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TARGETING T LYMPHOCYTES FOR IMMUNE MONITORING
AND INTERVENTION IN AUTOIMMUNE DIABETES

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Abstract

Recognition of a peptide-MHC complex by the T cell receptor (TCR) is a key interaction which initiates T lymphocyte activation or silencing during an immune response. Fluorochrome-labeled recombinant MHC Class II-peptide reagents function as soluble mimetics of this interaction, bind to their specific TCR, and allow for detection of antigen-specific CD4⁺ T cells. These reagents are now under scrutiny for “immune staging” of patients at risk for type 1 diabetes, in an effort to diagnose islet autoimmunity early enough to block immune-mediated β cell destruction. Several issues are currently being addressed to improve the performance of these T cell assays: enrichment steps for better sensitivity, multiplexing of several islet epitopes, simultaneous monitoring of CD4⁺ and CD8⁺ responses, detection of low avidity T cells, combination of quantitative (number of positive cells) and qualitative (cytokine secretion, naïve/memory phenotype) readouts. CD4⁺ T cells are key effectors of autoimmunity, and these MHC Class II-peptide reagents, through their signaling properties, might also provide therapeutics to block the autoimmune process at its onset, analogous to the use of OKT3₁(AlaAla) anti-CD3 antibody but in an antigen-specific fashion. The aim of such therapeutics is to potentiate different physiological control mechanisms to restore immune tolerance. Mechanisms initiated by this pathway may be capable of triggering elimination of pathogenic T cells through antigen-specific apoptosis and anergy, combined with the induction of regulatory T cells with broad suppressive function.

Key words: immune tolerance, apoptosis, anergy, regulatory cell, tetramer.

Introduction

T lymphocytes in the human body are estimated to be able to recognize about 25 million different antigen (Ag) specificities. Understanding this diverse potential is key to the design of specific targeted therapies which are alternatives to generalized immunosuppressive or immunostimulating agents. Thus, it is necessary to find feasible approaches to specifically follow the immune response to a given Ag in the human body. The analysis and detection of specific antibodies (Abs) is well developed, but the investigation of the other branch of the adaptive immune response, Ag-specific T cells, has been much more difficult. The challenge for this kind of endeavor is a formidable one: the Ag-specific T cell needle is usually as rare as 1 in a 5,000-100,000 T cell haystack. Nonetheless, the challenge has been widely taken up following different approaches.

The first approach relies on the identification of Ag-specific T cells based on their functional response to their cognate Ag. This is the concept behind Elispot assays, where whole peripheral blood mononuclear cells (PBMCs) are exposed to the Ag of interest, usually in a solid medium containing an anti-cytokine monoclonal antibody (mAb), most commonly anti-interferon (IFN)- γ . The cells are stimulated in this medium for a certain time and, if Ag-specific T cells are present, they can recognize the Ag displayed by Ag-presenting cells (APCs). Upon encounter, T cells become activated and start producing cytokines, which are bound by the mAb and can subsequently be detected by a second, fluorochrome-conjugated anti-cytokine mAb. This technique is extremely sensitive, and allows for the identification of very rare T cells (approximately 1 epitope-specific CD4⁺ T cell per 60,000 PBMCs). However, some limitations hamper the clinical applicability

of this technique: 1) the procedure is not readily adaptable for large scale screening purposes; 2) the identification of Ag-specific T cells is an indirect one, and does not allow for a precise enumeration of the cytokine-producing cells; 3) variability in the functional response of different T cells, even within the same Ag specificity (e.g., anergy, production of different cytokines) can give false negatives; 4) cytokines released by one cell may have bystander effects on neighboring cells.

A different approach to some of these issues is offered by MHC tetramers (TMrs). The underlying idea is to exploit the physiological recognition mechanism between a given T cell receptor (TCR) and a given peptide-MHC complex (Fig. 1) to bind the T cells of interest. Recombinant MHC molecules can be produced and either covalently linked or loaded with the peptide of interest. Labeling of the reagent with a fluorescent probe allows for the detection of binding and subsequent identification of Ag-specific T cells. Of the two primary classes of T lymphocytes, CD8⁺ T cells are directed toward peptide-MHC Class I complexes (Fig. 1B), while CD4⁺ T cells preferentially recognize peptide-MHC Class II complexes (Fig. 1A).

MHC Class II multimers

Lymphocyte monitoring with MHC multimers began with the study of CD8⁺ T cell responses to influenza virus and HIV using recombinant human MHC Class I multimers (1). There are several reasons why the development of this new research armamentarium started with CD8⁺ rather than CD4⁺ T cells. The first reason is that the number of CD8⁺ T cells responding to Ag present in circulation in human blood is higher than that of CD4⁺ T cells. Thus, the number of circulating CD8⁺ T cells customarily

greatly exceeds that of CD4⁺ counterparts targeted for most Ags, inviting the use of MHC Class I TMs. A second reason lies in the fewer technical difficulties encountered in the production of MHC Class I TMs: MHC Class I molecules have only one highly polymorphic \forall chain which is associated with the invariant \exists_2 -microglobulin; moreover, the peptide-binding groove is embedded solely in the \forall chain (Fig. 1B), so that the loading of the peptide once the recombinant MHC molecule is produced is relatively straightforward, and the correct refolding of the molecule is actually facilitated in the presence of the peptide.

To the contrary, the MHC Class II molecule is composed of an \forall and a \exists chain which are both highly polymorphic; and the peptide-binding groove is formed by the association of the two chains together (Fig. 1A), so that correct folding and peptide-loading is much more problematic. In the case of MHC Class I TMs, peptides are introduced during the refolding of the \forall chain during a denaturation-renaturation reaction. In the case of MHC Class II TMs, peptides are introduced using a detergent-facilitated peptide exchange reaction, or through a covalent linkage with the amino-terminus of the \exists chain. Moreover, 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, so that the same peptide may bind in two or more registers. MHC Class II reagents may thus display the same peptide in different conformations, allowing for the detection of T cells with related, yet distinct, Ag specificities.

Murine soluble MHC Class II molecules with covalently bound single peptides were described in 1994 by the group of J. Kappler and P. Marrack. These constructs were shown to activate T cell hybridomas but at that time were not engineered for staining purposes (2). They were later modified to allow detection of CD4⁺ Ag-specific T cells (3). As in the case of MHC Class I reagents, the low affinity interaction of the peptide-MHC complex with its cognate TCR did not allow for a stable binding using monomeric forms. Therefore, the overall avidity (which can here be viewed as the affinity of the single peptide-MHC/TCR interaction multiplied by the valency, i.e. the number of interactions) was increased through the use of multimeric complexes. Most commonly, the monomeric MHC molecules are biotinylated and later reacted with streptavidin (SA). Since SA has 4 biotin-binding sites, these multimeric reagents were christened “tetramers”, since they should contain four MHC monomers. However, it has become apparent that this definition is a misnomer, for at least two reasons: 1) the coupling ratio of MHC monomers with SA is less than perfect, and a mixture of free monomers, dimers, trimers and tetramers is generated (4); 2) SA has a natural tendency to aggregate; moreover, the large phycoerythrin (PE) protein fluorochrome commonly used for detection is difficult to conjugate to SA without causing some degree of SA-SA cross-linking, thus leading to large cross-linked complexes of multiple SA molecules; consequently, reagents with a much higher order of valency are generated (4). Moreover, only 3 of the 4 MHC arms can engage on a single T cell due to the tetrahedral configuration of the biotin-binding sites (5). A more appropriate name for these reagents would therefore be “multimers” or “oligomers”.

