Soluble MHC-peptide complexes induce apoptosis

SOLUBLE MAJOR HISTOCOMPATIBILITY COMPLEX-PEPTIDE OCTAMERS
WITH IMPAIRED CD8 BINDING SELECTIVELY INDUCE FAS-DEPENDENT
APOPTOSIS

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SUMMARY

Fluorescent labeled soluble major histocompatibility complex class I–peptide “tetramers” constitute a powerful tool to detect and isolate antigen-specific CD8⁺ T cells by flow cytometry. Conventional “tetramers” are prepared by refolding of heavy and light chains with a specific peptide, enzymatic biotinylation at an added C-terminal biotinylation sequence and “tetramerization” by reaction with phycoerythrin or allophycocyanin labeled avidin derivatives. We show here that such preparations are heterogeneous and describe a new procedure that allows the preparation of homogeneous tetra- or octameric major histocompatibility complex – peptide complexes. These compounds were tested on T1 cytotoxic T lymphocytes, which recognize the PbCS peptide 252-260 (SYIPSAEKI) containing photoreactive 4-azidobenzoic acid on K259 in the context of H-2Kᵈ. We report that mutation of the CD8 binding site of Kᵈ greatly impairs the binding of tetrameric, but not of octameric or multimeric Kᵈ-PbCS(ABA) complexes to cytotoxic T lymphocytes. This mutation abolishes the octamer’s ability to elicit significant phosphorylation of CD3, intracellular calcium mobilization and cytotoxic T lymphocyte degranulation. Remarkably, however, this octamer activates efficiently cytotoxic T lymphocytes for Fas (CD95)-dependent apoptosis.
INTRODUCTION

CD8⁺ T cells and thymocytes recognize with their T cell antigen receptor (TCR)¹ cognate MHC-peptide complexes on the surface of antigen presenting cells (1). CD8 by binding to the constant domain of MHC class I molecules can increase the avidity of TCR-ligand binding, but also act as an adhesion molecule and strengthen CTL – target cell conjugate formation (1-3). The coordinate binding of CD8 to TCR-associated MHC molecules brings CD8-associated Lck to TCR/CD3, which promotes tyrosine phosphorylation of its immunoreceptor tyrosine-based activation motifs (ITAM), which is an initial crucial event of TCR-mediated T cell activation (1-4). The CD8 binding site on MHC class I molecules contains an acidic loop (residues 222-229) and charge inversion in position 227 (e.g. KdD227K), impairs CD8 binding by about 85% (3, 4).

The recognition of sensitized target cells by CD8⁺ CTL involves rapid and avid conjugate formation, followed by CTL degranulation in the contact zone, resulting in perforin/granzyme-mediated target cell killing (5). With a slower kinetic CTL also express surface FasL, which by interacting with Fas on other cells induces Fas-mediated cytotoxicity (5, 6). Although perforin and Fas-mediated cytotoxicity are both induced by TCR triggering, the activation requirements are very different. For example, certain altered peptide ligands that are unable to elicit perforin-dependent cytotoxicity or cytokine production, can efficiently induce Fas-mediated killing (7, 8). We observed previously that blocking of CD8 greatly impairs calcium mobilization, degranulation of CTL and IFNγ release, but has no effect on Fas-dependent cytotoxicity (8). More recently it has been shown that antigen presenting cells or microspheres expressing MHC-peptide complexes with ablated CD8 binding, selectively induce Fas-mediated apoptosis of CTL (9).
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The idea to eradicate antigen-specific CD8+ T cells or thymocytes via Fas-dependent apoptosis is attractive, because it takes place in the absence of full, potentially harmful T cell activation. Since regarding in vivo application blocking of CD8 by antibodies or the use of MHC-peptide coated microspheres are risky, we investigated here whether soluble MHC-peptide complexes with impaired CD8 binding can be used to the same end.

Soluble fluorescent labeled MHC class I-peptide multimers, so called “tetramers”, are widely used for the detection and isolation of antigen-specific CD8+ T cells (10, 11). The conventional way to prepare such molecules involves enzymatic biotinylation of an added C-terminal biotinylation sequence (BSP) with the biotin ligase BirA (12). The biotinylated MHC-peptide monomers are then reacted with phycoerythrin (PE) or allophyco-cyanin labeled avidin derivatives. Although PE or allophyco-cyanin have very high fluorescence intensities, their conjugates with avidin are heterogeneous. Because of their large size, their conjugation with streptavidin yields ill defined mixtures of conjugates having different stochiometries and configurations (the Mr of PE is about 240 kDa, of allophyco-cyanin about 104 kDa and of streptavidin about 60 kDa). By consequence saturation of such conjugates with biotinylated MHC-peptide complexes gives heterogeneous MHC-peptide complexes, referred to as multimers.

To prepare well defined soluble MHC class I-peptide complexes of different sizes, we introduced by point mutation a free cysteine at the C-terminus of the MHC heavy chain, which can be alkylated with biotin containing iodoacetamide or maleimide derivatives. For oligomerization we used homogeneous streptavidin conjugates containing the low molecular weight fluorochrome Cyochrome Cy5. By using a branched peptide containing one
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biotin and two maleimide moieties (DMGS), this strategy allows the preparation of octameric MHC class I-peptide complexes. Here we describe the preparation of well defined soluble tetrameric and octameric MHC-peptide complexes that co-engage or not CD8 and their ability to activate perforin and Fas-dependent cytotoxicity on cloned T1 CTL. T1 CTL recognize the PbCS peptide 252-260 (SYIPSAEKI) containing photoreactive 4-azidobenzoic acid on K259 (PbCS(ABA)) in the context of K\textsuperscript{d} (4, 13). We find that the binding of tetrameric, but not octameric or multimeric K\textsuperscript{d}\textsubscript{D}227K-PbCS(ABA) complexes to T1 CTL is greatly reduced when CD8 co-engagement is ablated by the charge inversion K\textsuperscript{d}\textsubscript{D}227K. Although K\textsuperscript{d}\textsubscript{D}227K-PbCS(ABA) complexes fail to elicit significant tyrosine phosphorylation, calcium mobilization and degranulation, they efficiently induced Fas-dependent cytotoxicity.
EXPERIMENTAL PROCEDURES

Chemicals - The branched DMGS-biotin peptide was synthesized using conventional solid phase Fmoc strategy. Biotin was introduced by using Fmoc-K(εAhx-biotin) (Bachem AG, Bubendorf, Switzerland). The deprotected GS-biotin peptide was purified by GFC on a Superdex peptide column (1 x 30 cm; Amersham Biosciences, Lausen, Switzerland) and reacted with N-γ-maleimidobutyryloxy succinimide ester (GMBS, Pierce Chemical Co, Rockford, IL) in DMSO containing 1% di-isopropyl-ethylamine. The resulting di-maleimide conjugate was purified by HPLC on a C18 reverse phase column (2 x 30 m, Machery & Nagel, Oensingen, Switzerland). The column was eluted with 0.1 TFA and a linear gradient of acetonitril rising in one hour from 0 to 75%. The DMGS-biotin peptide eluted at 26.4 min and the mono maleimide derivative at 25.3 min. All products had the correct molecular weight as assessed by mass spectrometry. Iodoacetyl-PEO-biotin was from Pierce and SYIPSAEK(ABA)I (PbCS(ABA)) was prepared by the conventional solid phase Fmoc strategy using Fmoc-Lys(ABA) (Bachem) following published procedures (13, 14).

