

EDITORIAL

Clinical & Experimental Allergy

CD4 allergen tetramers

This editorial discusses the findings of the paper in this issue by M. Bonvalet et al. [2] pp. 821–829.

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The introduction of class II tetramers for identifying antigen-binding CD4⁺ cells has lagged behind the use of class I tetramers because of difficulties in producing the recombinant MHC scaffolds. Instead of requiring a single MHC chain fused to the invariant β 2-microglobulin it has required the expression and combining of two polymorphic chains, which are often unstable without a peptide in the peptide-binding groove. They were reported in 1999 but refinements in their expression have been ongoing and each class II molecule has required customized procedures. It has been difficult to refold the MHC class II complex from *Escherichia coli* inclusion bodies as used for the class I so the production of soluble complexes in eukaryotic cells has been favoured [1]. The adoption of the technology for allergy research has been cautious but is now clear that it has much to offer and, as shown by Bonvalet et al. [2], may circumvent the shortcomings that have bedevilled *in vitro* studies of T cell responses.

The initial report of allergen class II tetramer binding was performed with rye-grass allergy [3] with a Lol p 1 epitope restricted by an HLA-DRB1 allele. The tetramers bound T cells from cultures of allergen-stimulated peripheral blood mononuclear cells (PBMC) from allergic but not non-allergic donors. Dual staining showed that IL-4 could be induced in the tetramer-staining cells. There was, however, no increase in tetramer staining in the pollen season, when T cell responses are known to increase and the tetramer binding could not be detected when cells were examined immediately *ex vivo*.

Far more encouraging results were found with the cat allergen Fel d 1 [4]. Using a single epitope presented by HLA DRB1*0101 tetramer binding to CD4⁺ cells

was achieved without the need for earlier *in vitro* allergen stimulation. The frequency of detection was 0.014–0.053% for the patients with atopic dermatitis and 0.003% for controls. The frequencies increased after *in vitro* stimulation with peptide showing the veracity of the initial staining. The tetramer⁺ cells were central memory cells. Seventy-seven percent expressed CCR7 and/or CD62L but with high expression of the co-stimulatory molecules CD27 and CD28 suggesting that could be readily activated and many did express the activation marker CD38. There was a high prevalence of the Th2-associated chemokine receptors CCR4 and CCR8 and the skin homing marker CLA increased, which might be expected for cells from atopic dermatitis patients. Cytokine production by tetramer⁺ cells was not examined but IL-4 production in peptide-stimulated cultures correlated with the frequency of tetramer staining with a similar trend for IFN- γ .

These results have been independently confirmed with modifications to improve the detection of *ex vivo* binding [5]. Six epitopes of Fel d 1, each presented by a different HLA-DR molecule [5], were selected by a procedure called tetramer-guided epitope mapping. Here T cells are stimulated with a pool of peptides representing an antigen and the T cell expansion read out by tetramer binding itself. A two-step tetramer cell-analysis was also introduced where cells were incubated with phycoerythrin-conjugated tetramers and enriched on anti-phycoerythrin coupled beads before being examined by flow cytometry. A high frequency of tetramer binding was found in cells from allergic subjects, typically about 0.01%. Tetramer staining of cells from non-allergic donors was barely detectable. Similar to the study by Bateman and colleagues, most of the tetramer⁺ cells were Th2-type central memory cells. Most cells were negative for the Th1 chemokine receptor CXCR3 and the Th17-associated CCR6 receptor.

Ex vivo staining was also readily accomplished for tetramers made with a wasp Ves v 5 peptide [6]. Similar to Fel d 1 about 0.05% of CD4⁺ cells from wasp allergic subjects showed binding and about 0.01% of the CD4⁺ cells from non-allergic subjects. The tetramers were able

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to track an increase and then a decrease of epitope-binding cells during immunotherapy and to show that effectors and regulators most likely developed from the differentiation of a single precursor.

A tetramer has also been developed for the major house dust mite (HDM) allergen Der p 1. It bound 5–23% of CD4⁺ T cells from peptide-stimulated cultures [7] but attempts to detect the binding without culture were not reported. The binding frequency for individual atopic dermatitis patients [8] correlated with IL-4 production and disease exacerbation but the severity of the disease of different patients could not be predicted by the tetramer staining.

