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Abstract

Selective expansion and activation of a very small number of antigen-specific $CD4^+$ T cells is a remarkable and essential property of the adaptive immune response. Antigen-specific T cells were until recently identified only indirectly by functional assays, such as antigen-induced cytokine secretion and proliferation. The advent of MHC Class II tetramers has added a pivotal tool to our research armamentarium, allowing the definition of allo- and autoimmune responses in deeper detail. Rare antigen-specific $CD4^+$ cells can now be selectively identified, isolated and characterized. The same tetramer reagents also provide a new mean of stimulating T cells, more closely reproducing the MHC-peptide/TCR interaction. This property allows the use of tetramers to direct T cells towards the more desirable outcome, i.e. activation (in malignancies and infectious diseases) or Th2/T regulatory cell deviation, anergy and deletion (in autoimmune diseases). These experimental approaches hold promise for diagnostic, prognostic and therapeutic applications.

Keywords: immune tolerance, multimer, oligomer, artificial antigen presenting cell, diabetes, GAD65.

From MHC Class I to MHC Class II Tetramers: where similarities end

The binding between any given TCR and MHC-peptide complex is fairly specific, but is characterized by a low affinity and fast off-rates. It is now argued that during T cell recognition, these low affinity and fast off-rates are necessary to enable serial contacts of each TCR molecule with multiple MHC-peptide ligands (1). Such characteristics were assumed to be too unfavorable for direct staining of T cells by means of MHC-peptide reagents. Indeed, fluorescent-labeled single MHC-peptide molecules are not capable of stable binding to the cell. Such limitation has been circumvented by multivalently complexing MHC molecules, typically in the form of tetramers. The low affinity of the single MHC units is thus compensated for by the higher avidity gained by cooperative binding.

Since their first description in 1996 (2), the innovation of MHC Class I tetramers has revolutionized our understanding of virus- and tumor-specific T cells. To generate this class of reagents, MHC Class I molecules are made in *Escherichia coli* and peptides are introduced during the refolding of the Class I α chain. The approach is made easier by the fact that only the α chain, coupled with the invariant β_2-microglobulin structure, binds the peptide. On the contrary, successful MHC Class II tetramer production requires interaction of three components – α and β chains (both polymorphic) and the peptide – making the task more complex.

The group of J. Kappler and P. Marrack first described an approach in designing soluble MHC Class II molecules for murine alleles, where the peptide of interest is covalently linked to the β chain of the MHC molecule to ensure its placement in the peptide-binding groove during the synthesis process (3,4). Peptide-MHC multimers produced in this manner have been used to identify T cells from mice transgenic for an α/β TCR specific for moth cytochrome c (4). Because of the introduction of the TCR transgene, the majority of T cells are bound by the Class II tetramer in this system. In contrast, frequencies of epitope-specific T cells are significantly lower in humans, necessitating a much more sensitive system to successfully follow $CD4^+$ T cell responses.

Moreover, the main disadvantage is that a separate molecular construct must be produced for every Class II-peptide tetramer designed.

The drawback of a peptide that had to be engineered into the construct similarly arose with MHC Class II multimers of the human molecules (5-8). We first reported the production of human MHC Class II tetramers (9); notably, this construct is expressed in empty form and only subsequently is the peptide loaded, without any covalent binding. This approach allows greater flexibility, since different peptides can be loaded in the same MHC molecules. The structure of this tetramer construct is illustrated in Fig. 1. Recombinant Class II monomeric molecules are produced that incorporate leucine zipper motifs in place of the native transmembrane and cytosolic domains to stabilize the α/β complex. Flexible linkers on either side of leucine zippers provide structural flexibility, which likely allow better clustering of TCRs upon interaction. This molecule is produced in stably transfected *Drosophila* cells, purified by affinity chromatography and subsequently biotinylated on the terminal portion of the MHC β chain (9). The monomers thus obtained can be stored empty and later loaded with the peptide of interest, using a detergent-facilitated exchange reaction. The loaded monomers are subsequently assembled into tetramers by the addition of streptavidin, which has four biotin-binding sites. The use of fluorochrome-labeled streptavidin (typically phycoerythrin, for its bright emission and limited self-quenching) permits detection of the binding of the tetramers to target T cells.

