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Anti-CD8 Antibodies Can Inhibit or Enhance Peptide-MHC Class I (pMHCI) Multimer Binding: This Is Paralleled by Their Effects on CTL Activation and Occurs in the Absence of an Interaction between pMHCI and CD8 on the Cell Surface¹

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Cytotoxic T lymphocytes recognize short peptides presented in association with MHC class I (MHCI) molecules on the surface of target cells. The Ag specificity of T lymphocytes is conferred by the TCR, but invariable regions of the peptide-MHCI (pMHCI) molecule also interact with the cell surface glycoprotein CD8. The distinct binding sites for CD8 and the TCR allow pMHCI to be bound simultaneously by both molecules. Even before it was established that the TCR recognized pMHCI, it was shown that CTL exhibit clonal heterogeneity in their ability to activate in the presence of anti-CD8 Abs. These Ab-based studies have since been interpreted in the context of the interaction between pMHCI and CD8 and have recently been extended to show that anti-CD8 Ab can affect the cell surface binding of multimerized pMHCI Ags. In this study, we examine the role of CD8 further using point-mutated pMHCI Ag and show that anti-CD8 Abs can either enhance or inhibit the activation of CTL and the stable cell surface binding of multimerized pMHCI, regardless of whether there is a pMHCI/CD8 interaction. We further demonstrate that multimerized pMHCI Ag can recruit CD8 in the absence of a pMHCI/CD8 interaction and that anti-CD8 Abs can generate an intracellular activation signal resulting in CTL effector function. These results question many previous assumptions as to how anti-CD8 Abs must function and indicate that CD8 has multiple roles in CTL activation that are not necessarily dependent on an interaction with pMHCI. *The Journal of Immunology*, 2003, 171: 6650–6660.

T lymphocytes recognize short peptides bound to MHC molecules on the surface of target cells. The Ag specificity of T lymphocytes is conferred by the TCR, whose highly variable complementarity-determining regions interact with the peptide-binding platform of the MHC molecule (1, 2). Peptide-MHC (pMHC)³ also interacts with the cell surface glycoproteins CD8 and CD4, which bind to invariable regions of the MHC class I and II molecules, respectively (3–7). The binding sites for CD8 and CD4 are separate from the TCR-recognized (2), peptide-binding domains of MHC molecules and allow a single MHC molecule to be bound simultaneously by both TCR and CD8 or CD4 (4, 7).

Numerous recent studies have used multimeric pMHC ligands, often in tetrameric avidin-biotin form, to address whether the pMHC/coreceptor interaction assists in the formation of the TCR/pMHC interaction (8–13). All reports examining peptide-MHC

class II (pMHCI) show that CD4 does not aid the stabilization of the TCR/pMHCI interaction (9, 12, 13). Other evidence also indicates that the pMHCI/CD4 interaction does not significantly aid the TCR/pMHCI interaction and that the two are independent. First, recent surface plasmon resonance (SPR) data show that TCR binding to a pMHCI ligand is kinetically distinct from and independent of CD4 (14). Second, studies with altered peptide ligands suggest that the CD4 coreceptor binds sequentially to pMHCI provided the initial TCR/pMHCI interaction is of sufficient duration (15). Third, studies in which CD4 and mutant CD4 without a capacity for cytoplasmic signaling were expressed in T cell hybridomas lacking endogenous CD4 have concluded that CD4 has a very minor role as an adhesion molecule in T cell activation (16).

In contrast to studies with pMHCI and CD4, studies examining the role of CD8 in the binding of peptide-MHC class I (pMHCI) multimers have reached different conclusions (8, 10, 11). Daniels and Jameson (10) used anti-CD8 Abs and pMHCI tetramers to show that CD8 plays a critical, and in some cases obligatory, role in Ag-specific binding of murine TCRs. These results were interpreted in the context of the pMHCI/CD8 interaction. Anti-CD8 Abs have similarly been found to block the binding of human pMHCI tetramers to cell surface TCRs (11), and the results were taken as evidence that CD8 is recruited and participates directly in TCR/pMHCI interactions before the pMHCI complex has stably bound to the TCR (11). A further recent study examining the effects of anti-CD8 Abs on the binding of human pMHCI tetramers also concluded that the CD8 interaction with pMHCI is a crucial event in TCR/pMHCI binding (8). However, current SPR data indicate that the human (17) and murine (18) TCR/pMHCI interaction is independent of the pMHCI/CD8 interaction and do not support the notion of cooperative binding.

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³ Abbreviations used in this paper: pMHC, peptide-MHC; MIP, macrophage-inflammatory protein; SPR, surface plasmon resonance.

We have shown that human pMHCI multimers with point mutations that reduce or abrogate the pMHCI/CD8 interaction can stably bind to cell surface TCR, thus questioning an absolute requirement for this interaction in the binding of these reagents (19). We further used immunodominant human, antiviral CTL, and mutations in pMHCI that significantly reduce the pMHCI/CD8 interaction to demonstrate that CD8 maintains the majority of its biological function even when the affinity of the pMHCI/CD8 interaction is reduced by >15-fold (20). These data indicate that the energy provided by CD8 binding to pMHCI does not play a dominant role in the activation of such CTL. These data are consistent with studies using truncated murine CD8 molecules, without a cytoplasmic tail, that have concluded that any role of the extracellular domain of CD8 in stabilizing the TCR/pMHCI interaction is minor in comparison with the role of this coreceptor in signal transduction (21).

In this study, we use mutated pMHCI with an abrogated pMHCI/CD8 interaction to test previous assumptions that anti-CD8 Abs block the binding of cell surface TCR to pMHCI multimers by blocking this interaction. In agreement with studies examining the role of the pMHCI/CD4 interaction in the binding of pMHCI multimers to cell surface TCR, we show that both the human and murine pMHCI/CD8 interactions are not critical for the stable binding of pMHCI multimers to cell surface TCR. We further demonstrate that the blockade of pMHCI multimer binding observed with some anti-CD8 Abs does not result from interference with the pMHCI/CD8 interaction. Similarly, disruption of the pMHCI/CD8 interaction is not responsible for the blockade of T cell activation by CD8 coreceptor-specific Abs.

Materials and Methods

Cell lines and clones

The HLA-A2-restricted clone 003 specific for the HIV-1 p17–8 Gag-derived epitope SLYNTVATL (residues 77–85) has been described previously (22). The D1 clone specific for the human T cell leukemia virus-1 Tax epitope LLFGYPVYV (residues 11–19) was similarly generated by limiting dilution culture of a peptide-specific T cell line from an infected individual. The HLA-A2-restricted CTL clones 3G10 and 3F7 (tyrosinase-specific; epitope YMDGTMSQV), 2D10 (melan-A-specific; epitope ELA-GIGILT), 3F6 (Her-2/neu-specific; epitope KIFGSLAFL), and 2G7 (EBV-specific; epitope GLCTLVAML) were generated by tetramer staining and FACS cloning and maintained as described previously (23, 24). Briefly, cells were stained with PE-labeled tetramer before addition of Tricolor anti-CD8 (Caltag Laboratories, Burlingame, CA), followed by extensive washes and analysis on a FACSVantage (BD Biosciences, Mountain View, CA). Single cells were sorted directly into U-bottom 96-well plates, previously coated with anti-CD3 and anti-CD28, both at 100 ng/ml in PBS, containing 10^5 irradiated B-lymphoblastoid cells in CTL medium (Iscove's medium + 5% human serum + 100 U/ml IL-2). Cloning plates were incubated at 37°C in 5% CO₂ for 10–14 days without any manipulation. Proliferating clones were then expanded in CTL medium, followed by restimulation using 5 µg/ml PHA with irradiated allogeneic PBL and B-lymphoblastoid cells as feeders (5×10^6 PBL and 5×10^5 B cells in 2 ml for a 24-well tissue culture plate). The CIR cells expressing full-length HLA-A2 and HLA-A2 (D227K, T228A) are described elsewhere (19). Murine Melan-A_{26–35} CTL lines were produced as previously described (25).

pMHCI multimers

pMHCI multimers of HLA-A2, HLA-A2 A245V, and HLA-A2 D227K/T228A were made, as previously described (19, 26). pMHCI was multimerized either by the addition of PE-conjugated extravidin (Sigma-Aldrich, St. Louis, MO) or Cy3-conjugated streptavidin (Caltag Laboratories) at a pMHCI:streptavidin molar ratio of 4:1. Concentration of pMHCI multimer, as expressed throughout this work, refers only to the pMHCI component. A recent study has determined that avidin-biotin-based tetrameric molecules can exhibit some heterogeneity (27). To reflect this, we have used the term multimer rather than tetramer when referring to these molecules. All human pMHCI multimers used in this study were made fresh for the week of use from pMHCI monomers stored at –80°C to avoid previously re-

ported (20) effects due to differences in protein stability. A2K^b tetramers are described elsewhere (25).

