Chapter 4

MHC multimer technology: current status and future prospects

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The detection of antigen-specific T cell responses by MHC multimer staining is rapidly becoming one of the core immunological techniques, and the technology to produce MHC multimers has been optimized substantially in recent years. Furthermore, recent work demonstrates the potential of high-throughput detection of T cell responses and suggests that manipulation of T cell responses through the use of multimeric MHC reagents is also feasible.

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Introduction

T cell receptors (TCRs) are capable of singling out specific peptide–MHC (pMHC) complexes on target cells amidst a wide variety of other pMHC complexes. By exploiting the specificity of this interaction, multimeric forms of MHC molecules (MHC multimers) have been designed with the aim of detecting antigen-specific T cells amidst a multitude of unrelated T cells. The first MHC multimer used for specific T cell analysis, a human MHC class I tetramer, was described in 1996 by John Altman [1]. Today, MHC multimers can range from dimers to octamers, consisting of either MHC class I, MHC class II or nonclassical MHC molecules, from species including mouse, monkey and man.

In this review we discuss the value of currently available MHC class I and class II multimer technologies in terms of valency, expression system and peptide loading strategy. In addition, we provide a roadmap for the future development of multimeric MHC technology, and high-throughput multimer systems in particular, as well as potential clinical applications.

MHC multimer technologies

A great number of strategies has been developed for the production of MHC multimers. As illustrated in Table 1, the major differences between MHC multimers are found in just three parameters: the valency of the multimeric complex, the expression system through which the molecules are produced, and the peptide-loading strategy used to achieve occupancy of the peptide-binding groove with the desired antigenic peptide.

Valency

TCRs have a low affinity for their cognate pMHC counterparts, with an off-rate of only a few seconds [2]. Monomeric pMHC–TCR interactions are therefore too unstable to be exploited as an effective labelling technique, but — as for any multivalent interaction — combining multiple MHC molecules into one complex greatly increases binding stability [3,4]. In their landmark paper, Davis and colleagues [1] approached this need for multivalency by designing tetrameric forms of MHC molecules. In this strategy, soluble MHC monomers are biotinylated and converted to tetravalent structures by binding to (fluorochrome-conjugated) streptavidin or avidin, which both have four biotin binding sites. The resulting MHC tetramers remain by far the most popular reagents for the detection of antigen-specific $CD4^+$ and $CD8^+$ T cells by flow cytometry.

What's in a name?

In spite of their name, however, it is quite unclear whether binding of MHC tetramers to T cells occurs in a tetravalent fashion. First, due to the rigid tetrahedral configuration of these complexes only three out of the four available MHC molecules are likely to bind simultaneously to the T cell surface [5]. Second, the conjugates of (strept)avidin with the proteinaceous fluorochromes phycoerythrin (PE) and allophycocyanin (APC) that are used for MHC tetramer production are prepared by chemical crosslinking and therefore also contain multimers of (strept)avidin [6]. These higher order oligomers appear to make an important contribution to T cell binding, as evidenced by the fact that streptavidin–PE 'tetramers' show increased binding over Cy5-labeled (true) tetramers, when tested for CD8-independent binding to human cytotoxic T lymphocytes (CTLs) [6]. These data not only suggest that the valency of the standard PE conjugates exceeds four, but also that — at least for (strept)avidin-based multimers — a valency greater than four might be preferred for optimal binding. Other and arguably better defined multimers with valencies greater than four exist, such as the commercially available MHC pentamers, where five pMHC complexes face the same direction through the use of a five-stranded coiled coil as oligomerization domain. On the other side of the spectrum, MHC multimers with a valency of two (i.e. MHC

Table 1

Distinct MHC multimer formats.

Peptide-binding strategy	Dimer[a]		Tetramer[a]		Pentamer[a]		Octamer[a]		Polymer/aAPC[a]	
	Euk.[b]	Bact.[b]	Euk.[b]	Bact.[b]	Euk.[b]	Bact.[b]	Euk.[b]	Bact.[b]	Euk.[b]	Bact.[b]
Exchange	[**8**]		[*18*[c],*24*]						[**10**,*11*]	
Bound during refolding				[1[c],*4*[d],*21*]		[e]		[**6**]		[**9**,**43**]
Linked peptide	[**16**,*44*]		[*17*[c],*42*]						[*12*]	

Only selected references are cited. MHC class I multimers are shown in bold; MHC class II multimers are shown in italics. [a]Valency. [b]Expression system. [c]Most widely used formats. [d]Monomers and tetramers have been produced, but higher order oligomers are also possible. [e]Limited peer-reviewed data available at present. Euk., Eukaryotic; Bact., bacterial.

