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Critical Role for CD8 in Binding of MHC Tetrarmers to TCR: CD8 Antibodies Block Specific Binding of Human Tumor-Specific MHC-Peptide Tetrarmers to TCR1

Galit Denkberg,² Cyril J. Cohen,² and Yoram Reiter³

There are conflicting opinions about the role that the T cell coreceptors CD4 and CD8 play in TCR binding and activation. Recent evidence from transgenic mouse models suggests that CD8 plays a critical role in TCR binding and activation by peptide-MHC complex multimers (tetrarmers). Here we show with a human CTL clone specific for a tumor-associated MHC-peptide complex that the binding of tetrarmers to the TCR on these cells is completely blocked by anti-human CD8 Abs. Moreover, the staining of CTLs with specific MHC-peptide tetrarmers simultaneously with anti-CD8 Abs was completely blocked with three different anti-CD8 Abs. This blockage was mediated by anti-CD8 Abs but not anti-CD3 Abs and was dose dependent. The blocking effect of the anti-CD8 Abs was attributable to directly inhibiting tetrarm binding and was not attributable to Ab-mediated TCR-CD8 internalization and down-regulation. Our results have important implications in TCR binding to MHC-peptide tetrarmers. MHC-peptide tetrarmers are widely used today in combination with anti-CD8 Abs for the phenotypic analysis of T cell populations and in the study of T cell responses under various pathological conditions such as infectious diseases and cancer. Our results indicate that also in the human system CD8 plays a critical role in the interaction of MHC-peptide multimers with TCR.


CD4 and CD8 are coreceptors known to bind class II and class I MHC molecules, respectively and to be involved in the activation and biological functions of T cells (1–3). With a Th cell response, the presence of CD4 greatly augments the amount of cytokine produced and sometimes determines whether there is a response at all (1–3). Much of the effect of CD4 is apparently attributable to the recruitment of lck to the TCR-CD3 complex, which results in efficient T cell activation (4). In addition, there also is a significant positive effect on T cells, even with CD4 molecules that are unable to bind lck, and there appears to be an effect on TCR-ligand interaction as well (1–3). CD8 also greatly augments the response of class I MHC-specific T cells (5, 6) and is thought to stabilize TCR-peptide-MHC complexes by about 10-fold (7). Overall, each of these coreceptor molecules apparently has two roles: to stabilize TCR-ligand interactions physically and to aid in signaling by recruiting other molecules. Consistent with these roles is evidence that the presence of CD4 can convert an antagonist peptide into a weak agonist (8, 9); however, other studies have shown that although weak agonist peptides are made almost as potent as the best peptides by the presence of CD4, little or no effect is seen on antagonism (10). These and other results suggest that CD4 engagement is not automatic and concomitant with TCR binding, but instead that CD4 is recruited later into preexisting TCR-peptide-MHC complexes or oligomers (11–15). Sometimes antagonist-MHC complexes will be stable long enough for CD4 to have an effect; here, the T cell can be stimulated, but in others cases, the TCR-ligand association does not last long enough for CD4 to affect the outcome.

Little has been reported on CD8. A recent work analyzing the interaction between CD8 and TCR suggested that TCR and the CD8 coreceptor bind peptide-MHC independently and with distinct kinetics (16). However, CD8 binding to class I MHC-peptide complexes is known to be enhanced by signaling events. Moreover, activated CD8 binding to class I MHC mediated by the TCR results in activation and signaling (17, 18). The wealth of evidence suggests that similar to CD4, CD8 is recruited and participates in TCR-peptide-MHC interactions only after the TCR already has been stably bound to the MHC-peptide complex.

Recently, a study with transgenic mouse models revealed that CD8 plays a critical role in TCR binding and activation by peptide-MHC complex multimers (18). In this study, with mouse MHC class I tetramers and two well-defined class I-restricted TCR-transgenic systems (OT-1 and 2C), it was shown that CD8 was pivotal for both tetramer binding to TCR and the subsequent activation of T cell response mediated by the tetramers (18).