Stimulation of CTL for subsequent immunoblotting

CTL were washed twice in RPMI 1640 supplemented with antibiotics and glutamine and incubated overnight in RPMI 1640 with 10% FCS. The following day, FCS was removed with two washes of RPMI 1640, and 5×10^5 CTL were resuspended in 5 µl of RPMI 1640. After 10 min at 37°C in 5% CO₂, CTL were stimulated by incubation with 10 µg/ml anti-CD3, or anti-CD8 Ab at specified concentrations, for 1 min. The reaction was stopped by washing once with 0.5 ml ice-cold PBS, and resuspending the pellet in cold lysis buffer (140 mM NaCl, 20 mM Tris, pH 8.0, 10 mM NaF, 2 mM EDTA, 20% glycerol, 1% IGEPAL, 1 mM Na₃VO₄, 10 µg/ml aprotinin, 10 µg/ml leupeptin) at 5×10^7 cells/ml.

Antiphosphotyrosine immunoblots

Cells were lysed on ice for 30 min, then the nuclear fraction was pelleted by centrifugation at $16,000 \times g$ for 15 min. The remaining lysate was aspirated and added to an equal volume of SDS loading buffer (125 mM Tris, pH 6.8, 140 mM SDS, 20% glycerol, 200 µM DTT, 300 µM bromophenol blue). The sample was boiled for 6 min with agitation and then loaded into a 12% SDS-PAGE protein gel for electrophoresis at 100 V for 16 h. The gel, filter papers (Bio-Rad, Hercules, CA), and nitrocellulose (Amersham, Arlington Heights, IL) of matching size were equilibrated in ice-cold transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol) for 10 min. Protein was transferred from the gel by electrophoresis at 25 V for 50 min. The gel was stained with Coomassie blue, and the membrane was stained with Ponceau S, to verify transfer and equal protein loading. Ponceau S was washed off with distilled water and then wash buffer (PBS, 0.05% Tween). The membrane was blocked for 1 h with wash buffer containing 1% BSA, washed, and incubated for 4 h with mouse anti-phosphotyrosine Ab clone 4G10 (Upstate Biotechnology, Lake Placid, NY; 1 µg/ml in wash buffer, 0.1% BSA). The membrane was washed again with three changes of wash buffer for 10 min each, and incubated with sheep anti-mouse peroxidase-linked secondary Ab (Amersham; 0.25 µg/ml in wash buffer, 2.5% milk powder) for 1.5 h. After three further washes, the blot was developed using chemiluminescent substrate Supersignal Pico (Pierce, Rockford, IL). All washes and incubations with Ab were performed at 4°C. At least 48 h after development, blots were reprobed for total cellular ZAP 70 to control for protein loading. To probe, the membrane was rehydrated in wash buffer, then incubated with rabbit anti-human ZAP 70 primary Ab (Autogen Bioclear, Wiltshire, U.K.; 0.2 µg/ml in wash buffer, 2.5% milk powder) for 4 h. The blot was washed three times and incubated with peroxidase-linked anti-rabbit secondary Ab (Autogen Bioclear; 0.4 µg/ml in wash buffer, 2.5% milk powder) for 1.5 h, then washed again and developed, as above.

ELISA for soluble cytokines

CTL were washed twice in RPMI 1640 and brought to a concentration of 5×10^5 cells/ml in RPMI 1640 medium supplemented with 2% FCS. A total of 5×10^5 CTLs were incubated with 100 µg/ml CD3 or CD8 Ab in 100 µl final volume for specified time at 37°C, 5% CO₂ in 96-well U-bottom plates. Supernatant was harvested with care not to disturb cells, and assayed for macrophage-inflammatory protein (MIP)-1β, RANTES, and IFN-γ by ELISA (R&D Systems, Minneapolis, MN). SD from the mean of two duplicate assays is shown.

Antibodies

The following human anti-CD8 Abs were used in this study: MCD8 unconjugated (IQP, Groningen, The Netherlands), 3B5-Tricolor (Caltag Laboratories), SK1-PerCP (BD Biosciences), OKT-8 unconjugated (kindly provided by Avidex (Abingdon, U.K.)), and DK-25-FITC or DK-25 unconjugated (DAKO, Carpinteria, CA). All anti-CD8 Abs have been shown to reduce wild-type pMHCI multimer binding of CTL clones to a varying degree (8, 11), except for OKT-8, which has been shown to enhance wild-type pMHCI multimer staining (8). Both conjugated and unconjugated forms were used to rule out any fluorochrome effect. Anti-murine CD8 Abs CT-CD8a and CT-CD8b (Caltag Laboratories) were used with murine CTL.

Flow cytometry

For FACS analysis, 1×10^5 CTLs were resuspended in 20 µl PBS and preincubated with or without anti-CD8 Abs at a 1/5 dilution (i.e., 20 µg/ml MCD8, 40 µg/ml 3B5, 1.25 µg/ml SK1, 10 µg/ml OKT-8, or 10 µg/ml DK-25) on ice for 20 min to reproduce the conditions of previous studies (8, 11). Following preincubation, either A2 wild-type, A2 245V, or A2

227/8KA pMHC I multimers folded around peptides specific for each CTL clone were added at a final concentration of 50 $\mu\text{g/ml}$ (pMHC I content) and incubated for a further 45 min on ice. Samples were washed twice in PBS, then resuspended in PBS, and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software. In a separate set of experiments, 10^5 CTL resuspended in 20 μl PBS and preincubated with or without anti-CD8 Abs, as described above, were subsequently stained with a pan- $\alpha\beta$ TCR-FITC-conjugated Ab at 20 $\mu\text{g/ml}$ (clone BMA 031; Serotec, Oxford, U.K.), then washed twice in PBS, and resuspended in PBS before analysis, as above.

Confocal microscopy

For each condition to be analyzed, 2.5×10^5 003 CTLs were resuspended in 20 μl FACS buffer (0.5% BSA in PBS) with or without HLA-A2-SLYNTVATL or D227K/T228A HLA-A2-SLYNTVATL Cy3 multimers at 50 $\mu\text{g/ml}$. Samples were then incubated either on ice or at 37°C. After a 1-, 2-, 5-, or 10-min incubation, samples were washed once in ice-cold azide buffer (0.5% BSA, 0.1% azide in PBS) and resuspended in a 20 μl vol of ice-cold azide buffer. Each sample was then stained with anti-CD8 FITC at 5 $\mu\text{g/ml}$ (clone DK-25; BD Biosciences) for 20 min on ice. Cells were washed twice in azide buffer, then fixed in 50 μl 3% paraformaldehyde at 4°C overnight. Unstained samples and samples stained with anti-CD8 only were also included. Cells were resuspended in 2% FCS at 0.5×10^6 CTL/ml. A total of 100 μl of each sample (i.e., 50,000 CTL) was loaded into a cytofunnel and spun at 550 rpm for 5 min onto superfrost slides (BDH, Poole, U.K.) using a cytospin (Shandon, Pittsburgh, PA). Slides were allowed to air dry for 5–10 min. Each sample was then covered with 10 μl of Vectorshield (Vector, Peterborough, U.K.) containing Toto-3 (Molecular Probes, Eugene, OR) at 1/1000 dilution and 4',6'-diamidino-2-phenylindole (Molecular Probes) at 1/5000 dilution (i.e., 1 $\mu\text{g/ml}$), then further covered with a large coverslip while making sure no air bubbles were present. Samples were then examined using a Bio-Rad 2000 confocal microscope and analyzed using Lasersnap software.

Results

Some anti-CD8 Abs block the binding of multimeric human pMHC I to cell surface TCR

Two recent reports have shown that some anti-CD8 Abs inhibit the binding of human pMHC I multimeric complexes (8, 11). Both reports show data from a single HLA-A2-restricted, melanoma-specific CTL clone. We confirmed and extended these observations by examining the staining of six different CTL clones that recognize a total of five different Ags (Fig. 1). The anti-CD8 Abs 3B5, SK1, and DK-25 all inhibited tetramer staining (Fig. 1). The DK-25 Ab appeared to be most potent in this effect at the concentrations used. Curiously, DK-25 Ab only partially blocked the binding of the YMDGTMSQV-HLA-A2 multimer to the surface of clone 3F7 (Fig. 1). Multimer staining of clone 3G10, which recognizes the same Ag, appeared to be most sensitive to anti-CD8 Abs. This indicates that CTL with the same Ag specificity can vary in their sensitivity to anti-CD8 Ab-mediated blockade of pMHC I multimer binding to cell surface TCRs.

