

Interleukin-17A Promotes IgE Production in Human B Cells

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Recently the Th1/Th2 concept has been revised and Th17 cells have been implicated in allergy. Despite clear correlative evidence, the cellular and molecular basis for the connection between increased IL-17A and IgE in allergy has not been elucidated. Here we show using flow cytometry that allergic patients have higher numbers of IL-17A⁺ cells compared to nonallergic donors. The selective removal of IL-17A⁺ cells from peripheral blood mononuclear cells of allergic donors after an IL-17A secretion assay reduces IgE levels, whereas re-addition of recombinant IL-17A restores it, as measured by ELISA, showing their important functional implication for IgE production. In addition, IL-17A directly promotes the differentiation of IgE-secreting cells and IgE production upon anti-CD40/IL-4 costimulation, as shown by enzyme-linked immunospot technique and ELISA. IL-17A triggers rapid degradation of IκBα and subsequent translocation of NF-κB into the B-cell nucleus, followed by transcription of epsilon germ-line, activation-induced cytidine deaminase, and IFN regulatory factor 4, as analyzed by flow cytometry, western blot, and quantitative real-time RT-PCR, respectively. Our study shows that IL-17A⁺ cells promote IgE production and that IL-17A exerts its pro-allergic effect directly at the level of B cells. Therefore, IL-17A might be a target for the treatment of IgE-dependent diseases, including atopic dermatitis.

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INTRODUCTION

The key component of type I allergy is IgE directed against innocuous environmental antigens (Poulsen and Hummelshoj, 2007). IgE production proceeds in three tightly regulated stages: germ-line gene transcription, class switch recombination (CSR), and plasma cell differentiation (Gould and Sutton, 2008). Epsilon germ-line transcription (εGLT) is a prerequisite for the initiation of IgE CSR, by marking the sites for enzyme activation induced (cytidine) deaminase (AID), responsible for the DNA breaks (Geha *et al.*, 2003). At least two independent signals are required for IgE CSR and IgE plasma cell differentiation (Poulsen and Hummelshoj, 2007). The classical pathway involves IL-4 (or IL-13) and CD40L, operating through simultaneous activation of the STAT6 and NF-κB pathway, respectively. Various other cytokines influence IgE production by providing promoting or inhibitory signals. However, none of the supplementary factors can normally replace either of the two “dominant” signals required, with the exceptions of BAFF, APRIL, C4 binding protein, and corticosteroids under certain circumstances (Geha *et al.*, 2003). In addition, IL-21 was also recently

described to enhance IgE production in humans as reviewed by Konforte *et al.* (2009).

In contrast to their protective role in host defense against extracellular organisms, Th17 cells promote tissue destruction in autoimmune diseases (Boniface *et al.*, 2008; Pernis, 2009). Their role in allergy is less well defined, although emerging evidence has associated IL-17A and allergic diseases as reviewed by Tesmer *et al.* (2008) and Louten *et al.* (2009). To our knowledge, previously unreported models revise the role of Th1/Th2 and highlight the interaction of Th17 with Th2 cells, keratinocytes, and fibroblasts in the pathogenesis of atopic dermatitis (AD), but the exact role of IL-17A in AD is controversial. Further indication for a pro-allergic, IgE-promoting function of IL-17A comes from the analysis of the IL-17A^{-/-} mice (Nakae *et al.*, 2002; He *et al.*, 2007; Wakashin *et al.*, 2008). In particular, ovalbumin-sensitized IL-17A^{-/-} mice have reductions specifically in IgG1 and, most profoundly, IgE levels compared to those in IL-17A^{+/+} mice. Moreover, Th17-mediated autoimmune gastritis is accompanied by high serum IgE levels (Stummvoll *et al.*, 2008). However, the mechanisms behind the changes in IgE production in the context of IL-17A hyper- or hypoexpression have remained unexplored so far.

Interestingly, IL-17A promotes B-cell activity and function in germinal centers of BXD2 mice, suggesting that B cells might be a substantial target for IL-17A (Hsu *et al.*, 2008). A most recent report showed that B cells display increased survival, proliferation, and differentiation into plasma cells upon BAFF/IL-17A-mediated costimulation (Doreau *et al.*, 2009).

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Abbreviations: AD, atopic dermatitis; AID, activation-induced (cytidine) deaminase; CSR, class switch recombination; εGLT, epsilon germ-line transcript; PBMC, peripheral blood mononuclear cell

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On the basis of the above evidence, we hypothesized that IL-17A has a role in IgE production by B cells. Therefore, we studied the contribution of Th17 cells to the maintenance of elevated IgE levels encountered in allergic subjects and analyzed whether IL-17A acted directly on B cells to induce IgE CSR and the differentiation of IgE-producing cells.