Cells and antibodies - Cloned T1 CTL were cultured and used as described previously (13, 14). In brief, the CTL were re-stimulated weekly using γ-irradiated, PbCS(ABA) pulsed P815 cells and Balb/c feeder cells and DMEM supplemented with 5% fetal calf serum, 5 nM 2-mercaptoethanol and 30 U/ml of recombinant IL-2. Normal and Fas transfected mastocytoma P815 cells were cultured and used as described previously (8). The following antibodies were used: Cy5 labeled annexin V, and anti-Fas antibody were from Beckton and Dickinson Biosciences (Paolo Alta, California), anti-CD8β mAb H35 and anti-TCRβ mAb H57 from ATCC (American Type Culture Collection, Manassas, VA), anti-phospho-tyrosine (pY) mAb 4G10 was from Upstate Biologicals (Lake Placid, NY) and anti-CD3ε antibody M-20 from
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Santa Cruz Biotechnology (Santa Cruz, CA). All stainings for FACS were performed at 4 °C for 30 - 60 min.

Production of β2m and Kd-heavy chains containing a free cysteine - The cDNA encoding the Kd heavy chain was cloned into the pET3a vector by PCR amplification, using the primers: 5'-GCCATATGGGCCCATATTGCTGAG-3' (forward primer), and 5'-GCGGATCCTCAAGCCAGCTTCCATCTCA-3' (reverse primer). The restriction sites NdeI and BamHI used for cloning are underlined. A free cysteine was introduced by point mutation in position 273, 275, and 277 (R273C, K275C and A277C) of the Kd heavy chain, using the QuickChange mutagenesis kit (Stratagene). For PCR amplification the following primers were used: 5'-GAGCCTCTCACCCTGCTGGGAGCTCGTTGA-3' (forward primer) and 5'-TCAAGCCAGCTTCCAAGGATGGTGAGGCTTC-3' (reverse primer), 5'-CCTCTACCCTGAGATGGCTGGCTTGAGGATCCGGC-3' (forward primer) and GCCGGATCCTCAAGCCAGGAGCACCATAAGGCTTGAGAGG-3' (reverse primer), 5'-CCCTGAGATGGAAGCTGTGCTGCTGGAGATCCGGCTGC-3' (forward primer) and 5'-GCAGCCGGATCCTCAACACAGCTTCATCTCAGGG-3' (reverse primer). After digestion of the parental DNA template with Dpn I, the mutated strands were transformed in BL21 DE3 pLysS bacteria. For expression of recombinant Kd heavy chain and human β2m, BL21 DE3 pLysS bacteria were grown at 37 °C in Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin. The expression of the recombinant proteins was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) (1 mM final). The recombinant proteins were produced as insoluble inclusion bodies, and extracted as described (15). As judged by SDS-PAGE and Coomassie blue staining, the purity of both proteins was 80-90%. Aliquots of these solutions were stored at – 80%.
Refolding, purification and alkylation of K\textsuperscript{d}-peptide monomers - K\textsuperscript{d}-BSP heavy chain and human β2m were refolded in the presence of the peptide using the dilution method essentially as described (15). In brief, the K\textsuperscript{d} heavy chain and β2m containing urea solutions were added at 4 °C under agitation within two hours in a 100-fold larger volume of refolding buffer (100 mM Tris, pH 8.1, 400 mM L-arginine, 2 mM EDTA, 5 mM glutathion, 0.5 mM oxidized glutathion, 0.5 mM PMSF) containing 5 µM of the specific peptide. After stirring for 72 h at 4 °C, insoluble components were removed by filtration on 0.2 µm membrane filters (Nalgene) and the mixture concentrated about 40-fold on an Amicon ultrafiltration concentrator equipped with a polyethersulfone membrane (10 kDa cutoff)(Amicon). The concentrate was passed over a 26/10 desalting column (Amersham Biosciences) in Tris buffer (20 mM Tris; pH 8.0) and the K\textsuperscript{d}-PbCS(ABA) monomers were purified by anion exchange chromatography using a Source Q15 fast flow column (Amersham Biosciences). The column was eluted in the same buffer with a NaCl gradient rising in 60 min from 0 to 500 mM. The average refolding efficiency for the PbCS(ABA) peptide was 20 %, with 100% being the amount of K\textsuperscript{d} heavy chain invested. The purified K\textsuperscript{d}-peptide complexes were supplemented with 2 mM of glutathion, 5 mM EDTA and 2 µg/ml of β2m.

The enzymatic biotinylation of K\textsuperscript{d}-peptide-BSP was performed over night at 25 °C with ATP, biotin and the biotin ligases BirA as described (12). For alkylation the K\textsuperscript{d}-peptide complexes (1 mg/ml) were incubated in Tris buffer (20 mM Tris; pH 8.0, 150 mM NaCl) for 1 hour at 4 °C with 15 mM of glutathion to reduce the C-terminal free cysteine. After GFC over a Superdex S75 column (1 x 30 cm; Pharmacia), the reduced monomers were reacted in Tris buffer containing 5 mM EDTA with a 5-fold molar excess of alkylation reagent under argon overnight at 4 °C. The alkylated K\textsuperscript{d}-peptide complexes were purified by gel filtration on a Superdex S75 column. Dimeric K\textsuperscript{d}-DMGS-biotin complexes were obtained by reacting...
monomeric Kd-DMGS-biotin with a two-fold excess of Kd-cysteine monomers. Dimeric Kd-peptide complexes were separated from monomers by anion exchange chromatography on a Resource Q fast flow anion exchange column (Amersham-Pharmacia Biosciences). The biotinylation efficiency was determined by SDS-PAGE (15 % non-denaturing) of 5 µg aliquots of Kd-peptide complexes that were reacted or not with a two-fold molar excess of avidin (Molecular Probes). The Coomassie blue stained gels were evaluated by densitometry and the alkylation efficiency calculated in percent was as: (amount of Kd-peptide-avidin-)/(amount of Kd-peptide) x 100.

