

ORIGINAL ARTICLE

Comparison between major histocompatibility complex class II tetramer staining and surface expression of activation markers for the detection of allergen-specific CD4⁺ T cells

M. Bonvalet¹, E. Wambre^{1,2}, H. Moussu¹, S. Horiot¹, W. W. Kwok², A. Louise³, D. Ebo⁴, C. Hoarau⁵, L. Van Overtvelt¹, V. Baron-Bodo¹ and P. Moingeon¹

¹Stallergenes SA, Antony, France, ²Benaroya Research Institute, Seattle, WA, USA, ³Plateforme de cytométrie de flux, Institut Pasteur, Paris, France, ⁴Department of Immunology, Allergy and Rheumatology, Antwerp University, Antwerp, Belgium and ⁵Unité transversale d'allergologie et Immunologie clinique, CHRU Tours, France

Clinical & Experimental Allergy

Summary

Background Major histocompatibility complex (MHC) class II tetramers (tetramers) allow to detect allergen-specific CD4⁺ T cells at a single-cell level. Limits to this technology include HLA restriction and the need to identify immunodominant T cell epitopes.

Objective Assessing the expression of various activation markers following allergen stimulation to replace tetramer staining.

Methods Peripheral blood mononuclear cells (PBMCs) from 25 birch pollen, grass pollen or house dust mite allergic individuals were stimulated with peptide mixes encompassing immunodominant epitopes from corresponding major allergens. After 2 weeks of *in vitro* amplification, cells were stained with both the appropriate tetramer and antibodies directed to CD25, CD30, CD39, CD69, CD137, CD154, GITR, HLA-DR and ICOS, before FACS analysis.

Results Following allergen stimulation, percentages of tetramer⁺ cells among CD4⁺ CD154⁺ cells range from 5% to 87%, depending upon donors. As for CD154, a large inter-individual variability is observed in terms of surface expression for all activation markers tested in allergen-stimulated PBMCs. T cells reactive with either tetramers (0.4–10.4% CD4⁺ T cells) or anti-marker antibodies (2.2–32.7% CD4⁺ T cells), but not both, are observed, reflecting the presence of anergic as well as non-specifically activated cells. Tetramer⁺/marker⁺, tetramer⁺/marker[−] and tetramer[−]/marker⁺ cells were compared for their capacity to express cytokines, demonstrating that only the former represent *bona fide* allergen-specific activated CD4⁺ T cells, based upon a higher expression of cytokines or corresponding genes in presence of the allergen.

Conclusion and Clinical Relevance No strict correlation exists between tetramer staining and the expression of multiple activation markers in stimulated CD4⁺ T cells. Dual staining allows to discriminate functional tetramer⁺/marker⁺ vs. anergic (tetramer⁺/marker[−]) allergen-specific T cells or non-specifically activated (tetramer[−]/marker⁺) T cells. Combining tetramer staining with the detection of activation markers helps understanding patient heterogeneity regarding specific CD4⁺ T cell responses. This approach has immediate relevance for monitoring immune changes induced during specific immunotherapy.

Keywords activation markers, CD154, MHC class II tetramers

Submitted 2 November 2010; revised 23 December 2010; accepted 11 January 2010

Correspondence:

Philippe Moingeon, Research and Development, Stallergenes SA, 6 rue Alexis de Tocqueville, 92183 Antony Cedex, France. E-mail: pmoingeon@stallergenes.fr

Introduction

Monitoring allergen-specific CD4⁺ T cells at a single-cell level allows a better understanding of the physiopathology of allergic immune inflammation, as well as the nature of protective responses observed in healthy individuals [1–3].

It is also needed in the course of immunotherapy studies, with the aim to establish immunological correlates of clinical efficacy. Various functional assays conducted following allergen stimulation are available to analyse specific CD4⁺ T cells, such as proliferation assessment, intracellular cytokine staining, cytokine secretion assay (CSA) and

ELISPOT [4, 5]. However, immunodetection using major histocompatibility complex (MHC) class II/peptide tetramers (tetramers) is currently considered as the most specific approach in order to identify allergen-reactive CD4⁺ T cells [6–8]. As of today, several tetramers have been successfully developed to detect T cells directed against allergens such as Art v 1, Bet v 1, Bos d 2, Der p 1, Der p 2, Fel d 1, Lol p 1 and Ves v 5 [3, 9–14]. Nonetheless, tetramer-based detection remains a cumbersome technique which can only be applied to the follow-up of responses against selected T cell epitopes in individuals bearing appropriate HLA haplotypes.

Thus, the purpose of our study was to find an alternative method for detecting allergen-specific CD4⁺ T cells within peripheral blood mononuclear cells (PBMCs) after specific *in vitro* amplification. CD154 (CD40L) was recently described as an activation marker selectively expressed by specific CD4⁺ T cells after antigen stimulation [15–17]. Herein, we assessed whether surface detection of CD154 as well as other various activation markers, could be used as a substitute to tetramer staining. These markers include CD25 (IL-2RA receptor) [18], CD30, CD137 and GITR (members of the TNF-receptor superfamily) [19–22], CD154 (a member of the TNF-ligand superfamily) [15–17], CD39 (ectonucleoside triphosphate diphosphohydrolase) [23], CD69 (a member of the calcium-dependent lectin superfamily) [18], as well as ICOS [24] and HLA-DR [18].

In this study conducted in PBMCs obtained from both healthy donors and patients suffering from allergic rhinitis to birch pollen (BP), grass pollen (GP) or house dust mite (HDM), we report a partial correlation between tetramer staining of CD4⁺ T cells and their expression of activation markers following allergen stimulation, thus pointing out to the presence of fully reactive as well as anergic cells among allergen-specific CD4⁺ T lymphocytes, together with non-specifically activated T cells.

Materials and methods

Patients and HLA-DR typing

Citrated peripheral blood was obtained after informed consent from BP-, GP- or HDM-allergic individuals recruited at Antwerp University Hospital (Belgium) and CHRU Tours (France). The study protocol was approved by an ethical committee (CPP idf7). Allergic individuals suffering from rhinitis, but without asthma or atopic dermatitis, were selected based on clinical symptoms, positive skin prick test, specific IgE levels as well as a positive *in vitro* basophil activation test performed in the presence of either BP, GP or HDM extracts (Stallergenes, Antony, France). Genomic DNA was extracted from blood using an Easy-DNA Kit (Qiagen, Courtaboeuf, France). HLA-DR typing was performed by PCR HISTO TYPE DR mini kits (Bag, Lich, Germany) and further with either Allset⁺ Gold SSP HLA-DRB1*01, HLA-DRB1*04 or HLA-DRB1*15/16 High Res Kits (Invitrogen, Carlsbad, CA, USA) [25].

