Brief Definitive Report

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-2K^d–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 K^d–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-K^d α3 monoclonal antibody SF1-1.1.1 or substitution of K^d 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/a and CD8$^{-/-}$ cells, respectively. SF1-1.1.1 Fab′ or K^d 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 p56^{lck} (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 cytoplasmatic portion of CD8β has a functional significance (14). The recent finding that CD8β considerably increases CD8–p56^{lck} 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* cirumsporozoite 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, K^d-restricted CTL clones were derived that recognized this conjugate as well as the one lacking the *IASA* group, but not the derivative lacking the *ABA* group (17, 18). Selective photoactivation of *IASA* permitted cross-linking of the peptide derivative to K^d molecules (16). Incubation of soluble, monomeric K^d–peptide derivative complexes with cloned CTL and photoactivation of the *ABA* group resulted in TCR photoaffinity labeling, which was proportional to TCR–ligand binding (16–18). This labeling was not dependent on integrins but dependent on CD8 participation in TCR–ligand binding.

We have previously prepared a T cell hybridoma by fusing cloned T1 CTL with a TCR$^-$ variant of the BW thymoma (19). This hybridoma expressed T1 TCR but no

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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 efficienty 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(*BA*)I were obtained by chloramine T–mediated iodination of *ASA*-Y(PO_3H_2)IPSAEK(*ABA*)I and ASA-Y(PO_3H_2) IPSAEK(*BA*)I, respectively. Before deprotection and cleavage from the resin, the peptides were NH_2-terminally acylated with 4-azidosalicyloy-*N*-hyrdroxysuccinimidyl ester. After iodination with ^{125}I (~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. K^d and TCR photoaffinity labeling experiments were performed as described (16, 17, 19). In brief, purified soluble K^d 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, 8×10^6 cells/ml were resuspended in DMEM supplemented with 2% FCS and 20 mM Hepes and incubated in 1 ml aliquots with 1.2×10^7 cpm of K^d–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 H57-297, 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 $TCR\alpha\beta^-$ (BW^-) thymoma as described (19). N9.1 cells were transfected with CD8α cDNA inserted in the pH pHβAPr-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 $CD8\beta^-$. 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 K^d or K^dD227K cDNA as described (23).