RESULTS

Allergic patients have increased numbers of IL-17A+ cells

Since increased levels of IL-17A have been found locally and systemically in patients suffering from asthma and AD (Koga *et al.*, 2008), we tested whether allergic patients may display increased amounts of Th17 cells. Nonallergic and allergic donors are characterized in Table 1. After a whole-blood assay with Staphylococcal enterotoxin B/anti-CD28 stimulation, followed by intracellular staining, flow cytometric analysis of IL-17A, CD4, and CD154 was performed. Th17 cells made up for 0.5–4.7% of the CD4+CD154+ population (activated T-helper cells) in nonallergic individuals. In allergic donors, this proportion increased to 1–13.7% (elevated by 1.5-fold) (Figure 1a). Six patients (two men and

four women) with the highest numbers of IL-17A+ cells were all asthmatic, between 26 and 40 years of age. There was no difference in the percentages of activated T-helper cells between allergic and nonallergic donors (data not shown), excluding that the higher numbers found in allergic donors were the result of their general increase.

Table 1. Characterization of nonallergic and allergic donors

	Nonallergic donors	Allergic patients
<i>n</i>	16	23
Female/male	11/5	15/8
Age (years) ¹	30.5 (23–37)	29 (23–51)
Total plasma IgE (ng ml ⁻¹) ¹	23.52 (0.70–191.40)	426.60 (0.70–2492.10)
Asthma	0	13
Allergic rhinitis	0	13
Atopic dermatitis	0	3

¹Median (range).

