

ORIGINAL ARTICLE

Distinct characteristics of seasonal (Bet v 1) vs. perennial (Der p 1/Der p 2) allergen-specific CD4⁺ T cell responsesE. Wambre^{1*†}, M. Bonvalet^{1*}, V. B. Bodo¹, B. Maillère², G. Leclerc¹, H. Moussu¹, E. Von Hofe³, A. Louise⁴, A.-M. Balazuc⁴, D. Ebo⁵, C. Hoarau⁶, G. Garcia⁷, L. Van Overtvelt¹ and P. Moingeon¹¹Stallergenes SA, Antony, France, ²CEA, Gif Sur Yvette, France, ³Antigen Express Inc., Worcester, MA, USA, ⁴Plateforme de cytométrie de flux, Institut Pasteur, Paris, France, ⁵Department of Immunology, Allergy and Rheumatology, Antwerp University, Antwerp, Belgium, ⁶Unité transversale d'allergologie et Immunologie clinique, CHRU Tours, Tours, France and ⁷Département de Pneumologie, Hôpital Bécélère, Clamart, France

Clinical & Experimental Allergy

Summary

Background A better understanding of allergen-specific CD4⁺ T cell responses is needed to help improving immunological therapies.**Objective** To compare CD4⁺ T cell responses against seasonal (Bet v 1) and perennial (Der p 1, Der p 2) allergens.**Methods** Major histocompatibility complex class II peptide tetramers were engineered to monitor allergen-specific T cell responses. After *in vitro* expansion, tetramer⁺ cells were tested for surface markers using cytofluorometry. Cytokine gene expression and production were assessed using quantitative PCR and cytokine surface capture assays, respectively.**Results** Tetramer⁺ cells were detected in 19 patients allergic to house dust mites (HDM), seven allergic to birch pollen, 13 allergic to both and nine non-allergics with either an HLA-DRB1*0101, *0301, *1501 or an HLA-DPB1*0401 background. High-avidity T cells are elicited against the immunodominant Bet v 1_{141–155} epitope, whereas broader low-avidity T cell responses are induced against Der p 1_{16–30,110–124,171–185} and Der p 2_{26–40,107–121} epitopes. Responses against Bet v 1 involve effector (CDL62 low, CCR7 low) or central (CD62L⁺, CCR7⁺) memory cells in allergic and non-allergic individuals, respectively, whereas central memory cells are mostly detected against mite allergens. In non-allergics, both mite and Bet v 1-specific T cells produce IFN-γ and IL-10. In contrast to Bet v 1-driven Th2 responses, mite allergens induce highly polymorphic responses in allergics, including Th1, Th2/Th17 or mixed Th1/Th2 profiles. Mite-specific T cell frequencies in the blood remain in the range of 1–6 × 10^{−4} CD4⁺ T cells throughout the year.**Conclusion** Different memory CD4⁺ T cell responses are elicited in the context of chronic vs. seasonal stimulation with the allergen(s). The heterogeneity in the patterns of CD4⁺ T cell responses observed in patients allergic to HDMs should be taken into account for specific immunotherapy.**Keywords** allergens, immunotherapy, MHC class II tetramers, T cell polarization, tolerance

Submitted 15 June 2010; revised 17 September 2010; accepted 27 September 2010

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Introduction

Allergen-specific CD4⁺ T cells play a central role in both the pathogenesis and the regulation of allergic inflammation, depending on which cytokines they produce [1–4]. Specifically, excessive production of Th2 cytokines is considered as the hallmark of allergic T cell responses [2,

3, 5, 6]. In contrast, IL-10-producing regulatory T cells as well as IFN-γ-secreting Th1 cells are rather associated with the establishment of tolerance, whether it be during successful immunotherapy [3, 6–10] or during the course of the natural immune responses observed in healthy individuals exposed to allergens [11–13]. Beyond these observations, it remains of interest to further characterize and compare allergen-specific CD4⁺ T cell responses in detail in both allergics and non-allergics in order to document potential patient heterogeneity on the one hand, and

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further, to better understand the mechanisms underlying natural protective responses in healthy individuals. The latter information should help designing optimized allergy vaccines recapitulating natural tolerogenic responses.

The identification within common allergens of immunodominant epitopes restricted to various HLA-DR or -DP haplotypes [11–15] facilitates the design and development of major histocompatibility complex (MHC) class II peptide tetramers as reagents to investigate allergen-specific CD4⁺ T cell responses [6, 16, 17]. Such tetramers classically consist of complexes composed of four MHC class II heterodimers loaded with a peptide representing a high-affinity T cell epitope [18–20]. MHC molecules coupled to biotin can be multimerized subsequently with fluorescently labelled streptavidin, thus providing high-avidity reagents to detect epitope-specific T cells by flow cytometry. Studies with MHC class II peptide tetramers to identify, quantify and characterize antigen-specific CD4⁺ T cells are often limited by low progenitor frequencies within peripheral blood mononuclear cells (PBMCs) [18–20], thereby necessitating the *in vitro* expansion of rare specific T cells before tetramer staining [17]. Tetramer-based approaches have now been applied successfully to the analysis of CD4⁺ T cell responses against several common allergens, including Lol p 1, Fel d 1, Bet v 1, Der p 1, Bos d 2 and Art v 1 [6, 13, 16, 17, 21–23].

Using such a tetramer-based approach, we established recently that birch pollen (BP)-allergic patients and healthy individuals mount distinct CD4⁺ T cell responses to the Bet v 1_{141–155} immunodominant epitope [13, 17]. Whereas allergics consistently elicit specific Th2 cells, healthy patients have rather Th1 and Foxp3⁺ IL-10-producing T Reg cells circulating in their blood [13]. In the present study, we developed MHC class II peptide tetramers to extend those comparative analyses to perennial Der p 1 and Der p 2 allergens from the house dust mite (HDM) *Dermatophagoides pteronyssinus* species. Using those tools, we demonstrate in single or double BP/mite allergic patients as well as in healthy individuals that distinct CD4⁺ memory T cell responses are induced to mite allergens when compared with Bet v 1, with most noticeably, highly variable patterns of cytokines produced in response to the former in allergic patients.