Our group first reported the production of human MHC Class II TMs (6). In these constructs, the peptide is not covalently bound to the β chain as in the Kappler-Marrack murine reagent, but rather it is loaded on the assembled MHC molecule through a detergent-facilitated exchange reaction. The disadvantage of this approach is that the exchange reaction needs to be carried out in the presence of excess peptide concentrations. Unbound peptide can subsequently be removed by dialysis or size exclusion chromatography, but the stability of the reagent, which can usually be kept at 4°C for several months, decreases dramatically after dialysis (R. Mallone and E. James, unpublished observations). Likely, the peptide-MHC interaction is a dynamic one and the peptide can be more easily released from the groove, and not replaced, when the medium is devoid of the unbound fraction. However, the peptide loading allows excellent versatility, in that the same MHC construct can be loaded with different peptides rather than having to produce a different reagent for every specificity of interest.

An intermediate approach has recently been reported by the group of K. Wucherpfennig. In this system, the HLA-DR molecules are expressed as precursors with a covalently linked CLIP peptide, which is subsequently cleaved with thrombin and exchanged with the peptides of interest. Such peptides are labeled with dinitrophenol, a feature that later allows for purification of only the peptide-loaded DR molecules through an anti-dinitrophenol HPLC affinity column (7).

Murine MHC Class II TMs have been applied to study mice transgenic for a particular TCR (3), where the introduction of a single TCR transgene expressed by all T cells shortcuts the cumbersome problem of Ag-specific T cell frequency problem. Such shortcut is not an option in human studies, thus necessitating a much more sensitive

system to successfully follow CD4⁺ T cell responses. We first used human MHC Class II TMrS to follow the immune response to a hemagglutinin (HA) immunodominant peptide in influenza-immune DR0401⁺ individuals (6) following in vitro expansion. The number of Ag-specific CD4⁺ T cells was then calculated by simultaneously tracking cell divisions of TMr⁺ cells with carboxyfluorescein diacetate succinimidyl ester (CFSE). This fluorescent dye binds to the cell and it is equally distributed between daughter cells at every cell division, with subsequent halving of the fluorescence intensity. Peaks of different fluorescence can later be visualized by flow cytometry, corresponding to different cell divisions (i.e., n peaks witness n cell division). The total number of TMr⁺ cells can therefore be divided by 2^n to obtain the number of precursors originally present in the blood prior to in vitro expansion. TMr⁺ cells can simultaneously be stained for other markers of interest using different fluorochromes, another advantage over Elispot assays.

Many other human Class II TMr reagents have been subsequently synthesized by different groups, using a variety of approaches and covering additional MHC alleles [(7-11); for a recent review see (12)]. An example of the use of TMrS to visualize and isolate specific T cells is shown in Figure 2, in which human class II TMrS containing a peptide Ag associated with autoimmune diabetes are used to identify CD4⁺ T cells in peripheral blood of diabetic subjects. The MHC Class I TMr technique has also been adapted for in situ visualization of Ag-specific T cell responses: the combined use of TMr reagents with confocal laser scanning microscopy allowed detection of both virus- and tumor-specific CD8⁺ T cells in lymphoid organs, peripheral tissues and tumor infiltrates (13). This in

situ staining application has recently been reported also for murine MHC Class II reagents in an experimental allergic encephalomyelitis (EAE) model (14).

Monitoring of autoimmunity with MHC class II multimers

Quantitative parameters

a) Sensitivity issues

The specificity of MHC multimers is due to both the genetic restriction of individual HLA molecules and to the precise Ag target. This necessitates HLA genetic matching for multimers used for monitoring, but for several of the major human autoimmune diseases, 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 1 diabetes in Caucasians, multimers with the HLA-DR0401, -DR0404 and -DR0301 molecules identify CD4⁺ T cells in over 80% of patients.

Nevertheless, detection of T cells directed to self Ags is challenging, due to the low frequency of circulating autoreactive cells, their generally low avidity for Ag recognition, and the multiplicity of different Ag specificities involved in complex autoimmune diseases. The vast majority of potentially autoAg-specific CD4⁺ cells are deleted in the thymus during development, and autoreactive cells that escape this process are rare. Moreover, the T cells which are more likely to survive thymic selection express low affinity TCRs, making the TMr binding even more problematic. In addition, Ags that bind to MHC with lower biochemical affinity are likely to favor escape from thymic selection, contributing to the overall low TCR avidity of surviving cells (15). This feature

also makes production of a stable tetramer reagent for studies of autoimmunity more troublesome.

Greater numbers of autoAg-specific CD4⁺ T cells can be found in the tissues affected by the autoimmune disease, but sampling from these tissues is rarely available and not practical for large scale studies. A related issue currently open to speculation is how the autoAg-specific T cells found in the peripheral blood are representative of those at the disease site: differential migration of different subsets of cells is a likely possibility. Indeed, a recent work in the non-obese diabetic (NOD) mouse model demonstrated that the presence of islet-reactive CD8⁺ T cells present in the peripheral blood correlated with the presence of a similar population of T cells in pancreatic islets. However, the exact percentage of islet-specific T cells in the peripheral blood did not correlate with the percentage present in the islet infiltrates, and there were instances where islet-reactive T cells were present in islets, but could not be detected in peripheral blood (16).

In our studies of glutamic acid decarboxylase (GAD)65-specific T cells in type 1 diabetic patients, preliminary in vitro expansion of peripheral blood lymphocytes on GAD65-pulsed APCs was necessary to overcome the problem of low precursor frequency (17). A large expansion of TMr⁺ cells could thus be obtained, representing both the accumulation of proliferating Ag-specific cells and the loss of unrelated T cells lacking appropriate antigenic stimulation during in vitro culture. Similarly, TMr 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 (11). With this preliminary step of in vitro expansion, a correlation between the number of GAD TMr⁺ T cells and disease state, either in terms of overt diabetes or of prediabetic condition (as

defined by the presence of HLA susceptibility alleles and anti-islet autoAbs) was observed (17) (Fig. 3). The predictive value of this type of “immune staging” has also been shown for CD8⁺ T cells specific for an immunodominant \exists cell epitope in the NOD mouse model. Mice that eventually developed diabetes accumulated a significantly larger proportion of islet-reactive TMr⁺ T cells over time, evident several weeks before the average age of diabetes onset. Moreover, the number of TMr⁺ cells progressively decreased in the blood after the development of diabetes (16). In another study of vitiligo patients, the number of TMr⁺ CD8⁺ cells specific for a melanocyte Ag in the peripheral blood was also found to correlate with the severity of disease (18).

The precursor frequency problem is not unique to autoimmunity studies. Direct ex vivo detection of Ag-specific CD4⁺ T cells has so far yielded very low TMr-binding peripheral blood populations even in an infectious context (10). The low number of Ag-specific CD4⁺ cells in peripheral blood is beyond the sensitivity limit of flow cytometry: frequencies of TMr⁺ cells below 0.2% (1:500) significantly overlap with the 0.1% background staining typically obtained using TMrs loaded with control irrelevant peptides. However, a recent study showed that, when a HA-specific DR0401-restricted T cell clone was mixed with PBMCs from a non-DR0401 donor, HA-specific T cell frequencies as low as 0.005-0.003% (1:30,000) still gave a HA TMr staining above the control TMr background (22-15 events counted versus 3 events in background staining). As undependable as these low numbers may seem, the same 1:30,000 precursor frequency was consistently within the range of HA TMr detection in staining of whole PBMCs from recently influenza-vaccinated patients. These frequency estimates were

confirmed by Elispot and by deriving the number of precursors from the number of cell divisions by CFSE after in vitro expansion (19).

