

Human eosinophils release IL-1 β and increase expression of IL-17A in activated CD4⁺ T lymphocytes

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Summary

Background Differentiation and activation of CD4⁺ T cells is controlled by various cytokines produced by innate immune cells. We have shown that eosinophils (EOS) have the potential to influence Th1 and Th2 cytokine generation by CD4⁺ cells, but their influence on IL-17A (IL-17) has not been established.

Objective The purpose of this study is to determine the effect of EOS on IL-17 production by lymphocytes.

Methods Pre-activated CD4⁺ T cells were cultured in the presence of either autologous EOS or EOS culture supernatants. Expression of IL-17 was determined by real-time quantitative PCR (qPCR) after 5 h and protein level was measured after 48 h. To determine the effect of allergen-induced airway EOS on IL-17, subjects with mild allergic asthma underwent bronchoscopic segmental bronchoprovocation with allergen (SBP-Ag) after a treatment with an anti-IL-5 neutralizing antibody (mepolizumab) to reduce airway eosinophilia. IL-17 mRNA was measured in bronchoalveolar lavage (BAL) cells by qPCR.

Results *In vitro*, EOS significantly increased IL-17 production by CD4⁺ T cells. Addition of exogenous IL-1 β increased expression of IL-17 mRNA by CD4⁺ T cells. EOS expressed and released IL-1 β . Furthermore, levels of IL-1 β in EOS supernatants highly correlated with their ability to increase IL-17 expression by CD4⁺ T cells, and neutralizing antibody to IL-1 β reduced expression of IL-17 mRNA. *In vivo*, reduction of EOS in the airway using mepolizumab was associated with diminished IL-17 expression after SBP-Ag.

Conclusions and clinical relevance Our data demonstrate that EOS can promote IL-17 production through the release of IL-1 β . Enhanced IL-17 cytokine production is another mechanism by which EOS may participate in pathogenesis of allergic airway inflammation in asthma.

Keywords allergy, asthma, cytokines, eosinophils, human, IL-17A, IL-1 β , lymphocytes

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Introduction

Whereas expression of IL-4 and IL-13 or IFN- γ characterizes either Th2 or Th1 lymphocytes, the expression of IL-17A (IL-17) identifies the Th17 subset. Th17 cells are paramount to autoimmune diseases [1, 2], but there is growing excitement for the potential role of Th17 cells in asthma [3–5]. There is evidence that IL-17 is elevated in bronchoalveolar lavage (BAL) fluid of asthmatic subjects [6]. In many studies, IL-17⁺ T cells and

IL-17 levels have been associated with a more severe asthma phenotype, characterized by airway neutrophilia [7–9]. In other studies, IL-17 correlates with eosinophils (EOS) [10] or IL-5 mRNA [11]. IL-17 likely has many functions in asthma including activation of stromal cells to release cytokines, chemokines and profibrotic mediators, and to increase the generation of extracellular matrix proteins and the expression of the dominant negative isoform of the glucocorticoid receptor (GRB) [6, 12–14]. In addition, IL-17 up-regulates angiogenesis

and endothelial cell invasion [15]. In animal models, IL-17 has been associated with increased mucus production by goblet cells and airway smooth muscle proliferation [16, 17].

Eosinophilia is typically associated with atopic asthma; however, EOS are also increased in autoimmune diseases, such as eosinophilic fasciitis, autoimmune pancreatitis, systemic sclerosis and bullous pemphigoid [18–21]. EOS can influence type 1 and 2 immune responses directly [22–24] and by producing T cell chemoattractants [25, 26]. In a mouse model, EOS have been associated with decreased IL-17 expression due to their effect on recruitment of dendritic cells to the lung draining lymph nodes [27]. However, a direct effect of human EOS on IL-17 expression has not been reported. Therefore, to evaluate the ability of EOS to regulate IL-17 during an inflammatory response in the airway, we developed a model in which CD4⁺ T cells with a memory/effector phenotype were cultured in the presence of EOS or EOS supernatants. In addition, the effect of EOS on IL-17 expression was further analysed *in vivo* in cells from BAL fluid after segmental broncho-provocation with allergen (SBP-Ag) and pre-treatment with an anti-IL-5 neutralizing antibody (mepolizumab) which decreases EOS numbers in the airways [28].

Materials and methods

Subjects

Studies were approved by the University of Wisconsin-Madison Center for Health Sciences Human Subjects Committee. Informed written consent was obtained from subjects prior to participation. All subjects were atopic and were skin prick test positive. For the mepolizumab (*in vivo*) study, subjects had a history of mild asthma with airway reversibility to albuterol. None of the subjects were using inhaled or oral corticosteroids.

Cell preparation for the *in vitro* study

EOS and CD4⁺ T cells were purified by negative selection as previously described [22]. Heparinized blood was centrifuged through Percoll (1.090 g/mL), peripheral blood mononuclear cells at the interface were used for isolation of T cells, and EOS were purified from the granulocyte-containing pellet. After lysis of RBCs, EOS were purified by negative selection using anti-CD16, anti-CD3 and anti-CD14 immunomagnetic beads (Auto-Mac system, Miltenyi Biotec Inc., Auburn, CA, USA). The EOS purity was >99%. CD4⁺ T cells were prepared by negative selection using the Miltenyi Biotec CD4⁺ T Cell Isolation Kit II. Purity was >98% as determined by flow cytometry.

