Review

Use of soluble MHC class II/peptide multimers to detect antigen-specific T cells in human disease

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Received: 20 November 2001 Revisions received: 1 February 2002 Accepted: 6 February 2002 Published: 28 February 2002

Arthritis Res 2002, **4**:261-265

Abstract

Most techniques that identify antigen-specific T cells are dependent on the response of these cells to the relevant antigen in culture. Soluble multimers of MHC molecules, when occupied with the same peptide, will bind selectively to T cells specific for that MHC/peptide complex. Techniques to produce fluorescent MHC class II/peptide multimers have recently been developed. These reagents provide a method to facilitate detection and isolation of antigen-specific $CD4^+$ T cells and they represent a new research tool to study these cells in patients with immune-mediated diseases.

Keywords: flow cytometry, MHC class II, MHC/peptide multimer, T cell, T-cell receptor

Background

$CD4^+$ and $CD8^+$ T cells, through their T-cell receptors (TCRs), recognize peptides bound to MHC class II and class I molecules, respectively. The peptide, derived from the protein antigen, and the restricting MHC molecule are both critical for specific binding of the TCR. Until recently, the identification or quantitation of antigen-specific T cells was possible only by assaying for their function. Classically, a population of T cells was cocultured with antigen and antigen presenting cells, which express surface MHC molecules. Several days later, tritiated thymidine was added to the culture and antigen-induced T-cell proliferation was quantitated by the amount of incorporated thymidine. Modifications of this basic antigen-stimulation technique include plating the cells at limiting dilution and counting the number of T-cell clones that are generated (limiting dilution analysis). T cells can also be stimulated with antigen *in vitro* and assayed for cytokine production either in bulk cultures or by enumerating individual cytokine-producing cells. These methods probably underestimate the true number of antigen-reactive T cells since some cells cannot proliferate or make the particular cytokines being measured.

Attempts to isolate and study antigen-specific T cells after a functional response (e.g. proliferation) in bulk culture or after cloning can also be problematic. For example, the TCR repertoire of responding cells can be remarkably altered compared with the starting population, and T-cell function is frequently changed by the *in vitro* response. Furthermore, some T cells are not able to proliferate in culture or die in culture after stimulation. Examples include expanded $CD8^+$ T-cell clones in the circulation of older individuals, expanded $CD4^+$ T-cell clones in the synovial fluid of patients with rheumatoid arthritis, and $CD4^+$ T cells in patients with systemic lupus erythematosus.

The development of fluorescently labeled MHC/peptide staining reagents now permits direct detection and isolation of antigen-specific T cells, independent of cellular function. The preparation and use of MHC class I/peptide multimers to study antigen-specific $CD8^+$ T cells was recently reviewed in this journal [1]. We now review the development of MHC class II/peptide multimers as a research tool.

Perspective (what it can do)

A single soluble MHC/peptide complex binds to a specific TCR with low affinity, usually with dissociation constants no better than 1–100 μM. The weak binding to TCR and fast dissociation prevents these molecules from being useful

CFSE = 5-carboxyfluorescein diacetate succinimidyl ester; gp39 = cartilage glycoprotein 39; MHC = major histocompatibility complex; TCR = T-cell receptor.

reagents to detect peptide-specific T cells. However, several studies have shown that when soluble MHC/peptide complexes are multimerized, they can achieve much higher avidity for the TCR on the T-cell surface, presumably via cooperative multivalent binding [2–4]. Stable interactions with cell surface TCRs were therefore possible.

Two innovations made this multimerization more feasible and reproducible experimentally. The first was the addition of a peptide tag to the MHC molecule that permitted precise biotinylation using the BirA enzyme [5]. The second innovation was the use of fluorescently conjugated streptavidin to oligomerize and label the MHC/peptide molecules [2]. These innovations were first accomplished for the development of MHC class I multimers, which have since been used by a variety of investigators to study antigen-specific $CD8^+$ T cells during viral infection, tumor immunity, and autoimmune disease [1].

Crawford *et al*. were the first to develop fluorescent multimers of MHC class II/peptide complexes [6]. Recombinant MHC class II α-chains and β-chains were expressed with the antigenic peptide covalently bound to the MHC class II β-chain via a linker peptide. This allowed the same peptide to bind to each peptide-binding region as the MHC molecules folded into the native configuration. The MHC class II/peptide multimers bound with appropriate specificity to T-cell hybridomas and to T cells (isolated from TCR transgenic mice) specific for the particular MHC/peptide combination. In studies analyzing antigen-specific T-cell hybridomas, the intensity of binding was shown to be dependent on two main factors: the number of TCRs expressed on the cell surface, and the affinity of the MHC/peptide complex for the particular TCR. If TCR expression is held constant, then the intensity of fluorescent staining with MHC/peptide multimers can be used as a measure of the affinity of the TCR for the MHC/peptide. Binding of the multimer was shown to be mostly independent of CD4 [6].