Materials and methods

Patients and HLA-DR typing

Citrated peripheral blood was obtained after informed consent from BP- or HDM-allergic patients recruited at Antwerp University Hospital (Belgium), Hospital Bécélère (Clamart, France) or CHRU Tours (France). Blood samples from healthy donors were obtained from Etablissement Français du Sang (Rungis, France). The study protocol was approved by the ethical committee (CPP) from Bicêtre

hospital, Kremlin Bicêtre, France. BP- and HDM-allergic patients were selected based on their clinical record, a positive skin prick test and documented IgE reactivity. All patients had allergic rhinitis and none of them had asthma or atopic dermatitis. For healthy patients, *in vitro* basophil activation assays were performed with BP and HDM extracts (Stallergenes, France) to confirm their non-allergic status.

Up to 220 individuals (including BP and/or HDM allergics as well as healthy individuals) were PCR-typed for their HLA-DR and -DP allotypes, using an Easy DNA kit (Invitrogen, Carlsbad, CA, USA). From this cohort, 125 individuals were selected for this study, based on their HLA-DRB1*0101, *0301, *1501 or HLA-DPB1*0401 background, allowing tetramer analysis.

Bioinformatics analysis and HLA-DR (or -DP) peptide *in vitro* binding assays

Epitopes from Der p 1 and Der p 2 restricted to HLA-DRB1 or HLA-DPB1 molecules were preselected by bioinformatics analyses performed using TEPITOPE and Propred (<http://www.imtech.res.in/raghava/propred>) algorithms. For each allergen, peptides spanning the whole protein sequence and preserving putative anchor motifs were synthesized by NeoSystem (Strasbourg, France) with a purity grade > 75% confirmed by HPLC. Peptide-binding assays to human HLA-DR or -DP molecules purified from human EBV-transformed B cell lines were performed as described elsewhere [24, 25]. Biotinylated reporter peptides were incubated in the presence of serial dilutions of each allergen-derived peptide tested and appropriate purified MHC class II molecules. After a 24-h or a 72-h incubation at 37 °C, peptide-MHC class II complexes were added for 2 h to 96-well plates coated with the L243 antibody directed to a monomorphic determinant of MHC class II molecules. Bound biotinylated peptides were detected as described elsewhere [24]. Data were expressed as a ratio between concentrations of tested peptides over reporter peptide inhibiting 50% binding (IC₅₀) of biotinylated reporter peptides [24].

Staining with major histocompatibility complex class II peptide tetramers and characterization of tetramer⁺ cells

PBMCs were isolated from peripheral blood by centrifugation over Ficoll-Paque plus (Amersham Biosciences, Orsay, France) [13]. For *in vitro* expansion of epitope-specific CD4⁺ T cells, PBMCs were stimulated with conjugate peptide mixes comprising complete or truncated versions of peptides corresponding to each of Bet v 1-, Der p 1- or Der p 2-derived T cell epitopes, conjugated with a polymethylene linker to the li-Key moiety of the MHC class II-associated invariant chain [17, 26]. Freshly isolated PBMCs (5 × 10⁶/mL) were stimulated in 12-well

Table 1. Binding capacities of Der p 1 and Der p 2 peptides to purified HLA-DR and HLA-DP molecules

Amino acid sequences		HLA-DRB1 alleles				HLA-DPB1 allele
		0101	0301	0401	1501	0401
(a) Der p 1 peptides						
p3-17	ACSINGNAPAEIDLR	3594	> 261	> 1 748	> 949	> 1895
p16-30	LRQMRTVTPIRMQGG	6	14	10	18	> 1895
p37-51	FSGVAATESAYLAYR	342	8	14	17	721
p44-58	ESAYLAYRNQSLDLA	5	> 261	29	1	699
p91-105	ESYYRYVAREQSCRR	13	281	54	321	> 1895
p110-124	RFGISNYCQIYPPNV	29	72	417	1	7
p113-127	ISNYCQIYPPNVNKI	1	> 261	26	388	> 1895
p138-152	IAVIIGIKDLDAFRH	63	37	1	0.1	> 1895
p147-161	LDAFRHYDGRTHQR	13	70	61	3	> 1895
p156-170	RTIIQRDNGYQPNYH	> 4722	55	1144	282	> 1895
p171-185	AVNIVGYSNAQGVYD	1757	> 261	3	1	> 1895
p207-221	NIDLMMIEEYPYVVI	122	> 261	1436	1	9
(b) Der p 2 peptides						
p10-24	NHEIKKVLVPGCHGS	198	187	> 1748	121	> 1895
P26-40	PCIIHRGKPFQLEAV	3	> 261	> 1748	12	> 1895
p32-46	GKPFQLEAVFEANQN	58	> 261	1	107	> 1895
p37-51	LEAVFEANQNTKTAK	784	261	35	> 949	> 1895
p58-72	IDGLEVDVPGIDPNA	> 4722	20	> 1748	> 949	> 1895
p77-91	KCPLVKGQQYDIKYT	762	> 261	> 1748	92	> 1895
p85-99	GQQYDIKYTWNVPKIAP	3200	172	649	206	> 1895
p107-121	TVKVMGDDGVLAACAI	1593	1	26	176	1221

Peptide-binding capacities of (a) Der p 1-derived and (b) Der p 2-derived peptides to immunopurified HLA-DR and HLA-DP molecules. Results are expressed as a ratio between the IC₅₀ (inhibitory concentration) values obtained with either tested peptides or the corresponding reference peptides out of two independent experiments, as described in 'Materials and methods'. The HA₃₀₆₋₃₁₈ peptide was used as a reporter peptide for both HLA-DRB1*0101 (IC₅₀ = 2 nM) and DRB1*0401 alleles (IC₅₀ = 6 nM). Peptides HLA-A3₁₅₂₋₁₆₆ and MT₂₁₃₋₂₃₆ were used as reference peptides for binding to either the HLA-DRB1*1501 (IC₅₀ = 10 nM) or the HLA-DRB1*0301 alleles (IC₅₀ = 200 nM), respectively, and the Oxy₂₇₁₋₂₈₇ peptide for the HLA-DPB1*0401 allele (IC₅₀ = 10 nM). High-affinity peptides (IC₅₀ ratio < 50) are in bold.

plates with appropriate conjugate peptide mixes (10 µg/mL) in 2 mL of complete RPMI 1640 medium (Invitrogen). After 5 days at 37 °C, 20 U/mL IL-2 (Roche, Meylan, France) was added to cell cultures every 3 days. On day 14, cells were restimulated with autologous PBMCs pulsed with the conjugate peptide mix plus IL-2.