Finally, MHC-peptide tetrarmers have caused a revolution in molecular immunology by enabling for the first time the phenotypic analysis of T cell responses (19–24). Several groups reported on the construction of MHC-peptide tetrarmers and their use to detect specific and in some cases very rare T cell populations (19–25). Recently, we also have constructed single-chain MHC (scMHC)4-peptide complexes in a bacterial expression system and generated functional scMHC-peptide tetrarmers (25). Staining with MHC-peptide tetrarmers is performed in the presence of anti-CD8 Abs (19–24). This indicates that tetramer binding can occur in the presence of anti-CD8 Abs; however, the effect of anti-CD8 Abs on
tetramer binding was not studied in detail in the staining experiments involving human T cells.

To determine the role of CD8 in the binding of MHC-peptide tetramers to TCR in a human T cell system, we analyzed the influence of anti-CD8 Abs on the binding of specific cancer-associated MHC-peptide tetramers to a CTL clone derived from PBMCs isolated from a melanoma patient who was treated with a cancer peptide vaccine (26). Importantly, we show that human CD8 has a pivotal role in tetramer binding to TCR, because anti-CD8 Abs completely blocked tetramer-specific binding to TCR.

Our results suggest that TCR binding to MHC-peptide tetramers is coreceptor dependent. Thus, CD8 is recruited and participates in TCR-peptide-MHC interactions before the tetramer has stably bound to the TCR. We have concluded that the use of anti-CD8 Abs in various experimental systems to characterize phenotypes and to isolate T cell populations can greatly influence the outcome of TCR binding to MHC-peptide ligands in their multimeric (tetramer) form.

Materials and Methods

Cells

R6C12 is a CTL clone derived from the PBMCs of a melanoma patient after immunization with the gp100-derived G9-209-2M peptide. This clone was kindly given by S. Rosenberg (Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD).

The R6C12 was cloned in several steps of limiting dilution from bulk cultures and was expanded to large numbers by a protocol using allogeneic irradiated PBMCs (26). Frozen cells were thawed in culture medium consisting of RPMI 1640 containing 20% AIM-V medium plus 10% heat-inactivated human serum, 1 mM HEPES, pH 7.0, 1 mM glutamine, 1 mM pyruvate, 120 IU/ml IL-2, and 10 IU/ml IL-4.

In vitro stimulation of cells was performed every 4–5 days with irradiated JY cells as APCs and the melanoma cell line FM3D, which expresses high levels of gp100 (27). JY and FM3D cells were grown in RPMI 1640 containing 10% FCS, 1 mM glutamine, and 1 mM pyruvate.

Peptides and MHC tetramers

The peptides G9-209-2M (IMDQYPVPSV) and 154 (KTWWGQYWQV), derived from gp100, and the peptide TAX (LLFGYPVYV), derived from human T cell leukemia virus (HTLV)-I, were synthesized by standard techniques by Genemed Synthesis (South San Francisco, CA) and were >95% pure.

Production of scMHC-peptide tetramers was performed as described previously (25). Briefly, the plasmid encoding single-chain β2-microglobulin-HLA-A2 genes, which are connected by a flexible linker, was transformed and overexpressed in Escherichia coli BL21 cells. In this construct, a stretch of 20 amino acids, the biotinylation by anti-BirA enzyme, the BirA enzyme was needed at the C terminus of the HLA-A2 gene. The overexpressed protein was purified from inclusion bodies, denatured, reduced, and mixed with a 5- to 10-fold excess of the appropriate peptide in a redox-shuffling refolding system for 24–48 h. Soluble monomeric MHC-peptide complexes were purified from the refolding solution by fast protein liquid chromatography with Q-Sepharose chromatography and subjected to biotinylation with BirA (Avidity, Denver, CO). Biotinylation efficiency was determined by an ELISA with avidin-conjugated peroxidase and biotinylated myelin basic protein peptide as a control (Avidity). Biotinylation efficiency was determined by an ELISA with avidin-conjugated peroxidase and biotinylated myelin basic protein peptide as a control (Avidity). Biotinylation efficiency was routinely estimated to be >80% by this method. The biotinylated monomeric complexes were subsequently mixed with streptavidin-PE (Jackson ImmunoResearch, West Grove, PA) at a 4:1 molar ratio. Tetramers were purified and separated from monomers by size-exclusion chromatography with a TSK3000 column. Multimers were tested for specific binding over a range of doses by flow cytometry and were typically used at a concentration of 20–40 ng/ml.

