



Brilliant VioletTM Antibody Conjugates
Superior Performance for the Violet Laser



Anti-CD8 Antibodies Can Trigger CD8⁺ T Cell Effector Function in the Absence of TCR Engagement and Improve Peptide–MHC I Tetramer Staining

This information is current as of January 16, 2012

Mathew Clement, Kristin Ladell, Julia Ekeruche-Makinde, John J. Miles, Emily S. J. Edwards, Garry Dolton, Tamsin Williams, Andrea J. A. Schauenburg, David K. Cole, Sarah N. Lauder, Awen M. Gallimore, Andrew J. Godkin, Scott R. Burrows, David A. Price, Andrew K. Sewell and Linda Wooldridge

J Immunol 2011;187:654-663; Prepublished online 15 June 2011;
doi:10.4049/jimmunol.1003941
<http://www.jimmunol.org/content/187/2/654>

Supplementary Data <http://www.jimmunol.org/content/suppl/2011/06/15/jimmunol.1003941.DC1.html>

References This article **cites 59 articles**, 30 of which can be accessed free at:
<http://www.jimmunol.org/content/187/2/654.full.html#ref-list-1>

Article cited in:
<http://www.jimmunol.org/content/187/2/654.full.html#related-urls>

Subscriptions Information about subscribing to *The Journal of Immunology* is online at
<http://www.jimmunol.org/subscriptions>

Permissions Submit copyright permission requests at
<http://www.aai.org/ji/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at
<http://www.jimmunol.org/etoc/subscriptions.shtml/>



Anti-CD8 Antibodies Can Trigger CD8⁺ T Cell Effector Function in the Absence of TCR Engagement and Improve Peptide–MHC I Tetramer Staining

Mathew Clement,* Kristin Ladell,* Julia Ekeruche-Makinde,* John J. Miles,* Emily S. J. Edwards,* Garry Dolton,* Tamsin Williams,* Andrea J. A. Schauenburg,* David K. Cole,* Sarah N. Lauder,* Awen M. Gallimore,* Andrew J. Godkin,* Scott R. Burrows,[†] David A. Price,* Andrew K. Sewell,*¹ and Linda Wooldridge*¹

CD8⁺ T cells recognize immunogenic peptides presented at the cell surface bound to MHC I molecules. Ag recognition involves the binding of both TCR and CD8 coreceptor to the same peptide–MHC I (pMHC I) ligand. Specificity is determined by the TCR, whereas CD8 mediates effects on Ag sensitivity. Anti-CD8 Abs have been used extensively to examine the role of CD8 in CD8⁺ T cell activation. However, as previous studies have yielded conflicting results, it is unclear from the literature whether anti-CD8 Abs per se are capable of inducing effector function. In this article, we report on the ability of seven monoclonal anti-human CD8 Abs to activate six human CD8⁺ T cell clones with a total of five different specificities. Six of seven anti-human CD8 Abs tested did not activate CD8⁺ T cells. In contrast, one anti-human CD8 Ab, OKT8, induced effector function in all CD8⁺ T cells examined. Moreover, OKT8 was found to enhance TCR/pMHC I on-rates and, as a consequence, could be used to improve pMHC I tetramer staining and the visualization of Ag-specific CD8⁺ T cells. The anti-mouse CD8 Abs, CT-CD8a and CT-CD8b, also activated CD8⁺ T cells despite opposing effects on pMHC I tetramer staining. The observed heterogeneity in the ability of anti-CD8 Abs to trigger T cell effector function provides an explanation for the apparent incongruity observed in previous studies and should be taken into consideration when interpreting results generated with these reagents. Furthermore, the ability of Ab-mediated CD8 engagement to deliver an activation signal underscores the importance of CD8 in CD8⁺ T cell signaling. *The Journal of Immunology*, 2011, 187: 654–663.

CD8⁺ T cells are essential for the control of viral infection and the natural eradication of cancer. CD8⁺ T cells recognize short peptides, 8–13 aa in length, presented at the target cell surface bound to MHC I molecules. T cell Ag recognition is unique in nature because it involves the binding of a single ligand (peptide–MHC [pMHC]) by two receptors (TCR and coreceptor) (1, 2). The CD8 glycoprotein, which serves as the coreceptor on MHC I-restricted T cells, acts to enhance the Ag sensitivity of CD8⁺ T cells by binding to a largely invariant region of MHC I at a site distinct from the TCR docking platform. CD8

has multiple enhancing effects on early T cell activation events, which include the following: 1) promotion and stabilization of TCR/pMHC I binding at the cell surface (3–5); 2) recruitment of essential signaling molecules to the intracellular side of the TCR/CD3/ζ complex (6–11); and 3) localization of TCR/pMHC I complexes within specialized membrane microdomains that act as potentially privileged sites for initiation of the TCR-mediated signaling cascade (12, 13). CD8 binding also controls the level of T cell cross-reactivity (14) and can differentially affect the deployment of CD8⁺ T cell effector functions (15).

Anti-CD8 Abs have been used widely to investigate the role of CD8 in CD8⁺ T cell activation. Early studies showed that preincubation with anti-CD8 Abs can block conjugate formation between effector and target cells (16) and inhibit CD8⁺ T cell activation in response to cognate pMHC I presented on the target cell surface (17–20). These findings provided key evidence that CD8 was important in the process of CD8⁺ T cell activation. However, considerable heterogeneity between different CD8⁺ T cells was apparent in terms of their ability to activate in the presence of anti-CD8 Abs, and as a result, these reagents were used as tools to classify CD8⁺ T cells as either CD8 dependent or CD8 independent (21, 22). Ab-mediated ligation of T cell surface molecules, such as CD2, CD3, and CD28 (23, 24), can result in effector function. In contrast, studies of Ab-mediated CD8 ligation in the absence of TCR engagement have yielded conflicting results. Early studies demonstrated that induction of CD8 cross-linking at the cell surface can result in p56^{lck} phosphorylation similar to that seen with anti-CD3 Abs (25) and elicit downstream effector functions, such as chemokine release (26) and potent cytotoxicity (27). However, in conflict with these data, more re-

*Cardiff University School of Medicine, University Hospital, Cardiff CF14 4XN, United Kingdom; and [†]Cellular Immunology Laboratory, Department of Infectious Disease and Immunology, Queensland Institute of Medical Research, Brisbane, Queensland 4029, Australia

¹L.W. and A.K.S. contributed equally to this manuscript.

Received for publication December 2, 2010. Accepted for publication May 9, 2011.

This work was supported by a Wellcome Trust Clinical Intermediate fellowship (to L.W.); M.C. and J.E.-M. are also funded by the Wellcome Trust. A.K.S. is funded by the Cardiff University Link Chair scheme. D.A.P. is a Medical Research Council (U.K.) senior clinical fellow; K.L. is also funded by the Medical Research Council (U.K.). D.K.C. is a Leverhulme Trust Early Career Fellow. J.J.M. is supported by a Welsh Office of Research and Development translational fellowship and a National Health and Medical Research Council Biomedical fellowship. S.R.B. is a National Health and Medical Research Council senior fellow.

Address correspondence and reprint requests to Dr. Linda Wooldridge, Cardiff University School of Medicine, Henry Wellcome Building, University Hospital, Cardiff CF14 4XN, United Kingdom. E-mail address: wooldridgel@cardiff.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: CBA, cytometric bead array; pMHC, peptide–MHC.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/\$16.00

cent studies suggest that CD8 ligation alone may actually deliver a negative signal (28, 29).

To date, a cohesive explanation for these widely disparate findings with anti-CD8 Abs has remained elusive. Furthermore, there has been no systematic study of the effects of multiple different anti-human CD8 Abs on CD8⁺ T cells with different specificities. In this article, we report on the ability of a panel of seven monoclonal anti-human CD8 Abs to induce chemokine/cytokine release and cytotoxicity by six different human CD8⁺ T cell clones specific for a total of five different pMHCI Ags. The data, supported by parallel observations in a mouse system, reveal that considerable heterogeneity exists in the ability of anti-CD8 Abs to activate CD8⁺ T cells. These results elucidate the apparent incongruity that has been observed in previous studies and mandate that the disparate effects of anti-CD8 Abs are considered in the interpretation of results generated with these reagents.

Materials and Methods

Cells

The following HLA A*0201-restricted CD8⁺ T cell clones were used in this study: 1) ILA1, specific for the human telomerase reverse transcriptase-derived epitope ILAKFLHWL (residues 540–548) (30, 31); 2) ALF3, specific for the influenza A matrix protein (M1)-derived epitope GILGFVFTL (residues 58–66); and 3) MEL5 and MEL187.c5, specific for the Melan-A–derived epitope ELAGIGILT V (residues 26–35) (32). The HLA B*0801-restricted CD8⁺ T cell clone LC13 is specific for the EBV EBNA3A-derived epitope FLRGRAYGL (residues 339–347) (33), and the HLA B*3508-restricted CD8⁺ T cell clone SB10 is specific for the EBV BZLF1-derived epitope LPEPLPQQQLTAY (residues 52–64) (34). The HLA DR*0101-restricted CD4⁺ T cell clone C6 recognizes the influenza A hemagglutinin-derived epitope PKYVKQNTLKLAT (residues 307–319). CD8⁺ T cell lines specific for the EBV BMLF1-derived epitope GLCTLVAML (residues 280–288), restricted by HLA A*0201, were generated as described previously (35). Naive mouse CD8⁺ T cells were obtained by harvesting splenocytes from transgenic F5 mice. A significant percentage of CD8⁺ T cells within the splenic population of these mice express the F5 TCR, which recognizes the H-2D^b-restricted influenza H17 nucleoprotein-derived epitope ASNENMDAM (36). C1R-A*0201 target cells were generated as described previously (37).

Anti-CD8 Abs

The following anti-human CD8 α Ab clones were used in this study: 1) unconjugated or allophycocyanin-conjugated OKT8 (eBioscience, Hatfield, U.K.); 2) unconjugated, FITC-conjugated, or R-PE-conjugated SK1 (BD Biosciences, Oxford, U.K.); 3) unconjugated MCD8 (IqProducts, Groningen, The Netherlands); 4) unconjugated 32/M4 (Santa Cruz Biotechnology, Heidelberg, Germany); 5) unconjugated C8/144B (Santa Cruz Biotechnology); and 6) allophycocyanin-conjugated DK25 (DakoCytomation, Stockport, U.K.). The anti-human CD8 β Ab clone 2ST8.5H7 was also used, either in unconjugated or in PE-conjugated form (Abcam, Cambridge, U.K.). In functional assays, the maximum possible Ab concentrations were used, determined by the concentration of the commercially available preparation in each case. For experiments with mouse cells, the following unconjugated anti-mouse CD8 Abs were used: 1) anti-CD8 α clone CT-CD8a (Caltag-MedSystems, Buckingham, U.K.); 2) anti-CD8 α clone 53.6.7 (BioLegend, Cambridge, U.K.); 3) anti-CD8 β clone KT112 (hybridoma provided by Prof. R. Zamoyka, School of Biological Sciences, University of Edinburgh); and 4) anti-CD8 β clone CT-CD8b (Caltag-MedSystems).