Anti-CD8 Abs do not block the binding of human pMHC I multimers by blocking the interaction between pMHC I and CD8

Previous studies have used anti-CD8 Abs and pMHC I multimers to show that the pMHC I/CD8 interaction plays a critical, and in some cases obligatory, role in Ag-specific binding to cell surface TCRs (8, 10, 11). We have previously used point mutations in the HLA H chain (19) or human β_2 -microglobulin (28) to abrogate or reduce the pMHC I/CD8 interaction. SPR confirms the effect of these mutations on the pMHC I/CD8 interaction and shows they do not affect the TCR/pMHC I interaction (19, 28). We have used multimeric forms of these pMHC I ligands to show that human CD8 plays a minimal role in the binding of these ligands to the cell surface TCR on immunodominant antiviral CTL (19). Our findings contrast with recent Ab-derived conclusions, which suggest that the human pMHC I/CD8 interaction is critical for the binding of

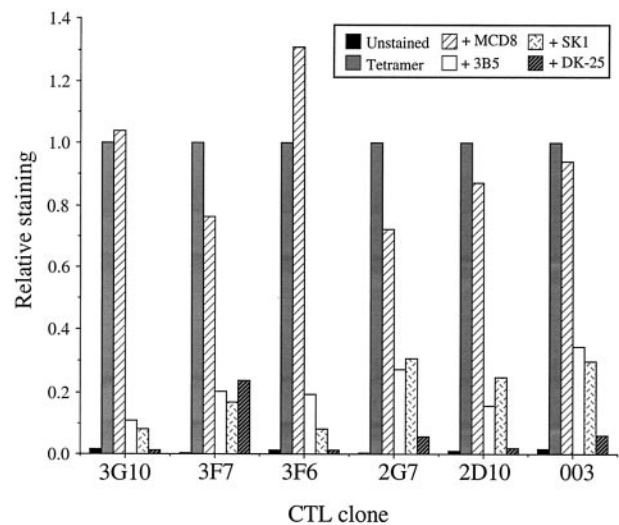


FIGURE 1. Anti-CD8 Abs can inhibit the binding of pMHC I multimers. PE-conjugated pMHC I multimers were used to stain six different CTL clones. A total of 1×10^5 CTLs were preincubated on ice in 20 μl of PBS \pm anti-CD8 Abs for 20 min before 45-min staining with 1 μg (with respect to pMHC I) of HLA-A2 multimer presenting the specific peptide recognized by the individual clones. Abs were used at a 1/5 dilution, as described previously (11), resulting in final concentrations of 20 $\mu\text{g/ml}$ MCD8, 40 $\mu\text{g/ml}$ 3B5, 10 $\mu\text{g/ml}$ DK-25, and 1.25 $\mu\text{g/ml}$ SK1. The CTL clones and their Ags are described in *Materials and Methods*. Tetramer staining of each clone varied from a mean fluorescence intensity of 267 (3G10) to 1778 (2G7). Results are plotted as staining relative to that without anti-CD8 Ab to allow a direct comparison between the clones. MCD8 Ab appeared to have little effect on the binding of tetramers and actually enhanced the staining of the 3F6 clone. Three other anti-CD8 Abs (3B5, SK1, and DK-25) inhibited the staining with all tetramers.

multimeric pMHC I (8, 11). We reasoned that anti-CD8 Abs might have pleiotropic effects on CTL.

Two recent studies have shown that anti-CD8 Abs can block the binding of melanoma-specific HLA-A2 pMHC I multimers to cell surface TCR (8, 11). Denzberg et al. (11) showed that preincubation of CTL with the anti-CD8 Ab DK-25 caused a total blockade of pMHC I multimer binding. We confirmed this observation with the HLA-A2-restricted, melanoma-specific CTL clone 3G10 (Figs. 1 and 2A).

The MHC class I molecule HLA-A*6801 (HLA-A68) differs from HLA-A*0201 due to a mutation in its $\alpha 3$ domain at position 245 (A245V). Previous studies have shown that HLA-A68 does not bind to CD8 (29) and that HLA-A68-restricted CTL are independent of the pMHC I/CD8 interaction (30). Molecular modeling based on the structures of HLA-A2, HLA-A68, and the HLA-A2/CD8 cocrystal predicts that the larger valine residue at position 245 in HLA-A68 distorts the $\alpha 3$ loop of the molecule, resulting in a less energetically favorable interaction with CD8 (4). A245V HLA-A2 pMHC I multimers have previously been shown to bind to cell surface TCR (31), even though they have a significantly reduced ability to bind to CD8 (28, 32). We used pMHC I multimers of A245V HLA-A2 to examine the role of the pMHC I/CD8 interaction in the stable binding of these reagents to cell surface TCRs. Both HLA-A2 and A245V HLA-A2 multimers bound stably to the surface of 3G10 CTL (Fig. 2). We further showed that the binding of A245V HLA-A2 mutant multimer could be completely blocked by DK-25 anti-CD8 Ab (Fig. 2B). The multimer blocking effect of DK-25 Ab titrated with Ab concentration (Fig. 2C). These results show that even pMHC I multimers with an extremely weak pMHC I/CD8 interaction can bind to cell surface

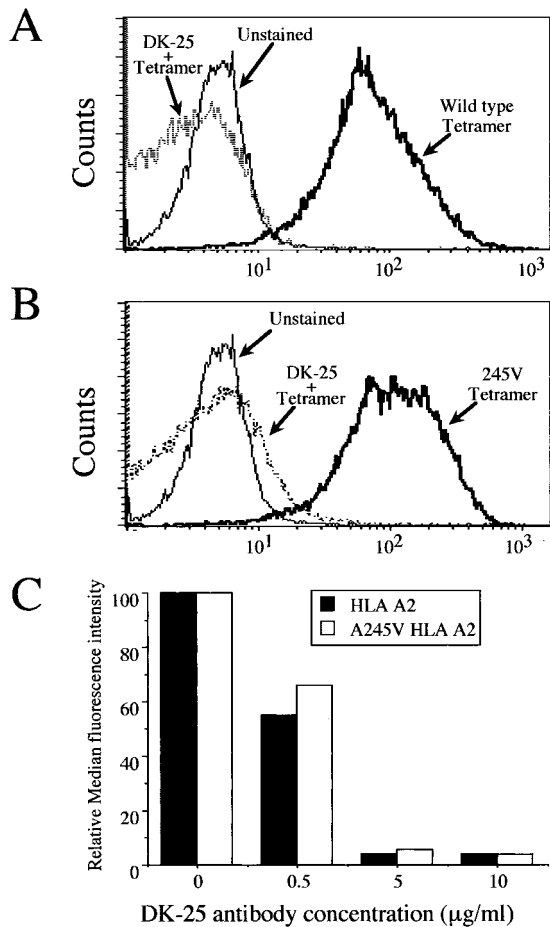


FIGURE 2. The effect of anti-CD8 Ab on pMHC I multimer staining of HLA-A*0201-restricted, YMDGTMSQV-specific CTL clone 3G10. Staining with 1 µg (with respect to pMHC I) of wild-type multimer (A) and A245V mutant multimer (B). The A245V substitution reduces the binding of CD8 to HLA-A2 by ~4-fold (32). Multimeric forms of YMDGTMSQV-HLA-A2 and YMDGTMSQV-HLA-A2 A245V bound to cell surface TCRs on clone 3G10 equally well. CTL were stained with multimer on ice for 45 min after a 20-min preincubation in 20 µl of PBS ± 10 µg/ml DK-25-FITC-conjugated anti-CD8 Ab (DAKO). C, 10⁵ 3G10 CTL were preincubated on ice in 20 µl PBS together with the indicated concentrations of DK-25 anti-CD8 Ab for 20 min before staining with 1 µg of wild-type or A245V multimer on ice for 45 min. Unstained 3G10 cells or those stained with a SLYNTVATL-HLA-A2 multimer were used as negative controls in all FACS stainings. Results are representative of at least two experiments. Data are shown as relative staining for comparison; the median fluorescence intensity of staining with wild-type and A245V tetramers without anti-CD8 Ab was 69.3 and 98.7, respectively (taken from stainings shown in A and B).

TCRs. They also show that anti-CD8 Abs that block pMHC I multimer binding to cell surface TCR may function independently of any interaction between pMHC I and CD8.

