Expansion of circulating follicular T helper cells associates with disease severity in childhood atopic dermatitis

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Highlights

- The ratio and absolute number of circulating T_{FH}-like cells were assessed in AD
- $ICOS^+$ T_{FH}-like cell ratios and numbers were increased mainly in children with AD
- IL-21^+ T_{FH}-like cell percentages were elevated in children compared to adults in AD
- SCORAD index correlated with the numbers of IL-21^+ T_{FH}-like cells in paediatric AD

Abstract

Follicular helper T (T_{FH}) cells play crucial role in B-cell differentiation and antibody production. Although, atopic dermatitis (AD) is often associated with increased serum IgE levels, B-cell mediated responses have not been studied thoroughly. The aim of our study was to investigate the proportion of T_{FH}-like cells in the disease. Twelve children and 17 adults with AD as well as 14 healthy controls were enrolled in the study. The frequency of $CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ T_{FH}-like cells and their IL-21 cytokine production were determined by flow cytometry. Immunohistochemical analysis was performed on skin biopsy specimens from AD patients for the detection of T_{FH} markers. The percentages and absolute numbers of circulating T_{FH}-like cells were significantly increased in children with AD compared to adult patients and healthy controls. IL-21 cytokine production of T_{FH}-like cells was also elevated and showed a strong positive correlation with paediatric patients' SCORAD index. The expression of T_{FH}-specific markers showed only a non-specific scattered pattern in skin biopsy specimens. This is the first study to demonstrate that T_{FH}-like cells expanded in the peripheral blood of children with AD compared to adults. These results reinforce the importance of further investigations on T_{FH}-like cells in different phenotypes and endotypes of AD.

Key words

dermatology; flow cytometry; circulating follicular T helper cells; interleukin-21; SCORAD

Abbreviations

AD: atopic dermatitis

CSR: class-switch recombination

CXCR5: chemokine (C-X-C motif) receptor type 5

DCs: dendritic cells

DEHP: di-(2-ethylhexyl)phthalate

FFPE: formalin-fixed, paraffin-embedded

GC: germinal center

ICOS: inducible T cell co-stimulator

Ig: immunoglobulin

IL: interleukin

PD-1: programmed cell death protein 1

PerCP-Cy5.5: Peridinin-chlorophyll protein-Cyanine dye 5.5

PMA: phorbol-12-myristate 13-acetate

SAP: signalling lymphocytic activation molecule (SLAM)-associated protein

SCORAD: SCORing Atopic Dermatitis

SHM: somatic hypermutation

STAT3: signal transducer and activator of transcription 3

T_{FH}: Follicular helper T

Th: T helper

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease. Clinically, it results in itchy, hyperaemic, swollen, excoriated plaques, which may also show lichenification. In about 80 % of patients, the disease associates with elevated levels of serum immunoglobulin (Ig) E [1]. The prevalence of the disease has strongly increased in developed countries during the last few years. According to epidemiological studies, it affects approximately 2-10 % of the adult and 15-30 % of the paediatric population worldwide [2]. Genetic predisposition as well as environmental factors exerts a profound influence on the disruption of intact epidermal barrier and on the development of abnormal immune responses. The interaction of above mentioned elements contribute to the sensitization and the development of unique clinical features of the disease [3, 4]. AD is known to be a complex heterogeneous disease with various phenotypes and endotypes which arises from age-related differences, acute or chronic phase of the disease and the presence or absence of allergen sensitizations may be associated with distinct branches of immune activation [5]. The pathomechanism of AD may be easier to understood as a biphasic system with the predominance of T helper (Th) 2 mediated responses with elevated interleukin (IL)-4 and IL-13 cytokines and Th22 cells in the acute phase followed by an expanded Th1 and Th17 guided responses in the chronic period [4, 6, 7]. Despite the complex interactions between keratinocytes, skin dendritic cells (DCs) and T cells, for the most part, generalized Th2 dysbalance and isotype switching of B cells to generate specific IgE is often associated with AD. However, the role of B cells and other participants in the regulation of humoral immunity in allergic or skin diseases has remained poorly examined. Although studies about the expansion of circulating B cells in patients with psoriasis and AD are reported, observations regarding B cell mediated

immune responses are limited [8, 9]. For that purpose, the profound examination of germinal center (GC) processes with a special emphasis on the regulation of IgE-producing plasma cell generation needs to be clarified in AD.

Follicular helper T (T_{FH}) cell, a specialized subset of $CD4^+$ T cells provides fundamental help to antigen specific B-cells in proliferation, differentiation as well as antibody production, are one of the most intensively studied cells [10, 11]. In the past few years, numerous research groups have described altered ratio of circulating T_{FH}-like cells in autoimmune and inflammatory diseases. Expansion of $CD4^+CXCR5^+$ T_{FH}-like cells was reported in autoimmune diseases, including systemic lupus erythematosus [12, 13], Sjögren's syndrome [14, 15], rheumatoid arthritis [16], juvenile dermatomyositis [17] and bullous pemphigoid [18]. However, in dermatology, investigations of T_{FH}-like cells are limited and occur mostly in cutaneous lymphomas [19] or inflammatory disorders such as psoriasis [20-22]. T_{FH} cells arise from activated T cells following the encounter with DCs, and migrate to the border of T and B cell areas in the lymph node under the guidance of Bcl-6 or c-Maf mediated chemokine receptors [23, 24]. GC localized T_{FH} cells are distinguished from other effector T cells by the intense expression of chemokine (C-X-C motif) receptor type 5 (CXCR5), inducible T cell co-stimulator (ICOS), programmed cell death protein 1 (PD-1) and the production of IL-21. Additional features include the elevated expression of CD40L, OX40L, signalling lymphocytic activation molecule (SLAM)-associated protein (SAP) and SLAM family receptors such as CD84 [25-27]. The interplay between T_{FH} and activated B cells is essential not only for GC reactions, including somatic hypermutation (SHM) and immunoglobulin class-switch recombination (CSR), but for the generation of extrafollicular responses as well [28].

The special capability of T_{FH} cells to direct B cell responses and antibody production proposed the possibility that they may play an important role in the pathogenesis of AD. Based on that potential concept, we investigated the distribution of T_{FH} cells and their markers not only in the peripheral blood, but in skin biopsy samples of patients with AD as well.