Generation of OKT8 Fab, F(ab')₂ and Fc' fragments

A total of 250 μ g of the anti-human CD8 Ab OKT8 or the anti-human CD3 Ab OKT3 were digested to yield Fab and Fc' fragments using a Pierce Fab micropreparation kit (ThermoScientific, Rockford, IL); F(ab')₂ fragments were produced similarly using a Pierce F(ab')₂ micropreparation kit (ThermoScientific). IgG fragmentation was performed according to the manufacturer's instructions.

CD8⁺ T cell effector function assays

T cells (3×10^4) were mixed with anti-CD8 Abs at the indicated concentrations, either with or without secondary cross-linking by the addition

of 5 μ l anti-mouse IgG Ab (Beckman Coulter, High Wycombe, U.K.), and incubated overnight at 37°C in a 5% CO₂ atmosphere. Positive controls included the following: 1) target cells pulsed with 10^{-7} M cognate peptide; 2) 10 μ g/ml anti-human CD3 Ab (UCHT1; BD Biosciences); or 3) 50 ng/ml PMA and 1 μ g/ml ionomycin (Sigma-Aldrich, Dorset, U.K.). Supernatants were harvested and assayed for MIP1 α , MIP1 β , and RANTES by ELISA (R&D Systems, Abingdon, U.K.) and for IFN- γ , TNF- α , and IL-2 by cytometric bead array (Th1/Th2 kit; BD Biosciences), according to the manufacturer's instructions in each case; mouse MIP1 β and IL-2 assays were performed by ELISA (R&D Systems). Cytometric bead array (CBA) data were acquired using a FACSCalibur flow cytometer and analyzed with CBA 6 Bead analysis software (BD Biosciences). CD107a mobilization was used to measure T cell degranulation by flow cytometry as described previously (38). For chromium release assays, 2×10^3 T cells were treated with anti-CD8 Abs at the indicated concentrations in 100 μ l RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with 100 U/ml penicillin (Life Technologies), 100 μ g/ml streptomycin (Life Technologies), 2 mM L-glutamine (Life Technologies), and 2% heat-inactivated FCS (Life Technologies) (R2 medium). C1R-A*0201 target cells (2×10^3), labeled with 30 μ Ci ⁵¹Cr (Perkin Elmer, Cambridge, U.K.) per 10^6 cells for 1 h previously, were subsequently added. Targets were also cultured alone (target spontaneous release) and with Triton X-100 (Sigma-Aldrich) at a final concentration of 5% (target total release). Cells were incubated at 37°C for 18 h in a 5% CO₂ atmosphere. For each sample, 20 μ l supernatant was harvested and mixed with 150 μ l OptiPhase Supermix Scintillation Cocktail (Perkin Elmer). Plates were analyzed using a liquid scintillator and luminescence counter (MicroBeta TriLux; Perkin Elmer) with Microbeta Windows Workstation software (Perkin Elmer). Specific lysis was calculated according to the following formula: (experimental release – target spontaneous release/target total release – target spontaneous release) \times 100.

pMHCI tetramer staining and flow cytometry

Soluble biotinylated pMHCI monomers were produced as described previously (3). Tetrameric pMHCI reagents (tetramers) were constructed by the addition of either PE-conjugated streptavidin (Life Technologies) or allophycocyanin-conjugated streptavidin (Prozyme, Hayward, CA) at a pMHCI:streptavidin molar ratio of 4:1. For human CD8⁺ T cell clones, 5×10^4 cells were preincubated with anti-CD8 Ab as indicated for 25 min on ice and then stained with cognate PE-conjugated tetramer (25 μ g/ml) at 37°C for 15 min (reviewed in Ref. 39) prior to staining with 5 μ l 7-aminoactinomycin D (Viaprobe; BD Biosciences) at 4°C for 30 min. For human CD8⁺ T cell lines, 5×10^4 cells were preincubated with anti-CD8 Ab as indicated for 25 min on ice and then stained with cognate PE-conjugated HLA A*0201 tetramer (25 μ g/ml) at 37°C for 15 min prior to staining with the amine-reactive fluorescent dye LIVE/DEAD Fixable Aqua (Life Technologies), Pacific Blue-conjugated anti-human CD14 (clone Tuk4; Caltag-MedSystems), Pacific Blue-conjugated anti-human CD19 (clone SJ25-C1; Caltag-MedSystems), PE-Cy5.5-conjugated anti-human CD4 (clone S3.5; Caltag-MedSystems), and FITC-conjugated anti-human CD8 (clone SK1; BD Biosciences) at 4°C for 20 min. For human PBMCs directly ex vivo, 1×10^5 cells were preincubated with anti-CD8 Ab as indicated for 25 min on ice and then stained with allophycocyanin-conjugated HLA A*0201 tetramer (25 μ g/ml) at 37°C for 15 min prior to staining with LIVE/DEAD Fixable Aqua (Life Technologies), Pacific Blue-conjugated anti-human CD14 (clone Tuk4; Caltag-MedSystems), Pacific Blue-conjugated anti-human CD19 (clone SJ25-C1; Caltag-MedSystems), FITC-conjugated anti-human CD3 (clone HIT3a; BD Biosciences), PE-Cy5.5-conjugated anti-human CD4 (clone S3.5; Caltag-MedSystems), and PE-Cy7-conjugated anti-human CD8 (clone RPA-T8; BD Biosciences) at 4°C for 20 min. For mouse experiments, 5×10^4 cells were preincubated with 100 μ g/ml CT-CD8a (Caltag-MedSystems), 53.6.7 (BioLegend), KT112 (in-house), or CT-CD8b (Caltag-MedSystems) for 25 min on ice and then stained with cognate PE-conjugated H-2D^b tetramer (25 μ g/ml) at 37°C for 15 min prior to staining with LIVE/DEAD Fixable Aqua (Life Technologies), Pacific Blue-conjugated anti-mouse CD4 (clone RM4-5; BD Biosciences), FITC-conjugated anti-mouse CD45R/B220 (clone RA3-6B2; BD Biosciences), and PerCP-Cy5.5-conjugated anti-mouse CD3 (clone 17A2; BD Biosciences) at 4°C for 20 min. Data were acquired using either a FACSCantoII or a modified FACSAriaII flow cytometer (both BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Intracellular cytokine staining

PBMCs were harvested from a healthy donor and cultured with or without 1 μ g/ml PHA (Alere, Cheshire, U.K.) and 25 ng/ml IL-15 (Promega, Hampshire, U.K.) for 7 d and then washed and cultured overnight in R2

medium. A total of 5×10^4 PBMCs (unstimulated or stimulated with PHA/IL-15) were resuspended in the presence of 1 μ l/ml brefeldin A (GolgiPlug; Sigma-Aldrich), 0.7 μ l/ml monensin (GolgiStop; BD Biosciences), and 5 μ l/ml anti-CD107a-FITC (clone H4A3; BD Biosciences) and then incubated with anti-human CD8 Abs at the indicated concentrations for 18 h at 37°C in a 5% CO₂ atmosphere. After washing with PBS, cells were stained with LIVE/DEAD Fixable Violet (ViViD; Life Technologies), Pacific Blue-conjugated anti-human CD14 (clone Tuk4; Caltag-MedSystems), and Pacific Blue-conjugated anti-human CD19 (clone SJ25-C1; Caltag-MedSystems) at room temperature for 15 min. Subsequently, cells were washed and stained with H7-allophycocyanin-conjugated anti-human CD3 (clone SK7; BD Biosciences) and PE-Cy5.5-conjugated anti-human CD4 (clone S3.5; Caltag-MedSystems) at 4°C for 20 min, then washed an additional three times, resuspended in 200 μ l BD Cytotfix/Cytoperm, and incubated at 4°C for 20 min. After three additional washes in Perm/Wash (BD Biosciences), cells were stained with PE-Cy7-conjugated anti-human IFN- γ (clone B27; BD Biosciences), allophycocyanin-conjugated anti-human TNF- α (clone MAb11; BD Biosciences), and PE-conjugated anti-human MIP1 β (clone D21-1351; BD Biosciences) at 4°C for 20 min, washed again three more times, and resuspended in 200 μ l Perm/Wash. Data were acquired using a modified FACSAriaII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Tetramer kinetics experiments

For tetramer association assays, 5×10^5 CD8⁺ T cells were washed twice and resuspended in 200 μ l PBS with or without anti-human CD8 Ab and then incubated with cognate tetramer (5 μ g/ml). At indicated time points, 12 μ l of the cell suspension was removed and acquired using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). Tetramer decay analysis was performed as described previously (3).

Surface plasmon resonance analysis

Soluble TCRs derived from the MEL5 and MEL187.c5 CD8⁺ T cell clones were manufactured as described previously (40–42). Binding analysis was performed using a BIAcore 3000 equipped with a CM5 sensor chip (43). Between 200 and 400 response units of biotinylated pMHCI was immobilized to streptavidin, which was chemically linked to the chip surface. The pMHCI was injected at a slow flow rate (10 μ l/min) to ensure uniform distribution on the chip surface. Combined with the small amount of pMHCI bound to the chip surface, this reduced the likelihood of off-rate limiting mass transfer effects. The MEL5 TCR and MEL187.c5 TCRs were purified and concentrated to ~ 100 μ M on the day of surface plasmon resonance analysis to reduce the likelihood of TCR aggregation affecting the results. For equilibrium analysis, eight serial dilutions were carefully prepared in triplicate for each sample and injected over the relevant sensor chips at 25°C. The TCRs were injected over the chip surface at a flow rate of 45 μ l/min. Results were analyzed using BIAevaluation 3.1, Microsoft Excel, and Origin 6.1. The equilibrium binding constant (K_D) values were calculated using a nonlinear curve fit ($y = [P_1x]/[P_2 + x]$).

Results

Anti-CD8 Abs can trigger T cell effector function in the absence of TCR engagement

Several studies suggest that Ab-mediated ligation of CD8 in the absence of TCR engagement can elicit downstream effector function (25–27); however, others have reported the delivery of negative signals with this manipulation (28, 29). To reconcile these apparently disparate findings, we conducted a systematic study of the effects of multiple different anti-human CD8 Abs on CD8⁺ T cells with several different specificities. For this purpose, we used a panel of anti-human CD8 Abs that comprised six anti-CD8 α Abs (OKT8, SK1, MCD8, 32/M4, C8/144B, and DK25) and one anti-CD8 β Ab (2ST8.5H7). Six of seven anti-human CD8 Abs from the panel (SK1, MCD8, 32/M4, C8/144B, DK25, and 2ST8.5H7) did not elicit any chemokine production when incubated with four different HLA A*0201-restricted CD8⁺ T cell clones (ILA1, ALF3, MEL5, and MEL187.c5) with a total of three different specificities in the absence of specific pMHCI Ag (Fig. 1). However, the anti-CD8 α Ab OKT8 induced MIP1 α , MIP1 β ,

and RANTES release from all four HLA A*0201-restricted CD8⁺ T cell clones (Fig. 1). Chemokine secretion was apparent over a range of OKT8 concentrations (Supplemental Fig. 1).