Cell culture

CD4⁺ T cells (2×10^6 /mL) were cultured in 1 mL of complete medium [RPMI plus 10% fetal bovine serum (FBS)] alone (resting) or were activated with either 1 µg/mL of plate-bound anti-CD3 or anti-CD3 plus 1 µg/mL of soluble anti-CD28 (clones 37407 and UCHT1, respectively; R&D Systems, Minneapolis, MN, USA) in a 24-well plate (Corning Costar, Lowell, MA, USA). After 48 h activation, CD4⁺ T cells were harvested and cultured for an additional 24 h in complete medium in the absence of anti-CD3. Simultaneously, autologous EOS (1×10^6 /mL) were maintained in complete medium with GM-CSF (100 pg/mL) added at the beginning of the culture and after 24 h to sustain viability. At 72 h, EOS supernatants were collected and EOS and CD4⁺ T cells were harvested. EOS and lymphocyte viability was 99%. CD4⁺ T cells were plated (10^5 cells in 100 µL per well) in a 96-well plate pre-coated with anti-CD3 (1 µg/mL). EOS (10^5 cells in 100 µL per well) or 100 µL of EOS supernatant were added to CD4⁺ T cells. As a negative control, CD4⁺ T cells were cultured alone in the absence of anti-CD3. For transwell experiments, 6×10^5 CD4⁺ T cells were added to the bottom chamber-well coated with anti-CD3, and 6×10^5 EOS were placed in the upper chamber of a 0.4 µm pore polycarbonate filter 24-well transwell plate (Costar). The recombinant human (rh) IL-1B was purchased from Endogen (Endogen/Pierce/Thermo Scientific, Rockford, IL, USA), whereas rhIL-6 and rhTGF-β1 were provided by R&D Systems (Minneapolis).

Bronchoscopy, SBP-Ag and anti-IL-5 administration

Detailed methods for bronchoscopy, SBP-Ag and BAL cell preparation have previously been described [25]. The allergen dose for SBP-Ag was 20% of the AgPD₂₀ (the provocative dose of Ag leading to a 20% fall in FEV₁). A baseline bronchoscopy with BAL (4 × 40 mL aliquots of sterile 0.9% NaCl) was performed followed by administration of Ag into the lavaged segment. Bronchoscopy with BAL was repeated 48 h later. One month after the first SBP-Ag, a single dose of anti-IL-5 (750 mg, mepolizumab) was administered intravenously. One month after mepolizumab treatment, the participant underwent a second SBP-Ag, and BAL was performed immediately before and 48 h later. WBC counts and differentials were performed by the hospital clinical laboratory. On the day of the bronchoscopies, WBC counts were determined in the research laboratory using a coulter counter and EOS were enumerated by hemacytometer using phyloxin staining. BAL cells were assessed by hemacytometer using Turk's counting solution containing acetic acid and methylene blue, and cell differentials were determined on

cytospin preparations stained with the Wright-Giemsa-based Hema-3 (ThermoFisher/Thermo Scientific, Rockford, IL, USA). Airway EOS were purified from BAL cells using a two-step Percoll gradient as previously described [22]. EOS were collected from the 1.085/1.100 g/mL interface. IL-17 mRNA levels were measured in EOS when purity was >99%.

Flow cytometry

Before co-culture, activated or resting CD4⁺ T cells were assessed using anti-CD25/PE (BD Biosciences, San Jose, CA, USA), -CD69/PE (eBioscience, San Diego, CA, USA) or -CD45RO/PE (BD Biosciences). Cells were acquired on a LSR II (BD Biosciences). Positive staining and gating strategy were determined by comparison to isotype control antibodies. Flow cytometry data were analysed using the FlowJo software (Treestar Inc., Ashland, OR, USA).

qPCR

Total RNA was extracted from BAL cells or from cells co-cultured for 5 h using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The reverse transcription reaction was performed using the Superscript III system (Invitrogen/Life Technologies, Grand Island, NY, USA). mRNA expression was determined by qPCR using SYBR Green Master Mix (SABiosciences, Frederick, MD, USA), and human IL-17A (forward: cgtccacctcaccttgga, reverse: tcccagatcacagaggatctctc), CD3ε (forward: gatgcagtctgggcactcactgg, reverse: ctgcccccaaacgccaactgat) and IL-1β (forward: tggacccttggttaaagaca, reverse: gaagaaatcagtagagctatgaacaataag) specific primers were designed using Primer Express 3.0 (Applied Biosystems, Carlsbad, CA, USA) and blasted against the human genome to determine specificity using <http://www.ncbi.nlm.nih.gov/tools/primer-blast>. The reference gene, β-glucuronidase [(GUSB), forward: caggacctgcgcaacagag, reverse: tcgcacagctggggtaag], was used to normalize the samples. Standard curves were performed and efficiencies were determined for each set of primers. Efficiencies ranged between 91 and 96%. Data are expressed as fold change using the comparative cycle threshold ($\Delta\Delta Ct$) method. For the *in vitro* study, $\Delta Ct = [Ct \text{ target gene (IL-17 or IL-1}\beta) - Ct \text{ reference gene (GUSB)}]$; $\Delta\Delta Ct = (\Delta Ct \text{ of anti-CD3 activated cells with or without EOS or EOS supernatants} - \Delta Ct \text{ of unstimulated cells})$; for the *in vivo* (mepolizumab) study, $\Delta Ct = (Ct \text{ IL-17} - Ct \text{ CD3}\epsilon)$; $\Delta\Delta Ct = (\Delta Ct \text{ of BAL cells after allergen challenge with or without mepolizumab treatment} - \Delta Ct \text{ of BAL cells before allergen challenge and mepolizumab})$. For both the *in vitro* and *in vivo* studies, the values presented are fold change = $(2^{-\Delta\Delta Ct})$.

