MHC multimers: expanding the clinical toolkit

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Introduction

T cell recognition of specific antigen is initiated by the interaction between TCR molecules and peptide–MHC complexes (pMHC) expressed on the surface of antigen-presenting cells. Soluble pMHC molecules can substitute as ligands in this interaction, binding specifically to T cells expressing the appropriate TCR. Because the monomeric interaction of TCR–pMHC is of low avidity [1–6], multimerization of the pMHC complex facilitates the binding reaction, and a large number of studies have now been performed in which pMHC multimers are used to detect and characterize specific T cells in patients and normal individuals. This technology, commonly referred to as "MHC tetramers," was originally developed for the analysis of class I restricted CD8^{+} T cells, in which the TCR recognizes a MHC class I–antigen complex [7]. Similar technology has been used for class I recognition by NK receptors [8–10], for CD1 recognition by NKT cells [11,12], for MIC recognition by γ-δ-TCR molecules [13], and for recognition of the peptide–HLA class II complex by CD4^{+} T lymphocytes in humans [14–19] and mice [20,21].

Tetramer technology comes of age

A primary objective for the use of fluorescent-labeled multimers is the ability to bind to T cells carrying the appropriate specific TCR, with sufficient avidity to assure detection by flow cytometry. The polyclonal T cell population responding to particular pMHC specificities is highly variable, representing a spectrum of TCR avidity. In studies of the anti-hemagglutinin (HA) response in people immunized with influenza virus, CD4^{+} responses to the immunodominant HA epitope have been marked by relatively high avidity for pMHC, such that almost all T cells responding ex vivo are bound by specific tetramers [22]. On the other hand, in studies of the anti-glutamic acid decarboxylase (GAD) response in patients with autoimmune diabetes, many antigen-responsive CD4^{+} T cells failed to bind tetramer, indicating low-avidity interactions [23]. In studies of anti-viral responsive CD4^{+} T cells associated with HSV infection, both high-avidity and moderate-avidity T cells were identified by tetramers, which represented different degrees of antigen sensitivity within the polyclonal responder population [24]. Understanding the biological significance of this type of variation is one of the current opportunities presented by using tetramers to analyze ongoing immune responses. It is intriguing that in the human autoimmune patients studied to date [23,25], low-avidity responses predominate and it is possible that fundamental insights into autoimmunity may arise from clarifying the distinctions between T cells selected for self- and nonself-antigens.

The polymorphisms which are characteristic of human class II genes lead to changes in solubility and stability of soluble class II molecules, as well as dictate specific peptide binding interactions. Different types of class II multimers attempt to circumvent this variation by incorporating accessory scaffold structures, such as leucine-zipper motifs or immunoglobulin domains, into the molecule. Dozens of distinct T cell specificities have been successfully visualized with these molecular tools, predominantly in infectious and autoimmune clinical settings. In these studies, some differences between HLA class I and HLA class II tetramer analyses have been noted, which may reflect important differences in CD8 and CD4^{+} T cell activation. For example, in many anti-viral responses, the frequency of antigen-specific CD8^{+} T cells in peripheral blood ranges from 1 in a few thousand to over I in 100 [26–29]. This is attributed to large "burst size" of the memory T cell response, result-

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ing in oligoclonal T cell expansions [30]. The CD4$^+$ T cell response, in contrast, generally has smaller burst size, with a resultant heterogeneous polyclonal distribution of low frequency specificities [31]. Because of this smaller CD4$^+$ T cell expansion in vivo, it is challenging to detect antigen-specific T cells within large heterogeneous cell populations, even using tetramers; the major limitation is the lower threshold for specific fluorescence detection, usually around 1 in 10,000 cells (0.01%). As the frequency of particular antigen-specific CD4$^+$ T cells in peripheral blood often is below this threshold, detection methods have been developed which utilize in vitro amplification to facilitate this analysis. Depending on the antigen and the likely frequency of responsive peripheral CD4$^+$ T cells, either a short in vitro culture of peripheral blood with antigen (e.g., for tetanus or influenza responses) [17,19] or a longer culture followed by restimulation of the T cells with specific monomer pMHC complexes (e.g., for autoimmune diabetes or relapsing polychondritis patients) [23,25] is required.

