

Accepted Article Preview: Published ahead of advance online publication



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Cite this article as: Lennart M Roesner, Annice Heratizadeh, Gabriele Begemann, Petra Kienlin, Susanne Hradetzky, Margarete Niebuhr, Britta Eiz-Vesper, Christian Hennig, Gesine Hansen, Véronique Baron-Bodo, Philippe Moingeon, Thomas Werfel, Der p1 and Der p2-Specific T Cells Display a Th2, Th17, and Th2/Th17 Phenotype in Atopic Dermatitis, *Journal of Investigative Dermatology* accepted article preview 28 April 2015; doi: [10.1038/jid.2015.162](https://doi.org/10.1038/jid.2015.162).

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Accepted article preview online 28 April 2015

Der p1 and Der p2-specific T cells display a Th2, Th17, and Th2/Th17 phenotype in atopic dermatitis

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This work was done in Hannover, Germany.

Short title: HDM-specific T cells in AD

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Abbreviations used:

AD: atopic dermatitis

DC: dendritic cell

Der p: *Dermatophagoides pteronyssinus*

HDM: house dust mite

SCORAD: scoring atopic dermatitis

TLR: toll-like-receptor

PBMC: peripheral blood mononuclear cells

MHC: major histocompatibility complex

LPS: lipopolysaccharide

TCR: T cell receptor

MD-2: myeloid differentiation protein 2

To the editor

Atopic dermatitis (AD) represents an inflammatory, relapsing, non-contagious and itchy skin disorder affecting up to 30% of children and 2-10% of adults in industrialized countries (Bieber, 2008). It is well-established that allergen-specific T cells display a Th2 polarization in allergic donors (Bateman *et al.*, 2006; Macaubas *et al.*, 2006; Wambre *et al.*, 2008) with a tendency to develop into Th1 in chronic atopic dermatitis (AD) (Thepen *et al.*, 1996; Werfel *et al.*, 1996). The high proportion of Th2-polarized T cells seems to be the key factor in allergic inflammation (Werfel, 2009), and allergen-specific Th2 cells are reduced after successful specific immunotherapy (Wambre *et al.*, 2012; Wambre *et al.*, 2014). However, the classical paradigm of a Th2-polarized allergen-specific T cell has been questioned, since Th17 and Th22 polarizations have been described in allergic diseases (Aggarwal *et al.*, 2003; Eyerich *et al.*, 2009; Langrish *et al.*, 2005). Especially, the responses to house dust mite (HDM) allergens differ between atopic diseases. While in allergic asthma this seems to be influenced by LPS-TLR4 signalling, in allergic rhinitis TLR2 responses may rather play a role (Ryu *et al.*, 2013).

High-titered HDM-specific IgE can be detected particularly often in older children, adolescents and adults with AD. This study aimed to characterize Der p 1 and Der p 2-specific T helper cells in patients suffering from AD. 30 adult consecutive patients with AD fulfilling the criteria of Hanifin and Rajka (Hanifin and Rajka, 1980) from our Department who were IgE-sensitized to HDM were included to this study. These showed various IgE-sensitizations and a mild to severe disease activity (SCORAD 2.5-70.5; mean 33.4, see supplemental table S1). Median CAP class of our patient cohort was class 5 (*D. pteronyssinus*) and class 4 (Der p 1 and Der p 2), respectively. One further AD patient was included who suffered from the intrinsic variant. Three non-atopic healthy control individuals served as controls.

Regarding the investigation of antigen-specific T cells, the MHC multimer-technology represents the state-of-the-art in detection and characterization of cells directly ex-vivo. HLA-DRB1*1501 Der p 1₁₆₋₃₀, Der p 1₁₇₁₋₁₈₅ and Der p 2₂₆₋₄₀ MHC class II tetramers used in this study had already been applied in patients with allergic rhinitis and showed specific staining to freshly isolated lymphocytes in PBMC fractions in every third HLA-matched patient and in a frequency of 1-2 in 40.000 CD4⁺ T cells (Wambre *et al.*, 2011). In our study, of all 31 AD patients, 18 matched the HLA-type of the tetramers. The three non-atopic, healthy individuals of this study were selected as appropriate controls since they also matched the wanted HLA-type HLA-DRB1*1501.

In order to gain detectable amounts of allergen-specific cells from PBMCs without pre-amplification generating cell culture artefacts, we applied magnetic-bead enrichment of tetramer-binding cells as described earlier (Day *et al.*, 2003). Subsequently, every single tetramer-positive cell was stained by Chipcytometry (Hennig *et al.*, 2009) with a series of markers and a vital stain inside a microfluidic chip (Figure 1a). This imaging technique bears advantages over flow cytometry whenever a large set of markers shall be assessed on a small numbers of cells. Detailed information about methods and control experiments can be found in the online supplement.