In vitro expansion of allergen-specific CD4⁺ T cell

PBMCs were isolated from peripheral blood by centrifugation over Ficoll-Paque plus (Abcys, Paris, France). For *in vitro* expansion, freshly isolated PBMCs from HLA-DRB1*15:01 and HLA-DRB1*01:01 individuals allergic to BP or HDM and from HLA-DRB1*04:01 individuals allergic to GP were seeded in 12-well plates in RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 10% heat-inactivated AB serum (PAA, Les Mureaux, France), 20 µg/mL gentamicin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 50 µM 2-mercaptoethanol and 1% non-essential amino acids (all from Invitrogen) at 37 °C, 5% CO₂. Cells were then stimulated for 14–16 days with either Bet v 1_{141–155}, Der p 1_{16–30}, Der p 1_{171–185} or Phl p 1_{194–208} peptides in the form of li-key conjugate peptide mixes (used at 10–20 µg/mL) as described elsewhere [3, 26, 27]. These conjugate peptide mixes comprise complete or truncated versions of Bet v 1_{141–155}, Der p 1_{16–30}, Der p 1_{171–185} or Phl p 1_{194–208} peptides conjugated with a polymethylene linker to the li-Key moiety of the MHC class II-associated invariant chain, thereby facilitating peptide exchange and binding [27, 28]. At day 5, and every 3 days thereafter, 20 UI/mL recombinant IL-2 (Roche, Meylan, France) was added to cell cultures. A resting period of three days was always respected between the last IL-2 addition and cytometry analysis.

Major histocompatibility complex class II tetramer staining and surface detection of activation markers

After epitope-specific CD4⁺ T cell amplification for 14–16 days, cells were stained with 1 µg/mL tetramers specific for either Bet v 1_{141–155}, Der p 1_{16–30} and Der p 1_{171–185} or Phl p 1_{194–208} in the context of HLA-DRB1*01:01, *04:01 and *15:01 molecules for 1 h at room temperature, and subsequently stained with antibodies directed against activation markers, for 20 min at 4 °C. Tetramers used in this study were made by Beckman Coulter (Villepinte, France) for Bet v 1_{141–155}, Der p 1_{16–30}, Der p 1_{171–185} [29] and by the Benaroya Research Institute for Phl p 1_{194–208} [30]. Empty or CLIP peptide tetramers were used as controls. All tetramers we used were coupled to phycoerythrin. Surface markers were detected using fluorescein isothiocyanate (FITC)-labelled anti-CD30, allophycocyanin (APC)-labelled anti-CD39, FITC-labelled anti-CD69, FITC- or APC-labelled anti-CD154 (BD Biosciences, Le Pont de Claix, France), FITC- or APC-labelled anti-CD25 (Beckman Coulter), FITC- or Alexa Fluor 647-labelled anti-ICOS (Biolegend, Saint Quentin Yvelines, France), APC-labelled anti-GITR (R&D Systems, Lille, France), FITC-labelled anti-CD137 and PC7-labelled anti-HLA-DR (eBiosciences, San Diego, CA, USA) antibodies. Cells were simultaneously stained with an APC- or PC7-labelled anti-CD4, ECD-labelled anti-CD14 and -CD19 antibodies (all from Beckman Coulter) and subsequently with Via-Probe (BD Biosciences) for 10 min at 4 °C to exclude

monocytes, B cells and dead cells, respectively [26]. The expression of all activation markers was assessed without allergen restimulation, except for CD137 and CD154 for which cells were restimulated with the initial li-key conjugate peptide mix at 37 °C for 24 and 5 h, respectively [16, 17, 21, 31]. For CD154 staining, allergen restimulation was performed in the presence of an anti-CD40-specific antibody (Beckman Coulter) at 1 µg/mL to stabilize its expression [15, 16]. When assessing ICOS expression among CD4⁺ T cells, only ICOS^{high} cells were considered as activated cells. Cells were analysed using a FC500 flow cytometer (Beckman Coulter), with at least 30 000 events acquired.

Functional characterization of CD4⁺ T cells subsets

Cytokine production by either tetramer⁺/marker⁺, tetramer⁺/marker⁻ or tetramer⁻/marker⁺ CD4⁺ T cells was assessed at a single-cell level by flow cytometry using CSA or quantitative RT-PCR on sorted cells (99% purity) using a MoFlo apparatus (Dako, Glostrup, Denmark), as

described elsewhere [3, 26]. Briefly, for CSA, before stimulation for 4–5 h at 37 °C with appropriate li-key conjugate peptide mixes, cells were stained with 1 µg/mL corresponding tetramers for 1 h at room temperature. Cells were then harvested, washed with cold phosphate-buffered saline containing 0.5% BSA plus 5 mM EDTA (washing buffer) and stained with 50 µg/mL anti-IL-5/CD45 or anti-IL-10/CD45 Ab–Ab conjugates (Miltenyi Biotec, Bergish, Germany) for 10 min at 4 °C. Cytokine secretion was allowed by incubating cells in medium at 37 °C for 45 min. After washing and resuspension in washing buffer, cells were stained with either APC-labelled anti-IL-5 or IL-10 and either FITC-labelled anti-CD25, -CD30, -CD154 or -ICOS and PC7-labelled anti-CD4, ECD-labelled anti-CD14 and anti-CD19 for 25 min on ice. Cells were washed and incubated with Via-Probe for 5 min on ice before FACS analysis of at least 50 000 events.

For quantitative RT-PCR analysis, cultured cells were stained with tetramer for 1 h at room temperature, stimulated with li-key conjugate peptide mixes for 1 h at 37 °C

Table 1. Detection of MHC class II-peptide tetramer⁺ CD4⁺ T cells in allergic and non-allergic individuals