All MHC class II peptide tetramers used in this study were made by Beckman Coulter (Marseille, France). Briefly, the α and β chains of HLA-DR or -DP molecules were produced as a fusion to biotin in a baculovirus expression system, as described elsewhere [27]. Tetramers prepared using streptavidin were complexed with either peptides corresponding to T cell epitopes from Der p 1 and Der p 2 (Table 1) or the li CLIP peptide (LPKPPKPVSKMRMATPLLMQALPM) or MAGE₃₄₈₋₆₃ used as negative controls. Tetramers engineered to assess CD4⁺ T cells responses to the Bet v 1₁₄₁₋₁₄₅ epitope have been described elsewhere [17]. All tetramers were conjugated to PE and obtained at a 100 µg/mL concentration. All conjugate peptide mixes as well as tetramers used in this study were tested for endotoxin content using the Limulus Amoebocyte Lysate test and found to be negative or to contain < 2 EU/mL. The DRB1*1501 Der p 2₂₆₋₄₀ tetramer contains 15 EU/mL endotoxin.

For tetramer staining, 10⁶ cells were incubated with 10 µg/mL MHC class II peptide tetramers for 2 h at 37 °C in RPMI 1640 medium. After washing in cold PBS, antibodies (i.e. FITC-labelled anti-CD4, ECD-labelled anti-CD14, PC7-labelled anti-CD3, all from Beckman Coulter) and ViaProbe reagent (BD Biosciences, San Diego, CA, USA) were added for 20 min in the dark at 4 °C. Cells were stained with the corresponding isotype-matched antibodies as controls. After washing, samples were analysed using an FC500 flow cytometer (Beckman Coulter), after excluding dead cells and CD14⁺ monocytes. In selected experiments, cells were further incubated with fluorescently labelled antibodies directed against various surface markers (i.e. CD45RO, CD62L, CXCR3 and CCR7, all from BD Biosciences; CD25, CD69 from Beckman Coulter; CTLA-4 from R&D Systems, Lille, France) or corresponding isotype-matched antibodies, before FACS analysis. A minimum of 50 000 cells were analysed, corresponding to at least 100 tetramer⁺ cells.

To assess the frequencies of Der p 1- or Der p 2-specific T cells, tetramer staining was performed after purification of CD4⁺ T cells from PBMCs, either using a MoFlo (Dako, Glostrup, Denmark) cell sorter or by negative selection using a Dynal CD4⁺ isolation kit (Invitrogen) according to

the manufacturer's instructions. The frequencies of allergen-specific cells were calculated as a ratio between the number of cells stained with the specific tetramer minus number of cells stained with the CLIP tetramer over the total number (at least 50 000) of CD4⁺ T cells analysed.

Cytokine surface capture assays

Cells were stained with 5 µg/mL MHC class II peptide tetramers at 37 °C in RPMI 1640 medium for 30 min. 2.5×10^6 cells were subsequently washed and stimulated in RPMI 1640 complete medium with 2.5×10^5 autologous PBMCs pulsed with li key peptide mixes. After 3–4 h at 37 °C, cells were harvested, washed in ice-cold buffer containing 0.5% BSA + 5 mM EDTA and labelled with 50 µg/mL of either anti-IFN-γ/CD45, anti-IL-5/CD45 or anti-IL-10/CD45 Ab-Ab conjugates (Miltenyi Biotec, Bergish Gladbach, Germany) for 10 min at 4 °C. Cells were resuspended in a complete culture medium and incubated for 45 min at 37 °C to allow cytokine secretion. Cells were then washed, resuspended in ice-cold buffer containing 0.5% BSA plus 5 mM EDTA and stained with PC7-labelled anti-CD4, ECD-labelled anti-CD14, ViaProbe reagent and either FITC-labelled anti-IFN-γ or APC-labelled anti-IL-10 or anti-IL-5 mAbs. After 30 min at 4 °C, cells were washed and analysed immediately by flow cytometry.

PCR analysis of purified major histocompatibility complex class II peptide tetramer⁺ CD4⁺ T cells

Both tetramer-positive and -negative CD4⁺ T cells were sorted from cultures obtained from allergic and non-allergic individuals, using a MoFlo (Dako). Total RNA was extracted from the various cell populations using the RNeasy Kit (Qiagen, Courtaboeuf, France) and cDNAs were synthesized using a TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. Messenger RNA expression corresponding to cytokine genes was evaluated by quantitative PCR on a 7300 Real-Time PCR System (Applied Biosystems) using pre-designed Taqman Gene Expression reagents (Applied Biosystems) [13]. Data were obtained after normalizing the target gene amplification value with an endogenous control (β-actin). The relative amount of target genes in each sample was assessed in comparison with tetramer⁻ CD4⁺ T cells purified from the same donor.

Statistical analyses

Data were analysed for statistical significance using a non-parametric Mann-Whitney test. A *P*-value ≤ 0.05 was considered as statistically significant.

Results

Design of major histocompatibility complex class II Der p 1 and Der p 2 tetramers

Using computer algorithms (TEPITOPE and ProPred) to predict HLA-DR- and -DP-(i.e., HLA-DRB1*0101, *0301, *0401, *1501 and HLA-DPB1*0401) binding motifs, potential MHC class II restricted T cell epitopes were identified within Der p 1 and Der p 2 allergens. Corresponding peptides were synthesized and tested for binding to those common Caucasian MHC class II molecules using an ELISA competition assay, in parallel with reporter peptides. This analysis revealed multiple epitopes within both Der p 1 and Der p 2 with a good affinity for MHC class II molecules (Table 1). For example, peptide Der p 1_{16–30} has a good affinity for both HLA-DRB1*0101, *0301, *0401 and *1501 alleles and Der p 1_{138–152} for DRB1*0401 and 1501. The Der p 1_{171–185} peptide was also selected, given its high affinity for HLA-DRB1*1501 alleles. Furthermore, two Der p 1 peptides, namely Der p 1_{110–124} and Der p 1_{207–221}, exhibit a high affinity for HLA-DPB1*0401. The Der p 2_{26–40} peptide has a high binding affinity for both DRB1*0101 and *1501 molecules, whereas Der p 2_{107–121} binds well to HLA-DRB1*0301 and Der p 2_{32–46} to HLA-DRB1*0401 (Table 1). These results are consistent with other studies establishing that Der p 1 epitopes are presented by DRB1 and DPB1, whereas Der p 2 epitopes are rather restricted to DRB1 but not DPB1. Among the peptides selected, we were successful in engineering-specific MHC class II peptide tetramers encompassing Der p 1_{16–30}, Der p 1_{110–124} and Der p 1_{171–185} as well as Der p 2_{26–40} and Der p 2_{107–121} epitopes. In parallel, synthetic peptides corresponding to such epitopes of interest were chemically conjugated with the li-Key peptide from the MHC invariant chain in order to produce reagents allowing to expand Der p 1- and Der p 2-specific CD4⁺ T cells *in vitro* from PBMCs, as described elsewhere [17, 26].