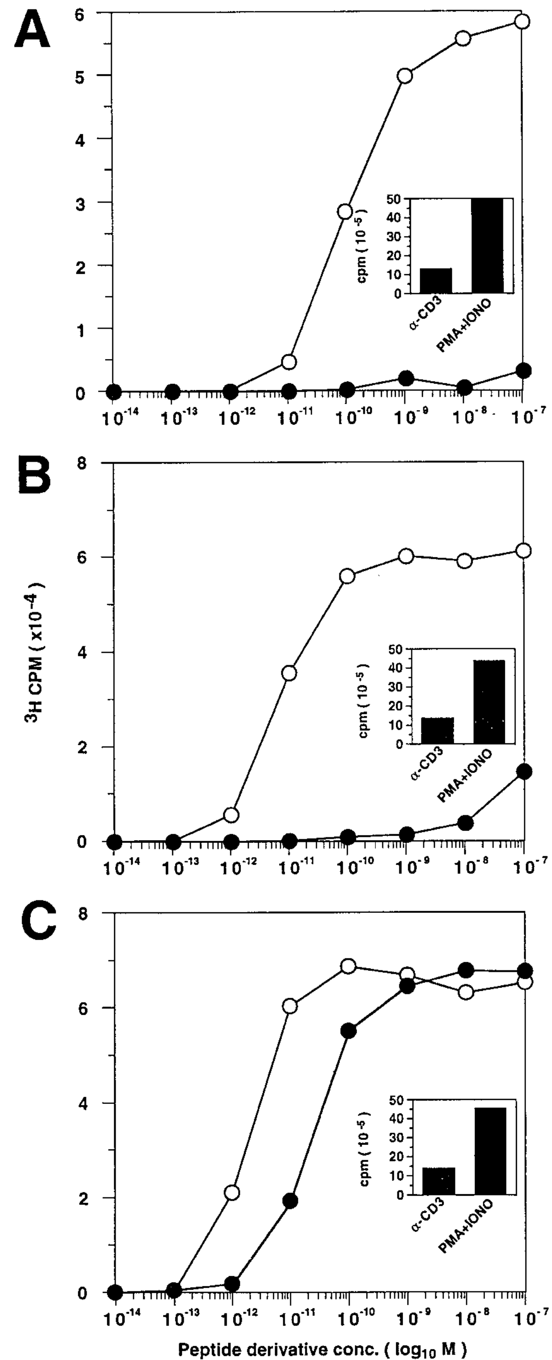

Figure 1. CD8α/β cells more efficiently recognize *IASA*-YIPSAEK (*ABA*)I and *IASA*-YIPSAEK(*BA*)I than CD8α/α or $CD8^{-/-}$cells. The IL-2 released by $CD8^{-/-}$ (*A*), CD8α/α (*B*), and CD8α/β (*C*) cells was measured as [3H]thymidine uptake by CTLL indicator cells after incubation with L-K^d cells sensitized with *IASA*-YIPSAEK(*ABA*)I (○) or *IASA*-YIPSAEK(*BA*)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 Cβ), 53-6-72 (anti-CD8α), H35-17 (anti-CD8β), SF1-1.1.1. (anti-$K^d\alpha3$), 20-8-4S (anti-$K^d\alpha1$), and anti-CD3 (145.2C11). For most experiments, single staining was performed with FITC- or PE-conjugated anti-CD8β and anti-K^d, PE-conjugated anti-CD8α, and anti-TCR (mAb). Samples were

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Table 1. *Flow Cytometry on Cell Lines under Study*

Cell line	TCR (H57-259)	CD8α (53.6.72)	CD8β (H35-17)	LFA1 (FD18.5)	K^d (20-8-4S)	ICAM1 (YN1.1.7)
	Surface marker (mean fluorescence)					
N9 CTL	259	272	155	316	ND	ND
N9.1$^{-/-}$	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-K^d wt	ND	ND	ND	ND	253	107
L-K^d D227K	ND	ND	ND	ND	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.

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

IL-2 Release Assay. L-K^d or L-K^d D227K cells (5×10^6 cells/ml) were incubated in DMEM supplemented with 0.7% FCS and 10 mM Hepes in 10 ml polypropylene 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 10^6 cells/ml, and plated in 100-μl aliquots into flat bottom 96-well microtiter plates (Falcon Plastics). The T cell hydridomas, resuspended in the same medium and at the same cell density, were added in 100-μl aliquots. Alternatively, hydridomas 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 (4×10^3 cells/100 μl/well). 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL), was added per well, and after incubation for an additional 12 h, the cells were harvested and the incorporated [^{3}H]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 K^d-*IASA*-YIPSAEK(*ABA*)I complexes and the released IL-2 was measured. The L-K^d cells were sensitized by incubation with the indicated concentrations of *IASA*-YIPSAEK(*ABA*)I and *IASA*-YIPSAEK(*BA*)I, respectively, followed by UV irradiation at ⩾350 nm, which produced covalent cell-associated K^d–peptide derivative complexes. As shown for a representative experiment in Fig. 1, all three hybridomas produced IL-2 upon incubation with L cells expressing K^d-*IASA*-YIPSAEK(*ABA*)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 ionomycine (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 *IASA*-YIPSAEK(*BA*)I, which lacks the azido function of the *ABA* 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 *ABA* group with *BA* reduced the efficiency of recognition by N9 CTL by ~50-fold (18). As assessed by TCR photoaffinity labeling (see below), K^d complexes with *IASA*-YIPSAEK(*BA*)I bound to the N9 TCR approximately seven times less efficiently than those containing *IASA*-YIPSAEK(*ABA*)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 CD8β broadens the range of antigen recognition by CD8$^+$ T cells (10, 11) and in addition provides a quantitative correlation between antigen recognition and TCR–ligand binding.