In addition, we measured chemokine release by two non-HLA A*0201-restricted CD8⁺ T cell clones following incubation with each anti-human CD8 Ab from the panel. Both of these non-HLA A*0201-restricted CD8⁺ T cell clones produced MIP1 α , MIP1 β , and RANTES in response to OKT8 but did not activate in the presence of the other Abs tested (Fig. 2). Remarkably, the highly Ag-sensitive HLA B*3508-restricted EBV BZLF1-specific CD8⁺ T cell clone SB10 released >2000 pg/ml of each chemokine in response to OKT8 (Fig. 2B). OKT8 was incapable of staining the HLA DR*0101-restricted CD4⁺ T cell clone C6 (Supplemental Fig. 2A) and failed to induce chemokine release from this clone (Supplemental Fig. 2B–D). Thus, the stimulatory effects of OKT8 appear to be CD8⁺ T cell specific.

The panel of seven anti-human CD8 Abs was further tested in cytotoxicity assays with four different CD8⁺ T cell clones (MEL187.c5, ALF3, LC13, and SB10). Anti-human CD8 Abs that were incapable of inducing chemokine release failed to elicit cytotoxic activity in any of these four CD8⁺ T cell clones (Fig. 3). In contrast, SB10 CD8⁺ T cells exhibited substantial cytotoxicity in response to stimulation with OKT8; lower levels of specific lysis

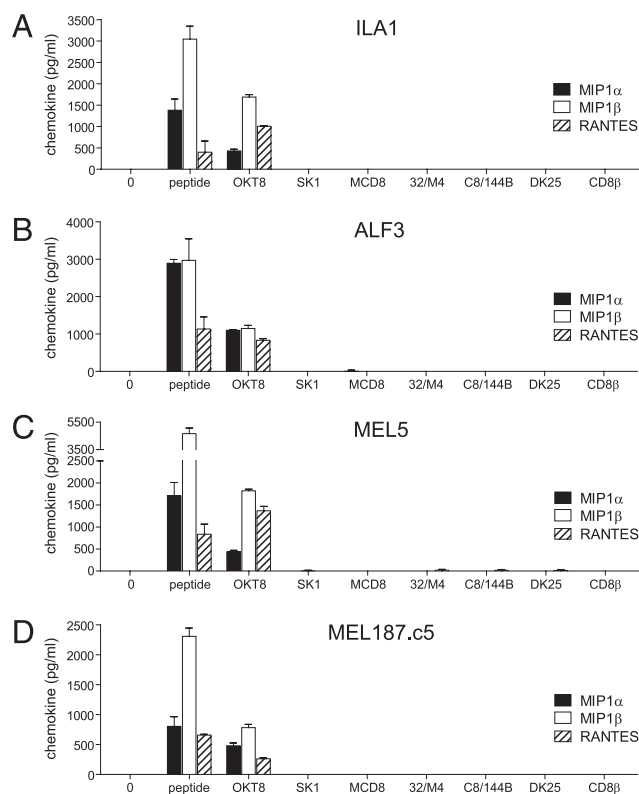


FIGURE 1. Ab-mediated CD8 ligation can trigger chemokine release from HLA A*0201-restricted CD8⁺ T cells. A total of 3×10^4 ILA1 CD8⁺ T cells (A), ALF3 CD8⁺ T cells (B), MEL5 CD8⁺ T cells (C), or MEL187.c5 CD8⁺ T cells (D) were incubated for 18 h with each of the following individual anti-human CD8 Abs in parallel: 100 μ g/ml OKT8, 6.25 μ g/ml SK1, 50 μ g/ml MCD8, 100 μ g/ml 32/M4, 100 μ g/ml C8/144B, 25 μ g/ml DK25, and 100 μ g/ml 2ST8.5H7 (CD8 β). The maximum possible Ab concentrations were used, determined by the concentration of the commercially available preparation in each case. For each CD8⁺ T cell clone, 3×10^4 C1R-A*0201 B cells pulsed with cognate peptide at 10^{-7} M were used as positive controls. 0 represents T cells only. Supernatant was harvested and assayed for MIP1 α , MIP1 β , and RANTES by ELISA. Error bars represent SDs.

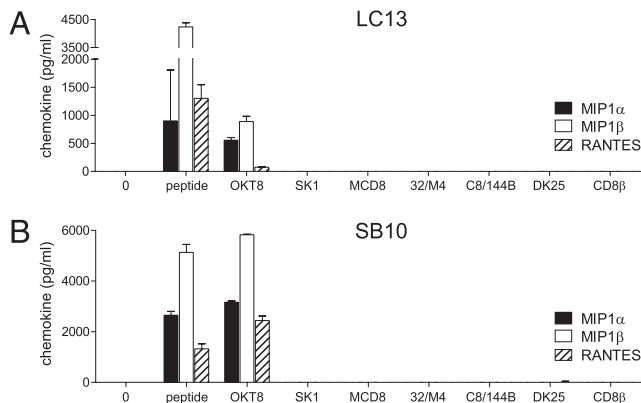


FIGURE 2. Ab-mediated CD8 ligation can trigger chemokine release from non-HLA A*0201-restricted CD8⁺ T cells. A total of 3×10^4 LC13 CD8⁺ T cells (A) or SB10 CD8⁺ T cells (B) were incubated for 18 h with each of the following individual anti-human CD8 Abs in parallel: 100 μ g/ml OKT8, 6.25 μ g/ml SK1, 50 μ g/ml MCD8, 100 μ g/ml 32/M4, 100 μ g/ml C8/144B, 25 μ g/ml DK25, and 100 μ g/ml 2ST8.5H7 (CD8 β). For each CD8⁺ T cell clone, 3×10^4 HLA-matched B cells pulsed with cognate peptide at 10^{-7} M were used as positive controls. Supernatant was harvested and assayed for MIP1 α , MIP1 β , and RANTES by ELISA. Error bars represent SDs.

were also induced in the CD8⁺ T cell clones LC13 (3.18%), ALF3 (5.1%), and MEL187.c5 (3.8%) (Fig. 3; data not shown). These results are consistent with a previous study that described a mouse anti-CD8 Ab, KT112, capable of inducing cytotoxicity (27). Collectively, these data indicate that considerable heterogeneity exists in the ability of anti-CD8 Abs to activate CD8⁺ T cells.

OKT8 induces chemokine secretion in the absence of cytokine secretion

Next, we examined the ability of Ab-mediated CD8 ligation to elicit cytokine release by CD8⁺ T cells in the absence of TCR engagement. As expected, the anti-human CD8 Abs that did not elicit chemokine release or cytotoxic activity (SK1, MCD8, 32/M4, C8/144B, DK25, and 2ST8.5H7) also failed to induce IFN- γ , TNF- α , or IL-2 release (Fig. 4). Interestingly, OKT8 similarly failed to elicit cytokine production from the majority of CD8⁺ T cell clones

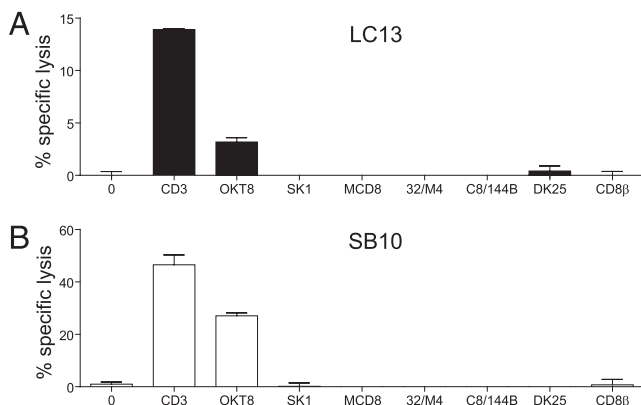


FIGURE 3. The anti-human CD8 Ab OKT8 can trigger cytotoxic activity. A total of 2×10^3 LC13 CD8⁺ T cells (A) or SB10 CD8⁺ T cells (B) were incubated with each of the following individual anti-human CD8 Abs in parallel: 100 μ g/ml OKT8, 6.25 μ g/ml SK1, 50 μ g/ml MCD8, 100 μ g/ml 32/M4, 100 μ g/ml C8/144B, 25 μ g/ml DK25, and 100 μ g/ml 2ST8.5H7 (CD8 β). The anti-human CD3 Ab UCHT1 (10 μ g/ml) served as a positive control. Cytotoxicity assays were then performed over a period of 18 h as described in *Materials and Methods* using 51 Cr-labeled C1R-A*0201 B cells as targets. Error bars represent SDs.

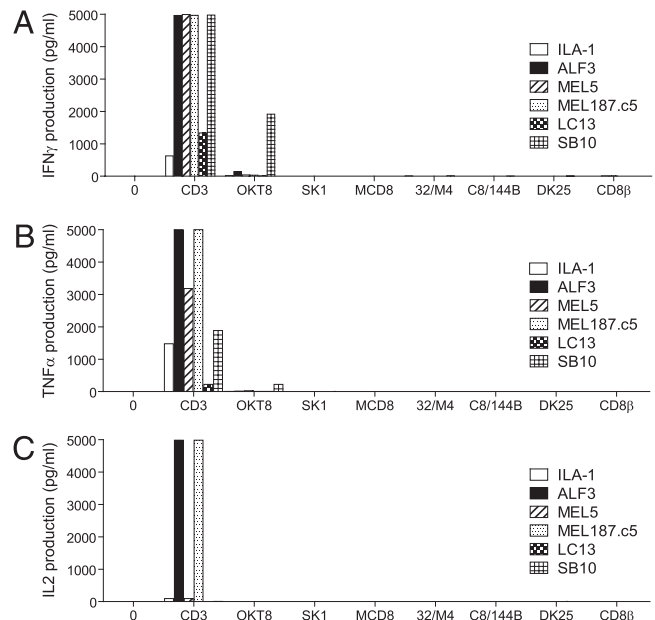


FIGURE 4. Anti-CD8 Ab-mediated chemokine release occurs in the absence of cytokine release. A total of 3×10^4 ILA1, ALF3, MEL5, MEL187.c5, LC13, or SB10 CD8⁺ T cells were incubated for 18 h with each of the following individual anti-human CD8 Abs in parallel: 100 μ g/ml OKT8, 6.25 μ g/ml SK1, 50 μ g/ml MCD8, 100 μ g/ml 32/M4, 100 μ g/ml C8/144B, 25 μ g/ml DK25, and 100 μ g/ml 2ST8.5H7 (CD8 β). The anti-human CD3 Ab UCHT1 (10 μ g/ml) served as a positive control. Supernatant was harvested and assayed for IFN- γ (A), TNF- α (B), and IL-2 (C) by CBA.

tested (Fig. 4). Importantly, chemokine and cytokine assays were performed using the same supernatant, thereby confirming that OKT8 stimulated CD8⁺ T cells to secrete chemokines in the absence of cytokine production; one exception to this dichotomy occurred with the CD8⁺ T cell clone SB10, which released IFN- γ in response to treatment with OKT8. These data suggest that OKT8-mediated CD8 ligation delivers a signal that falls below the threshold required for cytokine production in most CD8⁺ T cells.