Benefits of this in vitro expansion of the CD4$^+$ T cells are that detection by tetramer binding identifies the presence of specific cells, and flow cytometry makes it straightforward to clone and analyze the tetramer-positive population. A vexing problem with this type of expansion is that inferences about the original in vivo activity of the antigen-specific cells are challenged by possible in vitro changes. However, some information can be extrapolated from tetramer studies on the amplified cells which are informative; for example, the frequency of rare T cells in peripheral blood can be determined by coupling tetramer analysis with a measure of proliferation in response to antigen. Carboxy-fluorescein diacetate succinimidyl ester is a dye which can be added to peripheral blood lymphocytes to quantitate the number of cell divisions which occur during in vitro expansion; the original precursor frequency of antigen-specific T cells present in the sample can then be determined by calculation based on the residual fluorescence signal in each tetramer-binding T cell [17].

Tetramers enter the clinic

Most clinical trials and clinical management decisions regarding immune-mediated diseases and therapies occur today in the absence of any direct information about the antigen-specific T cell response. Surrogate markers for immune response to specific antigens, such as tetramer staining of T cells, will be a significant aid to the efficiency of clinical trial design and patient management. The introduction of this technology to distinguish responder and nonresponder populations in vaccine trials should become routine both for analysis of the CD8 T cell response (using class I tetramers) and for the CD4 response, using class II tetramers.

One of the most significant issues limiting the use of these techniques is the requirement to determine HLA phe-

notypes and dominant epitopes for the population being studied. In other words, selecting an appropriate HLA–peptide combination for the analysis of heterogeneous T cell responses in heterogeneous populations is a challenge. For most of the HLA class I tetramer studies of vaccine efficacy, investigators have studied HLA-A2-restricted responses, since this HLA specificity is highly prevalent in many parts of the world. Successful extension of these studies to other HLA class I molecules, such as HLA-B locus specificities, has required careful attention to the selection of HLA-compatible subjects. This issue is particularly significant in the design of HLA class II studies. There are over 200 HLA-DR alleles, of which more than a dozen are expressed in most populations, at highly variable frequencies. Studies of vaccine responsiveness in unselected populations, then, necessitates either the development of a large panel of suitable HLA-matched multimers or the limitation of study investigation to a subset of subjects selected for a common HLA type. In either event, improvements in the current reagents and technologies are anticipated before clinical use for vaccine efficacy with class II multimers becomes routine.

Monitoring patients with autoimmune diseases, however, is a special case. For several of the major human autoimmune diseases, such as autoimmune diabetes, multiple sclerosis, and rheumatoid arthritis, the HLA genetic associations with disease are of very high magnitude, such that a majority of patients express one or two common HLA class II alleles. Thus, for type I diabetes, multimers with the HLA-DRB1*0401, -DR0404, and -DR0301 molecules will identify CD4$^+$ T cells in over 80% of patients; in multiple sclerosis and rheumatoid arthritis, similar opportunities are present for use of the HLA-DRB1*1501, -DRB5*0101, and HLA-DR0401/04 molecules, respectively. However, even in these selected cases some additional issues must be considered. First, there are a variety of restriction elements present on each HLA haplotype, since most human haplotypes contain two HLA-DR molecules and one HLA-DQ molecule. The inference that the HLA-DRB1 molecule is the major restriction element to which patients mount CD4$^+$ T cell responses is based largely on observations of in vitro T cell activation, and it is possible that additional multimers with additional HLA specificities will be needed for comprehensive analysis, even in patients with HLA-associated autoimmunity. Second, even for a single HLA restriction element, there are several peptide epitopes within each antigenic protein associated with CD4$^+$ T cell responses in patients with these autoimmune diseases. For example, the HLA-DR0401 molecule binds three peptides from hGAD65, an autoantigen associated with diabetes, with high avidity, and binds another half dozen peptides with lower avidity [32]. Although in vitro studies have validated the use of the high-avidity epitopes for measuring T cell proliferative and cytokine responses [33], we do not currently know whether the immunodominant responses are the

most relevant for understanding, predicting, or monitoring disease progression.