Status of donors	Donor identification	HLA-DRB1	Percentages of tetramer ⁺ cells among CD4 ⁺ T cells				Month of blood collection
			Control	Bet v 1	Der p 1	Phl p 1	
BP allergic	BP3	01:01	0.1	4.5	ND	ND	June
	BP6	15:01	0.1	1.2	ND	ND	June
	BP8	15:01	0	1.4	ND	ND	September
	BP9	15:01	0.1	10	ND	ND	May
	BP10	15:01	0.1	39	ND	ND	April
HDM allergic	HDM15	15:01	0	ND	0.7*	ND	June
	HDM17	15:01	0	ND	0.6*	ND	November
	HDM19	15:01	0.1	2.6	ND	ND	June
BP and GP allergic	BG1	15:01	0	1.8	ND	ND	February
	BG2	01:01	0	2.1	ND	ND	February
	BG3	15:01	0	3.4	ND	ND	December
GP and HDM allergic	GH1	04:01	0.1	ND	ND	7.3	December
	GH2	04:01	0.2	ND	ND	4.4	February
BP, GP and HDM allergic	BGH1	15:01	0	6.3	ND	ND	November
	BGH2	15:01	0	4	ND	ND	June
	BGH3	15:01	0	55.5	ND	ND	February
	BGH4	15:01	0	0.5	ND	ND	March
	BGH5	15:01	0	2.5	ND	ND	March
	BGH6	15:01	0	0.2	ND	ND	March
	BGH7	15:01	0	7.1	ND	ND	April
	BGH8	15:01	0	0.4	ND	ND	September
	HB11	15:01	0	12	6.9*	ND	January
	HB12	15:01	0.1	15.1	ND	ND	July
Non-allergic	NA7	01:01	0	ND	0.5 [†]	ND	October
	NA8	15:01	0	ND	1.2*	ND	December

BP, birch pollen; GP, grass pollen; HDM, house dust mite; ND, not done.

PBMCs were stimulated *in vitro* with li-key conjugate peptides before tetramer staining as described in 'Materials and methods'. Percentages of Bet v 1_{141–155}-, Der p 1_{16–30}-, Der p 1_{171–185}- or Phl p 1_{194–208}-specific CD4⁺ T cells detected with corresponding MHC class II peptide tetramers in allergic and non-allergic individuals are indicated. The control tetramer is always made with the CLIP epitope, except for DRB1*04:01 patients for which an empty tetramer is used. Tetramers used were directed to:

*Epitope Der p 1_{171–185} for DRB1*15:01 patients;

[†]Epitope Der p 1_{16–30} for DRB1*01:01 patients.

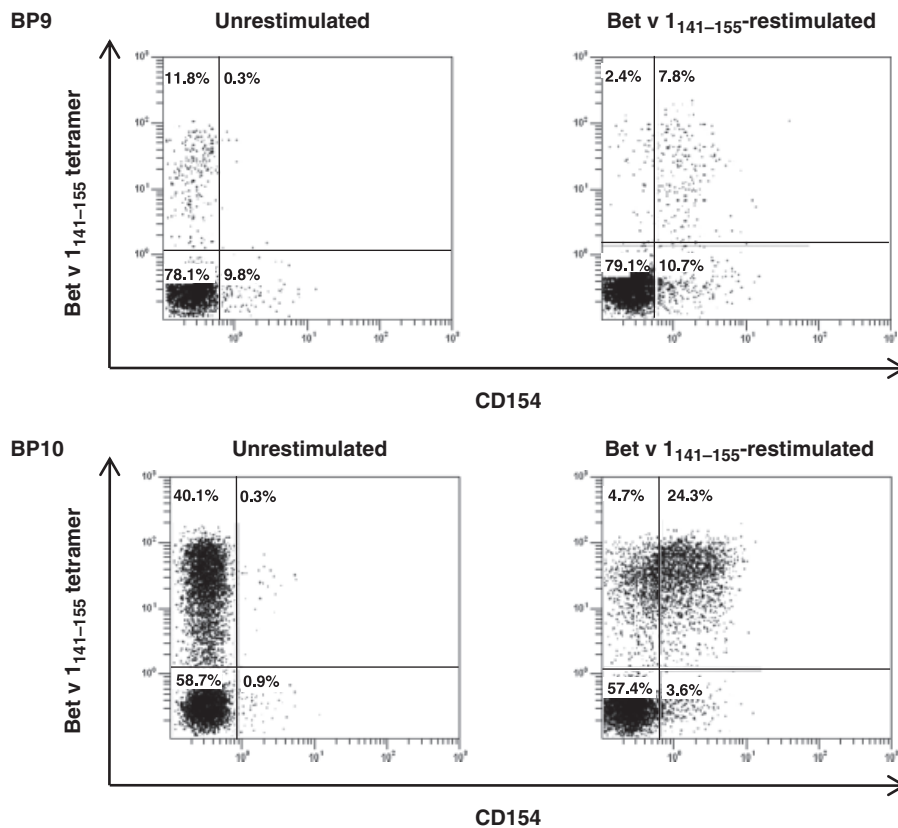


Fig. 1. Correlation between major histocompatibility complex (MHC) class II tetramer staining and CD154 surface expression. Peripheral blood mononuclear cells (PBMCs) from two representative HLA-DRB1*15:01 birch pollen allergic individuals out of eight tested were stimulated for 14–16 days with Bet v 1_{141–155} li-key conjugate peptide mixes (10 µg/mL). Before extracellular CD154 staining, cells were first stained with the appropriate tetramer and stimulated for 5 h with Bet v 1_{141–155} conjugated with li-key conjugate peptide mixes in the presence of CD40-specific blocking antibody. CLIP tetramer was used as a negative control and always stained <0.1% CD4⁺ T cells.

and stained with anti-marker antibodies, before cell sorting. RNA from sorted cell populations was harvested in RNeasy lysis buffer (Qiagen, Crawley, UK). Total RNA was extracted from each cell population with an RNeasy kit (Qiagen) and reverse transcribed as cDNAs using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. Messenger RNAs corresponding to IL-10, IL-13, IL-17A and IFN- γ were assessed by quantitative PCR using pre-designed Taqman Gene Expression reagents (Applied Biosystems) on a 7900 Real-Time PCR System (Applied Biosystems) [3]. Data were expressed as relative amounts of cytokine mRNA in sorted tetramer⁺/marker⁺ and tetramer⁻/marker⁺ CD4⁺ T cells in comparison with sorted tetramer⁻/marker⁻ CD4⁺ T cells from the same individual. Data were normalized based on relative amounts of β -actin mRNA.

Statistical analysis

Data were analysed for statistical significance using a Kruskal–Wallis test. A *P*-value < 0.05 was considered as statistically significant.

Results

Correlation between tetramer staining and surface expression of activation markers

Tetramer staining of allergen-specific CD4⁺ T cells was successfully carried out after 14–16 days of amplification in various healthy individuals as well as patients allergic to either BP, GP or HDM (Table 1). Such tetramer⁺ cells were subsequently co-stained as described in 'Materials and methods' with antibodies directed to various activation markers (i.e. CD25, CD30, CD39, CD69, CD137, CD154, GITR, HLA-DR, ICOS) before FACS analysis.