MHC class II/peptide multimers stained antigen-specific T cells in mice after immunization and could be used to track TCR selection during various stages of the immune response [7]. T cells from immunized mice demonstrated a range of multimer-binding levels, indicative of a range of TCR affinities for peptide. There was a narrowing of the TCR repertoire after secondary immunization, resulting from the loss of cells with lowest affinity and an increase in cells with higher affinity for peptide/MHC binding. Other studies with MHC class II/peptide multimers documented the presence (or absence) of self peptide reactive $CD4^+$ T cells before and after peptide immunization in animal models of autoimmune disease, such as the NOD mouse model of type 1 diabetes [8]. Together, these animal studies have set the stage for similar studies in humans after immunization and during the course of autoimmune disease.

Short technical description

Production of multimeric MHC class II/peptide staining reagents involves four basic steps: the expression of soluble monomeric MHC class II molecules, peptide loading, oligomerization, and fluorescent labeling. Most studies have used recombinant MHC molecules truncated proximal to the transmembrane domain to obtain soluble products in eukaryotic cell protein expression systems [7,9,10]. The expression of native molecules in these expression systems contrasts with that generally used for production of MHC class I/peptide complexes, which has relied on refolding denatured proteins expressed in a bacterial expression system [1,2].

Crawford *et al*. [6] described the use of MHC class II molecules with covalently attached peptides produced in a baculovirus expression system. MHC class II α-chains and β-chains are secreted into the supernatant of baculovirus-infected moth cells in the correctly folded, biologically active state. In addition, the constructs include a cassette encoding the MHC-binding peptide with a cleavable linker between the class II β-chain leader sequence and the β1 domain. The peptide-loaded, correctly folded molecules are purified by immunoaffinity chromatography. Biotinylation by the BirA enzyme is accomplished through an added peptide tag on the carboxy terminus of the β-chain, and the molecules are multimerized by adding phycoerythrin-labeled streptavidin. It is possible that multimers with covalently attached MHC-binding peptides [6] (versus those in which peptide has been added after MHC expression) may have greater stability and may better allow for the generation of complexes with peptides that have low affinities for MHC. However, covalent attachment of the peptide is not necessary for MHC class II/peptide multimer production [10,11].

Other expression systems have been used to generate MHC class II/peptide multimers. Boniface *et al*. [11] produced MHC class II molecules in *Escherichia coli* inclusion bodies, as they had for class I molecules. Following solubilization in guanidine, the molecules were refolded in the presence of excess peptide. Kwok, Nepom and colleagues have reported the successful production of several human MHC class II/peptide staining reagents using transfected *Drosophila melanogaster* (Schneider, S2) cells [10,12,13]. To foster correct HLA-DR (or DQ) α-chain and β-chain pairing and protein folding, these investigators also added a leucine zipper to compensate for the missing hydrophobic transmembrane regions [14]. The peptide can then be added to the secreted soluble molecules, prior to multimerization.

One of the issues related to both MHC class I/peptide staining reagents and MHC class II/peptide staining reagents is the actual extent of multimerization. These reagents were originally referred to as 'tetramers' because

of the theoretical binding of one streptavidin to four biotin molecules. Analyses have shown, however, that the multimers are generally mixtures of larger complexes [15,16]. The term 'multimer' is therefore preferred. The extent of multimerization that allows for optimal binding to TCR but maintains specificity is unknown.

Staining T cells with MHC class II/peptide multimers is accomplished by similar techniques compared with other staining reagents. However, studies have suggested that optimal staining of $CD4^+$ T cells may require prolonged incubation in media at 37°C [6,16]. Examination by confocal microscopy has shown that the labeled complexes have been mostly internalized [15,16]. Binding of MHC class II/peptide multimers to some, presumably low avidity, antigen-specific $CD4^+$ T cells can be enhanced by including a nonlabeled TCR crosslinking reagent during the staining, such as anti-TCR or anti-CD3 monoclonal antibodies [17]. The conditions used for staining with these reagents frequently make it imperative to exclude non-T-cell populations that nonspecifically bind the multimers, especially monocytes/macrophages [17].

