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Human CD8 co-receptor is strictly involved in MHC–peptide tetramer–TCR binding and T cell activation

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Abstract

Although there has been extensive analysis on the capacity of MHC–peptide tetramers to bind antigen-specific TCR, there have been comparatively few studies regarding the role of the CD4 and CD8 co-receptors in binding and activation by these multimeric molecules. Here, we start from the observation that different antibodies against human CD8 exert opposite effects on MHC–peptide tetramer binding to the TCR: tetramer staining was enhanced by OKT8 antibody, while it was blocked with SK1 antibody. We used these different anti-CD8 antibodies to modulate CD8 function during tetramer staining of Melan-A/MART1-specific CTL clones. We show that CD8 action could be variably modulated during all the phases of interaction, indicating that CD8 participates in both the initial association of the TCR with MHC–peptide tetramers and the stability of this interaction. While the blocking effect of anti-CD8 antibodies was mostly exerted during the initial binding of the TCR with MHC–peptide tetramers, the enhancing effect was exerted by augmenting the duration of this interaction. Blocking anti-CD8 antibodies were also capable of preventing tetramer-mediated T cell activation. The possibility of variably affecting MHC–peptide tetramer binding and T cell activation using anti-CD8 antibodies confirms the critical role exerted by the CD8 co-receptor in this interaction and supports the notion that TCR engagement by MHC–peptide ligands typically involves CD8.

Introduction

CD8 and CD4 are T cell surface glycoproteins that bind to the same MHC–peptide as the TCR, and are thus referred to as co-receptors (1). They are known to enhance T cell antigen recognition by binding to MHC class I and II directly, but the function of these molecules in TCR binding to MHC–peptide ligands remains unclear. The X-ray crystal structure of a few peptide–MHC class I complexes bound to their cognate TCR (2–4) has shown that all TCR recognize their ligands with a conserved topology, suggesting that the simultaneous binding of MHC–peptide to both TCR and CD8, leading to the apposition of CD8:Lck and the TCR–CD3:Zap-70 complex, is required for T cell activation (5). Indeed, CD8 molecules can increase the apparent affinity of MHC–peptide for the TCR (6,7).

In the past few years, the production of MHC–peptide tetramers has revolutionized the analysis of T cell responses thanks to their ability to label T lymphocytes according to their antigenic specificity (8–10). The availability of MHC–peptide multimers has encouraged studies on the function of the CD4 and CD8 co-receptors in TCR binding to MHC–peptide ligands. Surprisingly, for the CD4 co-receptor, the unanimous conclusion was reached that it is critical for signal transduction but not required for TCR binding to multimeric MHC–peptide ligands (11–13). The role of the CD8 co-receptor remains more debated: Wyer *et al*. (14) failed to detect any enhancement by CD8 of TCR binding to specific class I MHC–peptide ligands using surface plasmon resonance and several groups reported that multimer binding could

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occur in the presence of anti-CD8 antibodies (8,15–17). These findings led to the conclusion that CD8, like CD4, might participate in T cell responses only after the TCR has already stably bound and been activated by its ligand. On the contrary, other studies using MHC multimers indicated that CD8 is an active participant in the T cell recognition complex, rather than simply an accessory molecule (5,18). An interesting outcome of one of these studies (18) was the observation that different antibodies against murine CD8 exerted opposite effects on MHC–peptide multimer binding to the TCR. Enhancement of multimer staining was observed with some antibodies, while staining was blocked with others. Similar observations had been reported occasionally by other authors, again in the murine system (15,19). Although it was not clear why some anti-CD8 antibodies augmented multimer binding while others blocked it, it was hypothesized that the enhancing antibodies favor encounters between CD8 and class I, while the others block these interactions (18).

Here, we demonstrate that both enhancing and blocking effects on tetramer binding to TCR can be obtained with antibodies against human CD8, implying a variable effect of different antibodies on CD8 function. We used these antibodies to modulate CD8 function during tetramer binding to TCR to study how the co-receptor molecule contributes to the initial phase of interaction (binding), the duration of the interaction (stability) and the delivery of early signal transduction events following T cell activation.

Methods

Cells

Melan-A/MART-1-specific cytotoxic T lymphocyte (CTL) clones were derived in our laboratory from the peripheral blood mononuclear cells (PBMC) of both melanoma and vitiligo patients by limiting dilution as described (20,21). They were grown in complete RPMI 1640 supplemented with 5% human serum (EuroClone, Wetherby, UK) and 200 U/ml rIL-2, and periodically stimulated in the presence of irradiated PBMC (1×10^6/ml) and 2 μg/ml phytohemagglutinin.