Preparation of fluorescent labeled Kd-peptide oligomers – Kd-peptide tetramers and octamers were obtained by reacting the biotinylated Kd-PbCS(ABA) complexes with Cy5 labeled streptavidin (Amersham Biosciences) and the multimers by reacting biotinylated Kd-PbCS(ABA) monomers with Extravidin-PE (Sigma Chemical Co.) at a molar ratio of 4 : 1. Labeled oligomers were purified by GFC on a Superdex 200 (Pharmacia) column (1 x 30 cm), which was eluted in Tris buffer (20 mM, pH 8.0, 150 mM NaCl) at a flow rate of 0.7 ml/min.

Kd-peptide oligomer binding assays - For binding studies T1 CTL were washed with OptiMEM (Gibco) containing 1 % BSA, 0.02 % sodium azide, 15 mM HEPES and 2 µg/ml of human β2m. The cells (0.5 x 10^6/ml) were incubated for 30 min with fluorescent labeled Kd-peptide complexes in 50 µl aliquots at 18 oC or 37 oC, washed once in cold medium and analyzed by flow cytometry on a FACS Calibur (Becton Dickinson, Erembodegen, Belgium).

Bystander cytolytic assay - T1 CTL (5 x 10^5 cells/ml) were incubated in DMEM (Gibco), supplemented with 2 µg/ml of human β2m and 10 µM of PbCS 252-260 peptide at 37 oC with 25 nM of Kd-PbCS(ABA) octamers. After three washes the CTL were incubated in 96
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well plates (15,000 cells/well) with $^{51}$Cr labeled P815 cells (5,000 cells/well) at 37 °C for 4 h. The specific chromium release in percent was calculated as: (experimental release – spontaneous release) / (total release – spontaneous release) x 100. The total release was the esterase content following lysis of the cells with 1% Triton X-100.

Esterase release - T1 CTL were adhered in 96 well plates previously coated with super fibronectin (Sigma) and incubated in Optimem medium (Gibco) supplemented with 2 µg/ml of human β2m and 10 µM of PbCS 252-260 peptide for 90 min with 25 nM of Kd-PbCS(ABA) octamer in the absence or presence of 100 nM concanamycine A (Sigma), anti-CD8β mAb H35 (10 µg/ml) or PbCS(ABA) peptide (1 µM). Released esterases were measured in the supernatants as described previously (16). All incubations were performed in triplicates.

Immunoprecipitation and Western blotting – T1 CTL (1 x 10^7 cells/ml) were incubated or not with Kd-PbCS(ABA) complexes (25 nM) for 3 min at 37 °C. After washing with chilled PBS the cells were lysed on ice for one hour in PBS containing Brij78 (1 %) and protease inhibitor cocktail (Roche, Rotkreuz, Switzerland) and from the detergent soluble fraction TCR/CD3 was immunoprecipitated with anti-TCR mAb H57. The immunoprecipitates were resolved on SDS-PAGE (15 %, reducing) and Western blotted using the anti-phosphotyrosine mAb 4G10 or anti-CD3ε antibody. For the detection the enhanced chemoluminicence (ECL) Western-blotting detection kit (Amersham Biosciences) was used as recommended by the supplier.

Confocal microscopy- T1 CTL were incubated with Cy5 labeled Kd-PbCS(ABA), KdD227K-PbCS(ABA) or Kd-Cw3 170-179 octamer for 30 min at 37°C or 18°C. After washing, cells
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were fixed with 3% paraformaldehyde for 10 min at room temperature and laid onto poly-L-lysine-coated slides for 10 min and mounted. Internalization of Cy5-conjugated octamer was analyzed by confocal microscopy on an Axiovert 100 microscope (LSM510, Carl Zeiss, Jena, Germany) with a 63 x oil objective. Cy5 fluorescence was measured upon excitation with neon/helium laser at 633 nm. Each image was the average of four scans. Digital images were prepared using Adobe Photoshop.

Apoptosis assay – T1 CTL were resuspended in DMEM supplemented with 5% fetal calf serum and 20 mM HEPES (0.5 x 10^6 cells/ml) and incubated in 50 µl aliquots at 37°C for 30 min with 25 nM of K^d^-PbCS(ABA), K^dD227K-PbCS(ABA) or K^d^-Cw3 170-179 octamers or left untreated. After one wash, the cells were incubated for 4.5 h in the same medium and stained with Cy5 labeled annexin V and analyzed by FACS.

Intracellular calcium mobilization. T1 CTL (1 x 10^6 cells/ml) were incubated with 5 µM Indo-1 (Sigma, Buchs, Switzerland) at 37°C for 45 min, washed and incubated at 37°C with 25 nM of K^d^-peptide octamers or medium and calcium dependent indo-1 fluorescence was measured by FACS on a FACStar™ as described (14).
RESULTS

Preparation of fluorescent labeled soluble K<sup>d</sup>-peptide complexes – We prepared and examined three different types of soluble K<sup>d</sup>-peptide complexes: multimers, tetramers and octamers. All complexes were produced either with wild type K<sup>d</sup> or K<sup>d</sup>D227K, which has greatly impaired CD8 binding (4). Monomeric K<sup>d</sup>-PbCS(ABA) complexes were obtained by refolding of different K<sup>d</sup> heavy chains and human β2m in the presence of PbCS(ABA) peptide. The refolding efficiency of K<sup>d</sup>-PbCS(ABA) complexes containing the heavy chain comprising residues 1-277 or the heavy chain containing an added BSP sequence were in average 20 % (Fig. 1A). The BSP containing K<sup>d</sup>-PbCS(ABA) complexes were biotinylated by using the biotin ligase BirA (12). The efficiency of the biotinylation was 70 – 80 % and of the refolding of K<sup>d</sup>-PbCS(ABA) or K<sup>d</sup>-PbCS(ABA)-BSP complexes in average 20 % (Fig. I, Supplemental Data).

Alternatively biotinylation was accomplished by site-specific alkylation of a free cysteine introduced by point mutation at the C terminal portion of the K<sup>d</sup> heavy chain. To find out what position is most suitable, a free cysteine was introduced in position 273, 275 or 277 (Fig. 1B). The former two flank the conserved W274, which is the last residue of the folded α3 domain of MHC class I molecules (17). Refolding under the same conditions gave yields of 14 % for K<sup>d</sup>K275C, 12 % for K<sup>d</sup>R273C and 6 % for K<sup>d</sup>A277C (Fig. 1A). The refolding efficiency of K<sup>d</sup>K275C was increased to 20 % when 0.3 mM DTT was added to the urea buffer.

