Background: MHC-peptide multimers are used to analyze and sort antigen-specific CD8+ T cells.

Results: NTA-His tag containing multimers can be switched from stable binding to rapid dissociation.

Conclusion: These reagents allow improved analysis and sorting of bona fide T cells.

Significance: New tools for improved CD8+ T cells analysis.

MHC-peptide multimers containing biotinylated MHC-peptide complexes bound to phycoerythrin streptavidin (PE SA) are widely used for analyzing and sorting antigen-specific T cells. Here we describe alternative T cell staining reagents that are superior to conventional reagents. They are built on reversible chelate complexes of Ni²⁺ nitrilotriacetic acid (NTA) with oligo-histidines. We synthesized biotinylated linear mono, di and tetra NTA compounds using conventional solid phase peptide chemistry and studied their interaction with HLA-A*0201-peptide complexes containing a His₆, His₁₂ or a 2xHis₆ tag by surface plasmon resonance on SA coated sensor chips and equilibrium dialysis. The binding avidity increased in the order His₆ < His₁₂ < 2xHis₆ and NTA₁ < NTA₂ < NTA₄ respectively, depended on the configuration of the NTA moieties and increased to pico-molar Kᵦ for the combination of a 2xHis₆ His tag and a 2xNi²⁺NTA₂. We demonstrate that HLA-A2-2xHis₆-peptide multimers containing either Ni²⁺NTA₄-biotin and PE-SA or PE-NTA₄ stained influenza and Melan-A-specific CD8+ T cells equal of better than conventional multimers. While these complexes were highly stable, they very rapidly dissociated in the presence of imidazole, which allowed sorting of bona fide antigen-specific CD8+ T cells without inducing T cell death as well as assessment of HLA-A2-peptide monomer dissociation kinetics on CD8+ T cells.

Conjugation of proteins, DNA/RNA, fluorescent dyes to Qdots, microspheres, sensor or micro-array chips or ELISA plates is crucial for many applications in basic and applied sciences. The extremely strong binding of biotin to streptavidin (SA) (Kᵦ ~ 1 x 10⁻¹⁴ M) has been extensively used for such applications (1). While for some applications this practically irreversible binding is desirable, for others it is not, e.g. i) introduction of macro-molecules into cells by means of a delivery peptide reversibly conjugated to the molecules of interest (2), ii) purification of recombinant proteins via reversible adsorption on an affinity matrix, e.g. Ni²⁺ NTA column (3) or iii) reversible staining and sorting of antigen-specific T cells (4,5). Major histocompatibility complex (MHC) peptide tetramers are reagents that are widely used to enumerate, analyze and isolate antigen specific CD8+ T cells (6,7). CD8+ T cells express clonotypic T cell antigen receptors (TCR) that bind cognate MHC class I-peptide complexes. While the binding of monomers to T cells is unstable, the binding of tetramers is stable and allows detection, enumeration and isolation of antigen specific CD8+ T cells by flow cytometry. MHC class I -peptide monomers are produced by refolding of
MHC heavy and light chain in the presence of a peptide of interest and subsequent biotinylation at a C-terminal biotinylation sequence peptide (BSP) by means of the biotin-transferase BirA; they are subsequently tetramerized by incubation with phycocerythrin (PE) or allophycocyanine (APC) conjugated SA (6,7).

A major shortcoming of these tetramers is that they avidly bind to and cross-link cell-surface TCR and CD8, thereby inducing strong T cell activation, which frequently provokes T cell death (4,5,8,9). Thus, isolation of antigen-specific CD8+ T cells by tetramers harkens the risk of substantial T cell loss. To circumvent this, “reversible” tetramers have been developed, which contain low affinity biotin analogues and therefore dissociate upon addition of free biotin (4,5,9). However, although these reagents improved sorting and cloning of live antigen-specific CD8+ T cells, they are costly to produce and of limited stability, even at room temperature, commonly used for FACS sorting. Furthermore, there is strong evidence that the dissociation kinetic of TCR-MHC-peptide complexes is a key determinant for antigen-specific CD8+ T cell activation (10,11). Dissociation kinetics have been assessed by measuring tetramer dissociation on CD8+ T cells. However, results from such experiments are error prone, e.g. due to rebinding of dissociated tetramers and internalization that rapidly occurs at elevated temperatures. Based on our previous demonstration that dissociation kinetics can be accurately assessed on living CD8+ T cells by using MHC-peptide monomers and TCR photoaffinity labeling (12), we reasoned that reversible tetramers might provide an alternative, generally applicable means to conclusive measure of MHC-peptide monomer dissociation kinetics on living cells.

Here we describe the preparation of MHC-peptide multimers that are built on Ni²⁺ nitrilotriacetic acid (NTA) moieties and oligo-histidine tagged HLA-A*0201-influenza matrix peptide (A2/Flu) complexes. Mono Ni²⁺ NTA compounds form reversible coordination complexes with oligo-histidines; which for a hexa-histidine (His₆) have a Kᵣ of about 10⁻⁶ M (13-17). While this is sufficient for purification of His₆ tagged recombinant proteins from culture supernatants (3), it is not for the preparation of staining reagents which must be stable for months. Previous studies have shown that two His₆ tags joined by a flexible linker greatly strengthens their binding to mono- Ni²⁺ NTA groups (18,19). Moreover, it has been demonstrated that the affinity of linear or branched di-, tri- and tetra-Ni²⁺ NTA compounds for His₆ and His₆₉ tagged molecules increases substantially with the number of Ni²⁺NTA entities up to subnano-molar Kᵣ (2,20-24). However, the synthesis of such NTA compounds requires advanced organic chemistry, which limits their application to specialized laboratories. Here we describe the synthesis of biotinylated linear mono, di and tetra NTA compounds based on conventional, i.e. widely accessible solid phase peptide synthesis. We prepared A2/Flu monomers containing a C-terminal His₆, His₁₅ or a double hexa-histidine (2xHis₆) tag and studied their interactions with the different NTA compounds by surface plasmon resonance (SPR) and equilibrium dialysis. We report that the Kᵣ decreased in the order NT₄ > NT₆ > NT₉₄ and His₆ > His₁₅ > 2xHis₆, respectively, reaching 1.9 x 10⁻¹¹ M for the combination of NT₄ and 2xHis₆ A2/Flu complexes. While these complexes are remarkably stable, they dissociate rapidly upon addition of imidazole, which we demonstrate permits FACS sorting of bona fide antigen-specific CD8+ T cells and conclusive assessment of MHC-peptide monomer dissociation kinetics on CD8+ T cells.