2. Material and methods

2.1. Patients and healthy individuals

Peripheral blood was obtained from 29 patients with AD (19 female and 10 male; mean age 18.97 ± 11.03 years) for flow cytometric analysis. Fourteen healthy individuals (11 female and 3 male; mean age 22.64 ± 10.16 years) served as controls. Among control subjects, there were 4 individuals under 16 years (3 female and 1 male; mean age 8.50 ± 4.80 years). Since their cell ratios did not differ significantly, we included them into one control pool. AD patients were classified based on their age: one group comprised subjects with 12 children (10 female and 2 male; mean age 9.58 ± 3.82), while the other group consisted of subjects with 17 adults (9 female and 8 male; mean age 26.58 ± 7.99). Skin biopsy specimens were also collected from 5 patients at the Department of Dermatology, Clinical Center, University of Debrecen. All patients fulfilled the diagnostic criteria for AD established by Hanifin and Rajka [29]. The severity of the disease was determined by the SCORAD (SCORing Atopic Dermatitis) index. The mean SCORAD index of the patients was 43.21 ± 13.04 with the range of 24-72. In children, the mean SCORAD index was 43.67 ± 11.91 with the range of 28-68, while in adult it was 42.88 ± 13.82 with the range of 24-72. The median serum total IgE was 1453 kU/L with a range of 22-10000 kU/L. The titer of IgE was 637 kU/L (22-10000 kU/L) in children and 2537 kU/L (135-10000 kU/L) in adults. None of the patients received any antihistamines or topical corticosteroids for at least 5 days, or systemic immunosuppressive or immunomodulating medications for at least 4 weeks prior to the collection of blood samples. No patients or controls had ongoing or recent previous infections during the study. Data were obtained retrospectively from patients' records which contained detailed information about other possible allergic disease and

sensitization. Informed written consent was obtained from the subjects, and the study was approved by the Ethics Committee of the University of Debrecen. All experiments carried out were in compliance with the Declaration of Helsinki. Demographic data were summarized in Table 1.

2.2. Determination of circulating T_{FH}-like cells by flow cytometry

For the assessment of circulating T_{FH} cells, cells from 100 µl of heparinized whole peripheral blood were stained with CXCR5-Alexa Fluor 488 (clone: RF8B2)/ICOS-PE (clone: DX29)/PD-1-Peridinin-chlorophyll protein-Cyanine dye 5.5 (PerCP-Cy5.5) (clone: EH12.1)/CD4-APC (clone: RPA-T4) (all from BD Biosciences, San Jose, CA, USA) fluorochrome-labeled monoclonal antibodies. After the incubation period, erythrocytes were haemolysed. Stained cells were assessed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data analysis was performed by FlowJo Software (Treestar, Ashland, OR, USA). At least 40.000 $CD4^+$ events of each sample were analysed within the entire lymphocyte population. The absolute number of T_{FH}-like cells was determined from the percentage of these cells calculated by flow cytometry and from the absolute number of lymphocytes (x 10^9 L^{-1}) detected by a haematological cell counter.

2.3. Determination of single-cell cytokine production of circulating T_{FH}-like cells by intracellular cytokine analysis

For intracellular cytokine examination, diluted whole blood was incubated with phorbol-12-myristate 13-acetate (PMA) (25 ng/ml), ionomycin (1 µg/ml), and Golgi Stop brefeldin-A (10 µg/ml) (all from Sigma Aldrich, St Louis, MO, USA) at 37°C in 5% CO_2

milieu for 5 h. Cell surface staining was performed with fluorochrome-labeled monoclonal antibodies against CD4, CXCR5, PD-1 for 15 minutes at room temperature. Cells were then fixed and after erythrocyte lysation the membrane of the lymphocytes was permeabilized with Intraprep^{TM} permeabilization reagent (Beckman Coulter Inc, Miami, FL, USA) according to the manufacturer's instructions. Cytokines were stained with anti-IL-4-PE, anti-IL-10-PE and anti-IL-21-PE (all from BD Biosciences) for 30 minutes at room temperature. Measurements were performed and data were collected using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and data were analyzed using FlowJo Software (Treestar, Ashland, OR, USA). In case of intracellular measurements at least 20.000 $CD4^+$ events per sample were analysed within the whole lymphocyte population.

2.4. Immunohistochemistry

Immunostainings were performed on formalin-fixed, paraffin-embedded (FFPE) tissue blocks on lesional skin from AD patients' forearm (all severe AD; n=5). Four-µm thick serial sections of tissue specimens were prepared and stained with haematoxylin-eosin for conventional histopathological examination. Additional 4 µm thick FFPE sections were deparaffinized, rehydrated on descending ethanol dilutions and treated with H_2O_2 to block endogenous peroxidase. For antigen retrieval, sections were heated in boiling citrate buffer (pH 6.0) or Tris/EDTA buffer (pH 9.0) using a pressure cooker. After cooling, the slides were incubated with primary antibodies for 1 hour at room temperature or overnight at 4°C. The following antibodies were used in the procedure: mouse monoclonal anti-CD20 (clone L26, Dako, Glostrup, Denmark); rabbit monoclonal anti-CD84 (clone EPR8325, Abcam, Cambridge, UK); rabbit polyclonal anti-PD-1 (bs-

1867R, Bioss, Woburn, MA, USA); rabbit polyclonal anti-Bcl6/5 (bs-2734R, Bioss), rabbit polyclonal anti-IL-21 (Abcam). Super Sensitive One-Step Polymer-HRP Detection System (BioGenex, Fremont, CA, USA) as secondary antibody with Very Intense Purple peroxidase substrate (Vector Laboratories, Peterborough, UK) were used for detection. The sections were then counterstained with methyl green (Vector Laboratories). Images of skin tissue sections were taken using a Leica DM2000 LED (Leica Microsystems GmbH, Wetzlar, Germany) microscope equipped with an Evolution™ MP 5.0 Mega-pixel Camera Kit (Media Cybernetics, Inc., Silver Spring, MD USA) connected to a computer. For transferring and editing images, QCapture Pro 6 (QImaging, Surrey, BC Canada) and Adobe Photoshop CS5 were used.