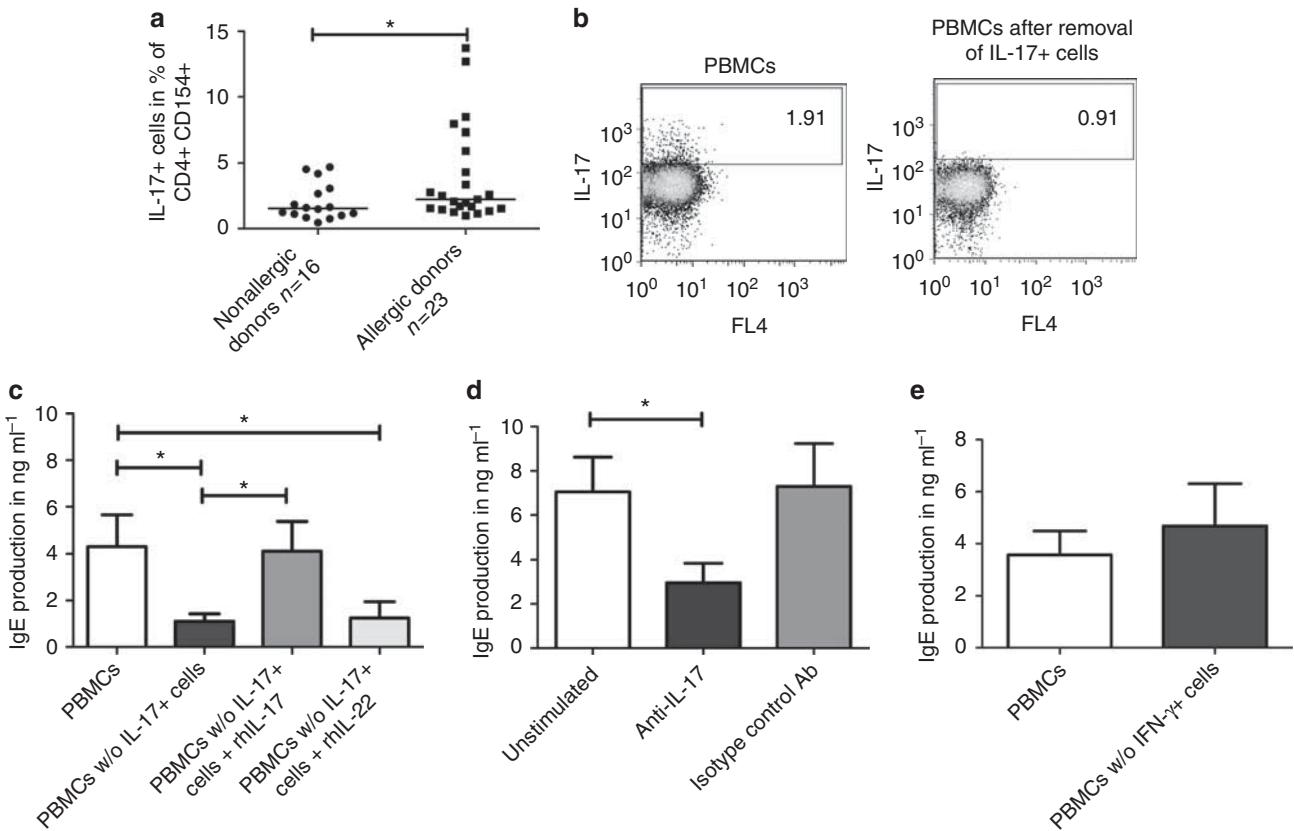


Figure 1. Allergic patients have increased numbers of IL-17A+ cells, which support IgE production. (a) Allergic patients have higher numbers of IL-17A+ cells than nonallergic donors. After a whole-blood assay, cells were subjected to intracellular staining of IL-17A, CD154, and CD4. Data are shown as single values with median. * $P < 0.05$; Mann-Whitney U -test. (b) Peripheral blood mononuclear cells (PBMCs) before and after separation of IL-17A-producing cells. (c) IL-17A+ cells promote IgE production in allergic patients. IgE production was compared before and after the removal of IL-17A-producing cells. In addition, IL-17A or IL-22 was added to cultures of PBMCs without IL-17A+ cells. Data are shown as mean $\pm$ SEM. * $P < 0.05$; Wilcoxon signed-rank test; $n = 6$ –14. (d) IL-17A promotes IgE production in allergic patients. PBMCs were cultured with/without an anti-IL-17A-blocking antibody. Data are shown as mean $\pm$ SEM; $n = 4$ –5. (e) IFN- γ + cells do not promote IgE production in allergic patients. IgE production was compared before and after the removal of IFN- γ -producing cells. Data are shown as mean $\pm$ SEM; $n = 5$.

IL-17A-producing cells support IgE production in allergic donors

We investigated whether the Th17 population contributes to the elevated IgE production in allergic patients with AD. It is known that B cells from allergic patients spontaneously produce IgE *in vitro* (Turner *et al.*, 1987). Peripheral blood mononuclear cells (PBMCs; stimulated with anti-CD2/anti-CD3/anti-CD28 for 24 hours) from which the IL-17A+ population had been removed (or not) by means of a secretion assay were analyzed for IgE production after 72 hours of culture. Upon the selective removal of IL-17A+ cells (a representative example is given in Figure 1b), IgE production declined to $25.86 \pm 7.26\%$ (Figure 1c; compare column 1 to column 2). To elucidate whether the IgE-promoting effect of Th17 cells was due to the production of IL-17A itself or mediated by other factors, we re-added recombinant IL-17A to Th17-depleted cultures, which completely restored IgE levels (Figure 1c; compare column 2 to column 3). Because Th17 also produce IL-22, we re-added IL-22 to Th17-depleted cultures as well, which could not restore IgE production (Figure 1c; compare column 2 to column 4). To exclude an influence of the separation method, we performed an IFN- γ secretion assay and depleted IFN- γ -producing cells from PBMCs. There was no significant difference in IgE production between the total PBMC population and PBMCs without IFN- γ + cells (Figure 1e).

In a second approach, an IL-17A-blocking antibody was added to resting PBMCs obtained from donors with AD. Anti-IL-17A was as effective as the removal of IL-17A+ cells (IgE production decreased to 42 ± 12.5 , Figure 1d), confirming that IL-17A was involved in IgE production. The specificity was proven by the use of an isotype control antibody. Interestingly, the blocking of IL-17A did not affect the spontaneous production of other Ig isotypes (Figure 2).

Due to the limiting cell numbers, the experiments shown in Figure 1c and d could not be performed with cells from the same individuals. However, the same criteria as listed in Table 1 were used for their selection.

IL-17A directly promotes IgE production and increases the number of IgE-producing cells

To investigate the direct impact of IL-17A on IgE production, we stimulated purified B cells with anti-CD40/IL-4 in the presence of IL-17A at different concentrations for 10 days. IgE production of unstimulated B cells was below the detection limit ($<20 \text{ pg ml}^{-1}$), as expected. IL-17A increased IgE production of IL-4/anti-CD40-stimulated cells in a dose-dependent manner (Figure 3a). The most effective concentration

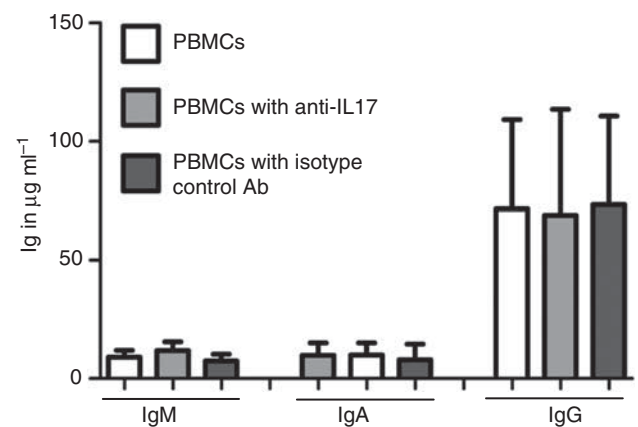


Figure 2. Blocking of IL-17A has no impact on IgM, IgA, or IgG production. Peripheral blood mononuclear cells (PBMCs) were cultured with/without an anti-IL-17A-blocking antibody. IgM, IgA, and IgG levels were determined in supernatants by ELISA. Data are shown relative to unstimulated PBMC samples as mean $\pm$ SEM; $n = 4-5$.