**EXPERIMENTAL PROCEDURES**

*Chemical synthesis of NTA compounds*

Detailed descriptions of the synthesis and characterization of the NTA compounds 1-4 (Fig. 1A) are provided in supplementary information. In brief, the compounds 1,2,4 were obtained by alkylation of the corresponding thio-peptide precursors with N-(5-(3-maleimidopropionylamino)-1-carboxypentyl)iminodiacetic acid (maleimido-C3-NTA) (Dojindo Laboratories (Kumamoto, Japan) in phosphate buffer (0.1 M; pH 7.2). The precursors were obtained by conventional solid phase peptide chemistry using chlorotriyl resins. Compound 3 was obtained by reacting the precursor peptide, which was synthesized on a rink resin and upon deprotection contained two orthogonal carboxyl groups, with H₂N-NTA(bu)₃ (23) in DMF. The thiopeptide used for alkylation of PE-maleimide.
was synthesized like compound 2, except that Fmoc-Cys(Acm)-OH was used instead of lysine-ε-amino-caproyl-biotin. The Acm protected group resisted TFA treatment and was removed with HgII in acetic acid (20%) for 1 h and then purified by HPLC (Rt: 13.9 min). The yield was 37%. ESI-MS calc. for C₄₅H₇₂N₆O₁₆S₂ [M+H]+ 1384.5, found 1385.3.

Surface plasmon resonance experiments

SPR experiments were performed on a BIACore 3000 instrument using SA coated, CM5 sensor chips (BIACore, GE Healthcare). The chips were loaded by injecting compounds 1-4 in flow cells 2, 3, 4 at a rate of 10 µl/min in running buffer (10 mM HEPES, 150 mM NaCl, 50 µM EDTA, pH 7.4). The average degree of loading was ~100 RU (range 75 to 130 RU). Free biotin binding sites were blocked with biotin. Cell 1 was used as control for changes in bulk refractive index. All experiments were performed in duplicate at 25°C and repeated two to four times. All buffers were filtered and degassed prior to use. For kinetic experiments A2-His6 Flu peptide complexes were diluted in running buffer in 1:2 serial dilutions starting from 22 µM, and A2-His12 or A2-2xHis6 peptide complexes in 1:3 serial dilutions, from 7.4 µM and passed over the Ni²⁺NTA-biotin-SA coated and control flow cells at 50 µl/min. As described previously (16,17) the steps were as follows: (1) rinse the flow cells with running buffer; (2) activation of peptide-NTA with Ni²⁺ (500 µM NiCl₂ in running buffer); (3) rinse in running buffer; (4) injection of His-tagged A2/Flu complexes at different concentrations and measuring changes in RU measured over 60 sec and then over an uninterrupted dissociation period of 6 min; (5) His-tagged protein removal by washing with imidazole solution (500 mM in water); (6) NiCl₂ removal with regeneration buffer (10 mM HEPES, 150 mM NaCl, 0.005% polysorbate 20, 350 mM EDTA, pH 7.4) and (7) rinse in dispenser buffer (10 mM HEPES, 150 mM NaCl, 0.005% polysorbate 20, 3 mM EDTA, pH 7.4). The kₐ and kₒff values were calculated assuming 1:1 Langmuir binding and data were analyzed using BIAevaluation 4.1 software and a global fit algorithm. For each concentration of His-tagged A2/Flu complexes the response of the control cell was subtracted.

Multimers

The preparation of the A2-BSP heavy chain has been described previously (4,25,26). Those containing the A2-His tags were prepared analogously using phosphorylated forward and reverse primers encoding the His₆, His₁₂ and 2xHis₆ tags with BamHI in 5’ and HindIII in 3’, complementary primers were annealed. A2/Flu monomers were obtained by refolding of the different A2 heavy chains in the presence of β2m and influenza matrix peptide58-66 (GLCTLVAML). A2/Flu58-66 multimers were prepared by mixing biotinylated A2/Flu monomers with SA-PE (Invitrogen). For the preparation of SA-PE-NTA multimers SA-PE was first incubated with compound 1, 2, 3 or 4 (five-fold molar excess) at 4°C for 1 h, followed by incubation for 30 min with NiSO₄ (10 mM). Excess of reagents were removed using spin columns (Zeba™ Spin Desalting Columns; Thermo Scientific). His-tagged A2/Flu monomers were incubated at 4°C with SA-PE-biotin-Ni²⁺NTA at a ten-fold molar excess for ≥ 16 h. Melan A and Epstein-Barr (EBV) monomers were prepared likewise using the Melan A peptide 26-35 (ELAGIGILTV) or EBV BMFl1 peptide 280-288 (GLCTLVAML). A2/Flu58-66 PE-streptamer was obtained from IBA (Lucerna Ltd, Lucerne, Switzerland). PE-NTA2 conjugates were prepared by first reacting PE (Sigma) (50 nM) in phosphate buffer (0.1 M, pH 7.2 with SM(PEG)2 ester (Pierce) (10 m) at room temperature for 2h. Excess reagents were removed by spin columns. The resulting PE-maleimide was incubated under argon in phosphate buffer (100 mM, pH 7.0) with cysteine-NTA₂ (50 mM) at room temperature for 2h. After incubation for 30 min with NiSO₄ (10 mM), excess reagents were removed by spin columns; the concentration of Ni²⁺NTA₂-PE was determined by Abs at 565 nm.

Cells under study

PBMC were stimulated with peptides as described (25). The Flu, Melan-A and EBV specific CD8+ T cell clones or lines were obtained by limiting dilution cloning of peptide stimulated PBMC from healthy donors or a melanoma patient Lau 1164 and cultured in RPMI 1640 medium supplemented with 8% human serum and rIL-2 (150 U/ml) (CTL medium).
**Multimer binding assays**

For binding studies peptide stimulated PBMC (5 x 10⁴) were incubated for 30 min at room temperature with graded concentrations of A2/Flu multimers in 50 µl of FACS buffer (PBS supplemented with 0.5% BSA, 15 mM HEPES, and 0.02% NaN₃). After washing, cell-associated fluorescence was measured on a LSR II flow cytometer (BD, Allschwil, Switzerland) using gating on live cells. Data were processed using the FlowJo software (Tree Star, Inc. Ashland, OR). For dissociation experiments PBMC were incubated for 60 min at 4°C with 8 nM multimer, washed and after different periods of incubation in FACS buffer containing or not ant-HLA2 mAb BB7.2 mAb (100 µg/ml; Serotec) at the indicated temperatures, cell-associated fluorescence was determined by flow cytometry. In some experiments washed cells were resuspended in FACS buffer supplemented with 100 mM imidazole or 10 mM biotin and re-incubated for different periods of time. For cell viability experiments CD8+ T cells (10⁵ cells) were incubated at 4°C for 1 h, and stained with 8 nM of multimers for 15 min at 4°C. After staining with conventional BSP multimers, Annexin V-FITC and DAPI (Molecular Probes, Basel, Switzerland) the cells were analyzed by flow cytometry. Live cells were enumerated as percent of AnnexinV- and DAPI cells in the live lymphocyte gate, using untreated cells as reference (100%).