In a first set of experiments, we focused on CD154, described by others as a reliable marker allowing to detect antigen-specific CD4⁺ T cells after antigen stimulation [15–17]. Out of eight individuals tested, we found a significant correlation between tetramer and CD154 staining in PBMCs from only one individual, with tetramer⁺ cells representing 87% CD154⁺ CD4⁺ T cells (patient BP10, Fig. 1, lower panel). In other individuals, percentages of tetramer⁺ cells among CD4⁺ CD154⁺ cells ranged from

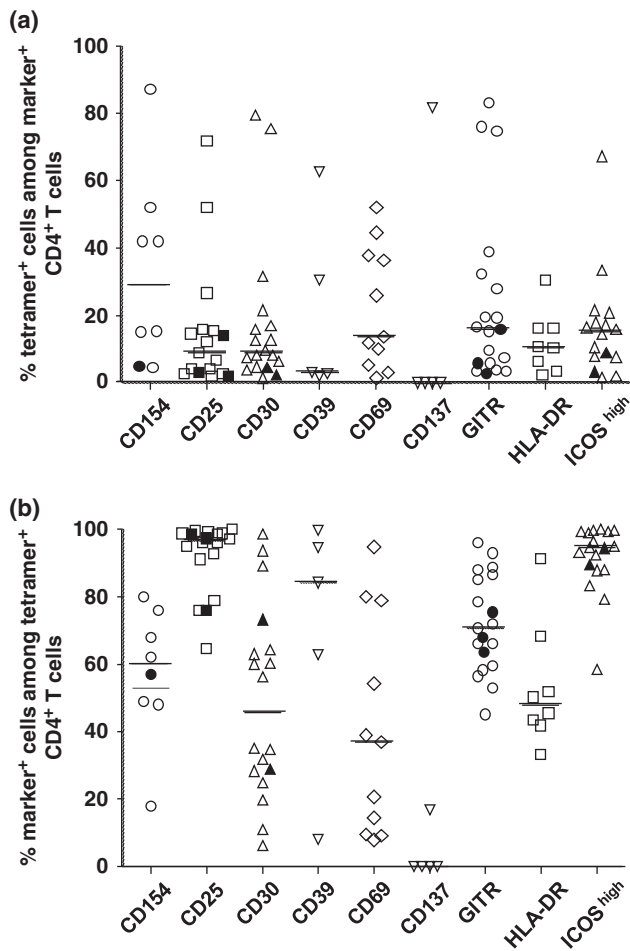


Fig. 2. Correlation between major histocompatibility complex (MHC) class II tetramer staining and surface expression of activation markers. Peripheral blood mononuclear cells (PBMCs) from 20 patients allergic to either birch pollen (BP), grass pollen (GP) or house dust mite (HDM) (open symbols) or three non Bet v 1-allergic donors (including two non atopic individuals as well as a patient allergic to HDM tested for Bet v 1 reactivity, black symbols) were stimulated for 14–16 days with appropriate li-key conjugate peptide mixes. Cells were co-stained with the appropriate tetramer and antibodies directed against two activation markers. The expression of markers was assessed without re-stimulation except for CD137, where cells were re-stimulated for 24 h after amplification. (a) Percentages of marker⁺ CD4⁺ T cells reactive with tetramers ($n = 5–20$) and (b) percentages of tetramer⁺ CD4⁺ T cells expressing given activation markers ($n = 5–20$).

5% to 52% (mean $\pm$ SEM: $25 \pm 7\%$, $n = 7$), with one representative patient (i.e. BP9), shown in Fig. 1 upper panel.

We subsequently extended these experiments to other activation markers, after determining in preliminary experiments the appropriate time-point for the optimal detection of each of these markers (data not shown). In those experiments conducted in samples from five to 20 individuals, the average percentage of marker⁺ CD4⁺ T cells reacting with specific tetramers ranged from 12% to 24%, depending upon the marker considered, with in addition a large inter-individual variability (Fig. 2a).

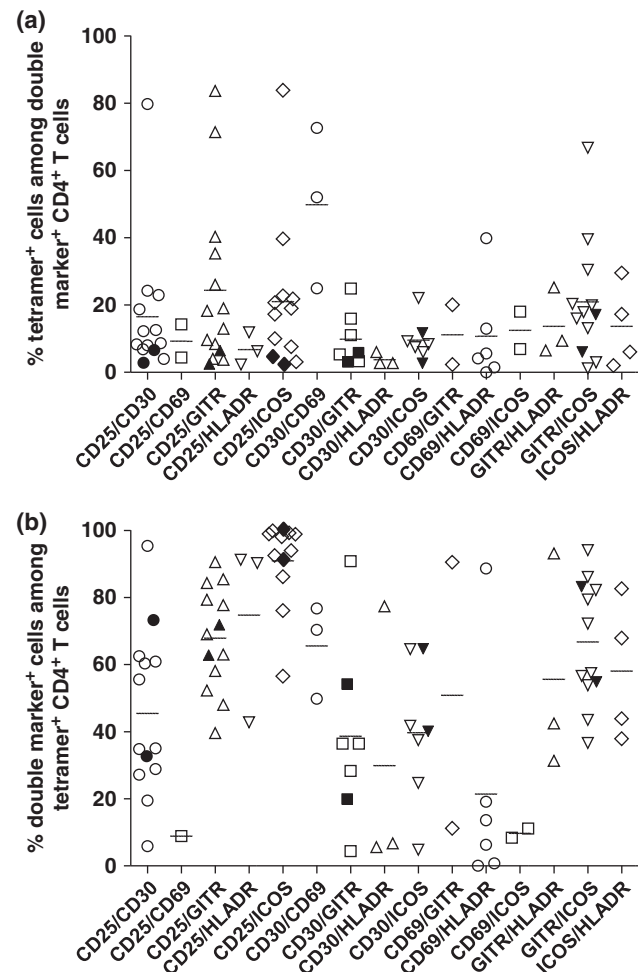


Fig. 3. Correlation between major histocompatibility complex (MHC) class II tetramer staining and surface expression of activation markers. Peripheral blood mononuclear cells (PBMCs) from 13 patients allergic to either birch pollen (BP), grass pollen (GP) or house dust mite (HDM) (open symbols) or two non Bet v 1-allergic donors (black symbols) were stimulated for 14–16 days with appropriate li-key conjugate peptide mixes. Cells were co-stained with the appropriate tetramer and two antibodies directed against various activation markers. (a) Percentages of double marker⁺ CD4⁺ T cells reactive with tetramers ($n = 1–13$) and (b) percentages of tetramer⁺ CD4⁺ T cells co-expressing given combinations of activation markers ($n = 1–13$).