MHC Class II Tetramers at work: identifying and characterizing antigen-specific CD4$^+$ T cells

The presence of antigen-specific T cells has traditionally been inferred by functional assays, i.e., as a readout of the activation induced by the antigen. The most sensitive assays of T cells function rely on the detection of cytokine synthesis, usually interferon-γ, by means of intracellular cytokine staining, surface capture or ELISpot. The main disadvantages of these techniques is that they are indirect and prone to considerable experimental variability.

On the other hand, the main disadvantage of both MHC Class I and Class II tetramers is that only known MHC-peptide specificities can be analyzed. This limitation is not critical in inbred mouse strains or human infections for which immunodominant peptides exist, but the problem arises in the most common situation where a complex set of epitopes is targeted by T cells. Further complexity is added when unknown epitopes need to be identified to load the appropriate peptide in the tetramer construct. To this aim, computer-assisted algorithms have been designed that predict potential MHC-binding epitopes by scanning the aminoacid sequence of whole antigens (10). We have devised a different approach named tetramer-guided epitope mapping (TGEM) (11,12), where the ability to load the MHC Class II molecule with different peptides allows to combine tetramer analysis with peptide array strategies for epitope identification. Different pools of peptides are loaded on the selected MHC Class II molecule: in this mixture, the peptides binding with higher affinity preferentially occupy the MHC groove. The corresponding pooled tetramers obtained are then used to stain T cells. In a second step, peptides from positively staining pooled tetramers are loaded individually onto MHC Class II molecules, and the staining of T cells with the corresponding tetramers is repeated to identify the individual T cell epitope(s). A panel of MHC Class II tetramers and different antigens containing relevant binding epitopes is shown in Table 1.

The application of human Class I tetramers to study self antigens has been most extensively developed in studies of tumor antigens. For example, peptides from melanoma-associated antigens loaded into HLA-A2 tetramers have been used for patient monitoring, phenotyping, and clinical

correlations in patients with melanoma and in cancer vaccine trials (13). Early uses of human MHC Class II multimers were concentrated primarily in the detection and monitoring of human T cell responses to infectious antigens, for which the $CD4^+$ T cell response is robust and epitope specificity is fairly predictable. Antigen-specific T cells from influenza-immune individuals were detected using Class II tetramers loaded with an immunodominant epitope from hemagglutinin (HA) (9). The use of tetramer staining to identify antigen-specific cells permits simultaneous analysis of cells using fluorochrome-labeled antibodies. This additional phenotypic analysis can provide important information about an antigen-specific response such as the type of T cell involved, presence of activation or other markers, and cytokine production through intracellular staining, thus differentiating, for instance, between Th1 and Th2 responses. With this approach, tetramer-positive cells which had been previously expanded in vitro with peptide-pulsed antigen-presenting cells were found to be $CD3^+CD4^{high}CD25^+$, a phenotype characteristic of activated T helper cells responding to antigen (9,14). Moreover, the concomitant use of MHC Class II tetramers and carboxyfluorescein diacetate succinimidyl ester (CFSE) staining allows to calculate precursor frequencies without the need for limiting dilution analysis. CFSE-labeled cells halve their dye content each time they divide, resulting in a parallel halving of their corresponding fluorescence. With each peak of progressively lower fluorescence intensity representing one cell division, the original number of $CD4^+$ T cell precursors can be derived (9).

Similar approaches have been used in identifying epitope-specific $CD4^+$ T cells from individuals infected with *Herpes simplex* virus type 2 (HSV-2) (15). In this latter work, tetramer-positive cells were subsequently sorted, cloned, and further characterized. The vast majority of the clones retained tetramer staining and proliferated when challenged with the same peptide used in the tetramer. This confirms the antigen-specific properties of peripheral blood T cells detected using tetramer staining (15). Tetramers can thus be used not only to identify antigen-specific cells, but also to isolate these cells by fluorescence sorting for further characterization. Different T cell clones have been obtained

from the same HSV-2-infected individual with this approach, showing different TCR gene usage and affinities (16).

