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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Der p1 and Der p2-Specific T Cells Display a Th2, Th17, and Th2/Th17 Phenotype in Atopic Dermatitis

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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 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, as Th17 and Th22 polarizations have been described in allergic diseases (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005; Eyerich *et al.*, 2009). Especially, the responses to house dust mite (HDM) allergens differ between atopic diseases. Although in allergic asthma this seems to be influenced by lipopolysaccharide (LPS)-toll-like receptor4 (TLR4) signaling, in allergic rhinitis TLR2 responses may rather have 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 *Dermatophagoides pteronyssinus* (Der p)-1– and Der p2–specific T-helper cells in patients suffering from AD. Thirty 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 in this study. These showed various IgE sensitizations and a mild-to-severe disease activity (scoring atopic dermatitis (SCORAD) 2.5–70.5; mean 33.4, see Supplementary Table S1). Median CAP class of our patient cohort was class 5 (*D. pteronyssinus*) and class 4 (Der p1 and Der p2), respectively. One further AD patient was included who suffered

Abbreviations: AD, atopic dermatitis; Der p, Dermatophagoides pteronyssinus*; HDM, house dust mite; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; SCORAD, scoring atopic dermatitis; TLR, toll-like receptor*

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from the intrinsic variant. Three non-atopic healthy individuals served as controls.

Regarding the investigation of antigen-specific T cells, the major histocompatibility complex (MHC) multimer-technology represents the state-of-the-art in detection and characterization of cells directly *ex vivo*. HLA-DRB1*1501 Der $p1_{16-30}$, Der $p1_{171-185}$, and Der $p2_{26-40}$ 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 peripheral blood mononuclear cell (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, as they also matched the wanted HLA-type HLA-DRB1*1501.

To gain detectable amounts of allergen-specific cells from PBMCs without pre-amplification generating cell culture artifacts, 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 number of cells. Detailed information about methods and control experiments can be found in the Supplementary Information online.

Specific staining was detected in six out of 18 HLA-matched individuals (Figure 1b). Of all the polarized T cells detected, 38.5% showed the Th2 marker pattern ($CD3^+/CD4^+/CRTh2^+/CCR6^-/IL\text{-}18R^-$), 23.1% displayed the Th17 surface markers ($CD3^+/CD4^+/CRTh2^-/CCR6^+/IL\text{-}18R^-$), and 19.2% a mixed Th2/Th17 phenotype ($CD3^+/CD4^+/CRTh2^+/CCR6^+/IL\text{-}18R^-$). Furthermore, a single cell (3.8%) with a Th1 marker pattern ($CD3^+/CD4^+/CRTh2^-/CCR6^-/IL\text{-}18R^+$) as well as four cells (15.4%) with other combinations of markers was observed (Figure 1b).

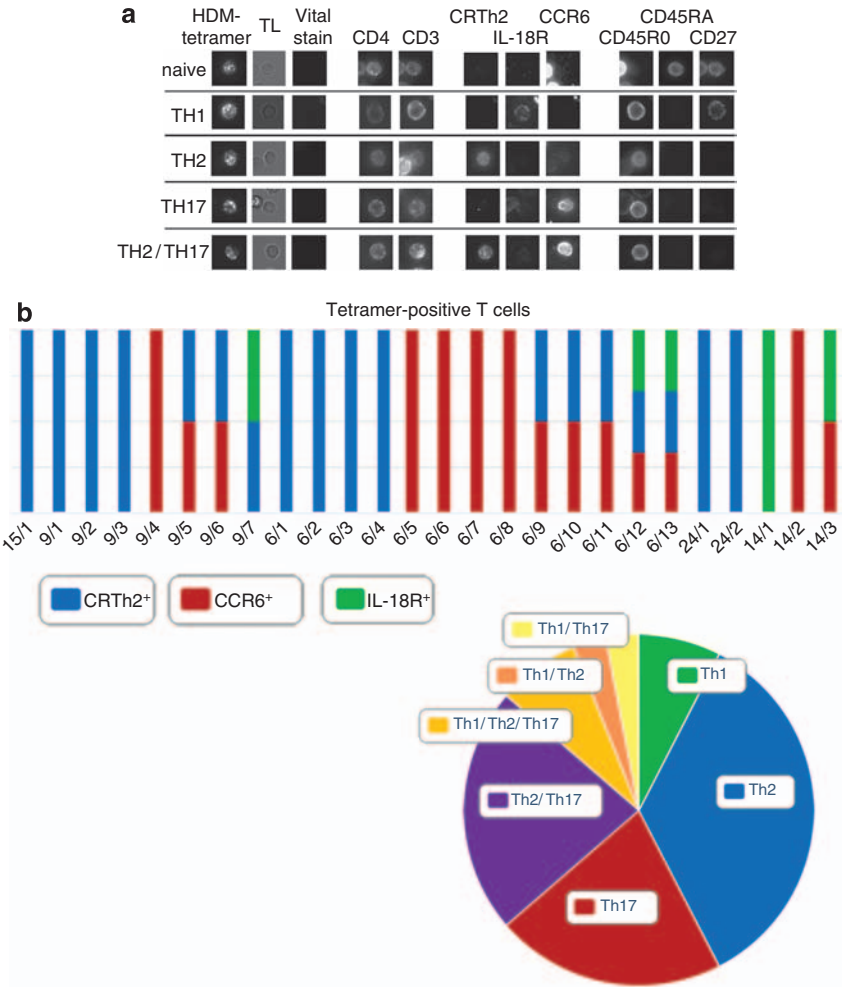

Figure 1. Tetramer$^+$ T cells were analyzed for the expression of surface markers as indicated and assigned to the corresponding polarization states. (**a**) Representative cells. HDM-tetramer, vital stain, and eight surface markers were analyzed per cell. TL, transmitted light. (**b**) Polarization pattern of HDM-specific T-helper cells identified. Cells expressed 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 colors, or no polarization markers (not shown). Patient/cell numbers below the bars correspond to patient numbers in Supplementary Table S1 online. TL, transmitted light.

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 naive 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 with those without detectable tetramer$^+$ T cells (Supplementary Figure S1 online). As 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