Although tetramer technology has been wildly successful for examination of MHC class I–recognizing T cells, the same hasn't been true for MHC class II reagents. A recent workshop at the US National Institute of Allergy and Infectious Diseases was convened to address this.

Frontiers in peptide–MHC class II multimer technology

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The capability to analyze antigen-specific CD4+ T cells in single-cell detail offers the potential for enormous advances in every area of research involving immune responses and immune homeostasis. In-depth characterization of major histocompatibility complex (MHC) class II–restricted T cells is important in such diverse undertakings as the design and testing of vaccines, monitoring therapy for autoimmune diseases, HIV research, organ transplantation and devising strategies to treat T cell–mediated hypersensitivities such as certain antibiotic allergies and poison ivy rashes. However, fishing out individual T cells, each specific for a particular peptide-MHC (pMHC) molecule combination, requires a very specialized set of hooks. Davis, McHeyzer-Williams and Altman established a practical design for the molecular tools that tag T cells in an antigen-specific manner in 1996[1]. Most familiarly known as "tetramers," four identical biotin-containing pMHC complexes are attached to fluorescently labeled avidin for binding to specific T cell receptors (TCRs) (**Fig. 1**). Tetramers, or as alternatives, pMHC multimers based on the immunoglobulin G (IgG) backbone[2], have rapidly become the gold standard for T cell analyses and manipulation, enabling the enumeration, isolation, stimulation or targeting of substances to T cells of known antigen specificity[3]. In addition to basic research objectives, pMHC multimers have potential as diagnostic and treatment modalities, including the selection of cells for adoptive transfer[4,5] (T. Brumeanu, New York, NY; G. Nepom, Seattle, WA).

The basic concept of pMHC multimers is simple: clonal T cells can be identified with a multivalent form of the specific complex of antigenic pMHC that is their cognate ligand. The engineered form of the pMHC complex provides specificity, and the Ig or tetramer construct offers increased avidity; this compensates for the relatively low affinity that is characteristic of monomeric pMHC-TCR interactions. Although MHC molecules come in two basic forms, MHC class I and II—which are recognized by CD8+ cytotoxic T cells and CD4+ helper T cells, respectively—most tetramers produced so far have been class I MHC–based reagents. Nevertheless, the very first tetramer made by Davis and coworkers was based on a class II MHC molecule, and a number of different class II reagents have led to important research findings in murine and human systems[6,7]. Although production of MHC class II–based reagents is not yet routine, many problems are now being overcome. These include the engineering of stable protein constructs, reliable production of recombinant class II MHC proteins in insect cell lines and the use of covalently attached peptides to overcome expression and external peptide-loading problems for certain alleles and peptide combinations[6,8]. However, the remaining problems—which include issues of TCR avidity and CD4+ T cell frequency—have created rough terrain, if not roadblocks, that hinder the widespread application of class II MHC tetramers.

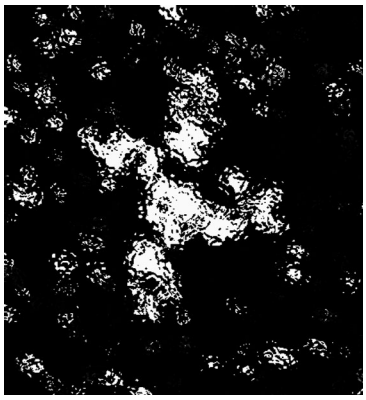

Figure 1. Electron micrograph of a class I MHC tetramer. Tetramers based on attachment of biotinylated pMHC monomers are excellent reagents for CD8+ T cells, but the class II counterparts do not perform with the same consistency on CD4+ T cells. The high rigidity of the tetramer arms may be a liability for such reagents in the class II system. (Courtesy of L. Teyton).

A workshop convened on 18–19 June 2002 by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health in Bethesda, MD, addressed major technical issues surrounding the production and use of pMHC class II multimers. The consensus emerged that whereas many pMHC multimers are already proving to be effective tools for the analysis and enumeration of MHC class II–restricted CD4+ T cells, determining exactly why others do not work remains an inexact science at present. Continued improvements in reagent design and specially tailored strategies for their application will emerge from basic studies of TCR-MHC interactions as well as from understanding lifestyle differences between CD4+ and CD8+ T cells.

