Establishment of the reversible peptide-major histocompatibility complex (pMHC) class I Histamer technology: tool for visualization and selection of functionally active antigen-specific CD8+ T lymphocytes

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Abstract

Multimers of soluble peptide-major histocompatibility complex (pMHC) molecules are used in both basic and clinical immunology. They allow the specific visualization and isolation of antigen-specific T cells from ex vivo samples. Adoptive transfer of antigen-specific T cells sorted by pMHC multimers is an effective strategy for treatment of patients with malignancies or infectious diseases after transplantation. We developed a new reversible pMHC multimer called ‘Histamer’ to enable the specific detection and isolation of antiviral T cells from peripheral blood. HLA-A*02:01/CMVpp65495–503 Histamer (A02/CMV Histamer) was generated by coupling 6xHis-tagged pMHC molecules onto cobalt-based magnetic beads. The specificity of the Histamer was evaluated by flow cytometry. Sorting of antiviral CD8+ cytotoxic T lymphocytes (CTLs) was performed by magnetic cell separation, followed by the monomerization of the Histamer after addition of the competitor L-histidine. Sorted T cells were analyzed for phenotype and function. The reversible pMHC Histamer proved to be highly specific and sensitive. CMV-specific T cells of up to 99.6% purity were isolated using the Histamer technology. Rapid and complete disassembly of the T-cell surface-bound A02/CMV Histamer followed by the subsequent dissociation of the pMHC monomers from CD8+ CTL receptors was achieved using 100 mM L-histidine. The function of CMV-specific T cells enriched by Histamer staining did not differ from CTLs induced by standard T-cell assays. This reversible T-cell staining procedure preserves the functionality of antigen-specific T cells and can be adapted to good manufacturing practice conditions. The pMHC Histamer technology offers full flexibility and fulfills all requirements to generate clinical-grade T lymphocytes.

Keywords: cytotoxic T lymphocytes, flow cytometry, human, immunomagnetic separation, pMHC Histamer

Introduction

Cellular immunodeficiency after allogeneic hematopoietic stem cell transplantation (HSCT) or solid organ transplantation (SOT) leaves the patient susceptible to a wide range of infections (1, 2). Viral and fungal infections are serious complications, which are difficult to control with drugs and are associated with high morbidity and mortality. Adoptive immunotherapy using antigen-specific T cells is an efficient tool to control viral infections after allogeneic HSCT or SOT, virus-induced malignancies and metastatic melanomas (3–7). The rationale behind T-cell transfer for infections is the hypothesis that a quantitative deficiency of antigen-specific T cells confers patient susceptibility to viral reactivation and primary infection of a variety of infectious agents, including Epstein-Barr virus (EBV), adenovirus (ADV) and cytomegalovirus (CMV) (8). Accumulating evidence indicates that effective antiviral drug therapy relies on specific immune reconstitution. Therefore, synergism between donor lymphocyte infusion (DLI) and antiviral drug therapy will have to be addressed by
further studies (9–11). However, the application for DLI in the treatment of infections is limited and associated with a significant risk of graft-versus-host disease (GvHD). Therefore, adoptive immunotherapy should be performed using antigen-specific T-cell infusions. In this context, peptide-major histocompatibility complex (pMHC) class I multimer technology is an aspiring powerful tool for obtaining antigen-specific CD8+ cytotoxic T lymphocytes (CTLs) for adoptive T-cell transfer in cancer or infectious disease patients after HSCT or SOT (12, 13).

In recent years, the direct visualization, quantification, phenotypical characterization and isolation of antigen-specific T cells by multimeric pMHC complex has attracted major attention (14, 15). The analysis of T-cell responses to autoantigens, infectious diseases and tumor cells using pMHC multimer staining reagents has been described previously (16). Altman et al. (1996) were the first to use the avidity effect of pMHC multimerization to stain T cells (17). Structurally, the heterotrimeric pMHC complex is composed of an MHC heavy chain (hc), the β2-microglobulin (B2M) light chain and an antigen-specific peptide, which is presented in a groove formed between the α1 and α2 domains (18). The pMHC staining technology exploits fluorescently tagged backbones to create pMHC multimers for visualization of antigen-specific T cells, whereas fluorophores are not necessary for separation of antigen-specific T cells. Several pMHC multimer techniques have been developed using dimers (19, 20), tetramers (17, 21), pentamers (13, 22–24), dextramers (25), octamers (26), streptamers (8, 27) and clinimers (28, 29) for visualization, characterization and sorting of antigen-specific T cells. All of these pMHC multimers use the natural T-cell receptor (TCR) ligand (the peptide–MHC complex) as the staining probe. Due to the low avidity of TCR/pMHC interactions, pMHC monomers have to be multimerized in order to raise the complex stability to a detectable level. The various means of pMHC multimerization, such as avidin/streptavidin-based tetramers, are reviewed elsewhere (12, 21).

As summarized by Constantin et al. (30) and Einsele et al. (8, 31), other techniques for T-cell isolation have various limitations by altering phenotype and function. Therefore, application of pMHC multimers seems to be a safe and promising tool for isolation of antigen-specific T cells without affecting their naivety. pMHC multimers proved useful in initial clinical trials for isolation and expansion of specific T cells for adoptive therapy after HSCT, especially in CMV infection (11, 13, 32, 33). Even though these studies are promising, T cells are manipulated during the enrichment process, and TCR/pMHC interactions could alter the functional status of enriched T cells. Such manipulation could reduce T-cell viability during isolation and expansion and even in vivo if the pMHC multimers remain on the T-cell surface (27). Moreover, the administration of pMHC multimer-enriched antigen-specific T cells into patients requires more complex protocols for production according to good manufacturing practice (GMP) guidelines. This poses financial and regulatory barriers to such cell treatments (11). The reversible conventional pMHC streptamer technique was devised in order to solve these problems (11, 27, 28). This novel reagent allows isolation of T cells without altering their original phenotype and functional status provided pMHC multimer staining is performed at 4°C (27).