Although, only a small number of samples from non-atopic individuals were included in the present study, no evidence was observed for differences between allergic (open symbols) and non-Bet v 1-allergic (filled symbols) donors. Importantly, based upon preserved staining with an anti-TCR $\alpha\beta$ antibody, we excluded tetramer[−]/marker⁺ CD4⁺ T cells that were not reacting with tetramers as a consequence of T cell receptor (TCR) down-regulation following antigen stimulation (data not shown). Thus, we conclude that such tetramer[−]/marker⁺ CD4⁺ cells represent activated cells, which are not allergen-specific. Conversely, we observed that an average of 67% (i.e. 3–92%) tetramer⁺ CD4⁺ T cells expressed surface activation

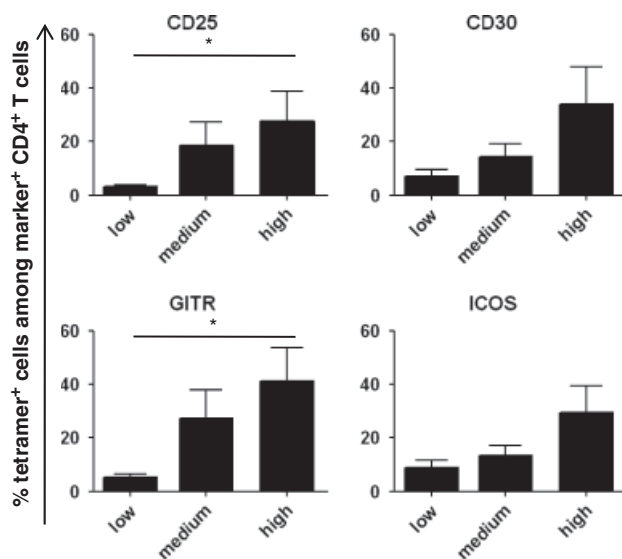


Fig. 4. Relationship between tetramer staining and allergen-specific expression of activation markers. After 14–16 days of amplification, cells were co-stained with the appropriate tetramer and antibodies directed against either CD25, CD30, GITR or ICOS, before FACS analysis. Samples were stratified in three equal groups, with respect to percentages of tetramer⁺ cells among CD4⁺ T cells as follows: low ($\leq 1.2\%$), medium ($1.2\text{--}4.5\%$) and high ($> 4.5\%$). Percentages of cells positive for a given activation marker among tetramer⁺ CD4⁺ T cells are represented in samples with low, medium or high percentages of tetramer⁺ CD4⁺ T cells. A *P*-value < 0.05 was considered as statistically significant.

markers, suggesting that not all tetramer⁺ cells were activated in the presence of the allergen (Fig. 2b). CD25 and ICOS were the only activation markers expressed by most tetramer⁺ CD4⁺ T cells. Furthermore, combining two activation markers did not improve such correlations (Fig. 3), with the best combination CD25/ICOS being expressed by 96% (SEM: 3.7, $n = 12$) tetramer⁺ CD4⁺ T cells (Fig. 3b), whereas only 22% (SEM: 6.5, $n = 12$) CD25⁺/ICOS^{high} cells reacted with tetramers (Fig. 3a). Although not shown, we failed to detect allergen-specific CD4⁺ T cells based upon CD25/OX40 (CD134) expression as proposed by others [32].

Noteworthy, when samples were subdivided into three groups based upon percentages of tetramer⁺ cells in the culture (i.e. low, medium and high), correlations between tetramer staining and marker expression were consistently superior in cell cultures containing high percentages of tetramer⁺ cells among CD4⁺ T cells, irrespective of the marker considered (Fig. 4).

Functional characterization of allergen-specific CD4⁺ T cell subsets

In this context, we further characterized functionally three subsets of CD4⁺ T cells (i.e. tetramer⁺/marker⁺, tetramer⁺/marker⁻ and tetramer⁻/marker⁺ CD4⁺ T cells) obtained from seven distinct allergic patients. To this aim, cells were

tested after *in vitro* amplification for both tetramer staining and surface expression of either CD25, CD30, GITR or ICOS activation markers. In all these cultures, IL-5 and IL-10 production was monitored in each of the three cell subsets using CSAs after 4–5 h of allergen stimulation. Beyond variations depending upon donors, the three cell subsets exhibited a distinct cytokine secretion profile, with tetramer⁺/marker⁺ (e.g. CD25⁺, CD30⁺, GITR⁺ or ICOS^{high}) cells producing in most cases higher amounts of cytokines when compared with other subsets (Fig. 5). Tetramer⁻/marker⁺ CD4⁺ T cells often produced significant levels of cytokines, thus confirming that these cells also represent truly activated CD4⁺ T cells. In contrast, tetramer⁺/marker⁻ cells consistently produced marginal levels of cytokines following allergen stimulation.

To confirm and extend these results, IL-10, IL-13, IL-17A and IFN- γ gene expression was assessed by quantitative PCR in sorted tetramer⁺/marker⁺ and tetramer⁻/marker⁺ CD4⁺ T cells purified (with more than 99% purity) out of PBMCs from three donors after 1 h of allergen stimulation. In these experiments, we failed to sort sufficient numbers of tetramer⁺/marker⁻ CD4⁺ T cells to conduct the analysis. Independently of the donor and the activation marker considered, tetramer⁺/marker⁺ CD4⁺ T cells always expressed cytokine genes at superior levels when compared with tetramer⁻/marker⁺ CD4⁺ T cells (Fig. 6).

Discussion

The analysis of allergen-specific CD4⁺ T cells is critical to better understand the physiology of immune responses in allergic and healthy individuals. Currently, tetramers are the most relevant tools to detect specific CD4⁺ T cells, but their use is restricted to the analysis of responses against immunodominant epitopes in individuals with appropriate HLA-DR haplotypes [33]. The induction of the activation marker CD154 following antigen stimulation was described as a promising alternative approach to tetramer staining in order to identify antigen-specific CD4⁺ T cells [15–17, 34]. More recently, the detection of CD154 expression was used to assess changes in CD4⁺ T cell polarization in ragweed allergic patients after immunotherapy using an Amb a 1-immunostimulatory oligodeoxynucleotide conjugate [35].