The alkylation efficiency of the different K<sup>d</sup>-PbCS(ABA) cysteine mutants was assessed following incubation at 4 °C overnight with a five-fold molar excess of iodoacetyl-PEO-
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biotin (Fig. 1C). For K\textsuperscript{d}R273C the alkylation efficiency was 16%, for K\textsuperscript{d}K275C 24 % and 12% for K\textsuperscript{d}A277C (Fig. IB). Based on these results K\textsuperscript{d}K275C was selected. Its alkylation efficiency was increased to about 80% upon reduction of K\textsuperscript{d}K275C-PbCS(ABA) complexes with 15 mM glutathion prior to the alkylation (Fig IC). The alkylation was selective for the free cysteine, as K\textsuperscript{d}-PbCS(ABA) wild type complexes were not significantly alkylated (Fig. IB and C). A slightly higher alkylation efficiency (about 85%) was obtained for the mono-alkylation of K\textsuperscript{d}K275C-PbCS(ABA) complexes with bi-maleimide-biotin reagent DMGS-biotin (Fig. 1D). The purified mono-alkylated K\textsuperscript{d}-DMGS-biotin complex were then reacted with a two-fold molar excess of reduced K\textsuperscript{d}K275C-PbCS(ABA). The efficiency for this reaction was 60 – 70 %. Together these results show that K\textsuperscript{d}K275C can be refolded and biotinylated by alkylation with the same efficiencies as by the BSP/BirA strategy. The same finding were obtained for HLA-A2 (data not shown).

Defined K\textsuperscript{d}-peptide tetramers and octamers were obtained by reacting K\textsuperscript{d}-peptide-PEO-biotin and dimeric K\textsuperscript{d}-peptide-DMGS-biotin complexes with homogeneous Cy5 labeled streptavidin. For multimeric K\textsuperscript{d}-peptide complexes K\textsuperscript{d}-BSP-biotin-peptide monomers were reacted with heterogeneous PE-extravidin. The different compounds were analyzed by gel filtration on a Superdex S200 column and anion exchange chromatography on a Source 15Q column, respectively. As shown in Fig. 2, the K\textsuperscript{d}-PbCS(ABA) monomers, dimers, tetramers and octamers were homogenous, except for some minor contaminants, and eluted in both types of chromatography as expected. By contrast, the PE labeled K\textsuperscript{d}-PbCS(ABA) multimers eluted in a heterogeneous manner from the anion exchange column. The majority of the PE labeled K\textsuperscript{d}-PbCS(ABA) multimers eluted from the Superdex S200 column at around 10 min, i.e. in the void volume. Since the size exclusion of this column is about 600 kDa and PE-
extravidin K\textsuperscript{d}-peptide tetramers have a Mr of about 470 kDa, this preparation contained mainly conjugates that were larger than tetramers containing one PE. In agreement with this is the late elution of these complexes from the anion exchange column. The same results were obtained for the K\textsuperscript{d}-peptide complexes containing the D227K mutation (data not shown).

**Binding of soluble K\textsuperscript{d}-peptide complexes to T1 CTL** - To study the binding of Cy5 labeled K\textsuperscript{d}-peptide complexes, we first measured the binding kinetics of the K\textsuperscript{d}-PbCS(ABA) octamers and multimers. As shown in Figs. 3A-D the binding of both complexes was rapid at 37, 18 and 4 °C, with ≥ 90 % of maximal binding reached within the first few minutes of incubation. For octamer at 37 °C (Fig. 3A), but not at 18 °C or 4 °C (Figs. 3C and D), a transient binding maximum was observed at about 10 min, followed by a modest decrease, to reach a stable plateau after one hour. A similar biphasic binding kinetics was recorded at 37 °C for K\textsuperscript{d}-PbCS(ABA) monomer and tetramer (data not shown and (4)). In all cases the binding of non-cognate K\textsuperscript{d}-Cw3 170-179 octamer or multimer was no more than a few percent of the binding of the corresponding cognate complexes, indicating that non-specific binding, namely binding to CD8 under these conditions is insignificant.

We next assessed the binding isotherms at 37 °C and 18 °C for the compounds under study (Figs. 3E-H). At 37 °C the binding of Cy5 labeled K\textsuperscript{d}-PbCS(ABA) octamers increased steeply in the concentration range of up to 30 nM and then gradually up to 100 nM, the highest concentration tested (Fig. 3E). By contrast, for the tetramer much lower levels of binding were observed and the increase in binding required higher concentrations. For the corresponding K\textsuperscript{d}D227K-PbCS(ABA) complexes the binding of the octamer was reduced by about 10%, but of the tetramer was close to the background binding, observed for the non-
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cognate K<sup>d</sup>-Cw3 171-179 peptide complexes. A similar binding pattern was observed at 18 °C, except that nearly maximal binding was reached already at 12.5 nM for the octamer and at 30 nM for the tetramer. This difference seems mainly accounted for by endocytosis, taking place at 37 °C, but not 18 °C (see below).

The binding of PE-labeled multimers increased continuously over the range of concentrations tested at 37 °C (Fig. 3F) and at 18 °C (Fig. 3H). Strikingly, at both temperatures K<sup>d</sup> wild type and K<sup>d</sup>D227K complexes exhibited about the same binding patterns. Since these multimer complexes contain higher order complexes (see above), these results argue that the CD8 dependence of the binding of MHC-peptide complexes decreases as their valence increases. Essentially the same findings were obtained on the PbCS(ABA)-specific S14 CTL clone.

K<sup>d</sup>-PbCS(ABA) and K<sup>d</sup>D227K-PbCS(ABA) octamers induce TCR and CD8 down-modulation and internalization — To assess whether octamers induce down modulation of TCR and CD8, T1 CTL were incubated with saturating concentrations of K<sup>d</sup>-PbCS(ABA) and K<sup>d</sup>D227K-PbCS(ABA) octamers for different periods of time and TCR and CD8 expression was measured by FACS. At 4 °C the surface expression of TCR and CD8 remained essentially unchanged (Figs. 4A and B). At 18 °C a scant reduction of TCR and CD8 expression was observed after 30 and 60 min of incubation (Figs. 4C and D). At 37 °C there was a time dependent down modulation of TCR and CD8 of about 40% after one hour of incubation with K<sup>d</sup>-PbCS(ABA) octamer (Fig. 4E). The K<sup>d</sup>D227K octamer induced the same TCR down modulation, but the down modulation of CD8 was less than half (18% after one hour) (Fig. 4F).
To visualize endocytosis of Cy5 labeled Kd-PbCS(ABA) and KdD227K-PbCS(ABA) octamers they were incubated with T1 CTL for 30 min at 18 °C or 37 °C and analyzed by confocal microscopy. As shown in Fig. 4G at 18 °C Kd-PbCS(ABA) complexes were distributed on the cell surface in a wide cap. This was also true for the Kd-D227K-PbCS(ABA) octamer, but the cap formation was less pronounced. By contrast at 37 °C the majority of Kd-PbCS(ABA) complexes were internalized in the form of a bright patch. For the KdD227K-PbCS(ABA) about half of the complexes were localized in a wide cap on the surface and half internalized in patch, distal to the cap. In the presence of Fab’ fragments of the anti-Kdα3 mAb SF1-1.1.1, which block residual CD8 co-engagement (4), internalization and patch formation of both complexes was strongly inhibited; especially the KdD227K-PbCS(ABA) complexes were localized predominately at the cell surface.