ELISA

Cytokines were measured in the 72 h EOS supernatants used on CD4⁺ cells or in the supernatants 48 h after the beginning of the co-culture of EOS and CD4⁺ T cells. IL-17, IL-1β and GM-CSF were measured utilizing 'in-house' sandwich ELISA [29]. Coating antibodies included anti-human IL-17A (clone 41809.111, R&D Systems), IL-1β (clone IL1B-H67, Endogen/Pierce/Thermo Scientific) and GM-CSF (clone 6804.11, R&D Systems). Detection antibodies included biotinylated polyclonal goat anti-human IL-17A (R&D Systems), IL-1β (clone ILB1-H6, Endogen) and GM-CSF (clone 3209.1, R&D Systems). The ELISA assay sensitivities were <3 pg/mL.

Statistical analysis

To compare expression of IL-17 in CD4⁺ T cells with or without EOS or EOS supernatants, and to compare IL-17 expression *in vivo* in BAL cells after SBP-Ag with or without mepolizumab treatment, data were log transformed and analysed using a Student's paired *t*-test. The Mann-Whitney Rank Sum test was used to test the effect of the recombinant proteins on IL-17 expression by CD4⁺ T cells. The correlations were analysed using the Spearman Rank Order Correlation. Statistical analyses were performed using the SigmaPlot 11.0 software package and significance was reached for $P < 0.05$.

Results

Characterization of *in vitro* activated CD4⁺ T lymphocytes

To analyse the effect of EOS on the expression of IL-17 by T lymphocytes, we developed an *in vitro* model where CD4⁺ T cells were cultured for 48 h with anti-CD3 and anti-CD28, harvested and 'rested' for 24 h. In parallel, part of the CD4⁺ T cells (resting) was cultured in medium for 72 h. After 72 h, activated CD4⁺ T cells expressed greater levels of CD69 compared with resting CD4⁺ T cells, and nearly 100% of the activated T cells were CD25⁺ and CD45RO⁺ (Fig 1). Activated CD4⁺ cells were then added to fresh anti-CD3-coated plates and co-cultured with EOS. These data imply that the activation step induces CD4⁺ T cell differentiation to a memory/effector phenotype.

Eosinophils alter IL-17 in activated CD4⁺ T lymphocytes

CD4⁺ T cells prepared as described above using anti-CD3 and anti-CD28 and activated again on anti-CD3 expressed 2- to 5-fold more IL-17 mRNA when co-cultured with EOS (Fig 2a). The requirement of cell-cell

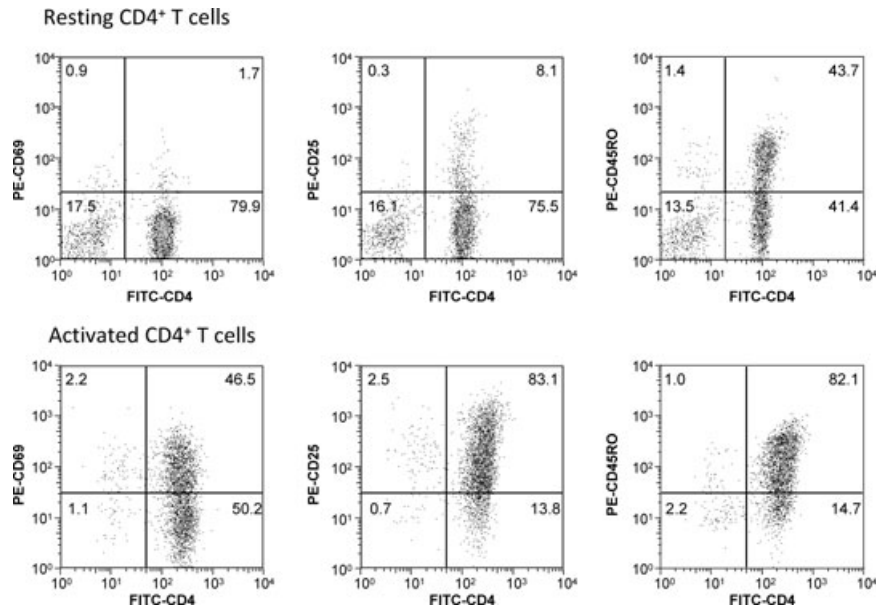


Fig. 1. In vitro activation of CD4⁺ lymphocytes leads to a memory/effector phenotype. CD4⁺ T cells were untreated (resting) or treated (activated) with anti-CD3 plus anti-CD28 for 48 h, washed, and then cultured in complete medium for 24 h. Cells were stained with FITC-anti-CD4⁺, PE-anti-CD69, PE-anti-CD25, or PE-anti-CD45RO, and analysed by flow cytometry.

