# MHC Class II Tetramers

Gerald T. Nepom

*J Immunol* 2012; 188:2477-2482; doi: 10.4049/jimmunol.1102398

http://www.jimmunol.org/content/188/6/2477

<table>
<thead>
<tr>
<th>References</th>
<th>This article cites 69 articles, 29 of which you can access for free at: <a href="http://www.jimmunol.org/content/188/6/2477.full#ref-list-1">http://www.jimmunol.org/content/188/6/2477.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Subscription</td>
<td>Information about subscribing to <em>The Journal of Immunology</em> is online at: <a href="http://jimmunol.org/subscription">http://jimmunol.org/subscription</a></td>
</tr>
<tr>
<td>Permissions</td>
<td>Submit copyright permission requests at: <a href="http://www.aai.org/About/Publications/JI/copyright.html">http://www.aai.org/About/Publications/JI/copyright.html</a></td>
</tr>
<tr>
<td>Email Alerts</td>
<td>Receive free email-alerts when new articles cite this article. Sign up at: <a href="http://jimmunol.org/alerts">http://jimmunol.org/alerts</a></td>
</tr>
</tbody>
</table>
MHC Class II Tetramers

Gerald T. Nepom

MHC class II tetramers have emerged as an important tool for characterization of the specificity and phenotype of CD4 T cell immune responses, useful in a large variety of disease and vaccine studies. Issues of specific T cell frequency, biodistribution, and avidity, coupled with the large genetic diversity of potential class II restriction elements, require targeted experimental design. Translational opportunities for immune disease monitoring are driving the rapid development of HLA class II tetramer use in clinical applications, together with innovations in tetramer production and epitope discovery. *The Journal of Immunology*, 2012, 188: 2477–2482.

The concept of using soluble labeled ligands to detect cell surface receptors is a standard approach for understanding molecular specificity and function. However, only in the last 15 y has this general strategy been successfully applied to the analysis of Ag-specific T cells through the use of soluble MHC-peptide ligands that engage the αβTCR. Originally developed for MHC class I recognition in the context of CD8 T cells (1, 2), over the last decade similar approaches have been successfully used for MHC class II recognition in the context of CD4 T cells for a wide variety of Ags. From this experience, it is now evident that interrogating CD4 T cells using soluble tetramers has matured into a robust technology, notably useful in numerous translational and clinical contexts. However, several constraints that limit their use are also now understood. In this Brief Review, key issues governing CD4 T cell identification by MHC class II tetramers are discussed, and examples from human immune monitoring studies illustrate the lessons learned.

**Adding specificity to the T cell flow cytometry toolkit**

Peptide-MHC (pMHC) class II multimers display an antigenic peptide in the class II-binding groove, functioning as a surrogate for recognition events that occur as part of the T cell interaction with APCs. Various methods for displaying pMHC class II molecules have been successfully used, ranging from monomeric pMHC bound to solid substrates or soluble in solution to higher-order multimers complexed with various scaffold molecules. The most prevalent form of multimer in use today consists of biotin-labeled pMHC displayed on streptavidin molecules, forming ostensibly tetravalent complexes; hence, the common use of the term “tetramers” for this detection method.

Tetramer assays are widely used for single-cell phenotyping and enumeration and offer an important advantage over other methods, such as ELISPOT and single-cell PCR, by enabling the recovery and further study of sorted cells based on fluorescent tetramer binding in flow cytometry. As a cytometry-based application, tetramers also provide the prospect of ease of use and short assay time, analogous to Ab-based flow cytometry studies. The major limitation to their use is the requirement to have some knowledge of the specific pMHC components involved in the recognition events being studied, because these are necessary to construct the tetramer reagents. A corollary to this element of MHC restriction is the consideration that humans have very diverse HLA class II molecules, so that random sampling or clinical monitoring studies may require the use of many different tetramers to include a broad representation of the population.

Straightforward solutions exist for both of these limitations—HLA diversity and epitope identification—by making the investment necessary to produce a universal set of class II tetramers for most HLA alleles, as well as by using functional selection of relevant epitopes as a criterion for study design. With these advances, discussed below, there has been a rapid expansion of studies using tetramers to study CD4 T cell response to vaccines, allergens, and a variety of Ags associated with autoimmune diseases. There has also been an improved understanding surrounding the potential use of tetramers, because as the technological problems have been largely solved, there is now realization of some important biological barriers associated with interrogating the specific TCR–tetramer interaction.