The group of K. Wucherpfennig recently proposed an interesting idea to tackle the precursor frequency problem. HLA-DR molecules were synthesized with a covalently bound CLIP peptide. This peptide was subsequently cleaved and exchanged with the peptides of interest. Notably, only the DR molecules loaded with the peptide were subsequently purified, taking advantage of an affinity column recognizing a dinitrophenyl tag attached to the peptide. This feature may endow these reagents with higher efficiency, since some empty HLA-DR molecules are likely to be expected in unpurified preparations. Individuals with spontaneously resolved hepatitis C virus (HCV) viremia mount a vigorous HCV-specific CD4⁺ T cell response that is maintained long-term. Whole PBMCs from such individuals were stained with PE-labeled TMr reagents, followed by an enrichment step using anti-PE microbeads. While no staining above background was visualized before enrichment, 15-100 TMr⁺ events were detected after enrichment as compared to 0-2 events with the control TMr. This low numbers correlated with the HCV-specific CD4⁺ T cell response: PBMCs from individuals with HCV infection which had become chronic due to the absence of an efficient CD4⁺ reaction did not significantly stain with HCV peptide TMrs even after anti-PE microbead enrichment (7). These two latter works, although obtained in a post-vaccination (19) or viral (7) immunity setting, are very encouraging for application to autoimmune monitoring. Indeed, frequencies of 1:30,000-1:100,000 fall within those expected for GAD-specific T cells in type 1 diabetes.

b) Multiple self antigens and epitopes

Autoimmune diseases are characterized by a complex cellular response to multiple autoAgs, often variable at different times during the disease process. Therefore, it is highly desirable to study several T cell epitopes from different Ags simultaneously on the same blood sample. Techniques for multiplexing several TMRs for analysis should provide a more comprehensive picture of immunologic activity. For example, TMRs for the islet autoAgs GAD65, proinsulin and IA-2 may all be valuable for following disease progression or response to therapy in autoimmune diabetes. The concept is the natural extension of the well-established use of multiple autoAb specificities to identify individuals at risk for type 1 diabetes. The potential of this multiplexing approach is exemplified by the predictive value of the cellular immunoblotting technique developed by Brooks-Worrell et al. (20). These authors identified reactivity of diabetic T cells to islet Ags in a wide range of molecular weights, by measuring ^3H -thymidine incorporation of PBMCs challenged with unidentified islet protein bands eluted from nitrocellulose blots. Despite the lack of information on the targeted Ags, the increase in the number of blot sections recognized by the T cells showed a correlation with the increasing number of autoAbs detected and with subsequent transition from an at-risk state to overt diabetes (21). The recent study by Peakman and colleagues combining Elispot readouts for proinsulin- and IA-2-specific T cells for higher sensitivity and specificity also underscores the potential of screening for multiple epitopes (22).

One limitation to this multiAg approach is the limited knowledge about relevant islet cell epitopes. Not only are a limited number of islet autoAgs (i.e., GAD, IA-2 and proinsulin) well characterized, but the relevant epitopes recognized within these molecules in the

context of different MHC Class II alleles need to be identified in order to prepare the corresponding peptide-loaded. Use of peptide epitopes rather than whole protein Ags also bears an advantage for the in vitro expansion phase (as well as for the Elispot format), since peptides bind surface HLA Class II molecules directly, minimizing the requirement for Ag processing and thus recruiting additional APCs such as B cells.

c) CD4⁺ and CD8⁺ T cell responses

In addition to this issue of multiple epitopes recognized by CD4⁺ T cells, multimer monitoring studies are challenged by considering the need to study both CD4⁺ and CD8⁺ immune responses to capture relevant immunologic profiles. The concurrent role of both CD4⁺ and CD8⁺ T cells in the pathogenesis of diabetes is widely established. Islet infiltrates in NOD mice contain both CD4⁺ and CD8⁺ T cells, and both are required for progression to diabetes. It appears that lymphocytic infiltrates occur in this animal model following a wave of physiological β cell death that takes place in the islets of juvenile rodents, peaking at 14-17 days after birth (23-25). An analogous perinatal wave, peaking at birth, has been demonstrated in humans (26). β cell Ags are thus released for APCs to prime islet reactive naïve T cells. Indeed, insulinitis begins abruptly in NOD mice at 15-18 days of age and, at this time, it is possible to detect the relevant β cell-derived Ags in pancreatic lymph nodes (PLNs). However, this availability of β cell Ags in PLNs could activate both CD4⁺ and CD8⁺ T cells, and it is likely that both of them participate with different mechanisms in the initial islet destruction. Some studies have suggested that the initial insult to β cells is mediated by CD8⁺ T cells, since NOD mice lacking MHC class I molecules do not experience insulinitis (27, 28). Some CD8⁺ T cell clones derived from

diabetic NOD mice can provoke diabetes on transfer into lymphocyte-deficient recipients (29, 30), and CD8⁺ T cells isolated from diabetic animals can attach to and lyse β cells in vitro. However, some CD4⁺ T cell clones can as well provoke diabetes in the absence of CD8⁺ cells, and splenocytes from diabetic donors can transfer disease into recipients lacking MHC Class I molecules on their islets (31). In our view, diabetes in an immunocompetent host is likely to involve CD8-mediated islet cytotoxicity, but the need for CD4⁺ T cells to orchestrate and regulate the attack is predominant. The strongest support for the central role of CD4⁺ T cells simply comes from the fact that genetic susceptibility and resistance to type 1 diabetes are profoundly affected by polymorphism of MHC Class II, and not Class I, genes. The potential of either T cell alone to provoke diabetes may derive from the fact that both lineages, once activated, can kill β cells through different mechanisms, i.e., cytotoxic T cell recognition, Fas/Fas ligand interactions, production of soluble mediators (IL-1, IL-6, TNF- α , IFN- γ , NO) or induction of the cytotoxic function of macrophages. In any event, better immune monitoring strategies might be achieved by targeting not only Ag-specific CD4⁺, but also Ag-specific CD8⁺ T cells by combined use of both MHC Class II and Class I TMrs. Current limitations for the latter are given by the scant information available on CD8 islet epitopes in humans. Nonetheless, the potential of MHC Class I monitoring for diabetes prediction when relevant epitopes are known has been shown in NOD mice (16).

One other current limitation to the simultaneous use of multiple TMrs lies in the number of fluorescence spectra available for conjugation to individual peptide-MHC complexes. Reversing the approach to these limitations, the groups of M. Davis and P. Brown have recently proposed a cellular array-based screening strategy using

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microarrays of immobilized peptide-MHC complexes. Cells are preliminarily labeled with lipophilic tracers and Ag-specific cells are captured onto an array of individual spots of peptide-MHC complexes, with cell capture at each spot being dependent on T cell specificity. Following this strategy, the authors were able to detect as few as 0.1% Ag-specific CD4⁺ or CD8⁺ T cells, simultaneously using MHC Class I and Class II constructs specific for two different peptides (32). This technique holds great promise for future large scale diagnostic application and screening of CD4⁺ and CD8⁺ T cell reactivity against multiple self Ags.

