Impact of CD8–MHC class I interaction in detection and sorting efficiencies of antigen-specific T cells using MHC class I/peptide multimers: contribution of pMHC valency

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Abstract

Recombinant soluble multimeric forms of MHC class I molecules loaded with antigenic peptides (pMHC) have turned out to be particularly useful to detect and isolate specific T cells. These applications both rely on the oligomerization of pMHC monomers in order to compensate for their inherent low affinity for the TCR. In this study, we have evaluated the precise contribution of CD8–pMHC interaction on the specificity and sorting efficiency of pMHC multimers according to their degree of oligomerization. To this end several wild-type versus mutated pMHC complexes (A*0201, B*0701, B*0801, B*3501) carrying point mutations known to reduce (245V mutants) or to abrogate (227,8KA mutants) CD8–pMHC interaction and showing various degrees of valency have been used. We show that irrespective of the HLA allele tested, 245V mutation strongly improves the binding specificity and immunomagnetic sorting efficiency of pMHC multimers. Our results also indicate that the contribution of CD8 to the binding of pMHC multimers to specific CD8⁺ T cells is inversely correlated to the degree of pMHC oligomerization. Consequently, efficient staining or specific sorting of high-affinity T cells (i.e. CD8 independent) can only be achieved using 227,8KA pMHC complexes with low-order oligomerization.

Introduction

The development of soluble fluorescent peptide–MHC class I (pMHC) complexes has opened new perspectives for the analysis of T cell biology, as these reagents allow (i) quantification of antigen-specific T cells without the need of ex vivo expansion (1), (ii) combined phenotypic and functional analyses of antigen-specific T cells using mAb against surface and intracellular proteins (2–5) and (iii) sorting of antigen-specific T cells by flow cytometric or immunomagnetic approaches (6, 7).

The conventional way to prepare fluorescent pMHC monomers involves enzymatic biotinylation of an added Tag sequence at the C-terminal end of the α MHC heavy chain (1). Biotinylated pMHC monomers are then oligomerized in order to compensate for their inherent low affinity for the TCR (8). To this end they are reacted with fluorescent, generally phycoerythrin-labeled streptavidin or with streptavidin-coated magnetic beads. Because of its large size (molecular mass: 240 kDa), the number of streptavidin units associated with this fluorochrome is generally not precisely defined. Only a tetramer grade streptavidin–PE guarantees the association of only one streptavidin with PE. As a consequence and depending of the grade of streptavidin–PE used, saturation of avidin–PE conjugates with pMHC monomers leads either to a ‘true’ tetramer or to pMHC complexes with heterogeneous valencies, referred to as multimers. In a same manner, association of pMHC monomers with streptavidin-coated magnetic beads leads to the generation of magnetic multimers.

Oligomerization of pMHC monomers results in their increased valency and avidity for antigen-specific CD8⁺ T cells. Besides, other factors influence the avidity of interactions...
between TCR and pMHC class I. In particular, binding of CD8 co-receptors decreases the off-rate of TCR–pMHC interactions (9–11). Binding of pMHC to CD8 mainly involves a small region in the α3 domain of the MHC heavy chain and previous reports have shown that mutations in this conserved region can either alter or abolish binding to CD8 molecules (12–14). In particular, a single mutation at position 245 (A–V) in the α3 CD8-binding loop of HLA-A*0201, referred to as 245V-A2 mutant, reduces without abolishing, binding to CD8 molecules (15, 16). At the opposite, the double mutation of residues 227 (D–K) and 228 (T–A), referred to as 227,8KA-A2 mutant (or CD8-null mutant), fully abrogates CD8–MHC class I interactions (9, 14, 17).