Identification of Der p 1- and Der p 2-specific CD4⁺ T cells in allergic and non-allergic patients

PBMCs from 220 HDM and/or BP allergic or healthy individuals were PCR-typed for their MHC class II haplotypes, and 125 subjects were selected based on a favourable MHC class II background allowing tetramer staining. Cells from such individuals were stimulated with appropriate Der p 1- or Der p 2-derived epitope li-Key conjugate mixes for 14–24 days (Table 2). After *in vitro* stimulation, Der p 1- or Der p 2-specific CD4⁺ T cells were detected in approximately 30% of the blood samples analysed (i.e. from 19 HDM allergics, seven BP allergics, 13 dual HDM and BP allergics and nine non-allergics) and such responses were directed to multiple epitopes restricted to various MHC class II haplotypes (Table 2, Fig. 1). Bet v

Table 2. Detection of MHC class II-peptide tetramer⁺ CD4⁺ T cells in allergic and non-allergic individuals

Status of donors	Identification of donors	HLA-DR or DP typing	% tetramer ⁺ cells among CD4 ⁺ T cells				Days <i>in vitro</i> stimulation	Month of blood collection
			Control*	Der p 1 [†]	Der p 2 [‡]	Bet v 1 [§]		
HDM allergic	HDM1	DP4	0.1	1.9	ND	ND	15	March
	HDM2	DR15	0.1	0.8	0.3	1.2	21	February
	HDM3	DR3	0	ND	0.3	ND	15	February
	HDM4	DR1	0.1	1.4	2.1	0.3	17	March
	HDM5	DR15	0	ND	0.3	0.5	14	August
	HDM6	DR1	0.1	ND	2	1.2	14	August
	HDM7	DR3	0.1	ND	0.8	ND	16	May
	HDM8	DR15	0	0.5	ND	0.2	17	June
	HDM9	DR15	0.1	0.7	ND	0.4	17	June
	HDM10	DR15	0	0.4	0.4	ND	16	July
	HDM11	DR15	0.1	0.7	0.4	ND	17	June
	HDM12	DR1	0	0.3	0.5	ND	21	November
	HDM13	DP4	0.2	2.8	ND	ND	10	May
	HDM14	DR15	0	0	0.4	ND	12	November
	HDM15	DR15	0	1.1	0.4	ND	14	November
	HDM16	DR15	0	0.4	ND	ND	19	December
	HDM17	DR15	0	0.4	0.2	ND	14	December
	HDM18	DR15	0	2	0	ND	14	January
	HDM19	DR15	0.2	ND	ND	1	14	June
BP allergic	BP1	DR3	0.2	ND	0.6	ND	18	September
	BP2	DR15	0.1	1	0.3	1.4	14	May
	BP3	DR15	0.1	0.3	0.2	0.9	14	June
	BP4	DR15	0	2.2	ND	ND	11	November
	BP5	DR1	0	ND	ND	4.5	6	June
	BP6	DR15	0.2	ND	ND	1.9	15	June
	BP7	DR1	0	ND	ND	1.5	14	December
HDM and BP allergic	HB1	DR15	0.2	0.6	0.5	0.6	23	December
	HB2	DR15	0.1	1.2	1.5	17.7	14	April
	HB3	DR15	0.1	0.4	0.9	18.5	14	July
	HB4	DR1	0	0.6	1.2	1.5	15	April
	HB5	DR15	0.1	0.4	ND	2.2	15	July
	HB6	DR15	0.1	0.4	0.8	1.9	21	November
	HB7	DR3	0.1	ND	2.9	ND	24	November
	HB8	DR15	0	ND	0.3	1.5	16	March
	HB9	DR15	0.1	0.6	0.8	3.8	20	March
	HB10	DR1	0.2	1.9	2.6	14.1	18	March
	HB11	DR1	0	ND	0.2	0	16	November
	HB12	DR15	0.2	ND	ND	15.1	15	July
	HB13	DR15	0.1	4.1	0	6.1	16	January
Non-allergic	NA1	DR15	0.1	0.2	0.8	ND	19	February
	NA2	DR1	0.1	0.9	ND	ND	21	February
	NA3	DR15	0	0.4	0.5	0.6	18	July
	NA4	DR15	0.1	0.8	0.4	1.4	18	June
	NA5	DR3	0.1	ND	0.7	ND	17	December
	NA6	DR3	0.1	ND	0.4	ND	17	December
	NA7	DR1	0.1	0.5	0	ND	17	November
	NA8	DR15	0	0.3	0	ND	13	December
	NA9	DR15	0	0.3	0	ND	16	January

Blood samples were obtained from individuals in Belgium and France. PBMCs were stimulated *in vitro* with conjugate peptide mixes before tetramer staining. Percentages of Der p 1-, Der p 2- or Bet v 1-specific CD4⁺ T cells detected with the corresponding MHC class II peptide tetramers in allergic and non-allergic individuals are indicated.

*Control epitope is always CLIP, except for DPB1*0401 patients, where MAGE-3₄₈₋₆₃ is used.

[†]Epitopes p16-30 for DRB1*0101 and *1501 (i.e. DR1 and DR15) patients; p110-124 for DPB1*0401 (i.e. DP4) patients and p171-185 for DRB1*1501 (i.e. DR15) patients.

[‡]Epitopes p26-40 for DRB1*0101 and *1501 (i.e. DR1 and DR15) patients and p107-121 for DRB1*0301 (i.e. DR3) patients.

[§]Epitopes p141-155 for all patients.

ND, not done.