In this context, the aim of our study was to determine whether tetramer technology could be replaced or complemented by the detection of activation markers including not only CD154, but also CD25, CD30, CD39, CD69, CD137, GITR, HLA-DR and ICOS. We first attempted to detect CD154⁺ allergen-specific CD4⁺ T cells after 18 h of GP extract or rBet v 1 stimulation using intracellular or extracellular CD154 staining [16, 17, 31, 33]. Because the low frequency of allergen-specific progenitors in peripheral blood does not allow their detection without

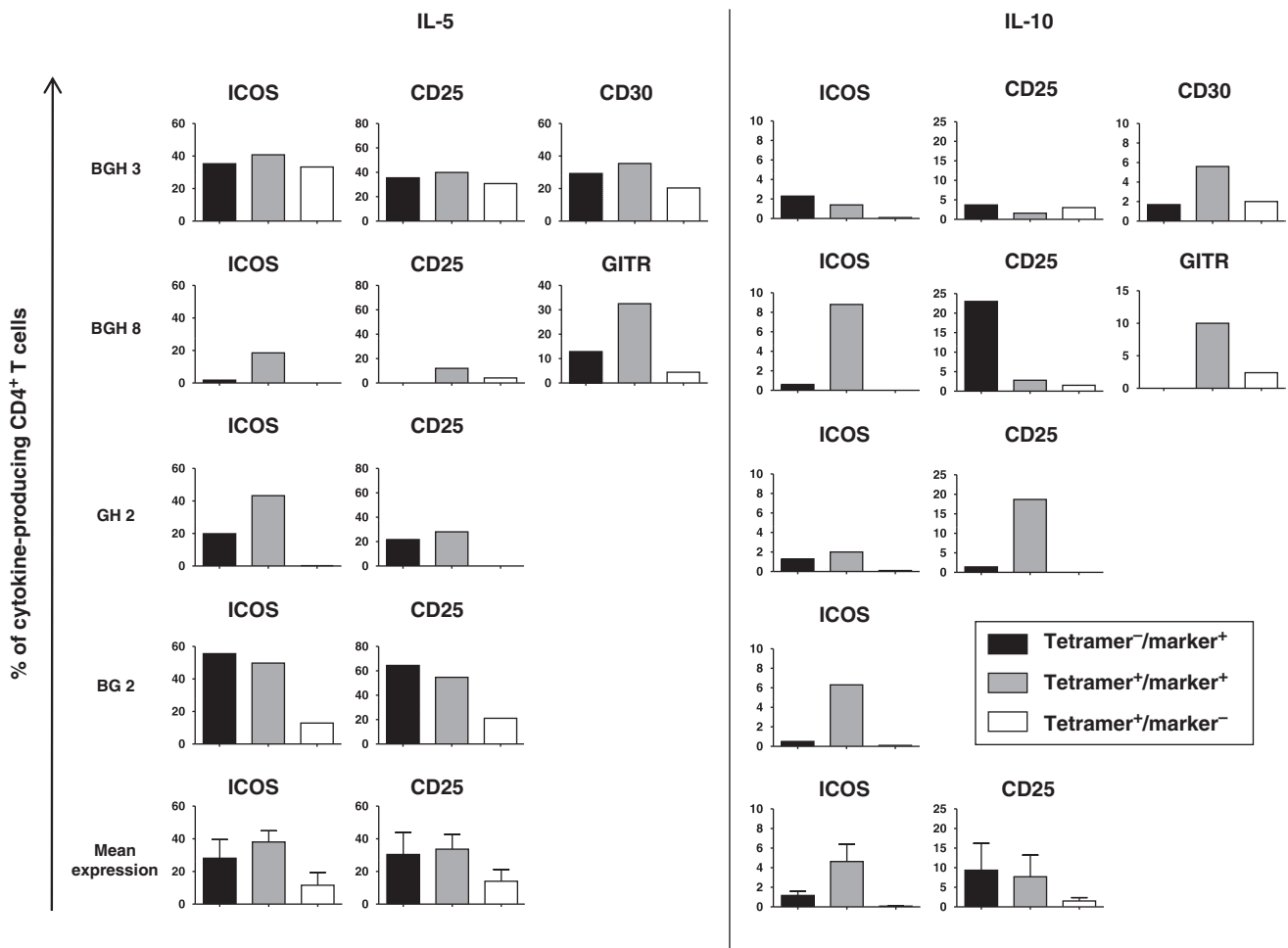


Fig. 5. Cytokine secretion by tetramer⁺/marker⁺, tetramer⁺/marker⁻ and tetramer⁻/marker⁺ CD4⁺ T cell subsets. Following 14–16 days of expansion, allergen-specific CD4⁺ T cells were stained with the appropriate tetramer before being specifically stimulated for 4–5 h with 10 µg/mL Ii-key conjugate peptide mixes. Cells were then analysed for IL-5 and IL-10 secretion at a single-cell level using a cytokine secretion assay and stained with various anti-activation marker antibodies. Data of mean expression ($n = 3-4$) ± SEM are represented in lower panels.

expansion, we recovered these cells from whole PBMCs after 14–16 days of *in vitro* allergen stimulation. In order to amplify epitope-specific CD4⁺ T cells only detected by our tetramers, we stimulated them with the Ii-key peptide conjugates. The latter were used preferentially to the whole protein because they provide a stronger expansion of well-defined epitope-specific T cells, whereas the rather whole allergen stimulates cells reactive with broader epitope repertoire [26, 27]. Importantly, in our experience, stimulation by Ii-key peptide conjugates does not skew the polarization of expanded CD4⁺ T cells [3].

We observed that 12–24% (average 18.5%) activation marker⁺ CD4⁺ T cells reacted with specific tetramers. Conversely, 3–92% (average 67%) tetramer⁺ cells were marker⁺ CD4⁺ T cells. These results highlighted three distinct populations of CD4⁺ T cells including (i) tetramer⁺/marker⁺, (ii) tetramer⁺/marker⁻ and (iii) tetramer⁻/marker⁺ cells.

Functional characterization of those populations confirmed that tetramer⁺/marker⁺ cells produce high levels

of cytokines and strongly express corresponding genes (e.g. IL-5, IL-10, IL-13 and IL-17A in allergic individuals), and thus represent 'functional' T cells specifically activated by the allergen. Tetramer⁻/marker⁺ CD4⁺ T cells represent also activated T cells based upon the significant cytokine production observed in the presence of the allergen. Having excluded a down-regulation of the TCR in tetramer⁻/marker⁺ cells, we conclude that this sub-population correspond to cells that are not specifically activated by the allergen, but may originate from a bystander activation due to cytokines secreted during allergen-specific activation [36–38]. This latter phenomenon complicates the functional analysis of allergen-specific CD4⁺ T cell responses *in vitro*, and raises as well the possibility that such non-specific T cell responses could nevertheless contribute to the amplification of allergic inflammation *in vivo*. Lastly, tetramer⁺/marker⁻ represent either anergic or 'exhausted' allergen-specific CD4⁺ T cells, unable to produce cytokines following