Wild-type (wt) pMHC oligomers show a concentration-dependent background binding to non-specific T cells by interacting with the CD8 co-receptor. We previously reported that 245V-A2 mutant multimers show a greatly diminished non-specific binding compared with wt complexes, and therefore allow accurate enumeration and efficient immunomagnetic sorting of antigen-specific T cells (7). In this study, we tried to generalize this observation to several other HLA–peptide complexes (B*0701, B*0801, B*3501). Moreover, we assessed the general impact of CD8–pMHC interaction on the binding specificity of pMHC–streptavidin conjugates according to their degree of oligomerization. To this end we have compared the staining behavior and sorting efficiencies of several wT versus mutated HLAs–peptide complexes carrying mutations known to reduce or to abrogate the CD8–MHC interaction and showing various valencies.

Our results indicate that introduction of the 245V mutation in the HLA α3 domain of various HLA alleles (A*0201, B*0701, B*0801 and B*3501) strongly decreases the background binding of pMHC complexes to CD8 co-receptors and allows efficient pMHC-based sorting and detection of antigen-specific T cells, irrespective of the avidity of pMHC complexes. Moreover, our results demonstrate that high pMHC complexes valency underlies the background binding observed with wt pMHC multimers to non-specific CD8+ T cells and can overcome CD8-dependent binding of pMHC oligomers to CD8+ T cell subsets.

Methods

T cell samples

Blood samples were obtained from donors with previously documented CD8+ T cell reactivities against Epstein-Barr virus (EBV) and/or human cytomegalovirus (HCMV): eight patients with rheumatic diseases (seven rheumatoid arthritis and one ankylosing spondylitis) and two kidney recipients (18, 19). PBMCs were separated by Ficoll density centrifugation (LMS Bioscience, Orsay, France). CD8-null monomers were freshly generated from PBMCs using anti-CD8 mAb. Cells were then expanded in vitro in IL-2/CM supplemented with leukoagglutinin at 1 µg ml−1, irradiated PBL and B lymphoblastoid cells as previously described (20). Cells were maintained for 3 weeks without re-stimulation prior functional analysis or pMHC immunomagnetic cell sorting.

Generation of recombinant pMHC complexes

Soluble pMHC monomers were synthesized as previously described (7). Briefly, recombinant HLA heavy chain and β2-microglobulin were produced as inclusion bodies in Escherichia coli, dissolved in 8 M urea and refolded in the presence of 15 µl M−1 of each synthetic peptide (Genosys, Montigny le Bretonneux, France). The folded pMHC complexes were filtered and concentrated in 10 mM Tris pH 8 on a prep scale 3-kDa concentration cassette (Millipore, Saint Quentin en Yvelines, France). pMHC complexes were biotinylated by recombinant BirA enzyme, purified by anion exchange on a MonoQ column (Pharmacia, Saint Quentin en Yvelines, France) with a 0- to 0.5-M NaCl gradient, and checked by gel filtration on a Superdex column (Pharmacia).

The following antigenic peptides were chosen: the HLA-A*0201-restricted epitope NLVPVMATV495–503 (NLV/A2) and the HLA-B*0701-restricted epitope TPRVTGGGAM417–426 (TRP/B7) derived from the HCMV pp65 tegument protein; the HLA-B*0801-restricted epitope RAKFKQLL190–197 (RAK/B8) and the HLA-B*3501-restricted epitope EPLPGQLTAY54–64 (EPL/B35) derived from the EBV BZLF1 immediate early protein.

The pMHC monomers were provided by Beckman Coulter and comprised either wt or mutated HLA I heavy chains carrying an A1a to Val substitution in the α3 domain at position 245. We also produced as described above double mutated HLA-A*0201 monomers (Asp to Lys and Thr to Ala substitutions, respectively, at position 227 and 228) loaded with the pp65 NLV/A2 peptide. Such pMHC complexes (referred to as 227,8KA mutants or CD8-null complexes) are no longer able to bind to human CD8 co-receptors and can be used to identify T cell subsets bearing high-affinity TCR (17). The cDNA encoding this CD8-null monomer was provided by P. Romero (Ludwig Institute for Cancer Research, Lausanne, Switzerland).

pMHC monomers were oligomerized either with PE-labeled streptavidin (BioSource, Nivelles, Belgium) to form an hetero-functional analysis or pMHC immunomagnetic cell sorting. pMHC monomers were oligomerized either with PE-labeled streptavidin (BioSource, Nivelles, Belgium) to form an hetero-functional analysis or pMHC immunomagnetic cell sorting.

