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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.

A affinities of normal TCR:peptide-MHC interactions are relatively weak ($K_D = 1–100 \mu M$) (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, according 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 $\sim 1 \mu M$ $K_D$ (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 $30 \mu M$, 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 $\mu M$, respectively) for a class I MHC (Ld) 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

SIYRYGL (SIY), SIINFEKL (OVA), and QLSPFPFDL (QL9) peptides were synthesized by the Macromolecular Core Facility at Penn State University (Hershey, PA). T cell hybridomas [58\(^{3}H\)]-, as described (11). 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.

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10^{-8} \text{M and } 1.1 \times 10^{-8} \text{M, respectively). However, the } 2C^{\text{int}} \text{ CD4}^+ \text{ T cell line was almost 500,000-fold less sensitive than the } 2C^{\text{high}} \text{ co-receptor-negative cell line (SD}_{50} = 5.4 \times 10^{-7} \text{ M and } 1.1 \times 10^{-8} \text{ 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 expression by sorting the CD8-transfected cell lines of 2C^{\text{high}}, 2C^{\text{int}}, and wild-type 2C (2C^{\text{low}}) to near or below the threshold required for signaling.

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

![Figure 2](http://www.jimmunol.org/) Effect of anti-CD8 Ab on the activity of CD8^- T cells that express high affinity TCRs. A, TCR, CD8α, and CD8β levels on 2C^{low} CD8^- enriched cells (solid lines), 2C^{high} CD8^- enriched cells (dashed lines), 2C^{high} CD8^- enriched cells (dotted lines), and parental 58^- cells (filled histogram). B, Binding of PE-labeled SIY/Kb tetramer in the absence (solid lines) or presence (dashed lines) of 10 μg/ml anti-CD8α blocking Ab, CT-CD8α. 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-CD8α Ab. Results are representative of two experiments.

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^- cells (supplemental Fig. 3). Although it is generally accepted that Lck is better able to associate with CD4 compared with CD8 (21), this 20-fold increase in the amount of CD8 could allow Lck to be sequestered by CD8 at greater levels than by CD4 in these experiments. This would result in a large reduction in the pool of free Lck able to associate with the TCR/CD3 complex, consistent with the notion that some threshold level of free Lck is required for activity. Thus, even a TCR that is extremely high affinity and clearly CD8 independent is not able to overcome Lck sequestration from high densities of coreceptor. This finding points to the exquisite ability...
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 1.5 μM, 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 58T− 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 αα 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 and both the sensitivity and the level of activity was virtually identical between the cell lines that expressed CD8 or did not express CD8 (Fig. 3B). In contrast, there was a significant difference in both the sensitivity and magnitude of activity between 2C and 2C CD8+ cells, as well as between m6 and m6 CD8+ cells (Fig. 3C) when activated by m31-QL9/Ld (α1/α2).

Although we have no direct evidence for what explains reduced sensitivity (i.e., SDαβ) vs the maximal amount of IL-2 released in these assays, we believe that the reduced sensitivity could be due to the need for a higher number of ligand/TCR interactions (i.e., due to lower levels of free Lck in the CD8+ cells). Reduced maximal IL-2 release could be due to the heterogeneity of cells that express CD8 (i.e., some cells with lower CD8 levels could fully secrete IL-2, whereas those with higher CD8 levels would fail to secrete IL-2).

To assess whether these results could be extended to normal T cells, we used splenic T cells from a 2C TCR transgenic mouse and the full-length Ld or truncated m31-Ld (Fig. 3D). Activated 2C-transgenic T cells released IFN-γ in response to the QL9/Ld Ig dimer but, in contrast, no IFN-γ was released when incubated with the αβm-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.
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-MHC interactions and the range of affinities likely for TCR-non-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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Disclosures

The authors have no financial conflict of interest.

References

Supplemental Figure 1. Characterization of TCR and co-receptor cell surface levels on 2C$^{\text{high}}$ and 2C$^{\text{int}}$ T cell lines. (A) 2C$^{\text{high}}$ panel of T cell hybridomas. Top panel - TCR surface levels as measured by anti-C$\beta$ staining of 2C$^{\text{high}}$ cells (solid line), 2C$^{\text{high}}$ CD4$^+$ cells (dashed line), 2C$^{\text{high}}$ CD8$^+$ cells (dotted line), and parental 58$^{-/-}$ cells (filled histogram). Middle panel - CD4 surface levels of 2C$^{\text{high}}$ CD4$^+$ cells (open histogram) and parental 58$^{-/-}$ cells (filled histogram). Bottom panel – CD8$\beta$ surface levels of 2C$^{\text{high}}$ CD8$^+$ cells and parental 58$^{-/-}$ cells (filled histogram). (B) 2C$^{\text{int}}$ panel of T cell hybridomas. Top panel - TCR surface levels as measured by anti-C$\beta$ staining of 2C$^{\text{int}}$ cells (solid line), 2C$^{\text{int}}$ CD4$^+$ cells (dashed line), 2C$^{\text{int}}$ CD8$^+$ cells (dotted line), and parental 58$^{-/-}$ cells (filled histogram). Middle panel - CD4 surface levels of 2C$^{\text{int}}$ CD4$^+$ cells (open histogram) and parental 58$^{-/-}$ cells (filled histogram). Bottom panel – CD8$\beta$ surface levels of 2C$^{\text{int}}$ CD8$^+$ cells and parental 58$^{-/-}$ cells (filled histogram). (C) 2C$^{\text{high}}$ cells (solid lines), 2C$^{\text{high}}$ CD4$^+$ cells (dashed lines), or 58$^{-/-}$ cells (filled histogram) were incubated with various concentrations of APC-labeled SIY/K$^b$ tetramers and analyzed by flow cytometry. (D) 2C$^{\text{int}}$ cells (solid lines), 2C$^{\text{int}}$ CD4$^+$ cells (dashed lines), or 58$^{-/-}$ cells (filled histogram) were incubated with various concentrations of APC-labeled SIY/K$^b$ tetramers and analyzed by flow cytometry.

Supplemental Figure 2. Inhibition of SIY/K$^b$ binding to CD8$^+$ hybridoma. 58$^{-/-}$, 2C$^{\text{low}}$, 2C$^{\text{int}}$, 2C$^{\text{high}}$ CD8$^+$ hybridomas were stained with 1 μM SIY/K$^b$:PE tetramer in the presence or absence of 10 μg/mL of the CD8 blocking antibody CT-CD8$\alpha$. Numbers inside histograms represent mean fluorescence units. Bold numbers represent percent inhibition of SIY/K$^b$ binding by CT-CD8$\alpha$. 

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Supplemental Figure 3. Quantitation of coreceptor levels on transduced T cells. 5 x 10^5 2C^int CD4^+, 2C^int CD8^+ and 2C^high CD4^+, 2C^high CD8^+ cells were stained with biotinylated anti-CD4 (GK1.5) or biotinylated anti-CD8α (53−6.7) antibodies in excess (10 μg/mL). To ensure both primary antibodies contained equivalent levels of biotin, the amount of biotin per molecule was quantitated by HABA displacement using the Pierce Biotin Quantification Kit. Anti-CD4 antibody had an average of 1.5 biotins/antibody molecule and anti-CD8α had an average of 1.6 biotins/antibody molecule. Thus, the 20-fold differences in signal can not be due to differences in the two antibody probes. Streptavidin:APC was added to all cell lines at the same concentration and surface levels were measured by flow cytometry.
Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3

- 58−/− CD4+
- 58−/− CD8α

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