So far, only pMHC streptamers are available as commercially reversible pMHC multimers for the separation of functional active T cells (8, 11, 27). In this study, we designed a reversible pMHC-class-I-His-tagged multimer, herein referred to as ‘Histamer’, for the visualization and separation of antigen-specific CD8+ T cells. The proof of principle of this technology was performed using the immunodominant HLA-A*02:01 restricted CMV peptide (CMVpp65495–503) of the lower matrix phosphoprotein 65 (pp65) (34) to detect CMV-specific CTLs in the blood of healthy HLA-A*02:01 platelet donors. Comparable to previous pMHC multimers, the specific reversible A*02:01/CMVpp65495–503 Histamer (A02/CMV Histamer) was designed to stain and isolate CMV-specific CD8+ CTLs without altering their differentiation and functional status. Such protocol could be employed to isolate highly pure antigen-specific T-cell sub-populations that can be used immediately or after in vitro expansion for adoptive antigen-specific T-cell transfer according to GMP. Moreover, as the number of pMHC complexes multimerized exceeds by the majority of multimers described so far, a higher efficiency in capturing the desired T cells is anticipated.

Methods

Generation of pMHC Histamer

To generate reversible human multimeric A02/CMV Histamer, we modified the pMHC multimer generation method described by Altman et al. (17). Specifically, a histidine-tagged HLA-A*02:01 hc was generated instead of a biotinylated HLA-A*02:01 hc. The basic principle of the reversible A02/CMV Histamer staining technology is illustrated in Fig. 1(A). Briefly, total RNA was isolated from peripheral blood mononuclear cells (PBMCs) of a healthy donor carrying HLA-A*02:01 using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Amplification of exons 2 to 4 of the HLA-A*02:01 hc was performed by RT-PCR with the primers HLA-A-E2-TAS (5’-ATG GGC TCC CAC TCC ATG AGG TA-3’) and HLA-E4-WAS (5’-CCA TCT CAG GGT GAG GGG CT-3’) using the OneStep RT-PCR Kit (Qiagen). The primers B2M-TAS (5’-ATG ATC CAG CGT ACT CCA AAG-3’) and B2M-WAS (5’-CAT GTC TCG ATC CCA CCT ACC T-3’) were used for amplification of B2M exon 2. The B2M plasmid (pET30) was kindly provided by Dr. L. Kjer-Nielsen (Melbourne University, Australia). The PCR products were ligated into the prokaryotic pCR7-CT/TOPO® (B2M) and pEXP5-CT/TOPO® (HLA-A*02:01 hc) expression vectors using the pCR7-CT/TOPO® TA Expression Kit and the pEXP5-CT/TOPO® TA Expression Kit, respectively (both Invitrogen, Karlsruhe, Germany). After IPTG-induced protein expression [strain BL21(DE3)] and purification of the hc and B2M, MHC molecules were folded in vitro with CMVpp65495–503 peptide (NLVPMVATV, purity >99%; EZ Biolabs, Carmel, IN, USA).

The refolding mixture was incubated for 44 h at 4°C and afterwards purified by size exclusion chromatography...
The HLA-A*02:01/CMVpp65 495-503 peptide complex was concentrated to 2 mg ml⁻¹ and labeled with the Alexa Fluor 647 (AF647) Protein Labeling Kit (Invitrogen). The multimeric A02/CMV Histamer was produced by adding cobalt-based Dynabeads according to the manufacturer’s instructions (Dynabeads® His-Tag Isolation & Pulldown, Invitrogen) and stored in sterile phosphate-buffered saline (PBS; pH 7.2) at 4°C (Fig. 1A). The magnetic beads were dissociated from 6xHis-tagged A02/CMV Histamer by adding 80 mM imidazole (Sigma-Aldrich, Hamburg, Germany) or 100 mM L-histidine (Sigma-Aldrich) (disassembly, Supplementary Figures 3B and C, 1B). The specificity of A02/CMV Histamer was checked by CMV-specific T-cell staining and flow cytometry (FACSCanto A, BD Biosciences, Heidelberg, Germany).

**Establishment of A02/CMV Histamer staining**

Histamer staining was performed with freshly isolated PBMCs and CMVpp65 495-503 peptide-stimulated PBMCs from healthy HLA-A*02:01 CMV-seropositive platelet donors (n = 17) with no prior history of blood transfusion. PBMCs from
CMV-seronegative donors \((n = 17)\) were used to exclude non-specific staining of A02/CMV Histamer. Informed consent was obtained from all donors as approved by the local ethics committee of Hannover Medical School. CMVpp65-specific CTLs were induced \textit{in vitro} as described elsewhere using the HLA-A*02:01-restricted CMVpp65_{495–503} peptide (NLVPWMVAT, purity >99%; EZ Biolabs) (35–37). Specific multimer staining was validated using different staining buffer compositions for staining, washing and resuspension of the cells (PBS with 50–280 mM imidazole as competitor for metal affinity or PBS/5% AB serum; C.C.pro, Neustadt, Germany; Supplementary Figure 1A and B). Furthermore, 0.1–4 µg A02/CMV Histamer-AF647 per 1 × 10^6 cells and staining temperatures at 4 and 37°C were tested to identify the optimal concentration and temperature (Supplementary Figure 1C–F) for staining purposes. Analysis of A02/CMV Histamer and A02/CMV Pentamer staining was carried out using standard lymphocyte gates on forward scatter and side scatter profile, followed by gating on populations positive for CD8. As control, staining with either phycoerythrin (PE)-conjugated A02/CMV Pentamer (Pro5® MHC Class I Pentamer, HLA-A*02:01 CMVpp65_{495–503} Pentamer; Proimmune, Oxford, UK) or PE-conjugated A02/CMV Streptamer (MHC class I Streptamer, IBA BioTAGnology, Göttingen, Germany; Supplementary Figure 1A and B) was performed according to the manufacturers’ instructions. Because best staining results for specific binding and low background signals were obtained at 4°C using 0.2 µg A02/CMV Histamer-AF647 per 1 × 10^6 cells in cold PBS/5% AB serum as staining buffer, these conditions were applied in all further experiments (Supplementary Figure 1A–F). Briefly, 1 × 10^6 cells were washed in staining buffer and incubated with 0.2 µg A02/CMV Histamer-AF647 for 45 min on ice, followed by staining with FITC-conjugated anti-CD8 mAb (Beckman Coulter, Krefeld, Germany) for 25 min. Cells were analyzed by flow cytometry (FACSCanto A), whereas data acquisition and analysis were realized using BD FACSDiva V6.1.2 (BD Biosciences) and FlowJo V7.6.1 software (Treestar, San Carlos, CA, USA). At least 100 000 events within the live gate or 50 000 events within the CD8+ population were acquired for each analysis. Gates were set based upon the scatter properties of lymphocytes and cells were further gated on CD8+ cells.