2.5. Statistical analysis

Data were analyzed and statistically evaluated with GraphPad Prism 5 software (Graphpad Software, San Diego, USA). To assess the distribution of the data Kolmogorov-Smirnov test was used. In cases of normal distribution, if the F probe was granted we used unpaired two-sample t test, otherwise if it was not granted, we used Welch's t test for statistical comparison of the experimental data. In cases if distributions the data set was different from normal, the Mann–Whitney U test was used. The correlations between two variables were evaluated with Pearson's rank correlation coefficient. Differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Expression of T_{FH}-related cell specific markers on $CD4^+CXCR5^+$ T cells

According to the expression of CXCR5, we gated a $CD4^+CXCR5^+$ lymphocyte subgroup and determined the fractions of $ICOS^+$, $PD\text{-}1^+$ and $ICOS^+PD\text{-}1^+$ T cells in children as well as adults with AD versus healthy controls (Fig. 1A). The ratio of $ICOS^+$ cell among $CD4^+CXCR5^+$ lymphocytes were significantly increased in children with AD when compared to adults with AD and healthy controls (15.66 ± 7.723% vs. 7.618 ± 4.149%, respectively, p=0.0050 and 15.66 ± 7.723% vs. 7.616 ± 4.515%, respectively, p=0.0030) (Fig. 1A). The percentages of $PD\text{-}1^+$ cells were also significantly elevated in children with AD, compared to adults with AD and increased in healthy individuals as well (38.88 ± 6.568% vs. 28.18 ± 6.673%, respectively, p=0.0047 and 35.88 ± 6.568% vs. 30.72 ± 7.730%, respectively, p=0.0818) (Fig. 1A). We obtained the same tendency when analyzed $ICOS^+PD\text{-}1^+$ cell percentages, the ratio of double positive cells showed a significant expansion in children with AD in contrast to adults with AD and controls (9.539 ± 4.940% vs. 4.521 ± 2.648%, respectively, p=0.0059 and 9.539 ± 4.940% vs. 4.315 ± 2.430%, respectively, p=0.0044) (Fig. 1A). We did not find significant difference in the ratio of the aforementioned markers between adults with AD and control group; interestingly, the proportions of ICOS and PD-1 differed barely from control values.

To measure the nature and frequency of circulating $CD4^+CXCR5^+$ T cell subset, the *de novo* synthesized intracellular IL-21 cytokine production was also determined (Fig. 1B). Similarly to cell surface staining, the frequency of $PD\text{-}1^+$ cells were showed the same tendency and significantly elevated in children with AD compared those measured in adults with AD and controls (34.80 ± 7.149% vs. 26.91 ± 7.075%, respectively, p=0.0207 and 33.49 ± 7.149% vs. 26.61 ± 6.721%, respectively, p=0.0185) (Fig. 1B). The ratio of

$CD4^+CXCR5^+$ T cells producing IL-21 was increased in children with AD, however it did not differ significantly compared to adults with AD and controls (Fig. 1B). The frequency of $PD\text{-}1^+IL\text{-}21^+$ cells were non-significantly increased in children with AD compared to healthy controls (9.807 ± 3.732% vs. 7.104 ± 4.140%, respectively, p=0.0525) (Fig. 1B). These cell proportions also differed between children and adults with AD (9.807 ± 3.732% vs. 7.412 ± 2.913%, respectively, p=0.0628), but it was not significant (Fig. 1B).

3.2. Quantification of circulating T_{FH}-like cells

Circulating T_{FH}-like cells were quantified as their percentages within the $CD4^+$ lymphocytes of peripheral blood. According to our results, the percentages of $CD4^+CXCR5^+ICOS^+$ T cells were significantly increased in children with AD when compared to adults with AD and healthy controls (1.675 ± 0.8029% vs. 0.8860 ± 0.5281%, respectively, p=0.0035 and 1.675 ± 0.8029% vs. 0.9991 ± 0.6141%, respectively, p=0.0230) (Fig. 2A). Cell ratios in the group of adults with AD did not differ from control values. The proportions of $CD4^+CXCR5^+PD\text{-}1^+$ T cells were significantly decreased in adults with AD, compared to control results (3.203 ± 1.095% vs. 4.138 ± 1.356%, respectively, p=0.0422) (Fig. 2A). Although cell ratios were lower in children with AD than in healthy individuals, it did not differ significantly. The same tendency was observed between children and adults with AD. The percentages of $CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ T_{FH}-like cells were significantly increased in children with AD compared those quantified in adults with AD and controls (1.019 ± 0.4977% vs. 0.5196 ± 0.3253%, respectively, p=0.0029 and 1.019 ± 0.4977% vs. 0.5680 ± 0.3360%,

respectively, p=0.0113) (Fig. 2A). The ratio of T_{FH}-like cells was similar in adults with AD and healthy individuals.

In order to gain a better view on the distribution of T_{FH}-like cells in the peripheral blood of AD patients and controls, we quantified the absolute numbers of the investigated cell populations. Significantly elevated absolute numbers of $CD4^+CXCR5^+ICOS^+$ T cells were found in children with AD in comparison with adults with AD and controls (16.57 ± 8.215 x 10^6 L^{-1} vs. 7.159 ± 3.809 x 10^6 L^{-1}, respectively, p=0.0024 and 16.57 ± 8.215 x 10^6 L^{-1} vs. 10.13 ± 6.081 x 10^6 L^{-1}, respectively, p=0.0460) (Fig. 2B). Similarly, to cell ratios, the absolute numbers of this cell subset in adults with AD did not differ from values in control group. The absolute numbers of $CD4^+CXCR5^+PD\text{-}1^+$ T cells were slightly increased in children with AD compared to controls; however, the difference was not significant. On the other hand, the absolute numbers of these cells in adults with AD were significantly dampened in contrast to values measured in children with AD (26.70 ± 6.694 x 10^6 L^{-1} vs. 38.85 ± 14.40 x 10^6 L^{-1}, respectively, p=0.0165), moreover we found an intense significant decrease, when compared to results of control subjects (26.70 ± 6.694 x 10^6 L^{-1} vs. 40.68 ± 14.33x 10^6 L^{-1}, respectively, p=0.0098) (Fig. 2B). The absolute numbers of $CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ T_{FH}-like cells were also elevated significantly in children with AD, compared to values of adults with AD and controls (10.22 ± 5.248 x 10^6 L^{-1} vs. 4.158 ± 2.215 x 10^6 L^{-1}, respectively, p=0.0023 and 10.22 ± 5.248 x 10^6 L^{-1} vs. 5.790 ± 3.670 x 10^6 L^{-1}, respectively, p=0.0302) (Fig. 2B). We did not found any difference in the cell count between adults with AD and controls.