Abs and flow cytometry

CTLs (5 × 105) were stained in 30 µl of PBS with MHC-peptide tetramers (20–40 ng/ml) and anti-CD8 Abs (10–40 µg/ml). In blocking experiments, anti-CD8 Abs were added 15 min before tetramers, which then were incubated for 45 min. In other experiments, anti-CD8 Abs and tetramers were added simultaneously or tetramers were added first for 45 min followed by a 15-min incubation with anti-CD8 Abs. After incubation, Abs and tetramers were washed with PBS and the cells were analyzed with a FACStar flow cytometer (BD Biosciences, Mountain View, CA). The following anti-CD8 Abs were used: clone 3B5 (mouse IgG2a, k; Caltag, Burlingame, CA); clone DK-25 (mouse IgG1 k; Dako, Glostrup, Denmark); and clone MCD8 (mouse IgG1; k; IgP). Anti-CD3 Ab was obtained from clone UCHT1-9 (IgGl, k; Dako).

Results

Specific binding of MHC-peptide tetramers by R6C12 CTL clone

To study the effect and contribution of CD8 in the binding of multivalent tetrameric MHC-peptide complexes to class I-restricted TCR in a human system, we analyzed CTLs recognizing a tumor-associated Ag. A CTL clone, R6C12, was derived from PBMCs from a melanoma patient who was treated with the gp100-derived peptide vaccine G9-209-2M (26). Recently, we reported on the construction and generation of human recombinant scMHC-peptide complexes and tetramers made by in vitro refolding of E. coli-expressed β2-microglobulin and HLA-A2 fused into a single gene by a flexible peptide linker (25). We have synthesized scMHC tetramers with the gp100-derived G9-209-2M peptide and other control peptides and have determined whether the interaction of these multimers with R6C12 CD8+ T cells was dependent on the specificity of the TCR. In vitro-stimulated R6C12 CTLs were stained for 1 h at 37°C with the specific G9-209-2M peptide-containing tetramers and with control scMHC tetramers containing a different peptide derived from gp100, peptide 154, and also the HTLV-1-derived peptide TAX. These binding assays revealed that R6C12 bound only the specific G9-209-2M MHC tetramers and not the control multimers containing peptides 154 and TAX (Fig. 1). An average of 70–90% staining of R6C12 CTLs was observed with the specific 209-containing tetramers in comparison with a 2–4% background nonspecific staining observed with the control nonspecific peptides 154 and TAX (Figs. 1 and 2 and Table I). As shown in Fig. 1E, the specificity of staining with the cognate 209 peptide tetramers vs the control nonspecific tetramers was similar when performed at 37°C or 4°C. These results correlate well with reports of functional assays using these similar ligands on other CTL lines or PBMCs derived from melanoma patients (19–25).

CD8 Abs block specific binding of MHC-peptide tetramers to TCR

Next, we tested a panel of Abs that recognize human CD8 to determine their effect on the binding of scMHC-G9-209-2M peptide tetramers to TCRs on R6C12 cells. We found that all three anti-CD8 Abs (3B5 (Caltag), DK-25 (Dako), MCD8 (IgP)) caused a total blockage of scMHC-209 tetramer binding to R6C12 cells (Fig. 3 and Table I). In these experiments, we incubated the cells for 15 min with anti-CD8 Abs before staining for 45–60 min with the scMHC tetramers. We performed the binding of anti-CD8 Abs and tetramers at 37°C, as suggested previously (28). Under these conditions, a blocking effect of >95% was observed (Fig. 3 and Table I) when different anti-CD8 Abs at various concentrations were used. Importantly, no staining of R6C12 cells with the non-specific tetramers was observed regardless of the anti-CD8 used (data not shown), which indicates that the anti-CD8 Abs did not change the fine specificity of tetramer binding. In control experiments, we used an anti-CD3 instead of an anti-CD8 Ab, which had no influence on tetramer binding (Fig. 4C). Thus, the inhibition was the direct result of binding the Ab to CD8.

Also, the blocking results were not influenced by the dose of the tetramers used because a similar blockage was observed when we used lower or higher doses of the 209-containing tetramers (Fig. 3, B–D). Similar inhibition profiles of tetramer binding was observed with three different anti-CD8 Abs directed against the α- or β-chain of CD8 (data not shown).
The blocking effect of the anti-CD8 Abs was titratable and covered a similar dose range (Fig. 3E and Table I). At an Ab dilution of 1/20, corresponding to a concentration of $10^{-2.5}$ μg/ml (depending on Ab used), maximal inhibition was achieved. A dilution of 1/1000 exhibited a minor inhibition effect. The blocking effect of CD8 Abs was similar when FITC-conjugated or unconjugated Abs were used (data not shown).