contact and/or soluble mediators was evaluated by separation of EOS and CD4⁺ T cells using transwell plates or by addition of EOS-conditioned medium to T cells. CD4⁺ T lymphocytes expressed increased levels of IL-17 when co-cultured with EOS in transwell (Fig 2b). Furthermore, conditioned medium from EOS cultured for 72 h augmented IL-17 mRNA expression by CD4⁺ T cells (Fig 2c). Importantly, EOS supernatants did not contain detectable levels of IL-17, and IL-17 mRNA was not expressed by EOS cultured with CD4⁺ T cells on transwell plates, indicating that CD4⁺ T cells are the source of IL-17. In accordance with mRNA levels, more IL-17 protein accumulated in the supernatants of the co-cultures of CD4⁺ T cells plus EOS compared with CD4⁺ T cells alone (Fig 2d). These data suggest that EOS promote IL-17 expression in activated memory/effector-like CD4⁺ T cells through a soluble(s) mediator(s).

IL-17 mRNA expression in activated CD4⁺ T cells is up-regulated by rhIL-1β

In human T cells, expression of IL-17 is controlled by IL-1β, IL-6 and TGF-β1 [30, 31], which are all potentially released from EOS. To determine if these cytokines regulate IL-17 in our model, pre-activated CD4⁺ T cells were stimulated with anti-CD3 in the presence of rhIL-1β, rhIL-6 or rhTGF-β1. rhIL-1β increased IL-17 mRNA expression by more than 3-fold, whereas IL-6 and TGF-β1, used alone or in combination, did not affect IL-17 expression (Fig 3).

IL-17 expression is regulated by IL-1β released from eosinophils

Because IL-1β is a potent inducer of IL-17 in CD4⁺ T cells, we sought to determine the expression of IL-1β by EOS. IL-1β mRNA expression in EOS was compared with autologous CD4⁺ T cells when both were cultured for 5 h on immobilized anti-CD3 (Fig 4a). CD4⁺ T cells expressed little IL-1β, whereas high levels were expressed in EOS.

Protein amounts of IL-1β in supernatants of EOS cultured for 72 h highly correlated with the ability of the supernatants to increase IL-17 mRNA by activated CD4⁺ T cells (Fig 4b, $r = 0.964$, $P < 0.0001$, $n = 7$). Furthermore, addition of neutralizing anti-IL-1β antibody to EOS supernatants inhibited 70% of the increase in IL-17 (Fig 4c). Enhanced IL-17 expression did not correlate with levels of GM-CSF present in the conditioned media of EOS (3–168 pg/mL; $r = -0.0714$, $P = 0.843$, $n = 7$). Altogether, these data demonstrate that active IL-1β released from EOS enhances IL-17 expression by CD4⁺ T cells.

IL-17 expression after SBP-Ag in vivo was decreased by a mepolizumab treatment

To evaluate our *in vitro* data in an *in vivo* model, IL-17 and CD3ε expression was measured by qPCR in BAL cells from nine subjects before and 48 h after SBP-Ag with or without a mepolizumab pre-treatment. At base-

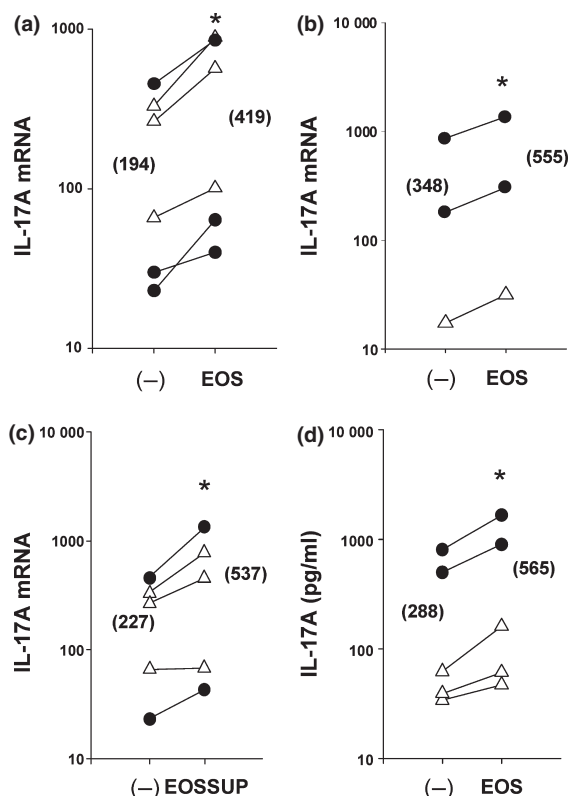


Fig. 2. EOS increase IL-17 by activated $CD4^+$ T cells. $CD4^+$ T cells were pre-activated with anti-CD3 (Δ) or anti-CD3 plus anti-CD28 ($\bullet$) for 48 h, rested for 24 h, and then reactivated with anti-CD3 in the absence (-) or presence of EOS (EOS) or EOS supernatants (EOSSUP). mRNA coding for IL-17 was measured by qPCR 5 h after restimulation (a, b, c) and IL-17 protein was measured by ELISA 48 h after restimulation (d). (a and d) $CD4^+$ T cells and EOS in contact. (b) $CD4^+$ T cells and EOS separated in transwell plates. (c) $CD4^+$ T cells in the absence or presence of EOS supernatants. mRNA values shown in a–c are fold change compared with non-reactivated $CD4^+$ T cells for each subject calculated as described in materials and methods. Means are shown in parenthesis. (* $P < 0.05$).