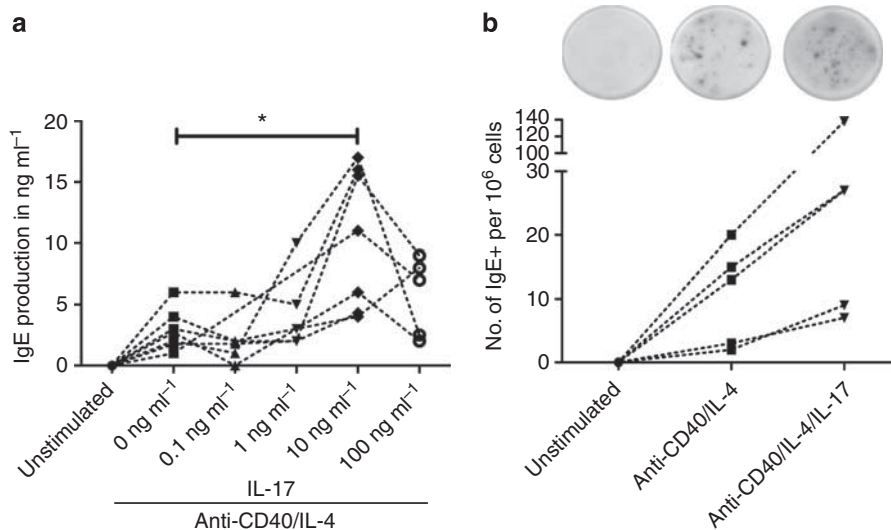


Figure 3. IL-17A provides IgE production in B cells and increases the number of IgE-producing cells. (a) IL-17A dose-dependently induces IgE production. B cells were stimulated with anti-CD40/IL-4 and costimulated with IL-17A for 10 days. IgE was determined in supernatants by ELISA. The data represent single values. $*P < 0.05$; Wilcoxon paired test; $n = 5-6$. (b) IL-17A contributes to the generation of IgE-secreting cells. B cells were stimulated with anti-CD40/IL-4 with/without IL-17A for 6 days. Number of antibody-secreting cells was determined by enzyme-linked immunospot (ELISpot) technique. The data show single values from five donors. The ELISpot wells are representative and are from one selected donor.

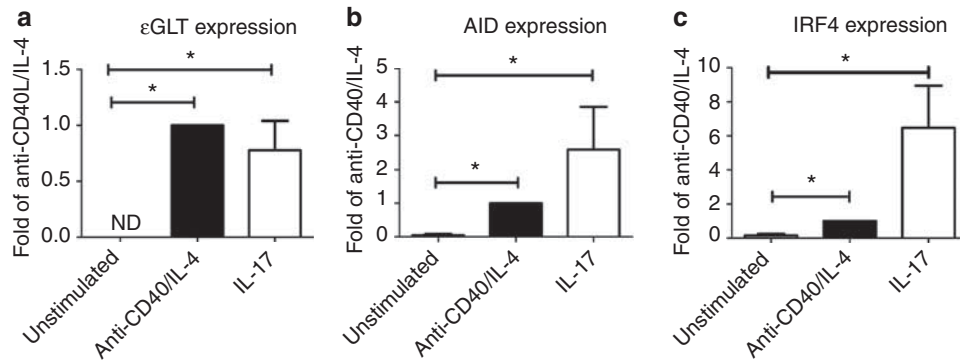


Figure 4. IL-17A induces IgE class switch recombination. IL-17A induces (a) epsilon germ-line transcript (εGLT), (b) activation-induced (cytidine) deaminase (AID), and (c) IFN regulatory factor 4 (IRF4) mRNA expression. B cells were stimulated with IL-17A for 72 hours. Anti-CD40/IL-4 stimulation was used as positive control. The measurement was performed using quantitative PCR. The data are shown as fold expression of anti-CD40/IL-4-stimulated samples and are shown as mean $\pm$ SEM. * $P < 0.05$; Wilcoxon signed-rank test; $n = 4-5$. ND = not detectable.

was 10 ng ml^{-1} of IL-17A and therefore this concentration was used for the subsequent experiments. IL-17A alone did not induce IgE secretion by B cells (data not shown).

