textsuperscript{high} or 2C\textsuperscript{int} (supplemental Fig. 1, C and D).

The functional responses (IL-2 release) of 2C\textsuperscript{low} and 2C\textsuperscript{int} TCRs in CD4\textsuperscript{+}, CD8\textsuperscript{−}, or parental 58–/– T cells were examined by incubation with T2-Kb cells in the presence of varying amounts of SIY peptide. The three lines that expressed the 2C\textsuperscript{high} TCR were activated with similar sensitivities (Fig. 1, A and C), although the magnitude of IL-2 released differed among the three lines. This difference in magnitude of IL-2 released may be due to the heterogeneity in protein expression of CD4 on a per cell basis that affects the “all or none” signaling response (9, 14) and the subsequent release of IL-2, as described by Altan-Bonnet and colleagues (15). In contrast, the three lines that expressed the 2C\textsuperscript{int} TCR were activated with markedly different sensitivities (Fig. 1, B and D). The 2C\textsuperscript{int} CD8\textsuperscript{+} T cell line was almost 100-fold more sensitive than the 2C\textsuperscript{int} coreceptor-negative cell line (median sensitivity dose (SD\textsubscript{50}) = 1.4 × 10\textsuperscript{6} median sensitizing dose (SD\textsubscript{50}) = 1.4 × 10\textsuperscript{6}).
$10^{-5}$ M and $1.1 \times 10^{-8}$ M, respectively). However, the $2C^{\text{int}}$ CD4$^+$ T cell line was almost 500,000-fold less sensitive than the $2C^{\text{low}}$ coreceptor-negative cell line ($SD_{50} = 5.4 \times 10^{-7}$ M and $1.1 \times 10^{-8}$ M, respectively).

The relationship between affinity and this inhibitory effect is likely a consequence of the amount of free Lck necessary for signaling through the TCR/CD3 complexes under the two different scenarios. In the case of the highest affinity TCR, a higher fraction of TCRs are in the bound state and signaling can occur as long as there is some threshold number of Lck recruited to the bound TCR/CD3 complex. In this model, we do not distinguish whether TCR multimerization, conformational change, or both are necessary for signaling, only that some threshold number of CD3/Lck interactions must occur (16). In the case of the intermediate affinity TCR, there are fewer TCRs bound, and the reduced number of free Lck available due to CD4 sequestration has lowered the number of CD3/Lck interactions to near or below the threshold required for signaling.

Anti-CD8 Abs inhibit high affinity, CD8-independent TCRs

We (17) and others (18) have shown that Abs to CD8 can inhibit T cell activity even when interactions between CD8 and a class I MHC target cell are not possible (i.e., CD8+class I interactions are absent). Because high-affinity TCRs do not require CD8, we were in a position to see whether similar results are observed with TCR affinities that are up to 1000-fold greater than typical wild-type TCRs. For these experiments, the cell lines were enriched for CD8$\alpha$ expression by sorting the CD8-transfected cell lines of $2C^{\text{high}}, 2C^{\text{int}},$ and wild-type $2C (2C^{\text{low}})$ and staining for levels of TCR, CD8$\alpha$, and CD8$\beta$ (Fig. 2A).

