CD8β Increases CD8 Coreceptor Function and Participation in TCR–Ligand Binding

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Summary
To study the role of CD8β in T cell function, we derived a CD8α/β− (CD8−/−) T cell hybridoma of the H-2Kd–restricted N9 cytotoxic T lymphocyte clone specific for a photoreactive derivative of the Plasmodium berghei circumsporozoite peptide PbCS 252–260. This hybridoma was transfected either with CD8α alone or together with CD8β. All three hybridomas released interleukin 2 upon incubation with L cells expressing Kd–peptide derivative complexes, though CD8α/β cells did so more efficiently than CD8α/α and especially CD8−/− cells. More strikingly, only CD8α/β cells were able to recognize a weak agonist peptide derivative variant. This recognition was abolished by Fab’ fragments of the anti-Kd α3 monoclonal antibody SF1-1.1.1 or substitution of Kd D-227 with K, both conditions known to impair CD8 coreceptor function. T cell receptor (TCR) photoaffinity labeling indicated that TCR–ligand binding on CD8α/β cells was ~5- and 20-fold more avid than on CD8α/α and CD8−/− cells, respectively. SF1-1.1 Fab’ or Kd mutation D227K reduced the TCR photoaffinity labeling on CD8α/β cells to approximately the same low levels observed on CD8−/− cells. These results indicate that CD8α/β is a more efficient coreceptor than CD8α/α, because it more avidly strengthens TCR–ligand binding.

MHC class I–restricted TCRα/β+ T cells express heterodimeric CD8 consisting of a disulfide linked α and β chain, whereas other cell types, such as NK cells or extrathymic intraepithelial T cells, express homodimeric CD8α (1, 2). Whereas CD8α can be surface expressed as CD8α/α homodimers, CD8β is expressed only as CD8α/β heterodimers (3). X-ray crystallography showed that CD8α is folded in an Ig-like manner (2, 4). For CD8α/α, a major CD8 binding site on MHC class I molecules is the acidic loop 222–229, located in the center of the α3 domain (5, 6). Moreover, the cytoplasmic tail of CD8α has been shown to associate with the T cell–specific tyrosine kinase p56ck (7, 8) and becomes phosphorylated on serine residues upon cell activation (8, 9).

Less is known about CD8β. It has only ~30% sequence homology with CD8α, and its hinge region is 13 amino acids shorter than the one of CD8α (1, 2). CD8β broadens the range of antigen recognition, e.g., CD8α/β expressing T cell hybridomas were able to recognize ligand variants, but CD8α/α expressing ones did not (10, 11). CD8β also plays a decisive role in thymic differentiation and maturation, since CD8β “knock out” mice have dramatically reduced numbers of mature CD8+ cells (12, 13). Transgenic mice expressing “tailless” CD8β also exhibited a reduced number of mature CD8+ cells, indicating that the cytoplasmic portion of CD8β has a functional significance (14). The recent finding that CD8β considerably increases CD8–p56ck association suggests that the tail of CD8β directly or indirectly interacts with this enzyme (15).

We have previously developed a system that allows assessment of TCR–ligand interactions by TCR photoaffinity labeling (16, 17). To this end, the Plasmodium berghei circumsporozoite peptide PbCS 262–260 (SYIPSAEKI) was modified by replacing PbCS S-252 with photoreactive iodo-4-azidosalicylic acid (IASA) and conjugating K-259 with 4-azidobenzoic acid (ABA). From mice immunized with this derivative, Kd–peptide derivative complexes, though

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CD8, because of a fusion mediated silencing of the CD8α promoter and the inability of CD8β to be surface expressed in the absence of CD8α (3, 20). As assessed by TCR photoaffinity labeling, TCR–ligand binding on this hybridoma was >95% weaker than on T1 CTL (19). CD8α transfection of the hybridoma resulted in high level expression of CD8α/α and weak CD8α/β expression (CD8β being contributed by the endogenous gene). This transfection only partially restored TCR–ligand binding, suggesting that CD8α/β (19) may strengthen TCR–ligand binding more efficiently than CD8α/α.