3.3. Measurement of individual cytokine production of circulating T_{FH}-like cells

To confirm the cytokine profile of circulating T_{FH}-like cells, we assessed the intracellular IL-21 cytokine production beside PD-1 expression on $CD4^+CXCR5^+$ T_{FH}-like cells. Although the ratio of $CD4^+CXCR5^+PD\text{-}1^+$ T cells, $CD4^+CXCR5^+IL\text{-}21^+$ T cells and IL-21 producing T_{FH}-like cells did not differ significantly in the measured subgroups with AD and controls (Fig. 3A). Interestingly, when we quantified the cell subsets between children and adults with AD, we revealed that the absolute numbers of both $CD4^+CXCR5^+PD\text{-}1^+$ T cells ($32.73 \pm 10.55 \times 10^6$ L^{-1} vs. $22.61 \pm 5.896 \times 10^6$ L^{-1}, respectively, p=0.0088) and $CD4^+CXCR5^+IL\text{-}21^+$ T cells ($16.53 \pm 6.771 \times 10^6$ L^{-1} vs. $12.31 \pm 4.738 \times 10^6$ L^{-1}, respectively, p=0.0580) were elevated in children in comparison with adults, although the latter did not differ significantly (Fig. 3B). On the other hand, the absolute numbers of IL-21 producing T_{FH}-like cells showed a distinct significant expansion in children with AD compared those analysed in adults with AD ($10.17 \pm 4.302 \times 10^6$ L^{-1} vs. $5.891 \pm 2.327 \times 10^6$ L^{-1}, respectively, p=0.0068), while it was only mildly elevated in contrast to control values (Fig. 3B). There were no significant differences in intracellular IL-4 and IL-10 positivity between patients and controls.

3.4. Correlation analysis between circulating T_{FH}-like cells and SCORAD score in patients with AD

We investigated the possible associations between the ratio of T_{FH}-like cells and the SCORAD values in the two subgroups of patients with AD. We discovered an obvious significant positive correlation between the absolute numbers of $CD4^+CXCR5^+PD\text{-}1^+IL\text{-}21^+$ T_{FH}-like cells and SCORAD index in children with AD (R=0.6105, respectively, p=0.0350), while our observations in adults with AD did not reveal any connection with the severity of the clinical symptoms (Fig. 3C).

3.5. Localization of T_{FH}-related marker expression in skin biopsies

Skin samples from patients with AD contained only a few cells expressing T_{FH}-specific markers and they displayed a scattered distribution tendentiously in the dermis (Fig. 4). We detected some $CD20^+$ B cells in the biopsy specimens, although this population form a small proportion of the dermal infiltrates. Interestingly, PD-1 marker was strongly expressed in the stratum granulosum. It is possible, that the applied antibody could cross-react with the ligand of PD-1 (PD-1L) which is expressed on activated keratinocytes.

4. Discussion

AD is a multifactorial, immune mediated inflammatory disease characterized by increased inflammatory cell migration to the skin lesions, elevated and abnormal production of inflammatory cytokines as well as higher amount of Th2 cells and frequently increased IgE level in the periphery [3]. According to the literature, IgE responses originate from two sources. Early, low-affinity antibodies produced by short-lived plasma cells arise from extrafollicular sources, whereas late IgE responses with high-affinity antibodies derive from GC reactions [30]. Direct switching to IgE results lower affinity IgE antibodies produced by short-lived plasma cells which generally form the major part of IgE responses [31]. However, sequential CSR in GCs yields IgEs that show hypermutations and affinity maturation which could bind environmental antigens with higher affinity [30, 32]. The elevated level of IgE in the serum of patients with AD raised a question, whether GC processes and T_{FH} cells are responsible for their production and regulation. The possible role of T_{FH} cells in allergic diseases has arisen recently, but no papers have been published focusing on the ratio and function of these cells in AD yet [33, 34].

In the last few years, multiple studies specialized to understand the origin and function of blood $CD4^+CXCR5^+$ T cells. These observations indicated that in contrast to GC T_{FH} cells, circulating T_{FH} cells express T_{FH}-related markers at lower intensity, however retain functional characteristic to promote B cell differentiation and Ig production [17, 35]. At the periphery, T_{FH}-like cells appear to represent a memory $CD4^+$ T cell subset and according to the expression of certain molecules, including ICOS, PD-1, CCR7, CXCR3 and CCR6, could be subdivided into several subsets [17, 36]. In the present study, we assessed the distribution of circulating T_{FH}-like cells, moreover we examined the presence

of their markers at the site of the inflammation in patients suffering from AD. Interestingly, we found significantly elevated ratio of $ICOS^+$ and $PD\text{-}1^+$ and increased ratio of $IL\text{-}21^+$ cells among the peripheral $CD4^+CXCR5^+$ T cell subset only in children with AD, while the values of these markers in adults with AD were similar those measured in healthy individuals. Next, we analysed not only the proportions of T_{FH}-like or IL-21 producing T_{FH}-like cells but the absolute numbers of the cells as well. We revealed a significant expansion only in paediatric patients with AD compared mainly to adults with AD. Our results are consistent with those recent observations showing that the initial pathogenic processes and T cell polarization in AD are different in children and adults [5]. However, our current knowledge about the pathogenesis of AD generally comes from studies on adult population, although the disease prevalence is higher in earlier ages. Early AD is often associated with other allergic diseases, including food allergy, allergic bronchial asthma as well as allergic rhinoconjunctivitis and from childhood this sequential disease development is called the atopic march [37]. Atopy has the aptitude to develop heightened IgE levels since T cell-B cell interactions seem to associate with this phase [38]. Most studies investigated immune-competent cells in cohort of children as well as adults did not compare the results simultaneously. Recently, Czarnowicki *et al.* compared the activation markers and cytokine production of skin-homing polarized T cell subsets both in children and adults with AD revealing a marked difference between the groups. Higher ICOS expression of $CD4^+$ cells were showed among paediatric patients with AD. As a next step, T helper cells were distinguished into systemic and skin-homing T cell populations, which revealed that ICOS expression was characteristic to non-cutaneous T cell subset [39]. Recently, the same group revealed that ICOS activated T cells not only participate in the disease maintenance but also correlate