The CD8$^+$ cells were all capable of binding to the PE-labeled SIY/K$^b$ tetramer (Fig. 2B). To assess qualitatively the extent to which CD8 contributed to this binding, the anti-CD8 Ab CT-CD8 was used at $10 \mu$g/ml to measure the extent of inhibition of tetramer binding (19, 20). As might be expected, the magnitude of inhibition was related to the affinity of the TCR (Fig. 2B, dashed line histograms; supplemental Fig. 2). $2C^{\text{low}}$ ($K_D = 30 \mu$M) showed an 11.5-fold decrease in staining, consistent with previous reports that the $2C$ TCR is dependent on the CD8 coreceptor for binding to the SIY/K$^b$ tetramer (19). $2C^{\text{int}}$ showed a 5.5-fold decrease in binding in the presence of the anti-CD8 Ab, and $2C^{\text{high}}$ showed a 2.4-fold reduction in binding. It was also of interest that the signal (in mean fluorescent units) obtained from the staining of CD8$^+$ cells with the intermediate and high affinity TCRs were slightly less (1.3 and 2.3-fold, respectively) than that from the low affinity TCR. This effect is clearly due to the binding contribution of CD8, as T cell hybridomas with these TCRs but without CD8 have the expected result of higher binding to the highest affinity TCR. We speculate that this result in the presence of CD8 is due to the complex binding properties of tetramer preparations, where it is possible that a greater number of TCRs per cell with the high-affinity TCRs have been occupied by a single tetramer. We are currently examining in more detail the quantitative equilibrium and kinetic features of this observation.

As there was significant binding of tetramers, especially to higher affinity TCRs, even in the presence of the anti-CD8 Ab at $10 \mu$g/ml, we examined whether the different CD8$^+$ T cell lines would be inhibited at this concentration of CT-CD8 in IL-2 activation assays with SIY and T2-K$^b$ (Fig. 2C). The $2C^{\text{low}}$ cell line was inhibited by anti-CD8 Abs as expected. However, both the $2C^{\text{int}}$ and $2C^{\text{high}}$ cell lines were also completely inhibited by the anti-CD8 Ab. Thus, despite the fact that these TCRs are fully capable of binding to the SIY/K$^b$ tetramer (even in the presence of the anti-CD8 antibody) and are stimulated by SIY/K$^b$ in the absence of CD8, Ab binding to CD8 acts as a very potent inhibitor of T cell activity when CD8 is expressed.

The result described here with CD8 Abs and CD8$^+$ cells bearing the highest affinity TCR differs from the observation with CD4, where there was no observable effect on activity. We suggest that the explanation for this difference lies in the amount of CD4 and CD8 that were expressed in the 58 lines that express high affinity TCRs.

FIGURE 2. Effect of anti-CD8 Ab on the activity of CD8$^+$ T cells that express high affinity TCRs. A, TCR, CD8$\alpha$, and CD8$\beta$ levels on $2C^{\text{low}}, 2C^{\text{int}},$ and $2C^{\text{high}}$ enriched cells (solid lines), $2C^{\text{low}}$ CD8$^+$ enriched cells (dashed lines), $2C^{\text{high}}$ CD8$^+$ enriched cells (dotted lines), and parental 58$^+$ cells (filled histogram). B, Binding of PE-labeled SIY/K$^b$ tetramer in the absence (solid lines) or presence (dashed lines) of $10 \mu$g/ml anti-CD8 blocking Ab, CT-CD8$\alpha$. C, Response of the indicated T cell lines in the presence of the T2-K$^b$ APCs, incubated with various concentrations of SIY peptide, in the absence (filled squares) or presence (open squares) of anti-CD8$\alpha$ Ab. Results are representative of two experiments.
of coreceptor levels to be able to “tune” signaling through the TCR and further explains why CD8 levels of TCR-transgenic, class I-restricted T cells in some transgenic mice are lower after thymic selection (22–24). The finding also suggests that the common approach of using anti-CD8 Ab inhibition to judge “CD8-dependence” is complicated by the Lck sequestration mechanism (i.e., even truly CD8-independent interactions can be inhibited).