Non-specific binding of A02/CMV Histamer to T cells was excluded by analyzing CMV-specific T cells in a HLA-A*02:01-restricted CMV-seropositive (Supplementary Figure 2A) and -seronegative donor (Supplementary Figure 2B), respectively. To strengthen this observation, A02/CMV Histamer staining was compared to conventional A02/CMV multimers (A02/CMV Streptamer and A02/CMV Pentamer; Supplementary Figure 2C and D). These data indicate that all three A02/CMV multimer technologies yield comparable results (Fig. 2A and B). The specificity of A02/CMV Histamer to bind only HLA-A*02:01-restricted CMV-specific CD8+ CTLs was further evaluated by staining with A02/CMV Histamer-AF647, human hematopoietic lineage FITC cocktail (lin; eBioscience, San Diego, CA, USA) and anti-human CD8-PE (BD Bioscience). The lineage cocktail consists of a mixture of antibodies that stain specific human T, B and NK cells and monocyte, myeloid and erythroid lineages (CD2/3/14/19/25/56). Aliquots of the cells were stained under similar conditions with A02/CMV Pentamer-PE, lin-FITC and allophycocyanin (APC)-conjugated anti-CD8 (BD Biosciences) for comparison. For analysis, cells were gated on lymphocytes.

A02/CMV Histamer-guided isolation of CMV-specific T cells

CMV-specific T cells were isolated either from PBMCs directly or after stimulation with the CMVpp65_{495–503} peptide. Therefore, 1 × 10^6 cells were incubated for 45 min with A02/CMV Histamer-AF647. Histamer+ T cells were isolated on a DynaMag magnet. Sample tubes were placed on a magnet until the Histamer+ T cells had collected at the wall of the tube and the supernatant was clear. The cells were washed three times with staining buffer by discarding the supernatant and resuspending the cells between each washing cycle. All steps were performed on ice. After isolation, CMV-specific CD8+ T cells were analyzed by flow cytometry. A02/CMV Pentamer-guided isolation was performed using anti-PE beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers’ instructions.

A02/CMV Histamer disassembly and culture of enriched CMV-specific CD8+ T cells

In the disassembly experiments, buffer conditions (PBS with 40–200 mM L-histidine) were validated and the isolated T cells were analyzed by flow cytometry using FITC-conjugated anti-CD8. Prior to this, proliferation assays were performed to evaluate the effects of 40–200 mM L-histidine on CD8+ T-cell proliferation (Supplementary Figure 3A). PBS/400 mM imidazole was also tested since imidazole is recommended for dissociation by the manufacturer. Because PBS/100 mM L-histidine achieved the best results for the disassembly of A02/CMV Histamer from CMV-specific CD8+ CTLs, it was used as the disassembly buffer in all further experiments (Supplementary Figure 3B and C).

Disassembly experiments were performed with cells obtained after 7 days of stimulation with CMVpp65_{495–503} peptide. Histamer-stained CTLs were washed four times with cold disassembly buffer on a DynaMag until the beads were collected at the tube wall. The supernatant containing the dissociated CMV-specific CD8+ CTLs was collected and washed with PBS. Briefly, 2.5 × 10^5 dissociated CD8+ CTLs (Histamer-treated) were cultured in 96-well plates (V-bottom, Sarstedt, Nümbrecht, Germany) in the presence of irradiated (30 Gy) autologous PBMCs pulsed with the CMVpp65_{495–503} peptide at a responder-to-stimulator ratio of 10:1 in 200 µl culture medium (RPMI1640/10% AB serum) containing 100 U/ml IL-2 (PeproTech, Hamburg, Germany). After 1 week, the T cells were analyzed by multimer staining and screened for lytic activity. Non-Histamer-treated CMV-specific CD8+ T cells (non-treated) from same donors were cultured under similar conditions and used as controls.