Neither secondary Ab cross-linking nor PHA/IL-15 treatment alter the functional phenotype of anti-human CD8 Abs

To probe the possibility that the degree of cross-linking mediated by each of the anti-human CD8 Abs tested could explain the functional heterogeneity observed between these reagents, we performed activation experiments with the addition of secondary Abs. Secondary cross-linking of OKT8 increased the level of MIP1 α , MIP1 β , and RANTES release by ILA1, ALF3, MEL5, MEL187.c5, LC13, and SB10 CD8⁺ T cells above that observed with OKT8 alone (Fig. 5; data not shown). However, secondary Ab-mediated cross-linking did not reverse the phenotype of the nonactivating anti-human CD8 Abs (Fig. 5).

We also examined the effect of PHA/IL-15 treatment on the ability of anti-human CD8 Abs to elicit effector function from CD8⁺ T cells in healthy donor PBMCs. PHA is capable of cross-linking glycosylated proteins at the T cell surface. The seven anti-human CD8 Abs tested did not substantially activate CD8⁺ T cells in untreated PBMCs (Fig. 6A). The six nonactivating anti-human CD8 Abs also failed to induce substantial levels of CD8⁺ T cell activation in PBMCs cultured for 7 d in PHA/IL-15 (Fig. 6B). In contrast, OKT8 activated CD8⁺ T cells in PHA/IL-15-stimulated PBMCs to release MIP1 β and degranulate as measured by surface mobilization of CD107a. Interestingly, OKT8 also induced IFN- γ

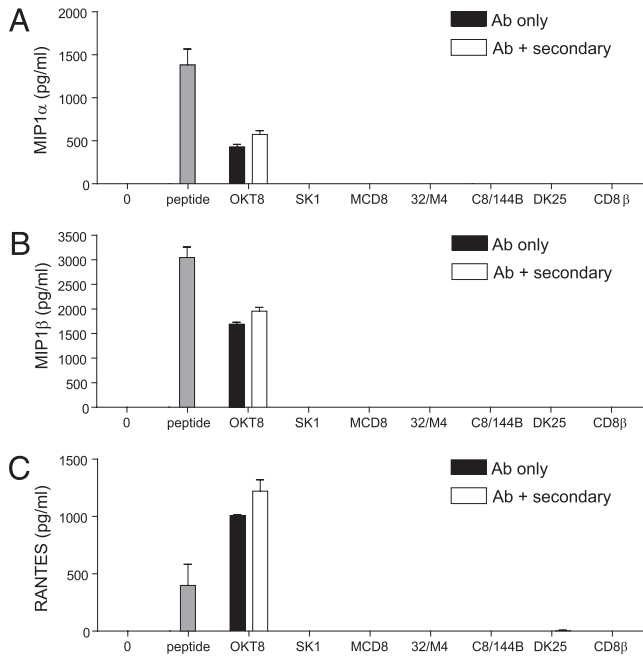


FIGURE 5. Secondary crosslinking does not alter the functional phenotype of anti-human CD8 Abs. A total of 3×10^4 ILA1 CD8⁺ T cells were incubated with each of the following individual anti-human CD8 Abs in parallel: 100 $\mu\text{g/ml}$ OKT8, 6.25 $\mu\text{g/ml}$ SK1, 50 $\mu\text{g/ml}$ MCD8, 100 $\mu\text{g/ml}$ 32/M4, 100 $\mu\text{g/ml}$ C8/144B, 25 $\mu\text{g/ml}$ DK25, and 100 $\mu\text{g/ml}$ 2ST8.5H7 (CD8 β). The positive control comprised 3×10^4 C1R-A*0201 B cells pulsed with cognate peptide at 10^{-7} M. Abs were then cross-linked with the addition of 5 μl anti-mouse IgG Ab (serum IgG) and incubated for 18 h at 37°C in a 5% CO₂ atmosphere. Supernatant was harvested and assayed for MIP1 α (A), MIP1 β (B), and RANTES (C) by ELISA. Secondary cross-linking of OKT8 increased the levels of all analytes measured; this also applied to anti-CD3 Ab-induced chemokine release (data not shown). Similar results were obtained with all other CD8⁺ T cell clones tested: ALF3, MEL5, MEL187.c5, LC13, and SB10 (data not shown). Error bars represent SDs.

and TNF- α production by CD8⁺ T cells in PHA/IL-15-stimulated PBMCs, thereby suggesting that this treatment regimen synergistically lowered the activation threshold of the responding cells. OKT8 failed to activate CD4⁺ T cells in PHA/IL-15-stimulated

FIGURE 7. Anti-human CD8 Abs can either enhance or inhibit the binding of pMHC tetramers. A total of 5×10^4 ILA1 (A), ALF3 (B), MEL5 (C), or MEL187.c5 (D) CD8⁺ T cells were preincubated at 4°C for 25 min with each of the following individual anti-human CD8 Abs in parallel: 100 $\mu\text{g/ml}$ OKT8, 6.25 $\mu\text{g/ml}$ SK1, 50 $\mu\text{g/ml}$ MCD8, 100 $\mu\text{g/ml}$ 32/M4, 100 $\mu\text{g/ml}$ C8/144B (144B), 25 $\mu\text{g/ml}$ DK25, and 100 $\mu\text{g/ml}$ 2ST8.5H7 (CD8 β). CD8⁺ T cells were subsequently stained with cognate PE-conjugated HLA A*0201 tetramers (25 $\mu\text{g/ml}$) and 7-aminoactinomycin D as described in *Materials and Methods*. Data were acquired using a FACSCalibur flow cytometer and analyzed with FlowJo software. Relative median fluorescence intensity (MFI) values with respect to pMHC tetramer staining in the absence of preincubation with anti-CD8 Ab are shown. Fluorescence in the absence of added cognate tetramer (con) is shown in each case. Data are representative of four separate experiments using ILA1 and ALF3 CD8⁺ T cells, and six separate experiments using MEL5 and MEL187.c5 CD8⁺ T cells.

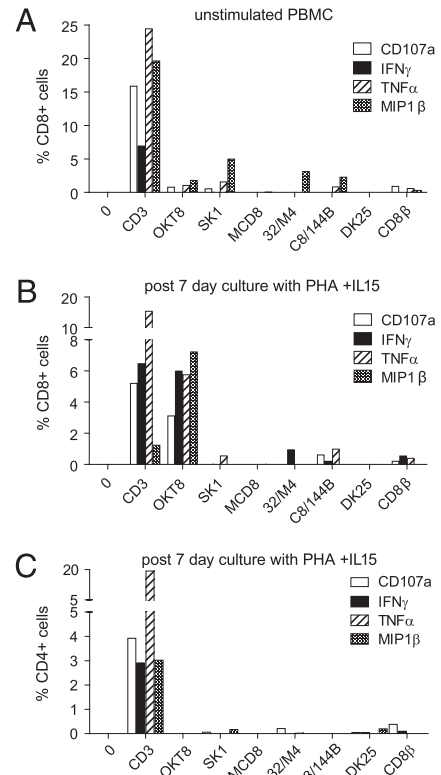
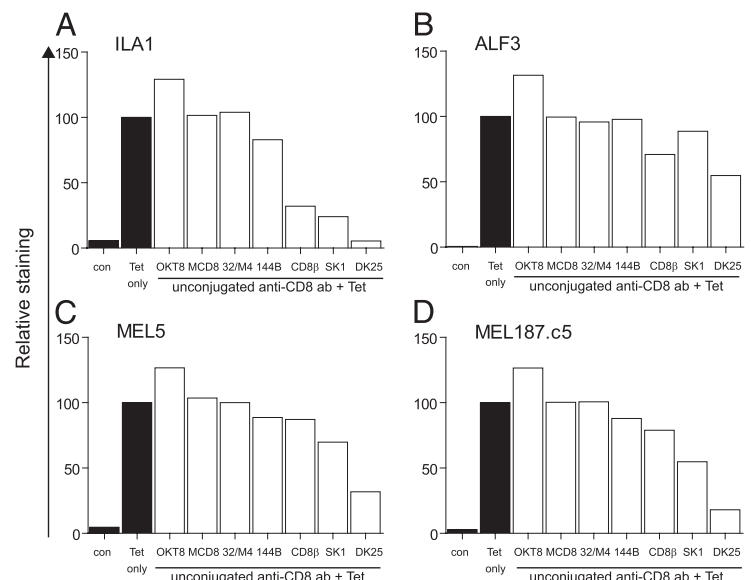


FIGURE 6. PHA/IL-15 treatment does not alter the functional phenotype of anti-human CD8 Abs. PBMCs were harvested from healthy donors and cultured either without (A) or with (B, C) 1 $\mu\text{g/ml}$ PHA and 25 ng/ml IL-15 for 7 d and then washed and cultured overnight in R2 medium. A total of 5×10^4 PBMCs were then incubated for 18 h with each of the following individual anti-human CD8 Abs in parallel: 100 $\mu\text{g/ml}$ OKT8, 6.25 $\mu\text{g/ml}$ SK1, 50 $\mu\text{g/ml}$ MCD8, 100 $\mu\text{g/ml}$ 32/M4, 100 $\mu\text{g/ml}$ C8/144B, 25 $\mu\text{g/ml}$ DK25, and 100 $\mu\text{g/ml}$ 2ST8.5H7 (CD8 β). The anti-human CD3 Ab UCHT1 (10 $\mu\text{g/ml}$) served as a positive control. CD8⁺ T cell effector functions were measured by intracellular cytokine staining and surface CD107a mobilization as described in *Materials and Methods*. Data were acquired using a modified FACSAriaII flow cytometer and analyzed with FlowJo software. Results obtained by gating on either the CD3⁺CD4⁻ (A, B) or CD4⁺ (C) population are shown for a representative experiment ($n = 2$). Minor differences in background levels of CD8⁺ T cell activation were observed with the non-OKT8 anti-human CD8 Abs (A, B); this may reflect heterogeneity within the CD8⁺ PBMC population.