Peptides and tetramers

Peptides Melan-$A_{27–35}$ (AAGIGILTV) and A27L (an analogue of the Melan-A/$MART1_{26–35}$ epitope carrying a substitution of Ala for Leu at position 2 from the N-terminus, ELAGIGILTV) (22–24) were purchased from Neosystem (Strasbourg, France). They were >90% pure as indicated by analytical HPLC. Lyophilized peptides were diluted in DMSO and stored at –20°C. A2/Melan-A tetramers built around these two peptides were produced as described (8). Briefly, recombinant A2 heavy chain and β_2-microglobulin (β_2m) were produced as inclusion bodies in *Escherichia coli* XA90F′LacQ1. After extensive washing, inclusion bodies were dissolved in 8 M urea, and human leukocyte antigen (HLA) monomeric proteins were refolded in the presence of 10–40 μg of the respective peptides by dilution in 100 mM Tris, 0.4 M Arginine, 2 mM EDTA, pH 8.0, 0.5 mM GSSG, 5 mM GSH and protease inhibitors. Monomers were concentrated, dialyzed against Tris 10 mM, pH 8.0, and biotinylated with 6 μg/ml of BirA enzyme for 4 h at 30°C. Biotinylated complexes were dialyzed and purified by ion-exchange chromatography (monoQ; Pharmacia, St Quentin-en-Yvelines, France) to remove free biotin. Tetramerization was achieved by adding phycoerythrin (PE)-conjugated streptavidin (Immunotech, Marseille, France) at a 4:1 ratio, and controlled by gel filtration on a Superdex 200 (Pharmacia). A mutated A2/Melan-A tetramer (A2m/Melan-A), containing the substitution Ala to Val at position 245 known to alter the interaction between class I and CD8 (25,26) was produced as described in (27).

Antibodies and flow cytometry

Clones were stained with PE-labeled tetramers (0.5 μg$\times10^6$ cells) for either 45 min on ice or 15 min at 37°C; after 2 washes, an indirect double staining was performed using an anti-human CD8α mAb (SK1, IgG1; Becton Dickinson, Mountain View, CA; or OKT8, IgG2a) and FITC-labeled goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) as second antibodies. In parallel, similar stainings were performed by adding the anti-human CD8 antibodies either before the tetramers or simultaneously. In some experiments, a fluorochrome-conjugated anti-human CD8α mAb (SK1-FITC, IgG1; Becton Dickinson) was used. Cells were also stained with a PE-labeled anti-human CD3 antibody (PharMingen, San Diego, CA) either in the presence or in the absence of the anti-CD8 antibodies. For the tetramer-mediated T cell activation experiments, cells were stained with FITC-labeled anti-CD69 (IgG1; Becton Dickinson) and CyChrome-labeled anti-CD25 mAb (IgG1; PharMingen) for 30 min on ice. Cells were analyzed on a FACSCalibur (Becton Dickinson), using the CellQuest software. Lymphocytes were gated by forward and side scatter profile. Dead cells were excluded by propidium iodide (PI) staining.

Tetramer binding and dissociation assays

Tetramer binding and dissociation assays were performed essentially as already described by Savage *et al*. (28), Amrani *et al*. (29) and Reichstetter *et al*. (30), with minor modifications. Briefly, for the analysis of tetramer staining at equilibrium, 10^5 cells were stained for 3 h at room temperature with subsaturating concentrations of tetramers ranging from 0.04 to 2 μg/ml, and with saturating concentrations of anti-CD8 antibodies. Stained cells were washed 3 times, resuspended in staining buffer (PBS, 1% FBS and 0.01% sodium azide) containing 1 μg/ml PI and analyzed by flow cytometry. The two-way ANOVA test was used to evaluate whether the binding differences obtained with or without anti-CD8 treatment were statistically significant. To investigate the tetramer binding kinetics, 4×10^5 cells were stained with a subsaturating concentration of tetramer (1.2 μg/ml) and saturating concentrations of the anti-CD8 antibodies for 30 min on ice. Aliquots were taken every 5 min, the cells fixed for 15 min on ice with 2% paraformaldehyde and then analyzed by flow cytometry.