The class I MHC transmembrane protein consists of a single heavy chain that contains the complete peptide-binding groove and is stable in the soluble form complexed with its essentially invariant light chain β_2-microglobulin. In contrast, the peptide-binding site of MHC class II has contributions from both of its two membrane-anchored chains. When not associated with the cell membrane or complexed with a particularly high-affinity peptide, these chains tend to dissociate, so most tetramer designers engineer molecular "zippers" to keep the class II MHC chains together. More significantly, the MHC class I peptide-binding groove has closed ends that define and limit the size of peptides that can bind, whereas the open-ended groove of the MHC class II molecule accommodates core peptides with flanking regions of considerably different lengths. In addition, the peptide-binding pockets of the class II molecule seem to be less stringent in their preference for particular amino acid side chains. Even well defined peptides may bind to the same MHC class II molecule in two or more frames, producing conformational ambiguity that

undermines the specificity of a distinct molecular reagent. Thus, it can be very difficult to define peptide-binding motifs because a class II molecule can elicit an array of T cells with related, but distinct, antigen specificities[9]. Recreating that complexity in tetramer reagents is a challenge specific to the MHC class II system.

Are there also fundamental differences between MHC class I and II molecules in the strength of their binding interactions with peptides or TCRs? Binding studies of peptides with isolated class II MHC molecules indicate that, at least for certain strong-binding peptides, the IC_{50} (concentration needed to competitively inhibit 50% of the binding of a standard peptide) can be in the nanomolar range (L. Teyton, La Jolla, CA), similar to other reported pMHC class I and II interactions[10,11]. Dissociation constants of TCR–MHC class II interactions in the range of $10 \times 10^{-6} - 35 \times 10^{-6}$ M (L. Stern, Cambridge, MA; E. Ward, Dallas, TX) are also similar to those reported for MHC class I–TCR binding[12]. There remains some disagreement as to whether T cells stain better with tetramer reagents at higher temperatures that permit metabolic internalization of TCR or at lower temperatures that reflect the entropic barriers to stable binding. But different practical applications need pMHC class II reagents to function at both higher and lower temperatures—at 37 °C to drive proliferation or to anergize T cells (D. Busch, Munich, Germany; T. Brumeanu; G. Nepom) and at cooler temperatures to stain cells in frozen tissue sections (L. Teyton)—so a single condition will not fit all applications. Stronger affinities of particular peptides for their MHC-presenting element results in more stable tetramer reagents, and better pMHC-TCR interactions generally correlate with better tetramers. Thus, engineering approaches that would maximize pMHC affinity as well as improve pMHC-TCR affinity without affecting specificity could result in better tetramers.

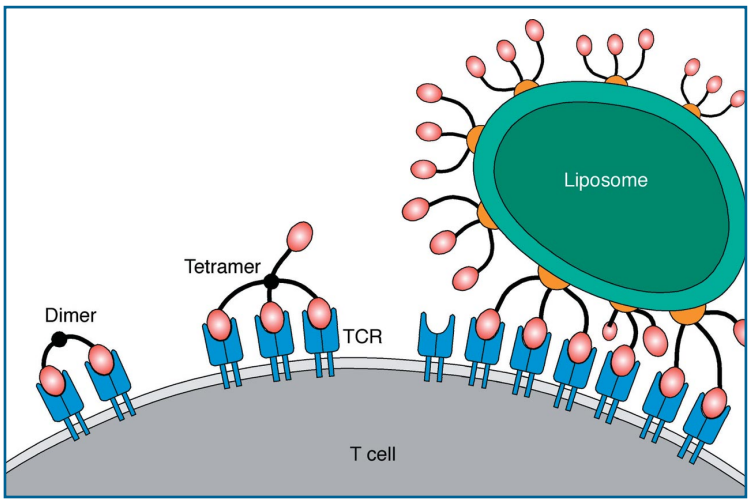

Figure 2. Flexible multivalent pMHC class II reagents. Dimers based on IgG as well as higher order multimers produced with cross-linking molecules more flexible than avidin may have distinct advantages for binding TCR on $CD4^+$ T cells. Liposomes with pMHC molecules clustered in microdomains are promising to become important reagents for further study.

However, certain important types of TCR–MHC class II interactions may tend to cluster at the weaker end of the MHC-TCR affinity spectrum. One broad category in which some suspect that low-strength pMHC-TCR interactions may predominate is the autoantigens, perhaps related to their escape from thymic selection through low-avidity interactions[13]. Autoreactive T cells have been difficult to find with class II multimers in several human autoimmune diseases and animal models, including diabetes, Lyme arthritis, autoimmune gastritis and multiple sclerosis (D. Hafler, Boston, MA; C. Liu, Duarte, CA; K. Wucherpfennig, Boston, MA; W. Kwok, Seattle, WA; D. Margulies, Bethesda, MD; G. Nepom; T. Brumeanu).