In this study we tested N9 CTL-derived hybridomas that expressed either no CD8 (CD8−/−), only CD8α (CD8α/α), or CD8α/β for antigen recognition (IL-2 release) and TCR–ligand binding (TCR photoaffinity labeling). The results indicate that CD8α/β cells more efficiently recognized antigen, especially a weak antagonist variant, because CD8α/β avidly strengthened TCR–ligand binding.

Materials and Methods

Peptide Derivatives and Photoaffinity Labeling Procedures. All synthetic and analytic procedures were performed essentially as described (16–18, 21). In brief, IASA-YIPSAEK(ABA)I and IASA-YIPSAEK(B4)I were obtained by chloramine T–mediated iodination of AASA-Y(P03H2)IPSAEK(ABA)I and ASA-Y(P03H2)IPSAEK(B4)I, respectively. Before deprotection and cleavage from the resin, the peptides were NH2-terminally acylated with 4-azidosalicyloy-L,N-hydroxysuccinimidyl ester. After iodination with 125I (>2,000 Ci/mMol) or nonradioactive iodine, the peptides were dephosphorylated with alkaline phosphatase and purified by reverse phase HPLC on a C-18 column. Kd and TCR photoaffinity labeling experiments were performed as described (16, 17, 19). In brief, purified soluble Kd was incubated with freshly radiolabeled peptide derivatives at ambient temperature for 2 h, followed by UV irradiation at >350 nm and FPLC gel filtration. For TCR photoaffinity labeling, 8 × 106 cells/ml were resuspended in DMEM supplemented with 2% FCS and 2 mM Hepes and incubated in 1 ml aliquots with 1.2 × 106 cpm of Kd peptide derivative complex for 1 h at 26°C. After UV irradiation at 312 ± 40 nm, the cells were washed and lysed on ice in PBS supplemented with NP-40, Hepes, and protease inhibitors. Immunoprecipitation was performed with anti-TCR mAb H57-259, and the immunoprecipitates were analyzed by SDS-PAGE (10% linear, reducing conditions). The dried gels were evaluated by autoradiography and densitometry. TCR photoaffinity labeling experiments were performed in triplicate and repeated at least twice.

Cell Lines. The T cell hybridoma N9.1 (CD8−/−) was obtained by fusing cloned N9 CTL (18) with the BW5147 TCRαβ− (Bw−) thymoma as described (19). N9.1 cells were transfected with CD8α cDNA inserted in the pHpβApr-1-neo expression vector (8) and selected in the presence of G418 (2.5 mg/ml). Various stable CD8α transfectants were tested by flow cytometry for expression of CD8β, and one clone was found that was CD8β−. This clone was transfected with a CD8β BamHI genomic fragment DNA inserted in the pSV2-his expression vector (pCA257.10). A representative clone (WB1.2) was selected in the presence of histidinol (3 mM). All transfections were performed using protoplast fusion (22). Murine fibroblast L cells were transfected with Kd or KdD227K cDNA as described (23).

Figure 1. CD8α/β cells more efficiently recognize IASA-YIPSAEK (ABA)I and IASA-YIPSAEK(B4)I than CD8α/α or CD8−/− cells. The IL-2 released by CD8−/− (A), CD8α/α (B), and CD8α/β (C) cells was measured as [3H]thymidine uptake by CTLL indicator cells after incubation with L-Kd cells sensitized with IASA-YIPSAEK(ABA)I (○) or IASA-YIPSAEK(B4)I (●). In insets, the IL-2 responses are shown as observed after incubation with anti-CD3 mAb or PMA and ionomycin.

mAbs and Flow Cytometry. The following mAbs were used: H57-259 (anti-TCR Cδ), 53–6–72 (anti-CD8α), H35–17 (anti-CD8β), SFI–1–1.1.1. (anti-Kαα), 20–8–4S (anti-Kαβ), and anti-CD3 (145.2C11). For most experiments, single staining was performed with FITC- or PE-conjugated anti-CD8β and anti-K4, PE-conjugated anti-CD8α, and anti-TCR (mAb). Samples were
Table 1. Flow Cytometry on Cell Lines under Study