The mugwort allergen unusually induces a dominant response to the peptide p25–36 restricted by a number of related HLA-DRB1 alleles. A tetramer made with the peptide bound T cells from Art v 1 stimulated cultures and more promisingly the authors stated in discussion that *ex vivo* detection appeared possible [9].

Some important aspects of tetramer binding have been illustrated with the p127–142 epitope of the major cow allergen Bos d 2 [10, 11]. The degree of proliferation of the T cell clones induced by *in vitro* stimulation with p127–142 correlated with the avidity of tetramer binding and a single amino substitution that increased the activation also increased tetramer binding. A clone that was able to proliferate and produce cytokine in response to p127–142, however, did not bind the tetramer but bound the substituted high avidity tetramer. Strategies to detect lower affinity binding are required and this concurs with observations of autoimmunity and virus infections in mice where low affinity T cells produced at high frequency are important for effector function [12]. Like the studies with grass pollen cultures the cells had an effector memory phenotype.

In a forerunner to the study of Bonvalet and colleagues, tetramer binding in birch pollen allergy was studied using the epitope p141–155 of Bet v 1 [13, 14]. For most of the study, PBMC were stimulated with li-Key-fused peptides and cultured for 2–4 weeks. li-Key is a small sequence (LRMK) from the MHC class II-associated invariant chain, which facilitates the loading of epitopes to the MHC class II groove. It has been shown to enhance T cell activation by allergens [14] and other antigens and appears not to bias differentiation along different cytokine pathways. Typically 1–2% of the CD4⁺ cells bound the tetramer and after longer culture tetramers were found for non-allergic subjects. The phenotype of the tetramer⁺ cells from the allergic subjects were, however, mainly CD62L[−] CCR7[−], indicating an effector memory, while most cells from the non-allergic subjects were CD62L⁺ CCR7⁺ indicating central memory. The cells from allergic patients produced Th2 cytokines with a low percentage of cells producing IFN- γ , while cells from the non-allergic subjects had a high proportion producing IFN- γ . IL-10 was similar in

both. In keeping with the cytokine profile, CXCR3 was found more frequently on cells from non-allergic subjects while CCR4 was found on 10% of cells regardless of allergic status. Although not studied in detail the tetramer-binding frequency rose from about 0.0002 out of pollen season to a very remarkable 0.1% during season. Interesting the frequency also rose impressively for non-allergic subjects.

Bonvalet *et al.* [2] have now used the li-Key peptide stimulation strategy to compare tetramer binding and CD154 activation in cultures maintained with epitopes from Bet v 1, Der p 1 and Phl p 1. The main aim was to determine if staining for CD154, the CD40 ligand, could be used as a marker for epitope responsive cells. CD40L is immediately and transiently up-regulated upon antigen activated and has been used in other antigen systems to identify allergen-specific T cells. Contrary to this, the correlation of tetramer and CD154 staining was very poor. Only 12–25% of CD154⁺ cells bound the tetramers. An average of 70% of tetramer⁺ cells bound CD154 but this could be as low as 3% for some patients. Staining of the CD154⁺ tetramer[−] cells with anti-TCR showed that they were expressing abundant TCR, presumably of other specificities. Peptide-induced cytokine production showed that the CD154[−] tetramer⁺ cells were anergic and rarely released IL-5 or IL-10. CD154⁺ tetramer[−] cells did release IL-5 and IL-10 and RT-PCR for IFN- γ , IL-10, IL-13 and IL-17 gene expression showed few transcripts for IFN- γ but transcripts were detected for the other cytokines and they were markedly more abundant in the tetramer⁺ CD154⁺ cells. Other activation markers CD25, CD30, GITR and ICOS were also not confined to the tetramer⁺ cells.

The *in vitro* culture might be responsible for the disconnect between activation markers and epitope binding. The cytokine data showed that CD154⁺ tetramer[−] cells were not activated for allergic effector functions so the most logical conclusion is that over 75% of cells in the allergen-stimulated cultures were activated by bystander interactions. The finding that a large and highly variable percentage of the tetramer-staining cells were anergic or exhausted has similar repercussions. Similar exhaustion has been demonstrated following prolonged antigen stimulation of TCR transgenic mice so the CD154[−] tetramer⁺ cells in allergen cultures may be of interest and as pointed out [2] could be a new measure of changes induced during immunotherapy.