line, BAL cells are typically composed of macrophages. SBP-Ag leads to accumulation of EOS and activated lymphocytes after 48 h. To account for the variation in the % of T lymphocytes in the BAL cells, levels of IL-17 mRNA were related to CD3 ϵ mRNA. Treatment with mepolizumab significantly reduced IL-17 levels after SBP-Ag (Fig 5). The reduction in IL-17/CD3 ϵ was more pronounced (~2.5-fold) for the subjects displaying higher expression of IL-17 after SBP-Ag and before mepolizumab treatment. While percentages of EOS were reduced by mepolizumab (~57%; Table 1), purified BAL EOS did not express any detectable IL-17 mRNA (Ct values >40) and cannot account for the loss of IL-17. These data suggest that, in individuals with allergic asthma, EOS may be associated with airway IL-17 up-regulation in T lymphocytes.

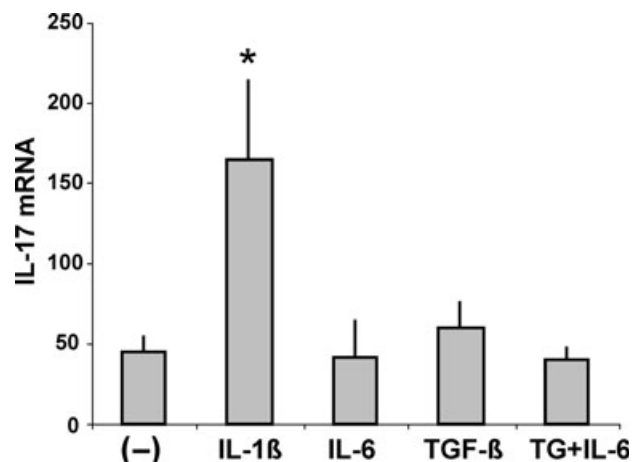


Fig. 3. rhIL-1 β increases IL-17 expression in $CD4^+$ T cells. $CD4^+$ T cells were pre-activated with anti-CD3 plus anti-CD28 for 48 h, rested for 24 h, and then activated with anti-CD3 in the presence of different recombinant cytokines (1 ng/mL) as indicated for 5 h. Data represent mean $\pm$ SD for 3–5 experiments. (* $P < 0.05$, $n = 5$, compare to cells without cytokine (-)).

Discussion

Our study demonstrates that EOS can enhance the production of IL-17 in $CD4^+$ T lymphocytes through the release of IL-1 β . Our data suggest that EOS interaction with effector/memory $CD4^+$ T cells can contribute to inflammation and remodelling via IL-17 production during an adaptive immune response. Because IL-17 is often associated with neutrophilia or neutrophil activation, eosinophilia might be a prelude to a neutrophilic inflammation, at least in some individuals with allergic asthma.

The potential impact of EOS on lymphocyte phenotypes has been studied in the context of Th1/Th2-type responses. We previously reported that EOS co-cultured with staphylococcal enterotoxin B-activated $CD4^+$ T cells enhance production of type 1 and type 2 cytokines [22]. Using a co-culture model whereby $CD4^+$ T cells are 'primed' with anti-CD3/CD28 and then activated again with anti-CD3 in the presence of EOS, we now show that EOS can augment the expression of the type 17 cytokine, IL-17, by $CD4^+$ T lymphocytes. In this model, $CD4^+$ T cells displayed a similar phenotype as activated memory/effector T cells ($CD25^+CD45RO^+$) that are predominant in the airways of asthmatics [32]. The enhancement of IL-17 correlated with EOS release of IL-1 β . The release of IL-1 β is consistent with reports that EOS are a source of IL-1 β [33, 34] and express a constitutive, inducible, active IL-1 β converting enzyme protease(s) [35, 36].

In previous studies, IL-1 β release was due to EOS activation with either toll-like receptor ligands or uric acid [33, 34]. In our co-culture model, the mediators

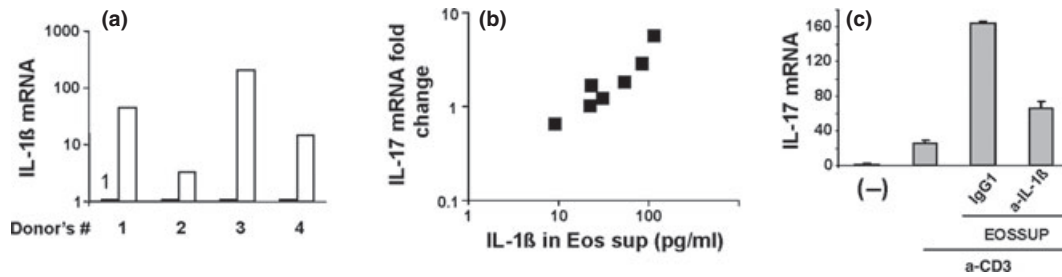


Fig. 4. *IL-1 β released from EOS regulates IL-17 expression by CD4⁺ T cells.* (a) Pre-activated T cells and EOS were prepared as for the co-culture experiments described above. At 72 h, IL-1 β mRNA was measured in CD4⁺ T cells or EOS by qPCR. For all four donors, very high levels of IL-1 β mRNA were measured in EOS compared with CD4⁺ T cells. $\Delta\Delta$ Ct method was used to compare the IL-1 β level in EOS with the level in CD4⁺ T cells, which was fixed at one for each donor. (b) IL-1 β protein levels in EOS supernatants prepared at 72 h from seven different donors were measured by ELISA. IL-1 β levels correlated with the ability of the EOS supernatants to increase IL-17 expression in pre-activated CD4⁺ T cells cultured for 5 h on anti-CD3 ($r = 0.964$, $P < 0.000001$, $n = 7$). (c) The activity of the EOS supernatant that increased IL-17 by 6-fold was neutralized by an anti-IL-1 β antibody.

