

Interleukin-17 as a Recruitment and Survival Factor for Airway Macrophages in Allergic Airway Inflammation

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Recent data indicate that the proinflammatory cytokine, interleukin (IL)-17, stimulates certain effector functions of human macrophages. We evaluated whether IL-17 mediates allergen-induced accumulation of airway macrophages and, if so, whether such an effect relates to the control of macrophage recruitment and survival. BALB/c mice were sensitized and challenged with ovalbumin. Three hours before challenge an anti-mouse IL-17 mAb (a-IL-17) was administered. Sampling was conducted 24 h after the allergen challenge. *In vitro* chemotaxis assay for blood monocytes and culture of airway macrophages, immunocytochemistry for Fas-antigen, and matrix metalloproteinase-9 (MMP-9) were used to determine the effect of IL-17 on the recruitment, survival, and activity of airway macrophages. A-IL-17 reduced the number of airway neutrophils and macrophages after allergen challenge. *In vitro*, recombinant IL-17 induced migration of blood monocytes and prolonged survival of airway macrophages. A-IL-17 also increased the expression of Fas-antigen in airway macrophages *in vivo*. Finally, the expression of MMP-9 by airway neutrophils and macrophages *in vivo* was down-regulated by a-IL-17. This study indicates that endogenous IL-17 mediates the accumulation of macrophages during allergen-induced airway inflammation. IL-17 exerts its effects by acting directly on airway macrophages by promoting their recruitment and survival. Furthermore, IL-17 is involved in controlling the proteolytic activity of macrophages and neutrophils in allergen-induced airway inflammation.

Keywords: mice; asthma; neutrophil; matrix metalloproteinase-9; apoptosis

Allergen-induced airway inflammation is characterized by a local increase in the number of various inflammatory cells including T-lymphocytes, eosinophils, neutrophils, and macrophages (1, 2). In allergic asthma there is also activation of T-lymphocytes, cells that via release of proinflammatory cytokines mediate the mobilization of other inflammatory cells in the airway (1, 3). It is also known that persistent airway inflammation, such as in asthma, leads to injury and abnormal repair of airway tissue (4).

Macrophages are predominant cells in bronchoalveolar space in individuals with and without asthma (5, 6). Moreover, the absolute number of airway macrophages is significantly increased in asthma (7). This accumulation of macrophages in asthma may be due to an increased recruitment, proliferation, or an enhanced survival of cells within the airway. In line with this, the concentration of macrophage chemotactic factors such as monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein

(MIP)-1 α , and granulocyte/macrophage colony-stimulating factor (GM-CSF) is increased in asthmatic airway (8, 9). However, it remains to be determined whether these cytokines are participating in allergen-induced enhanced accumulation of the macrophages in asthmatic airway. Furthermore, there is now evidence of enhanced survival of airway macrophages in the asthmatic airway (10), and this may be regulated by survival factors such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and GM-CSF (11). Yet another possibility is that the enhanced survival of airway macrophages in asthma relates to the degree of stimulation of the Fas receptor (12), which engagement by its ligand leads to cell death through apoptosis (13).

For at least two reasons, the control of macrophage accumulation may be important for determining the severity of airway inflammation. First, airway macrophages constitute a potentially powerful source of pro- and anti-inflammatory cytokines as well as of tissue-degrading proteinases and antiproteinases in subjects with asthma (14–17). Second, airway macrophages are involved in the removal of cells undergoing apoptosis. Importantly, removal of apoptotic cells occurs before cell lysis (18), and so release of intracellular contents in the surrounding tissue is avoided (19, 20). Thus, any endogenous factor that controls the accumulation of airway macrophages bears the potential to determine the severity of airway inflammation such as in allergic asthma.