Qualitative parameters

a) T cell avidity

One of our findings from studies of subjects with autoimmune diabetes is that both high and low avidity T cells specific for the same autoAg co-exist in the peripheral blood. It seems likely that high avidity T cells are pathogenetically relevant to the onset of autoimmunity (15); however, the high avidity fraction is also the one more likely to contain islet-specific T cells with regulatory functions, given the higher TCR affinity on which they are selected in the thymus (33-35). Such cells, although autoreactive, might be part of a normal T cell repertoire.

On the other hand, it has been suggested that low avidity T cells are expected to be abundant at earlier stages of disease (36). Moreover, in NOD mice T cell autoreactivity has been shown to spread from a \exists cell determinant with both the largest precursor pool and the highest proportion of high avidity T cells to determinants

displaying progressively smaller precursor pools and lower T cell avidities (37). This may indicate a scenario in which high avidity T cells require less Ag and costimulation, so that they can be the first and faster to expand. However, such expansion promotes an early wave of inflammation, thus creating a microenvironment more favorable for the activation of lower avidity T cells at a later stage. Therefore, the detection of low avidity T cells of different islet Ag specificity at different stages of (pre)diabetes would be particularly meaningful for immune monitoring purposes.

These issues relate to the conditions used for preliminary *in vitro* expansion: high Ag concentrations favor the growth of high numbers of low avidity T cells, while low Ag concentrations favour the raise of lower numbers of high avidity T cells (15, 38, 39). Moreover, it is felt that most low avidity T cells identified as autoreactive are in fact naïve T cells marginally cross-reactive to the autoAg and commonly detectable in healthy individuals (15). However, only the relevant autoreactive T cells would be expected to originate from *in vivo* activated T cells (40), i.e., to be CD45RA⁻/RO⁺. Concomitant staining for CD45RA and CD45RO may therefore improve the predictive value of TMr staining.

We have recently characterized GAD-specific T cell clones of different avidity derived from the same diabetic patient. At the opposite ends of the avidity spectrum, we were able to isolate GAD TMr^{high} (∃95%) cells and GAD TMr^{low} (#5%) T cells. These GAD TMr^{low} T cells could easily remain undetected while screening a whole CD4⁺ population after *in vitro* expansion. Nonetheless, different GAD TMr staining was accompanied by measurable activation outcomes induced by the same GAD TMr reagent. GAD TMr^{low} T cells upregulated CD69 and secreted IFN- γ (as measured by capture

assays) when incubated with their cognate TMr. Functional TCR avidity, as evaluated by these activation responses, correlated with the intensity of GAD TMr staining, as previously reported (3, 41-44). A readout of functional interaction (i.e., GAD TMr signaling) combined with that of a structural interaction (i.e., GAD TMr binding) could therefore increase the sensitivity of the detection towards the low avidity range of T cells (R. Mallone et al., manuscript in preparation). A similar observation has been reported in a model of EAE, where TMs containing a myelin proteolipid protein induced the release of Ca^{2+} in 36% of T cells as compared to only 4% of the cells stained by the same TMs (45). Remarkably, the activation event measured by these authors is a very early one. In our experiments, GAD TMr^{low} T cells did not display a Ca^{2+} response when stimulated with TMs. The lower avidity likely does not allow for a binding rate sufficiently fast to occupy the critical number of TCRs needed to trigger Ca^{2+} fluxes. The use of late activation readouts, such as IFN- γ secretion or proliferation through CFSE staining (46), is probably more suitable to improve detection sensitivity.

b) Pathogenic and regulatory autoreactive T cells

Not all the islet autoreactive T cells that escape central tolerance in NOD mice differentiate into diabetogenic T cells. For example, transgenic NOD mice expressing a TCR specific for a GAD peptide contained significant numbers of GAD TMr⁺ cells, yet the mice did not develop insulinitis or diabetes. In fact, the TCR-transgenic T cells of these mice, which expressed CTL Ag (CTLA)-4 and produced interleukin (IL)-10 as well as IFN- γ and IL-2, displayed anti-diabetogenic activity in adoptive T cell transfer experiments, suggesting that some autoreactive T cells acquire immunoregulatory properties during development

(47). The group of M. Peakman has also recently used Elispot assays on proinsulin- and IA-2-specific T cells to provide evidence that the quality of autoreactive T cells shows a distinct polarization in diabetic patients versus healthy controls. While a proinflammatory Th1 phenotype with IFN- γ production was predominant in patients, the majority of HLA-matched controls also manifested a response, but with extreme T regulatory (Treg) bias and IL-10 production (22). Interestingly, patients with type 1 diabetes who made IL-10 responses tended to be significantly older at diagnosis, suggesting a partial protection afforded by this kind of response.

Perhaps combining quantitative readouts of Ag-specific T cells (i.e., number of TMr⁺ cells) with qualitative parameters (i.e., T cell avidity, naïve/memory phenotype, pattern of cytokine secretion) will further improve the predictive value of this type of studies.

Therapy of autoimmunity with MHC Class II multimers.

As with mAbs, both MHC Class I and Class II multimers have been widely shown to be not only valuable detection reagents, but also potent stimulatory tools. Several groups showed the potent stimulatory effect of immobilized MHC Class II reagents, superior to that of anti-CD3 mAbs (2, 48, 49). This higher potency appeared to be due to the ability of TMrs to serially engage multiple TCRs (50, 51), due to the low binding affinity (hence less stable interaction) for the TCR. A second reason resides in the more efficient recruitment of the CD4 coreceptor with MHC Class II constructs (48). The formation of a TCR dimer is the minimal requirement for initiation of T cell signaling, and a higher valency achieves a higher stimulatory effect (52, 53). These

additional properties are widely exploited in the generation of artificial APCs, which are substrates of different origins (either cellular or synthetic) incorporating MHC Class I or Class II molecules and various combinations of costimulatory ligands. These reagents are mainly used for ex-vivo expansion of T cells for adoptive therapies or in investigations of T cell-mediated immunity [for a recent review, see (54)].

T cell activation using MHC-based soluble reagents also has the potential to induce therapeutically advantageous outcomes in pathogenic T cells. This is conceptually analogous to current therapies utilizing anti-CD3 mAbs, which similarly activate T cells through the TCR. In recent clinical trials, infusions of a modified humanized form of anti-CD3 mAb, OKT3₁(AlaAla), led to transient improvement in β cell function in new-onset type 1 diabetes (55). The effect is likely achieved through a combination of activation-induced cell death (AICD), Th2 polarization with IL-10 production (55, 56) and/or induction of transforming growth factor (TGF)- β -secreting CD4⁺CD25⁺ Treg cells [(57); for reviews, see also (58, 59)].

Several in vitro and mouse in vivo studies with MHC Class II reagents suggest the feasibility of a similar approach, in which multimers are used to target only discrete Ag-responsive T cells — a much higher degree of specificity compared to use of anti-CD3 mAb. Administration of a dimeric peptide/MHC Class II chimera to TCR transgenic mice specific for an HA peptide in the context of I-E^d induced differentiation of CD4⁺ cells towards a Th2 response. This Th2 polarization had subsequent bystander inhibitory effects on CD8⁺ T cell function as a result of IL-2 deprivation (60). The same dimeric peptide/MHC chimera was shown to have anti-diabetogenic properties in mice transgenic both for HA/I-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, apparently through induction of anergy in autoreactive $CD4^+$ T cells in the spleen and stimulation of IL-10-secreting T regulatory type 1 (Tr1) cells in the pancreas (61). A similar induction of IL-2-reversible anergy was obtained in vitro in human myelin basic protein (MBP)-reactive MHC-DR2-restricted T cells from multiple sclerosis patients by a dimeric DR2/MBP chimera (62). Similar results, with induction of anergy and/or Th2/Treg cells have been obtained in murine models of collagen-induced arthritis (63) and EAE (64). Dimeric Class I MHC molecules have also been shown to induce Ag-specific T cell unresponsiveness in CTLs in vitro and in vivo (65). In vivo tolerance induction was also obtained by Bluestone and colleagues by treating NOD mice with a peptide-MHC Class II dimer after adoptive transfer of diabetogenic BDC2.5 TCR transgenic $CD4^+$ T cells. The dimer therapy induced activation and increased cell death of transferred BDC2.5 $CD4^+$ T cells, and the T cells surviving this effect were hypoproliferative and produced increased levels of IL-10 and decreased levels of IFN- γ . This switch was critical to the observed therapeutic effect, since anti-IL-10 receptor administration reversed the tolerogenic effect of the dimer. However, targeting the single BDC2.5 specificity with the same dimer was not sufficient to reverse or prevent diabetes in wild-type NOD mice when BDC2.5 cells were not transferred (66).