More clear is that low avidity only compounds another fundamental problem: $CD4^+$ T cells of a given antigen specificity seem to be less abundant than their $CD8^+$ counterparts. This may be because peptides from some antigens are generated at low efficiency, which results in low density on antigen-presenting cells, or it might reflect localized tissue-specific expression. Locating antigen-specific T cells with tetramers is largely a numbers game: it is difficult to detect cells at a frequency lower than 0.2%[6]. Whereas $CD8^+$ T cells expand to large numbers in response to microbial infection or vaccination[14] and maintain high numbers for prolonged periods of time, there is no such surge for $CD4^+$ cells[6]. $CD4^+$ tetramer-binding cell frequencies in the blood or normal lymphoid tissues are in the range of 1 in 30,000. Greater numbers of antigen-specific $CD4^+$ T cells can be found in the tissues affected by autoimmune diseases or in an infection. But presently, the most practical approach for detecting $CD4^+$ T cells is *ex vivo* antigen-driven expansion of the T cells, after which one back-calculates the precursor frequencies. Lymphocytes labeled with the fluorescent molecule carboxyfluorescein diacetate succinimidyl diester (CFSE) distribute the dye evenly to daughter cells, permitting calculation of the number of divisions in the tetramer-binding cells. Calculation of a $CD4^+$ T cell precursor frequency of 1 in 30,000 after seven *in vitro* divisions is feasible (G. Nepom). Despite the promise of this approach, the difficulty of meaningful interpretation of numbers extrapolated from days 7 or 10 back to day 0, and how these evaluations can be applied practically in clinical settings, remains a complex task. One alternative, explored in the enumeration of MHC class II–restricted HIV antigens in the peripheral blood, is to exploit high-throughput fluorescence analysis to count low frequency events with higher statistical precision (R. Sekaly, Montreal, Canada). Peripheral mononuclear blood cells sampled from patients during primary HIV infection showed as many as 1.5% $CD4^+$ T cells stained at 37 °C by an individual tetramer (B. Yassine-Diab, Montreal, Canada). Also, there is a need to explore other body tissues and fluids, besides peripheral blood, that may contain distinctive populations of $CD4^+$ T cells; for example, T cells at the site of inflammation may be of greater frequency or higher affinity than peripheral T cells (D. Hafler).

What if pMHC class II reagents could be devised that bind strongly to specific $CD4^+$ T cells, stain them intensely and have insignificant nonspecific background binding—wouldn't that make up for weak avidity and low frequency? The prospects are promising. pMHC tetramers based on the avidin scaffold are rigid molecules that may not engage multiple TCRs freely[15]. The immunoglobulin hinge region, perhaps an overlooked feature of pMHC reagents based on the IgG framework, may in fact play a key role in allowing strong simultaneous binding to multiple TCR molecules (J. Schneck, Baltimore, MD; N. Glaichenhaus, Valbonne, France; K. Wucherpfennig; T. Brumeanu). Also, chromatographic analyses of many "tetramer" preparations indicate that considerable aggregation may inadvertently be present that could contribute significantly to binding results (L. Stern).

Nonspecific background binding is frequently the limiting factor in approaches based on multivalency, but exciting new observations that

well constructed membrane arrays of pMHC class II complexes may overcome that problem potentially open up a new type of reagent for studying T helper cells. Artificial membrane spheres—liposomes—that incorporate the murine MHC class II complex I-A^d complexed with an ovalbumin peptide strongly and specifically stain T cell lines (**Fig. 2**). Liposomes outperformed tetramers in the detection of $CD4^+$ cells taken directly from lymphocytic choriomeningitis virus–infected mice; with an irrelevant peptide, liposomes served as a negative control (L. Teyton). Much manipulation is possible in the liposome system, including pMHC incorporation (as monomer, trimer or tetramer), the lipid composition, liposome size and the fluorescent label. Liposomal "artificial antigen-presenting cells" have also been devised that mimic the high-density micromembrane domains observed in the immunological synapse[16] (S. Albani, San Diego, CA). With this approach, liposomes that focused HLA DR*1101 complexed with an influenza hemagglutinin peptide into lipid domains with biotinylated cholera toxin inserted in the bilayer were successful in detecting human polyclonal antigen-specific T cells after a relatively short (72-h) expansion *in vitro*. T cell detection was better with the high-density domains than with randomly distributed class II MHC complexes in liposomes. Like tetramers, liposomes can be used to stain T cells at room temperature or lower, whereas under culture conditions at 37 °C, they stimulate T cells. Other pMHC multimers also can trigger T cells, but liposomes may offer a platform for incorporating costimulatory molecules or other ligands and/or receptors to finely control T cell responses.