The proinflammatory cytokine IL-17 has previously been forwarded as a link between activated T-lymphocytes and the recruitment and activation of neutrophils in various types of airway inflammation (21). Recently it has been shown that the concentration of IL-17 is increased in bronchoalveolar lavage fluid (BALF), sputum, and blood from patients with asthma (22, 23). In a mouse model of allergic airway inflammation, systemic blockade of IL-17 inhibits the allergen-induced accumulation of neutrophils in the airway (24). In IL-17 knockout mice, the allergen-induced airway hyperreactivity to methacholine is significantly reduced (25). In line with this, increased immunoreactivity for IL-17 in the airway submucosa is associated with impaired lung function in patients with asthma (23).

Interestingly, there is now evidence that recombinant IL-17 protein stimulates the release of cytokines such as TNF- α , IL-1 β , and IL-6 in human blood-derived macrophages *in vitro* (26). In addition, IL-17 also stimulates the release of the tissue-degrading enzyme matrix metalloproteinase-9 (MMP-9) in the same type macrophages (27, 28). However, it is not known whether IL-17 is involved in control of allergen-induced accumulation of macrophages and their activity in allergic airway inflammation. In the current study, we therefore evaluated whether IL-17 mediates the accumulation of macrophages in allergic airway inflammation in mice and, if so, whether such an effect relates to the control of macrophage recruitment and survival.

MATERIALS AND METHODS

Mice

This study was approved by the Ethical Committee for Animal Studies in Gothenburg, Sweden (Diary nr. 298/01). BALB/c mice were purchased

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from B&K Universal AB (Sollentuna, Sweden). The mice were 5–6 wk old and were maintained under conventional animal housing conditions and provided with food and water *ad libitum*.

Allergen Sensitization, Challenge, and IL-17 Blockade

BALB/c mice were sensitized by intraperitoneal injections of 0.5 ml aluminum-precipitated antigen containing 8 µg of ovalbumin (OVA; Sigma Aldrich Sweden AB, Tyresö, Sweden) bound to 4 mg of aluminum hydroxide (Sigma) in phosphate-buffered saline (PBS) twice, 5 d apart. Eight days after the second sensitization, the animals were briefly anaesthetized using isoflurane (Schering-Plough, Welwyn Garden City, UK), and challenged intranasally with 5 µg of OVA dissolved in 25 µl of PBS. Three hours before OVA challenge, 200 µg of either an anti-mouse IL-17 mAb (a-IL-17, clone 50104.11; R&D Systems, Abingdon, UK) or its isotype control antibody rat IgG_{2a} (clone R35–95; BD Biosciences Europe, Erembodegem, Belgium) were administered intravenously into the lateral tail vein. The total volume of injected solution was matched between active treatment and control group, being 200 µl.

Collection and Processing of Cell Samples

All samples were collected 24 h after the allergen challenge. The animals were anesthetized with a mixture of xylazine (130 mg/kg, Rompun; Bayer, Leverkusen, Germany) and ketamine (670 mg/kg, Ketalar; Pfizer AB, Täby, Sweden). First, blood was obtained by puncture of the heart right ventricle. Second, bronchoalveolar lavage (BAL) was performed through the tracheal cannula by instillation of 0.25 and 0.20 ml of PBS. Finally, bone marrow (BM) was harvested by excising one femur, which was cut at the epiphyses and flushed with 2 ml of PBS.

Blood. A mixture was made of 200 µl of blood with 800 µl of 2 mM ethylenediaminetetraacetic acid (Sigma) in PBS. Red blood cells were lysed using 0.1% potassium bicarbonate and 0.83% ammonium chloride solution for 15 min at 4°C. The white blood cells were resuspended in PBS containing 0.03% bovine serum albumin (BSA; Sigma).

BALF and BM. BALF and BM samples were centrifuged at $300 \times g$ for 10 min at 4°C and cell pellet was resuspended in 0.03% BSA in PBS. The cell-free BAL supernatant was collected for cytokine measurements using commercial ELISA kits (R&D Systems).

The total cell numbers in blood, BALF, and BM were determined using standard hematologic procedures. Cytospins of blood, BALF, and BM samples were prepared and stained according to the May-Grünwald-Giemsa method for differential cell counting, performed by counting 400 cells, using a conventional light microscope (Zeiss Axioplan 2; Carl Zeiss, Jena, Germany). The cells were identified using standard morphologic criteria.