Different individuals may need to be studied with different tetramers, customized for HLA type and peptide response pattern. It is possible to "stage" patients with autoimmunity at the time of clinical presentation, screening the peripheral T cell response against a panel of potential epitopes which will determine which multimers are most informative for clinical markers in each patient. In this scenario, diagnostic HLA-epitope staging could become part of the laboratory evaluation of patients with autoimmunity, at least for those enrolled in clinical trials. Subsequent monitoring of each patient would then use the appropriate multimer reagents. In addition, techniques for multiplexing several tetramers for analysis are desirable, as it is likely that monitoring several epitope-specific responses will give a more useful picture of immunologic activity: for example, tetramers for the islet antigens GAD65, insulin, and IA2 may all be valuable for following disease progression or response to therapy in autoimmune diabetes. The simultaneous use of multiple tetramers, however, is currently limited, so that improved fluorescent multiplexing techniques are needed.

Looking ahead to the routine use of multimers in clinical studies, it is also evident that following the phenotype of $CD4^+$ T cells in patients with autoimmunity is at least as important as identifying or quantitating the epitope-specific cells. Thus, methods such as intracellular cytokine staining coupled with highly sensitive tetramer binding may require new and creative technologies to overcome the challenges of low specific frequency and bystander activation which characterize the peripheral lymphocyte pool.

One additional important issue has become evident in recently described examples of tetramer analysis of patients with type 1 (autoimmune) diabetes. Islet antigen-specific $CD4^+$ T cells restricted by both the DR3 and the DR4 class II molecules have been expanded from peripheral blood, by stimulation in vitro with proteins and peptides from hGAD65 or insulin, documenting the presence of multiple autoantigen specificities in a single individual [33]. In addition to multiple restriction elements, multiple epitopes within the hGAD65 protein are recognized, as shown by in vitro proliferation assays. When we tested supernatants from these in vitro cultures for cytokines, highly variable responses were seen, with both TH1 (gIFN, TNF, IL2) and TH2 (IL5, IL10, IL13) types present. Despite this huge diversity in the response, we were able to identify epitope-specific responses when we cultured PBMC with a specific hGAD65 peptide ($hGAD_{555-567}$), followed by stimulation with this same peptide bound by the HLA-DR4 monomer [23]. $CD4^+$ T cells showed a polyclonal activation response to this specific stimulus, and sequencing of TCR within the activated $CD4^+$ population showed a highly diverse repertoire. Tetramer-binding studies showed that this repertoire included T cells which, when expanded in vitro, were recognized by soluble pMHC tetramers containing the $hGAD_{555-567}$ peptide. Control tetramers, and T cells from normal individuals, are negative in this assay [23].

These studies demonstrated that it is indeed possible to identify, recover, and study islet antigen-specific $CD4^+$ T cells from peripheral blood. However, it is notable that these cells were undetectable prior to in vitro expansion, indicating their presence in peripheral blood at very low frequency, and it is also clear that additional epitopes were immunogenic which were not detected using a single pMHC tetramer. Furthermore, several antigen-activated cells were recovered from the in vitro expansion culture which failed to stain with tetramer, indicating the presence of low-avidity $CD4^+$ T cells specific for the same epitope. Thus, while it is now possible to interrogate antigen-specific T cells present within a complex milieu of diverse cells, it is quite likely that a complete understanding of the autoimmune response profile will require technologies to optimize the detection of low-avidity TCR and to increase the sensitivity to be able to detect rare events.

Applications for using pMHC multimers are rapidly expanding and include exciting new possibilities for ex vivo cell expansion [34] and for immunotherapy [35,36], as well as for improved patient monitoring [37,38]. These improvements are a work in progress, in which close collaboration between clinical science and laboratory science is key to rapid translation of HLA immunobiology into informed clinical decisions.

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