To confirm the above observations, we looked at CTL with differing specificity and used other HLA-A2 H chain mutations. We confirmed that anti-CD8 Abs can block the binding of pMHC I multimers to cell surface TCRs in a further melanoma-specific CTL and in a HIV-1, p17 Gag-specific CTL (Fig. 3). We also found that D227K/T228A-substituted HLA-A2 multimers bind to cell surface TCRs (Fig. 3). We have previously shown that the D227K/T228A substitution completely abrogates the pMHC I/CD8 interaction without affecting the TCR/pMHC I interaction (19). Anti-CD8 Abs that block the binding of pMHC I multimers also blocked the binding of our CD8 null (D227K/T228A) multimers.

It is thus clear that these blocking Abs do not function by blocking the pMHC I/CD8 interaction. Pretreatment with anti-CD8 Ab did not alter the level of the TCR on the CTL surface (Fig. 3D). Curiously, anti-CD8 Abs appeared to be better at blocking the binding of pMHC I multimers that enabled CD8 binding than at blocking the binding of CD8 null reagents (Fig. 3C).

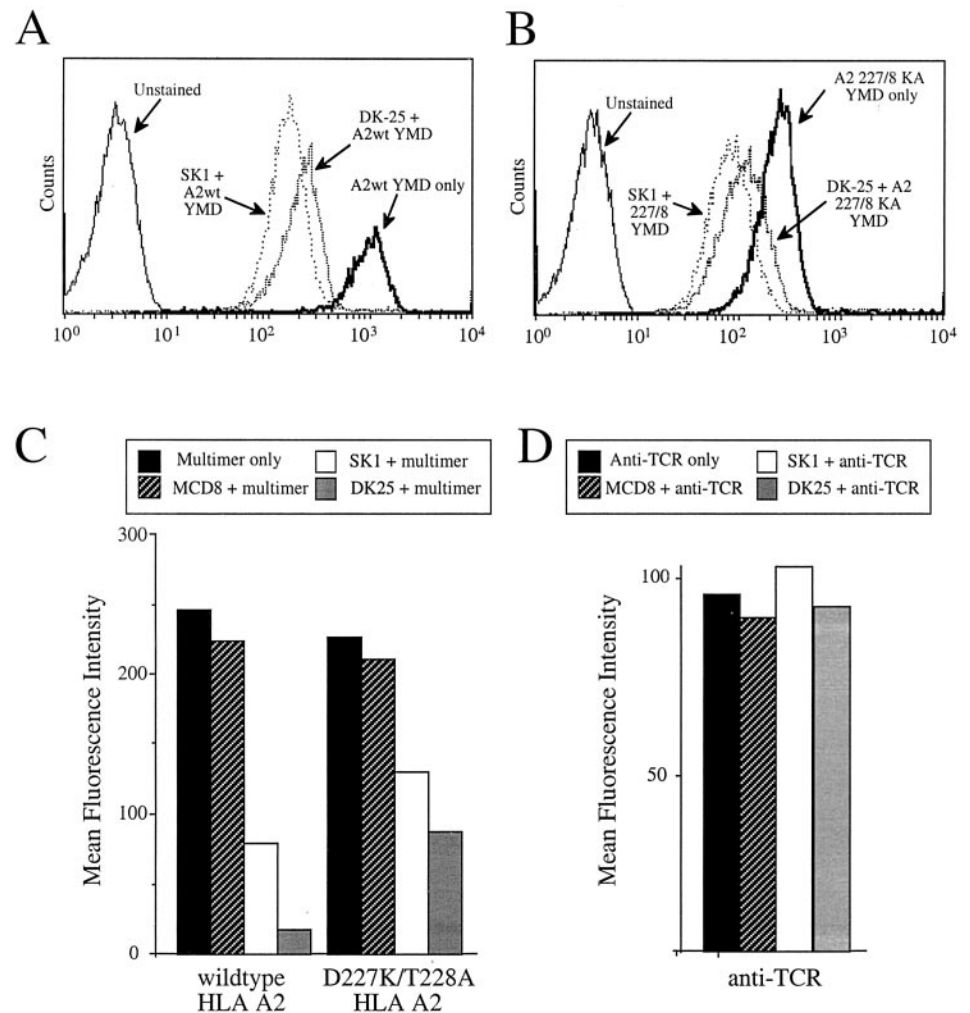
Anti-CD8 Abs do not block the binding of murine pMHC I multimers by blocking the interaction between pMHC I and CD8

There are significant differences in the human and murine pMHC I/CD8 interaction (19, 33, 34). Our results show that the murine pMHC I/CD8 interaction is four times stronger than the equivalent human interaction (19). Consequently, it is likely that the murine pMHC I/CD8 interaction might have a greater role in the stable binding of murine pMHC I multimers to cell surface TCR. We examined the role of the murine pMHC I/CD8 interaction in the binding of pMHC I tetramers using A2.1/K^b transgenic mice (35). We have previously determined that HLA-A2 does not bind to murine CD8 (19). Comparison of pMHC I multimer staining with HLA-A2 and chimeric A2K^b reagents, manufactured to have a murine α3 domain, shows that the latter reagents are able to stain a larger population of CTL in mice vaccinated using a DNA-prime/recombinant vaccinia virus-boost strategy (25). This difference is presumably due to the fact that the A2K^b reagents allow engagement with murine CD8 (25). Despite this advantage, it was observed that >10% of the total lymphocytes in transgenic mice expressing an HLA A2/Db fusion molecule immunized with DNA and vaccinia encoding the Melan A_{26–35} epitope could be stained with HLA-A2 pMHC I multimer containing this peptide (25, 36). Almost twice this number could be stained with A2K^b multimer, demonstrating the enhanced sensitivity of reagents with a murine α3 domain. As HLA-A2 cannot bind to murine CD8 (19), this result clearly demonstrates that, even in the murine system, the pMHC I/CD8 interaction need not be critical for the stable binding of multimerized pMHC I. We further used this system to examine the blocking of multimer binding by anti-CD8 Abs. In agreement with a previous study (10), we observed that an anti-CD8 Ab (CT-CD8a) could block the binding of a multimer with a murine α3 domain (Fig. 4). This Ab also partially blocked HLA-A2 pMHC I multimer binding, which cannot bind murine CD8 (19) (Fig. 4, rows 2 and 4). However, as in the human system, this effect was far less pronounced than the blockade of wild-type multimers that enable CD8 binding (Fig. 4, rows 1 and 3).

Anti-CD8 Abs can enhance the binding of pMHC I and CTL activation

It has previously been shown that some anti-CD8 Abs can enhance the binding of murine pMHC I multimers to the cell surface of some CTL (10). Campenelli et al. (8) have extended this observation to the human system by showing that the anti-CD8 Ab OKT8 (37) can slightly enhance the binding of pMHC I multimer to the surface of HLA-A2-restricted, melanoma-specific CTL. We observed a similar increase with one of our CTL clones, 3G10, and anti-CD8 Ab OKT8 (Fig. 5A). Interestingly, OKT8 Ab was also able to increase activation in this clone (Fig. 5B). OKT8 Ab did not increase the activation, or tetramer binding to the cell surface, of other CTL clones tested (data not shown). We also examined this phenomenon in the A2.1/K^b transgenic mouse. Anti-CD8 Abs CT-CD8a and CT-CD8b had opposing effects on the pMHC I multimer staining of murine CTL line m33 that recognizes the NY-ESO-1_{157–165} epitope. The CT-CD8b Ab enhanced pMHC I multimer staining, while the CT-CD8a Ab inhibited staining (Fig. 5C, top panel). This CTL line did not stain with the HLA-A2 CD8 null reagent (Fig. 5C, bottom panel). The anti-CD8 Ab CT-CD8b was

FIGURE 3. Anti-CD8 Ab inhibits the binding of human CD8 null tetramers. Staining of YMDGTMSQV-specific CTL clone 3F7 with wild-type (A) and D227K/T228A (B) pMHC multimers after prestaining with or without indicated anti-CD8 Abs, as described in Figs. 1 and 2. SPR shows that D227K/T228A substitution of HLA-A2 completely abrogates the pMHC/CD8 interaction without affecting the interaction with two HLA-A2-restricted TCRs (19). The 3F7 CTL stained with both wild-type and CD8 null tetramer. Preincubation with anti-CD8 Abs SK1 and DK-25 partially inhibited the binding of both tetramers to cell surface TCR on this clone. The 3B5 Ab behaved like DK-25 on this clone, and the MCD8 Ab did not reduce tetramer binding (data not shown). C, Comparison of the mean fluorescence intensity of staining of 003 CTL with wild-type and D227K/T228A HLA-A2-SLYNTVATL pMHC multimers with and without preincubation with indicated anti-CD8 Abs, as described in Figs. 1 and 2. Both pMHC multimers stain with almost equal intensity. Preincubation with SK-1 and DK-25 anti-CD8 Abs inhibits the binding of both pMHC multimers, although, curiously, the binding of the CD8 null reagent appears to be less affected. D, As in C, except anti-human $\alpha\beta$ TCR-FITC (clone BMA 031; Serotec) was used to stain cell surface TCR instead of pMHC multimers. Preincubation with anti-CD8 Ab does not significantly alter cell surface expression of TCR.



able to increase staining of this line with the HLA-A2 pMHC multimer (Fig. 5C, bottom panel). This reagent fails to bind murine CD8 (19). As in the human system, Abs that inhibited pMHC multimer binding also inhibited the recognition of Ag-bearing target cells (Fig. 5D). Anti-CD8 Ab CT-CD8b was observed to increase both pMHC multimer binding (Fig. 5C) and CTL activation (Fig. 5D).