For the dissociation assay 4×10^5 cells were stained with saturating amounts of both tetramers (66 μg/ml for 4×10^5 cells) and anti-CD8 antibodies for 45 min at room temperature. Cells were washed 3 times, cooled to 4°C and resuspended in 400 μl of cold staining buffer. To prevent rebinding of tetramers, cells were incubated with saturating amounts of an anti-human HLA-A2 antibody (BB7.2; IgG2b). At appropriate time points, cells were resuspended, removed and

analyzed by flow cytometry. Indirect double staining using an anti-human TCR PAN α/β (Immunotech) and FITC-labeled goat anti-mouse IgG2b (Southern Biotechnology Associates) as second antibody was performed in parallel to normalize tetramer fluorescence against the expressed TCR. Half-lives ($t_{1/2}$) were determined by calculating the (ln2)/mean slope value of plots of the corrected natural logarithm (ln) of the percentage normalized fluorescence for TCR. The slope is equivalent to $\ln(F_a/F_b)/t$, where F_a is the normalized fluorescence at the start of the interval, F_b is the normalized fluorescence at the end of the interval and t is the length of the interval (minutes).

Tetramer-mediated T cell activation

Cells (1.5×10^5) were stimulated with A2/Melan-A and A2m/Melan-A tetramers (1 μg/ml) in flat-bottomed 96-well plates, either in the absence or in the presence of saturating concentrations of anti-CD8 mAb. Cells were incubated at 37°C, 5% CO_2, and, at the indicated times, placed on ice and stained with anti-CD69 and anti-CD25 mAb.

Results and discussion

We previously established a panel of HLA-A*0201-restricted, $CD8^+$ human T cell clones directed against the Melan-A/MART1 antigen (20,21). We stained these clones with the relevant MHC–peptide multimer (a tetramer of HLA-A*0201 built around the A27L peptide, referred to as A2/Melan-A tetramer throughout the text) and two different anti-human CD8α antibodies and compared the effects of the two anti-CD8 antibodies on tetramer binding under different conditions: (i) when anti-CD8 antibodies were allowed to bind first and the cells were subsequently incubated with MHC–peptide tetramers, (ii) when antibodies and tetramers were added simultaneously or (iii) when tetramers were added before the anti-CD8 antibodies. We observed that tetramer staining was greatly decreased when the blocking anti-CD8 antibody (SK1) was added either before or during tetramer binding (Fig. 1A). In some clones there was also significant loss of tetramer when SK1 antibody was added only in the second incubation and this displacement effect could be observed kinetically, in that tetramer binding was not reduced to the same extent after shorter incubation times with the blocking anti-CD8 antibody (not shown). This blocking effect was not due to steric hindrance, as a similar CD8 mean fluorescence intensity was obtained when CTL clones were stained either with the SK1 antibody alone or following pre-staining with the specific tetramers (not shown). On the other hand, the enhancing anti-CD8 antibody (OKT8) greatly increased tetramer staining when added either before or during multimer binding, but it did not show appreciable effects on pre-bound tetramers (Fig. 1B). The enhancing effect was still observed when a tetramer containing a mutation in the α3 domain that prevents binding to CD8 (27) was used, but not with a tetramer complexed with an unrelated peptide (not shown). This demonstrates that clustering of CD8 molecules through the OKT8 antibody, eventually leading to a peptide-independent tetramer binding mediated by the CD8–HLA-A2α3 interaction, could not be responsible for the enhanced tetramer staining. When a mixture of equimolar amounts of SK1 and OKT8 anti-CD8 antibodies were used to treat the cells before staining, the blocking effect predominated and tetramer staining appeared to be decreased (Fig. 1C). The same effect was observed when SK1 antibody was added before OKT8 and vice versa, both before the tetramer staining (not shown). On the contrary, the CD3 staining profile was unaffected by exposure to anti-CD8 antibodies, indicating that modulation of the TCR (e.g. TCR internalization following tetramer-induced activation) could not be the cause of the different effects exerted by these two anti-CD8 antibodies on MHC–peptide tetramer binding (Fig. 1D). These results were highly reproducible in all the clones analyzed ($n = 17$), when fluorochrome-conjugated as well as unconjugated CD8 antibodies were used, when tetramer staining was performed at 37°C instead of 4°C, and when a different MHC–peptide multimer specific for the same clones (a tetramer of HLA-A*0201 built around the natural immunodominant Melan-A/$MART1_{27–35}$ peptide) was used (data not shown).