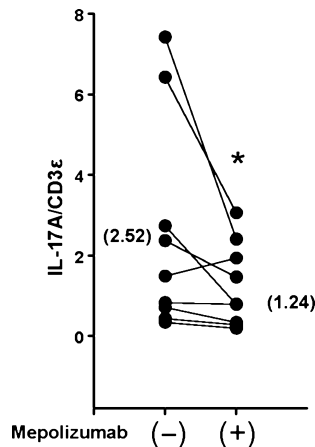


Fig. 5. *IL-17 levels after airway allergen challenge is diminished after mepolizumab treatment.* mRNA levels in BAL cells were determined by qPCR at baseline and 48 h after SBP-Ag performed before (–) and 1 month after (+) administration of mepolizumab. Fold change in IL-17/CD3 ϵ ratios compared with before baseline are shown for nine subjects. (* $P < 0.05$).

Table 1. The percentage of EOS in BAL cells was decreased by 57% (from 71.7 to 30.7%) by mepolizumab treatment after SBP-Ag ($n = 10$)

	Pre-mepolizumab		Post-mepolizumab	
	D0	D2 SBP-Ag	D0	D2 SBP-Ag
EOS % (Mean)	0.54	71.7	0.15	30.7
$\pm$ SD	0.30	8.1	0.12	12.8

used by CD4⁺ T cells to rapidly activate EOS remain unknown, but might involve a variety of cytokines. Spencer and colleagues demonstrated that EOS can quickly respond to TNF- α , IFN- γ , IL-10, or IL-4 to release multiple cytokines [37]. Surprisingly enough, in our study, EOS polarization by lymphocytes required CD4⁺ T cells to be activated again on anti-CD3 (not

shown). This suggests that a secondary T cell activation leads to a fast (within several hours) release of mediators that cannot involve newly transcribed proteins but might rather implicate post-transcriptional or post-translational mechanisms.

Our data are consistent with results by Liu *et al.*, demonstrating that IL-1 β increases IL-17 production by activated Th17⁺ memory T cells [31]. In that study and others, IL-23 and IL-6 amplified IL-1 β -induced IL-17. In our study, IL-23 was never detected in EOS at the mRNA level or as protein in EOS supernatant. IL-6 is also known to be released by EOS [30]; however, rhIL-6 alone did not increase IL-17 in our activated CD4⁺ T cells. We have not ruled out the possibility that EOS release of IL-6 enhances IL-17 induction by other factor(s). Also, in accordance with published studies of differentiated human Th17 cells [38, 39], TGF- β 1 was not required for IL-17 production in our model. It is important to note that our complete medium contained 10% FBS in which exogenous TGF- β 1 may be present and could thus interfere with the effects of rhTGF- β 1.

The heterogeneity and plasticity of Th17 is an area of active research and controversy. IL-17 can be expressed in Forkhead box P3⁺ T cells [40] and co-expressed with both IFN- γ (Th17/Th1) and IL-4 (Th17/Th2) [41]. In addition to IL-17, our CD4⁺ T cells that were activated again on anti-CD3 also produced high levels of Th2 (IL-13) and Th1 (IFN- γ) (data not shown). Future studies are required to determine the effect of EOS on various 'subsets' of IL-17⁺ T cells.

The biological significance of EOS regulation of IL-17⁺ cells during asthma remains speculative. We demonstrated that despite high levels of BAL EOS, antigen-induced increases in IL-17 mRNA only occurred in a subset of subjects. Notably, antigen-induced IL-17 was mitigated after administration of mepolizumab and subsequent reduction in Ag-induced BAL EOS. It is not clear why EOS would increase IL-17 expression in only

a subset of asthmatics. Our *in vitro* study suggests that the amount of IL-1 β produced, stored and released by EOS could explain the expression and the heterogeneity of IL-17 expression. The cause for elevated IL-1 β in EOS and its association with IL-17 expression remain unknown. Importantly, it is plausible that EOS-induced IL-17 is an important link to a more severe asthma phenotype that is observed in only 5–10% of the asthmatic population. As an important inducer of neutrophil chemoattractants, IL-17 is associated with neutrophilia in the airway [42]. Whereas mild allergic asthma is often associated with eosinophilia, more severe asthma can be characterized by the presence of both EOS and neutrophils [43].

Patients with nocturnal asthma have elevated levels of EOS, IL-1 β and CD4⁺ T lymphocytes in the airway at night-time [44–47], suggesting a link between eosinophilia and IL-1 β in symptomatic asthma. Other reports have demonstrated elevated levels of IL-1 β in BAL and sputum of asthmatics [48, 49] and that IL-1R1 is a potential therapeutic target for asthma [50]. Also importantly, IL-1 β and IL-17 have synergistic functions on epithelial cells and airway smooth muscle cells by enhancing mucin production and the neutrophil chemoattractant CXCL-8 and therefore participate in airway obstruction and inflammation [51, 52].