\textit{T-cell cytotoxicity assay}

Cytotoxicity assays were performed to determine whether the enriched CMV-specific T cells (Histamer-treated)
Fig. 2. A02/CMV multimers for the detection and isolation of CMV-specific CD8⁺ T cells. (A–D) PBMCs derived from a healthy HLA-A*02:01 CMV-seropositive donor were stimulated for 1 week with CMVpp65-495–503 peptide and stained with (A) A02/CMV Histamer and (B) A02/CMV Pentamer followed by staining with anti-CD8. Dot plots are shown on gated CD8⁺ lymphocytes for CD8-FITC versus A02/CMV Histamer–AF647 staining and for CD8-FITC versus A02/CMV Pentamer–PE staining. CMV-specific T cells were sorted with (C) A02/CMV Histamer or (D) A02/CMV Pentamer in high purity. (E and F) Freshly isolated PBMCs (day 0) and stimulated multimer-non-treated PBMCs (day 7) isolated from 17 HLA-A*02:01 CMV-seropositive and 17 HLA-A*02:01 CMV-seronegative donors (controls) were stained with (E) A02/CMV Histamer and (F) A02/CMV Pentamer. (G and H) Bivariate plots of frequencies of A02/CMV Histamer+/CD8⁺ T cells versus A02/CMV Pentamer+/CD8⁺ T cells in freshly isolated PBMCs (day 0) and CMVpp65-495–503 peptide stimulated PBMCs (day 7) from identical (G) HLA-A*02:01 CMV-seropositive and (H) CMV-seronegative donors. Asterisks indicate statistically significant differences between CMV-specific T-cell levels in HLA-A*02:01 CMV-seropositive and -negative donors (*P < 0.05, **P < 0.01, ***P < 0.001).
Fig. 3. Reversible A02/CMV Histamer staining and effects on T-cell function. (A) PBMCs isolated from a healthy HLA-A*02:01 CMV-seropositive donor were stimulated with CMVpp65495–503 peptide for 7 days. CMV-specific Histamer+/CD8+ T cells are shown as a part of the CD8+ lymphocytes after staining and after Histamer disassembly with PBS/100 mM l-histidine. Dot plots are shown on gated CD8+ lymphocytes for CD8-FITC versus A02/CMV Histamer-AF647. (B and C) For disassembly, CMVpp65495–503-stimulated T cells (day 7) from 14 healthy HLA-A*02:01 CMV-seropositive donors were stained with A02/CMV multimers (before disassembly). Histamer-treated (stained and disassembled) and non-treated T cells were re-stimulated for 1 week (day 14) and analyzed by A02/CMV multimer staining. (D) For the cytolytic activity assay, Histamer-treated (after reversible Histamer staining) and non-treated cells from six CMV-seropositive donors were re-stimulated for 7 days (day 14) and analyzed in a standard 4-h non-radioactive flow cytometric assay. CFSE-labeled non-pulsed PBMCs and PBMCs pulsed with either the relevant HLA-A*02:01-restricted CMVpp65495–503 peptide or the irrelevant minor antigen HA-1H were used as target cells. The cytolytic level of CMV-specific T cells against non-pulsed target cells (D) was subtracted from the lytic level of effector cells against the CMVpp65495–503 Peptide-pulsed target cells. (E) For cytokine secretion assay, Histamer-treated and non-treated cells from six CMV-seropositive donors were stimulated with CMVpp65495–503 overnight. Enriched IFN-γ-secreting cells were stained with CD4-FITC, CD8-APC and IFN-γ-PE followed by flow cytometric analysis. Dot plots are shown on gated lymphocytes and CD8+ or CD4+ T cells for CD8-APC versus IFN-γ-PE and for CD4-FITC versus IFN-γ-PE. The absolute number of CD3+/IFN-γ+ T cells among CD3+ lymphocytes and CD8+/IFN-γ+ T cells among CD8+ T lymphocytes was determined. The results of independent experiments are expressed as mean ± SD (n.s., not significant).
were still functional after dissociation of pMHC Histamer from their TCR. Additionally, cytolytic activity of A02/CMV Histamer-non-treated cells (non-treated) from same donors was analyzed. A standard 4-h non-radioactive flow cytometric assay was therefore performed using autologous CFSE (5- or 6-[N-succinimidyloxycarbonyl]-3′,6′-O′-diacetylfluorescein; Invitrogen)-labeled PBMCs as target cells (36, 38). The target cells were pulsed with 10 µg ml⁻¹ CMVpp65₄₉₅₋₅₀₃ peptide and placed in V-bottom 96-well tissue culture plates. To determine unspecific killing, non-pulsed CFSE-labeled PBMCs and PBMCs pulsed with the HLA-A*02:01-restricted minor histocompatibility antigen HA-1H peptide (VLHDDLLEA, purity >95%; EZ Biolabs) were used as target cells in the negative controls. T cells were added to target cells at effector-to-target (E:T) ratios of 3:1 and 30:1 in 200 µl per well culture medium in the presence of 100 U ml⁻¹ IL-2 for 4 h. Target cell lysis was then assessed by flow cytometry using 7-AAD (BD Bioscience) staining. Specific lysis was calculated by subtracting values of control-pulsed target cells.

**IFN-γ secretion assay**

Freshly isolated PBMCs from healthy HLA-A*02:01 CMV-seropositive platelet donors (n = 6) were treated with unlabeled A02/CMV Histamer and analyzed by IFNy-secreting assay (CSA; Miltenyi Biotec). IFN-γ CSA was performed to determine the impact of Histamer on functionality of CMV-specific T cells. Briefly, 3 × 10⁶ PBMCs were either stimulated with 10 µg ml⁻¹ CMVpp65₄₉₅₋₅₀₃ peptide, 1 µg ml⁻¹ SEB (Staphylococcus aureus enterotoxin B, positive control; Sigma-Aldrich) or without antigen (negative control). IFN-γ-secreting cells were detected using the IFN-γ secretion assay according to the manufacturer’s instructions. After CSA, enriched IFN-γ-secreting cells were stained with PE-conjugated anti-IFN-γ (Miltenyi Biotec), FITC-conjugated anti-CD4 (Beckman Coulter) and APC-conjugated anti-CD8 (BD Biosciences) and analyzed on the flow cytometer with gating on lymphocytes during acquisition. At least 10,000 events were acquired. The absolute number of CD3⁺ CD8⁺ T cells among CD3⁺ lymphocytes and CD8⁺/IFN-γ⁺ T cells among CD8⁺ T lymphocytes was determined. Quadrants were set based upon the negative controls.

**Expression of activation markers after A02/CMV Histamer-guided staining and isolation of CMV-specific T cells**

Expression analysis of the early activation markers CD137, CD69 and CD25 was performed to determine whether Histamer-stained or -dissociated CMV-specific T cells did not underlie functional alteration. Additionally, A02/CMV Histamer-non-treated CTLs (non-treated) from identical donors were analyzed and used as control. They were stimulated with the CMVpp65₄₉₅₋₅₀₃ peptide for 1 week (n = 6) and used for Histamer staining and dissociation of CMV-specific T cells. All steps were performed on ice. Early activation markers were analyzed on CMV-specific CD8⁺ T cells before (day 0) and after staining or disassembly of the Histamer into monomeric MHC molecules (days 1 and 2). Briefly, CTLs were incubated with peridinin chlorophyll protein complex–cyanin 5.5 (PerCP-Cy5.5)-conjugated anti-CD8 (BD Biosciences), phycoerythrin–cyanin 7 (PE-Cy7)-conjugated anti-CD137 (BioLegend, San Diego, CA, USA), FITC-conjugated anti-CD69 (BD Biosciences) and APC-H7-conjugated anti-CD25 (BD Biosciences) mAbs for 25 min. Gates were set based upon the scatter properties of lymphocytes, and cells were further gated on CD8⁺ T cells.

**Fluorescence microscopy**

PBMCs (1 × 10⁶) were stained with freshly prepared A02/CMV Histamer-AF647 (Fig. 1C and E) or 3-month-old Histamer (Fig. 1D and F) followed by anti-CD8-FITC. For nuclear staining, PBMCs were incubated for 5 min with 10 µg per millilitre of blue fluorescent diaminido-2-phenylindole (DAPI; Invitrogen). Stained cells were washed with staining buffer and examined with an Olympus-IX81 microscope (Olympus, Center Valley, PA, USA) equipped with a standard green, blue and red fluorescence filter set and a ×20–60 objective set. Images were acquired using a CCD camera (Olympus) and analyzed using Olympus cell⁴⁰ and cell⁰ image 3.0 software (Olympus).