We used these two anti-CD8 antibodies to modulate CD8 function during tetramer staining, and analyzed how CD8 can influence tetramer binding and dissociation. CTL clones were first tested for their tetramer-binding capacity by incubation in the presence of increasing, subsaturating concentrations of MHC–peptide multimers, either alone or after the addition of anti-CD8 antibodies (Fig. 2A). There was a linear relationship between the mean staining intensity and the tetramer concentration for low tetramer concentrations. In the representative case shown in Fig. 2, the slopes of the line were 140.7 in the absence of anti-CD8 antibodies, 353.7 in the presence of the enhancing OKT8 antibody and as low as 21.3 in the presence of the blocking SK1 antibody, meaning that comparable staining intensities as obtained with tetramers alone required a concentration of <0.5 and >6 times as much tetramer in the presence of the enhancing and blocking antibodies, respectively (Fig. 2A). In other words, at the same concentration, the tetramer in the presence of the blocking anti-CD8 antibody occupied far fewer TCR than did the tetramer alone. The opposite was true in the presence of the enhancing antibody, although the overall effect appeared to be less conspicuous.

Tetramer binding kinetics analysis indicated that in the presence of the blocking antibody a staining plateau corresponding to ~25% of the original fluorescence intensity was approached at the end of the time course (Fig. 2B). The same staining level was instead reached and overcome after only 5 min by both the tetramers alone and the tetramers in the presence of the enhancing antibody (Fig. 2B). These results confirmed that in the presence of the blocking anti-CD8 antibody only a minor fraction of TCR tetramer molecules was associated at the end of the binding phase, suggesting that cumulative tetramer binding was impeded by sustained blockade of certain CD8-combining sites.

To assess the stability of the TCR–tetramer complexes in the presence of the two antibodies, the amount of tetramer dissociation from the surface of stained cells was determined (Fig. 2C). Saturating concentrations of tetramers were used and unbound reagent was washed off. Stained cells were then incubated for 5 h at 4°C, a temperature at which TCR internalization is avoided, in the presence of the two anti-CD8 antibodies. To prevent rebinding of detached tetramers during