Human studies

Novak *et al*. [10] used HLA-DRB1*0401/peptide multimers to identify and quantitate influenza hemagglutinin peptide-specific $CD4^+$ T cells in two individuals. In both cases, antigen-specific T cells could only be detected following 7 days of *in vitro* culture with peptide. The number of divisions that multimer-staining cells had undergone in culture was estimated using 5-carboxyfluorescein diacetate succinimidyl ester (CFSE). Using multimer and CFSE staining in parallel, Novak *et al*. calculated the precursor frequency of peripheral blood antigen-specific T cells to be in the range of 3–5 per 100,000 cells. This frequency is well below the detection limit for staining freshly isolated cells with multimer.

This assay (using multimer and CFSE) also requires that the T cells are capable of proliferation in response to antigen *in vitro*. Thus, while it may be a more convenient way to estimate precursor frequency, this assay should detect about the same number of antigen-specific T cells compared with conventional limiting dilution analyses.

The same investigator group [12,13] has also used this approach to quantitate the frequency of herpes simplex virus reactive T cells in the peripheral blood of DQB1*0602-positive individuals with chronic infection. Again, they arrived at the very low estimate of 2 per 100,000 cells. These and other results (see below) indicate that the frequency of virus-specific $CD4^+$ T cells is likely to be much lower than that of virus-specific $CD8^+$ T cells.

The first use of peptide/MHC class II multimers to detect autoreactive T cells in human autoimmune disorders was reported by Kotzin *et al*. [17]. They examined blood and synovial fluid of patients with rheumatoid arthritis for T cells stainable with multimers of HLA-DRB1*0401 complexed with dominant epitopes of type II collagen and cartilage glycoprotein 39 (gp39). The DR4/peptide multimers stained in a specific manner to peptide-reactive hybridomas derived from HLA-DR4 transgenic mice. However, no stainable cells were found in the synovial fluid or peripheral blood of DRB1*0401 patients with an estimated limit of detection of 1 in 1000. Studies have suggested that T cells with these specificities may be present at low frequency in the blood of rheumatoid arthritis patients, and it had been thought that the true frequency would be much higher in synovial fluid. The results with multimer staining do not support these hypotheses. It is possible that synovial T cells are not enriched for cells directed to type II collagen, gp39, or other cartilage proteins.

Using DRB1*0401/peptide multimers, Meyer *et al*. [18] were able to find *Borrelia burgdorferi* peptide (outer surface protein A 164–183) reactive $CD4^+$ T cells in the synovial fluid of two out of three patients with treatment-resistant Lyme disease (0.5% and 3.1% of $CD4^+$ T cells). However, there was no staining above background in the peripheral blood of these patients or in three additional patients. These investigators went on to demonstrate that sorted multimer-positive synovial cells contained nearly all of the *B. burgdorferi* peptide-reactive $CD4^+$ T cells as determined by T-cell cloning. By sorting with the DR4/outer surface protein A multimer, they were also successful at deriving peptide-reactive T-cell clones from the peripheral blood of two out of four patients who did not have detectable levels of multimer-positive T cells.

Sensitivity/limitations

One striking feature of the studies so far performed with MHC class II/peptide multimers is that the frequency of detectable peptide-specific $CD4^+$ T cells is low. This seems to be true even when studying draining lymph node cells in immunized animals. For example, Savage *et al*. [7] studied T cells from draining lymph nodes following one and two immunizations with cytochrome c. They found that only ~1% of $CD4^+$ T cells stained with the I-Ek/cytochrome c multimer after the first immunization, and found that this frequency only marginally increased following the second immunization.

Similar observations have been made in other studies using different types of antigens and including studies of autoimmune and virus-infected animals [8,19,20]. In most studies of humans for peptide-specific $CD4^+$ T cells, multimer-positive cells have not been detected in freshly isolated peripheral blood cells. In nearly every case, *in vitro* expansion of antigen-reactive cells has been required to document the existence of circulating antigen-specific $CD4^+$ T cells and to accomplish additional analyses.

These findings question the original premise that cells staining positive with class II/peptide multimers would significantly outnumber those that proliferate in response to the particular peptide/MHC combination.