**Statistical analysis**

Statistical analysis was performed using the Mann-Whitney t test and linear regression run on GraphPad Prism v5.02 software (GraphPad Software, San Diego, CA, USA). Levels of significance were expressed as P values (*P < 0.05, **P < 0.01, ***P < 0.001).

**Results**

**Optimization of A02/CMV Histamer staining and dissociation conditions**

AF647-conjugated (for phenotypical analysis; Fig. 1A) and non-conjugated A02/CMV Histamers (for disassembly; Fig. 1B) were generated with approximately 100% recovery by adding cobalt-based magnetic Dynabeads to the A02/CMV Histamer. Immunofluorescence microscopy (Fig. 1C and D) and flow cytometric analysis (Fig. 1E and F) revealed typical surface staining pattern of A02/CMV Histamer. Specific labeling was strong and photostable enough for long examination. In the present study, A02/CMV Histamer remained stable for up to 3 months (Fig. 1D and F), but it is likely that the stability of pMHC/Histamer complexes varies with different pMHC complexes.

In the first series of experiments, buffer conditions for washing and staining were validated (Supplementary Figures 1 and 2). Overall, the staining and washing procedures performed with 0.2 µg Histamer per 10⁶ cells and PBS containing 5% AB serum resulted in the most sensitive and specific labeling of CMV-specific CD8⁺ CTLs combined with a low non-specific background, while the cell distribution was comparable to that observed when using the conventional A02/CMV Pentamer. Incubation of CD3⁺ and CD8⁺ T-cell populations with Dynabeads followed by magnetic removal indicated no non-specific binding (<0.01%) of beads to T cells, and recovery of the CD3⁺ (99.99%) and CD8⁺ T cells (99.97%) was nearly 100% (Supplementary Figure 2).
Flow cytometry using the human hematopoietic lineage flow cocktail (lin) was performed to assess the specificity of A02/CMV multimers to bind only CMV-specific CD8+ T cells. The lin cocktail consists of a mixture of antibodies against CD2, CD3, CD14, CD16, CD19, CD56 and CD235a.

Gates were set based upon the scatter properties of lymphocytes. Shown are the flow cytometric results using freshly isolated PBMCs from five HLA-A*02:01 CMV-seropositive donors, expressed as mean ± SD.

In the second step, the specificity of A02/CMV Histamer to bind only CMV-specific CD8+ T cells was further determined by using a human hematopoietic lineage cocktail (lin; Table 1). If there is specific multimer staining, an equivalent frequency of multimer+/CD8+ T cells and multimer+/lin+ cells gated on lymphocytes is expected. A02/CMV Histamer staining achieved specificities of 97.9% (lymphocyte population; Table 1) and 99.5% (Table 1), which was comparable to the results obtained for A02/CMV Pentamer staining. Bivariate plots of frequencies of multimer+/CD8+ T cells and multimer+/lin+ T cells, respectively, showed minimized scatter of points around the linear regression line. The bivariate plot of frequencies of multimer+/CD8+ T cells for A02/CMV Histamer staining versus A02/CMV Pentamer staining had a regression coefficient of 1.983, which was similar to the regression coefficient in the bivariate plot of multimer+/lin+ T cells (1.301). High regression coefficient and minimized scatter of points around the linear regression line indicated that both staining techniques yielded similar results with respect to CMV-specific T-cell visualization.

As shown in Fig. 1(B), Dynabeads can be easily disassembled from pMHC Histamer-stained T cells by adding the competitor -histidine. Treatment with 40–200 mM -histidine did not result in a significant decrease in CD8+ T-cell proliferation, while the most optimal viable CD8+ T-cell proliferation (4.9%) was achieved with 100 mM -histidine (Supplementary Figure 3A). After Histamer disassembly, dissociation of low-affinity HLA/His-tagged monomers from the TCRs of the CMV-specific CTLs occurs spontaneously (27). Reversibility was reflected by the loss of T-cell staining following A02/CMV Histamer removal and the detection of dissociated CMV-specific CD8+ T cells. Based on a nearly complete (99.6%) reversibility of T-cell staining, PBS/100 mM histidine (disassembly buffer) was used for all disassembly experiments (Supplementary Figure 3B and C).

Detection and isolation of CMV-specific T cells with A02/CMV Histamer

Both freshly isolated PBMCs and PBMCs expanded for 7 days with CMVpp65 peptide from 17 CMV-seropositive HLA-A*02:01 donors were analyzed to verify the ability of the A02/CMV Histamer to detect CMV-specific T cells (Fig. 2). PBMCs and expanded T cells from 17 HLA-A*02:01 CMV-seronegative donors (controls) were used to exclude nonspecific A02/CMV Histamer staining.

Figure 2A–D shows the frequency of CMV-specific T cells after multimer staining (A02/CMV Histamer versus A02/CMV Pentamer) and multimer-guided isolation for one representative donor stimulated with CMVpp65 peptide for 7 days. Histamer+/CD8+ T cells were detected at a frequency of 6.9% (Fig. 2A) and isolated via magnetic Dynabeads with a purity of 99.6% among CD8+ T cells (Fig. 2C). The amount of CMV-specific T cells and their purity after isolation was comparable to that observed with the A02/CMV Pentamer (Fig. 2B and D). Thus, magnetic enrichment with A02/CMV Histamer achieved a mean purity of 98.6% ± 1.4 of CMV-specific CD8+ T cells (n = 17 seropositive donors) compared to 98.9% ± 1.1 with A02/CMV Pentamer. The results of Histamer and Pentamer staining in all HLA-A*02:01 donors tested on day 0 and after 7 days of peptide stimulation (without foregoing MHC multimer-guided isolation) are shown in Fig. 2E and F. CMV-specific CD8+ T cells were detected at mean frequencies of 3.0% (0.3–8.3%) and 7.4% (0.3–29.4%) in freshly isolated (day 0) and stimulated PBMCs (day 7), respectively, from CMV-seropositive donors (n = 17) treated with A02/CMV Histamer (Fig. 2E). These results are in concordance with those obtained after A02/CMV Pentamer staining (Fig. 2F; day 0: mean 3.0%, range 0.5–5.3%; day 7: mean 5.6%, range 0.5–19.6%). Limited frequencies of Histamer+/CD8+ T cells (day 0: mean 0.4%; day 7: mean 0.5%), Pentamer+/CD8+ T cells (day 0: mean 0.3%; day 7: mean 0.4%) or any distinct multimer+/CD8+ T-cell populations were detected in the CMV-seronegative donors (n = 17). In summary, the efficiency of A02/CMV Histamer to visualize and isolate CMV-specific CD8+ T cells was comparable to that of conventional A02/CMV Pentamer.