with activated and memory B cells [38]. These results along with our findings underline the role of ICOS in regulating T-B cell interaction by augmenting their accumulation in draining lymph nodes and their importance in Th2 immunity in the early disease phenotype [40]. We also investigated the associations between the absolute numbers of IL-21 producing T_{FH}-like cells and the SCORAD scores. Although, no significant association was found between serum IgE levels and the severity of skin inflammation, nevertheless, the SCORAD index showed a strong positive correlation with the absolute numbers of IL-21$^+$ T_{FH}-like cells in children. Of note, clinical features of AD show a variable pattern according to the phase of the disease and the ages of the patients. In children, AD is characterized mainly by acute inflammatory lesions, whereas adults generally express skin thickening with eczematous lesions and lichenifications [41]. Our correlation analysis supports the role of IL-21 producing circulating T_{FH}-like cells in the participation of disease pathogenesis in paediatric patients. Regarding the effect of IL-21 in antibody production, former studies revealed that IL-21, IL-4 and CD40L could act synergistically to enhance the isotype-switching of IgE in human B cells via signal transducer and activator of transcription 3 (STAT3) dependent manner [42, 43]. Furthermore, an elevated IL-21 and IL-21R expression in skin biopsy of patients with psoriasis or AD was published recently [44, 45]. In addition, latter studies revealed an altered B cell distribution in AD with elevated transitional B cells, IgE expressing memory B cell subsets and plasmablasts; moreover, an elegant work with ovalbumin-sensitized CD19-deficient mice shed light on the role of B cells in the pathogenesis of AD [9, 46]. In another study, low numbers of non-switched memory B cells were showed in paediatric AD compared to adult AD confirming the dominance of T-cell dependent mechanisms in early childhood [38]. Aforementioned evidences suggest that upon

secondary antigen exposure and activation in inflammatory environment, circulating memory T_{FH} cells could migrate to draining lymphoid nodes and gain fully matured effector T_{FH} phenotype to support GC responses [47, 48]. Additionally, concerning their potential plasticity and the current status of the immune system, as highlighted recently, they may promote Th2 immunity or gain the characteristics of Th2 cells at the periphery [49-51].

Considering the dermal infiltration of T cells in AD, we examined biopsy samples from lesional skin biopsies of patients for the potential expression of T_{FH}-related markers. T_{FH} markers either were not detected or showed a non-specific scattered pattern in skin biopsy. Although, other authors reported that higher IL-21 mRNS expression could be detected in skin biopsy of AD patients, perhaps it was secreted by other sources, for example Th17 cells. We suppose that blood T_{FH} cells could migrate to regional lymph nodes rather than traffic to the skin. This statement is consistent with a study which demonstrate a divergent expression design for IL-4 and IL-13 during allergic condition, because it state that lung $CD4^+$ T cells do not express PD-1 and CXCR5, as well as T_{FH} cells in the lymph node express significant amounts of both IL-4 and IL-21 mRNA, but not IL-13 [52]. Another group examined the effects of di-(2-ethylhexyl)phthalate (DEHP) on humoral immunity in splenocytes and found that it acts as an adjuvant to increase the secretion of allergy-related IgE and IgG1 as a result of the stimulation of IL-21 and IL-4 production via the over-expression of transcription factors Bcl-6 and Maf in T_{FH} cells [53].

In conclusion, our results revealed an expansion in the ratio and number of circulating T_{FH}-like cells and IL-21 producing T_{FH}-like cells in children with AD compared to adult patients, moreover a clear correlation between these elevated T cell subsets and the

severity of skin inflammation. However, we also have to mention some limitations of this study. Since AD is a clinically heterogeneous disease and we worked with a relatively small sample size, our results should be confirmed in an independent set of patients and controls as well. Nevertheless, these results reinforce the importance of further investigations of T_{FH}-like cells with special attention to the interaction of B cells and certain T_{FH} cell subsets in different phenotypes and endotypes of AD.

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Conflict of interest

The authors declare that they have no competing interests.