The application of MHC Class II tetramer technology to autoimmune diseases faces additional problems. The number of antigen-specific $CD4^+$ cells present in peripheral blood is even lower than with allo-responses. Moreover, autoreactive T cells must harbor lower affinity TCRs in order to escape thymic negative selection. An initial study was conducted in rheumatoid arthritis patients, taking advantage of synovial fluid sampling, an enriched starting material not available in other autoimmune diseases. This study failed to directly detect cartilage antigen-specific $CD4^+$ T cells, finding DR4 tetramer-positive fractions above background only in a marginal subset of individuals (5). In our study on GAD65-specific T cells in type 1 diabetic patients, preliminary in vitro expansion of peripheral blood lymphocytes on GAD65-pulsed antigen-presenting cells was necessary to overcome this problem (17). A large expansion of tetramer-positive cells can thus be obtained, representing both the accumulation of proliferating antigen-specific cells and the loss of unrelated T cells lacking appropriate antigenic stimulation during in vitro culture. Similarly, tetramer staining of gliadin-specific T cells obtained from intestinal biopsies of patients with celiac disease was accomplished after in vitro expansion and generation of clones (8).

This problem is not unique to autoimmunity studies. The size of clonal expansion is considerably lower for $CD4^+$ than for $CD8^+$ T cells, resulting in low frequencies of antigen-specific $CD4^+$ T lymphocytes in peripheral blood, in the range of 1:6,000 to 1:100,000. While it is reasonable to attempt direct detection strategies in human studies when the antigen challenge is robust, as in vaccine trials, direct detection of antigen-specific $CD4^+$ T cells has so far yielded very low tetramer-binding peripheral blood populations even in an infectious context (18). The low number of antigen-specific $CD4^+$ cells in peripheral blood is beyond the sensitivity limit of flow cytometry: frequencies of tetramer-positive cells below 0.2% (1:500) significantly overlap with the 0.1% background staining tipically obtained using tetramers loaded with control irrelevant peptides. Coupling in vitro amplification with CFSE staining circumvents one problem, allowing to calculate

the original precursor frequency without the need for direct detection (9). Nonetheless, the requirement for in vitro amplification prior to tetramer detection prompts some caution in interpreting the data. Changes in T cell phenotype and preferential expansion of cells either with higher affinity TCRs (in the presence of limited peptide availability) or with lower affinity TCRs (in the presence of excess peptide concentration) may occur during culture.

Combining tetramer fluorescence sorting with more sensitive techniques such as gene expression array analysis and real-time PCR might help to fill the gap and to better characterize the functional profile of antigen-specific cells.

Beyond staining: tickling the TCR with MHC Class II Tetramers

As with monoclonal antibodies (mAbs), MHC Class II tetramers can be used not just as staining reagents, but also as stimulating tools for T cell activation. There are several ways in which T cells can be activated for in vitro studies (Table 2): typically, anti-CD3 mAbs (either plate-bound or in soluble form) have been used for this purpose. These reagents bind to the ε chain of the CD3 complex and supply a potent surrogate signal mimicking the MHC-peptide-TCR trimolecular interaction. Several features limit the fidelity of this system: 1) the affinity of mAb binding is approx. five-fold higher than the MHC Class II/TCR interaction, with different on-rate and off-rate characteristics; 2) anti-CD3 stimulation is not dependent on recognition by the TCR of the processed antigen in the MHC Class II groove, and 3) there is no CD4 contribution to the signal delivered. A more physiological approach is to use antigen-presenting cells (APCs) pulsed with the peptide of interest. This system closely mimics physiology but is subject to the vagaries of less controllable conditions and lacks the flexibility required to follow the whole range of signals delivered.