Statistical analyses were performed using bivariate plots of frequencies of A02/CMV Histamer+/CD8+ T cells versus A02/CMV Pentamer+/CD8+ T cells to compare A02/CMV Histamer with A02/CMV Pentamer staining for freshly isolated (day 0) and peptide-stimulated PBMCs (day 7), respectively, from same CMV-seropositive (Fig. 2G; n = 17) and CMV-seronegative donors (Fig. 2H; n = 17). Bivariate plots showed minimized scatter of points around the linear regression line, while the regression coefficient on day 0 (CMV-seropositive 1.019; CMV-seronegative 1.170) was comparably high to that on day 7 (CMV-seropositive 1.366; CMV-seronegative 1.327). Regression analysis indicated a high statistical similarity with a low standard deviation between A02/CMV Histamer and A02/CMV Pentamer staining.

Table 1. Specificity of A02/CMV multimers for the detection of CMV-specific CD8+ T cells

<table>
<thead>
<tr>
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<th>A02/CMV Histamer</th>
<th>A02/CMV Pentamer</th>
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<tr>
<td>Multimer+/lin+ T cells</td>
<td>0.9% ± 0.5</td>
<td>0.8% ± 0.4</td>
</tr>
<tr>
<td>Multimer+/CD8+ T cells</td>
<td>0.8% ± 0.6</td>
<td>0.7% ± 0.3</td>
</tr>
<tr>
<td>Specificity</td>
<td>97.9% ± 7.2</td>
<td>94.8% ± 8.6</td>
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The results of reversible staining (Fig. 1B) with A02/CMV Histamer are summarized in Fig. 3. CMV-specific T cells expanded for 1 week were stained and then dissociated from A02/CMV Histamer to evaluate the effects of reversible A02/CMV Histamer staining on T-cell function in terms of expansion and cytotoxicity. Figure 3A shows the result of a

A02/CMV Histamer disassembly and analysis of isolated CMV-specific T cells

The results of reversible staining (Fig. 1B) with A02/CMV Histamer are summarized in Fig. 3. CMV-specific T cells expanded for 1 week were stained and then dissociated from A02/CMV Histamer to evaluate the effects of reversible A02/CMV Histamer staining on T-cell function in terms of expansion and cytotoxicity. Figure 3A shows the result of a
representative experiment. CMV-specific CTLs were visualized at a frequency of 29.6% among CD8+ T cells after 7 days of stimulation with the CMVpp65 peptide by A02/CMV Histamer staining. After incubation with disassembly buffer, A02/CMV Histamer was completely removed from CMV-specific CD8+ T cells as indicated by the complete loss of the Histamer+/CD8+ T-cell population.

Figure 3B and C summarizes the results of CMV-specific CD8+ T-cell expansion obtained in 14 independent experiments. T cells expanded for 1 week were stained with A02/CMV Histamer (Fig. 3B) and A02/CMV Pentamer (Fig. 3C). On day 7, expanded cells were equally divided to perform disassembly and control experiments. After dissociation of A02/CMV Histamer, CD8+ T cells (Histamer-treated) were re-stimulated in the presence of CMVpp65 peptide-pulsed autologous PBMCs for 1 week (day 14) and analyzed again by multimer staining. Histamer-non-treated cells (non-treated) re-stimulated for 1 week with peptide-pulsed autologous PBMCs served as controls. The frequencies of Histamer-treated CTLs increased significantly from day 7 (5.0% ± 5.2) to day 14 (41.4% ± 18.2) and was comparable to the increase observed in the control (non-treated cells) with 5.0% ± 5.2 to 42.5% ± 18.9. The results were confirmed by A02/CMV Pentamer staining (Histamertreated: 4.6% ± 3.9 to 44.4% ± 20.1; non-treated: 5.0% ± 5.2 to 45.3% ± 21.9). The frequencies of CMV-specific CD8+ T cells correlated with the obtained cell numbers of CMV-specific CD8+ T cells (Fig. 3B and C). From day 7 to day 14, the absolute number of Histamer-treated CTLs increased from 5.0 × 10^5 to 1.3 × 10^6 per 2.6-fold. The absolute number of non-treated CTLs (control) increased to a specific extent (5.0 × 10^5 to 1.5 × 10^6 per 3.0-fold) indicating that disassembly has no negative impact on further expansion of CMV-specific T cells.

To substantiate these findings, the cytolytic activity of Histamer-treated CTLs was determined and compared to that of non-treated CTLs (Fig. 3D). Generally, there is a correlation between the frequency of specific cell lysis and the percentage of CMV-specific CTLs present during the cytotoxicity assay (39). Lysis of HA-1H-pulsed control target cells by CMV-specific CTLs was comparable to that by non-pulsed target cells. Histamer-treated CTLs (after reversible Histamer staining) achieved a specific lysis rate of 35.1% ± 13.2 at an effector-to-target ratio of 3:1, which was comparable to that of non-treated controls (35.8% ± 13.4). At an effector-to-target ratio of 30:1, 47.4% ± 5.4 of the target cells were lysed by Histamer-treated CTLs compared to 48.2% ± 4.7 by non-treated CTLs. Thus, there was no significant difference between Histamer-treated and non-treated CTLs in terms of cytolytic activity.

