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*J Immunol* 2009; 183:7639-7643; Prepublished online 18 November 2009; doi: 10.4049/jimmunol.0901664
http://www.jimmunol.org/content/183/12/7639

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/11/18/jimmunol.0901664.4.DC1

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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 (KD = 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 (KD = 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 ~1 μM KD (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 (KD 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 (KD = 14 nM and 1.5 μM, respectively) for a class I MHC (L4) 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), SIINFKE(L, OVA), and QLSPFPFDL (QL9) peptides were synthesized by the Macromolecular Core Facility at Penn State University (Hershey, PA). T cell hybridomas (58/1– line) used in SIY/Kb experiments (2Clow, 2Cint, 2Chigh; where int is intermediate) were transduced with Moloney sarcoma virus retroviral vectors that contained each TCR; 2Cint refers to the wild-type 2C TCR, 2Chow refers to the SIY1/Y48 mutant of the m33 TCR; and 2Chigh refers to the Y26 mutant of the m33 TCR (9). Independently, 58/1– T cells were transfected with plasmids that contained CD8α and β, as described (11). The 58/1– T cells with 2Chow and 2Chigh TCRs were transfected with a retroviral vector that contains CD4. The 58/1– T cells used in the QL9/Ld experiments expressed 2C TCR and m6 TCR with or without CD8αβ, as described (11).

The following Abs were used: PE-labeled H57–597 anti-mouse TCR Cβ (BD Pharmingen); PE-labeled 53–67 anti-mouse CD8α (BD Pharmingen); CT-CD8α (Cedarlane); PE-labeled 53–58 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 protein was expressed and purified as described (12). Protein was biotinylated via a C-terminal biotinylation signal peptide sequence using a BirA ligase (Avidity). A QL9/Ld Ig dimer (BD Biosciences) was generated by incubation of the BD DimerX Ld IgG1 dimer (BD Biosciences) with a 40-fold molar excess of QL9 peptide.

T cell activation assays

T cells (10⁵) and T2-Kb cells (10⁵) 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 Ig dimers containing 10 μM excess QL9 peptide were immobilized at various concentrations to 96-well plates (Corning) in PBS. For biotinylated m31-Ld, plates were coated with 10 μg/ml streptavidin before the addition of biotinylated m31-QL9/Ld with 10 μM excess QL9 peptide. T cells (10⁵) were added and, after 24 h at 37°C, supernatants were assayed for IL-2 as described above. For activation of transgenic 2C T cells, splenocytes were isolated from a 2C TCR transgenic mouse and activated in vitro with the SIY peptide for 4 days. Activated 2C T cells were then added to wells containing various concentrations of the QL9/Ld Ig dimer or m31-QL9/Ld for 24 h and assayed for IFN-γ release by capture ELISA (eBioscience).

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 Kb 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 (2Clow TCR, Kd = 30 μM; the wild-type 2C TCR), intermediate affinity (2Cint, Kd = 540 nM), and high affinity (2Chigh, Kd = 17 nM), as measured by surface plasmon resonance using soluble TCR and immobilized SIY/Kb (9). The 2Cint TCR has been shown previously to mediate activity in 58/1– T cell hybridoma cells only in the presence of CD8, whereas 2Cint and 2Chigh 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 58/1– (13). Independently, these TCRs were also introduced into a CD8αβ+ 58/1– cell line (11). The six cell lines (coreceptor negative, CD4+, and CD8− for each of the TCRs) were sorted for TCR expression with anti-CD4 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).4 The CD4+ and CD8− cell lines (2Cint and 2Chigh) 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 2Chigh or 2Cint (supplemental Fig. 1, C and D).

The functional responses (IL-2 release) of 2Chigh and 2Cint TCRs in CD4+, CD8−, or parental 58/1– 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 2Chigh 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 release 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 2Cint TCR were activated with markedly different sensitivities (Fig. 1, B and D). The 2Cint CD8+ T cell line was almost 100-fold more sensitive than the 2Cint coreceptor-negative cell line (median sensitivity dose (SD50) = 1.4 ×

4 The online version of this article contains supplemental material.
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 CD8α expression by sorting the CD8-transfected cell lines of 2C<sup>high</sup>, 2C<sup>int</sup>, and wild-type 2C (2C<sup>low</sup>) to near or below the threshold required for signaling. 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.

Both the 2C<sup>int</sup> and 2C<sup>high</sup> cell lines were completely inhibited by the anti-CD8 Ab. Thus, despite the fact that these TCRs are fully capable of binding to the SIY/K<sub>b</sub> tetramer (even in the presence of the anti-CD8 antibody) and are stimulated by CD4, where there was no observable effect on activity. 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 long-term CD8<sup>+</sup> T cell lines, we examined whether the different CD8<sup>+</sup> T cell lines would be inhibited at this concentration of CT-CD8α in IL-2 activation assays with SIY and T2-K<sub>b</sub> (Fig. 2C). The 2C<sup>low</sup> cell line was inhibited by anti-CD8 Abs as expected. However,
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 αLβ2 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 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 58B cells (Fig. 3A), 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 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 (αLα2).

Although we have no direct evidence for what explains reduced sensitivity (i.e., SD50) 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 αL/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 58B− cells (filled histogram). B, Functional responses of 2C, 2C CD8+, m6, and m6 CD8+ T cell lines to QL9/Ld Ig absorbed at the indicated concentration to wells of a 96-well plate. C, Functional responses of 2C, 2C CD8+, m6, and m6 CD8+ T cell lines to m31-QL9/Ld (biotin labeled), which lacks the αL/β2m domains, immobilized at various concentrations. D, 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-peptide-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.

Acknowledgments

We thank Herman N. Eisen, Arup K. Chakraborty, and Maxim N. Artyomov for their help with this work. We thank Herman N. Eisen, Arup K. Chakraborty, and Maxim N. Artyomov for providing the CD4-GFP construct.

Disclosures

The authors have no financial conflict of interest.

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