To delineate whether the IgE-promoting effect of IL-17A was isotype specific, we analyzed the other Ig isotypes. There was no significant change in IgM, IgA, and IgG production upon IL-17A/anti-CD40/IL-4 stimulation, although a tendency toward increased levels of IgM and IgG isotypes was noted (data not shown).

An enzyme-linked immunospot assay was performed to verify whether the enhanced amount of IgE in supernatants was due to a higher number of plasma-like cells. The addition of IL-17A to anti-CD40/IL-4-stimulated B cells indeed increased the numbers of IgE plasma-like cells. Although the level of increase was donor dependent, the number of IgE secreting cells increased in all donors upon IL-17A costimulation (Figure 3b). Together the data indicate that IL-17A supports the differentiation of B cells into IgE plasma cells.

IL-17A induces IgE CSR

We addressed the effect of IL-17A on the expression of key factors required for IgE CSR. Anti-CD40/IL-4 stimulation of B cells, used as positive control, induced εGLT, AID, and IFN regulatory factor 4 mRNA expression, as expected.

Interestingly, the stimulation with IL-17A alone led to a significant increase in εGLT, AID, and IFN regulatory factor 4 mRNA expression (Figure 4) in the same range as anti-CD40/IL-4. This implies that IL-17A may have a similar biological effect as anti-CD40/IL-4 on the molecular events preceding CSR to IgE.

IL-17A induces IκBα degradation and p50 translocation into the nucleus of B cells

The most important downstream target of IL-17A is NF-κB (Gaffen *et al.*, 2006; Huang *et al.*, 2007) and NF-κB itself is crucial for IgE CSR (Geha *et al.*, 2003).

To explore whether IL-17R triggering leads to NF-κB activation in B cells, we analyzed IκBα by flow cytometry. IκBα degradation was initiated by IL-17R stimulation (Figure 5), achieving the maximal response 15 minutes after IL-17A addition (mean fluorescence intensity decreased to

$77 \pm 14\%$, Figure 5a). IκBα decreased to $75 \pm 20\%$ upon anti-CD40 stimulation (used as positive control), but after 45 minutes. Thus, our data show that IL-17A elicits IκBα degradation more rapidly than anti-CD40.

The study of B-cell subsets revealed that IL-17A exerts comparable effects on IκBα degradation in both naive (gated on CD27 $^{-}$) and memory B cells (gated on CD27 $^{+}$) (data not shown).

To investigate whether the increased IκBα degradation by IL-17A was accompanied by p50 translocation, we analyzed its nuclear translocation by western blot. Stimulation with anti-CD40 (positive control) caused robust translocation of p50 into the nucleus (Figure 5c). The same pattern was observed with IL-17A, which resulted in rapid and prominent p50 shuttling into the nucleus (Figure 5c). Taken together, IL-17A (similar to anti-CD40) induces IκBα degradation, followed by profound translocation of p50 into the B-cell nucleus.

DISCUSSION

Our data show that IL-17A exerts a pro-allergic action on B cells by triggering IgE. We show that Th17 cells are increased in allergic subjects and that these cells contribute to IgE production by PBMCs. Because Th17 cells produce an array of mediators other than IL-17A, it was important to assess whether IL-17A directly induces IgE production. We show that this is indeed the case. Th17-depleted cultures showed a strongly reduced IgE response that was restored by exogenous IL-17A, but not by IL-22. Furthermore Th17 removal was mimicked by the addition of blocking anti-IL-17A antibody. By using highly purified B cells and recombinant IL-17A, we finally established that IL-17A enhances IgE production and the differentiation of IgE plasma cells by direct action on B cells.

IL-17A effects are mediated through IL-17A receptor complexes composed of at least two IL-17RA subunits and at least one IL-17RC subunit (Shen and Gaffen, 2008). The major signaling intermediates include Act1/TAK1/TRAF6, coordinating NF-κB, and JAK1-associated phosphatidylinositol-3-kinase signaling (Huang *et al.*, 2007). We show that B-cell stimulation with IL-17A leads to a rapid degradation of