Antibodies and flow cytometry

Staining and washing were performed in 0.1% BSA–PBS. T cells were stained with 10 ng µl−1 PE-labeled pMHC oligomers (except for titration experiment) and FITC-conjugated mAb against CD3 (Immunotech, Roissy, France) at 4°C for 30 min. After staining, cells were washed and analyzed immediately on an LSR (Becton Dickinson, Grenoble, France). Anti-human CD8 antibody used in this study to perform immunomagnetic
cell sorting with wt pMHC magnetic multimers and blocking experiments of TCR-independent CD8–pMHC interaction was B9.11 (Immunotech) at a concentration of 6 μg ml⁻¹. Data analysis was performed using the CellQuest Pro software (Becton Dickinson).

Immunomagnetic cell sorting

Sorting of antigen-specific T cells using recombinant MHC–peptide complexes was performed on CD8⁺ T lymphocytes as previously described (7). In brief, 6.7 × 10⁶ streptavidin-coated immunomagnetic beads (Dynabeads M-280 streptavidin; Dynal, Compiegne, France) were incubated with 1 μg of biotinylated pMHC monomers for 1 h at room temperature to form magnetic multimers, washed once and added to 5 × 10⁶ to 10⁷ CD8⁺ T cells in 500 μl 0.1% BSA–PBS. Cells were rotated 4 h at room temperature, washed eight times as previously described (7) and expanded as described above. The purity of antigen-specific T cells was checked by tetramer staining.

Tumor necrosis factor-α release assays

CD8⁺ T cells (3 × 10⁶) were incubated for 6 h with peptides at a concentration of 10 μM and the amount of tumor necrosis factor (TNF)-α released in the supernatant was estimated by the Wehi 164 cytotoxicity assay (22).

Results

Improved specific CD8⁺ T cell sorting efficiency of pMHC magnetic multimers carrying a 245V point mutation

We first compared specific CD8⁺ T cell sorting efficiencies of either wt or 245V pMHC magnetic multimers. To this end several HLA-A*0201, -B*0701, -B*0801 and -B*3501 wt or 245V mutated monomers loaded with peptides derived from the HCMV pp65 tegument protein (TPRVTGGGAM for HLA-B7) or the EBV immediate early protein BZLF1 (RAKFQKQLL for HLA-B8 and EPLPQGQLTAY for HLA-B35), abbreviated, respectively, TPR/B7, RAK/B8, EPL/B35, were generated. Immunomagnetic sorting of specific CD8⁺ T cells was performed on several CD8⁺ polyclonal T cell samples derived from three donors expressing HLA-B*0701, HLA-B*0801, HLA-B*3501 or irrelevant HLA alleles, in which we previously documented the presence of CD8⁺ T cells reactive against EBV or HCMV (18, 19, Table 1). The frequency of specific CD8⁺ T cells before and after sorting was evaluated using wt monomers conjugated with tetramer grade streptavidin–PE, hereafter referred to as pMHC tetramers. After only one round of sorting, the frequency of specific T lymphocytes was in most cases above 80% when using 245V magnetic multimers (Fig. 1), with an enrichment factor ranging from 15 to 471 (Table 1). The specificity of sorted cells was confirmed by cytokine release (Table 1) and cytotoxicity assays (data not shown). In contrast, most cells sorted with wt magnetic multimers corresponded to irrelevant T cells, even in situations where specific T cells were present at high frequency before sorting. For instance, only 10% of cells were stained by wt-EPL/B35 tetramer within CD8⁺ T cells from donor RA9 sorted with wt magnetic EPL/B35 multimers, although these cells represented 6% of the unsorted CD8⁺ T cell population (Table 1). As previously described for HLA-A*0201, our results indicate that 245V magnetic multimers are much more efficient and sensitive than wt magnetic multimers in sorting specific T cells, irrespective of the HLA allele studied. Indeed, in all cases, a single sort with 245V magnetic multimers allowed the recovery of highly enriched specific T cell lines, even when specific cells represented <0.2% of the initial polyclonal CD8⁺ population.