Anti-CD8 Abs that block CTL activation do not function by blocking the pMHC/CD8 interaction

It has long been known that anti-CD8 Abs can block the activation of some CTL (3, 38). The effects of such blocking Abs have been used to divide CTL into those that are CD8 dependent and those that are CD8 independent. It has often been assumed that anti-CD8 Abs function by blocking the interaction between CD8 and pMHC. Our findings that anti-CD8 Ab blocking of pMHC multimer binding to cell surface TCRs can be independent of the pMHC/CD8 interaction question this assumption. Our results showing that the function of anti-CD8 Abs on the binding of pMHC multimers is independent of the pMHC/CD8 interaction (Figs. 2–5) led us to question the role of this interaction in the functional blocking of CTL activation by such Abs. We have compared the activation requirements of 10 different HLA-A2-restricted CTL, with a variety of TCRs differing in affinity for pMHC, using C1R target cells expressing HLA-A2 and D227K/T228A HLA-A2 (L. Wooldridge, A. Lissina, P. Dunbar, F. Mirza, V. Cerundolo, and A. Sewell, manuscript in preparation). These

targets express identical amounts of MHC I and have been described previously (19). Although the level of dependency on the pMHC/CD8 interaction varies, all CTL appear to exhibit some dependency on this interaction at physiological levels of Ag. Like the majority of human CTL specific for immunodominant antiviral epitopes, we observe that HIV-1 Gag-specific CTL clone 003 exhibits only partial dependency on the pMHC/CD8 interaction, particularly at high Ag concentrations (Fig. 6, and our unpublished observations). Preincubation with the anti-CD8 Ab DK-25 not only blocked the activation of 003 CTL by targets presenting peptide in the context of wild-type pMHC, but also blocked the activation of CTL by targets that do not allow a pMHC/CD8 interaction (Fig. 6). Thus, anti-CD8 Abs that block CTL activation need not function by blocking the pMHC/CD8 interaction.

Blocking of pMHC multimer binding and CTL activation by anti-CD8 Ab correlate, but are independent of the pMHC/CD8 interaction

Some anti-CD8 Abs can block pMHC multimer binding to cell surface TCR (Figs. 1–5). Anti-CD8 Abs can also block CTL activation (Fig. 6). Both phenomena appear to be largely independent of the interaction between pMHC and CD8 (Figs. 2–6). Not all anti-CD8 Abs block the binding of pMHC multimers to cell surface TCRs (Fig. 1). Anti-CD8 Ab MCD8 only slightly inhibits pMHC multimer binding (Fig. 1) to CTL clone 003. Of the human anti-CD8 Abs tested, DK-25 appears to be the best at blocking pMHC multimer binding to this, and other, CTL. We examined

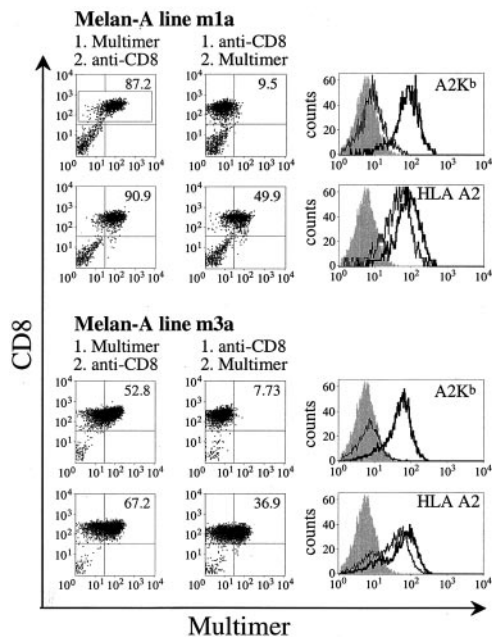


FIGURE 4. The murine pMHC/CD8 interaction need not be critical for the binding of murine pMHC multimers. Melan-A_{26–35}-specific murine CTL lines m1a and m3a were stained with PE-labeled A2K^b (top and third row) or A2 tetramers (second and bottom row) at 37°C for 30 min either before (left panels) or after (middle panels) incubation with anti-CD8α Ab CT-CD8α on ice for 20 min. The mean fluorescence intensity of the CD8⁺ population (gate shown in upper left panel) is indicated at the top of the upper right quadrant of the dot plots. Histograms of pMHC multimer on the right show staining with a control pMHC multimer (shaded) and Melan-A_{26–35} pMHC multimers with (thin line) and without (thick line) preincubation with anti-CD8 Ab.

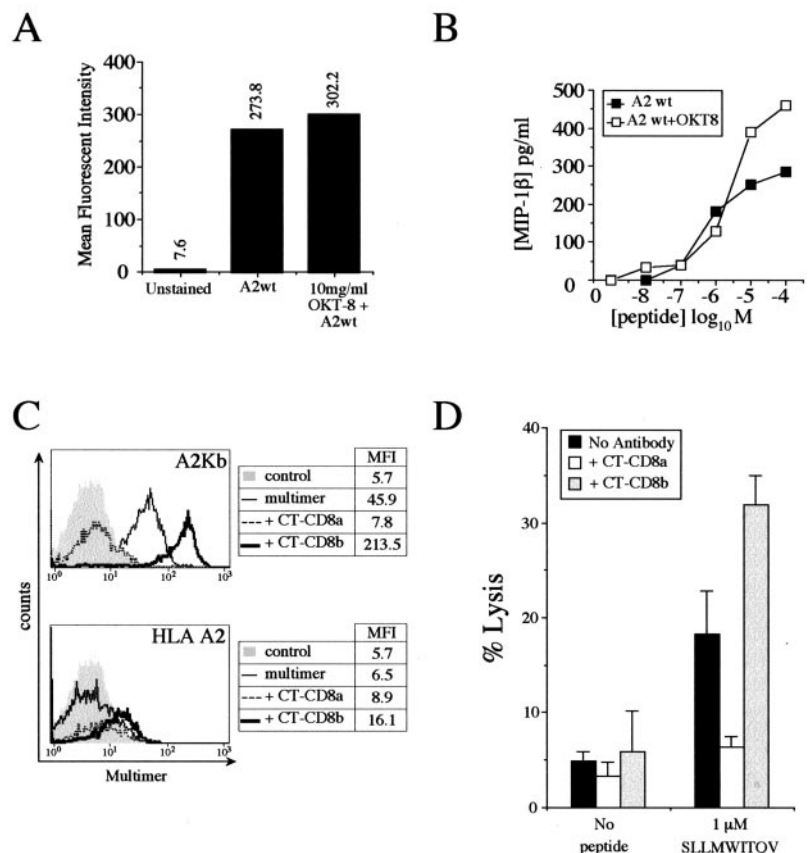
FIGURE 5. Anti-CD8 Ab can enhance multimer staining and CTL activation. *A*, PE-conjugated multimeric forms of YMDGTMSQV-HLA-A2 were used to stain the tyrosinase-specific CTL clone 3G10 following a 20-min preincubation on ice ± 10 μg/ml OKT8 Ab. The data show that the anti-CD8 Ab OKT-8 can enhance multimer binding to clone 3G10. *B*, OKT-8 Ab increases the activation of 3G10 CTL (MIP-1β ELISA). A total of 2.5 × 10⁴ CIR A2 targets per well was added to a 96-well plate and pulsed for 1 h with a range of concentrations of YMDGTMSQV tyrosinase peptide. After washing, 2.5 × 10⁴ 3G10 CTL per well were added to the same plate, followed immediately with OKT-8 Ab at a final concentration of 30 μg/ml. A total of 50 μl cell culture supernatant was withdrawn after 4-h incubation at 37°C, and assayed by ELISA for MIP-1β content. Assays were performed in duplicate. *C*, Murine NY-ESO-1_{157–165}-specific CTL line m33 from the A2K^b transgenic mouse was stained with A2K^b or A2 multimers for 20 min at 37°C, either alone (thin line) or after preincubation with 15 μg/ml anti-CD8 Abs CT-CD8a (dotted line) or CT-CD8b (thick line) on ice for 20 min. *D*, NY-ESO-1_{157–165}-specific CTL line m33 was incubated with peptide-pulsed ⁵¹Cr-labeled EL4-A2K^b cells in the absence (■) or presence of 20 μg/ml CT-CD8a (□) or CT-CD8b (▣) anti-CD8 Abs. The histogram shows SD from the mean of two replicate lysis assays at an E:T ratio of 2:1.