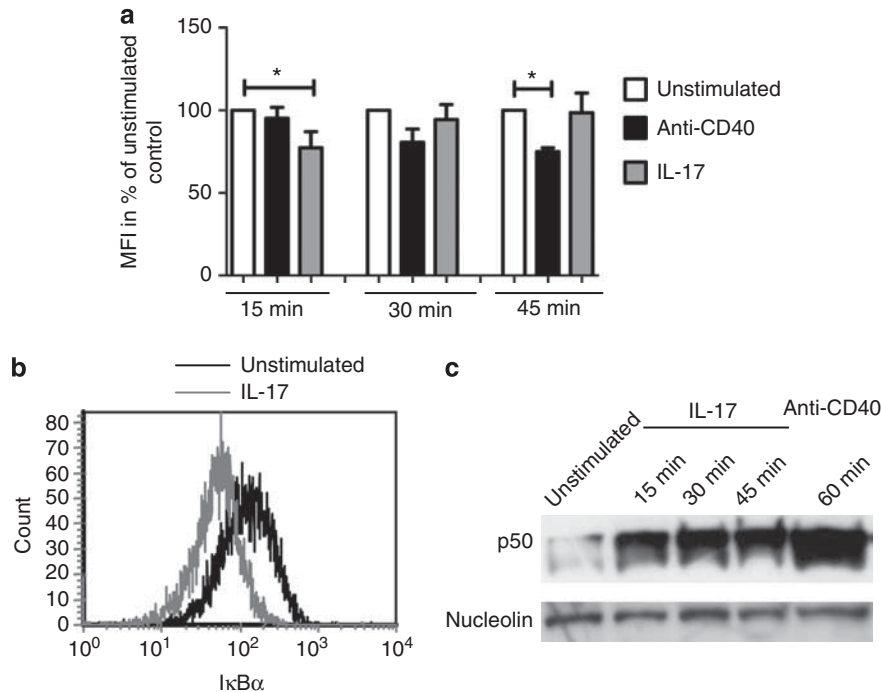


Figure 5. IL-17A induces IκBα degradation and subsequent p50 translocation into the B-cell nucleus. Purified B cells were stimulated with IL-17A or anti-CD40 (positive control). (a) After fixation, cells were permeabilized and stained with anti-IκBα and CD19. At least 30,000 gated B cells were collected for each sample and the geometric mean fluorescence was analyzed. Amount of IκBα in total B-cell population is shown as mean ± SEM. * $P < 0.05$; Wilcoxon signed-rank test; $n = 3-5$. (b) Histogram is a representation of stimulated vs. unstimulated IL-17A. Cells were gated on CD19+ population. (c) Nuclear extracts of B cells were analyzed by p50 and nucleolin western blot. The blot is a representative of six similar experiments.

IκBα to a comparable degree as with anti-CD40. This is followed by p50 translocation (and to a lesser degree p65, data not shown) into the B-cell nucleus. The importance of NF-κB signaling for CSR is indisputable. NF-κB binding sites have been identified in the promoters of molecules required for CSR (Geha *et al.*, 2003). Functional NF-κB binding sites are found in the promoters of εGLT, AID, and IFN regulatory factor 4, and their importance was shown in p50 knockout mice, failing to upregulate AID and εGLT (Messner *et al.*, 1997; Geha *et al.*, 2003).

In general, IgE CSR and plasma cell differentiation require the simultaneous triggering of the CD40 and the IL-4R (Poulsen and Hummelshoj, 2007). The concurrent activation of NF-κB and STAT6 initiates a cascade culminating in the AID and εGLT transcription. We show here that IL-17A directly induces εGLT and AID. Thus, the concomitant IL-17A stimulation increases IgE CSR in purified B cells and augments the generation of IgE plasma cells.

However, IgE production can be maintained even in the absence of Th17 cells, as seen in the hyper IgE syndrome. Although this seems to be contradictory, a recent report showed the crucial role of STAT3 signaling for memory B-cell formation and the differentiation of germinal center B cells into plasma cells, by acting in a B-cell intrinsic manner (Avery *et al.*, 2010). B cells with STAT3 mutations exhibit less Bcl-6. As Bcl-6 is a negative regulator of IL-4-induced STAT6 signaling and consequently εGLT expression (Harris *et al.*, 1999), this might explain why patients suffering from hyper IgE syndrome have increased IgE levels. Taken together,

STAT3 mutations result in both a dysregulation of T cell (Ma *et al.*, 2008) and B-cell intrinsic functions. In the context of allergy, a clear supportive role of Th17 cells for IgE production could be uncovered here, providing the molecular link between enhanced IL-17A activity and increased IgE levels found in allergic subjects.

IL-17A is a cofactor contributing to the enhanced IgE production in allergic individuals, but it does not clearly act on its own. This is also emphasized by the fact that enhanced Th17 function, encountered in various inflammatory diseases, does not predispose *per se* in enhanced IgE production, e.g. in multiple sclerosis, Crohn's disease, arthritis, and also psoriasis (Louten *et al.*, 2009). Interestingly, increased IgE levels have been reported in some patients suffering from psoriasis (Negosanti *et al.*, 1981) and our data may explain these previous observations. The fact that not all patients develop increased IgE may depend on the genetic background as well (Wilsmann-Theis *et al.*, 2008). Under such circumstances, IgE production is not promoted possibly because other stimulatory cytokines (such as IL-4 or IL-13) are not present in sufficient quantities. Our assays rather mimic the conditions encountered in allergic subjects, either by using PBMCs derived from AD patients, which spontaneously produce high IgE levels (Figure 1), or by simulating the T-cell help of a Th2 cell by addition of anti-CD40/IL-4. In a Th2-like environment, IL-17A clearly promotes IgE production.