Kd-PbCS(ABA) octamer elicit Fas-mediated cytotoxicity - To assess the ability of Kd-PbCS(ABA) octamers to elicit Fas-dependent cytotoxicity, T1 CTL were pulsed with different Kd-peptide complexes, washed and then incubated for four hours with 51Cr labeled P815 cells transfected with Fas. As shown in Fig. 5A, tetrameric, octameric and multimeric Kd-PbCS(ABA) and Kd-D227K-PbCS(ABA) complexes induced bystander cell killing. The most efficient killing was observed for Kd-D227K-PbCS(ABA) octamer (65 %). The Kd-PbCS(ABA) octamer induced slightly less efficient killing, but for both tetramers and multimers target cell killing was about 20 % lower. T1 CTL pulsed or not with the corresponding Kd-Cw3 170-179 complexes exhibited only faint background lysis. By contrast, very strong lysis was observed in the presence of anti-Fas antibody, which by cross linking of Fas on the target cells induces their apoptosis.
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A concern of the present experiments was that during the assay K\textsuperscript{d}-PbCS(ABA) complexes decay and that liberated PbCS(ABA) peptide binds to cell-associated K\textsuperscript{d} and induces target cell killing. To consolidate this we assessed the stability of K\textsuperscript{d}-PbCS(ABA) monomers at 37 °C. As shown in Fig. II (Supplemental Data) after 2.5 h of incubation approximately 50 % and after 3.5 h nearly 80 % of the K\textsuperscript{d}-PbCS(ABA) complexes were decayed. However, in the presence of β2m the dissociation was greatly reduced; after 3.5 h only 10 % of dissociation took place. The same results were obtained for K\textsuperscript{d}D227K-PbCS(ABA) complexes (data not shown). Thus to reduce the liberation of PbCS(ABA) peptide, β2m was added to all incubations. In addition, in all assays 10 µM of PbCS 252-260 peptide was added to prevent free PbCS(ABA) peptide from binding to cell-associated K\textsuperscript{d}. Under these conditions 10 nM of PbCS(ABA) peptide, caused no significant lysis. However, at very high concentration (10 µM) free PbCS(ABA) caused strong lysis (Fig. 5A).

The same experiment performed on normal P815 cells showed no significant lysis, except in the incubation where a high concentration of free PbCS(ABA) peptide was used (Fig. 5B). This is consistent with the finding that normal P815 cells, which express low amounts of Fas, in four hour cytolytic assays are sensitive to perforin/granzyme-mediated, but not Fas-dependent killing (8). Taken together these results indicate that CTL pulsed with soluble K\textsuperscript{d}D227K-PbCS(ABA) octamer induce strong Fas, but no perforin/granzyme dependent bystander cell killing.

K\textsuperscript{d}-PbCS(ABA), but not K\textsuperscript{d}D227K-PbCS(ABA) complexes induce degranulation of adherent CTL – We next examined the ability of the different soluble K\textsuperscript{d}-peptide complexes to elicit
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degranulation of T1 CTL, which reflects perforin/granzyme dependent lysis. Because
degranulation requires adhesion and polarization of CTL (18 - 20), we adhered T1 CTL to
immobilized fibronectin and incubated them with the soluble Kd-peptide complexes. As
shown in Fig. 6, all Kd-PbCS(ABA) complexes elicited CTL degranulation. The strongest
degranulation was observed for PE-labeled Kd-PbCS(ABA) multimers (75 %) and Kd-
PbCS(ABA) octamer (68 %); Kd-PbCS(ABA) tetramers induced about 48% esterase release.
In the presence of concanamycine A, which blocks CTL degranulation (21), the strong
degranulation induced by Kd-PbCS(ABA) tetramers was reduced to background levels, as
observed for the Kd-Cw3 170-179 complexes.

Remarkably, the corresponding KdD227K-PbCS(ABA) complexes elicited no or scant
esterase release, which was ablated upon blocking of the residual CD8 co-engagement by
anti-CD8 mAb H35 (Fig. 6 and ref. 4). Together these results indicate that soluble Kd-
PbCS(ABA) complexes, namely octamer and multimers efficiently elicit CTL degranulation
by soluble MHC-peptide complexes under the condition they co-engage CD8. The same
observations were made on clones S14 CTL (data not shown).

Kd-PbCS(ABA), but not KdD227K-PbCS(ABA) octamers induce calcium mobilization and
strong tyrosine phosphorylation – A hallmark of TCR/CD8 mediated T cell activation is a
rapid increase in intracellular calcium and tyrosine phosphorylation of CD3. To assess the
ability of Kd-PbCS(ABA) octamers to elicit intracellular calcium mobilization, indo-1
labeled T1 CTL were incubated with Kd-PbCS(ABA) and KdD227K-PbCS(ABA)
complexes and calcium flux measured by FACS. As shown in Fig. 7A, wild type octamer
induced strong calcium mobilization that was maximal about two min after addition of
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octamer. By contrast, $K_{d}^{D227K}$-PbCS(ABA) octamer elicited a calcium flux close background levels, i.e. unpulsed CTL. The same findings were obtained on S14 CTL (data not shown). This is consistent with our previous finding that soluble MHC-peptide complexes elicit intracellular calcium mobilization only when they co-engage CD8 (14, 22).

Moreover, T1 CTL upon brief incubation with $K_{d}^{d}$-PbCS(ABA) octamers exhibited strong tyrosine phosphorylation of CD3ε and ζ chain, including its pp23 phospho form, as is typically observed upon TCR triggering with agonists (Fig. 7B and (14, 23, 24)). By contrast, $K_{d}^{D227K}$-PbCS(ABA) octamers induced only faint phosphorylation of the pp21 phospho form of ζ chain, as typically occurs upon T cell triggering by weak agonists or antagonists (Fig. 7B and (14, 23, 24)). Blotting with anti-CD3ε antibody showed that equal amounts were loaded in all lanes. The soluble $K_{d}^{d}$-PbCS(ABA) complexes used were homogeneous according to SDS-PAGE (Fig. 7C). The same results were obtained on S14 CTL (data not shown). Taken together these results show that soluble $K_{d}^{d}$-PbCS(ABA) octamers efficiently induce intracellular calcium mobilization and CD3 phosphorylation, given they co-engage CD8.