References

[1] C.A. Akdis, M. Akdis, T. Bieber, C. Bindslev-Jensen, M. Boguniewicz, P. Eigenmann, Q. Hamid, A. Kapp, D.Y. Leung, J. Lipozencic, T.A. Luger, A. Muraro, N. Novak, T.A. Platts-Mills, L. Rosenwasser, A. Scheynius, F.E. Simons, J. Spergel, K. Turjanmaa, U. Wahn, S. Weidinger, T. Werfel, T. Zuberbier, E.A.o. Allergology., A.a.I. Clinical Immunology/American Academy of Allergy, Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report., J Allergy Clin Immunol. 118(1) (2006) 152-169.
[2] D.Y. Leung, M. Boguniewicz, M.D. Howell, I. Nomura, Q.A. Hamid, New insights into atopic dermatitis., J Clin Invest. 113(5) (2004) 651-657.
[3] T. Bieber, Atopic dermatitis., Ann Dermatol. 22(2) (2010) 125-137.
[4] K. Eyerich, N. Novak, Immunology of atopic eczema: overcoming the Th1/Th2 paradigm., Allergy 68(8) (2013) 974-982.
[5] D.Y. Leung, Atopic dermatitis: Age and race do matter!, J Allergy Clin Immunol. 136(5) (2015) 1265-1267.
[6] K. Eyerich, J. Huss-Marp, U. Darsow, A. Wollenberg, S. Foerster, J. Ring, H. Behrendt, C. Traidl-Hoffmann, Pollen grains induce a rapid and biphasic eczematous immune response in atopic eczema patients., Int Arch Allergy Immunol. 145(3) (2008) 213-223.
[7] T. Werfel, J.P. Allam, T. Biedermann, K. Eyerich, S. Gilles, E. Guttman-Yassky, W. Hoetzenecker, E. Knol, H.U. Simon, A. Wollenberg, T. Bieber, R. Lauener, P. Schmid-Grendelmeier, C. Traidl-Hoffmann, C.A. Akdis, Cellular and molecular immunologic mechanisms in patients with atopic dermatitis., J Allergy Clin Immunol. 138(2) (2016) 336-349.
[8] J. Lu, Y. Ding, X. Yi, J. Zheng, CD19+ B cell subsets in the peripheral blood and skin lesions of psoriasis patients and their correlations with disease severity., Braz J Med Biol Res. 49(9) (2016) e5374.
[9] T. Czarnowicki, J.B. Gonzalez, K.M. Bonifacio, A. Shemer, P. Xiangyu, N. Kunjravia, D. Malajian, J. Fuentes-Duculan, H. Esaki, S. Noda, Y. Estrada, H. Xu, X. Zheng, J.G. Krueger, E. Guttman-Yassky, Diverse activation and differentiation of multiple B-cell subsets in patients with atopic dermatitis but not in patients with psoriasis., J Allergy Clin Immunol. 137(1) (2016) 118-29.e5.
[10] C.G. Vinuesa, S.G. Tangye, B. Moser, C. Mackay, Follicular B helper T cells in antibody responses and autoimmunity., Nat Rev Immunol. 5(11) (2005) 853-865.
[11] G. Papp, K. Szabó, Z. Szekanecz, M. Zeher, Follicular helper T cells in autoimmune diseases., Rheumatology (Oxford) 53(7) (2014) 1159-1160.
[12] X. Zhang, E. Lindwall, C. Gauthier, J. Lyman, N. Spencer, A. Alarakhia, A. Fraser, S. Ing, M. Chen, T. Webb-Detiege, J. Zakem, W. Davis, Y.S. Choi, R. Quinet, Circulating CXCR5+CD4+helper T cells in systemic lupus erythematosus patients share phenotypic properties with germinal center follicular helper T cells and promote antibody production., Lupus. pii: 0961203314567750 (2015).
[13] C. Le Coz, A. Joublin, J.L. Pasquali, A.S. Korganow, H. Dumortier, F. Monneaux, Circulating TFH subset distribution is strongly affected in lupus patients with an active disease., PLoS One. 8(9) (2013) e75319.
[14] K. Szabo, G. Papp, S. Barath, E. Gyimesi, A. Szanto, M. Zeher, Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome., Clin Immunol. 147 (2013) 95-104.
[15] K. Szabó, G. Papp, A. Szántó, T. Tarr, M. Zeher, A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus., Clin Exp Immunol. 183(1) (2016) 76-89.
[16] J. Ma, C. Zhu, B. Ma, J. Tian, S. Baidoo, C. Mao, W. Wu, J. Chen, J. Tong, M. Yang, Z. Jiao, H. Xu, L. Lu, S. Wang, Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis., Clin Dev Immunol., 2012, p. 827480.

[17] R. Morita, N. Schmitt, S. Bentebibel, R. Ranganathan, L. Bourdery, G. Zurawski, E. Foucat, M. Dullaers, S. Oh, N. Sabzghabaei, E. Lavecchio, M. Punaro, V. Pascual, J. Banchereau, H. Ueno, Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion., Immunity 34 (2011) 108-121.
[18] Q. Li, Z. Liu, E. Dang, L. Jin, Z. He, L. Yang, X. Shi, G. Wang, Follicular Helper T Cells (Tfh) and IL-21 Involvement in the Pathogenesis of Bullous Pemphigoid, Plos One 8(7) (2013) e68145.
[19] M.S. Ally, R.Y. Prasad Hunasehally, M. Rodriguez-Justo, B. Martin, R. Verdolini, N. Attard, F. Child, A. Attygalle, S. Whittaker, S. Morris, A. Robson, Evaluation of follicular T-helper cells in primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma and dermatitis., J Cutan Pathol. 40(12) (2013) 1006-1013.
[20] J. Niu, Z. Song, X. Yang, Z. Zhai, H. Zhong, F. Hao, Increased circulating follicular helper T cells and activated B cells correlate with disease severity in patients with psoriasis., J Eur Acad Dermatol Venereol. 29(9) (2015) 1791-6.
[21] D. Shin, D.S. Kim, S.H. Kim, J.H. Je, H.J. Kim, D. Young Kim, S.M. Kim, M.G. Lee, Decreased PD-1 positive blood follicular helper T cells in patients with psoriasis., Arch Dermatol Res. 308(8) (2016) 593-599.
[22] Y. Wang, L. Wang, Y. Shi, F. Wang, H. Yang, S. Han, Y. Bai, Altered Circulating T Follicular Helper Cell Subsets in Patients with Psoriasis Vulgaris, Immunol Lett. 181 (2017) 101-108.
[23] S. Hardtke, L. Ohl, R. Förster, Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help., Blood 106 (2005) 1924-1931.
[24] M. Kroenke, D. Eto, M. Locci, M. Cho, T. Davidson, E. Haddad, S. Crotty, Bcl6 and Maf cooperate to instruct human follicular helper CD4 T cell differentiation., J Immunol. 188(8) (2012) 3734-3744.
[25] T. Chtanova, S.G. Tangye, R. Newton, N. Frank, M.R. Hodge, M.S. Rolph, C.R. Mackay, T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells., J Immunol. 173(1) (2004) 68-78.
[26] S. Crotty, E. Kersh, J. Cannons, P. Schwartzberg, R. Ahmed, SAP is required for generating long-term humoral immunity., Nature 421 (2003) 282-287.
[27] H. Akiba, K. Takeda, Y. Kojima, Y. Usui, N. Harada, T. Yamazaki, J. Ma, K. Tezuka, H. Yagita, K. Okumura, The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo., J Immunol. 175 (2005) 2340-2348.
[28] U. Klein, R. Dalla-Favera, Germinal centres: role in B-cell physiology and malignancy., Nat Rev Immunol. 8 (2008) 22-33.
[29] J.M. Hanifin, G. Rajka, Diagnostic features of atopic dermatitis., Acta Derm Venereol. 92(suppl) (1980) 44-47.
[30] L.C. Wu, A.A. Zarrin, The production and regulation of IgE by the immune system., Nat Rev Immunol. 14(4) (2014) 247-59.
[31] O. Talay, D. Yan, H.D. Brightbill, E.E. Straney, M. Zhou, E. Ladi, W.P. Lee, J.G. Egen, C.D. Austin, M. Xu, L.C. Wu, IgE^{+} memory B cells and plasma cells generated through a germinal-center pathway., Nat Immunol. 13(4) (2012) 396-404.
[32] H. Xiong, J. Dolpady, M. Wabl, M.A. Curotto de Lafaille, J.J. Lafaille, Sequential class switching is required for the generation of high affinity IgE antibodies., J Exp Med. 209(2) (2012) 353-364.
[33] D.M. Kemeny, The role of the T follicular helper cells in allergic disease., Cell Mol Immunol. 9(5) (2012) 386-389.
[34] G. Varricchi, J. Harker, F. Borriello, G. Marone, S.R. Durham, M.H. Shamji, T follicular helper (Tfh) cells in normal immune responses and in allergic disorders., Allergy 71(8) (2016) 1086-1094.
[35] N. Schmitt, S.E. Bentebibel, H. Ueno, Phenotype and functions of memory Tfh cells in human blood., Trends Immunol. 35(9) (2014) 436-442.