**CD8+ T cell sorting**

Flu peptide stimulated PBMC were incubated for 1 h at 4°C, stained for 15 min at 4°C with 4 nM of conventional BSP, SA-PE-NTA-A2-2xHis₆ multimers or PE-streptamer. After washing, cells were FACS sorted on a FACS Aria sorter (BD, Allschwil, Switzerland). A2/Flu multimer⁺ cells (5x10⁵ cells) were collected in medium containing imidazole (100 mM) or 10 mM biotin, washed and plated in 96 wells plate. After incubation in CTL medium for 1,4,24 and 48 h, the number of viable cells was enumerated by flow cytometry upon staining with A2/Flu BSP multimer, gating on live lymphocytes. The number of tetramer⁺ live cells at time zero was referred to as 100%.

**Cytolytic assays**

Cytolytic activity was assessed in a 4 h chromium release experiment using as targets T2 cells (5x10⁵ cells in 100 µl FCS) previously incubated with 100 µCi ⁵¹Cr for 1 h at 37 °C, washed and then incubated with serial dilutions of specific peptide for an additional 1 h at room temperature. As negative control, ⁵¹Cr-labeled T2 cells were loaded with an irrelevant peptide. An E:T ratio 10/1 was used. After 4 h of coincubation at 37 °C, the released ⁵¹Cr was assayed in 100 µl of supernatant and the specific lysis calculated as % of specific lysis as follows: ([sample release − spontaneous release] / (maximal release− spontaneous release))×100.

**A2/Flu₅₈₆₆ monomer dissociation kinetics**

Cloned Flu-specific BC74 CTL were incubated with PE-Ni²⁺NTA₂-A2/Flu-2xHis₆ multimers (6 nM) containing Cy5 labeled β2m, prepared by alkylation of β2m containing the S88C mutation with Cy5-maleimide (GE Healthcare), at 4°C for 45 min, washed and incubated for variable periods at 4°C in FACS buffer. PE and Cy5 fluorescence was measured before and after addition of imidazole (100 mM, dilution factor: 60-fold).

**RESULTS**

**Synthesis of mono, di and tetra-NTA compounds.**

To indentify suitable NTA moieties, we synthesized linear mono, di and tetra NTA peptides using conventional peptide synthesis. Based on the observation that the affinity of His₆ tagged molecules for Ni²⁺NTA increases with their valence (20-24), we initially synthesized the NTA₂ compound 3 (Figs. 1A, S1A). The coupling of the precursor (P3) with tri-bu NTA-lysine in solution was inefficient. The sequence Ala-Glu or Glu-Glu sequences very poor coupling yields were obtained, presumably due to steric hindrance of adjacent bulky tri-bu lysines. Moreover, tri-bu NTA-lysine has to be prepared (23) and the carrier peptide cannot contain groups reactive to activated carboxyl residues (i.e. OH, SH or NH₂), which imposes constraints on their synthesis. We therefore established another strategy, in which peptides containing 1, 2 or 4 cysteines were first synthesized (Fig. S1B) and then alkylated in aqueous solution with commercially available maleimido-NTA. The corresponding NTA thioethers (compounds 1, 2 and 4) were in high yields and purity. The NTA₄ compound 4 contains two NTA₂ moieties (as...
compound 2) linked by a flexible GGSGGGGS spacer.

Preparation of His tagged A2/Flu monomers.
To find a highly stable Ni\textsuperscript{2+}NTA-His tag pair, we prepared A2/Flu monomers containing C terminal of α3 domain of the heavy chain a His\textsubscript{S6}, His\textsubscript{S12} or a 2xHis\textsubscript{S6} tag (Fig. 1B). These heavy chains were refolded in the presence of β2m and Flu matrix peptide\textsubscript{58-66}. The refolding efficiency of the 2xHis\textsubscript{S6} tagged complex was nearly as high (98%) as the one of the BSP complex (data not shown). For the His\textsubscript{S6} tagged complex the refolding efficiency was approx $85\%$, but for the His\textsubscript{S12} tagged complex only 60%. Essentially the same results were obtained when using other peptides or other HLA alleles (data not shown).

SPR binding studies on Ni\textsuperscript{2+}NTA complexes and His tagged HLA-A2/Flu complexes. To study the interaction of the different A2/Flu monomers and biotinylated NTA molecules (Fig. 1), the latter were adsorbed on SA coated sensor chips, using equal and very low degree of loading (~100 RU). After saturation with biotin and NiCl\textsubscript{2}, graded concentrations of the different His tagged A2/Flu complexes were passed over the chips and the changes RU measured for one min; thereafter the decrease of RU was recorded upon flowing wash buffer (Figs. 2A, S3). Because the binding of biotin to SA is exceedingly stable (1), we disregarded dissociation of biotin from SA and calculated the binding on (k\textsubscript{on}) and off-rates (k\textsubscript{off}) assuming a 1:1 Langmuir binding and from these the K\textsubscript{D}. A nearly five log difference between the binding of the His\textsubscript{S6} tagged A2/Flu complexes to immobilized Ni\textsuperscript{2+}NTA\textsubscript{1} and the binding of 2xHis\textsubscript{S6} tagged complexes to Ni\textsuperscript{2+}NTA\textsubscript{4} was observed (Fig. 2B, Table S1). Because the latter value is beyond the range of accurate SPR measurements, we verified this K\textsubscript{D} value by equilibrium dialysis using \textsuperscript{125}I labeled NTA\textsubscript{4} as radioactive ligand. This analysis gave a K\textsubscript{D} value of 1.96 x 10\textsuperscript{-11} M, i.e. ~3-fold higher than the one determined by SPR (Fig. S4), most likely explained by an overestimate of the k\textsubscript{off} by SPR, due to rebinding of analyte.

For the His\textsubscript{S6} tagged A2/Flu complex the K\textsubscript{D} decreased modestly in the order NTA\textsubscript{1} > NTA\textsubscript{2} > NTA\textsubscript{4}. However, for His\textsubscript{S12} tagged complexes the affinity increased more substantially, notably by three orders of magnitude in the case of NTA\textsubscript{4}. Consistent with previous reports, we observed that the K\textsubscript{D} was over 100-fold lower for A2/Flu complexes carrying a 2xHis\textsubscript{S6} tag compared to those carrying a single His\textsubscript{S6} tag (18,19).