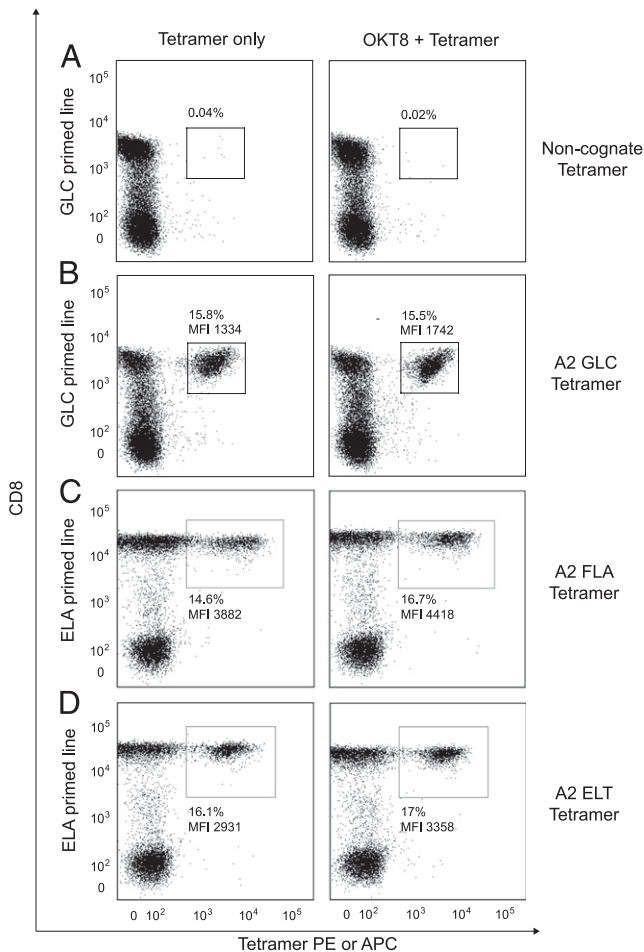


FIGURE 8. OKT8 enhances pMHC I tetramer staining in mixed cell populations. *A* and *B*, A total of 5×10^4 cells from a CD8⁺ T cell line primed with the EBV BMLF1-derived epitope GLCTLVAML (residues 280–288) were either mock treated or incubated with 100 μg/ml OKT8 at 4°C for 25 min and then stained with either noncognate HLA A*0201-ELAGIGILTV (*A*) or cognate HLA A*0201-GLCTLVAML (*B*) APC-conjugated tetramer (25 μg/ml each) at 37°C for 15 min. *C* and *D*, A total of 5×10^4 cells from a CD8⁺ T cell line primed with the Melan-A–derived epitope ELAGIGILTV (residues 26–35) were either mock treated or incubated with 100 μg/ml OKT8 at 4°C for 25 min and then stained with either HLA A*0201-FLAGIGILTV (*C*) or HLA A*0201-ELTIGIGILTV (*D*) PE-conjugated tetramer (25 μg/ml each) at 37°C for 15 min. Additional stains were performed as detailed in *Materials and Methods*. Data were acquired using a FACScan[™] flow cytometer and analyzed with FlowJo software.

PBMCs (Fig. 6C), consistent with previously discussed data (Supplemental Fig. 2).

OKT8 enhances pMHC I tetramer staining

Next, we tested the effects of anti-human CD8 Abs on the staining of ILA1, ALF3, MEL5, and MEL187.c5 CD8⁺ T cells with cognate pMHC I tetramers. Three anti-human CD8 Ab clones (SK1, DK25, and 2ST8.5H7) inhibited tetramer staining; clones MCD8, 32/M4, and C8/144B had little or no effect on staining. However, preincubation with OKT8 enhanced cognate pMHC I tetramer staining of all four CD8⁺ T cell clones (Fig. 7). Thus, OKT8 can enhance the binding of pMHC I tetramers in a range of systems. These findings suggested that OKT8 might facilitate the identification of Ag-specific CD8⁺ T cells within mixed cell populations. To test this idea, we examined pMHC I tetramer staining of CD8⁺ T cell lines raised against the HLA A*0201-restricted EBV BMLF1-derived epitope GLCTLVAML (residues 280–288). OKT8 enhanced the staining intensity of cognate CD8⁺ T cells with the relevant pMHC I tetramer without concomitant increases in noncognate HLA A*0201 tetramer binding (Fig. 8A, 8B). No increase in the percentage of tetramer⁺CD8⁺ cells was observed in the presence of OKT8 (Fig. 8A, 8B), which likely reflects the high-affinity TCR/pMHC I interactions that characterize antiviral CD8⁺ T cell populations (41).

We hypothesized that OKT8-mediated enhancement of TCR/pMHC I binding at the cell surface might have beneficial effects on pMHC I tetramer staining with low-affinity ligands, an effect that could prove very useful for the detection of CD8⁺ T cells with TCRs that bind weakly to cognate Ag, such as those that appear to predominate in anticancer and autoimmune responses (32, 41). To test this hypothesis, we used two monoclonal CD8⁺ T cell systems and a series of altered peptide ligands that vary in their affinity for cognate TCR by >5-fold (Table I, Supplemental Fig. 3). Preincubation with OKT8 enhanced staining efficiency with all variant pMHC I tetramers, including low-affinity variants (Table I, Supplemental Fig. 4). Consistent with this finding, OKT8 increased both the staining intensity and the percentage of Ag-specific events detected when CD8⁺ T cell lines raised against the HLA A*0201-restricted Melan-A–derived epitope ELAGIGILTV (residues 26–35) were stained with HLA A*0201 tetramers folded around the low-affinity peptide variants FLAGIGILTV or ELTIGIGILTV (Fig. 8C, 8D).

OKT8 enhances TCR/pMHC I on-rates at the cell surface

To examine how OKT8 enhances Ag binding at the CD8⁺ T cell surface in more detail, we examined the effects of this Ab on TCR/pMHC I kinetics using pMHC I tetramers. Differences in tetramer off-rates were minimal (data not shown). However, pretreatment of CD8⁺ T cells with OKT8 resulted in a significant increase in the TCR/pMHC I on-rate at the cell surface in each CD8⁺ T cell clone tested (Fig. 9). In contrast, DK25 inhibited pMHC I tetramer binding at the cell surface (Fig. 9B). OKT8 Ab-

Table I. OKT8 increases tetramer staining of MEL5 and MEL187.c5 CD8⁺ T-cells with low-affinity pMHC I ligands

Peptide ^a	MEL5 K _D (μM)	MEL5 Tetramer Only (MFI)	MEL5 OKT8 + Tetramer (MFI)	MEL187 K _D (μM)	MEL187 Tetramer Only (MFI)	MEL187 OKT8 + Tetramer (MFI)
ELAGIGILTV	17 ± 1	855	917	18 ± 1	353	418
<u>FL</u> AGIGILTV	92 ± 1	194	227	30 ± 2	300	373
EL <u>T</u> IGIGILTV	82 ± 4	36	87	37 ± 1	128	181
ELAGIGI <u>I</u> TV	77 ± 3	123	236	36 ± 3	195	257
<u>FL</u> AGIGI <u>I</u> TV	75 ± 3	367	426	47 ± 2	246	311

Summary of equilibrium binding analysis of MEL5 and MEL187.c5 TCRs with pMHC I variants and the effect of OKT8 on HLA A*0201 tetramer staining. Raw surface plasmon resonance data are shown in Supplemental Fig. 3; flow cytometry data are shown in Supplemental Fig. 4.

^aAmino acid residues marked in bold and underlined indicate substitutions made in the ELAGIGILTV peptide backbone.

MFI, median fluorescence intensity.

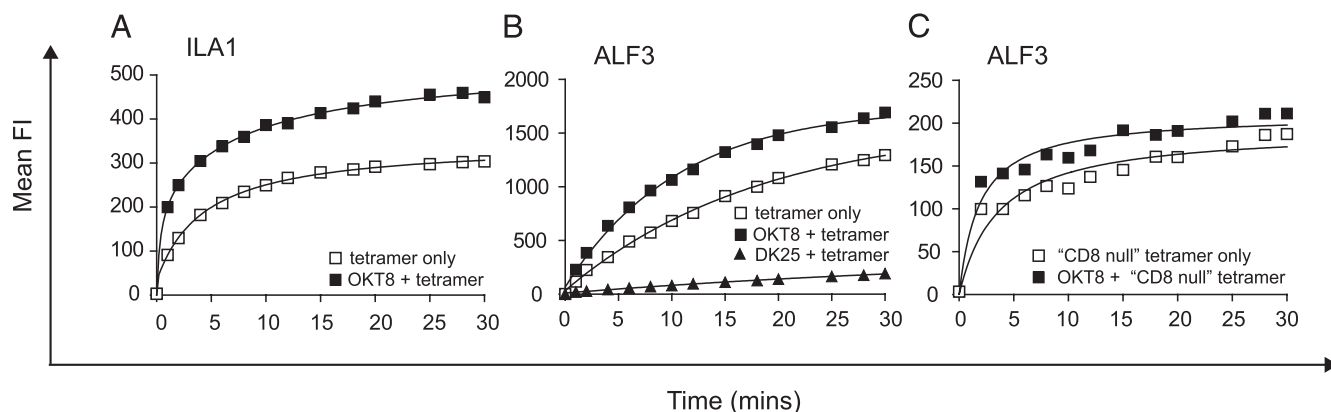


FIGURE 9. OKT8 increases TCR/pMHC I on-rates at the cell surface. A total of 5×10^5 ILA1 (A) or ALF3 (B, C) CD8⁺ T cells were removed from culture, washed twice, and resuspended in 100 μ l PBS with or without 100 μ g/ml OKT8 or 25 μ g/ml DK25 and then incubated at 4°C for 25 min. Cognate PE-conjugated HLA A*0201 tetramer was added in each case at 5 μ g/ml. At various time points as indicated, 12 μ l cell suspension was removed and acquired using a FACSCalibur flow cytometer and analyzed with FlowJo software. In C, the CD8-null (D227K/T228A) cognate HLA A*0201 tetramer was used (10).

induced enhancement of pMHC I tetramer on-rates was also apparent with CD8-null tetramers (Fig. 9C). Collectively, these data indicate that OKT8 enhances pMHC I tetramer staining by increasing the on-rate. Furthermore, OKT8 Ab-mediated augmentation of Ag binding at the cell surface occurs independently of the pMHC I/CD8 interaction.