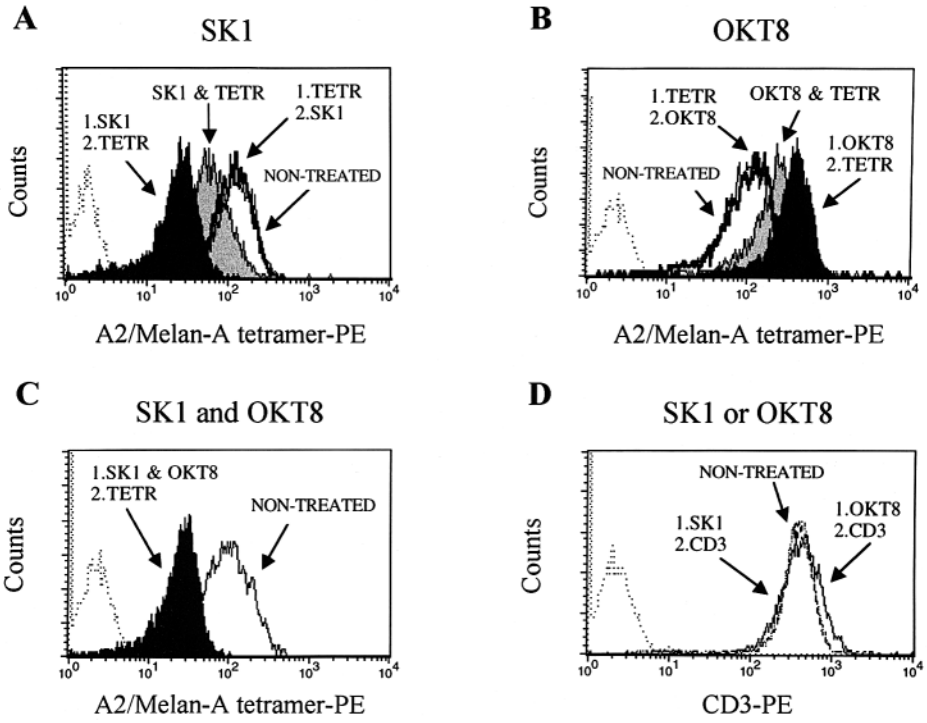

Fig. 1. Opposite effects of two anti-CD8 antibodies on MHC–peptide tetramer fluorescence intensity. T cell clones were treated with the anti-CD8 antibodies indicated on the top of each panel and stained with either A2/Melan-A tetramers (TETR) or anti-CD3 mAb. Cells were gated by forward and side scatter for lymphocytes. Negative controls are shown in each panel. (A and B) A representative experiment showing the effects of SK1 and OKT8 anti-CD8 antibodies when anti-CD8 treatment was performed before, during or after tetramer staining, as compared with untreated cells. (C) Blocking effect on tetramer staining when SK1 and OKT8 antibodies were contemporarily used to treat the cells. (D) No effects of SK1 and OKT8 antibodies on anti-CD3 staining.

the assay, we incubated the samples with saturating amounts of the BB7.2 mAb, which binds HLA-A2 independently of the complexed peptide. Samples were taken at increasing time intervals, fixed and analyzed by flow cytometry for the intensity of tetramer staining. In the representative case shown, we found that the tetramer label decayed with $t_{1/2} \sim 3.7$ h, but it decayed at a much slower rate, with $t_{1/2} = 13.6$ h, in the presence of the enhancing OKT8 antibody. This raises the possibility that binding by the OKT8 antibody lengthens the half-life of the MHC–peptide–TCR (–CD8) interaction by increasing its relative off-rate. A $t_{1/2} = 1.5$ h was obtained in the presence of the blocking SK1 antibody (Fig. 2C).

Together, these data confirm the model of a dynamic nature of tetramer binding to the TCR and support the notion that TCR binding by multimeric MHC–peptide complexes typically involves CD8. They also indicate that the blocking effect of anti-CD8 antibodies is mostly exerted during the initial association of the TCR with MHC–peptide tetramer, while the enhancing effect is exerted by augmenting the stability of this interaction.

We next investigated the effects of the two anti-CD8 antibodies on TCR stimulation by MHC–peptide tetramers. We treated the cells with an anti-CD8 antibody, either SK1 or OKT8, and then assessed the response to A2/Melan-A tetramers. As activation parameters, we studied the up-regulation of the early T cell activation marker CD69 (31) and of the intermediate/late activation marker CD25 (32). Our results indicated that activation was virtually unaffected by incubation with the OKT8 antibody, whereas it was highly blocked using the SK1 antibody (Fig. 3). In similar experiments, pretreatment with the SK1 antibody had no effect on the response to anti-CD3, suggesting that the blocking effect was specifically at the level of tetramer binding (not shown). The blocking effect was even more pronounced when we stimulated the cells with a modified tetramer (A2m/Melan-A) (27) containing a mutation known to alter the interaction between class I and CD8 (25,26) (Fig. 3). In this case, activation turned out to be almost completely blocked. These findings confirmed the important contribution of CD8–MHC interaction (CD8-mediated adhesion) in the early phases of tetramer-mediated T cell activation.

In conclusion, the different effects of antibodies against human CD8 on MHC–peptide tetramer binding and activation described in this work confirm the critical role exerted by the CD8 co-receptor in TCR binding by multimeric MHC–peptide complexes and support the notion that TCR engagement by MHC–peptide ligands typically involves CD8. Our data are in agreement with the model of a dynamic nature of tetramer binding to the TCR, as is demonstrated by the possibility of interfering with binding when antibodies were added either before or after the tetramers. The rationale of tetramer construction is that increased avidity compensates for the very low affinity of TCR for MHC–peptide ligands (8). Even in these conditions, however, the CD8 contribution to binding appears