the effects of different anti-CD8 Abs on the activation of this CTL clone (Fig. 6, and data not shown). Ab DK-25 was more potent at blocking CTL activation than SK-1. MCD8 only had minimal inhibitory affect on the activation of 003 CTL (data not shown). Thus, the effects of anti-CD8 Ab on tetramer staining (Fig. 1) and on CTL activation appear to correlate. In addition, anti-CD8 Abs that enhance the binding of pMHC multimers also enhance CTL activation (Fig. 5), suggesting that the effects of anti-CD8 Ab on pMHC multimer staining and CTL activation might be mediated through the same mechanism. This mechanism appears to be independent of the pMHC/CD8 interaction (Figs. 2–6).

pMHC Ag can alter the cell surface topography of CD8 in the absence of a pMHC/CD8 interaction

Recent evidence has suggested that the topological arrangement of TCRs on the cell surface is important for tetramer binding (39). The ability of TCR on the surface of functional CD8⁺ T cells to bind multimeric pMHC is dependent on the integrity of lipid rafts in the CTL membrane (39). Several studies have determined that there is a robust, direct interaction between the TCR and CD8 on the CTL surface in the absence of Ag (for examples, see Refs. 40 and 41). This linkage has been further highlighted by microscopy. When anti-TCR Abs are used to cap the TCR, cocapping of CD8 is observed (42). Conversely, capping with anti-CD8 Abs cocaps the TCR (42). It has also been reported that CD8 plays an essential role in recruiting TCR to lipid rafts (18). This recruitment is dependent on the palmitoylation of the CD8β chain and not on the pMHC/CD8 interaction (18, 43).

We extended the visual studies of the TCR/CD8 interaction (42) using multimerized forms of Ag with and without the ability to



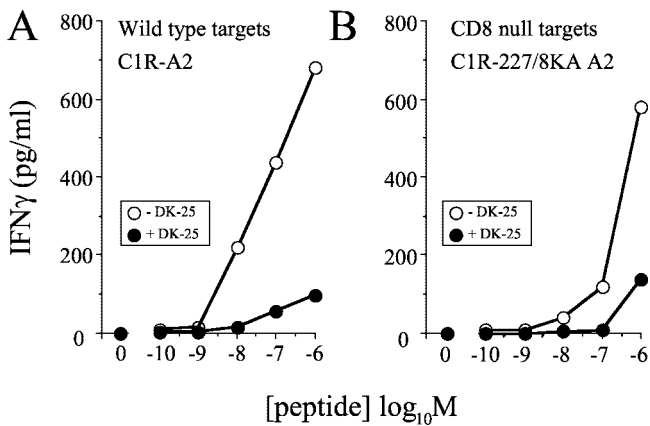
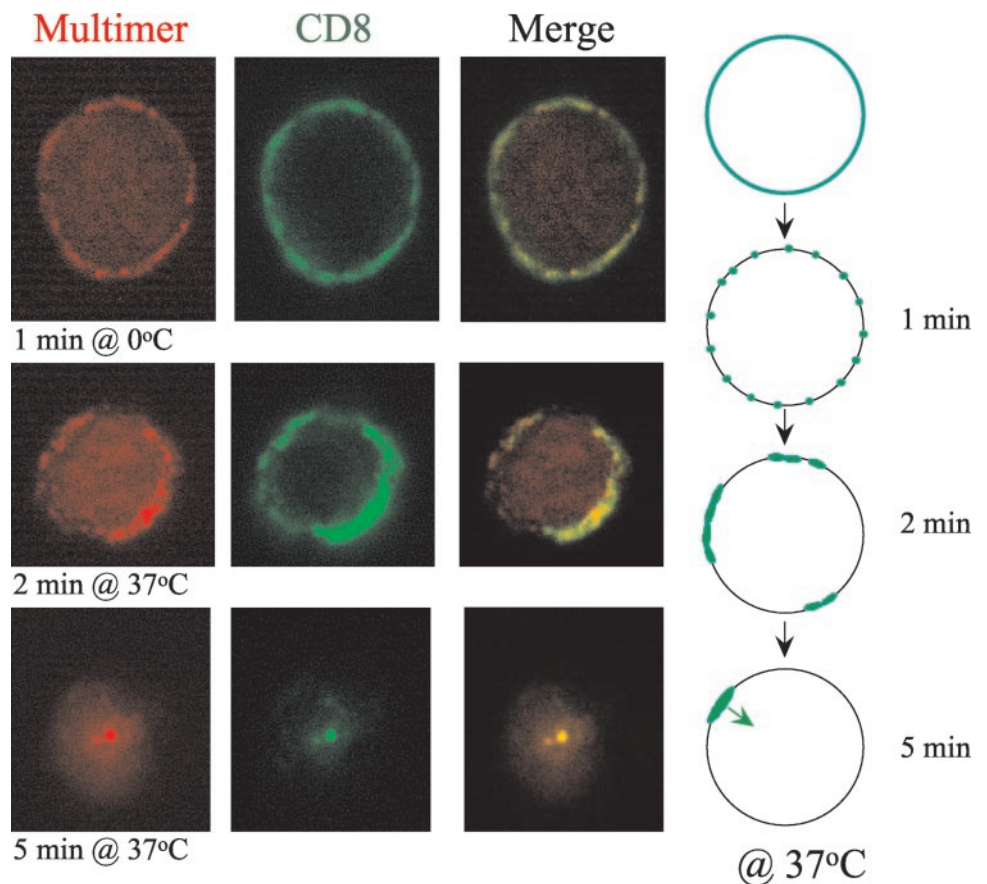


FIGURE 6. Anti-CD8 Abs that block CTL activation act independently of the pMHC/CD8 interaction. DK-25 Ab can block the production of IFN- γ by CTL 003 when Ag is presented on either C1R A2 or C1R 227/8KA A2 targets. The ability of different anti-CD8 Abs to inhibit CTL activation varied. A total of 10 $\mu\text{g/ml}$ DK-25 was more potent than 1.25 $\mu\text{g/ml}$ SK1 (data not shown). A total of 20 $\mu\text{g/ml}$ MCD8 Ab only gave very limited inhibition. This pattern of CTL inhibition correlated with the ability of these Abs to inhibit the binding of pMHC multimers to the cell surface of these CTL (Fig. 1).

interact with CD8. FITC-coupled anti-CD8 Ab was used to examine the distribution of CD8 on the surface of the SLYNTVATL-specific CTL clone 003 after TCR engagement of pMHC multimers (Fig. 7). We have previously shown that pMHC multimers are rapidly internalized by CTL at 37°C (44). Close analysis of CTL populations reveals that multimer engagement is rapidly followed by patch formation. These patches continue to aggregate,

FIGURE 7. pMHC multimers induce patch formation and capping of TCR and cocapping of CD8 independent of the pMHC/CD8 interaction. The 003 CTL were stained with Cy3 (red)-conjugated 227/8KA HLA-A2 SLYNTVATL multimer at 37°C. At 1-, 2-, and 5-min time points, multimer-stained cells were washed in 0.1% azide and stained with anti-CD8 FITC Ab DK-25 (DAKO) at 10 $\mu\text{g/ml}$ for 20 min on ice in the presence of azide to determine the organization of CD8 in relation to multimer-bound TCR. Analysis of cell populations at early time points shows that pMHC multimer engagement rapidly induces patch formation. These patches further aggregate so that in $\sim 30\%$ of cells they induce a single cap before internalization (44). CD8 appears to colocalize with TCR throughout this process. Both wild-type and D227K/T228A (CD8 null) pMHC multimers were observed to induce these similar effects. Patching and cap formation with CD8 null pMHC were judged to be $\sim 10\text{--}20\%$ slower than with the wild-type reagents. CTL did not stain for CD8 post-TCR internalization, indicating that all surface molecules were also internalized (data not shown).



and in $\sim 30\%$ of cells go so far as to produce a single cap before pMHC multimer internalization (Fig. 7). Multimerized Ag was also able to cocap CD8 (Fig. 7). The 227/8KA CD8 null multimer was also able to induce patch formation and capping, although this process was judged to be 10–20% slower. Curiously, this CD8 null Ag was able to cocap CD8 almost as efficiently as the wild-type HLA-A2 multimer, even though it cannot bind this glycoprotein. Thus, it appears that the majority of the TCRs that engage the Ag are associated with CD8 without any requirement for interaction between CD8 and pMHC. This finding is consistent with previous data suggesting that the TCR and CD8 are constitutively associated on resting CTL (40, 41).