The advent of recombinant MHC Class II molecules has opened new possibilities for T cell functional studies. Cochran et al. thoroughly investigated the valency required for TCR engagement in order to initiate signaling, using chemically defined MHC Class II oligomers (6). Monomeric MHC-peptide complexes did not induce T cell activation, while MHC dimers and tetramers stimulated T cells (6). Moreover, the extent of activation correlated with the number of TCR cross-linked, suggesting that clustering of two TCR molecules is necessary and sufficient for signaling. Another study performed with a series of oligomeric MHC-peptide complexes produced by streptavidin-mediated cross-linking of biotinylated MHC proteins reported that trimers were the minimal activating species (19). At odds with these results, some reports have suggested that monomeric MHC-peptide complexes may not trigger complete T cell activation, but can deliver early, transient Ca^{2+} signals (20). Two additional phenomena associated with MHC/TCR interactions further complicate the interpretation. First, at high concentrations MHC-TCR

complexes show a tendency to aggregate exhibited by neither MHC nor TCR alone, suggesting that binding of monomeric MHC peptide complexes could trigger oligomerization of TCR on the surface of T cells (21). Second, at low antigen densities single MHC-peptide complexes can serially engage multiple TCR molecules (1,22). In principle, such serial triggering could allow a single MHC molecule on an APC to cluster TCRs on the T cell membrane.

The activation events following the interaction of the TCR with recombinant soluble MHC molecules have been initially studied by means of dimeric peptide/MHC Class II ligands, obtained by fusing the MHC extracellular domains with the IgG Fc portion (23-25). In the study by Hamad et al., IE^k-peptide dimers adsorbed on plastic delivered a signal even more potent than anti-CD3 mAb, as assessed by TCR downregulation, IL-2 production and proliferation (23). Similar results were obtained with a human HLA-DR2-peptide dimer: this construct induced proliferation in myelin basic protein (MBP)-specific T cell clones when used in soluble form, while the same effect was not elicited by soluble anti-CD3 mAb (25). Monomeric MHC units coated on plastic have also been used in place of peptide-pulsed APCs to induce proliferation and support the growth of antigen-specific T cells (3,17).

More recently, we undertook a comprehensive evaluation of the TCR signaling cascades using MHC Class II tetramers as activating ligands. Stimulation with these reagents has several advantages over monomers: 1) tetramer is used in soluble form, allowing the amount of stimulus to be quantitated rather than just estimated as amount of MHC monomer offered for adsorption; 2) the signaling events can be correlated with tetramer binding by means of phycoerythrin-labeled streptavidin; 3) the short timeframes of early transduction steps can be studied more conveniently.

A simplified representation of the signaling pathways elicited by MHC/TCR interaction is depicted in Fig. 2. At the earliest time points, sequential phosphorylation cascades are activated. These events also lead to activation of phospholipase C (PLC)-γ_1, whose enzymatic activity produces diacylglycerol (DAG), which triggers protein kinase C (PKC) pathway, and inositol triphosphate (IP_3), which elicits Ca^{2+} release from intracellular stores. This first Ca^{2+} burst triggers a second

wave of Ca^{2+} influx from the extracellular space. All phosphorylation and Ca^{2+} signal transduction cascades ultimately lead to the formation of active transcription factor complexes, which initiate the expression of new genes. These early and late activation events were analyzed in glutamic acid decarboxylase (GAD65)-specific HLA-DR0401-restricted T cell clones derived from a diabetic patient (R. Mallone et al., manuscript submitted for publication). The initiation of tetramer-induced signal transduction was followed by real-time Ca^{2+} measurements. As shown in Fig. 3, panel A, optimal concentrations (10-20 ug/ml) of the tetramer loaded with the cognate GAD65 peptide (TMr-GAD) led to a consistent raise in intracellular Ca^{2+} concentration, which was paralleled by a smaller and low-onset signal delivered by the same tetramer loaded with an irrelevant MBP peptide (TMr-MBP). Fig. 3 panel B shows the Ca^{2+} fluxes registered with cross-linked anti-CD3 stimulation as compared to an isotype-matched IgG. The immediate onset of a potent signal reflects the much higher affinity of antigen/mAb interactions as compared to MHC-peptide-TCR ones. A similar picture was obtained when protein tyrosine phosphorylation was considered: TMr-GAD was capable of delivering an efficient signal, although delayed and not as high as the one elicited by cross-linked anti-CD3 mAb. However, this effect was promiscuous with respect to peptide specificity, since a smaller, transient shift in phosphorylation was also obtained by the non-cognate TMr-MBP. This lack of specificity was limited to early signaling events, and MBP-loaded tetramers were not capable of committing cells to full activation. As exemplified in Fig. 3, panels C-D, CD69 (the earliest newly synthesized surface protein following complete T cell activation) was readily upregulated by TMr-GAD, but not by TMr-MBP. This upregulation was accompanied by surface staining for TMr-GAD, while no binding was evident for TMr-MBP.