The sensitivity of immunofluorescence analysis with MHC class II/peptide multimers will probably vary depending on the intensity of fluorescence (i.e. avidity for TCR) and background (nonspecific) staining by other cells in the sample. In most experiments, the lower limit of detection is unlikely to be better than 0.1–0.2% of a definable subset (e.g. $CD4^+$ T cells or $CD4^+CD45RO^+$). Multimer staining is therefore much less sensitive than classical limiting dilution analyses or ELISPOT methods that identify cytokine-secreting cells after stimulation. From a sensitivity point of view, multimer staining can only outperform these other assays if there is a relatively large subset of peptide-specific $CD4^+$ T cells that cannot proliferate or secrete cytokine (which has not been demonstrated to date). As already discussed, *in vitro* stimulation with antigen followed by multimer staining has been useful to demonstrate that multimer positive cells were present at time zero. In conjunction with CFSE labeling, it can also provide an estimate of precursor frequency. However, these types of studies do not fulfill the promise that multimer technology would permit enumeration of antigen-specific T cells independent of their function.

The decreased binding of MHC class II/peptide multimers to TCRs with lower affinity raises the question of how much of the low-affinity T-cell population is below the limit of detection by multimer staining. In one study of NOD mice immunized to peptides derived from glutamic acid decarboxylase, T cells were tested for responses to peptide after separation with $I\text{-}A^{g7}$/peptide multimers [8]. Essentially all of the reactive clones appeared to be present in the multimer-positive pool. In more recent studies, HLA-DR4 transgenic mice were immunized with a dominant peptide from human gp39 [17], and peptide-specific T-cell hybridomas were derived from draining lymph node cells. Nearly all of the hybridomas that responded to peptide stimulation *in vitro* also were readily stained with the peptide-DR4 multimer (MT Falta *et al*., unpublished observations, 2001). These studies and others [20] suggest that peptide/MHC class II multimers are capable of detecting the great majority of the T cells that can respond to peptide *in vitro*.

A final limitation of this technology is probably the technical difficulty in generating particular MHC class II/peptide complexes by recombinant methods. For MHC class I multimers, the most common HLA-A and HLA-B molecules have been expressed as denatured proteins in bacteria, and if a peptide binds adequately the complex has been successfully folded, with a few exceptions. In contrast, MHC class II molecules with covalent peptides require a new construct in each individual case. In addition, certain HLA-DR and HLA-DQ molecules have been difficult to express in baculovirus or drosophila expression systems, either with or without covalent peptide, and despite the addition of 'zippers' to the α-chain and β-chain constructs. It almost seems that the expression of each molecule has its own rules, and the reasons for these technical problems remain unclear at this time.

Future development/direction

The use of MHC class II/peptide multimers will increase greatly in the near future, especially as more MHC/peptide complexes are successfully generated. Some new multimers, such as DQ8/glutamic acid decarboxylase peptide or DQ8/insulin peptide multimers for studying type 1 diabetes, or DR15/myelin basic protein peptide multimers for studying patients with multiple sclerosis, may be particularly insightful for studies of autoimmunity.

However, the available data suggest that the frequency of antigen-specific $CD4^+$ T cells in the peripheral blood of autoimmune disease patients may not be high enough to allow direct detection with multimers. Still, in combination with CFSE or other staining techniques, these multimers may facilitate estimates of precursor frequency of autoreactive $CD4^+$ T cells in longitudinal studies. If adequate numbers of cells are generated *in vitro*, multimer staining can be used directly to assess changes in TCR affinity and therefore TCR repertoire. In addition, sorting multimer-positive cells has worked well to enrich or deplete antigen-specific cells for subsequent analysis, and the use of multimers for enrichment can greatly facilitate analysis of antigen-specific T cells. In the cases where multimer-based cell sorting has been carried out, it is clear that the positively stained T cells can subsequently function in response to antigen, which argues against the idea that multimer binding causes apoptosis of the target T cells.

Other clinical situations, such as infection, cancer, and transplantation, will also be amenable to study with these multimers, although with the same limitations. MHC class II/peptide reagents may also be particularly useful to quantitate and evaluate $CD4^+$ T-cell immune responses after vaccination and to explore the repertoire and characteristics of responding cells.

Conclusion

MHC class II/peptide multimers have been used successfully to identify antigen-specific CD4+ T cells. The intensity of staining correlates with the affinity of TCR for the particular MHC/peptide. Although the frequency of antigen-specific $CD4^+$ T cells in human peripheral blood appears to be below the limit of direct multimer staining, these reagents, in conjunction with *in vitro* stimulation with antigen, can facilitate estimates of precursor frequency.

MHC class II/peptide multimers may be most useful to enrich antigen-specific T cells for further study.

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