In many cases, researchers must to be able to remove the pMHC reagents after their use in functional isolation of T cell populations. Leaving the multimers attached under culture conditions or adoptive transfer *in vivo* may lead to abnormal cell activation, anergy or unwanted effects of T cell activation on bystander cells induced by TCR cross-linking. A recently reported new technology allows for reversible removal of pMHC reagents from cell surface[17]. This approach replaces biotinylation of class I MHC molecules with a peptide sequence fused to the COOH terminus of β_2-microglobulin that binds a mutated streptavidin molecule. Addition of free d-biotin results in rapid dissociation of surface-bound class I MHC multimers from the cell surface. Whether this technology will work for dissociation of class II multimers is under investigation (D. Busch). Liposomes containing MHC multimers were removed with 200 mM imidazole (L. Teyton).

These are early days for liposomes as antigen-specific reagents for $CD4^+$ T cells. More studies are needed to determine whether the low backgrounds and high binding are typical. However, improved modeling of TCR-MHC multimeric binding[18], further understanding of increased antigen sensitivity by activated T cells[19] and more refined information about the immunological synapse—the site at which T cells interact with their targets—should support the development of reagents that match well the recognition needs of $CD4^+$ T cells. Future research needs to define how closely one must mimic the target to increase the number of catches on these molecular hooks.

Reagent resources, such as the NIH Tetramer Facility (http://www.niaid.nih.gov/reposit/tetramer/index.html), can promote MHC class II–based reagent design, refinement and application. Contributions of the NIH Tetramer Facility include providing standardized reagents to a wide range of investigators, facilitating collaborative studies, identifying appropriate positive and negative controls and serving as a clearinghouse for protocols and comparative binding data (J. Altman and J. Lippolis, Atlanta, GA). The NIH Tetramer Facility resources are freely available to all investigators, regardless of whether they have NIH funding. The Facility has supplied over 1400 class I MHC tetramers to researchers worldwide. Investigators in all areas of immunology research should avail themselves of the Facility's new class II MHC reagent offerings and help pioneer the widespread application and development of these important immunology tools.

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1. Altman, J. D. *et al. Science* **274**, 94–96 (1996).
2. Greten, T. F. *et al. Proc. Natl. Acad. Sci. USA* **95**, 7568–7573 (1998).
3. Doherty, P. C. & Christensen, J. P. *Annu. Rev. Immunol.* **18**, 561–592 (2000).
4. Casares. S. *et al. Nature Immunol.* **3**, 383–391 (2002).
5. Reijonen, H. *et al. Diabetes* **51**, 1375–1382 (2002).
6. Nepom, G. T. *et al. Arthritis Rheum.* **46**, 5–12 (2002).
7. McMichael, A. J. & Kelleher, A. *J. Clin. Invest.* **104**, 1669–1670 (1999).
8. Crawford, F., Kozono, H., White, J., Marrack, P. & Kappler, J. *Immunity* **8**, 675–682. (1998).
9. Latek, R. R., Petzold, S. J. & Unanue, E. R. *Proc. Natl. Acad. Sci. USA* **97**, 11460–11465 (2000).
10. Livingston, B. *et al. J. Immunol.* **168**, 5499–5506 (2002).
11. Sidney, J. *et al. Hum. Immunol.* **62**, 1200–1216 (2001).
12. Rudolph, M. G., Luz, J. G. & Wilson, I. A. *Annu. Rev. Biophys. Biomol. Struct.* **31**, 121–149 (2002).
13. Ober, B. T. *et al. Int. Immunol.* **12**, 1353–1363 (2000).
14. Murali-Krishna, K. *et al. Immunity* **8**, 177–187 (1998).
15. McMichael, A. J. & O'Callaghan, C. A. *J. Exp. Med.* **187**, 1367–1371 (1998).
16. Bromley, S. K. *et al. Annu. Rev. Immunol.* **19**, 375–396 (2001).
17. Knabel, M. *et al. Nature Med.* **8**, 631–637 (2002).
18. Stone, J. D., Cochran, J. R. & Stern, L. J. *Biophys. J.* **81**, 2547–2557 (2001).
19. Fahmy, T. M., Bieler, J. G., Edidin, M. & Schneck, J. P. *Immunity* **14**, 135–143 (2001).