dimers) were designed in the laboratory of Jonathan Schneck [7]. In these molecules, the extracellular domain of MHC molecules is expressed as a genetic fusion with an immunoglobulin scaffold, resulting in an MHC–Ig dimer [7,8]. Although these MHC dimers were originally used for T cell activation, these dimeric MHC molecules are now also used as staining reagents in flow cytometry [8]. Finally, several approaches have been developed for the generation of MHC-coated artificial antigen-presenting cells (aAPCs). In these systems, multivalency is achieved by coupling MHC monomers to magnetic beads [9,10], or by incorporating MHC monomers into liposomal vesicles [11,12].

What's your favourite number?

Faced with this multitude of multimers, which format should one choose to detect antigen-specific T cells by flow cytometry? The available data suggest that a valency of four or possibly even greater is optimal for the detection of high-avidity $CD8^+$ T cells with (strept)avidin-based MHC multimers. It is noted, however, that the scaffold used for multimerization is likely to influence the valency that is required, by affecting the conformational freedom of the attached MHC monomers. MHC dimers are used infrequently in comparison to MHC tetramers, although this could also be due to the more complicated production process (see below). MHC pentamers have the advantage of being more molecularly defined. At present, not many peer-reviewed data on these new molecules are available, but it seems likely that their binding strength will not differ substantially from the standard MHC tetramers.

A higher valency of the complex may become more important when aiming to detect antigen-specific $CD4^+$ T cells, as standard MHC class II tetramers appear to miss lower avidity cells present in the antigen-specific $CD4^+$ T cell repertoire [12,13]. Low-avidity $CD8^+$ T cells, such as T cells specific for self-antigens, can be detected by conventional MHC class I tetramers in at least some cases [14,15] (and one should therefore be aware that the detection of antigen-specific $CD8^+$ T cells by MHC tetramer staining is not necessarily indicative of a high-avidity interaction). It seems plausible, however, that the detection of low-avidity self-specific $CD8^+$ T cells will also be facilitated by the use of higher order MHC oligomers.

Expression system

Recombinant MHC molecules used for the production of multimeric MHC reagents have been produced in either bacterial cells or eukaryotic cells, such as insect cells and mammalian cells. A clear advantage of the bacterial expression systems is the ease with which large quantities of proteins can be generated. MHC molecules that are produced in *Escherichia coli* cells generally need to be refolded *in vitro*. This process is straightforward for the majority of MHC class I alleles and, with few exceptions [8,10,16], bacterial expression has indeed been the preferred system for MHC class I multimer production. By contrast, the efficiency with which MHC class II molecules can be refolded *in vitro* is notoriously low, and the majority of MHC class II production systems are therefore based on eukaryotic expression, such as baculovirus-infected insect cells [17] or stable Drosophila cell transfectants [18,19]. Although the yield of MHC class II molecules obtained in such eukaryotic expression systems can be optimized [20••], the development of *E. coli* expression strategies that provide higher yields of refolded MHC class II molecules than the current strategies [3,12,21] remains a laudable goal.

Peptide-loading strategy

The third — and rather important — parameter in the generation of MHC multimers is the method by which the MHC molecules are loaded with peptide. Three fundamentally different approaches have been used to date:

1. Antigenic peptides can be included during the *in vivo* process, through genetic linkage to one of the MHC chains.
2. Peptides can be included during the *in vitro* production process.
3. Peptides can be bound after MHC monomer, or even MHC multimer, production.

Low-throughput strategies

From a structural point of view, genetic fusion of the desired antigenic peptide with one of the MHC chains makes sense for MHC class II molecules, but less so for

MHC class I molecules. In the case of MHC class I molecules, the terminal $NH3^+$ and COO^- groups are normally buried in the MHC structure and contribute to peptide binding [22,23]; genetic fusion of MHC to one of the peptide termini is therefore bound to result in a local change in the structure of the pMHC complex. Genetic peptide fusions to MHC molecules have been used fairly extensively to produce human and murine MHC class II multimers [17,19]. High throughput synthesis of multimeric MHC molecules by this strategy, however, is precluded by the fact that a different construct and producer cell line is required for each peptide antigen. Production of MHC multimers with several different peptide antigens is more straightforward with the commonly used strategy for MHC class I multimer production, in which a synthetic peptide antigen is included during the *in vitro* refolding process. Because separate refolding and purification is still required for each single MHC class I multimer, the production of large collections of pMHC reagents remains a challenging task.

