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Commentary

Soluble MHC-peptide complexes: tools for the monitoring of T cell responses in clinical trials and basic research

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Soluble MHC-peptide complexes, commonly known as tetramers, allow the detection and isolation of antigen-specific T cells. Although other types of soluble MHC-peptide complexes have been introduced, the most commonly used MHC class I staining reagents are those originally described by Altman and Davis. As these reagents have become an essential tool for T cell analysis, it is important to have a large repertoire of such reagents to cover a broad range of applications in cancer research and clinical trials. Our tetramer collection currently comprises 228 human and 60 mouse tetramers and new reagents are continuously being added. For the MHC II tetramers, the list currently contains 21 human (HLA-DR, DQ and DP) and 5 mouse (I-A^b) tetramers. Quantitative enumeration of antigen-specific T cells by tetramer staining, especially at low frequencies, critically depends on the quality of the tetramers and on the staining procedures. For conclusive longitudinal monitoring, standardized reagents and analysis protocols need to be used. This is especially true for the monitoring of antigen-specific CD4+ T cells, as there are large variations in the quality of MHC II tetramers and staining conditions. This commentary provides an overview of our tetramer collection and indications on how tetramers should be used to obtain optimal results.

Keywords: human, mouse, MHC-peptide complex, tetramer, immunologic monitoring

Introduction

Because of their wide application and importance, tetramers, for both CD8+ (1-3) and CD4+ (4, 5) T cells, have been the subject of numerous reviews. Improvements in staining reagents, procedures and combinations with other monitoring techniques are continuously being reported (6-9). We therefore limit this commentary to a description of: (i) the new Ludwig Institute for Cancer Research (LICR) Tetramer Collection being published concurrently in Cancer Immunity (10), (ii) fundamental aspects of tetramer staining, (iii) critical parameters for tetramer staining protocols, (iv) MHC II tetramers and staining procedures and (v) monitoring of T cell responses in clinical trials. Additional general information, such as an overview of other types of staining reagents and special applications, can be found in the introduction of the LICR Tetramer Collection (10).

Overview of the LICR Tetramer Collection

The LICR Tetramer Collection (10) consists of an introduction, listings of available MHC class I and II tetramers, and related references with links to PubMed. The MHC I tetramers are all phycoerythrin (PE) or allophycocyanin (APC) streptavidin-based tetramers and are grouped in three lists. Because these reagents are heterogeneous, they are henceforth referred to as multimers (11).

The A list contains the nine most commonly used multimers for monitoring in clinical trials. These reagents are tested for staining on reference CTL clones and are shipped with quality control data. Over the years there were only a few bad batches that were discarded and, in some cases, staining differences were noted when switching to a new batch or commercial source of PE-streptavidin. Because of such variations, multimers used in clinical trials should imperatively be quality controlled and standardized by testing on well established reference CTL clones.

The B list currently contains 168 multimers; most of them contain HLA-A*0201, but 14 other alleles are represented as well (HLA-A*0101, HLA-A*0301, HLA-A*2301, HLA-A*2402, HLA-A*3101, HLA-A*6801, HLA-B*0702, HLA-B*1302, HLA-B*1801, HLA-B*3501, HLA-B*3503, HLA-CW*0304, HLA-CW*0602, and HLA-CW*0702). In addition we have inclusion bodies of HLA-A*1101, HLA-B*0801, HLA-CW*0303, HLA-CW*0401, and HLA-CW*1402, which allows rapid preparation of tetramers comprising these alleles. In contrast to the A list multimers, those on the B list were not tested on reference cell lines.

The C list contains 51 multimers for special applications such as:

1. **HLA-A*0201 multimers with reduced or deficient binding to CD8.** The A245V mutation in the α3 domain of HLA I molecule reduces binding to CD8 and the double mutation D227K and T228A abolishes CD8 binding (12). It has been reported that tetramers containing the A245V mutation exhibit reduced background binding, because it abolishes non-specific binding to CD8 (12). As shown in other systems, non-cognate multimer binding to CD8 is allele-specific and mainly observed on hyposialylated cells upon incubation in the cold (13, 14). With HLA-A*0201 multimers using our standard protocol (see below), non-specific staining is below 1%, usually less than 0.01%.
2. **Chimeric HLA-A*0201/K^b multimers.** In these chimeric multimers the α3 domain of HLA-A*0201 was replaced with the one of K^b. These reagents are used for monitoring HLA-A*0201-restricted CD8+ T cells in HLA-A*0201/K^b transgenic mice which use endogenous mouse CD8 as co-receptor (15).
3. **Reversible multimers.** As described in more detail in the next section, multimer binding to effector CD8+ T cells can elicit activation-depended cell death (16, 17). To avoid this, antigen-specific T cells can be isolated by FACS or MACS

using reversible multimers (18). In our reversible multimers we use desthiobiotin (DTB) for biotinylation of MHC I peptide complexes. Because DTB binds streptavidin less avidly than biotin, these complexes dissociate upon addition of free biotin (18). Because this dissociation is very rapid, such reagents can also be used for measuring dissociation kinetics on T cells.