This latter study exemplifies how in vivo therapeutic effects are obtained in monoclonal but not in polyclonal TCR settings. This limitation underlines the importance of targeting multiple specificities not only for monitoring but also for therapeutic purposes. However, a different and complementary interpretation is that the same MHC reagent might not

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have the same effect on all the T cells targeted, even within the same specificity. Differences in susceptibility among T cells could be dictated by several factors. In particular, the avidity of MHC multimer interaction with particular TCRs expressed by different T cells can result in different strengths of antigenic stimulation and subsequent different outcomes. We have addressed these issue by evaluating the response to MHC Class II TMr of GAD-specific T cells of different avidity. As expected, the activation induced upon GAD TMr treatment was lower in T cells harboring a lower affinity TCR. Consequently, also the AICD resulting after prolonged GAD TMr stimulation preferentially targeted higher avidity T cells, leaving the low avidity counterparts relatively unaffected (67). Based on these observations, a prediction could be that T cells of lower avidity might have a survival advantage following MHC-based therapy.

Therapy resulting in a survival advantage of low avidity T cells may be clinically efficacious, consistent with the avidity maturation model of diabetes proposed by the group of P. Santamaria (36). According to this model, progression of islet inflammation to overt diabetes in the NOD mouse is driven by so called “avidity maturation” of CD8⁺ T cells. In other words, the autoreactive T cell population is progressively enriched in high avidity T cells as the insulitis progresses to diabetes. This enrichment is critical in diabetes progression, since treatment of prediabetic NOD mice with a soluble mimotope peptide prevents diabetes by blunting the avidity maturation, preferentially deleting high avidity T cells and expanding low avidity clonotypes (36). This avidity maturation model has also been widely supported by data on CD4⁺ T cell responses to several types of model Ags. The group of M. Davis evaluated the avidity and dissociation kinetics of peptide/MHC Class II TMr binding to Ag-specific lymphocytes isolated following

primary or secondary immunization. Their findings revealed a narrowing of the secondary repertoire relative to the primary repertoire, largely resulting from the loss of cells expressing TCRs with the fastest dissociation rates for peptide/MHC binding. In addition, T cells in the secondary response expressed TCRs of higher average affinity for peptide/MHC than cells in the primary response (42). At variance with these reports, other studies described a shift towards TCRs of intermediate affinity, which involves the loss of both high and low avidity T cells, as an immune response develops (68). The partial discrepancies among these observations might be explained by the current technical limitations in identifying low avidity T cells. It is indeed likely that a delicate balance between low (ineffective/proliferative) and high (proliferative/apoptotic) signals trims both the low and the high avidity tails of the T cell repertoire (69).

However, AICD is not the only mechanism at work in the periphery, and other outcomes such as anergy induction or Th2/Treg polarization might not equally be subjected to the same avidity-related gradient of response. Several *in vivo* models of adaptive tolerance have shown that stimulation of Ag-specific T cells induces a first phase of expansion, followed by AICD of the majority of these responding T cells. However, there usually remains a cohort of cells that are unresponsive. This phenomenon has been observed following stimulation with bacterial superAgs (70), persistent viral infections (71) or in models where TCR transgenic T cells were transferred either into hosts that constitutively express the Ag recognized by the transferred T cells (72, 73) or into unirradiated syngeneic mice subsequently challenged intravenously with soluble peptide Ag [(74); for recent reviews, see (75, 76)]. In some cases, these remaining anergic T cells have been shown to acquire suppressive activity and/or IL-10 secretion

(66, 77). Similar observations have been made in vitro (78). Also in our system (67), a small fraction of T cells survive AICD following GAD TMr stimulation. We are currently focusing our attention on this small fraction of surviving T cells, investigating whether they become anergic and/or acquire regulatory properties, and whether this transition is avidity-dependent (R. Mallone et al., manuscript in preparation).

A role for T regulatory cells

The potential of MHC-based therapies encompasses a combination of peripheral tolerance mechanisms (79). In particular, a promising target would be the induction of anergic T cells with regulatory properties [Table 1; for reviews, see (80-83)]. Treg cells (Tregs) are divided in two broad families: “innate”, which are continuously produced by the thymus (so called naturally occurring CD4⁺CD25⁺ T cells); and “adaptive”, which arise as a result of tolerogenic encounters in the periphery (84). Naturally occurring CD4⁺CD25⁺ Tregs are thymus-derived anergic cells that act, at least in vitro, via a cytokine-independent mechanism of cell-cell contact between T cells that is APC-independent (85-87). However, there is probably an additional APC-dependent suppressive effect mediated through inhibition of DC maturation, with downregulation of HLA Class II, CD80 and CD86 (88, 89) as opposed to the upregulation induced by activated CD4⁺ cells (90). Maturation through CD40-CD40L interaction (91) or through innate stimuli rescues DCs from this inhibitory effect (92). The transcriptional repressor FoxP3 is selectively expressed by CD4⁺CD25⁺ T cells and has been shown to be critical for their regulatory function (34, 93-95). Naturally occurring Tregs (but also all kinds of induced Tregs), suppress in an Ag-nonspecific manner (85), but they need to be activated

(either by their cognate Ag or by anti-CD3 stimulation) to exert this function (96). The molecular interaction responsible for this contact-dependent suppression is controversial: it seems to act by inhibiting IL-2 production (97), but there are contradictory reports about the role of CTLA-4 (80, 81, 98-101) and/or membrane-bound TGF- \exists (80, 81, 96, 101-103).

In addition, several subtypes of induced (or adaptive) Tregs have been described, which could be suitable therapeutic targets for MHC based multimers:

1) T regulatory 1 (Tr1) cells: the case for cytokine-mediated suppression was made by Roncarolo and colleagues, who discovered that naïve human CD4⁺ T cells could be induced into an anergic state if stimulated in the presence of IL-10 (104). This effect is more pronounced if IFN- γ is present (105). These cells, later called Tr1 (106) produce copious amounts of IL-10 and TGF- \exists , moderate amounts of IFN- γ (but little or no IL-2 upon restimulation (107). The generation of Tr1-like cells have also been described following repetitive stimulation with immature DCs in vitro (108), continuous antigenic stimulation in vivo (109), activation through CD3 and CD46 (110), treatment with the immunosuppressive drugs vitamin D3 and dexamethasone (111).