The early signals delivered by non-cognate tetramers occurred in the absence of any detectable binding, likely due to fast, transient tetramer/TCR interactions not followed by stable binding and signal progression. Indeed, approx. 90% of the energy necessary for the interaction with the TCR is given solely by the MHC molecule (26). In line with a two-step model of TCR recognition (26), the GAD peptide delivers specificity and full T cell activation by turning this initial association into a

stable, longer binding. Transient peptide-independent TCR engagements are increasingly recognized as important contributors to effective signaling and antigen sensitivity (20,27,28). Co-stimulation was not a requirement for these T cell clones, likely due to their memory phenotype (29) and, possibly, to the high transducing efficiency of tetramers (19,30-32). Despite the limitations of a direct comparison, tetramer stimulation achieved stronger effects than APC stimulation, as judged by the induction of cytokine secretion.

The plethora of stimulatory effects observed is even more striking considering the non-saturating binding of MHC Class II tetramers, which occupied only a small fraction of the TCRs available. Using a quantitative flow cytometry approach, we estimated that, at optimal signaling concentrations, only 8-12% of the available TCRs were stably occupied by cognate tetramers. The potent signals delivered despite binding to so few TCRs could be partly related to a serial engagement effect as described by Valitutti et al. (1). In other words, each single MHC unit within the tetramer could transiently contact and scan a large number of TCRs by serial fast interactions. At the same time, the tetramer as a whole would still be stably bound to the cell surface, due to the increased avidity achieved by the multiple MHC interactions of the tetramer. In contrast, the high affinity of anti-CD3 mAbs allows them to bind a very large number of TCRs, but in a static rather than dynamic fashion.

A promising frontier for T cell activation studies will be the development of so-called “artificial” APCs. This approach has been mainly developed in the field of adoptive transfer of cytotoxic T lymphocytes (CTLs) (33,34). The infusion of antigen-specific $CD8^+$ cells is a potential immunotherapy against selected cancers (e.g., melanoma) and infectious diseases (e.g., HIV and *Cytomegalovirus* infections), but its broad use is challenged by the need to generate consistent numbers of autologous T cells directed against the selected epitopes. While beads coated with anti-CD3 and anti-CD28 mAbs are capable of supporting the long-term growth of $CD4^+$ T cells, additional requirements need to be met for $CD8^+$ T cells (34). To circumvent this problem, mouse fibroblasts have been transfected with single peptide-MHC Class I complexes along with B7.1,

ICAM-1 and LFA-3 costimulatory molecules (33). The induction of fully functional CTLs was more efficient than that obtained with autologous blood-derived dendritic cells, probably due to a higher availability of MHC and costimulatory molecules and absence of other MHC alleles in the artificial APC. Moreover, strong responses were induced not only against flu peptides, but also against autoantigens in the absence of autoimmune diseases. This suggests that there is not only a recall effect on primed CTLs, but also activation of naïve T cells present at very low frequencies (33). For expansion of antigen-specific $CD4^+$ T cells, MHC Class II tetramers have been used to engineer bead-based artificial APCs capable of activating human $CD4^+$ T cells in an antigen-specific manner (35). Recombinant MHC Class II molecules have also been incorporated into liposomes. Compared with bead-based artificial APCs, cell- and liposome-based systems exploit membrane fluidity to more closely mimic physiological interactions with T cells and have been shown to induce immunological synapse formation (36).