In conclusion, we demonstrated that EOS augment IL-17 during CD4⁺ T lymphocyte activation via IL-1 β release. The increase in pathogenic cytokines such as IL-1 β and IL-17 during an immune response could have dramatic effects *in vivo*. Understanding IL-1 β processing in EOS could further elucidate EOS function in pathologies such as asthma.

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Author contributions

All the authors contributed to the design and/or acquisition and analysis of the data. All the authors contributed to the drafting of the article or critically reviewed the manuscript. All authors approved the final version of the manuscript for submission.

Disclosures

The authors have no financial conflict of interest.

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References

- Gaffen SL. Recent advances in the IL-17 cytokine family. *Curr Opin Immunol* 2011; 23:613–9.
- Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. *Immunity* 2011; 34:149–62.
- Aujla SJ, Alcorn JF. T(H)17 cells in asthma and inflammation. *Biochim Biophys Acta* 2011; 1810:1066–79.
- Park SJ, Lee YC. Interleukin-17 regulation: an attractive therapeutic approach for asthma. *Respir Res* 2010; 11:78.
- Wang YH, Wills-Karp M. The potential role of interleukin-17 in severe asthma. *Curr Allergy Asthma Rep* 2011; 11:388–94.
- Molet S, Hamid Q, Davoine F *et al*. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001; 108:430–8.
- Zhao Y, Yang J, Gao YD, Guo W. Th17 immunity in patients with allergic asthma. *Int Arch Allergy Immunol* 2010; 151:297–307.
- Chakir J, Shannon J, Molet S *et al*. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF- β , IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003; 111:1293–8.
- Al Ramli W, Prefontaine D, Chouiali F *et al*. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* 2009; 123:1185–7.
- Ciprandi G, De AM, Murdaca G *et al*. Serum interleukin-17 levels are related to clinical severity in allergic rhinitis. *Allergy* 2009; 64:1375–8.
- Bullens DM, Truyen E, Coteur L *et al*. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res* 2006; 7:135.
- Henness S, van Thoor E, Ge Q, Armour CL, Hughes JM, Ammit AJ. IL-17A acts via p38 MAPK to increase stability of TNF- α -induced IL-8 mRNA in human ASM. *Am J Physiol Lung Cell Mol Physiol* 2006; 290:L1283–90.
- Wong CK, Cao J, Yin YB, Lam CW. Interleukin-17A activation on bronchial epithelium and basophils: a novel inflammatory mechanism. *Eur Respir J* 2010; 35:883–93.
- Vazquez-Tello A, Senglali A, Chakir J *et al*. Induction of glucocorticoid receptor- β expression in epithelial cells of asthmatic airways by T-helper