Low sorting efficiency with wt pMHC multimers is due to TCR-independent CD8–pMHC interactions

As shown by tetramer staining, most cells sorted with wt magnetic multimers were irrelevant (Fig. 1). These CD8⁺ T cells were not simply carried over because of insufficient washing, since we could not increase the frequency of specific

<table>
<thead>
<tr>
<th>pMHC complexes</th>
<th>Donors</th>
<th>% Tetramer-positive cells (enrichment factor)</th>
<th>TNF-α release (pg ml⁻¹)</th>
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<tr>
<td></td>
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<td><strong>Unsorted</strong></td>
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<td><em>wt multimers</em></td>
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<tr>
<td>EPL/B35</td>
<td>RA19</td>
<td>0.5</td>
<td>4.5 (9)</td>
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<td></td>
<td>AS1</td>
<td>0.2</td>
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<td></td>
<td>RA9</td>
<td>6</td>
<td>10.4 (2)</td>
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<td>Control</td>
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<td>RAK/B8</td>
<td>KR6</td>
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<td></td>
<td>RA3</td>
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<td>4.9 (16)</td>
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<td>TPR/B7</td>
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<td>47.5 (118)</td>
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<td></td>
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<td>0.2</td>
<td>0.6 (3)</td>
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<td>Control</td>
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Rheumatoid arthritis (RA), ankylosing spondylitis (AS) and kidney recipient (KR). Controls correspond to CD8⁺ T cell samples derived from healthy donors that do not express the relevant HLA allele. Percentages of positive cells were assessed by staining with the wt and/or 245V mutated monomers conjugated with tetragrade streptavidin–PE. TNF-α responses: results are expressed as pg ml⁻¹ TNF-α released by 10⁶ T cells after a 6-h incubation with relevant peptide at 10 μM in autopresentation assay. nt: non-tested, ne: no expansion, —: not applicable. Values highlighted in gray indicates enrichments in specific CD8⁺ T cells above 40%.

Table 1. Comparative sorting efficiencies of wt and 245V mutated magnetic multimers
T cells by serial sortings, irrespective of the wt pMHC magnetic multimers used (data not shown). By contrast, a dramatic enrichment for specific CD8+ T lymphocytes was achieved with wt magnetic multimers if sorting was performed in the presence of anti-CD8 antibodies (data not shown). This indicates that TCR-independent CD8–pMHC interactions underlie the low sorting efficiency achieved with wt pMHC magnetic multimers.

**TCR-independent CD8–pMHC interactions occur at high pMHC valencies**

We compared the binding specificity of wt and 245V monomers oligomerized either with tetragrade (tetramers) or non-tetragrade (multimers) streptavidin–PE using unsorted polyclonal CD8+ T cells or cell lines sorted with wt or 245V magnetic multimers. A representative experiment is shown in Fig. 2 for the pMHC complex RAK/B8. Whereas wt pMHC tetramers specifically stained the relevant T cells (Fig. 2A, left dot plots), wt pMHC multimers showed a much higher background staining (Fig. 2B, left dot plots). In agreement with previous reports, this background was strongly inhibited by blocking anti-CD8 antibody (Fig. 2B, middle dot plots). Nevertheless, the percentage of specific T cells was underestimated when compared with those obtained after staining with wt or 245V pMHC tetramers (Fig. 2B versus 2A, middle dot plots). The degree of oligomerization of fluorescent tetramers and multimers was evaluated by gel filtration. As shown in Fig. 3, oligomerization of monomers with tetramer grade streptavidin–PE yielded a homogeneous complex with an apparent molecular weight of 470 kDa, which corresponded in all likelihood to pMHC tetramers containing one streptavidin per PE molecule. By contrast, most pMHC complexes multimerized with non-tetramer grade streptavidin were harvested in the void volume, and thus corresponded to aggregates with high valencies. Altogether, our results strongly suggest that TCR-independent CD8–pCMH interactions observed with wt multimeric complexes occur at high valencies (i.e. above 4) and can be circumvented either by...
using anti-CD8 antibody or by decreasing the degree of pMHC oligomerization.