Binding isotherms of NTA\textsubscript{1}, NTA\textsubscript{2} and NTA\textsubscript{4} biotin-SA-PE multimers on Flu-specific CD8\textsuperscript{+} T cell populations. To examine the usefulness of the different Ni\textsuperscript{2+}NTA-His tag combinations for the preparation of CD8\textsuperscript{+} T cell staining reagents, SA-PE was loaded with the different biotin-NTA compounds and conjugated with His tagged A2/Flu\textsubscript{S6,66} multimers. The room temperature binding isotherms of A2/Flu multimers containing the 2xHis\textsubscript{S6} tag exhibited striking differences depending on the NTA moiety (Figs. 3A,D). The NTA\textsubscript{4} containing multimers displayed the most avid binding, followed by the NTA\textsubscript{2} (compound 2) multimers. The K\textsubscript{D} calculated from Scatchard analysis were 1.5 and 2.7 nM, respectively. Non-specific staining was essentially background (not shown). The multimers containing NTA\textsubscript{2} (compound 3) bound markedly less avidly and for the NTA\textsubscript{1} (compound 1) containing ones no K\textsubscript{D} could be conclusively determined. For comparison we included commercial A2/Flu\textsubscript{S6,66} streptamers, which exhibited less avid binding than the NTA\textsubscript{4} and NTA\textsubscript{2} (compound 2) containing multimers. We repeated this experiment using multimers containing His\textsubscript{S12} tagged A2/Flu complexes. The binding of all NTA containing multimers were reduced compared to the 2xHis\textsubscript{S6} containing counterparts (Figs. 3B,D). The difference was particularly pronounced for multimers containing NTA\textsubscript{2} compound 2. The results from the staining experiments correlated with those from the SPR experiments, i.e. the apparent K\textsubscript{D} observed for the multimer binding on cells showed the same hierarchy as those determined by SPR (Figs. 2B, 3D) and demonstrate that multimers containing Ni\textsuperscript{2+}NTA\textsubscript{2} (compound 2) and Ni\textsuperscript{2+}NTA\textsubscript{4} (compound 4) and 2xHis\textsubscript{S6} tagged A2/peptide complexes are highly stable and efficient CD8\textsuperscript{+} T cell staining reagents.

NTA-His tag built multimers are rapidly reversible. To examine the stability of the staining of Flu-specific CD8\textsuperscript{+} T cells by different A2/Flu multimers, polyclonal Flu-specific CD8\textsuperscript{+} T cells were incubated in the cold with equal concentrations of multimers, washed and incubated for different periods of time at ambient temperature. The stability of 2xHis\textsubscript{S6} tag containing multimers increased in the order NTA\textsubscript{1} (compound 1) < NTA\textsubscript{2} (compound 2) < NTA\textsubscript{4} (compound 4)
The dissociation of the NTA<sub>4</sub> and BSP multimers were remarkably slow, reaching about 92% of initial binding after 2 h. The sluggish dissociation of BSP multimers has been shown to be accounted for in part by rebinding (27); indeed in the presence of unlabeled tetramers or anti-A2 antibody considerably faster dissociation kinetics were observed (half-lives of 11.9 and 5.4 min, respectively; Fig. S5). For the NTA<sub>2</sub> (compound 2) containing multimer the dissociation was slightly faster, reaching about 82% after 2 h. However, for A2/Flu streptamer and NTA<sub>1</sub> containing multimers, the dissociation was considerably faster, reaching after 2 h approximately 60% and 27%, respectively. For multimers containing the His<sub>12</sub> tag the dissociations were slightly slower, notably in the case of NTA<sub>1</sub>, with half maximal dissociations (t<sub>1/2</sub>) of 34.5 versus 56.4 min (Figs. 4A,E).

When repeating this dissociation experiment at 37°C experiment, significantly accelerated kinetics were observed, especially for the streptamer (t<sub>1/2</sub> 14.6 min) and the multimers containing NTA<sub>1</sub> (t<sub>1/2</sub> 9.0 and 10.1 min for 2xHis<sub>6</sub> and His<sub>12</sub> complexes, respectively (Figs. 4B,E). The dissociations of the His<sub>12</sub> containing multimers were slower compared to the 2xHis<sub>6</sub> containing ones, which seems at odd with the SPR experiments, according to which the k<sub>off</sub> were lower for 2xHis<sub>6</sub> than for His<sub>12</sub> containing A2/Flu complexes (Fig. 2B). However, in the latter experiments monomeric complexes were examined, whereas in the former multimeric ones and their dissociation from cells may depend on additional parameters.

We then investigated the multimer dissociation in the presence of imidazole, which disrupts Ni<sup>2+</sup>NTA-histidine complexes (11-18). To find out the maximal imidazole concentration tolerated by our polyclonal CD8+ T cells, we incubated these with graded concentrations of imidazole for 1 h at 37°C and assessed their viability by trypan blue exclusion counting. Upon incubation with 100 mM imidazole the cell viability was ≥ 99%, however, it significantly decreased at > 200 mM (data not shown). In the presence of 100 mM imidazole the dissociations of the 2xHis<sub>6</sub> tag containing multimers at room temperature were remarkably rapid. For the NTA<sub>1</sub> and NTA<sub>2</sub> (compound 2) containing multimers dissociations with t<sub>1/2</sub> of ~ 0.2 min were barely measurable by flow cytometry (Figs. 4C,D,F). For the NTA<sub>4</sub> containing multimers the dissociation was slower with t<sub>1/2</sub> of 0.5 and 1 min for 2xHis<sub>6</sub> and His<sub>12</sub> containing multimers, respectively. It is noteworthy that in the presence of EDTA (20 mM) the Ni<sup>2+</sup>NTA<sub>4</sub>-2xHis<sub>6</sub> A2/Flu complexes were stable, arguing that NTA<sub>4</sub> (compound 4) more avidly chelated Ni<sup>2+</sup> than EDTA (data not shown). For comparison we assessed the dissociation kinetics of A2/Flu streptamers. Upon addition of 10 mM biotin the staining of the polyclonal CD8+ T cells decreased at room temperature with a t<sub>1/2</sub> of 1.1 min, i.e. slower than the NTA containing multimers (Figs. 4C,D,F).

Collectively these results demonstrate that MHC I-peptide multimers built on NTA<sub>2</sub> (compound 2) or NTA<sub>4</sub> (compound 4)-2xHis<sub>6</sub> tag chelate complexes stain Flu-specific CD8+ T cells as efficiently as or better than conventional BSP multimers. Importantly, they rapidly dissociated in the presence of imidazole, while being highly stable in its absence.

Reversible NTA multimers minimally affect CD8+ T cell viability. Numerous immunological studies require sorting of antigen-specific CD8+ T cells by FACS (or MACS). Because conventional BSP multimers affect CD8+ T cell viability (4-7), we examined what impact reversible staining with SA-PE-NTA<sub>4</sub> containing multimers has on cell viability. Randomly chosen cloned CTL specific for Flu matrix peptide 58-66, Epstein Barr peptide EBV 280-288 or a CTL line specific for Melan-A 26-35 were incubated at 4°C with the corresponding multimers containing BSP or SA-PE-NTA<sub>4</sub>, and cell-associated fluorescence analyzed by flow cytometry. The staining with SA-PE-NTA<sub>4</sub> multimers was slightly higher as compared to conventional multimers (Fig. 5A). The stained cells after washing with 100 mM imidazole were incubated at 37°C for different periods of time and viable cells enumerated by flow cytometry. The percentages of live, i.e. DAPI and Annexin V cells were markedly lower on cells treated with conventional as compared to reversible multimers (Fig. 5B). To assess the functional integrity of thus treated CTL, we examined their cytotoxicity. As shown in Fig. 5C the CTL treated with BSP, but not SA-PE-NTA<sub>4</sub> multimers exhibited reduced lysis, both in terms of maximal lysis and EC50. Remarkably, BSP multimer treated CTL displayed increased nonspecific lysis, which is probably explained by
transfer of peptide from BSP multimers to cell-associated MHC I molecules, giving rise to aberrant CTL-CTL and CTL-target cell interactions (28,29).