The compilation of data from different allergies should be viewed with the knowledge they induce very different types of immune response. HDM allergens induce high IgE and high IgG titres [15], which are restricted to allergic subjects and for IgG, vary with lower responses in adults and in children [15] susceptible to exacerbation [15, 16]. The anti-Fel d 1 IgE titres in adults who develop rhinoconjunctivitis after exposure to cats are typically 100-fold less than those to HDM [17] but the IgG titres are high and

are also found in non-allergic subjects [18]. Grass pollen allergic subjects produce similar IgE titres to HDM but the IgG titres, which are mainly restricted to allergic subjects, are very low [19]. These differences are presumable underpinned by differences in T cell responses. There are also differences between individuals responding to the same allergens. Now that pure allergens are available for quantitative studies, it would seem prudent to profile the donors before embarking on complex experiments.

A dream outcome for the tetramers would be to profile the phenotype of allergen-reactive T cells without the artefacts induced by *in vitro* culture. The feedback of IL-4 on Th1 responses in cell culture [20] has always been a contentious issue and it is now known that both Th1 and Th2 cytokines feed back on Th17 responses [21]. The discrepancy between the dominant central memory responses found in the *ex vivo* CD4⁺ cells of allergic subjects and effector memory response found in cultures of cells from pollen- and cow-allergic subjects obviously needs to be resolved. Is it a culture artefact or are there differences in responses to different allergens? It can be readily envisaged that the distribution of allergen-specific T cells within different functional and differentiation compartments can be informative and that modest quantitative differences might be important. As well as providing an avenue to assess the relationship between T cell activation and disease these studies could assess the importance of individual allergens for sensitization and to monitor treatments including immunotherapy. Developments to enable the application of tetramers to more patients are therefore critical. These will include the ongoing improvement in the production of the MHC scaffolds for the presentation of peptides with a wider range of MHC restrictions and more specifically for allergy, identifying a wider range of epitopes that can be studied with the existing technology. Tetramer-guided epitope mapping could have application here as well as the use of multiplexing for different epitopes [5]. It also appears that more can be done to harness the power of flow cytometry, which can currently analyse cell subpopulations as small

as 0.0001% [22] with speeds of 100 000 cells per second. As shown in cow dander allergy further development to detect lower avidity binding would be required to interrogate the full allergen-reactive repertoire. Investigators in the tetramer area have already flagged the use of higher order oligomers.

The current tetramer technology has much to offer. Limit dilution studies with allergen extracts have shown that T cells responding to allergens are present at high frequency in PBMC. Typical results from studies for HDM show frequencies of 0.05% for allergic subjects and 0.01% for non-allergic subjects [23]. A more recent estimate for birch and grass pollen extracts using dye dilution analysis found a median frequency of 0.56% of CD4⁺ cells in allergic subjects and a median of 0.13% on non-allergic subjects [24]. These high values concur with the tetramer study of Van Overtvelt *et al.* [13]. For comparison the frequencies of T cell precursors of immunologically naïve subjects have been shown to be in the region of 0.001% for KLH [25] and hepatitis B [26] and these rise to about 0.02% after vaccination. Thus even non-allergic subjects show evidence of a large degree of expansion when compared to the effect of deliberate immunization. At least for cat and HDM allergens the high frequency of T cells concurs with the presence of IgG antibody [15, 18] with the same titres found for responses to bacterial infections [16] and to vaccination [27]. Anti-allergen responses should accordingly be just as amenable to tetramer analysis as anti-microbial responses. The epitopes of cat and wasp allergens have T cell-binding frequencies of 0.01–0.05% that can be directly analysed from modest samples of blood and it was considered that mugwort allergen could be similarly studied. The astonishing frequency of 0.1% for a single birch tetramer found in the pollen season further emphasises the scope for *ex vivo* analysis. As shown by Bonvalet *et al.* [2] combined tetramer binding and CD154 activation has the potential to be a very informative combination along with chemokine receptors and other markers to define T cell differentiation pathways.

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