**Rare CD4 T cell detection using class II pMHC tetramers**

A major challenge for studies using HLA class II tetramers is the low frequency of Ag-specific CD4 T cells in peripheral blood. Highly boosted responses, such as tetanus-specific or influenza-specific CD4 T cells after immunization, display transient levels of Ag-specific cells in the 1:1,000–1:20,000 range; specific memory T cells without recent boosting are more often found in the 1:20,000–1:100,000 range; and rare responses or autoantigen-specific responder cells are detectable...
in the 1:100,000–1:1 million range. Naïve T cell frequencies to particular pMHC are also ~1:200,000–1:1 million. Because of these low cell frequencies, the first generation of tetramer-based immunoassays used in vitro activation and/or expansion to enable T cell detection (3), somewhat analogous to the historically used techniques of limiting dilution-proliferation assays and ELISPOT analysis. Of course, a major problem with such an approach was the potential for phenotypic changes introduced by in vitro manipulation; therefore, these studies were limited primarily to the estimates of cell frequency and specificity.

Current second-generation tetramer assays avoid the use of in vitro expansion and are either based on single-cell capture technology (4–6) or on direct flow cytometry staining analysis (7–10). In the latter, stringent “dumping” criteria and enrichment with magnetic bead-trapping procedures are used to remove cells that lack tetramer specificity, enriching the Ag-specific population as much as 10,000-fold, allowing for detection of rare events (e.g., 10 tetramer-binding T cells from 20 ml of peripheral blood). In addition, multiparameter flow cytometry analysis using a variety of mAbs simultaneously with tetramer assays allows for direct phenotyping of the Ag-specific cells, a major advance toward discriminating T cell functional subsets directed to a single Ag.

HLA class II tetramers are now widely used in studies of pathogen immunity and vaccine development, in evaluation of antitumor responses, in allergy monitoring and desensitization studies, and in autoimmunity (Fig. 1). Examples of each are summarized below and illustrate particular issues faced in these different applications.

**Monitoring immunity to infectious pathogens.** Class II tetramers have been used for analysis of a variety of human CD4 T cell responses to pathogens, including influenza A, *Borrelia*, EBV, CMV, *Mycobacterium tuberculosis*, human T-lymphotropic virus 1, hepatitis C, anthrax, severe acute respiratory syndrome virus, human papillomavirus, and HIV (3, 7, 11–30). Ag-specific T cells are detected in peripheral blood, with a very broad range of frequency, depending on prior Ag exposure, vaccination history, and timing of immunization. The most significant limitation for these studies is simply the large number of potential epitopes present in complex organisms; not only are there multiple potential protein targets within each pathogen, but a huge number of processed and presented peptides are potentially targets for CD4 T cell recognition. For example, within the protective Ag of *Bacillus anthracis*, multiple epitopes are present; as expected, the dominance of each epitope varies depending on the HLA genotype of the individual subject (23). Therefore, selection of tetramers for studies of the T cell response to anthrax or similarly complex agents involves matching the HLA class II genotype of each subject with appropriately tailored pMHC tetramers (31). In contrast, occasionally there are immunodominant epitopes from pathogens that bind promiscuously to multiple HLA class II molecules. When this occurs, production and use of appropriate pMHC tetramers becomes straightforward. For example, the hemagglutinin protein of influenza A H1N1 contains a peptide epitope (aa 306–319) that binds to at least five common human class II molecules and is recognized by T cells in each case. Most people, through either natural exposure or periodic flu vaccination, contain peripheral CD4 T cells recognizing this epitope (14). A similar strategy has been adopted to search for conserved epitopes in pandemic strain H5N1, after vaccination (32). T cells recognizing additional influenza epitopes can be found simultaneously in the same blood samples using other tetramers but appear to be more specific to a given individual (12, 33). An interesting exception was seen in tetramer analysis with pMHC corresponding to the H5N1 influenza strain hemagglutinin, with cross-reactivity from pre-existing immunity to other influenza strains, allowing detection of T cells reactive to H5N1, even in the absence of direct exposure to this strain (24).

With hundreds of potential peptide epitopes to choose from, selecting pMHC tetramers for studying infectious pathogens or vaccines is challenging. Traditional methods involving functional testing of individual peptides from large libraries are laborious and, when based on a particular functional parameter (e.g., IFN ELISPOT analysis), may omit epitopes that are important for other T cell subsets or lineages. To address this issue, an unbiased platform has been developed for a tetramer-binding and flow cytometry screening method that directly determines suitable pMHC tetramers. This technique, called tetramer-guided epitope mapping (TGEM), involves the display of large peptide libraries loaded into HLA class II molecules (Fig. 2). Pools of tetramers are used to probe the T cell population of interest, and positive tetramer binding, assessed by flow cytometry, is deconvoluted in a second flow cytometry step into individual pMHC targets (34, 35). This approach has been applied to a variety of infectious agents, with epitope identification information deposited in the National Institute of Allergy and Infectious Diseases-sponsored Immune Epitope Database and Analysis Resource, IEDB.org.