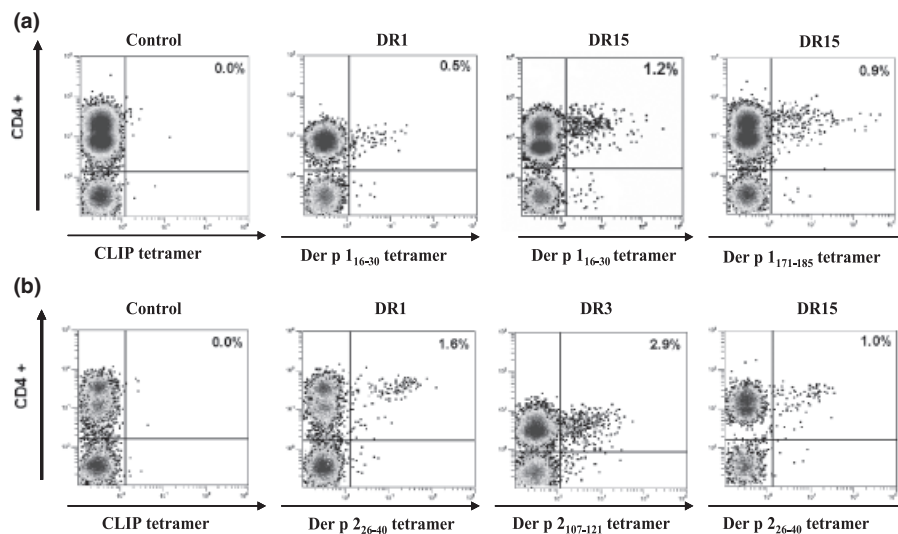


Fig. 1. Detection of (a) Der p 1- or (b) Der p 2-specific CD4⁺ T cells with various major histocompatibility complex class II peptide tetramers. Freshly isolated PBMCs from various HDM-allergic individuals were stimulated for 14–24 days with Der p 1- or Der p 2-derived epitope li-Key conjugates (10 µg/mL). Percentages of CD4⁺ tetramer-positive cells within CD3⁺ cells are indicated in the upper right quadrant. CLIP tetramer staining was used as a negative control.

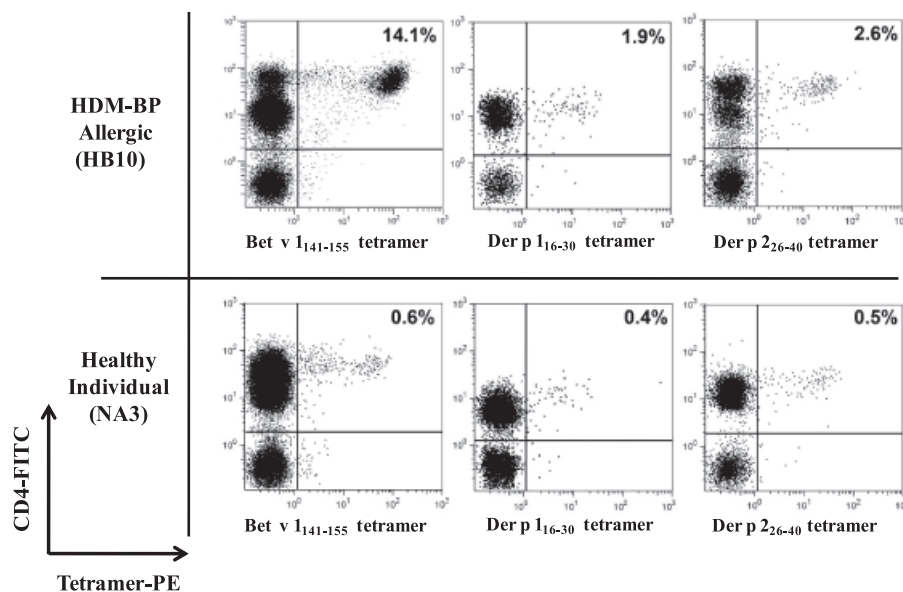


Fig. 2. Detection of either Bet v 1-, Der p 1- or Der p 2-specific CD4⁺ T cells in allergic and healthy individuals. PBMC from HLA-DRB1*1501 or HLA-DRB1*0101 individuals were stimulated for 18 days with either Bet v 1₁₄₁₋₁₅₅-, Der p 1- or Der p 2-derived epitope li-Key conjugates (10 µg/mL). Percentages of CD4⁺ tetramer-positive cells within CD3⁺ cells are indicated in the upper right quadrant. Background staining with the corresponding CLIP tetramers was always ≤0.1% CD4⁺ T cells.

1₁₄₁₋₁₅₅-specific CD4⁺ T cells were detected in most of the individuals tested (Table 2, Fig. 2), in agreement with our previous studies [13, 17]. Such tetramer staining is specific as only background reactivity (0.2% or less) is observed with irrelevant tetramers (i.e. corresponding HLA molecules associated with the CLIP or MAGE-3 peptides). Also, most tetramer staining is obtained within CD4⁺, but not CD4⁻ T

cells (Fig. 1). Interestingly, Der p 1- and Der p 2-specific CD4⁺ T cells were also detected in healthy individuals, as well as in BP-allergic patients with no clinical history of HDM allergy, although often in lower quantities when compared with HDM allergics (Table 2 and Fig. 2, lower panel). As shown in Fig. 2 (upper panel), analyses conducted with PBMCs from a double HDM-BP allergic patient

confirm that a much stronger staining, reflecting superior tetramer avidity, is observed in Bet v 1_{141–155}-specific CD4⁺ T cells (MFI = 81.9) when compared with mite-specific T cells (with MFI = 8.3 and 13.2 for Der p 1_{16–30} and Der p 2_{26–40}-specific T cells, respectively).

Characterization of allergen-specific CD4⁺ T cells against Bet v 1, Der p 1 and Der p 2 in both allergic and healthy individuals

In a first set of experiments, we assessed the expression of surface markers by allergen-specific CD4⁺ T cells detected with either Bet v 1-, Der p 1- or Der p 2-specific MHC class II peptide tetramers. As shown in Fig. 3 (left panel), most Der p 1- and Der p 2-specific CD4⁺ T cells from mite-allergic patients ($n=8$) are CD45RO⁺, and a significant fraction are CD62L⁺ and CCR7⁺, corresponding to a central memory phenotype. In contrast, Bet v 1-specific CD4⁺ T cells from BP-allergic individuals ($n=7$) rather exhibit an effector memory profile (CD45RO⁺, CD62L low, CCR7 low), in agreement with our previous findings [13]. Interestingly, a central memory profile is always found for BP- and HDM allergen-specific CD4⁺ T cells in healthy individuals ($n=6$, Fig. 3, right panel). Importantly, two markers are better expressed by tetramer⁺ cells from non-allergic individuals when compared with allergics, independent of the allergen specificity. The latter include Cytokine T Lymphocyte Antigen-4 (CTLA-4), which may provide a regulatory signal during immune responses [28]. Although not statistically significant, a trend is also observed for CXCR3, considered as a Th1 marker [29]. Surprisingly, significant differences are also found within allergics in terms of CXCR3 expression between mite and Bet v 1-specific responses.