| Cell line     | TCR (H57-259) | CD8α (53.6.72) | CD8β (H35-17) | LFA1 (FD18.5) | Kd (20-8-4S) | ICAM1 (YN1.1.7) |
|---------------|---------------|----------------|---------------|---------------|--------------|-----------------|
| N9 CTL        | 259           | 272            | 155           | 316           | ND           | ND              |
| N9.1-1/-      | 187           | 4              | 3             | 308           | ND           | ND              |
| N9.1 α/α      | 143           | 753            | 11            | 229           | ND           | ND              |
| N9.1 α/β      | 194           | 792            | 95            | 187           | ND           | ND              |
| L-Kd wt       | ND            | ND             | ND            | ND            | ND           | ND              |
| L-Kd D227K    | ND            | ND             | ND            | ND            | 253          | 107             |
|               |               |                |               |               | 765          | 145             |

Cells were stained with the antibodies indicated in parenthesis. The mean fluorescence intensities for a representative experiment are shown. The histograms were monophasic for all cell lines/antibody combinations.

analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA) equipped with LYSSIS II software.

**IL-2 Release Assay.** L-Kd or L-Kd D227K cells (5 × 10⁶ cells/ml) were incubated in DMEM supplemented with 0.7% FCS and 10 mM Hepes in 10 ml polystyrene tubes (Falcon Plastics, Oxnard, CA) with graded concentrations (10⁻⁷–10⁻¹⁴ M, in 10-fold dilutions) of peptide derivative at 26°C for 2 h. After UV irradiation at ≥350 nm (16), cells were washed three times, resuspended in DMEM supplemented with 5% FCS and 10 mM Hepes at 10⁶ cells/ml, and plated to 100-μl aliquots into flat bottom 96-well microtiter plates (Falcon Plastics). The T cell hybridomas, resuspended in the same medium and at the same cell density, were added in 100-μl aliquots. Alternatively, hybridomas were incubated either with anti-CD3 mAb (145.2C11, absorbed on plates) or PMA (2.5 ng/ml) and ionomycin (0.5 μg/ml). After 24 h of incubation at 37°C, supernatants (100 μl) were transferred into fresh microtiter plates and incubated for 36–48 h with CTLL indicator cells (4 × 10⁵ cells/100 μl/well). 1 μCi of [³H]thymidine (Amersham Corp., Arlington Heights, IL), was added per well, and after incubation for an additional 2 h, the cells were harvested and the incorporated [³H]thymidine was measured by β counting (Inotech Harvester and Trace 96 β counter). Each experiment was performed in triplicate and was repeated at least twice.

**Results and Discussion**

To assess the antigen recognition of the hybridomas under study, they were incubated with L cells expressing covalent Kd-IAβA-YP PSAEK (AB4)I complexes and the released IL-2 was measured. The L-Kd cells were sensitized by incubation with the indicated concentrations of IAS4-YP PSAEK (AB4)I and IAS4-YP PSAEK (BA4)I, respectively, followed by UV irradiation at ≥350 nm, which produced covalent cell-associated Kd-peptide derivative complexes. As shown for a representative experiment in Fig. 1, all three hybridomas produced IL-2 upon incubation with L cells expressing Kd-IAβA-YP PSAEK (AB4)I, but CD8α/β cells more efficiently, especially at low degrees of sensitization. Because all three hybridomas expressed comparable levels of TCR and LFA1 (Table 1) and responded similarly upon stimulation with anti-CD3 mAb or PMA and ionomycin (Fig. 1, insets), these results indicate that CD8α/β, but less CD8α/α, increased the efficiency of antigen recognition.

More strikingly, the peptide derivative variant IAS4-YP PSAEK (BA4)I, which lacks the azido function of the AB4 group, was efficiently recognized only by CD8α/β cells (Fig. 1). Half-maximal IL-2 release was observed at >10-fold higher degree of sensitization than the wild-type conjugate. Substitution of the AB4 group with BA4 reduced the efficiency of recognition by N9 CTL by ~50-fold (18). As assessed by TCR photoaffinity labeling (see below), Kd complexes with IAS4-YP PSAEK (BA4)I bound to the N9 TCR approximately seven times less efficiently than those containing IAS4-YP PSAEK (AB4)I (see Fig. 3 D). The observation that this weak agonist was significantly recognized only by CD8α/β cells is in accordance with reports indicating that CD8α/β complex binding is more avidly strengthens TCR–ligand binding.