To find out why CD8α/β cells efficiently recognize *IASA*-YIPSAEK(*BA*)I, we performed the previous experiment (Fig. 1 *C*) in the presence of Fab′ fragments of the anti-K^d 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 *IASA*-YIPSAEK(*BA*)I. Similarly, CD8α/β cells failed to recognize significantly this conjugate on L cells expressing mutant K^d D227K, though they well recognized the wild type conjugate (Fig. 2 *B*). This K^d 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 *IASA*-YIPSAEK(*BA*)I suggests that CD8α/β more avidly strengthens TCR–ligand binding than CD8α/α.

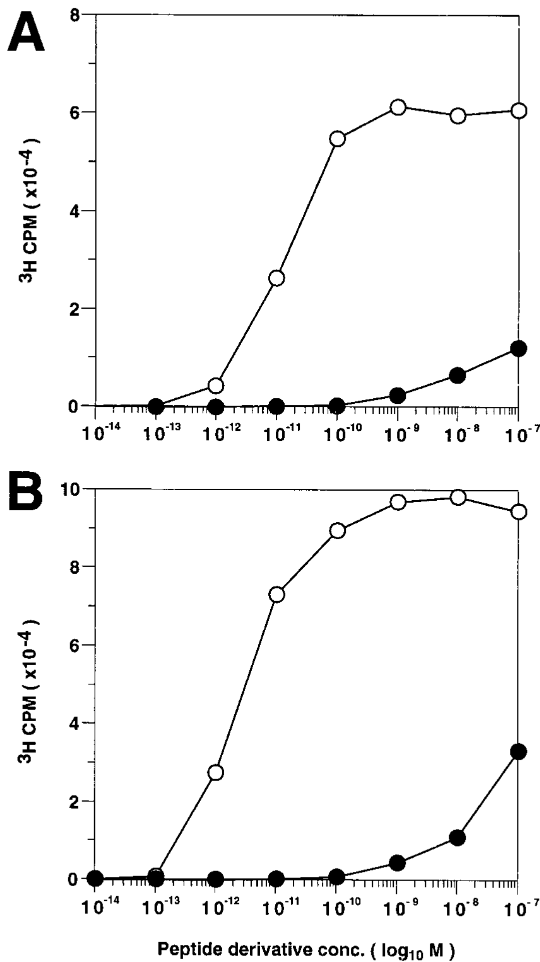

Figure 2. The recognition of *IASA*-YIPSAEK(*BA*)I by CD8α/β cells is abrogated by SF1-1.1.1 Fab′ and K^d mutation D227K. The IL-2 release by CD8α/β cells was measured after incubation with L-K^d sensitized with *IASA*-YIPSAEK(*BA*)I in the absence (○) or presence (●) of SF1-1.1.1 Fab′ (*A*), as described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

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 K^d–peptide derivative complexes (16, 17, 19). TCR photoaffinity labeling with K^d-^{125}*IASA*-YIPSAEK (*ABA*)I on CD8α/β cells was ∼5 and 20 times more efficient than on CD8α/α cells and $CD8^{-/-}$ cells, respectively (Fig. 3, *A* and *B*). The background, e.g., nonspecific labeling, was 1–2%, as observed in the presence of the α-K^dα1 mAb 20-8-4S, which blocks specific TCR–ligand binding (16, 19). Because CD8α/α cells expressed more CD8α than N9 CTL or CD8α/β 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 preceeding experiments, in which various CD8α and CD8α plus CD8β transfectants of $CD8^{-/-}$ N9.1 cells or subclones of CD8α/β WB1.2 were likewise tested (unpublished results).