2) Th3 cells: they are generated in vivo after oral administration of Ag; their suppressive effect is mediated by TGF- \exists . These cells have been described in an EAE model, where oral administration of the pathogenic Ag MBP suppresses EAE. This protective effect was found to be mediated by the induction of CD4⁺ cells isolated from mesenteric LNs, structurally identical to Th1 pathogenic CD4⁺ clones in terms of TCR usage, MHC

restriction and epitope recognition but producing TGF- \exists and various amounts of IL-4 and IL-10 (112).

3) Induced CD4⁺CD25⁺ T cells: Walker et al. recently described that, in contrast to mouse studies, activation of human CD4⁺CD25⁻ T cells with anti-CD3 and anti-CD28 mAbs led to expression of FoxP3 and acquisition of an anergic state and cell contact-dependent, cytokine-independent suppressor properties, similar to the thimically derived CD4⁺CD25⁺ Tregs described in mice (113). Conversion of murine and human naïve peripheral CD4⁺CD25⁻ T cells into CD25⁺ FoxP3⁺ contact-dependent anergic/suppressor cells has also been described following TCR stimulation in the presence of TGF- \exists (114, 115). The implication of these findings is that during the normal activation processes of CD4⁺CD25⁻ T cells in an immune response, both effector and regulatory populations probably arise.

4) Anergic T cells: it is sometimes difficult to reconcile earlier studies with recent work due to differences in nomenclature or molecular markers. Nevertheless, Ag-initiated phenomena of functional unresponsiveness have been seen under different circumstances for a variety of T cells, a set of outcomes which we view as representing different aspects of anergy. In some cases, such anergic T cells are also capable of suppressing other neighboring lymphocytes, thus further linking the anergic and regulatory phenotypes (116-118). This T cell suppression acts through T cell contact with the APC (118) and delivery of an inhibitory signal to APCs, which is dependent on the presence of a cognate peptide/MHC-TCR interaction and is cytokine-independent (119). This negative

conditioning of the APCs acts either by downregulating the expression of MHC Class II and costimulatory molecules in immature dendritic cells (120, 121) or by apoptosis induction through the Fas/FasL pathway in mature dendritic cells (121). The lack of CD40L (CD154) upregulation on anergic T cells, and subsequent lack of triggering of CD40 expressed on DCs, may be responsible at least for the apoptotic effect; indeed, the suppressive effect on mature DCs could be mimicked or blocked by the addition of blocking or agonistic anti-CD40 mAbs, respectively (121).

5) Cytotoxic CD4⁺CD25⁺ T cells: these cells have been described in a mouse in vitro system by the group of J.-M. Saint-Remy (122), and were found to lyse Ag-presenting B cells, but not DCs, via Fas-FasL and only in the presence of the cognate peptide. Although cytotoxic CD4⁺ T cells have been widely described both in vitro and ex vivo (123-125), this subtype was considered to be a new subset of CD4⁺CD25⁺ regulatory cells because they were unresponsive to stimulation with their cognate peptide, did not make any IL-2 but were dependent on it for their growth, constitutively expressed CD25 and could down-regulate the proliferation of bystander T cells of different specificities through their cytotoxic action on APCs. The phenotype of cytotoxic activity despite a lack of proliferation has been previously described both in CD8⁺ T cells (126) and in cytotoxic CD4⁺ T cells (124).

The relationships among natural CD4⁺CD25⁺ T cells and different adaptive Tregs are a matter of debate, but they do not seem to act independently. Therefore, a potential amplification of the therapeutic effect of Ag-elicited regulatory mechanisms could further

increase efficacy, since it has been shown that CD4⁺CD25⁺ Tregs can induce additional populations of suppressor T cells that exert distinct but complementary regulatory mechanisms (96, 127, 128). Moreover, the suppressive effect of IL-10 and TGF- β is mediated not only by their anti-proliferative effects on T cells, but also by their interference on DC maturation. Therefore, several types of IL-10- or TGF- β -secreting Tregs could induce immature tolerogenic DCs that could then promote the differentiation of naïve CD4⁺ T cells into Tr1 cells. CD4⁺CD25⁺ T cells have also been shown to inhibit maturation of DCs (88, 89, 92), with potential further amplification of the tolerogenic effect. TGF- β signaling is also capable of inducing IL-10 production (129) and of converting CD4⁺CD25⁻ naïve T cells into CD25⁺ FoxP3⁺ regulatory cells (114). These feedback loops between natural CD4⁺CD25⁺ and adaptive Tregs might also be the key to interpret in vivo evidence supporting the role of cytokines (IL-10 and TGF- β) for suppression, at variance with in vitro observations (130).

Current issues

The induction of AICD and anergy by MHC-based reagents could therefore not only act to quench pathogenic autoreactive T cells, but also through active regulatory mechanisms. However, the therapeutic deletion of T cells by TMs should be contemplated with caution, because self-reactive T cells could potentially be activated and gain full effector function before the death of these cells is induced (131-136). Indeed, we showed that autoreactive T cells which eventually undergo apoptosis are the ones which proliferate the most (67). Passage through an activation phase has also been demonstrated for T cells anergized in vivo (72, 73, 137, 138). This general principle

would be true not only at a single clonotypic level, where activation is a necessary preamble to AICD, but also for T cell clones of different avidity. A strong apoptotic stimulus for a high avidity clone could easily be only an activation stimulus for a low avidity clone (38). Whether this avidity-based hierarchy stands true also for other tolerogenic outcomes is currently under investigation, and may have broader implications for T cell regulation. Different types of T cell anergy have been shown to be induced by different Ag doses: suboptimal doses induced hyporesponsiveness, optimal doses induced an anergic state with acquisition of suppressive properties, while supraoptimal doses led to a persistent anergic/suppressive phenotype not reversed by IL-2 (139).

As discussed, not all the islet autoreactive T cells that escape central tolerance differentiate into diabetogenic T cells. In fact, some autoreactive T cells acquire immunoregulatory properties during development (47). MHC multimers could similarly be used to induce such properties. Perhaps the most interesting feature of all (natural and adaptive) Treg cells is their Ag-nonspecific suppression once activated. This is a potentially major therapeutic advantage, especially compared to the alternative solution of targeting several autoimmune specificities for deletion (Fig. 4). If we depict autoimmunity as an imbalance between autoreactive pathogenic and regulatory T cells, with a predominance of the former (140, 141), we could intervene therapeutically by eliminating pathogenic T cells through mechanisms such as AICD and anergy (therapy 1 in Fig. 4). This approach is however difficult, because, as discussed, it would require targeting of several specificities, most of which are likely unknown. Even if the “deletional” intervention is attempted early when the autoimmune process has not yet spread to other autoAgs that could propagate disease, autoreactivity might recur directed towards different Ags. A

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different approach would be to potentiate regulatory mechanisms with the induction of different kinds of Tregs, for example through IL-10 or TGF- β treatment (therapy 2 in Fig. 4). This is also the main effect probably induced by anti-CD3 treatment (55-59). However, potential side effects due to generalized immune suppression might arise in the long term, including the induction of tumor growth.

A third approach could be to exploit the advantages of Ag-specific immune modulation through induction of both deletional and regulatory effects (therapy 3 in Fig. 4). In this respect, MHC-based reagents could be better candidates than self peptide or whole protein vaccinations because of their longer half life (142, 143) and more uniform Ag presentation and subsequent TCR signaling. Moreover, delivery of the peptide epitope already loaded on the MHC Class II molecule would avoid the undesired effect of activating cytotoxic CD8⁺ T cells triggered by peptides containing additional binding motifs for Class I alleles (144). The deletional effects of MHC multimers would affect only that fraction of autoreactive T cells specifically targeted. Nonetheless, it would help decreasing the bulk of pathogenic T cells, thus easing the subsequent suppressive effect of induced Tregs (73). Furthermore, the concomitant Ag-specific induction of Tregs would likely deliver not only a direct suppressive effect on the targeted specificity, but also bystander suppression to other pathogenic T cells present locally. The selective targeting of islet specificities should at the same time ensure a fairly site-specific bystander suppression, with fewer systemic effects.