Because these experiments showed considerable variations between clones, we next used polyclonal, Flu58-66 peptide stimulated PBMC. The cells were stained by A2/Flu58-66 BSP, SA-PE-NTA4 multimers or streptamer to comparable extents (Fig. 6A). A constant number of annexin V$^+$ cells was FACS sorted at 4°C and after different periods of incubation at 37°C the percentage of dying cells assessed by annexin V staining. Cells sorted with BSP exhibited extensive apoptosis, cumulating after 4 h with 57% of annexin V$^+$ cells (Fig. 6B). By contrast cells sorted with the reversible NTA4 multimer displayed only about 6% of apoptotic cells, marginally less than streptamer sorted cells. To assess the functional integrity of the sorted cells they were tested in a $^{51}$Cr release assay. For sake of fair comparison we pre-incubated the BSP multimer sorted cells with anti-A2 mAb BB7.2 to achieve maximal multimer dissociation (supplementary Fig. S5, ref. 27). As shown in Fig. 6C, BSP multimer sorted cells exhibited significantly reduced target cell killing compared to SA-PE-NTA4 multimer of streptamer sorted cells and increased non-specific lysis, as observed on clones (Fig. 5C). For further analysis we cloned sorted cells by limiting dilution. Compared to BSP multimer sorted cells about 1.8 and 1.7-fold more clones were obtained from NTA4 multimer and streptamers sorted cells, respectively (Fig. 6D). A2/Flu58-66 BSP multimer staining of clones showed that those derived from NTA4 multimer and to a lesser degree streptamer sorted cells contained a fraction of high avidity specificites (Fig. 6E). This was also observed when CD8 binding deficient A2227/228/Flu58-66 multimers were used (Fig. 6F). Upon incubation with A2/Flu58-66 BSP multimer at 37°C, the brightly stained clones became annexin V$^+$ (Fig. 6G). These results indicate that sorting of Flu-specific CTL with BSP multimers results in selective loss of high avidity, cell death prone CD8+ T cells.

**PE-NTA$_2$-A2/potide multimers – a new type of T cells staining reagents.** To directly conjugate MHC-peptide complexes with PE, we activated it with maleimido-NHS and reacted the resulting maleimido–PE with NTA$_2$-Cys(SH) (Fig. 7A).

After incubation with NiCl$_2$, PE-Ni$^{2+}$NTA$_2$ was conjugated with A2/Flu58-66-2xHis$_6$ monomers, resulting in multimers containing 7-8 MHC-peptide complexes per PE. The staining of Flu peptide stimulated PBMC by A2/Flu58-66 multimers was strikingly different, whether these were conventional or PE-NTA$_2$ containing ones (Fig. 7B). While the binding of the conventional multimer increased steadily with the multimer concentration, it reached a plateau at about 5 nM of PE-NTA$_2$ multimer. As seen from Scatchard analysis the $K_D$ value for the PE-NTA$_2$ multimer was about 12-fold lower than the one for the conventional multimer; however, the maximal binding ($B_{max}$) was modestly higher (1.6 fold) for conventional multimer. We tested these reagents also on Flu peptide stimulated PBMC from a different donor as well as on Melan-A$_{26-35}$ peptide stimulated PBMC from a melanoma patient, using the corresponding A2/Melan-A multimers. As shown in Figs. 7E-H, PE-NTA$_2$ multimers efficiently stained antigen-specific CD8$^+$ T cells, typically with slightly lower MFI compared to conventional multimers and similar backgrounds.

**Assessment of A2/Flu monomer dissociation kinetics on cloned, Flu-specific CTL.** The staining of PE-NTA$_2$ multimers was as very rapidly reversed upon addition of imidazole (100 mM) as shown for NTA$_2$-biotin-SA-PE at room temperature and at 4°C (Fig. 4C and data not shown). We therefore examined whether PE-NTA$_2$ MHC-peptide multimers allow assessment of monomer dissociation kinetics as depicted in Figs. 8A,B. To this end we prepared A2/Flu58-66-2xHis$_6$ complexes containing Cy5 labeled β2m (Fig. 7A) and with these PE-NTA$_2$ multimers, were used to stain in the cold cloned Flu-specific BC74 CTL cold. The washed cells were analyzed by flow cytometry, recording the PE (Fig. 8C) and Cy5 fluorescence before and after addition of imidazole (Fig. 8D). While the PE fluorescence reached background within 20-30 sec, the Cy5 fluorescence only after 300-350 sec. The Cy5 fluorescence decrease exhibited half maximal dissociation in the range of 98-118 sec. Importantly the monomer dissociation reached background levels, which is in contrast to dissociation experiments with BSP multimers, in which complete dissociation is typically not reached due to multimer rebinding and/or internalization (8,9). The experiments gave well
reproducible results when performed in the cold; at elevated temperatures the monomer dissociation were to rapid to be reliably measured (data not shown). Moreover, for these experiments it is preferable to use 2xHis$_6$-NTA$_2$ multimers, because they dissociate faster upon addition of imidazole than those containing NTA$_4$ and/or the His$_{12}$ tag (Fig. 4).

**DISCUSSION**

A range of immunological studies require sorted antigen-specific CD8+ (and CD4+) T cells. While FACS is technically highly efficient, a recurrent problem is that MHC-peptide multimers (or antibodies) used for staining persist on sorted cells and provoke cell death and functional alterations (4-9). The present study demonstrates that reversible, highly effective MHC-peptide multimers can be prepared based on Ni$^{2+}$NTA-His tag chelate complexes. Previously branched tri-NTA complexes have been described that have up to sub-nanomolar K$_D$ for His$_6$ and His$_{10}$ tags (22-24). Our results demonstrate that highly stable chelate complexes can be formed with linear peptides carrying orthogonal NTA moieties; the binding of Ni$^{2+}$NTA$_4$ peptide (compound 4) to 2xHis$_6$ tagged A2/Flu complexes with a K$_D$ of 19.6 pM is the highest affinity ever reported for NTA-His tag complexes (Fig. 2 and supplemental Fig. S3). While previously described tri and tetra NTA compounds allow diverse applications, their difficult chemical synthesis make these accessible only to specialized labs (20-24). In only one previous study a tri-NTA compound was synthesized as a peptide derivative (2). However, as building block Fmoc-Asp($\beta$-amido-Ne-Lys(tri-bu'-NTA)) was used, which is neither commercially available, nor facile to synthesize. Moreover, we obtained remarkably poor yields for the coupling of Ne-Lys(tri-bu'-NTA) with peptides containing free orthogonal carboxyl groups, especially when these are adjacent (compound 3; Fig. 1A, supplemental Fig. S1A and unpublished results); and thus argue that coupling of remarkably bulky Ne-Lys(tri-bu'-NTA) or derivatives thereof tend to be inefficient due to steric hindrance. Consistent with this view, we obtained excellent yields for couplings performed in solution on fully deprotected molecules using site-specific alkylation (supplemental Fig S1B). The strategy to synthesize linear oligo cysteines peptides by conventional solid phase peptide synthesis and to alkylate these with commercially available maleimido-NTA renders accessible a wide range oligo-NTA compounds to non-specialized labs.