**Allergy tetramers: a tool for improved immunotherapy?** Translation from a laboratory tool to a clinically useful biomarker is a difficult transition for any technology, but in the area of allergy desensitization therapy, human class II tetramers have their most immediate translational potential. Similar to the study of infectious pathogens, tetramer development for allergens is complicated by the diversity and number of potential CD4 T cell epitopes. However, for many of the major allergic disorders, including allergies to foods, pollen, and dander, there is a large body of knowledge narrowing the field of target allergen proteins. The TGEM technique has

---

**FIGURE 1.** Molecular design schematic of a class II tetramer molecule, illustrating four pMHC domains tethered to a central streptavidin molecule through flexible linker sequences and leucine zipper motifs to provide structural stability. Some of the major areas of tetramer analysis in human disease applications are listed.
Epitope identification using a tetramer-guided mapping strategy. Large peptide libraries, separated into sets of pooled sequences, are loaded into the binding groove of recombinant class II molecules and assembled into tetramers. Direct flow cytometry analysis of T cell populations identifies peptides containing actual epitopes representative of the natural immune response.

FIGURE 2. Epitope identification using a tetramer-guided mapping strategy. Large peptide libraries, separated into sets of pooled sequences, are loaded into the binding groove of recombinant class II molecules and assembled into tetramers. Direct flow cytometry analysis of T cell populations identifies peptides containing actual epitopes representative of the natural immune response.
also posttranslational modification of target Ags, class II tetramers composed of gliadin pMHC complexes were shown to bind peripheral CD4 T cells; however, these cells were only detectable when the subjects consumed gluten-containing bread for 3 d (53). This finding strongly suggests that mobilization of the Ag-specific cells from tissue to blood was necessary for visualization, and it may provide a generalizable strategy, involving Ag challenge, for improving the detection threshold for rare autoreactive cells.

A related issue, particularly relevant to the analysis of low-viability T cell responses in autoimmunity, is the definition of a precise peptide-binding register for docking within the class II molecule. The presence of more than one binding register within long peptides generated during Ag processing can result in presentation of pMHC ligands to distinct TCR, with potentially different roles in selection and autoreactivity (54, 55).

Tetramer studies of tumor Ags are similar to the autoantigenic approach, with an opportunity to help inform both disease mechanism and therapy. Although most tumor Ags are self-Ags or modified self-Ags, intentional immunization with these Ags is conducted for therapeutic benefit. For example, class II tetramer studies in patients with malignant melanoma after experimental vaccine therapies documented evidence of immunogenicity, measured the effects of adjuvant on T cell lineage, and have been used to evaluate shifts in T cell subsets (56–59). Indeed, because the pMHC complex is itself a surrogate for Ag recognition, it is feasible that tetramers might be considered for therapeutic expansion of Ag-specific effector T cells (60).

**Variations on the tetramer theme**

Biophysical studies and off-rate analysis suggest that multivalent binding of pMHC to the TCR is essential for successful tetramer imaging of Ag-specific T cells. Monomeric and dimeric forms of pMHC are much less efficient than tetramers (61), whereas higher-order multimers, such as octamers, decamers, or pMHC-coated beads, are all successful reagents, generally comparable to tetramers. However, a caveat to this interpretation is that streptavidin aggregates during storage, so that experiments ostensibly using tetrameric forms of pMHC linked to streptavidin may, in fact, be using higher-order complexes, at least in part.

A variety of methods has been successfully used to manufacture pMHC class II tetramers, with expression of HLA class II molecules as “empty” (without intentional loading of peptide into the class II Ag-binding groove); “loaded,” in which the expression system is coupled to a facilitated peptide-loading process, resulting in production of complete pMHC; or “covalent,” in which an MHC expression vector is engineered to cotranslationally express the peptide, with a flexible and/or removable linker sequence allowing for 1:1 stoichiometry of peptide/MHC and directed peptide binding into the class II-binding groove. Expression systems have included mammalian, insect cell, or bacterial sources (62). There are advantages to each system, depending on the intended type of tetramer assay planned. For example, covalent pMHC are well suited for consistent production systems and for situations in which a small number of specific pMHC combinations are used. They are not well suited to screening assays involving hundreds of different potential peptide Ags, and neoepitope exposure due to the presence of linker sequence or a too-stringent binding register is a potential concern. An important use of noncovalent peptide-loaded pMHC production systems is the ability to assess many different tetramer-binding specificities at the same time. This has been exploited in the TGEM technique described above, in which large mixtures of peptides, most commonly representing the entire protein sequence of potential antigenic targets, are interrogated to determine which peptides within the mixture contain actual T cell epitopes.