How much activation is right?

The capability of recombinant MHC Class II reagents for activating $CD4^+$ lymphocytes is a double-edged sword that can be differently used in distinct disease situations. For malignancies and infectious diseases, it may be desirable to stimulate these cells, thus allowing better cooperation between cytotoxic and helper lymphocytes. Trials of adoptive immunotherapy have focused on the infusion of $CD8^+$ cells as direct effectors of tumor cell killing. However, several studies have shown that $CD4^+$ cells are required in the optimal induction of human tumor-specific $CD8^+$ cells (37,38). In murine models, $CD4^+$ cells play an important role in eradicating tumors and can sometimes do so even in the absence of $CD8^+$ T cells (39). The limited success of trials of adoptive transfer with $CD8^+$ T cells has been partially attributed to a need for continuing $CD4^+$ T cells help to sustain the anti-tumor response (40,41).

In the setting of autoimmunity (and of transplantation immunity as well), different goals are therapeutically pursued. $CD4^+$ T cells can either be skewed towards more protective phenotypes (e.g., Th2 versus Th1, stimulation of $CD4^+CD25^+$ regulatory T cells) or can be turned off. These outcomes can be achieved by changing the quality or quantity of the signal delivered through the TCR. Administration of a dimeric peptide/MHC Class II chimera to TCR transgenic mice specific for an HA peptide in the context of $I\text{-}E^d$ induced differentiation of $CD4^+$ cells towards a Th2 response through negative signaling on the STAT4 pathway of Th1 differentiation. This Th2 polarization had subsequent bystander inhibitory effects on $CD8^+$ T cell function as a result of IL-2 deprivation (24). The same dimeric peptide/MHC chimera was shown to have anti-diabetogenic properties in mice transgenic both for HA/$I\text{-}E^d$-specific TCR and for selective expression of the HA protein in pancreatic β cells. This treatment prevented autoimmune diabetes and reversed it in animals that were already diabetic through induction of anergy in autoreactive $CD4^+$ T cells in the spleen and stimulation of IL-10-secreting T regulatory type 1 cells in the pancreas (42). A similar induction of IL-2-reversible anergy was obtained in vitro in human MBP-reactive MHC-DR2-restricted T cells from multiple sclerosis patients by a dimeric DR2/MBP chimera (32). In a murine

model of collagen-induced arthritis, a bivalent form of a single I-A^q chain presenting a peptide from type II collagen delayed the onset and reduced severity of disease by induction of antigen-specific hyporesponsiveness (43). Dimeric Class I MHC molecules have also been shown to induce antigen-specific T cell unresponsiveness in cytotoxic T lymphocytes in vitro and in vivo (44).

Another way to silence autoreactive T cells may be to quantitatively alter the TCR signal to induce activation-induced cell death (AICD) (45). In our diabetes model of GAD65-specific T cell clones, sustained stimulation with GAD65-loaded Class II tetramers induced massive apoptosis, as readily detectable even by morphological parameters alone (Fig. 4, panel A). This apoptotic process was characteristic of AICD, being inhibited by blocking the Fas/Fas ligand interaction (Fig. 4, panel B) (R. Mallone et al., manuscript submitted for publication). Gene expression arrays also confirmed that the apoptosis was mainly antigen-driven, following the classical death receptor pathway (46). Increased mRNA expression was detected for: 1) members of the tumor necrosis factor (TNF) ligand family: Fas ligand (47,48), CD40 ligand (49), lymphotoxin α (50) (but not TNF-α, likely for the short half life of its mRNA) (51); 2) members of the TNF receptor family (52,53): TNF receptor 2 (but not FAS, which is already highly expressed in this clones) and 4-1BB (54); 3) downstream mediators, more notably IAP-1 (downstream of TNF receptor 2) (53), caspase-3 (46) and caspase-14 (55); 4) members of the Bcl-2 family: Bcl-x (56), suggesting some contribution of a cytokine withdrawal mechanism (57) (R. Mallone and E.M. Laughlin, unpublished observations).