- type 17 cytokines. *Clin Exp Allergy* 2010; **40**:1312–22.
- 15 Moran EM, Connolly M, Gao W, McCormick J, Fearon U, Veale DJ. Interleukin-17A induction of angiogenesis, cell migration, and cytoskeletal rearrangement. *Arthritis Rheum* 2011; **63**:3263–73.
 - 16 Wang Q, Li H, Yao Y, Xia D, Zhou J. The overexpression of heparin-binding epidermal growth factor is responsible for Th17-induced airway remodeling in an experimental asthma model. *J Immunol* 2010; **185**:834–41.
 - 17 Lukacs NW, Smit JJ, Mukherjee S, Morris SB, Nunez G, Lindell DM. Respiratory virus-induced TLR7 activation controls IL-17-associated increased mucus via IL-23 regulation. *J Immunol* 2010; **185**:2231–9.
 - 18 Bachmeyer C, Monge M, Dhote R, Sanguina M, Aractingi S, Mougeot-Martin M. Eosinophilic fasciitis following idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia and Hashimoto's disease. *Dermatology* 1999; **199**:282.
 - 19 Simon D, Hoesli S, Roth N, Staedler S, Yousefi S, Simon HU. Eosinophil extracellular DNA traps in skin diseases. *J Allergy Clin Immunol* 2011; **127**:194–9.
 - 20 Sah RP, Pannala R, Zhang L, Graham RP, Sugumar A, Chari ST. Eosinophilia and allergic disorders in autoimmune pancreatitis. *Am J Gastroenterol* 2010; **105**:2485–91.
 - 21 Simon D, Simon HU. Eosinophilic disorders. *J Allergy Clin Immunol* 2007; **119**:1291–300.
 - 22 Liu LY, Mathur SK, Sedgwick JB, Jarjour NN, Busse WW, Kelly EAB. Human airway and peripheral blood eosinophils enhance Th1 and Th2 cytokine secretion. *Allergy* 2006; **61**:589–98.
 - 23 Akuthota P, Wang H, Weller PF. Eosinophils as antigen-presenting cells in allergic upper airway disease. *Curr Opin Allergy Clin Immunol* 2010; **10**:14–9.
 - 24 Odemuyiwa SO, Ghahary A, Li Y *et al.* Cutting edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase. *J Immunol* 2004; **173**:5909–13.
 - 25 Liu LY, Bates ME, Jarjour NN, Busse WW, Bertics PJ, Kelly EA. Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF- α . *J Immunol* 2007; **179**:4840–8.
 - 26 Jacobsen EA, Ochkur SI, Pero RS *et al.* Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *J Exp Med* 2008; **205**:699–710.
 - 27 Jacobsen EA, Zellner KR, Colbert D, Lee NA, Lee JJ. eosinophils regulate dendritic cells and Th2 pulmonary immune responses following allergen provocation. *J Immunol* 2011; **187**:6059–68.
 - 28 Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am J Respir Crit Care Med* 2003; **167**:199–204.
 - 29 Kelly EA, Rodriguez RR, Busse WW, Jarjour NN. The effect of segmental bronchoprovocation with allergen on airway lymphocyte function. *Am J Respir Crit Care Med* 1997; **156**:1421–8.
 - 30 Burgler S, Ouaked N, Bassin C *et al.* Differentiation and functional analysis of human T(H)17 cells. *J Allergy Clin Immunol* 2009; **123**:595.
 - 31 Liu H, Rohowsky-Kochan C. Regulation of IL-17 in human CCR6⁺ effector memory T cells. *J Immunol* 2008; **180**:7948–57.
 - 32 Robinson DS, Bentley AM, Hartnell A, Kay AB, Durham SR. Activated memory T helper cells in bronchoalveolar lavage fluid from patients with atopic asthma: relation to asthma symptoms, lung function, and bronchial responsiveness. *Thorax* 1993; **48**:26–32.
 - 33 Wong CK, Cheung PF, Ip WK, Lam CW. Intracellular signaling mechanisms regulating toll-like receptor-mediated activation of eosinophils. *Am J Respir Cell Mol Biol* 2007; **37**:85–96.
 - 34 Kobayashi T, Kouzaki H, Kita H. Human eosinophils recognize endogenous danger signal crystalline uric acid and produce proinflammatory cytokines mediated by autocrine ATP. *J Immunol* 2010; **184**:6350–8.
 - 35 Hebestreit H, Dibbert B, Balatti I *et al.* Disruption of fas receptor signaling by nitric oxide in eosinophils. *J Exp Med* 1998; **187**:415–25.
 - 36 Simon HU, Yousefi S, Dibbert B *et al.* Role for tyrosine phosphorylation and Lyn tyrosine kinase in fas receptor-mediated apoptosis in eosinophils. *Blood* 1998; **92**:547–57.
 - 37 Spencer LA, Szela CT, Perez SA *et al.* Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. *J Leukoc Biol* 2009; **85**:117–23.
 - 38 Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1 β and 6 but not transforming growth factor- β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007; **8**:942–9.
 - 39 Wilson NJ, Boniface K, Chan JR *et al.* Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007; **8**:950–7.
 - 40 Liu T, Song CH, Liu AM *et al.* Forkhead box P3⁺ T cells express interleukin-17 in nasal mucosa of patients with both allergic rhinitis and polyposis. *Clin Exp Immunol* 2011; **163**:59–64.
 - 41 Cosmi L, Maggi E, Santarlasci V *et al.* Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. *J Allergy Clin Immunol* 2010; **125**:222–30.
 - 42 Laan M, Cui ZH, Hoshino H *et al.* Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* 1999; **162**:2347–52.
 - 43 Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999; **160**:1532–9.
 - 44 Jarjour NN, Busse WW. Cytokines in bronchoalveolar lavage fluid of patients with nocturnal asthma. *Am J Respir Crit Care Med* 1995; **152**:1474–7.
 - 45 Kelly EAB, Houtman JJ, Jarjour NN. Inflammatory changes associated with circadian variation in pulmonary function in subjects with mild to moderate asthma. *Clin Exp Allergy* 2004; **34**:227–33.
 - 46 Panzer SE, Dodge AM, Kelly EA, Jarjour NN. Circadian variation of sputum inflammatory cells in mild asthma. *J Allergy Clin Immunol* 2003; **111**:308–12.
 - 47 Esnault S, Fang Y, Kelly EA *et al.* Circadian changes in granulocyte-macrophage colony-stimulating factor message in circulating eosinophils. *Ann Allergy Asthma Immunol* 2007; **98**:75–82.

- 48 Baines KJ, Simpson JL, Wood LG, Scott RJ, Gibson PG. Transcriptional phenotypes of asthma defined by gene expression profiling of induced sputum samples. *J Allergy Clin Immunol* 2011; 127:160.
- 49 Borish L, Mascali JJ, Dishuck J, Beam WR, Martin RJ, Rosenwasser LJ. Detection of alveolar macrophage-derived IL-1beta in asthma. *J Immunol* 1992; 149:3078–82.
- 50 Lee JH, Wang LC, Yu HH, Lin YT, Yang YH, Chiang BL. Type 1 IL-1 receptor (IL-1RI) as potential new therapeutic target for bronchial asthma. *Mediators Inflamm* 2010; 2010:567351.
- 51 Fujisawa T, Velichko S, Thai P, Hung LY, Huang F, Wu R. Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. *J Immunol* 2009; 183:6236–43.
- 52 Dragon S, Rahman MS, Yang J, Unruh H, Halayko AJ, Gounni AS. IL-17 enhances IL-1beta-mediated CXCL-8 release from human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2007; 292:L1023–9.