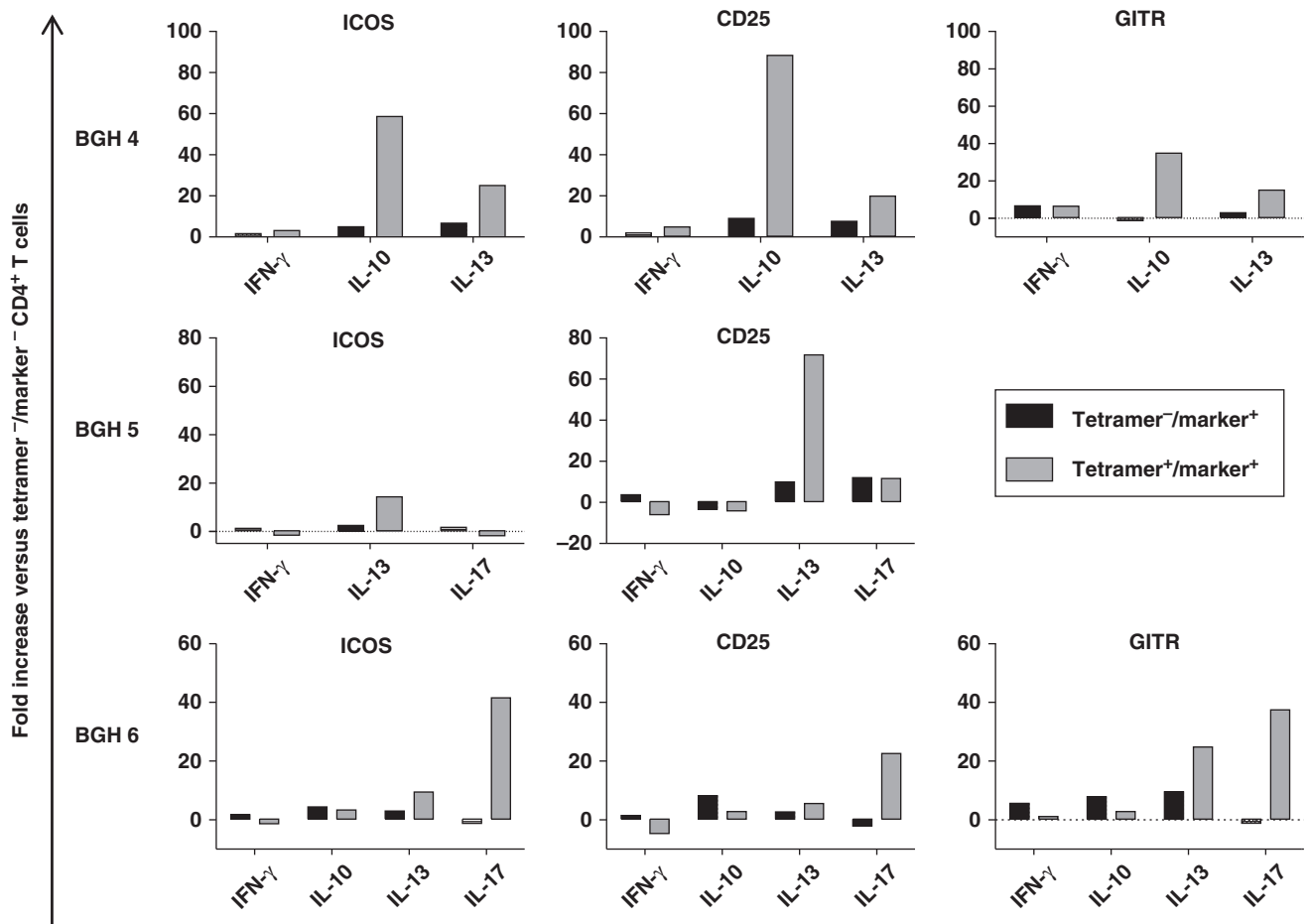


Fig. 6. Cytokine expression levels in tetramer⁺/marker⁺ and tetramer⁻/marker⁺ CD4⁺ T cell subsets. Following 14–16 days of expansion, allergen-specific CD4⁺ T cells were stained with the appropriate tetramer, stimulated for 1 h, labelled with anti-marker antibodies before being sorted using a MoFlo apparatus. Gene expression was analysed by quantitative RT-PCR as described in 'Materials and methods' and normalized to levels of β -actin mRNA. Data are expressed as relative amounts of IL-10, IL-13, IL-17A and IFN- γ mRNA in sorted tetramer⁺/marker⁺ and tetramer⁻/marker⁺ CD4⁺ T cells in comparison with sorted tetramer⁻/marker⁻ CD4⁺ T cells from the same individual.

allergen re-exposure [39, 40]. The induction of such a phenotype in T lymphocytes could be critical as a mechanism for tolerance induction [41], but is clearly overlooked by conventional functional analyses of CD4⁺ T cell responses.

Altogether, the detection of surface-expressed activation markers following allergen-derived peptide stimulation does not allow to detect specific CD4⁺ T cells in a reliable way, at least under our present culture conditions, and tetramer staining remains the gold standard to establish the specificity of allergen recognition. Combin-

ing the two approaches allows to investigate potentially important functional differences among CD4⁺ T cell subsets.

Acknowledgements

Conflict of interest: Some authors (M. B., H. M., S. T., L. V. O., V. B., P. M.) are employees at Stallergenes SA.

The authors declare no financial or commercial conflict of interest.

References

- 1 Akdis M, Verhagen J, Taylor A *et al*. Immune responses in healthy and allergic individuals are characterized by a Fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004; 199:1567–75.
- 2 Bohle B, Kinaciyan T, Gerstmayr M *et al*. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol* 2007; 120:707–13.
- 3 Van Overtvelt L, Wambre E, Maillere B *et al*. Assessment of Bet v 1-specific CD4⁺ T cell responses in allergic and nonallergic individuals using MHC class