In stark contrast, mutated 245V pMHC complexes allowed an accurate detection of CD8⁺-specific T cells irrespective of the streptavidin used for conjugation (Fig. 2A and B, right dot plots). Besides, the percentage of specific T cells detected using 245V tetramers or multimers were at least as high as those obtained with wt pMHC tetramers. This result provides further evidence that the single 245V mutation within the HLA class I CD8-binding site still allows staining of virtually all T cells irrespective of their CD8 dependency. Moreover, 245V multimers yielded a brighter fluorescence intensity of positive cells and no background staining, when compared with wt or 245V tetramers (Fig. 2). This observation could have important implications for the detection of rare specific T cells within a polyclonal T cell line.

**Fig. 3.** Characterization of the oligomerization degree of fluorescent pMHC complexes. pMHC monomers (42 kDa) oligomerized either with tetragrade or non-tetragrade streptavidin–PE (streptavidin: 60 kDa; PE: 240 kDa) were analyzed by gel filtration on a Superdex 200 column. A representative elution profile is shown for NLV/A2 streptavidin–PE conjugates. Elution was performed in PBS at a flow rate of 0.5 ml min⁻¹. The absorbance was measured at 280 nm.

**Fig. 4.** The CD8 dependence of multimer binding to CD8⁺ T cells is associated to the degree of pMHC oligomerization. (A and B) Control staining of NLV/A2-specific T cell clones expressing either a high- (A) or a low-affinity TCR (B). Note that clones were already described in (24); CD8⁺ T cell clones were stained by 245V mutated (white histograms) and 227,8KA-A2 (shaded histograms) tetramers loaded with pp65 peptide. As a control, cells were stained by an irrelevant A2 tetramer (dashed histograms). (C and D) 245V (panel C) or 227,8KA (panel D) NLV/A2 mutated monomers oligomerized either with tetragrade or non-tetragrade streptavidin–PE were used to stain CD8⁺ T cell populations (derived from donor RA15) sorted either with 245V (top row) or 227,8KA (bottom row) NLV/A2 mutated magnetic multimers.

**CD8 contribution to the binding of pMHC multimers to specific T cells is tightly related to the degree of pMHC oligomerization**

It was recently shown that CD8 dependence of pMHC multimer binding decreases as their valency increases (23). It was therefore anticipated that only CD8-null pMHC complexes with low-order oligomerization could allow staining or specific sorting of high-affinity T cells. In order to test this, 227,8KA-A2 mutant monomers loaded with the HCMV pp65 peptide NLVPMVATV (referred to as NLV/227,8KA-A2) were used to perform staining and immunomagnetic sorting of antigen-specific T cells. While NLV/227,8KA-A2 tetramers brightly stained T cell clones expressing a high-affinity NLV/A2-specific TCR [Fig. 4A and (24)] but not clones expressing low-affinity TCR [Fig. 4B and (24)], they stained only 0.7% of an almost pure pp65/A2-specific polyclonal CD8⁺ T cell population, presumably composed of ‘low-affinity’ CD8-dependent T cells (Fig. 4D). By contrast, nearly all NLV/A2-specific T cells were stained by NLV/227,8KA-A2 multimers. Therefore, these results suggest that a high pMHC valency can overcome CD8-dependent binding of pMHC complexes to a CD8-dependent T cell population. Accordingly, sorting with NLV/227,8KA-A2 magnetic multimers did not allow enrichment for high-affinity T cells specific for pp65/A2, as only 0.5% of sorted cells were stained by NLV/227,8KA-D null tetramers (Fig. 4D). The increased avidity associated with pMHC oligomerization by non-tetramer grade streptavidin–PE or on streptavidin-coated magnetic beads thus precludes the use of CD8-null multimers to selectively stain or isolate high-affinity T cells.