OKT8 F(ab')₂ fragments can enhance tetramer staining and elicit CD8⁺ T cell effector function

Abs can be digested by papain or pepsin to produce Fab or F(ab')₂ fragments, respectively. These enzymatically generated fragments have been used extensively in the past to study the structure and function of Abs. We examined the ability of OKT8 Fab and F(ab')₂ fragments to enhance pMHC I tetramer staining and to induce chemokine release in the absence of TCR engagement. Not surprisingly, Fab fragments of OKT8 failed to activate CD8⁺ T cells or enhance pMHC I tetramer staining (Fig. 10A, 10B). Interestingly, however, OKT8 F(ab')₂ fragments retained some ability to enhance pMHC I tetramer staining and elicit chemokine release (Fig. 10A, 10B). OKT8-mediated effects were diminished by pepsin digestion, but anti-human CD3 Abs were similarly impaired functionally after digestion with this enzyme (Fig. 10C); this latter effect has been described previously (44–46). Thus, it

appears that intact OKT8 exerts effects on pMHC I tetramer binding and CD8⁺ T cell activation more efficiently than derived F(ab')₂ fragments. However, cellular activation by OKT8 F(ab')₂ fragments confirms that this effect is not entirely Fc' dependent.

Anti-mouse CD8 Abs can trigger CD8⁺ T cell effector function in the absence of TCR engagement

To extend these findings beyond human systems (summarized in Table II), we examined the effects of the anti-mouse CD8 α Ab CT-CD8a and the anti-mouse CD8 β Ab CT-CD8b on pMHC I tetramer staining and CD8⁺ T cell activation in the absence of TCR engagement. We observed that CT-CD8a inhibited tetramer staining of mouse transgenic F5 CD8⁺ T cells, whereas CT-CD8b enhanced tetramer staining of the same Ag-specific population (Fig. 11A). These results are consistent with our previous findings (26). Interestingly, despite opposing effects on pMHC I tetramer staining, both of these anti-mouse CD8 Abs induced MIP1 β production in the absence of TCR engagement from both naive and Ag-exposed F5 CD8⁺ T cells (Fig. 11B; data not shown). These effects were shown to be CD8-specific and occurred in the absence of any concomitant IL-2 release (data not shown). The anti-mouse CD8 Abs 53.6.7 and KT112 both enhanced pMHC I tetramer staining and induced small amounts of MIP1 β production

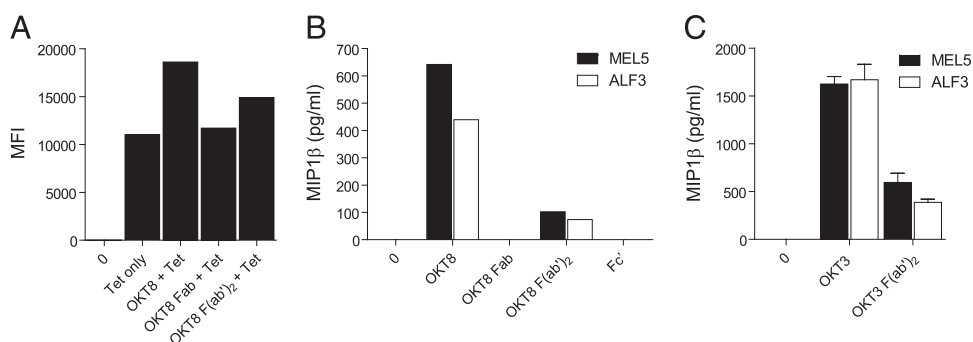


FIGURE 10. OKT8 F(ab')₂ fragments can enhance pMHC I tetramer staining and elicit CD8⁺ T cell effector function. A, A total of 5×10^4 MEL5 CD8⁺ T cells were either mock treated or incubated with 100 μ g/ml OKT8, 100 μ g/ml OKT8 Fab, or 100 μ g/ml OKT8 F(ab')₂ and then stained with PE-conjugated HLA A*0201-ELAGIGILTV tetramer (25 μ g/ml) as described in *Materials and Methods*. Data were acquired using a FACSCantoII flow cytometer and analyzed with FlowJo software. B, A total of 3×10^4 MEL5 or ALF3 CD8⁺ T cells were incubated with 100 μ g/ml OKT8, 100 μ g/ml OKT8 Fab, 100 μ g/ml OKT8 F(ab')₂, or 100 μ g/ml OKT8 Fc' for 18 h. Supernatant was harvested and assayed for MIP1 α , MIP1 β , and RANTES by ELISA. C, A total of 3×10^4 MEL5 or ALF3 CD8⁺ T cells were incubated with either 10 μ g/ml OKT3 or 10 μ g/ml OKT3 F(ab')₂ for 18 h. Supernatant was harvested and assayed for MIP1 α , MIP1 β , and RANTES by ELISA (only MIP1 β is shown). Data in A–C are representative of three separate experiments. Error bars represent SDs.

Table II. The heterogeneity of anti-human CD8 Abs

Ab Clone	α or β	Tetramer Binding	MIP1β	MIP1α	RANTES	IFN-γ	TNF-α	IL-2	Cytotoxicity
OKT8	α	Enhance	Yes	Yes	Yes	No ^a	No ^a	No	Yes
SK1	α	Inhibit	No	No	No	No	No	No	No
MCD8	α	Neutral	No	No	No	No	No	No	No
32/M4	α	Neutral	No	No	No	No	No	No	No
C8/144B	α	Neutral	No	No	No	No	No	No	No
DK25	α	Inhibit	No	No	No	No	No	No	No
2ST8.5H7	β	Inhibit	No	No	No	No	No	No	No

Summary of the effects exerted by anti-human CD8 Abs on pMHC1 tetramer binding and CD8⁺ T cell activation in the absence of TCR engagement.

^aOKT8 was shown to elicit IFN-γ and TNF-α release by SB10 and PHA/IL-15-stimulated PBMC.

(Fig. 11A, 11B). Thus, three different phenotypes were identified within a panel of four different anti-mouse CD8 Abs (Table III), which further underscores the considerable heterogeneity that exists within this group of reagents.

Discussion

Anti-CD8 Abs are integral to the flow cytometric detection of pMHC1-restricted T cells and have been used extensively in the past to identify an important role for CD8 in CD8⁺ T cell activation (16–18, 20). Most studies have concluded that anti-CD8 Abs inhibit the recognition of cognate Ag (19, 21, 22). Furthermore, a recent study provided evidence that a single anti-CD8 Ab could deliver a negative signal to a CD8⁺ T cell clone in the absence of cognate Ag (29). In contrast, however, earlier studies concluded that anti-CD8 Abs could activate CD8⁺ T cells (25, 27). Thus, contradictory effects of Ab-mediated CD8 ligation have been reported and the overall picture remains unclear. To clarify this issue, we examined the ability of seven different monoclonal anti-human CD8 Abs to activate six different human CD8⁺ T cell clones specific for a total of five different pMHC1 Ags.

In the absence of cognate Ag, the anti-human CD8 Ab OKT8 induced chemokine release from all six human CD8⁺ T cell clones tested and cytotoxic activity in all four human CD8⁺ T cell clones tested (Figs. 1–3). Interestingly, this activation appeared to occur

in the absence of any detectable cytokine release, with the exception of CD8⁺ T cell clone SB10, which released IFN-γ (Fig. 4). It is well established that a hierarchy of CD8⁺ T cell effector functions exists with respect to Ag sensitivity (47, 48); thus, each function exhibits a distinct activation threshold that must be exceeded for triggering to occur. Our findings suggest that OKT8 delivers a positive signal to CD8⁺ T cells that is generally sufficient to exceed the activation threshold required for chemokine release and cytotoxic activity but is not sufficient to trigger cytokine release in the majority of CD8⁺ T cells. In contrast to OKT8, the anti-human CD8α Ab clones SK1, MCD8, C8/144B, 32/M4, and DK25 as well as the anti-human CD8β Ab clone 2ST8.5H7 did not induce any measurable T cell effector functions in the absence of cognate Ag. It was not possible to reverse the phenotype of the nonactivating anti-human CD8 Abs by secondary Ab-mediated cross-linking or PHA/IL-15 treatment (Figs. 5, 6). Thus, we conclude that anti-CD8 Abs can exert differential effects on CD8⁺ T cells. These findings help to reconcile disparate observations and suggest that previous reports in the literature may not be intrinsically contradictory but rather reflective of the considerable heterogeneity that characterizes the ability of anti-CD8 Abs to induce CD8⁺ T cell effector function.

Anti-CD8 Ab-mediated activation of CD8⁺ T cells is consistent with a recent report, in which we demonstrated that MHC1 molecules with superenhanced CD8 binding properties can also activate CD8⁺ T cells in the absence of a specific TCR/pMHC1 interaction (49). Furthermore, thymus leukemia Ag interacts strongly ($K_D = 12 \mu\text{M}$) with cell surface CD8αα expressed by intraepithelial lymphocytes and can modulate T cell responses independently of the TCR (50–52). These studies all demonstrate that the engagement of CD8 in the absence of cognate Ag binding to the TCR can activate CD8⁺ T cells and, collectively, underscore the importance of CD8 in T cell signaling.

To examine the effects of OKT8 on Ag binding at the CD8⁺ T cell surface, we used soluble pMHC1 tetramer technology, which has transformed the study of Ag-specific CD8⁺ T cells by

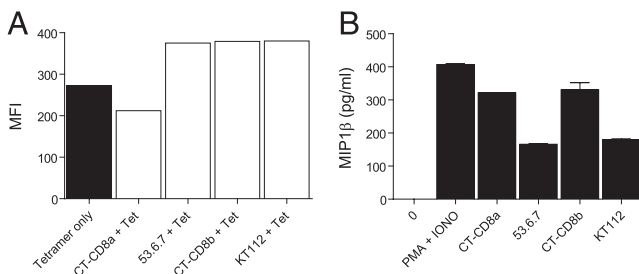


FIGURE 11. Anti-mouse CD8 Abs can exhibit the same phenotype as OKT8. *A*, A total of 5×10^4 naive mouse transgenic F5 T cells were either mock treated or incubated with 100 μg/ml CT-CD8a, 100 μg/ml 53.6.7, 100 μg/ml CT-CD8b, or 100 μg/ml KT112 at 4°C for 25 min and then stained with cognate PE-conjugated H-2D^b tetramer (25 μg/ml) as described in *Materials and Methods*. No staining was observed under any of the conditions shown with a control H-2D^b tetramer folded around the lymphocytic choriomeningitis virus GP1-derived epitope KAVYNFATC (residues 33–41). Data were acquired using a modified FACSAriaII flow cytometer and analyzed with FlowJo software. Results were obtained by gating on the CD3⁺CD4⁻ population. *B*, A total of 3×10^4 naive mouse transgenic F5 T cells were incubated at 37°C for 18 h with 100 μg/ml CT-CD8a, 100 μg/ml 53.6.7, 100 μg/ml CT-CD8b, 100 μg/ml KT112, or 50 ng/ml PMA and 1 μg/ml ionomycin. Supernatants were harvested and assayed for MIP1β by ELISA. Error bars represent SDs.