High-throughput strategies

In cases in which a multitude of MHC multimers with different antigens bound to the same MHC allele is required, the preferred strategy is to bind peptide ligands to preformed MHC monomers or multimers. For MHC class II molecules, such 'exchange' strategies have been developed, using either presumed peptide-free [18] or class II-associated invariant chain peptide (CLIP)-bound [24] MHC class II molecules as starting material. Although the CLIP-based strategy is conceptually somewhat more appealing, as it mimics the *in vivo* binding of ligands to MHC class II, no comparison between the two strategies has been made.

Because of the greater number of identified MHC-class-I-associated epitopes, a similar type of exchange strategy would be of considerable use for the generation of MHC class I multimers [8,10,25]. Because of the instability of peptide-free MHC class I molecules [26,27], however, the conditions that can be used to promote the release of bound ligands, such as low pH, also affect the MHC structure itself, and this type of exchange technology has not gained widespread use for MHC class I. To circumvent this issue, we have recently developed so-called 'conditional' MHC class I ligands. These ligands can be made to dissociate from MHC class I upon exposure to defined triggers, such as ultraviolet light, that do not by themselves destabilize MHC class I molecules (M Toebes *et al.*, personal communication). Using such conditional ligands, a large array of MHC multimers can be generated from preformed MHC complexes in one or two hours, and the resulting complexes appear to bind to antigen-specific T cells with comparable avidity and specificity as conventional MHC class I tetramers. This technique might prove to be of substantial use for the generation of large collections of pMHC multimers, for conventional MHC multimer flow cytometry, and for high-throughput systems for T cell analysis (see below).

Future strategies and challenges

High throughput analysis with MHC multimers

At present, the detection of antigen-specific T cells by MHC tetramer staining is a technology with a highly limited throughput, as only a single T cell specificity is analyzed per sample. For the definition of novel pathogen- or tumour-associated epitopes, or for the comprehensive screening of T cell responses in blood samples, the simultaneous monitoring of a large number of T cell specificities in a single sample would be highly desirable.

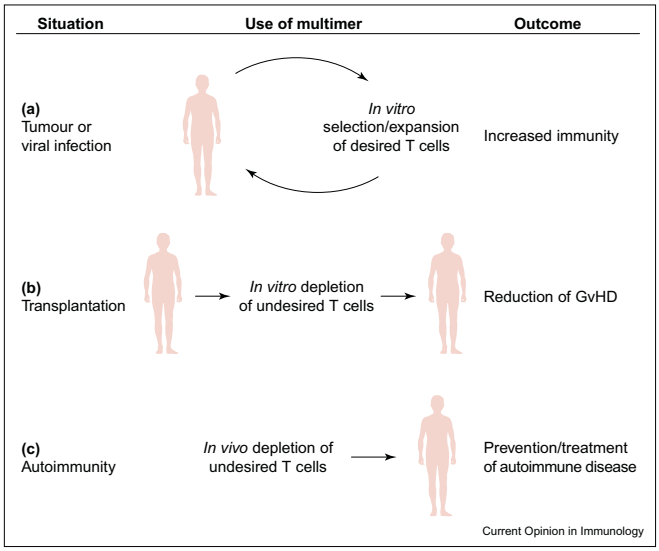
Multiparameter flow cytometry

If MHC multimers with multiple specificities are to be used in a single flow cytometric analysis, this requires that MHC multimers of each separate specificity carry an individual label [28]. The low diversity of fluorochromes routinely used in conjunction with MHC multimers (i.e. PE and APC) has severely limited the potential for multiparameter screening. Although the non-proteinaceous fluorochromes have not worked well with the classical MHC tetramer format, this problem can be overcome by the use of octameric MHC reagents [6]. Perhaps more important for the long-term development of multiparameter MHC multimer analysis is the recent interest in fluorescent nanocrystals called quantum dots (qdots) [29••]. Qdots are more stable than organic fluorochromes, and — importantly for their potential use with MHC multimers — quantum dots are available in a wide fluorescent spectrum and exhibit very narrow emission spectra. On the basis of these properties, we consider it plausible that MHC multimers, built on qdot-coupled streptavidin, for example [30], will enable the simultaneous measurement of 10 or more T cell responses in a single sample in the not-too-distant future. Furthermore, if qdot-coupled MHC multimers can be made to contain defined combinations of qdots, the combinatorial power of the method could increase even further [31].