[36] J. He, L.M. Tsai, Y.A. Leong, X. Hu, C.S. Ma, N. Chevalier, X. Sun, K. Vandenberg, S. Rockman, Y. Ding, L. Zhu, W. Wei, C. Wang, A. Karnowski, G.T. Belz, J.R. Ghali, M.C. Cook, D.S. Riminton, A. Veillette, P.L. Schwartzberg, F. Mackay, R. Brink, S.G. Tangye, C.G. Vinuesa, C.R. Mackay, Z. Li, D. Yu, Circulating precursor CCR7(lo)PD-1(hi) CXCR5$^+$ CD4$^+$ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure., Immunity. 39(4) (2013) 770-781.
[37] R. Fölster-Holst, Management of atopic dermatitis: are there differences between children and adults?, J Eur Acad Dermatol Venereol. 28(Suppl 3) (2014) 5-8.
[38] T. Czarnowicki, H. Esaki, J.B. Gonzalez, Y. Renert-Yuval, P. Brunner, M. Oliva, Y. Estrada, H. Xu, X. Zheng, S. Talasila, I. Haugh, T. Huynh, S. Lyon, G. Tran, H. Sampson, M. Suárez-Fariñas, J.G. Krueger, E. Guttman-Yassky, A.S. Paller, Alterations in B-cell subsets in early pediatric atopic dermatitis., J Allergy Clin Immunol. S0091-6749(16)31452-X (2016).
[39] T. Czarnowicki, H. Esaki, J.B. Gonzalez, D. Malajian, A. Shemer, S. Noda, S. Talasila, A. Berry, J. Gray, L. Becker, Y. Estrada, H. Xu, X. Zheng, M. Suárez-Fariñas, J.G. Krueger, A.S. Paller, E. Guttman-Yassky, Early pediatric atopic dermatitis shows only a cutaneous lymphocyte antigen (CLA)(+) TH2/TH1 cell imbalance, whereas adults acquire CLA(+) TH22/TC22 cell subsets., J Allergy Clin Immunol. 136(4) (2015) 941-951.
[40] A.G. Tesciuba, R.A. Shilling, M.D. Agarwal, H.S. Bandukwala, B.S. Clay, T.V. Moore, J.V. Weinstock, A.A. Welcher, A.I. Sperling, ICOS costimulation expands Th2 immunity by augmenting migration of lymphocytes to draining lymph nodes., J Immunol. 181(2) (2008) 1019-1024.
[41] S. Weidinger, N. Novak, Atopic dermatitis., Lancet 387(10023) (2016) 1109-1122.
[42] D.T. Avery, C.S. Ma, V.L. Bryant, B. Santner-Nanan, R. Nanan, M. Wong, D.A. Fulcher, M.C. Cook, S.G. Tangye, STAT3 is required for IL-21-induced secretion of IgE from human naive B cells., Blood 112(5) (2008) 1784-1793.
[43] S. Kobayashi, N. Haruo, K. Sugane, H.D. Ochs, K. Agematsu, Interleukin-21 stimulates B-cell immunoglobulin E synthesis in human beings concomitantly with activation-induced cytidine deaminase expression and differentiation into plasma cells., Hum Immunol. 70(1) (2009) 35-40.
[44] R. Caruso, E. Botti, M. Sarra, M. Esposito, C. Stolfi, L. Diluvio, M.L. Giustizieri, V. Pacciani, A. Mazzotta, E. Campione, T.T. Macdonald, S. Chimenti, F. Pallone, A. Costanzo, G. Monteleone, Involvement of interleukin-21 in the epidermal hyperplasia of psoriasis., Nat Med. 15(9) (2009) 1013-1015.
[45] H. Jin, M.K. Oyoshi, Y. Le, T. Bianchi, S. Koduru, C.B. Mathias, L. Kumar, S. Le Bras, D. Young, M. Collins, M.J. Grusby, J. Wenzel, T. Bieber, M. Boes, L.E. Silberstein, H.C. Oettgen, R.S. Geha, IL-21R is essential for epicutaneous sensitization and allergic skin inflammation in humans and mice., J Clin Invest. 119(1) (2009) 47-60.
[46] K. Yanaba, M. Kamata, Y. Asano, Y. Tada, M. Sugaya, T. Kadono, T.F. Tedder, S. Sato, CD19 expression in B cells regulates atopic dermatitis in a mouse model., Am J Pathol. 182(6) (2013) 2214-2222.
[47] L.M. Tsai, D. Yu, Follicular helper T-cell memory: establishing new frontiers during antibody response., Immunol Cell Biol. 92(1) (2014) 57-63.
[48] N. Chevalier, D. Jarrossay, E. Ho, D.T. Avery, C.S. Ma, D. Yu, F. Sallusto, S.G. Tangye, C.R. Mackay, CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses., J Immunol. 186(10) (2011) 5556-5568.
[49] J.M. Coquet, M.J. Schuijs, M.J. Smyth, K. Deswarte, R. Beyaert, H. Braun, L. Boon, G.B. Karlsson Hedestam, S.L. Nutt, H. Hammad, B.N. Lambrecht, Interleukin-21-Producing CD4(+) T Cells Promote Type 2 Immunity to House Dust Mites., Immunity 43(2) (2015) 318-330.
[50] R. De Bruyne, P. Gevaert, M. Van Winckel, N. De Ruyck, A. Minne, D. Bogaert, S. Van Biervliet, S. Vande Velde, F. Smets, E. Sokal, F. Gottrand, J. Vanhelst, B. Detry, C. Pilette, B.N. Lambrecht, M. Dullaers, Raised immunoglobulin A and circulating T follicular helper cells are linked to the development of food allergy in paediatric liver transplant patients., Clin Exp Allergy. 45(6) (2015) 1060-1070.