Present and future clinical applications: diagnosis, prognosis, therapy

The use of MHC Class II tetramers for diagnostic and prognostic evaluations is currently the focus of clinical application. In mouse models of infection, Class II tetramers specific for lymphocytic choriomeningitis virus-derived peptides have been used to track the expansion of $CD4^+$ T cell populations, showing that the increase in the $CD4^+$ compartment starts later, achieves a lower maximum level and is less stable than that of $CD8^+$ T cells (58). Results are very promising in human infectious diseases, where, despite the limited feasibility of direct detection (18), influenza A and HSV-2 antigen-specific $CD4^+$ T cells have been detected following in vitro expansion (9,15,16). This area of application is particularly relevant for infectious diseases such as AIDS and hepatitis C, for which $CD4^+$ T cell responses are crucial to the outcome. The translation of these tools to the field of tumor immunology could also open new avenues for the development and monitoring of cancer vaccines and, at the same time, for better understanding the relative contribution of $CD4^+$ cells.

Lower precursor frequencies in peripheral blood and lower TCR affinities have made advancement slower for autoimmune diseases. The recent report by Reijonen et al. filled this gap by demonstrating the possibility to detect significant numbers of GAD65-reactive T cells in patients with new-onset type 1 diabetes (17). GAD65-loaded MHC DR0401 and DR0404 tetramers were capable of detecting 4-28% antigen-specific $CD4^+$ T cells from peripheral blood lymphocytes previously expanded in vitro on GAD65-pulsed APCs. More importantly, GAD-reactive cells were found in all type 1 diabetic patients and some at risk subjects, but not in normal control subjects (17). The prognostic significance of tetramer staining in at risk subjects, i.e., whether they have an increased probability of developing diabetes in the short term, deserves further study. Indeed, the prediction of diabetes development obtained using MHC Class I tetramers to quantify β cell epitope-specific $CD8^+$ T cells in the peripheral blood of NOD mice is encouraging (59).

The relevance of the functional properties of recombinant MHC Class II reagents in stimulating T cells is not limited to in vitro activation studies. The possibility of exploiting these properties for

therapeutic purposes is intriguing. While in oncologic and infectious diseases the aim would be to bolster the CD4 response in order to provide sustained help for the cytotoxic effector phase (40,41), the more promising preclinical studies using immunomodulatory MHC Class II multimers come from autoimmunity (24,32,42,43). In this field, immune intervention has proved to be a formidable task, so far rewarded with little success. The recent publication of the first clinical trial using a non-activating (60) humanized form of OKT3 mAb in new-onset type 1 diabetes is a notable exception (61). This treatment obtained a consistent, although transient, improvement in β cell function, likely acting through Th2 polarization and induction of $CD4^+CD25^+$ regulatory T cells (62,63). The possibility of using immunodominant peptides or altered peptide ligands for more antigen-specific immunomodulatory therapies has been extensively explored. Several animal studies have shown that prevention of experimental autoimmune encephalomyelitis (a murine model of multiple sclerosis) or diabetes can be achieved by the downregulation of autoreactive T cells after administration of immunodominant peptides derived from MBP (64,65) or from the major β cell antigens, i.e., insulin B chain (66), GAD65 (67), heat-shock protein (hsp)60 (68). However, some studies have shown a lack of protection by peptide-based therapy, and the translation to clinical trials using different routes of administration has been disappointing at best (69-73). This inefficacy may partly be due to the fast proteolytic degradation of peptides, with lifespans in the order of 0.5-10 minutes in the bloodstream (74). The threat of exacerbating autoimmunity rather than inducing tolerance adds further uncertainty (75). New clinical trials with insulin B chain and GAD65 peptides are under way, supported by the Immune Tolerance Network (http://www.immunetolerance.org).