The observation that NTA$_2$ dimer compound 3 exhibited considerably weaker binding than NTA$_2$ dimer compound 2, especially for the His$_{12}$ and 2xHis$_6$ tagged HLA-A2/Flu complexes (Figs. 2-5), indicates that the chelate complex stability critically depended on the configuration of NTA compound. The main difference between these two NTA dimers is the length of the side chains carrying the NTA groups (y and y’) and the distance by which these attached to the peptide backbone (x and x’, Fig. 1A). In compound 2 this distance is shorter than in compound 3. A previous study has shown that the avidity of branched tri- Ni$^{2+}$NTA molecules for His$_6$ tagged molecules increased with decreasing length of the spacer linking the NTA moieties to a central nitrogen (24). This argues that the NTA dimer compound 2 exhibited more avid binding than dimer compound 3, because of the shorter spacing distance x’. On the other hand, the longer side chain y’ in compound 2 may permit better interactions of individual Ni$^{2+}$NTA moieties with histidine residues on the His tags, namely on the long 2xHis$_6$ tag. While it has been shown that the avidity and stability of Ni$^{2+}$NTA-histidine complexes increases with the valence of Ni$^{2+}$NTA moieties and the length of the His tags (18-24), our study demonstrates for the first time that the combination of a double His$_6$ tag and a double NTA dimer (compound 4) provides a dramatic gain of avidity and stability. We suggest that the long and flexible GGSGGGS linkers contained in both entities allows a two register binding mode, in which two His$_6$ tags and two NTA$_2$ moieties interact in a zipper-like fashion. However, interactions of oligo-Ni$^{2+}$NTA moieties with His tags are complex, involving diverse kinetic, entropic and enthalpic aspects and more extended studies.
will be needed to fully understand the underlying structure-binding relations (20-24, 30-32).

It is interesting to note that the conventional A/Flu BSP multimers displayed a modestly lower staining of Flu-specific PBMC than those containing NTA<sub>6</sub> and a 2xHis<sub>6</sub> tag (Fig. 3A). Because the BSP-SA binding is more stable than the Ni<sup>2+</sup>NTA<sub>6</sub>-2xHis<sub>6</sub> tag complex, this difference may be explained by different configurations of the two types of multimers. Indeed, we have shown previously that soluble MHC I-peptide complexes depending on their configurations can have strikingly different K<sub>D</sub> and B<sub>Max</sub> values (25).

Avid and stable binding of MHC-peptide multimers allows efficient staining of antigen-specific CD8<sup>+</sup> T cells and at the same time results in extensive cross-linking of TCR and CD8, which at elevated temperature induces strong T cell activation and rapid T cell death (Figs. 5, 6; ref. 4, 5, 8, 9, 26, 33, 34). It also results in internalization of MHC-peptide complexes and transfer of peptide to cell-associated MHC I molecules, which can cause aberrant CTL functions, e.g. fratricide and bystander killing (26, 28, 29). Because of this, FACS sorting of CD8<sup>+</sup> T cells using BSP multimers is prone to infer loss of antigen-specific cells, namely selectively high avidity ones and alteration of the functional integrity of surviving cells (Figs. 5, 6 and refs. 4, 5, 9). This can be avoided by using reversible MHC-peptide multimers, the effectiveness of which depends on: 1) their stability, which determines the staining performance and shelf-life. The here described Ni<sup>2+</sup>NTA<sub>6</sub>-2xHis<sub>6</sub> tag based multimers are considerably more stable than streptamers and DTB multimers, especially at elevated temperatures and their shelf-life is the same as for BSP multimers (Figs. 4, ref. 4 and data not shown). 2) Their reversibility, which determines how rapidly and quantitatively they can be removed from sorted cells. The induced dissociation of NTA-His tag multimers is clearly faster than the ones of streptamers and DBT multimers (Figs. 4, S5, ref. 4). As we have shown that the induced dissociation of DTB multimers from CTL is sufficiently rapid to prevent intracellular Ca<sup>2+</sup> mobilization (4), the same is expected for NTA-His tag multimers. Intracellular Ca<sup>2+</sup> mobilization is required for most CTL functions, including multimer induced CD8<sup>+</sup> T cell death (8, 26, 33).

The here described NTA-2xHis<sub>6</sub> chelate complexes allow the preparation of a wide range of conjugates. An attractive application is to couple NTA moieties onto PE (or APC), which allows facile and stable conjugation with MHC-peptide-2xHis<sub>6</sub> complexes (Fig. 7A). CD8<sup>+</sup> T cell staining by these conjugates is strikingly different compared to BSP multimers (Figs. 7B, C). While BSP multimers are heterogeneous, containing PE-SA conjugates of various stoichiometries and configurations (e.g., orientations of SA to PE), PE-NTA containing multimers are molecularly better defined, containing one central PE conjugated with 7-8 MHC-peptide complexes (26). This difference is reflected in the binding isotherms, which exhibited a clear saturation in the case of PE-NTA-2xHis<sub>6</sub> but not BSP multimers and hence a better linear regressions in Scatchard analysis (Figs. 7A-D). This also explains why the PE-NTA-2xHis<sub>6</sub> multimers bind with lower K<sub>D</sub> and B<sub>Max</sub> than BSP multimers. For these reasons PE-NTA-2xHis<sub>6</sub> multimers are better suitable for accurate binding studies. Furthermore and importantly, owing to the very rapid, imidazole induced disintegration of NTA-2xHis<sub>6</sub> multimers, they permit conclusive assessment of MHC-peptide monomer dissociations on living CD8<sup>+</sup> T cells (Fig. 8). PE-NTA<sub>6</sub>-2xHis<sub>6</sub> multimers are particularly suitable for this, because they provide stable staining and very rapid imidazole induced disintegration (Figs. 3A, 4A and unpublished data). In contrast to dissociation experiments using BSP multimers this strategy provides real kinetics of dissociation of MHC-peptide monomers from TCR and CD8 in the absence of artifactual residual rebinding (Fig. 8C, D, refs. 10, 11). Although this technique is less accurate than SPR measurements, it provides binding data on living cells, which substantially deviate from SPR binding experiments using recombinant TCR and MHC-peptide complexes, notably due to coordinate binding of CD8 to TCR-associated MHC-peptide (7, 12, 27). Recent 2D binding studies (e.g. intercellular) of TCR-MHC-peptide interactions have indicated binding parameters that were strikingly different than those measured in 3D (e.g. SPR) binding studies (35-37). This casted doubt on the value of the latter in terms of predicting T cell activation. This is especially
intriguing for CD8+ T cells, because CD8 can bind to TCR-associated cognate as well as to non-cognate MHC-peptide complexes and thereby substantially affect TCR-MHC-peptide binding and T cell activation (12,27,36). The here described assay (Fig. 8) and TCR photoaffinity labeling (12) constitute interesting alternatives, because they allow MHC-peptide binding studies on living cells in the absence of intercellular adhesion effects.