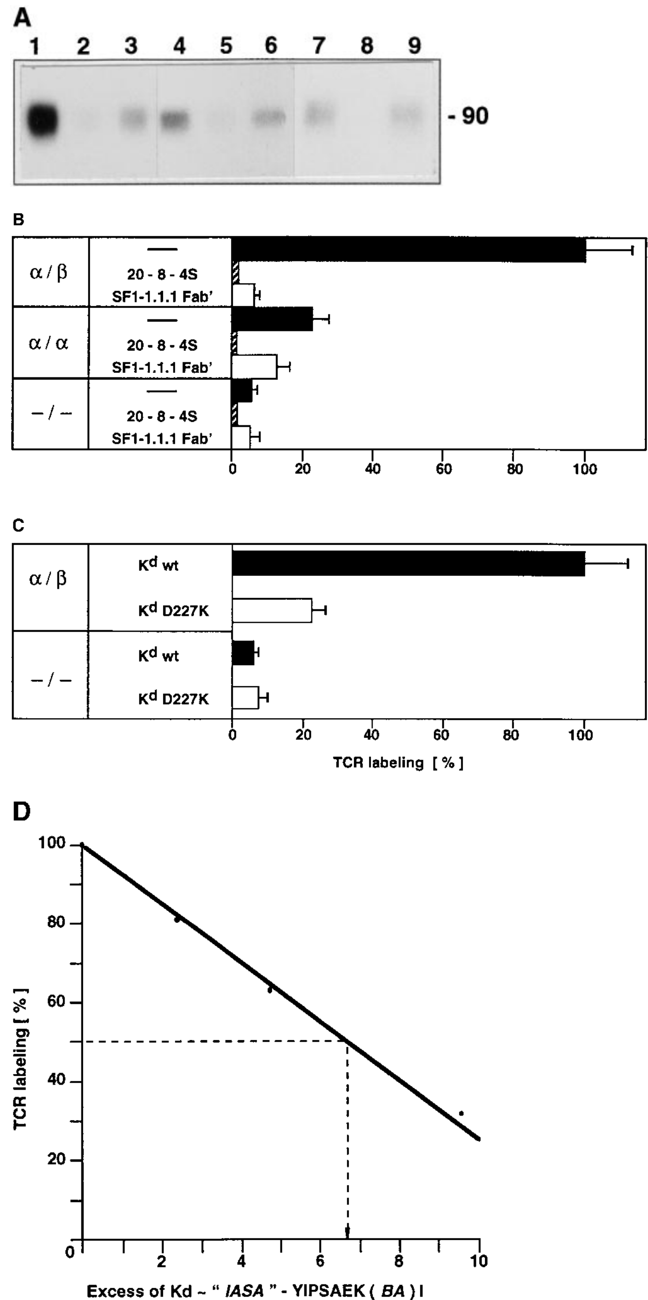

Figure 3. TCR photoaffinity labeling with soluble ligand K^d–peptide derivative complexes. CD8α/β, CD8α/α or $CD8^{-/-}$ cells were incubated with K^d-^{125}*IASA*-YIPSAEK(*ABA*)I in absence (*black bars*) or presence of SF1-1.1.1 Fab′ (20 μg/ml) (*white bars*) or 20-8-4S mAb (10 μg/ml) (*hatched bars*), and TCR photoaffinity labeling was evaluated by SDS-PAGE and autoradiography (*A*) and densitometry (*B*). Alternatively, CD8α/β and $CD8^{-/-}$ cells, respectively, were tested likewise, either with K^d-*IASA*-YIPSAEK(*ABA*)I (*black bars*) or mutant K^dD227K-*IASA*-YIPSAEK(*BA*)I (*white bars*) (*C*). In both experiments, 100% refers to the TCR labeling observed on CD8α/β cells with the wild type ligand. The TCR binding of K^d-*IASA*-YIPSAEK(*BA*)I was assessed by its ability to inhibit the TCR photoaffinity labeling by K^d-^{125}*IASA*-YIPSAEK (*ABA*)I on N9 CTL (*D*). Mean values and standard deviations were calculated from at least three independent experiments.

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The efficient TCR photoaffinity labeling on CD8α/β cells was reduced in the presence of SF1-1.1.1 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 SF1-1.1.1 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 K^d D227K-*IASA*-YIPSAEK(*ABA*)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 K^d 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 *IASA*-YIPSAEK(*BA*)I, lacking an orthogonal photoreactive group, can cross-link to K^d, but not to TCR; therefore, the binding of K^d-*IASA*-YIPSAEK(*BA*)I to N9 TCR was assessed by its ability to inhibit the TCR photoaffinity labeling by K^d-*IASA*-YIPSAEK(*ABA*)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 K^d-^{125}*IASA*-YIPSAEK(*BA*)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, SF1-1.1.1 Fab′ and K^d mutation D227K inhibited the efficient recognition of the conjugate variant *IASA*-YIPSAEK(*BA*)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 $p56^{lck}$ (15). In the CD4 system, $p56^{lck}$ 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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