The timing of Anti-CD8 Abs use in tetramer-CD8 double-staining experiments is critical to the inhibition effect

The binding of MHC-peptide tetramers to TCR is dynamic because the half-life of the TCR-MHC-peptide complex is relatively short, and the binding of the individual MHC-peptide complex units of the tetramer to the TCR is expected to be dynamic rather than static. Each MHC-peptide complex (here the scMHC-209 complex) dissociates and reassociates with the TCR over time. In such a case, the timing of the addition of anti-CD8 Abs in double-staining experiments may be very critical for observing the blocking effect of the anti-CD8 Abs. Therefore, to test this we compared the effects of anti-CD8 Abs on tetramer binding to R6C12 cells under two conditions: (1) when anti-CD8 Abs and scMHC-209 tetramers were added simultaneously (Fig. 3D), and (2) when scMHC-209 tetramers were allowed to bind first for 45 min and anti-CD8 was added to the cells only later, after the tetramers had already stably bound to R6C12 TCRs (Fig. 2 and Table I).

FIGURE 2. The influence of temperature on the blocking effect of CD8 Abs on the specific binding of MHC-peptide tetramers to R6C12 TCR. R6C12 CTLs ($5 \times 10^5$) were stained with PE-labeled G9-209-2M-containing MHC-peptide tetramers (20 μg/ml) at 37°C (A and B) or at 4°C (C and D) in the presence of FITC-conjugated anti-CD8 Abs (10 μg/ml; clone DK-25; Dako). A and C, Cells were incubated first with G9-209-2M-containing tetramers for 45 min followed by incubation with CD8 Abs for 15 min. B and D, Cells were incubated first with CD8 Ab (DK-25; 10 μg/ml) for 15 min followed by incubation for 45 min with the MHC-peptide tetramers. C and D, Cells also were incubated in the presence of 3 mM azide. Numbers represent the percentage of stained cells in respective quadrants.
As shown, when cells were incubated first with tetramers and only later with anti-CD8 Abs, no inhibition was observed, and multimer staining appeared to be as stable as staining with tetramers alone, without anti-CD8 Abs. Thus, although we expect that the affinity of the anti-CD8 Abs to CD8 is much higher than that of the MHC-peptide complex to TCR, the high avidity of the tetramers enables them to bind strongly enough to overcome the blocking effect of the anti-CD8 Abs in the final stage of the experiment when the Abs are added to the assay.

When tetramers and anti-CD8 Abs are added simultaneously, a complete blocking effect on tetramer binding was observed (Fig. 3D) that was indistinguishable from the blocking effect observed when anti-CD8 Abs and tetramers were added sequentially (first anti-CD8 Abs followed by tetramers, Fig. 3C).

### Blockage of tetramer binding to TCR by CD8 Abs is not mediated by receptor down-regulation

We postulated that the staining of CTLs at 37°C could allow T cell biological effects such as CD8 and/or TCR internalization. To control these activities and to rule out the possibility that the blockage of tetramer binding to TCR by anti-CD8 Abs is mediated by increased internalization of TCRs, we incubated with anti-CD8 Abs and stained with tetramers only at 4°C in the presence of 3 mM azide to prevent Ab-induced internalization and TCR down-regulation, as previously reported (29). The binding and specificity of multimer binding at 4°C to R6C12 was maintained and was similar or in several cases even somewhat improved than that obtained with staining at 37°C (Fig. 2, A and C). Seventy percent staining with the specific 209-containing tetramer was observed under both conditions, whereas the nonspecific tetramers (containing peptides 154 and TAX) did not stain the R6C12 cells at both temperatures (data not shown). However, the blocking effect of the anti-CD8 Abs on the binding of the cognate tetramers to the R6C12 cells was identical under both conditions: all showed a significant 70–90% blockage of tetramer binding at 4°C as well as at 37°C (Fig. 2, B). Table I.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Tetramer Staining (%)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Tetramer 209 (alone)</td>
<td>71.4</td>
<td></td>
</tr>
<tr>
<td>Tetramer 154 (alone, control)</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>1. CD8 (10 µg/ml)</td>
<td>2.7</td>
<td>96</td>
</tr>
<tr>
<td>2. Tetramer 209 (40 µg/ml)</td>
<td>33.5</td>
<td>53</td>
</tr>
<tr>
<td>1. αCD8 (0.5 µg/ml)</td>
<td>64.0</td>
<td>10</td>
</tr>
<tr>
<td>2. Tetramer 209 (40 µg/ml)</td>
<td>70.5</td>
<td>0</td>
</tr>
<tr>
<td>1. CD3 (10 µg/ml)</td>
<td>72.8</td>
<td>0</td>
</tr>
<tr>
<td>2. Tetramer 209 (40 µg/ml)</td>
<td></td>
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</table>