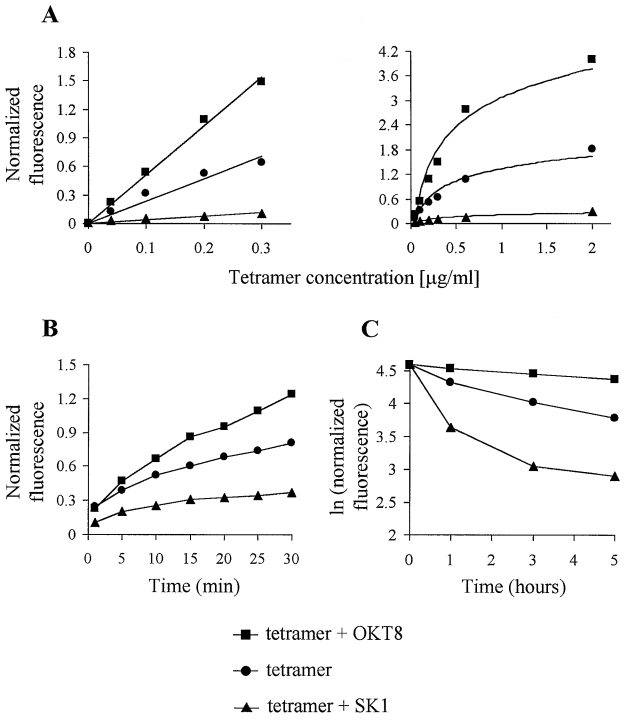

Fig. 2. Effects of anti-CD8 antibodies on tetramer binding and dissociation. The experiments were performed on different Melan-A/MART-1-specific CTL clones; one representative case is shown. The tetramer mean fluorescence intensities were always normalized for the TCR fluorescence. (A) Tetramer staining at equilibrium. Increasing, subsaturating concentrations of tetramer and saturating concentrations of the two anti-CD8 mAb were used. For low tetramer concentrations there was a linear relationship between the normalized fluorescence and the tetramer concentration. At higher concentrations of tetramers the relationship between the two parameters became logarithmic. The fluorescence difference among the three conditions (tetramer alone, tetramer plus SK1 and tetramer plus OKT8) as evaluated with the two-way ANOVA test was statistically significant ($P = 0.0082$). (B) Binding kinetics using a subsaturating concentration of tetramers and saturating concentrations of anti-CD8 mAb. The time interval ranged between 1 and 30 min. (C) Tetramer staining decay kinetics. The decay plot of the natural logarithm of the normalized fluorescence versus time is shown.

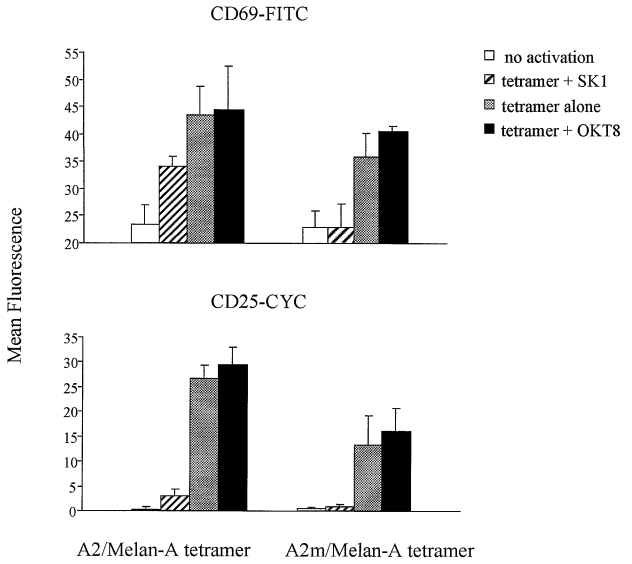

Fig. 3. Effects of two anti-CD8 antibodies on TCR activation by normal and modified MHC–peptide tetramers. T cell clones were activated by either a normal A2/Melan-A tetramer or a modified tetramer carrying a mutation in the A2 heavy chain known to alter CD8–MHC interaction (A2m/Melan-A tetramer). Flow cytometry analysis of CD69 (top panel) and CD25 (bottom panel) up-regulation at 6 and 24 h respectively is shown. The figure shows a comparison between activation obtained either in the absence or in the presence of saturating concentrations of the two anti-CD8 antibodies SK1 and OKT8. Non-activated cells were used as control. Bars represent the mean fluorescence ± SD. Data are representative of the different experiments performed.

to be essential. We could speculate that the role of CD8 might be even more important during physiologic interactions between a TCR and a monomeric MHC molecule loaded with the cognate peptide, as recently suggested (5). Human CD8 function appears to be very sensitive to modulation by anti-CD8 antibodies, and this results in profound alteration of TCR engagement and T cell effector function. A similar effect was observed using the soluble CD8αα receptor (33). It would be interesting to exploit this feature to design molecular therapeutics for prevention of graft rejection/treatment of autoimmune diseases, on the one hand, and for use as immunotherapy adjuvants, on the other.

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Abbreviations

β_2m	β_2-microglobulin
CTL	cytotoxic T lymphocyte
HLA	human leukocyte antigen
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PI	propidium iodide
TCR	T cell receptor

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