MHC Class II multimers could represent an appealing therapeutic alternative, endowed with remarkable specificity and better bioavailability (with half lives of approx. 50 h in vivo) (76). The selective nature of this approach is a potential drawback because it requires knowledge of the relevant T cell epitopes of several target proteins. It may also require intervention early in the disease process, before extensive epitope spreading has occurred (77). Dominant peptide epitopes

are likely to change during the progression of autoimmune disease (78), and successful tolerance induction will depend on correlating antigen hierarchy with disease progression. In this respect, a combined approach of disease "immune staging" and tolerogenic therapy could be envisioned. Periodic screening of at risk or newly-diagnosed individuals with the same tetramers, which could be conveniently loaded with any relevant peptide, may allow to choose the best timing for specific immunomodulatory intervention.

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Tables

Table 1. Human MHC Class II tetramers and relevant antigens

	Infectious antigens	**Autoimmune antigens**	**Tumor antigens**
DRB1*0401	Influenza A hemagglutinin Borrelia burgdorferi OspA Herpes simplex 2 VP16	RP collagen II Type 1 diabetes GAD65 MS myelin basic protein	Melanoma tyrosinase
DRB1*0101	Influenza A hemagglutinin Herpes simplex 2 VP16 Cytomegalovirus pp65 HIV gag	RP Collagen II	
DRB1*0404	Herpes simplex 2 VP16	Type 1 diabetes GAD65	
DRB1*0402	Herpes simplex 2 VP16		
DRB1*1104	Herpes simplex 2 VP16		
DRB1*1501	Herpes simplex 2 VP16	MS myelin basic protein	
DQB1*0602	Herpes simplex 2 VP16		
DQB1*0201		Celiac disease gliadin	

RP, relapsing polycondritis; MS, multiple sclerosis

Table 2. In vitro T cell activation systems

- Anti-CD3 monoclonal antibodies
- Peptide-pulsed antigen-presenting cells
- Plate-bound or soluble MHC dimers
- Plate-bound MHC monomers
- Soluble MHC tetramers
- Artificial antigen presenting cells

Figure Legends

Fig. 1. Structure of MHC Class II tetramers.

Fig. 2. TCR signaling events triggered following TCR/MHC interaction. TCR ligation leads to phosphorylation of Lck, which then phosphorylates ITAM domains on the ζ chains of the CD3 complex. Once phosphorylated, ITAM domains function as docking sites for ζ-associated protein (ZAP)-70, which is activated through phosphorylation by Lck and in turn phosphorylates adaptor proteins such as LAT. Further phosphorylation cascades are triggered, which interact with co-stimulatory signals and also lead to activation of phospholipase C (PLC)-γ_1. The enzymatic activity of PLC- γ_1 produces diacylglycerol (DAG), which triggers protein kinase C (PKC) pathway, and inositol triphosphate (IP_3), which elicits Ca^{2+} release from intracellular stores. This first Ca^{2+} burst triggers a second wave of Ca^{2+} influx from the extracellular space. All phosphorylation and Ca^{2+} signal transduction cascades ultimately lead to the formation of active transcription factor complexes, which initiate gene expression.

Fig. 3. Signals delivered upon tetramer (TMr) stimulation on GAD-specific DR0401-restricted T cell clones. (A-B) Ca^{2+} mobilization, as assessed by the fluorescence shift of the Ca^{2+}-sensitive dye Fluo-3. The indicated stimuli were added at t=0; the dotted lines indicate the basal Ca^{2+} level before stimulation. (C-D) CD69 upregulation. Cells were stimulated with the indicated tetramers for 3 h at 37ºC. Insets in panels show density plots of CD69 upregulation relative to tetramer binding.

Fig. 4. Cognate tetramer-induced apoptosis on GAD-specific DR0401-restricted T cells. (A) Three-dimensional plots of forward (FSC) and side scatter (SSC) distribution among cells treated with the same DR0401 tetramer loaded with either cognate GAD or non-cognate MBP peptide. (B) The apoptotic effect of the cognate TMr-GAD is due to activation-induced cell death: inhibition of TMr-GAD-induced apoptosis by a blocking anti-Fas ligand mAb.