Because of the high specificity and sensitivity of TCR recognition for pMHC, tetramer-binding studies can also be designed to identify unconventional antigenic epitopes. For example, many protein therapeutics that are administered to patients are potentially immunogenic. Analysis of the T cell response in these cases, using tetramers containing pMHC specificities from the therapeutic molecule, can readily identify the specific epitopes that trigger immunogenicity, as illustrated in a study of Factor VIII administration in hemophilia A (63).

Examples of technical variations that are designed to improve the efficiency of tetramer production include coexpression with chaperone proteins (64), biotinylation of the expressed MHC molecule during synthesis (65), modifications of peptide linkers used to dock covalent peptides in the coexpressed MHC groove (66), or the use of cleavable CLIP peptides (7), which take advantage of the natural role of invariant chain residues (CLIP) that are a functional intermediate in loading peptides into class II molecules. One of the most intriguing innovations is the use of entire libraries of class II pMHC molecules plated on arrays designed to capture Ag-specific T cells (67), similar to earlier studies using class I tetramers (68).

**Conclusions**

After more than a decade of experience, class II tetramer analysis has become a regular component of T cell-specificity studies across the entire landscape of immunological investigation. However, a decision to include class II tetramer studies, as well as how to use them, is not as routine as selecting Abs for flow cytometry. There are four key issues that should be considered and used as the basis for experimental choice.

First, what is the anticipated frequency of CD4 T cells recognizing immunodominant epitopes in the system? If there is no prior evidence for immunodominance and no information from previous T cell assays regarding prevalence, then it is a challenge to select a particular pMHC tetramer that will be representative of the T cell response profile. Either multiple different pMHC can be selected, individually or as a mixed tetramer pool, or a technique such as TGEM can be used in pilot studies to directly determine the appropriate pMHC combinations.

Second, what is the likely biodistribution of CD4 T cells of interest? In cases such as celiac disease, as described above, mobilization of the Ag-specific T cells into the bloodstream was accomplished by Ag challenge. This may have general applicability, supported by the observations in T1D that tetramer-positive cells are much higher after pancreas transplantation or after glutamate dehydrogenase immunization (H. Reijonen, personal communication) compared with unimmunized diabetic subjects. If the Ag-specific cells are at tissue sites of interest, more complex biopsy and in situ histology techniques are needed.
Third, avidity matters. Unlike foreign Ag responses in vaccine or allergy studies, self-Ag responses in autoimmunity or tumor immunology reflect a diverse spectrum of TCR avidity for pMHC complexes, including interactions that are near or below the threshold for robust tetramer-binding detection. If the experimental system requires detection of such low-avidity interactions, methods to amplify the tetramer-binding signals may be helpful. Alternatively, T cell activation triggered by tetramer binding can be used as a surrogate measure, with a threshold lower than conventional flow cytometry detection (62, 69).

Fourth, how genetically diverse is the population being studied? The requirement for pMHC cognate recognition by the CD4 T cell dictates that at least one of the class II molecules in each subject corresponds to the HLA molecule used for the tetramer. For populations that are highly enriched for particular HLA class II genes, such as in T1D, rheumatoid arthritis, multiple sclerosis, or celiac disease, this is not a limiting factor. However, for population-based vaccine studies or allergy monitoring trials, a large number of tetramers is needed or, alternatively, subjects are preselected based on HLA genotype. Either way, a significant complexity is added to the study. HLA class II tetramers for 25 of the most common genotypes are now available, so this is not an insurmountable barrier, if taken into account in experimental design (http://www.benaroyaresearch.org/our-research/core-resources/tetramer-core-laboratory).

Acknowledgments
The work of numerous colleagues at the Benaroya Research Institute and elsewhere, who have pioneered the II tetramer technology, is gratefully acknowledged, and any omissions from the citation list, limited by space, are inadvertent.

Disclosures
The author has no financial conflicts of interest.

References