We subsequently assessed cytokine production by tetramer⁺ cells. With the relatively low avidity of mite-specific tetramers as a limitation, we could perform a single-cell analysis of cytokine secretion using a surface capture assay in samples from nine individuals, including three

BP allergics, two mite allergics, four HDM/BP double allergics and one non-allergic (NA3) individual (Fig. 4a). These experiments confirmed that Der p 1- and Der p 2-specific cells from non-HDM-allergic individuals (Fig. 4a, patients BP4 and NA3) produce mostly IFN- γ , with or without IL-10 and IL-5, similarly to what is observed in non-BP allergics for Bet v 1_{141–155}-specific cells (e.g. Fig. 4a, patients HDM 19 and ref 13). Surprisingly, such a Th1 pattern of polarization biased towards IFN- γ production was also observed in Der p 1- or Der p 2-specific cells from 3 mite-allergic individuals (Fig. 4a, patients HDM15, HB10 and Fig. 4b, patient HB1) out of seven tested. Interestingly, T cell responses analysed in parallel in a double HDM-BP allergic individual (HB13) revealed that Th2 responses were stronger to Bet v 1 when compared with Der p 1. These experiments also confirmed that Bet v 1-specific CD4⁺ T cells in BP allergics (Fig. 4a, patients BP5, BP7, HB12, HB13) are mostly IL-5-producing *bona fide* Th2 cells. To further analyse CD4⁺ T cell polarization in mite allergics, we sorted tetramer-positive and -negative cells in four allergic patients for which cytokine surface capture assays were not possible, due to limited numbers of tetramer⁺ cells, and assessed, after RNA extraction, the expression of multiple cytokine genes by quantitative RT-PCR. As shown in Fig. 4b, significant differences were observed between donors, with mite-specific CD4⁺ T cells from allergic individuals expressing IFN- γ , IL-4, IL-10, IL-13 and occasionally IL-17 cytokine genes, thus resulting in Th1 (patient HB1), Th2/Th17 (patient HDM13) or Th1/Th2 (patients HB8 and HB9) patterns of polarization, respectively.

Frequencies of Der p 1- and Der p 2-specific CD4⁺ T cells in peripheral blood of mite-allergic patients

After purification of CD4⁺ T cells from PBMCs of mite-allergic patients, tetramer staining was performed without *in vitro* stimulation. In an analysis conducted in a blood sample collected in April, Der p 1- and Der p 2-specific

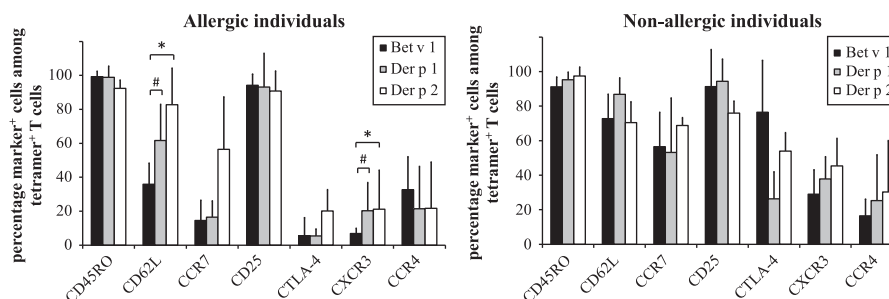


Fig. 3. Phenotypic analysis of either Bet v 1, Der p 1 or Der p 2 tetramer⁺ CD4⁺ T cells. PBMCs from allergic ($n=7$ for Bet v 1; $n=8$ for Der p 1; $n=4$ for Der p 2 tetramers) or non-allergic ($n=4$ for Bet v 1; $n=6$ for Der p 1; $n=3$ for Der p 2 tetramers) individuals were stained after 10–24 days of *in vitro* stimulation with appropriate major histocompatibility complex class II peptide tetramers together with a combination of antibodies directed against surface markers. Expressions of cell surface molecules by tetramer⁺ CD4⁺ T cells are indicated as median percentages $\pm$ SD. Statistical significance was determined using the Mann-Whitney test (* $P<0.05$; # $P=0.05$).