PE-NTA2 or 4 can be equally used for the preparation on MHC class II-peptide and antibody Fab multimers. In the former case the 2xHis<sub>6</sub> tag is fused C terminal of the α2 domain, which additionally allows universal and gentle purification of fragile, “empty MHC II molecules from culture supernatants; in the latter case a synthetic His tag is conjugated with Fab’ fragments by site specific alkylation, similarly as described previously (4). The stability and imidazole induced reversibility of NTA-His tag based conjugates depends on those of the underlying chelate complexes and not on the type of the conjugate (e.g. PE-NTA or NTA-biotin-SA-PE). In case of NTAC-2xHis<sub>6</sub> containing multimers the stability of the underlying chelate complex exceeds by far any of the previously described switchable conjugates, including streptamers and DTB multimers. At the same time their imidazole induced disintegration is faster than any of the previously reported reversible MHC-peptide multimers and their production is simpler, which affords a better accessibility (Figs. 2-4, refs 4,5,9,21-24).

In conclusion, the present work provides a framework of novel types of protein conjugates, namely fluorescent MHC-peptide multimers, built on reversible NTA-His tag chelate complexes. We describe the synthesis of linear NTA peptides that requires no specialized equipment or knowhow and that allow complex formation with 2xHis<sub>6</sub> tagged proteins of unprecedented stability, while being rapidly switchable upon addition of imidazole. MHC-peptide multimers built on NTA-His coordinate complexes outperform previously described reversible staining reagents and allow greater diversity of conjugate formation, e.g. PE-NTA based multimers and hence provide new opportunities to advance T cell analysis.

REFERENCES

FOOTNOTES

* We are indebted to Nicole Montandon for competent technical assistance, Dr. Marek Kosinski (Centre Hospitalier Universitaire Vaudois, Lausanne) for expert assistance with equilibrium dialysis experiments, and Dr. B. Robert (Centre de Recherche en Cancérologie de Montpellier) for critical reading of the manuscript. This work was supported by grants from the Swiss National Science Foundation (310030_12533/1) and from the Cancer Research Institute.

The abbreviations used are: A2, HLA-A*0201; β2m, beta 2-microglobulin; BSP biotinylation sequence peptide; CTL, cytotoxic T lymphocyte; K_D, dissociation constant; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NTA, nitrilo-triacetic acid; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; SA, streptavidin; SPR, surface plasmon resonance; TCR, T cell antigen receptor.

FIGURE LEGENDS

Figure 1. The NTA compounds and His tag under study. A) Structures of the four peptidic NTA compounds (1-4) under study. The approximate maximal distances are for x = 7 Å, y = 8 Å, x’ = 5 Å, y’ = 14 Å. B) Sequence of the different sequences added C-terminally at the HLA-A2 heavy chain, including the biotinylation sequence peptide BSP, His_{6}, His_{12} or two His_{6} tags (2x His_{6}) separated by a flexible GGGSGGGGS spacer (as in compound 4). The underlined residues (274-276) mark the C-terminal end of the α3 domain.

Figure 2. Binding of His tagged A2/Flu monomers to immobilized Ni^{2+}-NTA moieties assessed by SPR. A) Scheme of SPR binding assay. Streptavidin coated sensor chips were loaded with biotinylated NTA compounds (100 RU each), loaded with NiCl_{2} and changes in RU measured upon incubation with His tagged A2/Flu complexes. B) Summary of SPR binding parameters for the indicated Ni^{2+}-NTA compounds A2/Flu complexes. The lowest K_D is boxed in red. Mean values were calculated from at least three experiments. Standard deviations are shown in supplemental Table S1. The K_D indicated as footnote was determined by equilibrium dialysis (see mFig. S4).

Figure 3. Binding isotherms of SA-PE-NTA A2/Flu multimers on Flu-specific CD8+ T cell population. A) Flu_{58-66} peptide stimulated PBMC were incubated at ambient temperature for 30 min with graded concentrations of SA-PE A2/Flu multimers containing a 2xHis_{6} tag and biotinylated NTA_{1} (compound 1) (blue squares), NTA_{2} (compound 2) (dark green triangles), NTA_{2} (compound 3) (light green circles) or NTA_{4} (compound 4) (purple triangles). For comparison, conventional BSP multimers (red circles) and streptactin multimers (orange diamonds) were included. After washing, cell-associated fluorescence was assessed by flow cytometry. B) The same experiment was performed with SA-PE A2/Flu multimers containing a His_{12} tag. C) Comparison of the binding of SA-PE A2/Flu multimers containing either a 2xHis_{6} tag (solid lines, full symbols) or a His_{12} tag (dashed lines, empty symbols) and NTA_{2} (compound 2) (green triangles) or NTA_{4} (compound 4) (purple triangles), respectively. D) Dissociation constants (K_D) and maximal binding values (B_{Max}) observed for the indicated SA-PE A2/Flu multimers. Mean values and SD were calculated from > 3 experiments.
Figure 4. Dissociation kinetics of SA-PE A2/Flu complexes. A, B) Flu58-66 peptide stimulated PBMC were incubated at 4°C for 30 min with 8 nM of SA-PE A2/Flu complexes containing NTA1 (blue squares), NTA2 (compound 2, green triangles) or NTA4 (purple triangles) and either a 2xHis6 (solid lines) or His12 tag (dashed lines). For comparison BSP multimers (red circles) and streptamers (orange diamonds) were included. After washing cells were incubated at room temperature (A) or 37°C (B) and after the indicated period cell-associated fluorescence was assessed by flow cytometry. C, D) The experiment shown in A) was repeated for 2xHis6 (C) or His12 containing complexes (D) in the presence of imidazole (100 mM) or 10 mM biotin (for streptamer). The dashed gray lines indicate half maximal dissociation. E, F) Compilations of half lives (t1/2) observed in the absence (E) or presence (F) of imidazole or biotin. Mean values and SD were calculated from at least three experiments.

Figure 5. Impact of multimers on CD8+ T cell viability. A) HLA-A2-restricted CD8+ T cells specific for influenza matrix 58-66 (clone BC74), Melan-A 26-35 (melan A) or EBV BMLF1 280-288 (clone BC25) were stained at 4°C with 5 nM BSP or reversible SA-PE-NTA4 multimers containing the respective 2xHis6 tagged A2/peptide complexes. After washing with or without imidazole (100 mM) cell-associated fluorescence was measured by flow cytometry. The inserted values represent cell percentage for the indicated gates. B) The same cells were treated likewise with BSP (red) or NTA4 multimers (green) or left untreated (dark), incubated at 37°C for the indicated periods of time, stained with annexin V-FITC and DAPI and analyzed by flow cytometry. Live, annexinV- and DAPI- cells are represented as percentages, with 100% referring to untreated cells. One out of three experiments is shown. C) Alternatively, cells treated likewise with BSP (red) or NTA4 multimers (green) or left untreated (black) were tested in a 51Cr release assay using as targets T2 cells pulsed with graded concentrations of the respective specific or as negative control unspecific peptide. Mean values and SD of specific lysis were calculated from two experiments.