Fundamental aspects of tetramer staining

MHC-peptide multimers efficiently cross-link TCR and CD8, which results in cell activation and eventually cell death, especially of activated effector CTLs (16, 17). For example, multimers can induce a burst in cell respiration that causes mitochondrial damage and rapid CTL death (16). Alternatively, MHC-peptide oligomers can trigger up-regulation of FasL (CD95L) and induce Fas-depended apoptosis (11, 17). Such apoptosis occurs with slower kinetics and is equally induced by CD8 binding-deficient multimers, which allows elimination of CTLs of given specificities. One should be aware that isolation of differentiated, antigen-specific CD8+ T cells, especially CTLs, by means of MHC-peptide multimers results in loss of cells and that the surviving cells may not be representative of the original population.

MHC-peptide multimer binding to cells depends on several factors, such as (i) coordinate binding of CD8 to TCR-associated MHC-peptide complexes, which substantially strengthens the binding avidity (11, 19), and (ii) the state of cell activation and differentiation (20). It has been reported that multimer staining is also feasible in chemically fixed cryo-sections (21); however, in our experience, the efficiency of multimer staining decreases dramatically upon paraformaldehyde (PFA) fixation of cells.

In human and mouse systems, CTL activation can result in transient loss of multimer staining *in vitro* and *in vivo* (22-24). One study demonstrated that, on staining refractory cells, CD8 is segregated from TCR (22), while another study showed that certain anti-CD8 mAbs and enzymatic desialylation can restore tetramer binding, suggesting that altered glycosylation and CD8 are involved (24). Differentiation and activation can induce diverse and extensive changes in glycosylation and sialylation of T cell surface molecules, including TCR and CD8 (25). This can yield galectin (beta-galactoside binding lectins) binding sites, which can allow the formation of molecular lattices that interfere with MHC peptide multimer binding (22, 26, 27). Moreover, by testing well-defined dimeric, tetrameric and octameric MHC-peptide complexes containing linkers of variable lengths and flexibilities, we found striking differences in binding depending on the stage of differentiation of the CD8+ T cells (20). This argues that the state of glycosylation and sialylation of T cell surface molecules also impacts on the MHC-peptide complex induced, avidity enhancing the aggregation of TCR and CD8.

Staining protocols - what are the critical parameters?

Staining of T cells with MHC-peptide multimers is more complex than staining of T cell surface molecules with antibodies because multimer binding depends on various factors and can induce TCR down-modulation and/or cell death, which in turn affects multimer staining. In the following we list and comment on critical staining parameters:

1. **The multimer concentration.** Frequently only one multimer concentration is used for staining. Unless this concentration is high (e.g. 1.5 µg/ml / 3.16 nM or higher), it is possible that CD8+ T cells with low binding avidities (e.g. naïve cells or cells expressing low affinity TCRs) are missed or their proportions underestimated. To avoid this, different multimer concentrations should be tested.
2. **The incubation conditions.** Multimer binding at 37°C is rapid and results in cell activation and eventually cell death, multimer internalization, and TCR down-modulation (16-18, 22). Since these events can interfere with reliable enumeration of antigen-specific CD8+ T cells, we recommend staining at ambient temperature in the presence of EDTA (5 mM) and sodium azide (5 mM), which inhibit cell activation-dependent molecular events. Alternatively, it is feasible to stain CD8+ T cells at 0-4°C, but longer incubation periods should be used (1 h or more).
3. **Combined multimer and antibody staining.** For polychrome flow cytometric analysis, multimer staining is combined with antibody staining, which can interfere with one another. For example, anti-CD8 mAbs can have striking effects on multimer staining, ranging from strong inhibition to several-fold increases (6, 19, 28). Therefore anti-CD8 mAbs should be used that do not significantly affect multimer binding. In addition, performing CD8 staining in the cold after multimer staining reduces inference. Furthermore, if multimer staining is combined with intracellular staining (e.g. of granzymes or IFN-γ), the multimer staining should be performed before peptide stimulation, fixation and cell permeabilization, and controls should be included to account for the impact of the cytokine staining on multimer staining (9, 29).
4. **Cell viability.** Multimer staining strongly depends on cell viability and vitality, and hence on the handling of the cells. Dead or dying cells should be gated out, e.g. using staining with annexin V or propidium iodide (PI).