Table III. The heterogeneity of anti-mouse CD8 Abs

Ab Clone	α or β	Tetramer Binding	MIP1β	IFN-γ	IL-2
CT-CD8a	α	Inhibit	Yes	No	No
53.6.7	α	Enhance	Weak ^a	No	No
CT-CD8b	β	Enhance	Yes	No	No
KT112	β	Enhance	Weak	NT	NT

Summary of the effects exerted by anti-mouse CD8 Abs on pMHC1 tetramer binding and CD8⁺ T cell activation in the absence of TCR engagement.

^a53.6.7 elicited low levels of MIP1β production from naive F5 CD8⁺ T cells, F5 CD8⁺ T cell lines, and blasted BALB/c CD8⁺ cells but not from naive BALB/c CD8⁺ cells (data not shown).

NT, not tested.

enabling their visualization, enumeration, phenotypic characterization, and isolation from ex vivo samples. Preincubation with OKT8 enhanced the capture of cognate pMHC I tetramers from solution and produced higher intensity staining (Figs. 7–9). Accordingly, OKT8 enhanced the identification of CD8⁺ T cells with low-affinity TCR/pMHC I interactions (Fig. 8, Table I), such as those that typically predominate in tumor-specific and autoimmune responses (41). The other anti-CD8 Abs examined in this study either exerted inhibitory effects on pMHC I tetramer binding (SK1, DK25, and 2ST8.5H7) or displayed no biologically significant activity in this regard (MCD8, 32/M4, and C8/144B). Thus, OKT8 can be used as a tool to improve pMHC I tetramer staining; this property may be especially useful in the context of low-avidity Ag-specific CD8⁺ T cell populations.

The findings described above suggest that OKT8 has properties that are distinct from other anti-human CD8 Abs. Furthermore, these properties are not entirely Fc' dependent (Fig. 10). To extend these results, we conducted additional experiments with the anti-mouse CD8 α Ab CT-CD8a and the anti-mouse CD8 β Ab CT-CD8b. CT-CD8a was shown to inhibit pMHC I tetramer staining, whereas CT-CD8b enhanced pMHC I tetramer binding, consistent with a previous report (26). Despite their differential effects on pMHC I tetramer binding, both of these anti-mouse CD8 Abs activated CD8⁺ T cells efficiently (Fig. 11). These results demonstrate that the ability of anti-CD8 Abs to elicit CD8⁺ T cell effector function does not always correlate with their effect on pMHC I tetramer staining. This lack of correspondence was further supported by the identification of a third phenotype in the mouse system. The anti-mouse CD8 α Ab 53.6.7 and the anti-mouse CD8 β Ab KT112 both enhanced pMHC I tetramer staining but only activated CD8⁺ T cells weakly (Table III). Taken together, these data further underline the heterogeneity that exists within this group of reagents.

The mechanism by which anti-CD8 Abs exert either inhibitory or stimulatory effects on pMHC I recognition remains elusive. Previous studies have shown that anti-CD8 Abs retain their effects in the absence of a pMHC I/CD8 interaction (26, 53–55). In this study, we confirm that the enhancing effects of OKT8 on HLA A*0201 tetramer on-rate at the cell surface are still apparent in the context of CD8-null MHC I molecules (Fig. 9); thus, these effects are independent of any interaction between pMHC I and CD8. Subtle local rearrangements of the TCR relative to CD8 on pMHC I engagement are required for optimal CD8⁺ T cell activation (56, 57). By extension, it seems likely that anti-CD8 Abs exert their effects by interfering with, or enhancing, this surface receptor topology. The observation that anti-CD4 Abs can block cell surface intermolecular interactions essential for calcium flux and inhibit subsequent synapse formation is consistent with this hypothesis (58). Furthermore, we have previously demonstrated that anti-CD4 Abs can interfere with pMHC II tetramer binding even though the pMHC II/CD4 interaction does not stabilize TCR/pMHC II interactions (59).

In summary, we have shown the following: 1) heterogeneity exists in the ability of anti-CD8 Abs to activate CD8⁺ T cells; 2) Ab-mediated ligation of CD8 in the absence of TCR engagement can induce chemokine release and cytotoxic activity, largely in the absence of cytokine release; 3) the anti-human CD8 Ab OKT8 can enhance pMHC I tetramer staining; and 4) anti-mouse CD8 Abs (CT-CD8a and CT-CD8b) can activate CD8⁺ T cells in the absence of TCR engagement despite differential effects on pMHC I tetramer staining. Thus, anti-CD8 Abs can have potent effects on TCR/pMHC I binding kinetics and activation. These effects vary according to the Ab clone under investigation and should be taken into account when interpreting studies using these reagents. Fur-

thermore, the ability of Ab-mediated CD8 engagement to activate CD8⁺ T cells underscores the importance of coreceptor function in CD8⁺ T cell signaling.

Acknowledgments

We thank Rose Zamoyska for helpful discussions and provision of the KT112 hybridoma. We also thank Katherine Adams for the MEL187.c5 CD8⁺ T cell clone.

Disclosures

The authors have no financial conflicts of interest.

References

- Gao, G. F., J. Tormo, U. C. Gerth, J. R. Wyer, A. J. McMichael, D. I. Stuart, J. I. Bell, E. Y. Jones, and B. K. Jakobsen. 1997. Crystal structure of the complex between human CD8 α (α) and HLA-A2. *Nature* 387: 630–634.
- Gao, G. F., Z. Rao, and J. I. Bell. 2002. Molecular coordination of $\alpha\beta$ T-cell receptors and coreceptors CD8 and CD4 in their recognition of peptide-MHC ligands. *Trends Immunol.* 23: 408–413.
- Wooldridge, L., H. A. van den Berg, M. Glick, E. Gostick, B. Laugel, S. L. Hutchinson, A. Milicic, J. M. Brenchley, D. C. Douek, D. A. Price, and A. K. Sewell. 2005. Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface. *J. Biol. Chem.* 280: 27491–27501.
- Gakamsky, D. M., I. F. Luescher, A. Pramanik, R. B. Kopito, F. Lemonnier, H. Vogel, R. Rigler, and I. Pecht. 2005. CD8 kinetically promotes ligand binding to the T-cell antigen receptor. *Biophys. J.* 89: 2121–2133.
- van den Berg, H. A., L. Wooldridge, B. Laugel, and A. K. Sewell. 2007. Coreceptor CD8-driven modulation of T cell antigen receptor specificity. *J. Theor. Biol.* 249: 395–408.
- Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* 55: 301–308.
- Barber, E. K., J. D. Dasgupta, S. F. Schlossman, J. M. Trevilan, and C. E. Rudd. 1989. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc. Natl. Acad. Sci. USA* 86: 3277–3281.
- Zamoyska, R., P. Derham, S. D. Gorman, P. von Hoegen, J. B. Bolen, A. Veillette, and J. R. Parnes. 1989. Inability of CD8 α' polypeptides to associate with p56lck correlates with impaired function in vitro and lack of expression in vivo. *Nature* 342: 278–281.
- Chalupny, N. J., J. A. Ledbetter, and P. Kavathas. 1991. Association of CD8 with p56lck is required for early T cell signalling events. *EMBO J.* 10: 1201–1207.
- Purbhoo, M. A., J. M. Boulter, D. A. Price, A. L. Vuidepot, C. S. Hourigan, P. R. Dunbar, K. Olson, S. J. Dawson, R. E. Phillips, B. K. Jakobsen, et al. 2001. The human CD8 coreceptor effects cytotoxic T cell activation and antigen sensitivity primarily by mediating complete phosphorylation of the T cell receptor ζ chain. *J. Biol. Chem.* 276: 32786–32792.
- Hutchinson, S. L., L. Wooldridge, S. Tafuro, B. Laugel, M. Glick, J. M. Boulter, B. K. Jakobsen, D. A. Price, and A. K. Sewell. 2003. The CD8 T cell coreceptor exhibits disproportionate biological activity at extremely low binding affinities. *J. Biol. Chem.* 278: 24285–24293.
- Arcaro, A., C. Grégoire, N. Boucheron, S. Stotz, E. Palmer, B. Malissen, and I. F. Luescher. 2000. Essential role of CD8 palmitoylation in CD8 coreceptor function. *J. Immunol.* 165: 2068–2076.
- Arcaro, A., C. Grégoire, T. R. Bakker, L. Baldi, M. Jordan, L. Goffin, N. Boucheron, F. Wurm, P. A. van der Merwe, B. Malissen, and I. F. Luescher. 2001. CD8 β endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56^{lck} complexes. *J. Exp. Med.* 194: 1485–1495.
- Wooldridge, L., B. Laugel, J. Ekeruche, M. Clement, H. A. van den Berg, D. A. Price, and A. K. Sewell. 2010. CD8 controls T cell cross-reactivity. *J. Immunol.* 185: 4625–4632.
- Laugel, B., D. A. Price, A. Milicic, and A. K. Sewell. 2007. CD8 exerts differential effects on the deployment of cytotoxic T lymphocyte effector functions. *Eur. J. Immunol.* 37: 905–913.
- Normant, A. M., R. D. Salter, P. Parham, V. H. Engelhard, and D. R. Littman. 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* 336: 79–81.
- Shinohara, N., and D. H. Sachs. 1979. Mouse alloantibodies capable of blocking cytotoxic T-cell function. I. Relationship between the antigen reactive with blocking antibodies and the Lyt-2 locus. *J. Exp. Med.* 150: 432–444.
- Nakayama, E., H. Shiku, E. Stockert, H. F. Oettgen, and L. J. Old. 1979. Cytotoxic T cells: Lyt phenotype and blocking of killing activity by Lyt antisera. *Proc. Natl. Acad. Sci. USA* 76: 1977–1981.
- Janeway, C. A. Jr. 1992. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu. Rev. Immunol.* 10: 645–674.
- Miceli, M. C., and J. R. Parnes. 1993. Role of CD4 and CD8 in T cell activation and differentiation. *Adv. Immunol.* 53: 59–122.
- MacDonald, H. R., A. L. Glasebrook, C. Bron, A. Kelso, and J. C. Cerottini. 1982. Clonal heterogeneity in the functional requirement for Lyt-2/3 molecules