$K_{d}^{d}$-PbCS(ABA) octamers induce apoptosis of T1 CTL – Based on the observation that $K_{d}^{d}$-PbCS(ABA) octamers induce Fas-dependent cytotoxicity of bystander cells (Fig. 5), we investigated whether they also induce apoptosis of the CTL. As shown in Fig. 8, T1 CTL upon incubation with $K_{d}^{d}$-PbCS(ABA) and slightly less with $K_{d}^{D227K}$-PbCS(ABA) octamers exhibited a marked increase in annexin V, which is a marker for apoptotic cells (9). For CTL incubated with $K_{d}^{d}$-Cw3 170-179 octamer the annexin V expression was background level.
DISCUSSION

The present study shows that conventional “tetramers” are ill-defined mixtures of MHC-peptide conjugates (Fig. 2) and that this precludes precise binding studies (Fig. 3). The cause for this heterogeneity is the high molecular weight of PE (or allophyco-cyanine), which renders difficult, if not impossible, defined conjugation with the smaller avidin or avidin derivatives. We find that defined MHC-peptide complexes can be obtained by using Cy5 labeled streptavidin. Although the fluorescence intensity of Cy5 labeled streptavidin is 4 - 5-fold lower as compared to PE-streptavidin, it is unlike PE remarkably resistant to photo bleaching, which allows analysis other than FACS. Other low molecular weight fluorochromes can be used instead of Cy5, such as Cy3 or various Alexa dyes.

Moreover, the conventional strategy to derivatize monomeric MHC-peptide complexes by enzymatic biotinylation of an added BSP sequence permits only the preparation of avidin based MHC-peptide “tetramers”. To produce different soluble MHC-peptide complexes we investigated the derivatization of MHC-peptide monomers by site-specific alkylation. It has been reported that the heavy (25) or light chain (26) of MHC class I molecules can be biotinylated by alkylation of a free cysteine with maleimide containing biotin derivatives. Since on living cells under physiological conditions β2m is rapidly exchanged (27), we examined how best site-specific alkylation of the heavy chain is accomplished. Our results show that the position 275 of the heavy chain is most suitable. For Kd the refolding and the alkylation efficiency of the K275C heavy chain were higher as compared to the R273C and A277C mutants (Fig. I, Supplemental Data). This is consistent with the fact that the conserved Trp274 marks the end of the folded α3 domain (17). Our results further indicate that the efficiency of refolding and alkylation critically depend on appropriate reduction of the
introduced free cysteine (Fig. I, Supplemental Data). Since the same results were obtained for HLA-A2 (unpublished results), this may be generally applicable.

The biotinylation of MHC peptide complexes has several important advantages compared to the conventional enzymatic biotinylation. 1) The biotinylation can be performed in the cold, which is advantageous in particular in case of thermo-labile MHC-peptide complexes. 2) Significantly lower costs, as alkylation reagents are much cheaper than BirA. 3) The thioether bond formed by alkylation of a free cysteine is very stable and resist proteolytic and chemical degradation 4) The alkylation method is remarkably versatile. In addition to biotinylation of MHC class I-peptide complexes (Figs. 1, 2 and (25, 26)), site-specific alkylation allows the preparation of MHC-peptide complexes of diverse valence and configuration by using for alkylation branched, maleimide containing linkers. Also fluorescent labeled MHC-peptide complexes can be prepared by alkylation with fluorescent labeled maleimides or maleimide containing linkers.

Our MHC-peptide binding studies allow three conclusions. First, the increase of $K_d$. PbCS(ABA) binding to T1 CTL is dependent on the valence of the complexes. CD8 increased the binding of monomeric complexes about 10-fold at 37 °C (4, 14); about five-fold for tetrameric complexes, less than two-fold and hardly at all for multimeric complexes (Fig. 4). Concerning the CD8 dependence of multimer binding to CD8 positive T cells there exists a controversy in the literature. While according to some studies multimer binding is markedly CD8 dependent (28, 29), it is not according to others (23, 30). This discrepancy may be explained in part by differences in the MHC-peptide multimers composition used in the different studies. However, we observed that under the same conditions as described here, the multimer binding to HLA-Cw3-specific CTL, which express low affinity TCR, is
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substantially strengthened by CD8 (unpublished results). Similarly, Daniels and Jameson found that the CD8 dependence of multimer binding depends on the affinity of the TCR of the cells under study (28). It thus appears that the binding of MHC-peptide complexes to CD8+ T cells is essentially determined by the over-all binding avidity. Thus, the higher the affinity of the TCR and the higher the valence of the complexes, the less important is the contribution of CD8 to the binding. In our hands the binding of MHC-peptide octamers, but not of smaller complexes, is little CD8 dependent, except on CTL that express exceptionally low affinity TCR.

Second, the kinetics of Kd-PbCS(ABA) octamer binding to T1 CTL are remarkably rapid, taking place within few minutes at all temperatures tested (Figs. 3A,C and D). This was also true for tetrameric complexes and on S14 CTL (unpublished results). By contrast, the binding of monomeric Kd-PbCS(ABA) complexes were considerably slower, especially in the cold (4). This argues that the kinetics of MHC-peptide complex binding increases with their valence.

Third, the heterogeneity of MHC-peptide multimers (Fig. 2) precludes precise binding studies. For example, the binding of Kd-PbCS(ABA) multimers increased continuously with the concentration and this also at 18 °C, where internalization is scant; whereas the octamer binding at 18 °C reached saturation already at low concentrations (Figs. 3 and 4). It thus appears that low valence complexes in multimer preparations significantly bind only at higher concentrations, whereas high valence ones bind already at low concentrations. However, it is also conceivable that MHC-peptide complexes that have an appropriate configuration, can elicit TCR (and CD8) aggregation. Such aggregation effects may explain why tetrameric Kd-
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PbCS(ABA) complexes fail to reach the high levels of binding observed for octameric complexes (Fig. 4G) (tested up to 500 nM).