*R6C12 CTLs (5 × 10⁵) were stained with PE-labeled G9-209-2M-containing MHC-peptide tetramers or control tetramer at 37°C for 1 h with or without staining with saturating amounts of FITC-conjugated anti-CD8 Abs. In A, cells were stained with tetramers alone at 20 µg/ml. In B, cells were stained first for 15 min with anti-CD8 Ab (10 µg/ml; clone DK-25; Dako) followed by incubation for 45 min with the specific G9-209-2M MHC-peptide tetramer (20 µg/ml). In C, staining was similar to that performed in A, but tetramer concentration was 80 µg/ml. Anti-CD8 Abs (DK-25) were at 10 µg/ml. In D, anti-CD8 Abs (10 µg/ml; clone 3B5; Caltag) and specific tetramers (40 µg/ml) were added simultaneously to the R6C12 cells. The overlay of staining with (open histogram) and without (filled histogram) the presence of CD8 Abs is shown. In E, titration of the effect of anti-CD8 (DK-25) on the binding of the G9-209-2M specific MHC-peptide tetramers. The overlay of staining with various indicated anti-CD8 dilutions in comparison with staining without CD8 Ab is shown. The shaded histogram is a 1/1000 dilution. Similar results were obtained with Caltag clone 3B5 and IgP clone MCD8 anti-CD8 Abs. The stock solution of anti-CD8 Ab for the titration shown was 50 µg/ml; thus, the 1/1000 dilution represents a concentration of 0.05 µg/ml (see also Table I).
The inhibitory effect of the anti-CD8 Abs here was somewhat better at 4°C (96% inhibition, Fig. 3D), probably due to the improved binding of anti-CD8 Abs at 4°C. However, as shown in Fig. 3B–D, total inhibition of >95% of the tetramer binding by CD8 Abs was also observed at 37°C. Thus, the data indicate that the CD8 effects on tetramer binding are not dependent on T cell activation and are not caused by TCR-CD8 internalization.

Anti-CD8-mediated blockage of tetramer binding is not a result of steric interference

Finally, we tested the effect of tetramers on the binding of anti-CD8 Abs to rule out the possibility that the anti-CD8-mediated blockage of tetramer binding is the result of a steric interference. The binding of anti-CD8 Abs was not affected by the presence of tetramers, and in fact was identical with the binding of anti-CD8 Abs to R6C12 cells in the absence of tetramers (Fig. 4A). Moreover, there was no influence on the sequence of incubation, because similar staining with anti-CD8 was observed when anti-CD8 Ab was added first followed by the addition of tetramers, or tetramers were allowed to bind before incubating with anti-CD8 (Fig. 4A). As a control, we measured the influence of tetramers on the binding of CD3 Abs and showed that the tetramers compete with the binding of anti-CD3 Abs (Fig. 4B). Thus, the specific scMHC-209 tetramers blocked the binding of anti-CD3 Abs; however, when anti-CD3 Abs were added first before the addition of the 209-specific tetramers, neither inhibition in anti-CD3 binding (Fig. 5A) nor inhibition of tetramer binding was observed (Fig. 4C).

The evident tetramer blockade of anti-CD3 binding, as shown in Fig. 5A, can be accounted for by tetramer-induced TCR internalization and down-regulation because in a similar experiment performed at 4°C, the tetramer-mediated blockade of anti-CD3 binding was significantly diminished (Fig. 5B).