Flow cytometric analysis and data evaluation

Especially for samples containing low frequencies of multimer positive cells, flow cytometric results can be significantly improved as follows: (i) At least 100,000 CD8+ T cells should be analyzed in order to detect rare events with significant confidence. (ii) Negative controls should be included for the appropriate setting of gates to discriminate multimer positive and multimer negative cells. (iii) More than two colors should be used for staining and correct compensations should be made in the different channels.

Our staining protocol

CD8+ T cells (1×10^6 cells) are enriched from PBMCs by negative selection using a CD8+ T Cell Isolation Kit (Miltenyi Biotec) and incubated in 50 µl FACS buffer [OptiMEM (Invitrogen AG, Basel, Switzerland) supplemented with 0.2% BSA (Sigma-Aldrich), 15 mM HEPES (pH 7.4), 5 mM EDTA, and 5 mM NaN_3] with multimers (3, 10 and 30 nM, corresponding to 1.43, 4.7 and 14.3 µg/ml, respectively) for 30 min at ambient temperature. After one wash, the cells are incubated for 30 min at 4°C with antibodies specific for CD28 (APC labeled), CD27 (APC-Alexa750), CCR7 (PE-CY7), CD45RA (ECD) and CD127 (Pacific blue) (BD Bioscience) for example. The cells are washed twice, analyzed on an LSRII flow cytometer and data processed with the FlowJo software (Tree Star, Inc. Ashland, OR). Samples stained with non-cognate multimers are used for correct setting of the gates and compensations. For intracellular staining, the cells are first labeled with multimers, washed and fixed for 20 min at room

temperature in PBS containing 1% formaldehyde, 2% glucose and 5 mM NaN_3, followed by permeabilization with saponin (0.1%; Fluka AG, Buchs, Switzerland) in PBS containing BSA (0.2%) and EDTA (50 μM), and staining with the antibodies of interest for 20 min at 4°C.

Multimer staining of CD4+ T cells

MHC class II staining reagents

A general introduction to MHC II multimers is provided in the LICR Tetramer Collection (10). The biochemical and conformational integrity of MHC II-peptide monomers is *a priori* less certain compared to that of MHC I-peptide monomers obtained by refolding (3-5). Due to the added leucine zippers, the subunit α and β chains remain associated, as will tethered-on peptides, irrespective of the structural integrity of the complex. Therefore, if possible, MHC II-peptide multimers should be tested and validated on defined reference cell lines or clones; otherwise the use of MHC II multimers is on a trial and error basis, which is frustrating in the case of negative results.

In the case of MHC II-peptide complexes obtained by loading "empty" MHC molecules, we observed that efficient peptide binding is limited to native molecules. By adding a negatively charged, low molecular weight tag N-terminally to the peptide, correct MHC II-peptide complexes can be isolated by anion exchange chromatography. This is a reliable means to ensure the biochemical integrity of MHC II multimers.

Staining of antigen-specific CD4+ T cells with MHC II multimers

Although multimer staining of CD8+ and CD4+ T cells have much in common, some significant differences exist: (i) Staining with MHC II multimers should be performed at 37°C. Although some CD4+ T cells can also be efficiently stained in the cold, others are not or poorly stained at low temperature (30). (ii) Efficient staining usually requires longer incubation periods, which is explained, at least in part, by the accumulation of MHC II multimers by endocytosis (30). Therefore agents that affect cell vitality and cytoskeleton function inhibit MHC II staining. (iii) The avidity of MHC II multimer binding is on average lower than the binding of MHC I multimers. This is so mainly because CD4, unlike CD8, barely strengthens MHC-peptide binding (6, 19, 31). For conclusive analyses MHC II multimers therefore have to be titrated up to high concentrations (e.g. 100 nM / approx. 50 μg/ml). Even then some antigen-specific CD4+ T cells remain un- or barely detectable by MHC II multimer staining (4). This may be explained by a combination of very low TCR avidity and rapid TCR down-modulation. Dasatinib, an Src kinase inhibitor, has been reported to inhibit TCR down-modulation and hence to increase multimer staining (7). Moreover, we observed that enzymatic desialylation with neuraminidase from *Vibrio cholerae* (Roche Ltd.) can increase MHC II multimer staining several-fold (manuscript in preparation).