Figure 6. FACS sorting of Flu-specific CD8+ T cells using A2/Flu multimers. A) Flu58-66 peptide stimulated PBMC were incubated for 15 min at 4°C with 5 nM of A2/Flu58-66 multimers containing BSP, SA-PE-NTA4 and 2xHis6 or A2/Flu58-66 streptamer, washed and analyzed by flow cytometry. The inserted values represent cell percentage for the indicated gates. B) 100,000 BSP multimer+ cells (red), NTA4 multimer+ cells (green) or streptamer+ cells (blue) were FACS sorted, washed with imidazole or biotin containing medium and after different periods of incubation at 37°C analyzed by flow cytometry upon staining with annexin V and BSP multimers. Percentages of tetramer+ and annexin V+ cells are shown, with 100% being the input cell number. One of two experiments is shown. C) Alternatively the cells sorted with BSP multimers were incubated with anti-A2 mAb BB7.2 (100 µg/ml) at room temperature for 20 min (red) or sorted with SA-PE-NTA4 (green) multimers or streptamer (blue) and washed with imidazole or biotin containing medium were incubated with 51Cr labeled T2 cells in the presence of graded concentrations of Flu58-66 peptide for 4h at 37°C. Mean values and SD of specific lysis were calculated from two experiments. D) Sorted cells (300 cells each) treated as in C) were cloned by limiting dilution (0.5 cells/well) and after 14 days growing clones enumerated. Mean values and SD were calculated from two experiments. E, F) Randomly chosen clones from BSP (red), SA-PE-NTA4 (green) multimers or streptamer (blue) sorted cells were stained at room temperature for 30 min with conventional A2/Flu BSP (E) or A222722/Flu BSP multimer (F) (5 mM) and analyzed by flow cytometer. G) Alternatively the cloned cells were stained with A2/Flu BSP multimer (5 nM) and annexin V and analyzed by flow cytometry after incubation at 37°C for 30 min. The numbers indicate individual clones.
Figure 7. PE-NTA-A2/peptide multimers – a new type of T cells staining reagents. A) PE was reacted with maleimido-NHS (SM(PEG)$_2$) and the resulting maleimido-PE subsequently with NTA$_2$-Cys (SH). PE-NTA$_2$, after loading with Ni$^{2+}$ was conjugated with A2/peptide-2xHis$_6$ complexes to make biotin SA free PE-NTA$_2$ multimers. B) Flu$_{58-66}$-peptide stimulated PBMC were incubated at 4°C for 1 h with graded concentrations of A2/Flu BSP (red circles) or PE-NTA$_2$ multimers (blue squares) and cell-associated PE fluorescence measured by flow cytometry. C) The binding data of B) were subjected to Scatchard analysis. D) The mean values and SD of K$_D$ and B$_{max}$ values were calculated from three experiments. E-H) Flow cytometry analysis of Flu$_{58-66}$ peptide stimulated PBMC from a healthy donor (E, F) or Melan A$_{26-35}$ peptide stimulated PMBC from melanoma patient Lau 1164 G, H) were incubated at 4°C with 8 nM of the corresponding BSP (E, G) or PE-NTA$_2$ (F, H) multimers and anti-CD8 antibody.

Figure 8. Assessment of A2/Flu monomer dissociation kinetics. A) Cy5 labeled A2/Flu$_{58-66}$ monomers were prepared by refolding with blue β2m obtained by mutating S88C, indicated by spheres and alkylation with Cy5 maleimide. B) Scheme of the dissociation kinetic experiment. CD8+ T cells are stained at 4°C with A2/Flu$_{58-66}$ multimers containing PE (red)-NTA$_2$ (two hemi-spheres), 2xHis$_6$ tagged MHC (gray)-peptide (red dot) complexes and Cy5 labeled β2m (blue). Upon addition of imidazole the Ni$^{2+}$NTA-2xHis$_6$ tag complex very rapidly decays in PE-NTA$_2$ and A2/Flu$_{58-66}$ monomers, which subsequently dissociate from cell-associated TCR (green) and CD8 (orange). C, D) Cloned Flu-specific BC74 CD8+ T cells were stained at 4°C with multimers A2/Flu$_{58-66}$ multimers containing Cy5 labeled β2m and either biotin and PE-SA (C) or a 2xHis$_6$ tag and PE-NTA$_2$ (D) and analyzed by flow cytometry at 4°C recording PE (C) or Cy5 (D) fluorescence before and after addition of imidazole (100 mM) (time 0; arrows and gray lines). The half maximal dissociations are indicated by gray lines and by their t$_{1/2}$ values. Shown are three different experiments.
Figure 1

A

B

BSP : HLA-A*0201......WEPSLHHILDAQKMVNHR

His$_6$ : HLA-A*0201......WEPSHHHHHH

His$_{12}$ : HLA-A*0201......WEPSHHHHHHHHHHHHH

2xHis$_6$ : HLA-A*0201......WEPSHHHHHHHHGGSGGGGSGSHHHHHH
**Figure 2**

**A**

Coating with NTA-biotin peptides

1-Loading with Ni2+  
2-Binding of MHC

**B**

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* $K_D = (1.96 \pm 0.16) \times 10^{-11}$ M by equilibrium dialysis
Figure 4

E

\[
\begin{align*}
 & t_{1/2} \text{ RT (min)} \\
\text{SA-PE-NTA1 (1)} & \begin{array}{cc}
\text{A2-2xHis6} & 34.5 \pm 3.1 \\
\text{A2-His12} & 56.4 \pm 2.3
\end{array} \\
\text{t}_{1/2} \text{ 37°C (min)} \\
\text{SA-PE-NTA1 (1)} & \begin{array}{cc}
\text{A2-2xHis6} & 9.0 \pm 0.8 \\
\text{A2-His12} & 10.1 \pm 0.5
\end{array} \\
\text{PE-streptamer} & 14.6 \pm 1.5
\end{align*}
\]

F

\[
\begin{align*}
 & t_{1/2} \text{ RT (min)} \\
\text{SA-PE-NTA1 (1)} & \begin{array}{cc}
\text{A2-2xHis6} & 0.20 \pm 0.01 \\
\text{A2-His12} & 0.16 \pm 0.02
\end{array} \\
\text{SA-PE-NTA2 (2)} & \begin{array}{cc}
\text{A2-2xHis6} & 0.20 \pm 0.02 \\
\text{A2-His12} & 0.23 \pm 0.02
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\text{SA-PE-NTA4 (4)} & \begin{array}{cc}
\text{A2-2xHis6} & 0.55 \pm 0.05 \\
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Figure 7

A

B

C

D

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E

F

G

H

CD8
Reversible MHC I-peptide multimers containing Ni2+NTA peptides and his tags improve analysis and sorting of CD8+ T cells
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J. Biol. Chem. published online October 11, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.283127

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