It was reported previously that the competition of class I MHC-peptide tetrameric complexes with anti-CD3 provides evidence of the specificity of peptide binding to the TCR complex (30).

Discussion

In this study, we used multimeric forms of human class I MHC-peptide complexes to study the role of CD8 in binding to TCR. We chose a homogeneous population of cloned melanoma-specific CD8+ CTLs generated from PBMCs of a vaccinated patient. Previous studies in the mouse analyzing the role of CD8 in tetramer binding to TCR were performed with well-defined, class I-restricted TCR-transgenic systems (18). MHC-peptide tetramers are widely used today to study T cell responses including those in cancer patients; however, the role of CD8 binding in these studies was not determined. We wanted to analyze a human system relevant to a cancer-associated peptide that is restricted to the superhaplotype HLA-A2. For this purpose, the homogeneous population of a cloned HLA-A2-restricted CTL isolated from a patient is very
The order of incubation events with anti-CD3 Ab and tetramer is indicated. After the incubation with anti-CD3 Abs (15 min at 37°C; 17.5 μg/ml) of tetramers. The tetramers were added for 45 min at 37°C, either before or in the absence of G9-209-2M-containing MHC-peptide tetramers (20 μg/ml). Anti-CD8 Abs directed to the peptide tetramers to TCR. We used three different anti-CD8 Abs, a–b and γ-δ chains of CD8 and found that all of them are functional, as they can respond to TCR ligand but only at high doses. The results suggest that the CD8 coreceptor and its influence on tetramer binding was not determined. There are no studies for the human system reporting blocking effects of anti-CD8 Abs on the binding of MHC-peptide tetramers to CTLs under various pathological or normal conditions. Thus, our results suggest that the use of anti-CD8 Abs in flow cytometry analysis of T cell populations can drastically influence TCR binding to MHC-peptide tetramers. The order of incubation events with the reagents (anti-CD8 Ab and tetramers) used in these staining experiments is critical when an inhibitory Ab is used. Therefore, the influence of the commonly used anti-human CD8 Abs must be determined. The blocking effects shown here, with three commonly used Abs, raise a cautionary note as to the current strategies that are being used to stain with MHC-peptide tetramers and to detect low frequencies of T cell populations in various experimental systems and under different pathological conditions.

Our results have important implications for understanding the early molecular events occurring during TCR-peptide-MHC interaction and the consequences of these interactions on T cell activation and signaling. They show that CD8 is recruited and participates directly in TCR-peptide-MHC interactions before the MHC-peptide complex has stably bound to the TCR. However, they also shed new light on the use of peptide-MHC tetramers for the phenotypic characterization of various T cell responses. The ability to produce large quantities of MHC-peptide tetramers has caused a revolution in T cell biology, allowing the detection of Ag-specific T cell populations (sometimes very rare) by using the increased avidity of the tetrameric form to compensate for the very low affinity of the TCR for MHC-peptide complexes (19–24). Numerous reports have been published in recent years that show the ability of MHC-peptide tetramers to bind to Ag-specific TCRs. These T cell populations were analyzed under various pathological conditions such as after viral infections, cancer, and autoimmune diseases. All of these studies analyzing class I MHC-peptide tetramer binding used a double-staining procedure so that the ability to stain CD8+ T cells by the MHC-peptide tetramers was tested (19–24).

However, the role of the CD8 coreceptor and its influence on tetramer binding was not determined. There are no studies for the human system reporting blocking effects of anti-CD8 Abs on the binding of MHC-peptide tetramers to CTLs under various pathological or normal conditions. Thus, our results suggest that the use of anti-CD8 Abs in flow cytometry analysis of T cell populations can drastically influence TCR binding to MHC-peptide tetramers. The order of incubation events with the reagents (anti-CD8 Ab and tetramers) used in these staining experiments is critical when an inhibitory Ab is used. Therefore, the influence of the commonly used anti-human CD8 Abs must be determined. The blocking effects shown here, with three commonly used Abs, raise a cautionary note as to the current strategies that are being used to stain with MHC-peptide tetramers and to detect low frequencies of T cell populations in various experimental systems and under different pathological conditions.

A recent study contradicts these findings. With two well-defined transgenic mouse systems, it showed that at least in the mouse system, CD8 plays a critical and sometimes even an obligatory role in Ag-specific TCR binding and T cell activation (18). The results in these mouse systems contradict the view that the multimeric (tetramer) form of MHC-peptide ligands bind directly and solely to the TCR, but rather, highlight a pivotal contribution of CD8 for this association.