The role of IL-17A in psoriasis is well established (Louten *et al.*, 2009), whereas its role in AD has been controversially

discussed. It is currently believed that IL-17A is an enhancer rather than an immune polarizer in AD and that it is primarily associated with acute rather than chronic stages of the disease (Koga *et al.*, 2008). Epicutaneous sensitization through the compromised skin barrier induces a local and systemic Th17 response that accounts for the symptoms of asthma, frequently co-occurring in AD patients (He *et al.*, 2007). Our finding that IL-17A potentiates IgE production may contribute to the allergic responses in these patients. Moreover, the activation of dendritic cells by cross-linking of IgE molecules, leads to polarization of naive T cells into Th2 cells and their cytokine secretion, which are of major importance for the acute AD (Avgerinou *et al.*, 2008). It is also discussed that Th2-derived IL-4 suppresses IL-17A secretion during acute AD (Nogales *et al.*, 2009). Furthermore, during the acute AD recruited Th17 cells can activate eosinophils, by increasing their IL-8 and CXCL1 production (Louten *et al.*, 2009). Conversely, the chronic AD phase is dominated by Th1 cytokines and IL-22, but not by IL-17A (Toda *et al.*, 2003; Guttman-Yassky *et al.*, 2008; Koga *et al.*, 2008). It was recently established that T cells can independently express IL-22 (a Th17-related cytokine), and that these "T22" cells may be important for AD severity (Nogales *et al.*, 2009).

Taken together, our data imply a biological relevance of IL-17A for IgE production. Although future studies will have to assess in detail whether interference with the exaggerated Th17 activity in allergic patients might be a feasible treatment option, our findings provide a first hint that IL-17A may turn out a significant target for the prevention and/or therapy of allergic diseases.

MATERIAL AND METHODS

Cell preparation and cell culture

PBMCs were separated from heparinized blood obtained from nonallergic donors, if not stated otherwise, by density gradient centrifugation using Ficoll (PAA, Pasching, Austria). Nonallergic and allergic donors are characterized in Table 1. After density gradient centrifugation using Ficoll and two washing steps with phosphate-buffered saline, cells were labeled with anti-CD19 beads, washed, and positively selected to >99% purity by magnetic sorting (Miltenyi, Bergisch Gladbach, Germany) as described previously (Milovanovic *et al.*, 2009). B cells or PBMCs (10^6 per ml) were cultured in flat-bottomed 48-well plates in Roswell's Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal calf serum, glutamine (2 mM), penicillin (100 U ml^{-1}), and streptomycin ($100 \mu\text{g ml}^{-1}$) (all from Biochrom, Berlin, Germany). Cultures were carried out at 37°C in humidified air and 5% CO_2 . The study was approved by the ethics committee of the Charité Universitätsmedizin, was conducted according to the Declaration of Helsinki Principles, and all patients gave written informed consent.

Reagents and antibodies

Recombinant human IL-17A and IL-4 (5 ng ml^{-1} , both from ImmunoTools, Friesoythe, Germany), the anti-CD40 mAb

(100 ng ml^{-1} ; DRFZ, Berlin, Germany), IL-22 (10 ng ml^{-1}), the neutralizing anti-IL-17A mAb ($1 \mu\text{g ml}^{-1}$), and isotype control mouse IgG2B (all from R&D Systems, Wiesbaden, Germany) were used. The purity of recombinant human IL-17A is >98.0% as determined by RP-HPLC and SDS-PAGE silver stained gel, as stated by the manufacturer. Fluoro-chrome-coupled anti-IL-17A and anti-CD27 were from eBioscience (San Diego, CA); anti-CD19, anti-CD3, and anti-CD4 were from BD PharMingen (San Diego, CA); and anti-Ik $\text{B}\alpha$ was from Cell Signaling Technology (Boston, MA). Dead cells were excluded using propidium iodide staining (Sigma-Aldrich, Steinheim, Germany).

Flow cytometric analyses of IL-17A-expressing T cells

Heparinized blood was stimulated using Staphylococcal enterotoxin B (Sigma-Aldrich) and anti-CD28 (BD PharMingen) for 6 hours. Brefeldin A (Sigma-Aldrich) was added 2 hours after beginning. Cells were treated with lysing solution (BD PharMingen), followed by Perm II solution (BD PharMingen). Intracellular staining was performed with anti-human IL-17A, anti-CD154, and anti-CD4. The analysis was performed using flow cytometer (FACSCalibur; Becton Dickinson, Heidelberg, Germany) and FlowJo 7.2.2 (Tree Star, Ashland, OR).