Conclusions

A central problem of type 1 diabetes is that an irreversible destruction of insulin-producing β cells has already occurred once diagnosis is made. At this stage, we can only cure the consequences, not the causes, of autoimmunity. Hence the importance of immunologic tools for T cell analysis to monitor subjects with ongoing autoimmunity and intervene earlier. MHC multimers potentially provide such tools, and also offer novel activation-based therapeutics for directing and deviating T cell responses towards a regulated homeostasis.

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Figure Legends

Figure 1. The trimolecular interaction between a peptide-MHC complex and the T cell receptor (TCR). This is the molecular mechanism allowing CD4⁺ T cells to sense antigenic peptides presented by antigen-presenting cells (APCs) and to become activated during an immune response (**panel A**). A similar mechanism accounts for CD8⁺ T cell activation through binding of peptide-MHC Class I complexes (**panel B**). The ability of antigen-specific CD4⁺ and CD8⁺ T cells to recognize and bind a particular peptide-MHC complex is exploited to identify these T cells with MHC tetramer reagents.

Figure 2. A, peripheral blood mononuclear cells, here obtained from a diabetic patient, can be stained with GAD-loaded MHC Class II tetramers (TMrs) after in vitro expansion to visualize islet antigen-specific T cells as a population of TMr⁺ cells. **B**, TMr⁺ cells can subsequently be single cell-sorted to generate a clonal T cell population for further functional characterization.

Figure 3. Correlation between number of GAD TMr⁺ CD4⁺ T cells present in peripheral blood and disease state. Type 1 diabetic subjects, at risk individuals (i.e., positive for HLA Class II DR4 susceptibility alleles and for anti-islet aAbs) and healthy controls were studied. Adapted from (17).

Figure 4. Balance between autoreactive pathogenic and regulatory T cells in health and autoimmune disease, and possible therapeutic interventions through: elimination of

pathogenic T cells (therapy 1); induction of regulatory T cells (therapy 2); a combination of the two (therapy 3). See text for details.

	Naturally occurring CD4 ⁺ CD25 ⁺	Tr1	Th3	Peripheral CD4 ⁺ CD25 ⁺	Anergic	Cytotoxic CD4 ⁺ CD25 ⁺
Anergy	yes	yes	yes	yes	yes	yes
Specificity of suppression	nonspecific	nonspecific	nonspecific	nonspecific	nonspecific	nonspecific
Need for activation to suppress	yes	yes	yes	yes	yes	yes
Cytokine-mediated suppression	?	IL-10, TGF- βββ	TGF-βββ, IL- 10	?	no	no
Contact-mediated suppression	yes	no	no	yes	yes (T-APC)	yes (T-APC)
IL-2-dependent proliferation and reversal of suppression	yes	no	no	yes	yes	yes
CD25⁺	yes	yes	yes	yes	yes	yes
FoxP3⁺	yes	no	no	yes	?	?
Induction	/	immature DC IL-10, IFN-γ Vit. D ₃ /Dexa	oral antigens TGF-βββ, IL- 4	anti-CD3 or peptide-MHC with costim	anti-CD3 without costim T-T presentation	repetitive stimulation

Table 1. Different subtypes of naturally occurring and induced (adaptive) regulatory T cells.