A key finding of the present study is that soluble MHC class I-peptide complexes that are unable to co-engage CD8, induce strong Fas, but no perforin-mediated cytotoxicity (Figs. 5, 6, 8). This is in accordance with the previous observations that blocking of CD8 by antibody blocks perforin, but not Fas-dependent killing of target cells (8) and that target cells or microspheres expressing MHC class I-peptide with ablated CD8 binding induce Fas dependent apoptosis of human CTL (9). Common to these strategies is that CD8 coreceptor function is blocked. In the present study, where soluble MHC-peptide complexes are used, CD8-mediated adhesion and MHC-peptide binding or involvement of other auxiliary molecules is excluded (Fig. 3). It thus appears that engagement and cross-linking of TCR in the absence of CD8 co-engagement induces Fas-dependent cytotoxicity, including Fas-mediated apoptosis of the CTL, in the absence of other cell activation (Figs 5-8).

What implications has blocking of CD8 coreceptor function on CTL activation? On one hand the lack of CD8 co-engagement by MHC-peptide complexes impairs the avidity of TCR-ligand binding (4, 14, 28, 29). We show here that for soluble MHC-peptide complexes this can be compensated for by increasing their valence (Fig. 3). On the other hand, the lack of CD8 co-engagement impairs Lck-mediated phosphorylation of CD3. This is so, because normally the coordinate binding of MHC-peptide to CD8 and TCR brings CD8-associated Lck to CD3, which upon cross-linking mediated activation of Lck results in their phosphorylation (14, 22-24, 31). Once phosphorylated by Lck, CD3 and ζ chain ITAMs recruit ZAP-70 (and Syk), which upon phosphorylation by Lck phosphorylates LAT and other substrates, thus initiating various down-stream signaling cascades (32-34).
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The lack of CD8 co-engagement hence results in impaired Lck activation and by consequence reduced tyrosine phosphorylation of CD3 and recruitment and activation of Zap-70 (Fig. 7B and (8, 9, 23, 32)). As a result of this namely two downstream signaling pathways are compromised. The first is the recruitment and activation of PLC\(\gamma\), which is involved in observation that in the absence of CD8 co-engagement there is no significant the generation of IP3, which in turn mediates the release of intracellular calcium from stores (34, 35). In agreement with this is our intracellular calcium mobilization (Fig. 7A and (8)). It is well established that CTL degranulation requires a rapid intracellular calcium mobilization, followed by a sustained influx of extracellular calcium, whereas for Fas-dependent killing the latter is sufficient (36, 37).

The second signaling pathway that is compromised, involves the recruitment of PI3-K to phosphorylated CD3 and \(\zeta\) chain. This kinase phosphorylates PIP2 to IP3 and is critically involved in early TCR signaling, including calcium mobilization, activation of Rho family GTP-binding proteins and cytoskeleton function (38). Inhibition of PI3-K by wortmannin blocks CTL degranulation, but has no effect on Fas-dependent cytotoxicity (39). It thus appears that MHC-peptide complexes that do not co-engage CD8, are unable to significantly activate Lck and hence most CTL effector function, including degranulation, cytokine release and proliferation. The remarkable exception is Fas-dependent killing, which is Lck independent, which makes it possible to selectively induce this cytotoxicity in the absence of any other cellular response (8, 40).

The physiological significance of this is not clear. Since CTL once they express FasL are prone to apoptosis (Fig. 8 and (9), it is conceivable that this way CD8\(^+\) T cells with defective
CD8 coreceptor function are eliminated. Indeed it has been shown that misselected CD8$^+$ T cells, which express TCR that are not MHC class I-restricted, are eliminated this way (41). The observation that large soluble MHC-peptide complexes with ablated CD8 binding permit eradication of antigen-specific CTL (Fig. 8) gives them a therapeutical potential. They are more attractive to this end than the previously described use of anti-CD8 antibodies (8) or MHC-peptide coated microspheres (9), as such molecules can be produced in well defined form in adequate quantities and purity. Also since soluble MHC-peptide complexes with ablated CD8 binding are unable to elicit any other cell activation than Fas-dependent cytotoxicity, they harbor a minimal risk to induce unwanted, potentially harmful immunological reactions (Figs. 5, 6, 8 and (23)).
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FOOTNOTE

1) Abbreviations

ABA, 4-azidobenzoic acid; β2m, β2-microglobulin; BSP, biotinylation sequence; Cy5, Cychrome 5; DMGS-biotin: di-maleimide-di-glycine-serine-biotin linker; Fas, CD95; FasL, Fas ligand; FACS, flow cytometry; FCS, fetal calf serum; IAc-PEO-biotin, iodoacetyl-ethyleneglycol-biotin; ITAM, immunoreceptor tyrosine based activation motif; MHC, major histocompatibility complex; PbCS; Plasmodium berghei circumsporozoite; PbCS(ABA); SYIPAEK(ABA); PE, phycoerythrin; TCR, T cell antigen receptor; SF’, Fab’ fragments of anti-Kdα1 mAb SF1-1.1.1.

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LEGENDS TO FIGURES

Figure 1) **Linkers under study.** A) The sequences of the Kd α3 residues 270-271 with the BSP sequence, shown in underlined, added via a Gly-Ser spacer. The lysine in the BSP sequence is biotinylated by BirA. B) The C-terminal sequence (270-277) of the Kd heavy chain, in which residues 273, 275 or 277 (triangles) were mutated to cysteine. Structures of I-Ac-PEO-biotin (C) and DMGS-biotin (D).

Figure 2) **Characterization of the Kd-peptide complexes under study.** Kd-PbCS(ABA) monomers-PEO-biotin, DMGS-biotin dimers, Cy5 labeled tetramers, octamers and PE-labeled multimers were analyzed by gel filtration on a Superdex S200 column (A) or by anion exchange chromatography on Source Q15 column (B). The Superdex column was eluted with PBS at a flow rate of 0.7 ml/min and the Source Q15 at a flow rate of 1 ml/min with 20 mM Tris, pH 8.0 with a gradient of NaCl rising in 70 min from 0 to 500 mM. The OD of the effluent was measured at 280 nm.

Figure 3) **Binding of soluble Kd-PbCS(ABA) complexes to T1 CTL.** T1 CTL were incubated at 37 °C (A,B), 18 °C (C) or 4 °C (D) for the indicated periods of time (A-D) with 25 nM of Cy5-labeled Kd-PbCS(ABA) octamer (open circles), Kd-Cw3 170-179 octamer (filled diamonds), PE-labeled Kd-PbCS(ABA) multimer (open triangles) or Kd-Cw3 170-179 multimer (open diamonds). Cell-associated fluorescence was assessed by FACS as mean fluorescence intensity (MFI). Alternatively, T1 CTL were incubated for 30 min in E and G with the indicated concentrations of Cy5-labeled Kd-PbCS(ABA) octamer (open circles), KdD227K-PbCS(ABA) octamer (filled circles), Kd-PbCS(ABA) tetramer (open
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squares), KdD227K-PbCS(ABA) tetramer (filled squares), Kd-Cw3 170-179 octamer (open diamonds), Kd-Cw3 170-179 tetramers (filled diamonds) or in F and H with Kd-PbCS(ABA) multimer (open triangles), KdD227K-PbCS(ABA) multimer (filled triangles) or Kd-Cw3 170-179 multimers (gray diamonds) and cell-associated fluorescence assessed likewise. Representative experiments are shown. Each experiment was repeated two to five times.