**Discussion**

In the present study, we compared the staining behavior and sorting efficiencies of several wt versus mutated HLA–peptide complexes carrying point mutations known to reduce (245V mutants) or to abrogate (227,8KA mutants) the CD8–pMHC interaction. Our results demonstrate that mutated pMHC
multimers are more efficient and sensitive than wt pMHC multimers to sort out by immunomagnetic approach specific T cells, irrespective of the pMHC allele tested. They allow rapid and cost effective generation of T cell lines greatly enriched for specific T cell populations, even from cell samples containing <0.3% of specific T cells. This study thus extends our previous results obtained in the HLA-A*0201 context to three other MHC alleles (i.e. HLA-B*0701, -B*0801 and -B*3501), and suggests an extensive conservation of the CD8 binding sites in these various HLA alleles (7). This study also shows that the background staining to irrelevant CD8+ T cells observed with wt pMHC reagents is only observed with high-order oligomers, that is, conjugated to non-tetramer grade streptavidin–PE or to streptavidin-coated beads. This background staining is due to TCR-independent CD8–pMHC interactions and consequently accounts for the low sorting efficiency of wt CD8 magnetic multimers. These TCR-independent CD8–pMHC interactions could be circumvented either by decreasing the degree of pMHC oligomerization (i.e. using wt pMHC monomers oligomerized with tetramer grade streptavidin–PE) or by using anti-CD8 antibodies. However, both approaches have their caveats. Firstly, CD8 antibodies can directly impact on multimer binding (25, 26). Accordingly, the percentage of T cells stained with wt multimers in the presence of anti-CD8 antibodies was significantly underestimated, probably as a consequence of steric hindrance of TCR–pMHC class I interactions (rather than that of an inability to stain CD8-dependent T cell population under these conditions). Secondly, the fact that in some cell samples the frequency of specific T cells stained by pMHC tetramers (wt or 245V mutated) was lower than those stained by pMHC multimers (e.g. compare the lower dot plots in Fig. 2A versus 2B) suggests the existence of a presumably low-affinity T cell subset that is missed by pMHC tetramers. Besides, our results indicate that the CD8 dependence of pMHC multimer binding is primarily determined by the overall avidity of the pMHC–TCR interaction. Indeed, it appears that the higher the valency of pMHC complexes the lower the contribution of CD8 co-receptors to TCR–pMHC interaction. Consequently, CD8 binding to class I oligomers is not an absolute requirement for staining and sorting of antigen-specific T cells.

While CD8-null pMHC tetramers can discriminate between CD8+ T cells expressing low- versus high-affinity TCR, CD8-null pMHC multimers allow staining and/or sorting of specific T cells irrespective of their affinity and CD8 dependency. Therefore, the functional significance of binding studies using CD8-null pMHC complexes should be interpreted with caution in the absence of a careful assessment of pMHC complex valency. Similarly, the present observations indicate that CD8-null pMHC complexes cannot be used to sort out high-affinity T cells by immunomagnetic-based approaches. In any case, both CD8-null and 245V pMHC multimers appear well suited for sorting of specific T cells by immunomagnetic approaches.

Acknowledgements

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Abbreviations

EBV         epstein-barr virus
HCMV        human cytomegalovirus
pMHC        MHC class I molecules loaded with antigenic peptides
TNF         tumor necrosis factor
wt          wild type

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