- II peptide tetramers. *J Immunol* 2008; 180:4514–22.
- 4 Bercovici N, Duffour M-T, Agrawal S *et al.* New methods for assessing T-cell responses. *Clin Diagn Lab Immunol* 2000; 7:859–64.
 - 5 Gratama JW, Kern F, Manca F *et al.* Measuring antigen-specific immune responses, 2008 update. *Cytometry A* 2008; 73A:971–4.
 - 6 James EA, Kwok W, LaFond R *et al.* Visualizing antigen specific CD4⁺ T cells using MHC class II tetramers. *J Vis Exp* 2009; 323:1726–9.
 - 7 Vollers SS, Stern LJ. Class II major histocompatibility complex tetramer staining: progress, problems, and prospects. *Immunology* 2008; 123:305–13.
 - 8 Mallone R, Nepom GT. MHC Class II tetramers and the pursuit of antigen-specific T cells: define, deviate, delete. *Clinical Immunol* 2004; 110:232–42.
 - 9 Bateman EAL, Arderm-Jones MR, Ogg GS. Persistent central memory phenotype of circulating Fel d 1 peptide/DRB1*0101 tetramer-binding CD4⁺ T cells. *J Allergy Clin Immunol.* 2006; 118:1350–6.
 - 10 Kinnunen T, Jutila K, Kwok WW *et al.* Potential of an altered peptide ligand of lipocalin allergen Bos d 2 for peptide immunotherapy. *J Allergy Clin Immunol* 2007; 119:965–72.
 - 11 Kwok WW, Roti M, DeLong JH *et al.* Direct ex vivo analysis of allergen-specific CD4⁺ T cells. *J Allergy Clin Immunol* 2010; 125:1407–9.
 - 12 Macaubas C, Wahlstrom J, Galvao da Silva AP *et al.* Allergen-specific MHC class II tetramer+cells are detectable in allergic, but not in nonallergic, individuals. *J Immunol* 2006; 176:5069–77.
 - 13 Gadermaier G, Jahn-Schmid B, Vogel L *et al.* Targeting the cysteine-stabilized fold of Art v 1 for immunotherapy of *Artemisia* pollen allergy. *Mol Immunol* 2010; 47:1292–8.
 - 14 Aslam A, Chan H, Warrell DA *et al.* Tracking antigen-specific T-cells during clinical tolerance induction in humans. *PLoS One* 2010; 5:e11028.
 - 15 Chattopadhyay PK, Yu J, Roederer M A live-cell assay to detect antigen-specific CD4⁺ T cells with diverse cytokine profiles. *Nat Med* 2005; 11:1113–7.
 - 16 Frentsch M, Arbach O, Kirchhoff D *et al.* Direct access to CD4⁺ T cells specific for defined antigens according to CD154 expression. *Nat Med* 2005; 11:1118–24.
 - 17 Cohen GB, Kaur A, Johnson RP Isolation of viable antigen-specific CD4 T cells by CD40L surface trapping. *J Immunol Methods* 2005; 302:103–15.
 - 18 Ferenczi K, Burack L, Pope M *et al.* CD69, HLA-DR and the IL-2R identify persistently activated T cells in psoriasis vulgaris lesional skin: blood and skin comparisons by flow cytometry. *J Autoimmun* 2000; 14:63–78.
 - 19 Bengtsson A, Johansson C, Linder M *et al.* Not only Th2 cells but also Th1 and Th0 cells express CD30 after activation. *J Leukoc Biol* 1995; 58:683–9.
 - 20 Romagnani S, Del Prete G, Maggi E *et al.* CD30 and type 2 T helper (Th2) responses. *J Leukoc Biol* 1995; 57:726–30.
 - 21 Wehler TC, Karg M, Distler E *et al.* Rapid identification and sorting of viable virus-reactive CD4⁺ and CD8⁺ T cells based on antigen-triggered CD137 expression. *J Immunol Methods* 2008; 339:23–37.
 - 22 Nocentini G, Ronchetti S, Cuzzocrea S *et al.* GITR/GITRL: more than an effector T cell co-stimulatory system. *Eur J Immunol* 2007; 37:1165–9.
 - 23 Langston HP, Ke Y, Gewirtz AT *et al.* Secretion of IL-2 and IFN- γ , But Not IL-4, by antigen-specific T cells requires extracellular ATP. *J Immunol* 2003; 170:2962–70.
 - 24 Beier K, Hutloff A, Dittrich A *et al.* Induction, binding specificity and function of human ICOS. *Eur J Immunol* 2000; 30:3707–17.
 - 25 Zetterquist H Olerup O identification of the HLA-DRB1*04, -DRB1*07, and -DRB1*09 alleles by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. *Hum Immunol* 1992; 34:64–74.
 - 26 Wambre E, Van Overtvelt L, Maillère B *et al.* Single cell assessment of allergen-specific T cell responses with MHC class II peptide tetramers: methodological aspects. *Int Arch Allergy Immunol* 2008; 146:99–112.
 - 27 Kallinteris NL, Lu X, Blackwell CE *et al.* li-key/MHC class II epitope hybrids: a strategy that enhances MHC class II epitope loading to create more potent peptide vaccines. *Expert Opin Biol Ther* 2006; 6:1311–21.
 - 28 Bakker AH, Schumacher TNM. MHC multimer technology: current status and future prospects. *Curr Opin Immunol* 2005; 17:428–33.
 - 29 Fourneau J-M, Cohen H, vanEndert PM. A chaperone-assisted high yield system for the production of HLA-DR4 tetramers in insect cells. *J Immunol Methods* 2004; 285:253–64.
 - 30 Novak EJ, Masewicz SA, Liu AW *et al.* Activated human epitope-specific T cells identified by class II tetramers reside within a CD4^{high}, proliferating subset. *Int Immunol* 2001; 13:799–806.
 - 31 Chattopadhyay PK, Yu J, Roederer M Live-cell assay to detect antigen-specific CD4⁺ T-cell responses by CD154 expression. *Nat Protocols* 2006; 1:1–6.
 - 32 Zaunders JJ, Munier ML, Seddiki N *et al.* High levels of human antigen-specific CD4⁺ T cells in peripheral blood revealed by stimulated coexpression of CD25 and CD134 (OX40). *J Immunol* 2009; 183:2827–36.
 - 33 Rosa D, Ribeiro S, Cunha-Neto E. CD4⁺ T cell epitope discovery and rational vaccine design. *Arch Immunol Ther Exp* 2010; 58:121–30.
 - 34 Meier S, Stark R, Frentsch M *et al.* The influence of different stimulation conditions on the assessment of antigen-induced CD154 expression on CD4⁺ T cells. *Cytometry A* 2008; 73A:1035–42.
 - 35 Campbell JD, Buchmann P, Kesting S *et al.* Allergen-specific T cell responses to immunotherapy monitored by CD154 and intracellular cytokine expression. *Clin Exp Allergy* 2010; 40:1025–35.
 - 36 Genova GD, Savelyeva N, Suchacki A *et al.* Bystander stimulation of activated CD4⁺ T cells of unrelated specificity following a booster vaccination with tetanus toxoid. *Eur J Immunol* 2010; 40:976–85.
 - 37 Boyman O. Bystander activation of CD4⁺ T cells. *Eur J Immunol* 2010; 40:936–9.
 - 38 Botturi K, Vervloet D, Magnan A. T cells and allergens relationships: are they that specific? *Clin Exp Allergy* 2007; 37:1121–3.
 - 39 Schwartz RH. T cell anergy. *Annu Rev Immunol* 2003; 21:305–34.
 - 40 Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 2010; 129:474–81.
 - 41 Jutel M, Akdis M, Blaser K *et al.* Mechanisms of allergen specific immunotherapy – T-cell tolerance and more. *Allergy* 2006; 61:796–807.