Immunocytochemistry

All immunocytochemistry (ICC) procedures were performed at room temperature unless otherwise stated. Cells were determined by counting 400 cells using a light microscope (magnification $\times 1,000$, Zeiss Axioplan 2; Carl Zeiss).

ICC for Fas extracellular domain. Cytospin preparations were fixed with 2% formaldehyde during 30 min, followed by incubation in pre-heated basic antigen retrieval reagent (R&D Systems) at 94°C for 4 min. Unspecific binding was blocked using 10% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), and endogenous biotin was blocked with Biotin Blocking System (DAKO Corporation, Glostrup, Denmark). Slides were incubated with a purified anti-mouse Fas/TNFRSF6 mAb (R&D Systems) over night at 4°C. As secondary antibody a biotinylated F(ab')₂-fragment donkey anti-goat IgG (Jackson ImmunoResearch Laboratories) was used, followed by alkaline phosphatase-conjugated streptavidin (DAKO). Bound antibodies were visualized with Vector Red alkaline substrate kit (Vector Laboratories, Burlingame, CA). Mayer's Hematoxylin (Sigma) was used for counterstaining.

ICC for MMP-9. Cytospin preparations were fixed with 2% formaldehyde during 30 min. Unspecific binding and endogenous biotin were blocked as described above. Slides were incubated with a purified anti-mouse MMP-9 mAb (R&D Systems) during 1 h, followed by the same secondary antibody and detection system as described previously.

In Vitro Experiments

For *in vitro* experiments, mice were sensitized and challenged with OVA or PBS as described above. BAL was performed by instillation of 1 ml of PBS four times. Blood was obtained by puncture of the heart right ventricle. For each single experiment, BALF or blood from 15–20 mice was pooled to obtain a sufficient cell number. Cell enrichment was performed using a magnetic cell sorting system with indirect microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). BALF macrophages or blood monocytes were enriched using a biotinylated *Griffonia simplicifolia* lectin 1 antibody (Vector Laboratories) (29). Results from *in vitro* experiments are shown as the average from three separate and independent experiments.

Chemotaxis assay. Blood monocytes were brought to the final concentration of $0.5 \times 10^6/\text{ml}$ in RPMI medium containing 1% BSA. The chemotaxis assay was performed in a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD) as previously described (30). Briefly, the solutions and equipment were brought to 37°C before onset of the experiment. The bottom wells of the chamber (at least triplicate for each condition in each experiment) were filled with 28 µl of fluid containing either the 1% BSA/RPMI (negative control), the 10 ng/ml or recombinant MCP-1 (positive control; R&D Systems), or 10 ng/ml of recombinant IL-17 (R&D Systems). A polycarbonate filter with pore size of 8 µm (Nucleopore, Pleasanton, CA) was placed over the bottom wells. The silicon gasket and upper piece of the chamber were applied, and 50 µl of monocyte suspension was pipetted into upper wells. The chamber was incubated in humidified air with 5% CO₂ at 37°C for 1 h, then disassembled, and the filter was removed. The filter was then fixed in methanol, stained according to the May-Grünwald-Giemsa method, and mounted on a glass slide. In each well, monocytes that completely migrated through the filter were counted using a light microscope (magnification $\times 1,000$; Zeiss Axioplan 2, Carl Zeiss). The chemotactic response was expressed as a migration index. For each chemotactic stimulus (recombinant MCP-1 or IL-17 protein), a migration index was calculated by dividing the number of migrated cells in response to the cytokine by the number of cells that migrated randomly, that is, in response to 1% BSA/RPMI alone. Thus, a reference index exceeding 1 indicates chemotaxis.