Moreover, functionality of CMV-specific T cells after reversible A02/CMV Histamer staining was analyzed by the detection of the effector cytokine IFN-γ upon antigen-specific stimulation by performing CSA (Fig. 3E). The absolute number of CD8+/IFN-γ+ T cells among CD8+ T cells after reversible Histamer staining (Histamer-treated) was detected with a mean of 2.0 × 10^4 (range: 1.2 × 10^4 to 3.0 × 10^4), which was comparable to that obtained in non-treated CD8+ T cells (1.9 × 10^4; range: 1.4 × 10^4 to 2.9 × 10^4). Unspecific IFN-γ secretion of Histamer-treated cells compared to non-treated cells was determined by the absolute number of CD3+/IFN-γ+ T cells among CD3+ T cells. Both Histamer and non-treated cells were specifically stimulated with CMVpp65 peptide before CSA. Histamer-treated CD3+ T cells produced a significant level of IFN-γ (2.0 × 10^4; range: 1.2 × 10^4 to 3.0 × 10^4) comparable to non-treated CD3+ T cells (1.9 × 10^4; range: 1.4 × 10^4 to 2.9 × 10^4). The deviation between CD3+IFN-γ+ and CD8+/IFN-γ+ T cells was significantly low, indicating a specific cytokine production in both Histamer-treated and non-treated CD8+ CTLs.

Expression of early T-cell activation markers was further analyzed to exclude that reversible A02/CMV Histamer staining does not change the naivety of CMV-specific CD8+ T cells (Fig. 4). The expression of CD137, CD69 and CD25 on CD8+ lymphocytes was determined before (day 0) and after (days 1 and 2) A02/CMV Histamer-AF647 staining (b, Histamer-stained) or A02/CMV Histamer dissociation (c, Histamer-treated) of CMV-specific T cells (Fig. 4). As a control, non-treated CTLs (a, non-treated) were used. CD137+ cells were detected at mean frequencies of 2.4% (day 0: 1.9–4.0%), 12.6% (day 1: 2.3–32.1%) and 12.3% (day 2: 5.8–22.0%) in stimulated PBMCs after A02/CMV Histamer-AF647 staining, respectively (Fig. 4A). The frequencies of CD137+ cells in stimulated PBMCs after A02/CMV Histamer disassembly (Histamer-treated) were significantly lower [day 0: 2.4% (1.9–4.0%); day 1: 0.9 (0.5–1.7%); day 2: 1.0% (0.6–1.8%)] as in A02/CMV Histamer-AF647-stained cells, whereas the frequencies are in concordance with those obtained in non-treated CTLs [day 0: 2.4% (1.9–4.0%); day 1: 1.3% (0.6–2.3%); day 2: 0.9% (0.7–1.7%)]. The expression level of CD69 (Fig. 4B) and CD25 (Fig. 4C) for A02/CMV Histamer–AF647-stained cells were comparable low to A02/CMV Histamer-dissociated and non-treated CTLs. These results indicate that reversible Histamer staining does not cause phenotypic change of antigen-specific T cells.

In conclusion, performed functional assays (expansion, cytotoxicity assays, CSA and expression analysis of early T-cell activation markers) demonstrate that reversible staining with unlabeled A02/CMV Histamer does not alter the functional status of the specific CD8+ T-cell population.

Discussion

In the present study, we developed a new type of a reversible pMHC multimer, termed Histamer. Multimerization was performed by coupling refolded 6xHis-tagged pMHC class I molecules onto cobalt-based magnetic beads. A02/CMV Histamer was used for the efficient detection and separation of CMV-specific T cells without altering their functional status by surface staining at low temperatures. Disassembly of the A02/CMV Histamer followed by the complete dissociation of the monomeric MHC molecules from the TCR of CD8+ T cells was achieved using PBS/100 mM l-histidine. Furthermore, A02/CMV Histamer staining could be used to identify the appropriate number of memory T cells in addition to serological testing (33). Our results demonstrate that the A02/CMV Histamer is a specific and sensitive pMHC multimer for the visualization and sorting of functionally active CMV-specific T cells.
Multimeric scaffolds and characteristics of pMHC Histamer

Pentamer, tetramer and streptamer staining are the most common multimer technologies (12, 13, 21). Unlike single pMHC class I molecules, multimers can simultaneously bind a large number of TCR molecules. The binding of pMHC multimers to surface TCRs is associated with high avidity, which allows epitope-specific binding to T cells (27). All multimer technologies yield similar results with respect to antigen-specific T-cell visualization, while the tetramer technology results in the lowest background signal (33). The low affinity of single TCRs for their cognate pMHC counterparts results in an off-rate of only a few seconds (40). To apply an effective labeling technique, multiple pMHC monomers have to be multimerized into a stable complex that has higher binding avidity (12, 41, 42). Avidin/streptavidin-based tetramers are one of the most common means of pMHC multimerization (12, 21). We developed Histamers as reversible pMHC multimers using a backbone of magnetic Dynabeads displaying a cobalt-based metal affinity ligand on their surface. This backbone afforded to bind maximum quantities of A02/CMV monomers, which can then interact with specific TCRs on the cell surface. Due to multimerization, a high number of pMHC molecules of the Histamer would face in the same direction, resulting in a very high avidity (12, 13). The high valency stage of Histamers might enable a more sensitive detection of CD8+ T cells with relatively low-affinity characteristics of monomeric pMHC-TCR interactions (e.g. T cells specific for self-antigens) than tetramers or pentamers (12). Specific T-cell staining by pMHC multimers is particularly dependent on the staining conditions, which can have a dramatic effect on the specificity and sensitivity of T-cell staining (21, 27). As shown by our findings, optimization of staining conditions is necessary to achieve best staining results.

We selected AF647 as fluorophore for Histamer labeling. AF conjugates exhibit brighter fluorescence and greater photostability than conjugates of other spectrally similar fluorophores, allowing more time for image capture. However, chemical labeling of the pMHC multimers with fluorophores, such as AF647, may result in conceivable site damages of the multimer that are important for T-cell interactions. AF647 binds to primary amines of proteins and could theoretically influence both specificity and sensitivity of the pMHC multimer due to random labeling regions of the pMHC, which are important for pMHC–TCR interactions. To avoid this, AF647-labeled pMHC Histamers should only be applied for the detection of antigen-specific T cells, which will not be further used or expanded. Unlabeled pMHC Histamers should be used for the isolation of antigen-specific T cells to maintain their naivety. As shown by our findings, AF647 labeling does influence neither sensitivity nor specificity of the A02/CMV Histamer themselves in the detection of antigen-specific T cells.