MHC-microarrays

As an alternative to flow cytometric analysis of multiple T cell responses by MHC multimers, Soen and colleagues [32] have developed an MHC-microarray-based approach for T cell detection. In these arrays, each array spot contains MHC molecules complexed with a specific peptide, and T cell responses are measured by quantifying either T cell binding or T cell activity in distinct spots. Although only a modest number of pMHC specificities were spotted in the first arrays, the use of peptide-exchange systems for MHC class II [18,24] and MHC class I molecules (M Toebes *et al.*, personal communication), in which exchange reactions are performed on the slide, has the promise to produce microarrays with a

Figure 1



Potential clinical applications for MHC multimers. **(a)** MHC multimers can be used for the *ex vivo* selection and expansion of desired antigen-specific T cells. These T cells can then be (re-)infused to enhance reactivity against defined tumour- or virus-associated antigens. **(b)** MHC multimers can be used for the *ex vivo* depletion of undesired (e.g. graft-versus-host disease [GvHD]-associated) T cells from transplant material before transfer to the recipient. **(c)** MHC multimers can be used for the *in vivo* inactivation of undesired (e.g. autoimmune-associated) T cells for the prevention/treatment of autoimmune disease.

substantially larger number of specificities. In comparison to multiparameter analysis of T cell responses by MHC multimer flow cytometry, MHC microarrays are likely to have a somewhat lower sensitivity, but such arrays should be capable of visualizing T cell populations specific for a vast number of (possible) antigens.

Manipulating T cell responses with MHC multimers

A series of studies has provided proof-of-principle for the use of MHC multimers as reagents to boost desirable or suppress unwanted T cell responses (Figure 1). MHC multimers have been used for rapid and efficient *ex vivo* isolation and expansion of specific T cells [11,33,34], which should be of use for adoptive therapy following allogeneic stem cell transplantation, for example [35]. MHC multimer–TCR interactions during such enrichments could affect T cell viability, so to reduce the effects of these interactions, Knabel and colleagues [36] have devised a strategy for reversible multimer staining. This technique forms an elegant addition to the standard MHC multimer approach, but whether such reversible binding improves the *in vivo* activity of adoptively transferred T cells in human trials remains to be determined.

MHC multimers could also conceivably be used for the *ex vivo* removal of unwanted T cells, such as alloreactive T cells contained in peripheral stem cell transplants. Furthermore, several studies provide support for the use of MHC dimers for the *in vivo* inactivation of autoreactive T cells, thereby preventing type I diabetes and arthritis [37–39]. In addition to these non-conjugated MHC multimers that rely on TCR signalling for their biological effects, isotope-coupled MHC multimers have been generated [40•]. These ^{225}Ac-labeled MHC tetramers are promising reagents to induce killing of specific T cell populations. Although the *in vivo* activity of this type of 'suicide tetramer' remains to be established, the isotope does not seem to be overly toxic *in vivo* [41].

Because the quantity of MHC multimers required for *in vitro* enrichment or depletion is likely to be lower than the amount required for *in vivo* use, these *in vitro* technologies could perhaps be implemented more readily. Before MHC multimers can be used in any of these *ex vivo* or *in vivo* clinical settings, however, it will obviously be essential to develop procedures for the Good Manufacturing Practice production of these reagents, which will prove a challenge for translational researchers in the coming years.

Conclusions

The dissection of T cell responses by MHC multimer staining has become an established technique in preclinical research, and is becoming increasingly important for clinical trial monitoring. The parallel development of a variety of multimeric MHC strategies in the past decade has been important to discovering which formats work best and we expect that in the coming decade a small number of these formats will become standard for MHC multimer flow cytometry. More importantly, we speculate that the near future will bring the arrival of technologies that will enable high-throughput analysis of T cell responses, either by flow cytometry or on solid surfaces. In addition, MHC multimers might find increasing use as therapeutic agents, be it either for the enrichment of desired T cells, or the removal of rogue T cell populations.

Update

Elegant work has recently been published by the Stern laboratory on the generation of MHC microarrays [45••]. In this study, T cell responses against a number of pMHC complexes are analyzed in parallel by cytokine capture.

Cebecauer and colleagues [46•] have recently demonstrated that the distance between individual pMHC molecules of an MHC multimer affects the cytotoxic effect of such multimers on antigen-specific T cells: MHC molecules connected by short linkers induce rapid cell death, whereas MHC molecules connected by long linkers do not. This is valuable information for the development of MHC multimers for clinical use.

Finally, the first papers have been published that make use of the MHC class I pentamer technology to detect antigen-specific T cells by flow cytometry [47,48].

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