Our results in the human system presented here come to the same conclusion, namely that CD8 interaction with MHC-peptide tetramers is a crucial and pivotal step in the binding events occurring between the TCR and MHC-peptide complexes. Anti-CD8 Abs were able to completely block the specific binding of sCD8 MHC-peptide tetramers to TCR. We used three different anti-CD8 Abs directed to the a- and b-chain of CD8 and found that all of them blocked tetramer binding to TCR with similar dose response curves. Interestingly, in their study of the mouse system, Daniels and Jameson (18) found that one anti-CD8 Ab of the four tested enhanced TCR association by cognate (but not noncognate) MHC-peptide tetramer (18). The other three Abs tested were all inhibitory and blocked MHC-peptide tetramer binding to the transgenic TCR.

Our results have important implications for understanding the early molecular events occurring during TCR-peptide-MHC interaction and the consequences of these interactions on T cell activation and signaling. These T cell populations were analyzed under various pathological conditions such as after viral infections, cancer, and autoimmune diseases. All of these studies analyzing class I MHC-peptide tetramer binding used a double-staining procedure so that the ability to stain CD8+ T cells by the MHC-peptide tetramers was tested (19–24).

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Our recent study demonstrates the ability to differentiate between T cell subsets that have relative high or low affinity to MHC-peptide tetramers (18, 24). These subpopulations were designated tetramerhigh and tetramerlow populations, or in other cases, high-avidity and low-avidity populations. Interestingly, in the 2C TCR-transgenic mice, there is a natural population of T cells that are positive for the 2C TCR yet are negative for CD8 (18). These cells are functional, as they can respond to TCR ligand but only at high doses. The results suggest that the CD8+ population is perhaps coreceptor-independent and that it may differ in tetramer binding compared with the CD8- population. Analysis of tetramer binding
by these two populations revealed that all 2C cells stained specifically with the specific tetramer in the absence of CD8 Ab, but the staining profile was bimodal, with a tetramer_high and a tetramer_low population (18). The percentage of tetramerhigh and tetramerrlow populations was correlated with the percentage of CD8− and CD8+ cells, respectively. The use of inhibitory anti-CD8 Abs caused the disappearance of the tetramerhigh population, this group of cells becoming tetramerlow. When using another melanoma-specific CTL line that recognizes MHC-peptide tetramers containing the melanoma-derived MART-1 peptide 27–35, we have indications that a similar situation exists in a human system. These in vitro-stimulated cells contain two distinct populations that are CD8− or CD8+. The specific MHC-peptide tetramers bind to the CD8− cells and stain this T cell population with a pattern similar to the tetramerlow population observed by Daniels and Jameson and others (18, 24). Interestingly, the staining of this tetramerlow population was not affected by the various anti-CD8 Abs that were used in this study (C. J. Cohen, G. Denkberg, and Y. Retire, manuscript in preparation). Thus, we postulate that CD8 engagement and involvement in binding by MHC-peptide complexes and multimers to TCR enables the formation and detection of the tetramerhigh T cell population.

The staining intensities of the tetramerhigh T cell population are much weaker because of the absence of C8 infection with the TCR-peptide-MHC complex. Thus, anti-CD8 Abs will not exert any effect on the staining of the tetramerlow population by MHC-peptide tetramers. However, the tetramerhigh T cell population is CD8-dependent and thus, it is significantly influenced by the use of inhibitory CD8 Abs. With CD8-dependent binding, the inherent affinity of the tetramers is insufficient to allow multimeric ligand binding if CD8 participation is blocked.

It is possible that in many experimental systems where MHC-peptide tetramers have been used in combination with anti-CD8 Abs to analyze and isolate tetramer-specific T cell populations, the experimental procedures caused an undesirable preference for staining the tetramerlow population only, because of the blocking effects of the anti-CD8 Abs. Consequently, an important subpopulation of the tetramerhigh cells was not detected because of the presence of the inhibitory anti-CD8 Ab in the staining assays. These findings are supported by results showing that CD8 has a role in enhancing TCR-MHC-peptide interactions, including in the transgenic 2C TCR mouse model (18).

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References