**CD8** T cell activation mediated by high-affinity TCRs is reduced using a class I ligand that is unable to bind CD8

We have engineered a stable α1/α2 Ld molecule that lacks both the α3 domain of the class I H chain and β2-microglobulin (β2m) (12). This protein, called m31-Ld, completely lacks the CD8 binding site, yet retains the TCR binding site of peptide-Ld. This differs from the now often used class I mutants with amino acid substitutions that may eliminate detectable CD8 binding in solution but cannot be said to definitively lack effects associated with CD8 binding as a cell membrane protein (e.g., avidity effects). We have also described two TCRs with different affinities for the QL9/Ld complex: the wild type 2C TCR that binds to QL9/Ld with an intermediate affinity of 15 nM, and the mutant TCR m6 that binds to QL9/Ld with a high affinity of 14 nM (25). Both the 2C TCR and the m6 TCR were previously transfected into 58β2m−/− cells, and the lines were stimulated efficiently by QL9/Ld expressed on T2-Ld cells in the absence or presence of CD8 (11). By examining the comparative ability of m31-Ld to stimulate coreceptor-negative cell lines vs CD8+ T cell lines, we were in a position to directly assess the impact of stimulating T cells through TCRs that might bind to non-MHC ligands (i.e., those that cannot bind to either CD4 or CD8).

T cell lines expressing 2C or m6 TCRs, with or without the CD8αβ, had similar levels of TCR, and the CD8 + lines had similar levels of CD8α and CD8β (Fig. 3A). We compared the effect of m31-QL9/Ld, which lacks ability to bind CD8, with a full-length Ld construct called QL9/Ld-Ig, which contains the α3 domain and β2m for CD8 binding. The purified Ld proteins were immobilized in wells at various concentrations, incubated with the different T cell lines, and IL-2 release was measured after 24 h. Full-length Ld was capable of stimulating all of the cell lines that express CD8 or CD8−/− cells (Fig. 3B) when incubated with the α1/β2m-negative m31-QL9/Ld. Thus, despite the relatively high affinity of the 2C:QL9/Ld interaction compared with most characterized TCR systems (26), CD8 appears to act as a dominant-negative inhibitor for ligands that do not recruit CD8 to the TCR/CD3 complex.

**FIGURE 3.** Elimination of the CD8β binding domain in Ld reduces activity of CD8+ T cells expressing high-affinity, CD8-independent TCRs. A, TCR, CD8α, and CD8β levels in CD8-negative cells (top panels) or CD8-positive cells (bottom panels) that express the 2C TCR (solid lines), the m6 TCR (dashed lines), or parental 58β2m−/− cells (filled histogram). B, Functional response of T2-Ld T cell lines to QL9/Ld-Ig or m31-QL9/Ld (biotin labeled), which lacks the α/βm domains, immobilized at various concentrations. C, Functional response of activated 2C transgenic splenocytes to QL9/Ld-Ig or m31-QL9/Ld immobilized at various concentrations.
These findings again support the notion that coreceptor sequestration of Lck acts as an effective inhibitor of signaling through the TCR, even when the affinity of the interaction with a ligand is quite high. Thus, T cells that bear a TCR with cross-reactivity for the “noncognate” MHC, or any non-MHC ligand, are subject to regulation by this mechanism. Given that the high-affinity interactions shown in this study could be inhibited by this effect, it seems likely that the normal range of affinities found for TCR-peptidemHC interactions and the range of affinities likely for TCR-MHC interactions would be especially sensitive to inhibition by the coreceptor sequestration mechanism. These findings lend further support to the model proposed by Singer and colleagues (7) indicating that coreceptor Lck sequestration could be an effective mechanism for ensuring MHC restriction by TCRs. The process of “coreceptor inhibition” could be yet another way in which T cells maintain specificity for the correct MHC, promoting peripheral tolerance against non-MHC ligands.

These results also point to the dynamic interplay between TCR affinity and coreceptor density for controlling T cell activity, similar to the tuning mechanism that has long been appreciated in the development of thymocytes (22–24). For TCRs that might have both class I and class II reactivity, it is possible that the expression of low levels of the corresponding coreceptor (or both CD4 and CD8), perhaps even below the level of detection by conventional flow cytometry, could be sufficient to drive signaling and overcome the inhibitory effect of noncognate coreceptor expression (27, 28). Proper coreceptor matching with its MHC ligand not only enhances signaling, as has been known for some time, but inappropriate coreceptor matching acts as an inhibitor and possibly as an antagonist.

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