Anti-CD8 Abs can activate CTL

The results above, and those of Schott and Ploegh (41), suggest that the majority of TCRs that recognize Ag are cross-linked to CD8. It is well established that cross-linking the TCR with anti-TCR or anti-CD3 Abs can generate an intracellular activation signal. We examined whether anti-CD8 Abs are capable of cross-linking the TCR indirectly, generating an intracellular activation signal. Surprisingly, all the anti-CD8 Abs we tested induced a pattern of intracellular signaling similar to that induced with anti-CD3 Ab, albeit weaker (Fig. 8A). Of the Abs tested, OKT8 appeared to induce the greatest activation. Significant OKT8-induced activation was observed within a minute and titrated down to 1 $\mu\text{g/ml}$ of Ab (data not shown). This early activation signal was also capable of inducing CTL effector function in some CTL (Fig. 8B). This result is in accordance with a previous murine study that showed that some murine anti-CD8 Abs can trigger CTL activation as efficiently as anti-CD3 Ab (45).

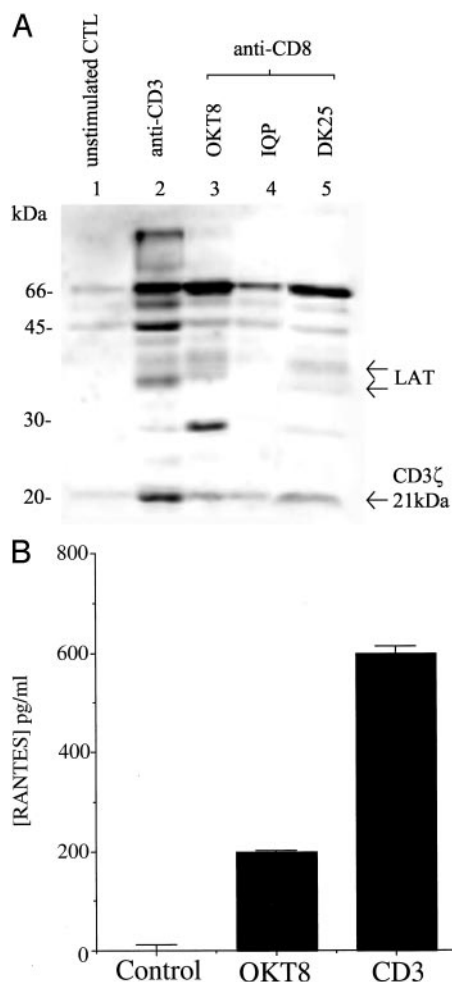


FIGURE 8. Induction of early signal transduction and CTL effector function by CD8 Abs. *A*, A2 Tax CTL clone D1 (5×10^5) was stimulated for 1 min at 37°C with 1 $\mu\text{g}/\text{ml}$ Ab and washed once in PBS before lysis. Lysates were run on SDS-PAGE and immunoblotted with anti-phosphotyrosine Ab, as described in *Materials and Methods*. Data were collected by FluorSMax Multiimager (Bio-Rad). Resting CTL (no Ab) are shown in *lane 1*, followed by CTL stimulated with anti-CD3 Ab (*lane 2*), or with anti-CD8 Ab clone OKT8 (*lane 3*), MCD8 (*lane 4*), or DK-25 (*lane 5*). Equal lane loading was confirmed as described in *Materials and Methods*. *B*, RANTES release by CTL in response to CD3 or CD8 Ab. A2 Tax CTL were incubated for 16 h with 100 $\mu\text{g}/\text{ml}$ Ab, and the cell culture supernatants were analyzed by RANTES ELISA. We have not observed RANTES release using Abs for other CTL surface molecules.

Discussion

Recent studies have shown that anti-CD8 Abs can affect the binding of multimeric pMHC complexes to cell surface TCRs (8, 10, 11). These results have been interpreted in the context of the pMHC/CD8 interaction. However, the use of anti-CD8 Abs does not allow discrimination between the interaction of CD8 with pMHC on the surface of the target cell, interaction with the TCR on the CTL surface, or other possible roles for CD8. We have therefore examined the role of the pMHC/CD8 interaction in the binding of pMHC multimers using mutations in the HLA H chain that reduce

or abrogate the pMHC/CD8 interaction without altering the TCR/pMHC interaction (19). Our key findings are that: 1) human or murine pMHC multimers can bind to cell surface TCR in the absence of a pMHC/CD8 interaction; 2) anti-CD8 Abs can either block or enhance the binding of pMHC multimers to cell surface TCRs; 3) anti-

CD8 Abs that block the binding of pMHC multimers do not function by blocking the pMHC/CD8 interaction; 4) Abs that block or enhance pMHC multimer binding have parallel effects on the activation of CTL by APCs; 5) pMHC Ag can recruit CD8 even when there is no pMHC/CD8 interaction; and 6) anti-CD8 Abs can generate an activation signal that leads to CTL effector function.

To date, we have examined the binding of CD8 null human pMHC multimers to eight different CTL clones with four different Ag specificities, restricted by two different MHC class I molecules (19, 28, 46) (our unpublished observations). CD8 null tetramers bind to cell surface TCR with >90% of the intensity observed with wild-type molecules with all CTL clones that recognize immunodominant human antiviral epitopes. Binding of wild-type and CD8 null multimeric pMHC remains the same over a range of pMHC concentrations (19, 28). Of the human CTLs we have examined to date, only antitumor CTLs, such as the 3F7 clone (Fig. 3 and Ref. 47), show a significant difference between the staining with wild-type and CD8 null tetramers. Interestingly, these clones appear to have the greatest dependency on the pMHC/CD8 interaction for activation (L. Wooldridge, A. Lissina, P. Dunbar, F. Mirza, E. Cerundolo, and A. Sewell, manuscript in preparation) and also appear to require high amounts of Ag for activation (47). It is thus tempting to speculate that the TCR/pMHC interaction of these CTL may be weaker. Experiments to test this hypothesis are in progress.

Although the findings of our study show that the pMHC/CD8 interaction can aid the binding of pMHC tetramers, they do not support the supposition that this interaction is critical for (10, 11) or strictly involved in (8) the binding of these reagents to the cell surface TCR of all CTL. Previous assertions of an obligatory role of CD8 in the stable cell surface binding of pMHC tetramers have involved costaining with anti-CD8 Abs (8, 10, 11). We confirm that some anti-CD8 Abs can completely block the binding of human pMHC multimers (Figs. 1 and 2), but also show that these Abs have inhibitory effects on the binding of CD8 null tetramers (Figs. 2 and 3). We thus conclude that anti-CD8 Abs that inhibit the binding of multimeric pMHC to cell surface TCR do not function by blocking the pMHC/CD8 interaction. Anti-CD8 Abs were better at blocking the binding of human wild-type pMHC multimers than they were at blocking CD8 null reagents (Fig. 3). The pMHC/CD8 interaction is thought to result in a rapid local rearrangement of CD8 with respect to the TCR/pMHC interaction (41). Any subtle rearrangement of CD8, and hence CD8 Ab when cells are pretreated with such, may increase the capacity of some anti-CD8 Abs to interfere with the TCR/pMHC interaction, thus leading to a greater blocking effect with pMHC multimers that enable CD8 binding. The reduced ability of anti-CD8 Abs to block the binding of CD8 null multimers was even more pronounced in the murine system (Fig. 4).