Compared to the conventional multimer technologies, Knabel et al. have designed a reversible streptamer staining technique that offers the advantage of selecting antigen-specific CTLs by preserving the functional status of the T lymphocytes (27). This is of great benefit for the in vitro investigation of T-cell functions and for the adoptive transfer of isolated cells. Streptamer is currently the only pMHC multimer used in adoptive immunotherapy, and according to medical law, streptamers are not considered as drugs but as adjuvants (8, 11). We demonstrated that the reversible Histamer technology (Fig. 1B) might also be applicable for effective isolation and separation of antigen-specific T cells without affecting their functional status. The disassembly procedure was performed using l-histidine at a low concentration instead of imidazole to disrupt the 6xHis-tag/cobalt binding. l-Histidine as an essential amino acid is known to be safe for clinical in vivo applications (43–45). Additionally, the amount of l-histidine that might be transferred with Histamer-isolated T cells is low and thus unlikely to be harmful.

In addition to the results of our functional assays, unchanged expression levels of the T-cell activation markers CD137, CD69 and CD25 were measured (Table 1). As expected, the expression levels of the T-cell activation markers CD137, CD69 and CD25 were increased upon A02/CMV Histamer treatment (Fig. 4A–C). However, no statistically significant differences were observed between A02/CMV Histamer–AF647-stained or A2/CMV Histamer dissociated CMV-specific CTLs and A02/CMV Histamer-non-treated CTLs (*P < 0.05, **P < 0.01, ***P < 0.001).

**Table 1. Effects of reversible A02/CMV Histamer staining on CD137, CD69 and CD25.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>CD137</th>
<th>CD69</th>
<th>CD25</th>
</tr>
</thead>
<tbody>
<tr>
<td>A02/CMV Histamer</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Non-treated</td>
<td>0.12 ± 0.03</td>
<td>0.08 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Histamer-treated</td>
<td>0.34 ± 0.05</td>
<td>0.20 ± 0.03</td>
<td>0.12 ± 0.02</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001. The results of independent experiments are expressed as mean ± SD (n.s., not significant).
CD137, CD69 and CD25 after A02/CMV Histamer disassembly (reversibilization) substantiate that treatment with AF647-unlabeled Histamers does not alter the naïvety of CMV-specific T cells after disassembly of the Histamer followed by dissociation of the monomeric MHC molecules from the TCR of CMV-specific CD8+ T cells. However, a moderately increased expression of CD137, but not CD69 and CD25, was identified on the cell surface of T cells after 1 to 2 days after staining with AF647-labeled Histamers. As long as A02/CMV Histamer-guided isolation is performed with unlabeled Histamers at 4°C, antigen-specific T cells can be purified without altering their original phenotype and function. TCR-MHC-mediated signaling events are not activated by Histamer-guided isolation. CD137 seems to be a more quantitative CTL activation marker compared to CD69 and CD25.

The present study clearly demonstrates that the pMHC Histamer technique is a powerful tool for a specific and effective detection of antigen-specific T cells, which is comparable to the conventional pentamer staining. Moreover, the Histamer technology affords efficient sorting of CMV-specific T cells either from whole PBMCs or after antigen-driven in vitro stimulation. Similar to the streptamer technology, Dynabead-based pMHC Histamers have the major advantage of an efficient separation of low-frequency antigen-specific T cells (39).

To investigate whether the Histamer technology might be applicable for various non-self peptides with different MHC molecules, we generated a specific Histamer to detect and isolate ADV-specific CD8+ T cells. For this purpose, the reversible human multimeric HLA-A*01:01/ADV5 hexon 886–894 peptide Histamer called A01/ADV Histamer was synthesized. ADV5 hexon 886–894 peptide pre-stimulated PBMCs from HLA-A*01:01 positive donors (n = 12) were used for the A01/ADV Histamer validation (Supplementary Figure 4). The highest sensitivity and specificity was obtained by staining at 4°C using 0.2 µg AF647-conjugated A01/ADV Histamer per 10⁶ cells. The frequencies of ADV-specific CD8+ T cells detected by staining with A01/ADV Histamer were comparable to that observed using the conventional A01/ADV Pentamer. This corroborates the Histamer technology to be an appropriate procedure for various MHC/peptide specificities.

Applications of pMHC

Adoptive transfer with donor-derived antigen-specific CTLs is an attractive way to restore immunity to viral infections and to improve T-cell reconstitution in immunosuppressed patients (1, 31, 46). However, in vitro generation of antigen-specific T cells is time-consuming and cost-intensive. In addition, ex vivo stimulation may affect the in vivo survival, proliferation and functionality of the transferred cells (11, 39).

In addition to the conventional streptamer technology, the pMHC Histamer-based sorting technology appears to be an elegant method for direct isolation of high-purity-specific T cells while maintaining their functional status. Thus, it is a promising tool for the direct transfer of antigen-specific T-cell populations into conditioned patients. Small amounts of highly pure antigen-specific T cells might be sufficient to achieve an efficient proliferation in vivo (47). High purity of the specific CTLs may also minimize the risk of GvHD (13). Selection of leukemia-antigen-specific T lymphocytes by pMHC multimers might strengthen the dissection of graft-versus-leukemia (GvL) effect from GvHD and could be an efficient way to eliminate leukemic cells (13).

The Histamer technology can be considered as a flexible platform technology that can easily be adapted to other cell subsets such as CD4+ T cells using pMHC class II Histamers. The pMHC Histamers take the advantage of magnetic particles that facilitate their use under GMP conditions. It is thus anticipated that the application of the pMHC Histamers can be adapted at a lower price to GMP conditions than currently available reversible streptamer technology for preparation of clinical-grade antigen-specific T lymphocytes.

These findings demonstrate the power of reversible pMHC Histamer technology, which can be used for the specific, sensitive and effective detection, selection and enrichment of antigen-specific T cells in very high purity without altering their function. In addition to the conventional streptamer, it is thus anticipated that this new cost-effective and rapid procedure has promising perspective for future clinical applications. The reversible pMHC Histamer technique offers a flexible and potent platform that should facilitate adoptive immunotherapy and bring the wider use of antiviral, antileukemic and anticancer T cells closer to reality.

Supplementary data

Supplementary data are available at International Immunology Online.

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Conflict of Interest

The authors declare that they have no competing interests.

References

Reversible pMHC class I Histamer technology