To find out why CD8α/β cells efficiently recognize IAS4-YP PSAEK (BA4)I, we performed the previous experiment (Fig. 1 C) in the presence of Fab fragments of the anti-Kd α3 mAb SF1-1.1.1. This reagent has been shown to impair participation of CD8 in TCR–ligand binding while leaving CD8 mediated adhesion unaffected (19). As shown in Fig. 2 A, this reagent abolished the efficient recognition of IAS4-YP PSAEK (BA4)I. Similarly, CD8α/β cells failed to recognize significantly this conjugate on L cells expressing mutant Kd D227K, though they well recognized the wild type conjugate (Fig. 2 B). This Kd mutation has been shown to dramatically impair CD8–MHC class I interactions (5, 6, 19). The finding that CD8α/β cells under conditions that prevent CD8 participation in TCR–ligand binding failed, as CD8α/α cells, to significantly recognize the weak agonist IAS4-YP PSAEK (BA4)I suggests that CD8α/β more avidly strengthens TCR–ligand binding than CD8α/α.
The utilized system allowed to test this possibility, because it permits direct assessment of CD8 participation in TCR-ligand binding by TCR photoaffinity labeling with soluble covalent Kd–peptide derivative complexes (16, 17, 19).

TCR photoaffinity labeling with Kd-125 IASA-YIPSAEK (ABA)I on CD8α/b cells was 5–20 times more efficient than on CD8α/a cells and CD8β/β cells, respectively (Fig. 3, A and B). The background, e.g., nonspecific labeling, was 1–2%, as observed in the presence of the α-Kd-1 mAb 20-8-4S, which blocks specific TCR–ligand binding (16, 19). Because CD8α/a cells expressed more CD8α than N9 CTL or CD8α/b cells expressed CD8α/β (Table 1), these results demonstrate that heterodimeric CD8α/β indeed more avidly strengthens TCR–ligand binding than homodimeric CD8α/α. This is in accordance with preceding experiments, in which various CD8α and CD8α plus CD8β transfectants of CD8β/β N9.1 cells or subclones of CD8α/β WB1.2 were likewise tested (unpublished results).

The recognition of IASA-YIPSAEK(BA)I by CD8α/b cells is abrogated by SF1-1.1.1 Fab’ and Kd mutation D227K. The IL-2 release by CD8α/b cells was measured after incubation with L-Kd sensitized with IASA-YIPSAEK(BA)I in the absence (C) or presence (●) of SF1-1.1.1 Fab’ (A), as described for Fig. 1. Alternatively, L-Kd cells expressing Kd D227K were used as presenting cells, which were sensitized either with IASA-YIPSAEK(ABA)I (C) or IASA-YIPSAEK(BA)I (●) (B).

Figure 2. The recognition of IASA-YIPSAEK(BA)I by CD8α/b cells is abrogated by SF1-1.1.1 Fab’ and Kd mutation D227K. The IL-2 release by CD8α/b cells was measured after incubation with L-Kd sensitized with IASA-YIPSAEK(BA)I in the absence (C) or presence (●) of SF1-1.1.1 Fab’ (A), as described for Fig. 1. Alternatively, L-Kd cells expressing Kd D227K were used as presenting cells, which were sensitized either with IASA-YIPSAEK(ABA)I (C) or IASA-YIPSAEK(BA)I (●) (B).