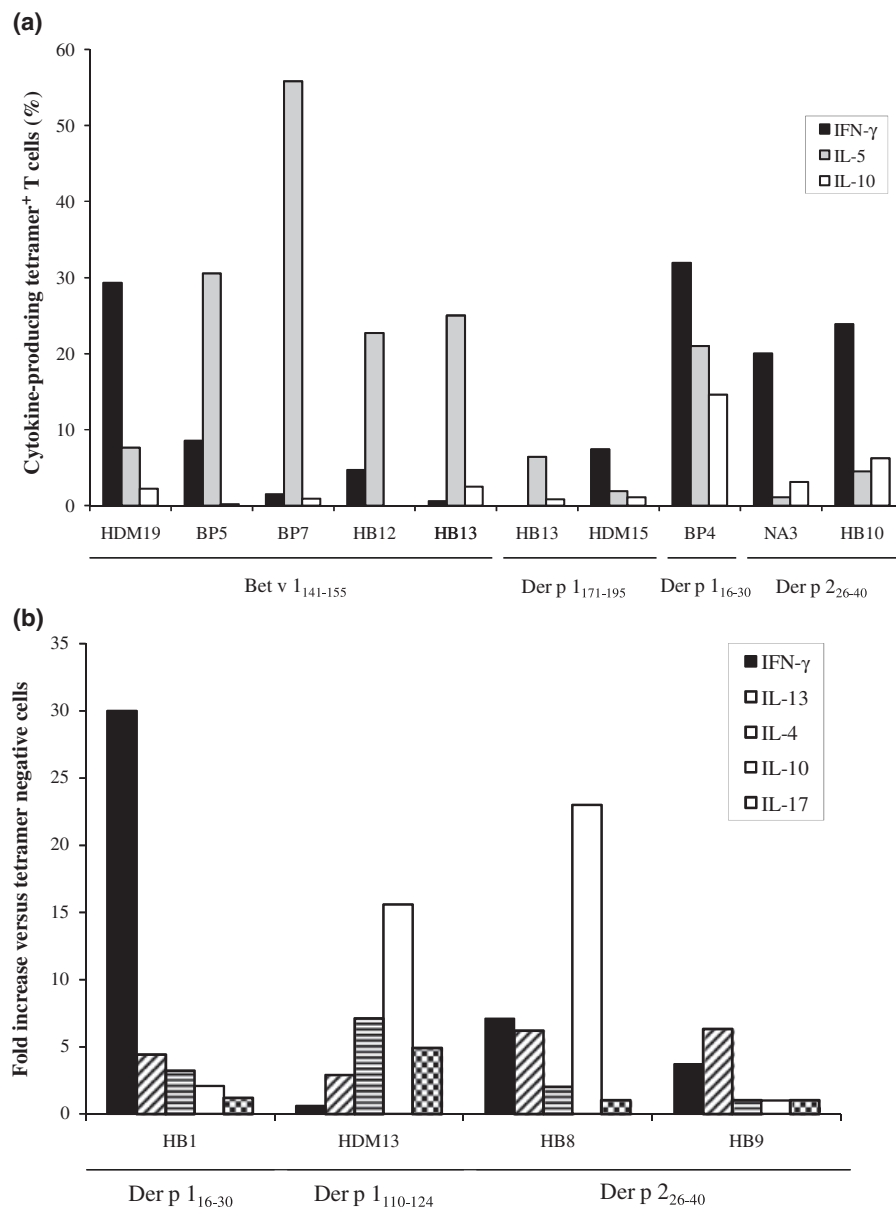


Fig. 4. Analysis of patterns of differentiation in Bet v 1-, Der p 1- or Der p 2-specific tetramer⁺ CD4⁺ T cells. (a) Bet v 1, Der p 1 or Der p 2 tetramer⁺ CD4⁺ T cells were analysed for IL-5, IL-10, IFN-γ production at a single cell level using a cytokine surface capture assay. (b) Tetramer⁺ CD4⁺ T cells from mite-allergic individuals were sorted using a MoFlo. Gene expression was analysed by quantitative PCR. Data are expressed as relative amounts of mRNA in sorted tetramer⁺ CD4⁺ T cells in comparison with sorted tetramer⁻ CD4⁺ T cells from the same donor. Data are normalized to amounts of β-actin.

CD4⁺ T cells were detected at frequencies between 1 and 2 cells among 10 000 CD4⁺ T cells, respectively, whereas none were stained with the control CLIP tetramer (Fig. 5). Circulating Der p 1_{16–30}, Der p 1_{171–185}, Der p 2_{26–40}, Der p 2_{107–121}-specific CD4⁺ T cells were detected at frequencies ranging from 1 to 6/10 000 cells in blood samples harvested throughout the year from 11 independent donors. Overall, as expected in the context of perennial exposure to the antigen, these results suggest that no major variation of circulating mite-specific CD4⁺ T cells occurs with time, besides a possible limited amplification in the last months of the year. We failed to detect

mite-specific T cells, and thus, to determine progenitor frequencies under those conditions in non-allergic individuals, consistent with our observation that the latter are usually present in lower quantities in the blood of non-allergics (Table 2).

Discussion

The present study was undertaken to compare CD4⁺ T cell responses against highly immunogenic seasonal Bet v 1 and perennial Der p 1 and Der p 2 allergens, in both allergic and non-allergic individuals. For this purpose, we

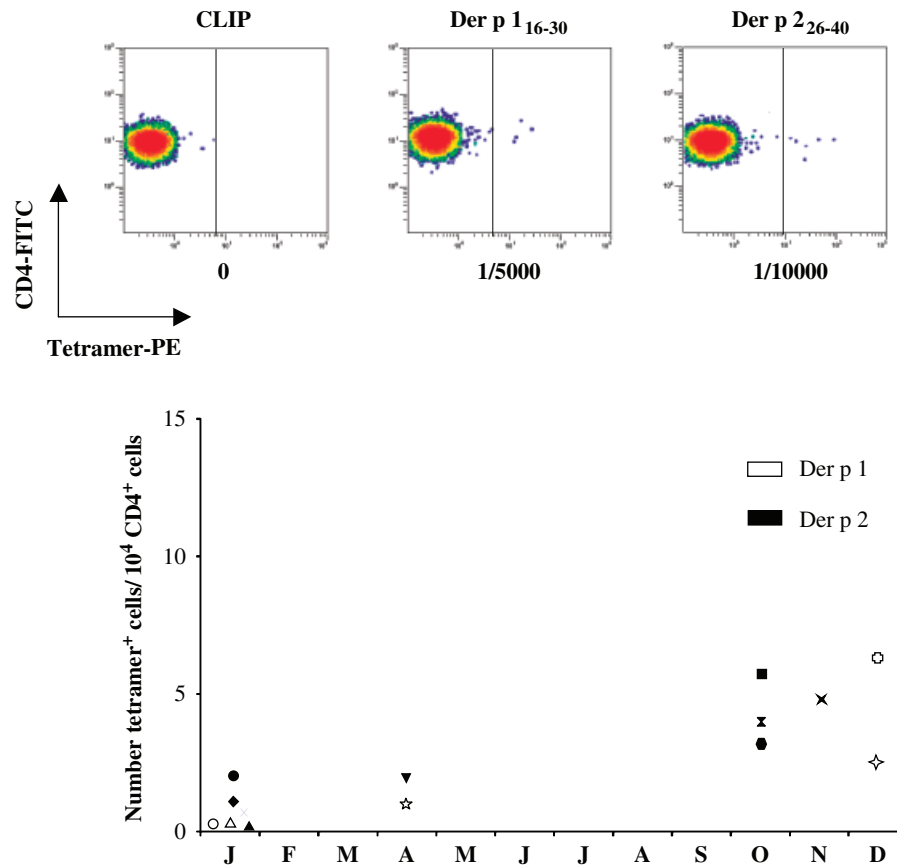


Fig. 5. Frequencies of Der p 1- and Der p 2-specific T cell progenitors. (a) CD4⁺ T cells were purified from PBMCs and stained with the following tetramer-peptide complexes: HLA-DRB1*0101-Der p 1₁₆₋₃₀, -Der p 2₂₆₋₄₀ or -CLIP as a control. The frequencies of specific T cells were evaluated as a ratio between the numbers of cells stained with specific tetramers minus the number of cells stained with CLIP tetramers over the number of CD4⁺ T cells analysed. (b) CD4⁺ T cells were purified from PBMCs from 11 independent donors at various time-points during the year and stained with tetramer-peptide complexes or -CLIP as above. The frequencies of specific T cells are expressed as the number of cells stained with specific tetramers minus the number of cells stained with CLIP tetramers per 10⁴ CD4⁺ T cells.

relied on MHC class II peptide tetramers as the most accurate tool to assess antigen-specific CD4⁺ responses. One prerequisite for developing stable high-avidity MHC class II peptide tetramers is to identify epitopes with a high affinity for HLA molecules within the allergen. To this end, we used computer algorithms to identify HLA-DR- or DP-restricted epitopes within Der p 1 (i.e. peptides 16–30, 37–51, 44–58, 110–124, 113–127, 138–152, 171–185, 207–221) and Der p 2 (peptides 26–40, 32–46, 107–121). We further confirmed, using *in vitro* binding assays, the presence of multiple MHC class II-restricted T cell epitopes within both Der p 1 and Der p 2 in agreement with previous studies [30–36]. Corresponding tetramers were subsequently engineered and successfully used to detect specific cells directed against some of those epitopes. Using these tools, we could detect mite-specific CD4⁺ T cells not only in allergic patients but also in non-allergics with various MHC class II haplotypes. This observation corroborates our previous findings observed with Bet v 1_{141–155} tetramers [13], with the difference that CD4⁺ T cell responses to mite allergens are directed to a broader

range of epitopes (i.e. Der p 1_{16–30}, 110–124, 171–185, Der p 2_{26–40}, 107–121), as opposed to a single immunoprevalent epitope within Bet v 1, in agreement with other reports from the literature [11, 14, 17, 30–36].