Specific staining was detected in six out of 18 HLA-matched individuals (Figure 1b). Of all polarized T cells detected, 38.5% showed the Th2 marker pattern ($CD3^+/CD4^+/CRTh2^+/CCR6^-/IL-18R^-$), 23.1% displayed the Th17 surface markers ($CD3^+/CD4^+/CRTh2^+/CCR6^+/IL-18R^-$), and 19.2% a mixed Th2/Th17 phenotype ($CD3^+/CD4^+/CRTh2^+/CCR6^+/IL-18R^-$). Furthermore, a single cell (3.8%) with a Th1 marker pattern ($CD3^+/CD4^+/CRTh2^+/CCR6^-/IL-18R^+$) as well as four cells (15.4%) with other combinations of markers were observed (Figure 1b). All of these cells displayed an effector/memory phenotype ($CD45RA^-/CD45R0^+/CD27^-$) with the exception of the single Th1-polarized cell, which displayed the memory T cell marker set ($CD45RA^-/CD45R0^+/CD27^+$). The intrinsic patient showed one tetramer-positive cell which turned out to be a naïve T cell (as shown in Figure 1A, $CD45RA^+/CD45R0^-/CD27^+$). In HLA-matched, healthy control individuals no tetramer-positive T cells were detectable.

Patients with detectable tetramer⁺ T helper cells showed a significantly higher median SCORAD but a comparable IgE-sensitization pattern compared to those without detectable tetramer⁺ T cells (Figure S1). Since all patients of the tetramer⁺ group have been investigated in our clinical department several times, the value of the SCORAD depicted here reflects a chronic moderate to severe course of AD and not an incidental flare-up. On the one hand this may suggest that despite the small size of the cohort, the presence of tetramer⁺ T cells in the blood reflects the disease severity. On the other hand this observation indicates that the presence of tetramer⁺ T cells is more probable in those individuals suffering from more severe AD.

To elucidate whether the T cell phenotypes identified by surface marker analysis lead to the corresponding Th2/Th17 cytokine milieu, we analyzed secreted cytokines in cell culture supernatants of T cell lines (TCL) from 26 HDM-sensitized AD patients which were generated in the presence of Der p 1 and Der p 2, respectively. Only T cell lines with proliferative capacity upon re-stimulation with the antigen assessed by 3H-thymidine uptake

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were regarded as specific. Successful proliferation of Der p 1- and Der p 2-specific cells in the TCL could also be observed by a shift towards CD4⁺ content of the cultures (Figure 2a). Incubation with these antigens led to increased amounts of secreted IL-4, IL-17 and IL-22, while IL-9 and IFN- γ production was not affected (Figure 2b). This effect was observed for both allergens, but not in LPS-control experiments (Figure S2). TCL without Der p 1- or Der p 2-specific proliferative capacity served as controls. Here, no increased cytokine secretion after allergen incubation was detectable (Figure 2c). We therefore conclude that HDM-specific T cells might be the cytokine source although we cannot exclude that bystander cells activated by specific T cells contributed to the results obtained by this approach. Our data mirrors results from other allergic diseases, since a Th2/Th17 double-positive subfraction of human T helper cells was detected in allergic rhinitis and asthma (Cosmi *et al.*, 2010; Wambre *et al.*, 2011; Wang *et al.*, 2010). Probably, the Th17 polarization occurs due to microbial compounds like LPS, endotoxins, and other natural adjuvants accompanying HDM allergens (Hammad *et al.*, 2009). Additionally, the C-type lectin receptor Dectin-2 on DCs has been recently reported to bind HDM extracts and to signal towards Th2 and Th17 in mice (Barrett *et al.*, 2011; Parsons *et al.*, 2014).

In summary, we demonstrate that Der p 1- as well as Der p 2-specific T cells elicit a Th2/Th17-response in patients with AD, although the allergens are structurally and functionally different. However, the existence of Th2, Th17 and Th2/Th17 polarized HDM-specific T cells in AD patients may indicate the requirement for further disease-specific anti-inflammatory treatment strategies. New data provided here on the HDM-specific T cell response deserves attention, since they may help to further optimize specific immunotherapy in sensitized patients suffering from AD. Of note, combined use of Chipcytometry and tetramer staining could serve as a valuable diagnostic tool for interventional studies. By this approach new details on the allergen-specific immune response in relation to the clinical course in patients with AD might be identified in future.

Conflict of Interest

C.H. has founded a company commercializing Chipcytometry. V.B. and P.M. are employees of Stallergenes SA.

Acknowledgements

This study was supported by a grant of Hannover Medical School (HiLF Programme).