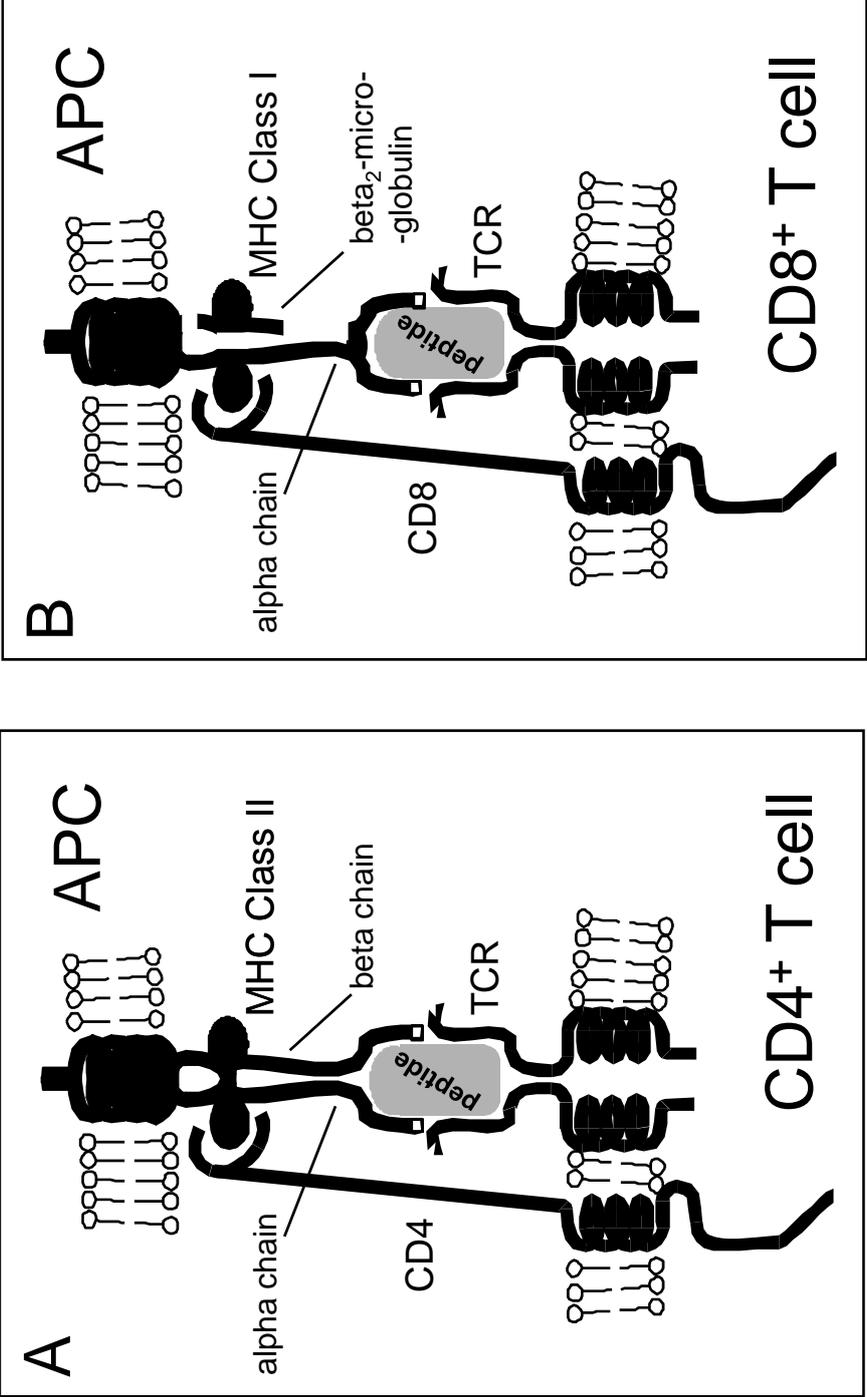


Fig. 1
R. Mallone and G.T. Nepom

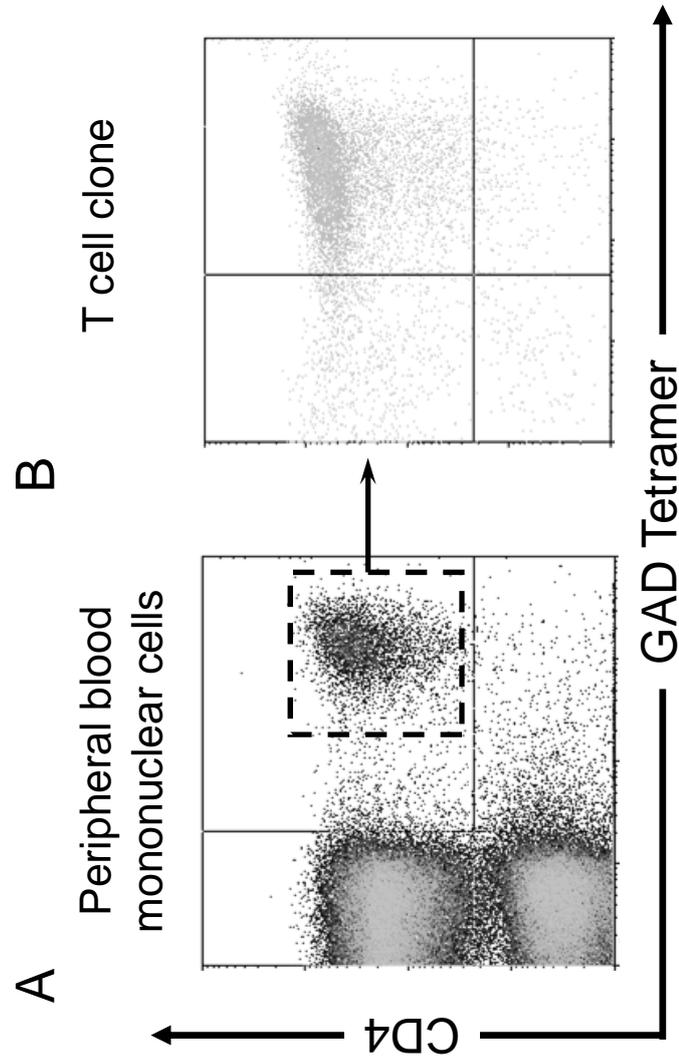


Fig. 2
R. Mallone and G.T. Nepom

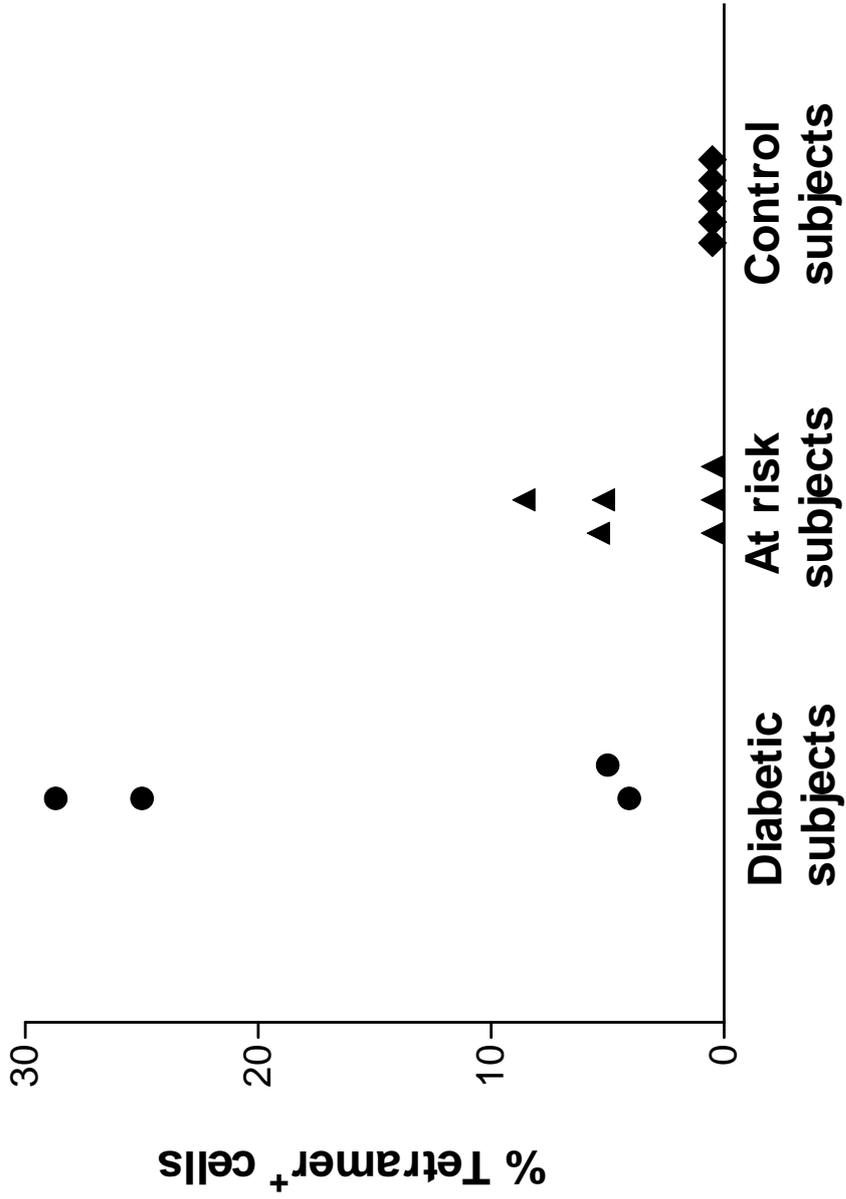


Fig. 3
R. Mallone and G.T. Nepom

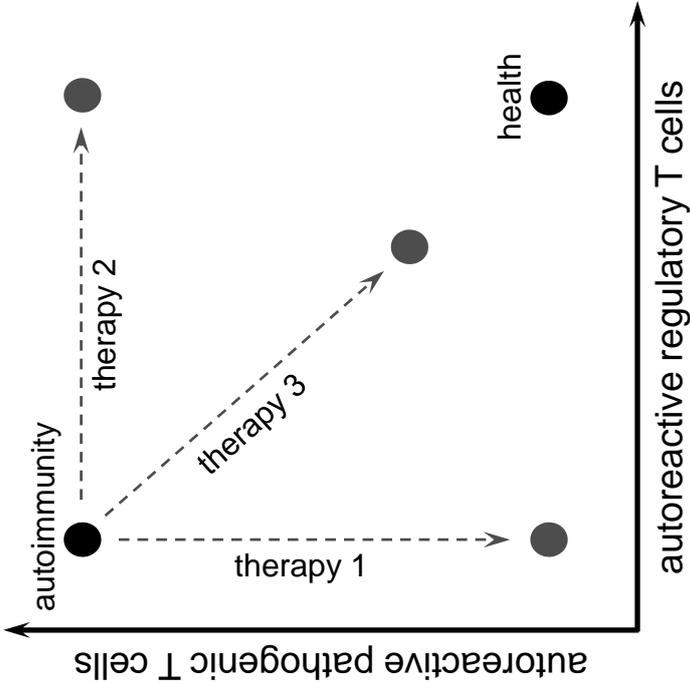


Fig. 4
R. Mallone and G.T. Nepom