[51] R. Kamekura, K. Shigehara, S. Miyajima, S. Jitsukawa, K. Kawata, K. Yamashita, T. Nagaya, A. Kumagai, A. Sato, H. Matsumiya, N. Ogasawara, N. Seki, K. Takano, Y. Kokai, H. Takahashi, T. Himi, S. Ichimiya, Alteration of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid association of allergic rhinitis with bronchial asthma., Clin Immunol. 158(2) (2015) 204-211.
[52] H.E. Liang, R.L. Reinhardt, J.K. Bando, B.M. Sullivan, I.C. Ho, R.M. Locksley, Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity., Nat Immunol. 13(1) (2011) 58-66.
[53] Y. Han, X. Wang, G. Chen, G. Xu, X. Liu, W. Zhu, P. Hu, Y. Zhang, C. Zhu, J. Miao, Di-(2-ethylhexyl) phthalate adjuvantly induces imbalanced humoral immunity in ovalbumin-sensitized BALB/c mice ascribing to T follicular helper cells hyperfunction., Toxicology 324 (2014) 88-97.

Tables

Table 1. Demographic data of AD patients and controls enrolled in the study.

Parameter	Healthy controls (total; n=14)	Healthy children (n=4)	Healthy adults (n=10)	Patients with AD (total; n=29)	Children with AD (n=12)	Adults with AD (n=17)
Age (years)						
range	4-37	4-15	23-37	4-42	4-16	18-42
mean ± SD	22.64 ± 10.16	8.50 ± 4.80	28.03 ± 4.14	18.97 ± 11.03	9.58 ± 3.81	26.58 ± 7.99
Sex (no.)						
female	11	3	8	19	10	9
male	3	1	2	10	2	8
SCORAD score						
range				24-72	28-68	24-72
mean ± SD				43.21 ± 13.04	43.67 ± 11.91	42.88 ± 13.82
IgE (kU/L)						
median (range)				1453 (22-10000)	637 (22-10000)	2537 (135-10000)

Figure legends

Figure 1. Comparison of the frequency of T_{FH}-related cell markers in the $CD4^+CXCR5^+$ T cell subpopulation from AD patients and healthy controls. Representative dot plots show the gating strategy (panels on the left), the expression of $ICOS^+PD\text{-}1^+$ cells (top panel on the right) and $PD\text{-}1^+IL\text{-}21^+$ cells (bottom panel on the right). **(A)** Percentages of $ICOS^+$, $PD\text{-}1^+$ and $ICOS^+PD\text{-}1^+$ cells in the $CD4^+CXCR5^+$ T cell subset from the two subgroups of AD patients (children with AD; n=12, black dots and adults with AD; n=17, grey dots) and healthy controls (n=14, empty dots). **(B)** Frequency of $PD\text{-}1^+$, $IL\text{-}21^+$ and $PD\text{-}1^+IL\text{-}21^+$ cells in the $CD4^+CXCR5^+$ T cell subset from the two subpopulations of AD patients and healthy donors. Each data point represents an individual subject; horizontal lines show the mean values with standard deviation (SD). Statistically significant differences are indicated by *, $p < 0.05$; **, $p < 0.01$; ns, no significant differences.

Figure 2. Determination of circulating T_{FH}-like cells in children and adults with AD versus healthy subjects. **(A)** Percentages of $CD4^+CXCR5^+ICOS^+$ T cells, $CD4^+CXCR5^+PD\text{-}1^+$ T cells and $CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ T_{FH}-like cells in children with AD (n=12, black dots), adults with AD (n=17, grey dots) and control subjects (n=14; empty dots). Peripheral T_{FH}-like cells were assessed as their percentage in the $CD4^+$ lymphocyte population. **(B)** Absolute numbers of $CD4^+CXCR5^+ICOS^+$ T cells, $CD4^+CXCR5^+PD\text{-}1^+$ T cells and $CD4^+CXCR5^+ICOS^+PD\text{-}1^+$ T_{FH}-like cells in children with AD (n=13, black dots), adults with AD (n=17, grey dots) and control subjects (n=11; empty dots). Each data point represents an individual subject; horizontal lines show the mean values with standard deviation (SD). Statistically significant differences are indicated by *, $p < 0.05$; **, $p < 0.01$; ns, no significant differences.

Figure 3. Determination of IL-21 producing circulating T_{FH}-like cells in children and adults with AD versus healthy subjects. **(A)** Frequency of $CD4^+CXCR5^+PD\text{-}1^+$ T cells, $CD4^+CXCR5^+IL\text{-}21^+$ T cells and $CD4^+CXCR5^+PD\text{-}1^+IL\text{-}21^+$ T_{FH}-like cells in patients with AD (children; n=12, black dots and adults; n=17, grey dots) and healthy controls (n=14; empty dots). **(B)** Absolute numbers of $CD4^+CXCR5^+PD\text{-}1^+$ T cells, $CD4^+CXCR5^+IL\text{-}21^+$ T cells and $IL\text{-}21^+$ producing T_{FH}-like cells in the two subgroups of AD patients and healthy individuals (n=11; empty dots). Each data point represents an individual subject; horizontal lines show the mean values with standard deviation (SD). Statistically significant differences are indicated by **, $p < 0.01$; ns, no significant differences. **(C)** Correlation between the absolute number of $CD4^+CXCR5^+PD\text{-}1^+IL\text{-}21^+$ T_{FH}-like cells and SCORAD (SCORing Atopic Dermatitis) index. Each data point represents an individual subject. The regression line is shown in each panel (R, Pearson's correlation coefficient; p, P-value).

Figure 4. Immunohistochemical analysis of skin biopsy specimens from AD patients with a special emphasis on the presence and distribution of T_{FH}-related molecules. CD3 pan-T cell, CD20 pan-B cell and T_{FH}-specific marker (PD-1, CD84, Bcl-6, IL-21) expression in lesional skin from patients with AD (n=5). Boxed areas indicate the localization of the zoomed-in images in the right. Scale bar on the slides are 200 μm (left images) and 50 μm (right images).