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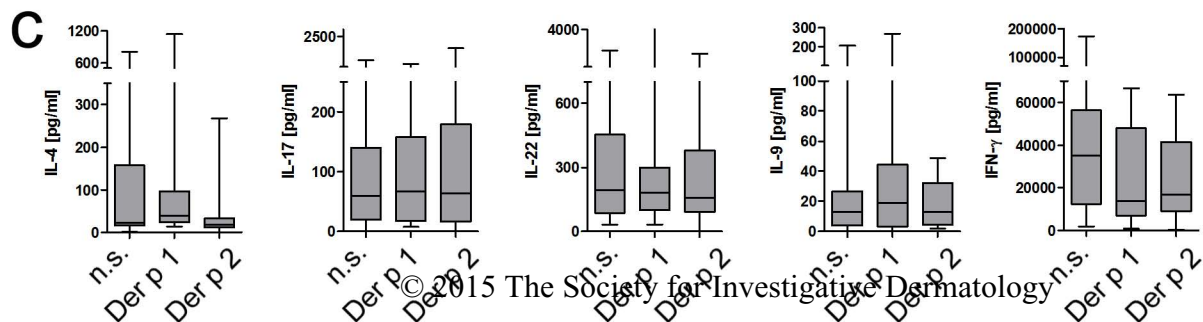
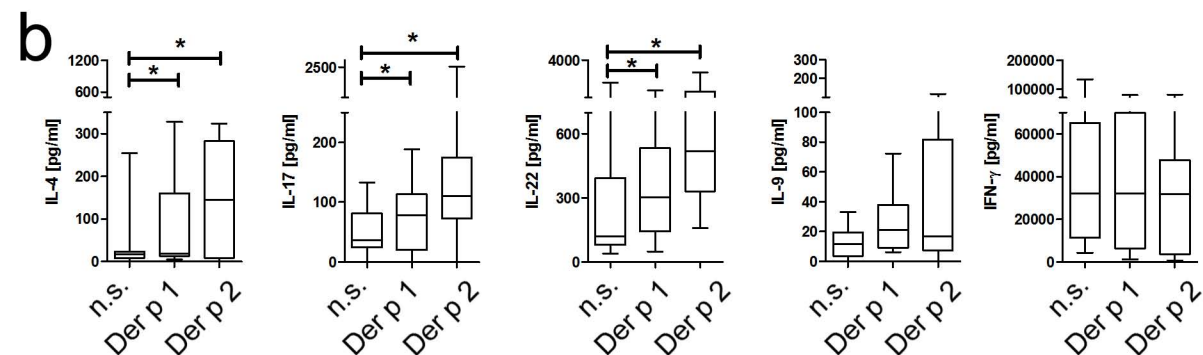
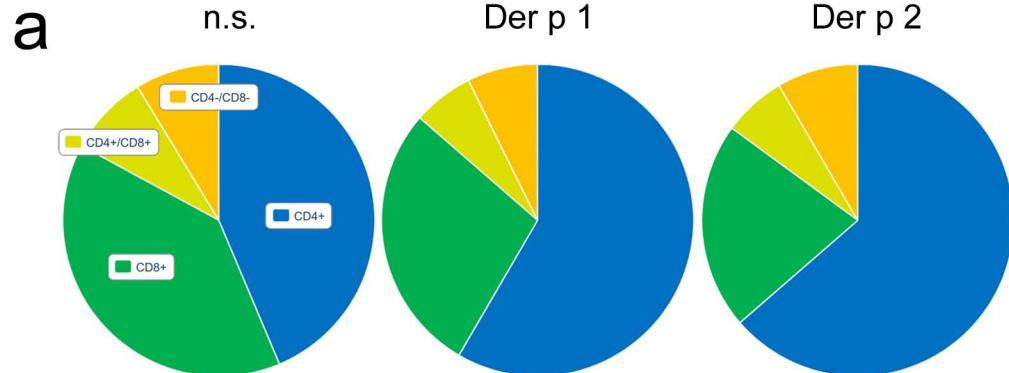
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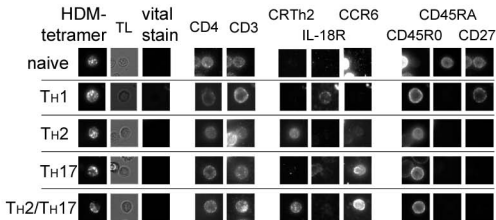
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Figure 1. Tetramer⁺ T cells were analyzed for the expression of surface markers as indicated and assigned to the corresponding polarization states. (a) Representative cells. HDM-tetramer, vital stain and eight surface markers were analyzed per cell. TL: transmitted light. **(b)** Polarization pattern of HDM-specific T helper cells identified. Cells expressed either CRTh2 (n=10/26), CCR6 (n=6/26), both CRTh2 and CCR6 (n=5/26), IL-18-R (n=1/26), or another combination of these (n=4/26) as indicated by the colours, or no polarization markers (not shown). Patient/cell numbers below the bars correspond to patient numbers in table S1.

Figure 2. T cell lines generated from *D. pteronyssinus*-sensitized patients with AD in the absence (n.s.) or presence of the antigen (Der p 1 and Der p 2, respectively). (a) Proportion of CD4⁺ cells was increased within the T cell lines generated in the presence of Der p allergens. **(b)** Cytokine production pattern of T cell lines that showed Der p 1 and Der p 2 specific proliferative capacities were intra-individually compared to unstimulated control cell lines. **(c)** Control experiments. T cell lines generated in the presence of Der p 1 and Der p 2, respectively, but negative in re-stimulation assay were compared to unstimulated control cell lines. *p<.05 Wilcoxon matched pairs test. Each group contained 9-17 patients.



a



b

tetramer positive T cells