- on cytolytic T lymphocytes (CTL): possible implications for the affinity of CTL antigen receptors. *Immunol. Rev.* 68: 89–115.
22. MacDonald, H. R., A. L. Glasebrook, and J. C. Cerottini. 1982. Clonal heterogeneity in the functional requirement for Lyt-2/3 molecules on cytolytic T lymphocytes: analysis by antibody blocking and selective trypsinization. *J. Exp. Med.* 156: 1711–1722.
 23. Clark, S. J., D. A. Law, D. J. Paterson, M. Puklavec, and A. F. Williams. 1988. Activation of rat T lymphocytes by anti-CD2 monoclonal antibodies. *J. Exp. Med.* 167: 1861–1872.
 24. Lühder, F., Y. Huang, K. M. Dennehy, C. Guntermann, I. Müller, E. Winkler, T. Kerkau, S. Ikemizu, S. J. Davis, T. Hanke, and T. Hünig. 2003. Topological requirements and signaling properties of T cell-activating, anti-CD28 antibody superagonists. *J. Exp. Med.* 197: 955–966.
 25. Veillette, A., J. C. Zúñiga-Pflücker, J. B. Bolen, and A. M. Kruisbeek. 1989. Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways. *J. Exp. Med.* 170: 1671–1680.
 26. Wooldridge, L., S. L. Hutchinson, E. M. Choi, A. Lissina, E. Jones, F. Mirza, P. R. Dunbar, D. A. Price, V. Cerundolo, and A. K. Sewell. 2003. Anti-CD8 antibodies can inhibit or enhance peptide-MHC class I (pMHC1) multimer binding: this is paralleled by their effects on CTL activation and occurs in the absence of an interaction between pMHC1 and CD8 on the cell surface. *J. Immunol.* 171: 6650–6660.
 27. Tomonari, K., and S. Spencer. 1990. Epitope-specific binding of CD8 regulates activation of T cells and induction of cytotoxicity. *Int. Immunol.* 2: 1189–1194.
 28. Grebe, K. M., R. L. Clarke, and T. A. Potter. 2004. Ligand of CD8 leads to apoptosis of thymocytes that have not undergone positive selection. *Proc. Natl. Acad. Sci. USA* 101: 10410–10415.
 29. Abidi, S. H., T. Dong, M. T. Vuong, V. B. Sreenu, S. L. Rowland-Jones, E. J. Evans, and S. J. Davis. 2008. Differential remodeling of a T-cell transcriptome following CD8- versus CD3-induced signaling. *Cell Res.* 18: 641–648.
 30. Laugel, B., H. A. van den Berg, E. Gostick, D. K. Cole, L. Wooldridge, J. Boulter, A. Milicic, D. A. Price, and A. K. Sewell. 2007. Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. *J. Biol. Chem.* 282: 23799–23810.
 31. Purbhoo, M. A., Y. Li, D. H. Sutton, J. E. Brewer, E. Gostick, G. Bossi, B. Laugel, R. Moyssey, E. Baston, N. Liddy, et al. 2007. The HLA A*0201-restricted hTERT(540–548) peptide is not detected on tumor cells by a CTL clone or a high-affinity T-cell receptor. *Mol. Cancer Ther.* 6: 2081–2091.
 32. Cole, D. K., E. S. Edwards, K. K. Wynn, M. Clement, J. J. Miles, K. Ladell, J. Ekeruche, E. Gostick, K. J. Adams, A. Skowera, et al. 2010. Modification of MHC anchor residues generates heteroclitic peptides that alter TCR binding and T cell recognition. *J. Immunol.* 185: 2600–2610.
 33. Argaet, V. P., C. W. Schmidt, S. R. Burrows, S. L. Silins, M. G. Kurilla, D. L. Doolan, A. Suhrbier, D. J. Moss, E. Kieff, T. B. Sculley, and I. S. Misko. 1994. Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus. *J. Exp. Med.* 180: 2335–2340.
 34. Green, K. J., J. J. Miles, J. Tellam, W. J. van Zuylen, G. Connolly, and S. R. Burrows. 2004. Potent T cell response to a class I-binding 13-mer viral epitope and the influence of HLA micropolymorphism in controlling epitope length. *Eur. J. Immunol.* 34: 2510–2519.
 35. Lissina, A., K. Ladell, A. Skowera, M. Clement, E. Edwards, R. Seggewiss, H. A. van den Berg, E. Gostick, K. Gallagher, E. Jones, et al. 2009. Protein kinase inhibitors substantially improve the physical detection of T-cells with peptide-MHC tetramers. *J. Immunol. Methods* 340: 11–24.
 36. Mamalaki, C., J. Elliott, T. Norton, N. Yannoutsos, A. R. Townsend, P. Chandler, E. Simpson, and D. Kiousis. 1993. Positive and negative selection in transgenic mice expressing a T-cell receptor specific for influenza nucleoprotein and endogenous superantigen. *Dev. Immunol.* 3: 159–174.
 37. Wooldridge, L., A. Lissina, J. Vernazza, E. Gostick, B. Laugel, S. L. Hutchinson, F. Mirza, P. R. Dunbar, J. M. Boulter, M. Glick, et al. 2007. Enhanced immunogenicity of CTL antigens through mutation of the CD8 binding MHC class I invariant region. *Eur. J. Immunol.* 37: 1323–1333.
 38. Betts, M. R., J. M. Brechley, D. A. Price, S. C. De Rosa, D. C. Douek, M. Roederer, and R. A. Koup. 2003. Sensitive and viable identification of antigen-specific CD8⁺ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* 281: 65–78.
 39. Wooldridge, L., A. Lissina, D. K. Cole, H. A. van den Berg, D. A. Price, and A. K. Sewell. 2009. Tricks with tetramers: how to get the most from multimeric peptide-MHC. *Immunology* 126: 147–164.
 40. Li, Y., R. Moyssey, P. E. Molloy, A. L. Vuidepot, T. Mahon, E. Baston, S. Dunn, N. Liddy, J. Jacob, B. K. Jakobsen, and J. M. Boulter. 2005. Directed evolution of human T-cell receptors with picomolar affinities by phage display. *Nat. Biotechnol.* 23: 349–354.
 41. Cole, D. K., N. J. Pumphrey, J. M. Boulter, M. Sami, J. I. Bell, E. Gostick, D. A. Price, G. F. Gao, A. K. Sewell, and B. K. Jakobsen. 2007. Human TCR-binding affinity is governed by MHC class restriction. *J. Immunol.* 178: 5727–5734.
 42. Cole, D. K., F. Yuan, P. J. Rizkallah, J. J. Miles, E. Gostick, D. A. Price, G. F. Gao, B. K. Jakobsen, and A. K. Sewell. 2009. Germ line-governed recognition of a cancer epitope by an immunodominant human T-cell receptor. *J. Biol. Chem.* 284: 27281–27289.
 43. Wyer, J. R., B. E. Willcox, G. F. Gao, U. C. Gerth, S. J. Davis, J. I. Bell, P. A. van der Merwe, and B. K. Jakobsen. 1999. T cell receptor and coreceptor CD8 α bind peptide-MHC independently and with distinct kinetics. *Immunity* 10: 219–225.
 44. Woodle, E. S., J. R. Thistlethwaite, I. A. Ghoobrial, L. K. Jolliffe, F. P. Stuart, and J. A. Bluestone. 1991. OKT3 F(ab')₂ fragments—retention of the immunosuppressive properties of whole antibody with marked reduction in T cell activation and lymphokine release. *Transplantation* 52: 354–360.
 45. Herold, K. C., J. B. Burton, F. Francois, E. Poumian-Ruiz, M. Glandt, and J. A. Bluestone. 2003. Activation of human T cells by FeR nonbinding anti-CD3 mAb, hOKT3 γ 1(Ala-Ala). *J. Clin. Invest.* 111: 409–418.
 46. Chatenoud, L., and J. A. Bluestone. 2007. CD3-specific antibodies: a portal to the treatment of autoimmunity. *Nat. Rev. Immunol.* 7: 622–632.
 47. Valitutti, S., S. Müller, M. Dessing, and A. Lanzavecchia. 1996. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. *J. Exp. Med.* 183: 1917–1921.
 48. Price, D. A., A. K. Sewell, T. Dong, R. Tan, P. J. Goulder, S. L. Rowland-Jones, and R. E. Phillips. 1998. Antigen-specific release of β -chemokines by anti-HIV-1 cytotoxic T lymphocytes. *Curr. Biol.* 8: 355–358.
 49. Wooldridge, L., M. Clement, A. Lissina, E. S. Edwards, K. Ladell, J. Ekeruche, R. E. Hewitt, B. Laugel, E. Gostick, D. K. Cole, et al. 2010. MHC class I molecules with superenhanced CD8 binding properties bypass the requirement for cognate TCR recognition and nonspecifically activate CTLs. *J. Immunol.* 184: 3357–3366.
 50. Leishman, A. J., O. V. Naidenko, A. Attinger, F. Koning, C. J. Lena, Y. Xiong, H. C. Chang, E. Reinherz, M. Kronenberg, and H. Cheroutre. 2001. T cell responses modulated through interaction between CD8 α and the nonclassical MHC class I molecule, TL. *Science* 294: 1936–1939.
 51. Tsujimura, K., Y. Obata, E. Kondo, K. Nishida, Y. Matsudaira, Y. Akatsuka, K. Kuzushima, and T. Takahashi. 2003. Thymus leukemia antigen (TL)-specific cytotoxic T lymphocytes recognize the α 1/ α 2 domain of TL free from antigenic peptides. *Int. Immunol.* 15: 1319–1326.
 52. Cole, D. K., and G. F. Gao. 2004. CD8: adhesion molecule, co-receptor and immuno-modulator. *Cell. Mol. Immunol.* 1: 81–88.
 53. Van Seventer, G. A., R. A. Van Lier, H. Spits, P. Ivanyi, and C. J. Melief. 1986. Evidence for a regulatory role of the T8 (CD8) antigen in antigen-specific and anti-T3-(CD3)-induced lytic activity of allospecific cytotoxic T lymphocyte clones. *Eur. J. Immunol.* 16: 1363–1371.
 54. Hoo, W. S., and D. M. Kranz. 1993. Role of CD8 in staphylococcal enterotoxin B-mediated lysis by cytotoxic T lymphocytes. *J. Immunol.* 150: 4331–4337.
 55. Campanelli, R., B. Palermo, S. Garbelli, S. Mantovani, P. Lucchi, A. Necker, E. Lantelme, and C. Giachino. 2002. Human CD8 co-receptor is strictly involved in MHC-peptide tetramer-TCR binding and T cell activation. *Int. Immunol.* 14: 39–44.
 56. Lee, P. U., and D. M. Kranz. 2003. Allogeneic and syngeneic class I MHC complexes drive the association of CD8 and TCR on 2C T cells. *Mol. Immunol.* 39: 687–695.
 57. Block, M. S., A. J. Johnson, Y. Mendez-Fernandez, and L. R. Pease. 2001. Monomeric class I molecules mediate TCR/CD3 ϵ /CD8 interaction on the surface of T cells. *J. Immunol.* 167: 821–826.
 58. Krummel, M. F., M. D. Sjaastad, C. Wülfing, and M. M. Davis. 2000. Differential clustering of CD4 and CD3 ζ during T cell recognition. *Science* 289: 1349–1352.
 59. Wooldridge, L., T. J. Scriba, A. Milicic, B. Laugel, E. Gostick, D. A. Price, R. E. Phillips, and A. K. Sewell. 2006. Anti-coreceptor antibodies profoundly affect staining with peptide-MHC class I and class II tetramers. *Eur. J. Immunol.* 36: 1847–1855.