Figure 3. TCR photoaffinity labeling with soluble ligand Kd–peptide derivative complexes. CD8α/β, CD8α/a or CD8β/β cells were incubated with Kd-IASA-YIPSAEK(ABA)I in absence (black bar) or presence of SF1-1.1.1 Fab’ (20 μg/ml) (white bar) or 20-8-4S mAb (10 μg/ml) (hatched bar), and TCR photoaffinity labeling was evaluated by SDS-PAGE and autoradiography (A) and densitometry (B). Alternatively, CD8α/β and CD8β/β cells, respectively, were tested likewise, either with Kd-IASA-YIPSAEK(ABA)I (black bar) or mutant KdD227K-IASA-YIPSAEK(BA)I (white bar) (C). In both experiments, 100% refers to the TCR labeling observed on CD8α/b cells with the wild type ligand. The TCR binding of Kd-IASA-YIPSAEK(BA)I was assessed by its ability to inhibit the TCR photoaffinity labeling by Kd-IASA-YIPSAEK(ABA)I on N9 CTL (D). Mean values and standard deviations were calculated from at least three independent experiments.
The efficient TCR photoaffinity labeling on CD8α/β cells was reduced in the presence of SF1-1.1 Fab’ to the same low levels as observed on CD8−/− cells (Fig. 3, A and B). The TCR photoaffinity labeling on CD8α/α was also reduced in the presence of this reagent, but for unknown reasons to a slightly lesser degree than on CD8α/β cells. The lack of a significant inhibition of the TCR photoaffinity labeling on CD8−/− cells in the presence of SF1-1.1 Fab’ showed that this reagent does not affect the TCR–ligand interaction per se, but rather the CD8 participation in TCR–ligand binding. Similarly, when TCR photoaffinity labeling on CD8α/β cells was performed with soluble KdD227K-L4SA-YIPSAEK(AB4)I, nearly five times weaker labeling was observed than with the wild type ligand (Fig. 3 C). In contrast, on CD8−/− cells, both ligands exhibited essentially the same weak TCR photoaffinity labeling, confirming our previous finding that this Kd mutation does not significantly affect the actual TCR–ligand binding, but rather its dependence on CD8 (Fig. 3 B and reference 19).

The peptide derivative variant L4SA-YIPSAEK(AB4)I, lacking an orthogonal photoactive group, can cross-link to Kd, but not to TCR; therefore, the binding of Kd-L4SA-YIPSAEK(AB4)I to N9 TCR was assessed by its ability to inhibit the TCR photoaffinity labeling by Kd-L4SA-YIPSAEK(AB4)I. As shown in Fig. 3 D, the TCR photoaffinity labeling on N9 CTL was inhibited in a linear fashion in the presence of graded amounts of Kd-L4SA-YIPSAEK(AB4)I. By extrapolation, 50% of inhibition was observed at ~6.8-fold molar excess of variant ligand.

Taken collectively, the results of the TCR photoaffinity labeling experiments correlate well with those of the IL-2 release experiments. Most strikingly, SF1-1.1 Fab’ and Kd mutation D227K inhibited the efficient recognition of the conjugate variant L4SA-YIPSAEK(AB4)I by CD8α/β cells, because they inhibited the TCR photoaffinity labeling (Figs. 2 and 3). We thus conclude that CD8α/β cells more efficiently recognize antigen than CD8α/α cells, because CD8α/β more avidly strengthens TCR–ligand binding. Although our results do not rule out that other factors may play a role as well (i.e., that CD8α/β has superior signaling capabilities or more efficiently mediates CD8-dependent adhesion), they clearly demonstrate that CD8β significantly increases CD8 participation in TCR–ligand binding, and this predictably is important for antigen recognition, especially of weak agonists.

It remains to be explained why CD8α/β more efficiently increases TCR–ligand binding than CD8α/α. It is conceivable that either CD8α/β more avidly binds MHC class I molecules or that it more efficiently “couples” with TCR/CD3, thus defining an orientation of CD8 relative to the TCR that favors coordinate ligand binding. Although CD8α/α and CD8α/β both have been shown to bind MHC class I molecules (5, 24), it is unknown whether they do so with different affinities. In support of the second possibility, we have previously observed that the dynamics of TCR–ligand interactions on CTL clones are modulated by CD8 in a time- and temperature-dependent manner (16, 19). Moreover, several reports indicate that CD8 interacts with TCR/CD3 complex (25, 26), which in view of our present data may imply that CD8β plays an important role in such interactions. Interestingly, CD8β has been shown to significantly increase the association of CD8 with the tyrosine kinase p56Lck (15). In the CD4 system, p56Lck is known to be involved in coupling CD4 with TCR/CD3 (27). If the same were true for CD8, it would explain why CD8α/β is a more efficient coreceptor than CD8α/α. The system described here, by including kinetic experiments and further genetic engineering of the hybridomas, should now permit detailed analysis of the role of CD8β in T cell function.

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