We considered it important to take interspecies differences in the pMHC/CD8 interaction (19, 33, 34) into account by also examining murine cells. The murine pMHC/CD8 interaction is more than four times stronger than the equivalent human interaction (19). There are no reports of significant differences between murine and human TCR/pMHC interactions. Consequently, it is likely the murine pMHC/CD8 interaction might have a greater role in the stable binding of murine pMHC tetramers to cell surface TCR. The evidence to date (25, 41) lends some support to this hypothesis. In addition, Daniels and Jameson (10) took advantage of the CD8⁻ CTL population in 2C transgenic mice to show that CD8⁻ CTL bind pMHC tetramers with a lower intensity (~50%) than the CD8⁺ population. This experiment is pertinent to the debate as, unlike most others examining the role of CD8 in Ag recognition, it does not use anti-CD8 Abs. However, caution should be exercised in reaching conclusions based on CD8⁻ CTL

(or CD8⁻ hybridomas). CD8 plays a pivotal role in the organization of cell surface TCR (18, 42), and this organization can affect the binding of pMHC I tetramers (39). Consequently, it is possible that an absence of CD8 on the cell surface affects cells in other ways beyond the disruption of the pMHC I/CD8 interaction.

We have previously shown that HLA-A2 complexed with human β_2 -microglobulin does not bind to murine CD8 (19). However, the majority of Melan-A₂₆₋₃₅-specific CTL induced in mice immunized with DNA and vaccinia encoding this epitope stain well with HLA-A2 reagents (25). This result confirms that, even in the murine system, the pMHC I/CD8 interaction is not obligatory for the binding of pMHC I multimers to all CTL. However, nearly one-half of the cells that stained with an A2K^b hybrid molecule, which enables the binding of murine CD8, did not stain with HLA-A2 reagents (25). This demonstrates that CTL populations exhibit heterogeneity in their requirement for a pMHC I/CD8 interaction to bind pMHC I multimers.

Our observations that anti-CD8 Ab-mediated blocking or enhancement of pMHC I tetramer binding to cell surface TCR is independent of the pMHC I/CD8 interaction led us to examine the effects of such Abs in CTL activation. It has long been established that CTL differ in their ability to activate in the presence of anti-CD8 Abs (38). Such assays have been used to divide CTL into those that are CD8 dependent and those that are CD8 independent. We show that these blocking Abs can inhibit the activation of CTL by targets that do not allow a pMHC I/CD8 interaction, and conclude that these Abs do not function by blocking this interaction. Because anti-CD8 Abs can block CTL activation and pMHC I multimer binding independently of the pMHC I/CD8 interaction, these Abs must be functioning through a mechanism distinct from this interaction. Interestingly, the ability of anti-CD8 Abs to inhibit or enhance the binding of multimeric pMHC I to cell surface TCRs appeared to correlate with their ability to inhibit or enhance the activation of CTL in both the human and murine systems (Fig. 5). This suggests that the mechanism for these phenomena may be the same, in addition to being independent of the pMHC I/CD8 interaction.

If anti-CD8 Abs do not affect CTL activation and pMHC I multimer binding via the pMHC I/CD8 interaction, then how do they exert their effects? Given the size of an Ab in comparison with CD8, the TCR, and pMHC I, the most obvious explanation is that blocking anti-CD8 Abs sterically interfere with the TCR/pMHC I interaction. This steric hindrance could explain the inhibition of both CTL activation by Ag-bearing targets and pMHC I tetramer binding. However, it is more difficult to invoke such a theory to explain the observed enhancement effect of some anti-CD8 Abs on tetramer binding (8, 10) (Fig. 5, A and C) and CTL activation (Fig. 5, B and D). This enhancement of activation and tetramer binding suggests that anti-CD8 Abs might function by altering the cell surface topography of the TCR.

Zamoyska and colleagues (42) have used confocal microscopy to show that anti-CD8 Abs induce the cocapping of TCR, but not other cell surface molecules. Conversely, it was observed that capping with anti-TCR Abs induced the cocapping of CD8 (42). These observations suggest that the TCR and CD8 can form stable associations on the cell surface that are independent of pMHC I. More recent data also confirm that CD8 is associated with the TCR independently of the CD8-binding capacity of the engaged pMHC I molecule (41). It is also becoming clear that the CD8 β chain plays a role in organizing the TCR within lipid rafts, further highlighting a functional association between the TCR and CD8 on the CTL surface (18). Other recent work has further characterized the TCR/CD3/CD8 interaction and serves to highlight that this interaction is both robust and functionally important for Ag recognition (48, 49). It should be noted that a recent study (50) indicates that constitu-

tive TCR/CD8 association is not a property of all CTL. Furthermore, Ag-experienced and naive CTL also differ in that only in the former population does CD8 efficiently associate with the key kinase *Lck* (51). However, it remains possible that on Ag-experienced CTL, the TCR and CD8 may form a single Ag receptor with two separate binding sites for pMHC I. Our results further support this possibility by showing that multimerized Ag efficiently colocalizes the TCR and CD8 on the CTL surface even when this Ag fails to interact with CD8. Although this colocalization is supported by other recent evidence of a direct interaction between the TCR and CD8 (41), it remains possible that our findings are the result of Ag-induced aggregation of membrane microdomains. Experiments are in progress to test this possibility. Although our experiments exclude a requirement for pMHC I/CD8 association for recruitment of CD8 to the TCR/pMHC I complex, they do not rule out the possibility that the physical linkage we observe between the TCR and CD8 occurs subsequent to TCR/pMHC I engagement. Consequently, our data could, for example, reflect a cytoplasmic association between CD8-bound *Lck* and intracellular components of the TCR/CD3/ ζ complex induced by TCR/pMHC I engagement. Regardless of the precise mechanism of TCR/CD8 association, the fact that anti-CD8 Abs are able to generate an early intracellular activation signal that, in some cases, can result in effector function in both human (Fig. 8) and murine (45) CTL serves to further highlight a functional TCR/CD8 interaction and indicates that such Abs can affect cells independently of interference with the pMHC I/CD8 interaction. We have further determined that pMHC I Ag with super-enhanced CD8 binding ($K_D < 5 \mu\text{M}$), but with unaltered TCR binding, can bypass the need for specific TCR engagement and activate any CD3⁺CD8⁺ cell (L. Wooldridge, J. Vernazza, S. Hutchinson, E. Choi, E. Gostick, V. Cerundolo, D. Price, and A. Sewell, manuscript in preparation).

Despite the potential caveats of using CD8⁻ cells discussed above, experiments examining the CD8 dependency of murine CTL hybridomas may also be relevant to the debate (52). Some hybridomas are able to respond well to Ag even though they fail to express CD8 (52). Ag-induced activation of these hybridomas must, therefore, occur independently of the pMHC I/CD8 interaction. Transfection of these hybridomas with the CD8 α chain results in only a small increase in Ag responsiveness (52), although other studies document that transfection with CD8 α results in a dramatic increase in Ag responsiveness (53, 54). Tellingly, the activation of CD8-independent hybridomas is rendered sensitive to blocking by anti-CD8 Ab by transfection with CD8 α (50). These experiments further emphasize that anti-CD8 Abs that block T cell activation do not function by blocking the pMHC I/CD8 interaction and show that anti-CD8 Ab-mediated inhibition of T cell activation can occur in the absence of CD8 β . It is further noteworthy that tailless CD8 α also renders the activation of CD8-independent CTL hybridomas sensitive to blocking by anti-CD8 Abs (50), indicating that the blocking effects of anti-CD8 Ab are independent of any CD8-mediated intracellular signaling. Our findings are also in accord with those showing that anti-CD8 Abs inhibit the CTL-mediated lysis of staphylococcus enterotoxin B-bearing target cells even when the targets are MHC I negative (55). In agreement with our own conclusions, the authors of this study conclude that anti-CD8 Ab-mediated inhibition of CTL activation is not due to the prevention of intercellular interactions between CD8 and MHC I molecules, and suggest it is probably a consequence of altered association between CD8 and the TCR (55).

In summary, our results confirm the observations of previous groups showing that anti-CD8 Abs can affect the binding of multimeric pMHC I to cell surface TCRs. The ability of anti-CD8 Abs to enhance or inhibit the binding of multimeric pMHC I appears to

correlate with their ability to enhance or inhibit CTL activation. In both these effects, anti-CD8 Abs can act independently of the pMHC/CD8 interaction. Our findings demonstrate that CD8 has multiple roles in the activation of CTL and have some broader implications. It is well established that Abs are invaluable tools for determining which molecules are present on the surface of a given cell. However, our results show that caution should be used when attempting to derive information on the function of a cell surface molecule based solely on the cellular effect of an extracellular Ab against this molecule.

Acknowledgments

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