Figure 4) TCR down-modulation and internalization of Kd-PbCS(ABA) and KdD227K-PbCS(ABA) octamers on T1 CTL. T1 CTL were incubated at 4 °C (A, B), 18 °C (C and D) or 37 °C (E and F) for the indicated periods of time with 25 nM of Cy5 labeled Kd-PbCS(ABA) (wt) (A, C, E) or KdD227K-PbCS(ABA) (227) (B, D, F) octamers. The cells were washed and stained with PE-labeled anti-TCR mAb H57 (filled bars) or FITC-labeled anti-CD8α mAb 53.6.72 (open bars) and cell-associated fluorescence was measured by FACS. Mean values and SD of the mean fluorescence intensities (MFI) were calculated from three experiments. G) Alternatively, T1 CTL were incubated in the absence or presence of Fab’ of antiKdα3 mAb SF1-1.1.1 (SF’; 20 µg/ml) with Cy5-labeled Kd-PbCS(ABA) (wt) or KdD227K-PbCS(ABA) (227) octamer for 30 min at 37°C or 18°C. After washing, cells were fixed and the distribution of Cy5 analyzed by confocal microscopy. Representative pictures from at least 100 cells analyzed are shown.

Figure 5) Soluble Kd-PbCS(ABA) complexes induce Fas-dependent killing of bystander cells. T1 CTL in suspension were incubated at 37 °C for 30 min with 25 nM of Kdwt, KdD227K PbCS(ABA) or Kd-Cw3 170-179 peptide tetramers, octamers or PE-labeled multimers, washed and incubated at 37 °C for 4 h with 51Cr labeled P815 cells over expressing Fas (P815Fas) (A) or normal P815 cells (B). In all incubations 10 µM of PbCS 252-260 peptide
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(competitor) and β2m (5 µg/ml) were present. As indicated, in some incubations free PbCS(ABA) peptide (10 nM or 10 µM) or anti-Fas antibody (2 µg/ml) were added. The specific lysis was calculated from the released chromium measured in supernatants. Mean values and SD were calculated from triplicate values from three different experiments.

Figure 6) $K^d$-PbCS(ABA), but not $K^dD227K$-PbCS(ABA) complexes elicit degranulation of adherent T1 CTL. T1 CTL adhered to fibronectin coated plates were incubated at 37 °C for 90 min with 25 nM of $K^d$-PbCS(ABA) (wt), $K^dD227K$-PbCS(ABA) or $K^d$-Cw3 170-179 (Cw3) tetramers, octamers or PE-labeled multimers and the released esterases measured in the supernatants. In some incubations anti-CD8β mAb H35 (10 µg/ml) were present (full bars). All incubations contained 10 µM of PbCS 252-260 peptide and 2 µg/ml β2m. As positive control 1 µM of free PbCS(ABA) or PbCS 252-260 peptide was used and as negative control an incubation containing concanamycine A (CMA) (100 nM), an inhibitor of CTL degranulation. Mean values and SD were calculated from three experiments each performed in triplicates.

Figure 7) $K^d$-PbCS(ABA), but not $K^dD227K$-PbCS(ABA) octamers elicit calcium mobilization and strong tyrosine phosphorylation in T1 CTL. A) Indo-1 labeled T1 CTL were incubated 37 °C with 25 nM of $K^d$-PbCS(ABA) (black line) or $K^dD227K$-PbCS(ABA) (gray line) octamers or medium (dotted line) and calcium dependent indo-1 fluorescence was measured by FACS. B) T1 CTL untreated (-) or incubated for 3 min at 37 °C with 25 nM of $K^d$-PbCS(ABA) (wt) or $K^dD227K$-PbCS(ABA) (227), were washed, lysed in 1% Brij78 and the detergent soluble fraction immunoprecipitated with anti-TCR mAb H57. The immunoprecipitates were resolved on SDS-PAGE (15 % reducing) and Western blotted with anti-phospho-tyrosine (pY) mAb 4G10 and anti-CD3 antibody,
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respectively. Representative experiments out three are shown. C) The purified Kd-PbCS(ABA) monomers and (lane 1) and DMGS-biotin dimers (lane 2) used in the preparation of tetramers and octamers were resolved on SDS-PAGE (15 %, reducing) and the gel stained with Coomassie blue.

Figure 8) KdD227K-PbCS octamers induce apoptosis of T1 CTL. A) T1 CTL were incubated or not with 25 nM of KdD227K-PbCS(ABA), Kd-PbCS(ABA) or Kd-Cw3 170-179 octamers at 37 °C for 30 min followed by an additional 4.5 h at 37 °C in medium and then analyzed by FACS following staining with Cy5 labeled annexin V. Data from one out of two experiments is shown.

Supplemental Data

Figure I) Optimal conditions for refolding and alkylation of soluble Kd-peptide complexes – A) Kd-PbCS(ABA) monomers were produced by refolding using β2m, PbCS(ABA) peptide and Kd heavy chains without (wt) or with an added BSP sequence (BSP) or free cysteines in position 273 (R273C), 275 (K275C) or 277 (A277C). The refolding efficiency is expressed in percent with 100% being the amount of Kd heavy chain used. B) The different Kd-PbCS(ABA) complexes were reacted with a 5-fold molar excess of I-Ac-PEO-biotin and the efficiency of the alkylation was determined in percent (see Materials and Methods). C) Kd-PbCS(ABA) and KdK275C-PbCS(ABA) were examined likewise without (filled bars) and with (open bars) prior reduction with 15 mM gluthation

Fig. II) Stability of Kd-PbCS(ABA) complexes at 37 °C - Kd-PbCS(ABA) complexes were incubated in Hanks balanced salt solution HBSS in the absence (full bars) or presence (open
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bars) of human β2m (2 μg/ml) at 37 °C for the indicated periods of time and the integrity of the complexes was assessed by gel filtration on a Superdex S75 column.
Fig. 1 Guillaume et al.
Figure 2, Guillaume et al.
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Figure 5, Guillaume et al.
Figure 6, Guillaume et al.
Figure 7, Guillaume et al.
Figure 8, Guillaume et al.
Figure 1, Guillaume et al. Supplemental Data
Figure II, Guillaume et al.
Supplemental Data
Soluble major histocompatibility complex-peptide octamers with impaired CD8 binding selectively induce FAS-dependent apoptosis

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