Allergen-specific CD4⁺ T cells from both allergic and non-allergic individuals are found within the CD45RO⁺ population. However, distinct memory profiles are observed between CD4⁺ T cells directed to seasonal Bet v 1 vs. perennial Der p 1 and Der p 2 allergens. At least two main categories of antigen-specific CD4⁺ T cells have been described, including (i) central memory cells, highly replicative, with lymph node-homing capacity and involved in long-lived responses to the antigen, and (ii) effector memory cells, rather poorly replicative, with tissue-homing capacity and effector function [37]. Both the nature and the timing of antigenic stimulation have been shown to influence the generation of memory T cells [38–40], raising the possibility that different conditions of allergen exposure may induce phenotypically and functionally distinct CD4⁺ T cell memory subsets. Indeed, we found that Bet v 1_{141–155}-specific CD4⁺ T cells exhibit a

higher TCR avidity for tetramers when compared with mite-specific T cells. Chronic exposure to HDM allergens likely explains the persistent central memory profile (CD62L⁺, CCR7⁺) observed most particularly in Der p 2 (and to a lesser extent in Der p 1)-specific CD4⁺ T cells, both in allergic and in non-allergic individuals. Interestingly, our results are consistent with the observation that CD4⁺ T cells directed to the perennial Fel d 1 cat allergen also maintain a central memory phenotype [16]. In contrast, BP allergics transiently exposed to Bet v 1 during the pollen season have rather circulating Bet v 1-specific CD4⁺ T cells with an effector memory profile (CD62L low, CCR7 low).

Irrespective of the allergen, specific CD4⁺ T cells from non-allergic individuals express higher levels of CTLA-4 and CXCR3 when compared with allergics. CTLA-4 plays a role in peripheral CD4⁺ T cell tolerance by negatively regulating T cell responses [28] and CXCR3 has been proposed to be a marker associated with Th1 responses [29]. Whereas we could detect Foxp 3⁺ bright Bet v 1-specific T cells in healthy individuals [13], such an analysis could not be conducted in Der p 1- or Der p 2-specific T cells due to the low avidity of the corresponding tetramers. Nonetheless, together with the observation that mite-specific CD4⁺ T cells express IFN- γ and to a lesser extent IL-10 in non-allergics (Fig. 4), our results are consistent with the hypothesis that the latter encompass predominantly Th1 and regulatory T cells, as also suggested by others [12]. Surprisingly, in contrast to *bona fide* Th2 responses directed to Bet v 1 observed in BP allergics, highly polymorphic CD4⁺ T cell responses are found in mite-allergic patients (Fig. 4). Specifically, CD4⁺ T cells directed to mite allergens often produce predominantly IFN- γ with or without Th2 cytokines, as reported in some studies [41, 42] but not others [43, 44]. In addition, some of these cells express the Th1 marker CXCR3 even in allergics, in contrast to Bet v 1-specific T cells (Fig. 3, left panel). The induction of such Th1 responses is surprising, most particularly for Der p 1, as the cysteine protease activity of this allergen has been linked to its propensity to elicit Th2 responses [45]. This discrepancy can possibly be explained by the fact that both Der p 1 and Der p 2 are presented within mites or dust samples in the context of high levels of endotoxin or other natural Th1 adjuvants [46, 47]. Alternatively, persistent exposure to high doses of antigen may lead to IFN- γ -producing CD4⁺ T cells, even in allergics [40, 42]. In addition, whereas most patients exposed to Bet v 1 develop allergic rhinitis linked with Th2 responses, mite-allergic patients with chronic exposure to such allergens also develop allergic dermatitis or asthma, possibly involving sustained Th1 or even Th17 responses [5, 48].

Using tetramers, we detected in HDM allergics circulating CD4⁺ T cells specific for Der p 1 and Der p 2 at frequencies in the range of 1 to 6/10 000 cells, in agreement with other studies relying either on limiting dilution

assays to quantify allergen-specific proliferating cells [41] or purification of cytokine-producing T cells [12]. Our results suggest that such frequencies do not vary significantly throughout the year, as expected for CD4⁺ T cells directed to perennial allergens. This result contrasts with our previous observation of a three-log transient expansion in BP-allergic patients as well as healthy individuals of circulating Bet v 1_{141–155}-specific CD4⁺ T cells during the BP season [13].

Altogether, with the limitation that only a few patients have been investigated, our study establishes that the CD4⁺ T cell responses differ between seasonal and perennial allergens, both in terms of the broadness and the spectrum of epitopes involved as well as the affinity of TCRs for corresponding antigens. We also observed that the Th2 vs. Th1/T Reg patterns of CD4⁺ T cell responses observed with Bet v 1 in allergics vs. non-allergics do not apply to mite allergens. Whereas it cannot be excluded that such differences are due to the inherent characteristics of Bet v 1 vs. Der p 1 and Der p 2 allergens, we hypothesize that distinct exposure conditions (i.e. sustained presence in the environment, association with natural Th1 adjuvants) lead to a variety of chronic inflammatory conditions, which in turn influence dramatically the nature of CD4⁺ T cells responses observed in peripheral blood. As an end result, whereas Bet v 1-specific CD4⁺ T cell responses are remarkably homogeneous in BP allergics or healthy individuals, mite-specific responses in HDM-allergic individuals are extremely variable. Immunotherapy protocols aiming to modulate existing immune responses to mite allergens should take into account such a diversity among patients, and efforts should be made to link selected patterns of T cell polarization with clinical benefit induced by such treatments.

Acknowledgements

We thank Martine Pinheiro for excellent secretarial assistance.

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

The authors declare no financial or commercial conflict of interest.

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