Detection and separation of IL-17A- and IFN- γ -producing cells

PBMCs were stimulated with a T-cell activation/expansion kit (anti-CD2, anti-CD3, anti-CD28; Miltenyi) for 24 hours. The secretion assay was performed using IL-17A or IFN- γ secretion assay kits (both from Miltenyi) under the manufacturers' instructions. Afterward, the cells were cultured for 72 hours and IgE production was measured.

ELISA

IgE levels were determined by ELISA as previously described (Heine *et al.*, 2009). For IgE detection, clones HP6061 and HP6029 (SouthernBiotech, Birmingham, AL) were used. The matched antibody pairs for IgA, IgG, and IgM ELISA (anti-IgA, anti-IgG, and anti-IgM) were purchased from Dianova (Hamburg, Germany). Immunoplates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with the primary antibodies. After blocking of unspecific binding, supernatants and standards were incubated for 2 hours. The second alkaline phosphatase-conjugated anti-Ig antibody was added for 1.5 hours. Because the secondary mouse anti-human IgE antibody was biotinylated (HP6029), another hour of incubation with alkaline phosphatase-conjugated streptavidin (Zymed, San Francisco, CA) was performed. After the final reaction with phosphatase substrate (Sigma-Aldrich), plates were read in a microplate ELISA reader (Dynatech, Chantilly, VA) at 405 nm and the amount of Ig was calculated according to the standard curve. Diluted human serum of an atopic individual served as standard curve preparation.

Enzyme-linked immunospot technique

MultiScreen High-Protein-Binding Immibilion-P Membrane plates (Millipore, Schwalbach, Germany) were coated with

mouse anti-human IgE (HP6061). B cells (3×10^6) were used for each stimulation. After 6 days of culture, each sample was serially diluted (1:3 into seven wells) on the precoated plate and incubated at 37°C overnight. Detection was performed with biotin-conjugated antibody mouse anti-human IgE antibody (HP6029). The reaction was developed with streptavidin-horseradish peroxidase (R&D Systems) and peroxidase substrate 33-amino-9-ethyl-carbazole/n,n-dimethyl-formamide (Sigma-Aldrich).

Antibody-secreting cells, appearing as red spots, were analyzed with the CTL ImmunoSpot S4 Analyzer (C.T.L. Cellular Technology, Cleveland, OH).

RNA extraction and PCR

RNA isolation of single-cell suspensions and synthesis of cDNA were performed according to the manufacturers' instructions using RNA isolation kit Nucleospin RNA II (Macherey-Nagel, Dueren, Germany) and TaqMan Reverse Transcription Reagent (Applied Biosystems, Darmstadt, Germany). Gene expression was analyzed using real-time quantitative PCR. The primers (TIB Molbiol, Berlin, Germany) were applied at a hybridization temperature of 65°C at concentration of 500 nM. Primers for HPRT were sense TggCTTATATCCAACACTTCgTg and antisense ATCAgACTg AAgAgCTATTgTAATgACCA; for ϵ GLT sense gACgggCCACA CCATCCACAggCACCAAATggACgAC and antisense CAggAC gACTgTAAGATCTTCACg; for AID sense AgAggCgTgACAgTg CTACA and antisense ATgTAgCggAggAAGAgCAA; and for IFN regulatory factor 4 sense CCAGgTgACTCTATgCTTTgg and antisense CTTTgCgCTCATAACgTCAG.

Flow cytometric analysis of I κ B α degradation

After fixation with 4% paraformaldehyde for 10 minutes at 37°C, permeabilizing with Perm II solution (BD PharMingen) for 30 minutes at 4°C, cells were stained with FITC-coupled anti-I κ B α (Cell Signaling Technology), anti-CD19 (BD PharMingen), and anti-CD27 (eBioscience), and analyzed using flow cytometry.

Protein isolation and western blotting

NE-PER extraction reagent (Pierce, Rockford, IL) containing a protease inhibitor cocktail (Roche, Penzberg, Germany) was used for protein extract preparation. After nuclear extracts separation on tris-glycine SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Freiburg, Germany). Membranes were incubated with anti-human p50 (Santa Cruz Biotechnology, Heidelberg, Germany) or anti-human nucleolin (used as nucleus loading control; Sigma-Aldrich). After incubation with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), antigen detection was performed with the chemiluminescent detection system ECL Plus (Amersham Biosciences).

Statistics

Statistical calculations were performed using GraphPad Prism (La Jolla, CA). Wilcoxon signed-rank test for nonparametrical, paired data was carried out to analyze the within-group

changes. The two-tailed Mann-Whitney test was used to compare results between groups.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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