Monitoring of T cell responses in clinical trials

A multicenter study, in which 27 different laboratories used commercially available multimers and their staining protocols, revealed striking differences in staining of influenza matrix$_{58-66}$ and Melan-A/Mart-1$_{26-35}$-specific CD8+ T cells in identical PBMC samples (32). This illustrates the need to establish harmonized, high performance T cell monitoring protocols for conclusive evaluation of multicenter cancer vaccine trials. Moreover, critical evaluation of the efficacy of clinical trials requires establishing a bridge between clinical endpoints and monitoring data (33). While longitudinal, quantitative and reliable monitoring of tumor-associated antigen (TAA)-specific T cell responses is important, additional information is required as well, such as: (i) detailed phenotyping of the TAA-specific T cells is needed to elucidate what impact the immune intervention (e.g. vaccine, chemotherapy or adoptive T cell transfer) has on the differentiation of TAA-specific T cell populations (20, 33-35). (ii) The functionality of the TAA-specific T cell populations needs to be assessed. In patients with metastatic cancer (or chronic viral infections, such as AIDS or hepatitis), partially differentiated CD8+ T cells with ablated effector functions typically occur and hence the impact of the immune intervention on T cell effector function needs to be determined (20, 36-38) Longitudinal changes in the TCR repertoire of TAA-specific T cell populations and their replicative history provide valuable information on the immune intervention-related expansions of TAA-specific clonotypes (36).

The advent of polychrome flow cytometry allows simultaneous assessment of various differentiation markers of TAA-specific T cell populations (20, 34-36, 38). To some extent it also allows the gauging of T cell effector functions, e.g. by combining multimer staining with intracellular cytokine staining or CD107 staining, a molecule expressed on cytolytically active CTLs (9, 29, 35, 39). However, for precise quantitative assessment of T cell functions (e.g. killing or cytokine release), it is better to use specialized assays such as the chromium release assay, ELISPOT or quantitative cytokine measurements (36, 37, 39-41). Because the results of such analyses depend on how they were performed and with what reagents, well-defined protocols (for analyses and evaluations) and the same reagents should be used for the monitoring of multicenter clinical trials. Moreover, for reasons of standardization and normalization, suitable controls need to be included. Because anti-TAA T cell responses are often close to the threshold, PMBCs from healthy donors should be used as negative control (33). The same PBMCs, which should be from a single source, can be used as a positive control upon determining their frequencies of T cells specific for irrelevant antigens such as influenza or tetanus.

For practical reasons longitudinal analyses are commonly performed on cryopreserved PBMCs (32, 33, 40). It is important to realize that monitoring of peripheral TAA-specific T cells is of ill-defined clinical relevance. Patients may exhibit good frequencies of TAA-specific CD8+ effector T cells in the periphery, yet show no tumor regression (42). Furthermore, the immune status in the periphery and inside the tumors can be strikingly different. The expression of chemokines and chemokine receptors plays crucial roles in trafficking and homing of subsets of T cells (and DCs), not only in the lymphatic system but also in tumors (43-45). In fact tumor cells and the tumor environment create chemokine milieus that control to a large extent leukocyte tumor infiltration. For example, tumors (e.g. pancreatic cancers, lymphomas, melanomas) can produce CCL5 (ligand for CCR5), CCL17 and CCL22 (ligands for CCR4), which can direct Tregs to the tumor (46, 47). Conversely, metastatic melanomas expressing CCL2, CCL3, CCL4, CCL5, CXCL9 (MIG) and/or CXCL10 (IP-10) were infiltrated by CD8+ effector T cells expressing the corresponding chemokine receptors (48). The latter two chemokines bind to CXCR3 and have been shown to attract

CD8+ T cells to tumors (49, 50). Moreover, several studies have demonstrated that tumors expressing CX3CL1 (lymphotactin or fractalkine) are infiltrated by CX3CR+, CD8+ T cells (and NK cells), resulting in strong tumor regression (51, 52). In addition chemokines and chemokine receptor expression play instrumental roles in the migration of DCs, tumor angiogenesis and metastasis formation (43, 44). Based on such observations, immune interventions have been used that target chemokine and chemokine receptor expression, e.g. by means of cytokines (e.g. rIL-7 or rIL-12), that enforce the expression of chemokines (e.g. CXCL9 and CXCL10) by tumors, thus promoting tumor infiltration by CD4+ Th1, CD8+ CTLs (49, 53). Because of the complex and fundamental roles of chemokines and chemokine receptors in controlling interactions of the immune system with tumors, they should be taken into account in future clinical trials and immune monitoring.

In recent publications critical aspects of immune monitoring have been reviewed such as the harmonization and standardization of multicenter clinical trials, state of the art statistical analyses, monitoring of antigen-specific T cells by multimers and their functional parameters (32, 40, 54-56).

In conclusion, reliable and integral monitoring of T cell responses in cancer vaccine trials is essential for critical evaluation of their efficacy. This task is complex, as it requires not only high quality and standardized reagents (e.g. peptides, multimers and antibodies), but also optimized monitoring procedures and highly qualified personnel. Since the number of variables is high and monitoring techniques and instruments are continuously evolving, one way to ensure a high degree of harmonization and proficiency of monitoring is to establish a core facility that performs the analysis of all samples from all participants. Such a facility could be located in one of the participating centers and could disseminate analytical data in a dynamic manner by electronic means.

Abbreviations

TAA, tumor-associated antigen

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