Culture of BALF macrophages. BALF macrophages were seeded in a concentration of $0.625 \times 10^6/\text{ml}$ in a 96-well plate (BD Biosciences) in RPMI media complemented with 10% fetal calf serum, 1% penicillin-streptomycin, 2 mM L-glutamine, and 1% sodium pyruvate (all obtained from Sigma) at 37°C in an atmosphere containing 5% CO₂ for 20 h. The seeded fraction contained $92.7 \pm 2.3\%$ macrophages ($n = 3$) as determined using May-Grünwald-Giemsa staining. For BAL macrophages from OVA- and PBS-exposed animals, the following treatment groups (at least triplicate for each condition in each experiment) were established: (1) Negative control (i.e., vehicle with no recombinant mouse IL-17 protein); (2) Recombinant IL-17 protein (R&D Systems) 0.1 ng/ml; and (3) IL-17 protein 1 ng/ml. The trypan blue exclusion test was performed at baseline before and at the end of the 20-h experiment. The baseline viability of the seeded cells from OVA- and PBS-exposed animals, respectively, was $76.5 \pm 1.9\%$ and $81.3 \pm 3.7\%$ ($n = 3$). After each experiment, the conditioned medium was collected for cytokine measurements.

Statistical Analysis

Data are presented as mean with SEM. Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis test) to evaluate variance among all groups. If a significant variance was found, an unpaired two-group test (Mann-Whitney test) was used to determine significant differences between individual groups. Spearman Rank correlation test was used for detection of the relationship between two variables. $P < 0.05$ was considered statistically significant.

RESULTS

BALF

Pretreatment with a-IL-17 decreased the number of macrophages and neutrophils in BALF from sensitized and allergen-challenged mice ($P = 0.009$ and $P = 0.03$, respectively; Figure 1). In contrast, a-IL-17 caused no statistically significant changes

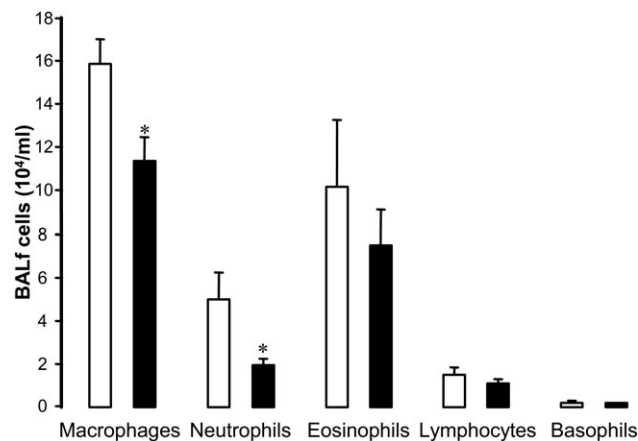


Figure 1. BALF differential cell counts in ovalbumin-sensitized and challenged BALB/c mice. Mice received systemic pretreatment with an anti-mouse IL-17 mAb (a-IL-17, filled columns) or an isotype control antibody (IgG_{2a}, open columns). Data are shown as mean with SEM from two independent experiments. * $P < 0.05$, $n = 12-13$.

in the corresponding number of eosinophils, lymphocytes, or basophils (Figure 1).

Pretreatment with a-IL-17 increased the expression of Fas antigen in BALF macrophages ($P = 0.0004$; Figure 2), but not within other cell populations in BALF (data not shown). A typical example of the ICC staining for Fas antigen in BALF cytoplasm is illustrated in Figure 3.

Pretreatment with a-IL-17 substantially decreased the expression of MMP-9 in BALF macrophages ($P = 0.004$; Figure 4) and in BALF neutrophils ($P = 0.04$; Figure 4), but not in BALF eosinophils (10 ± 7 versus $20 \pm 16\%$ for a-IL-17 and isotype control antibody, respectively, $P = 0.5$). A typical example of the ICC staining for MMP-9 in BALF macrophages and neutrophils is illustrated in Figure 5.

BALF Cytokines

The measurements of cytokines in BALF samples did not indicate any significant differences in the concentration of MIP-2 ($n = 7$, 27 ± 4 versus 24 ± 2 pg/ml, $P = 0.5$), TNF- α ($n = 12-13$, 39 ± 4 versus 41 ± 2 pg/ml, $P = 0.6$), IL-6 ($n = 5$, 269 ± 79 versus 241 ± 57 pg/ml, $P = 0.7$), macrophage colony-stimulating

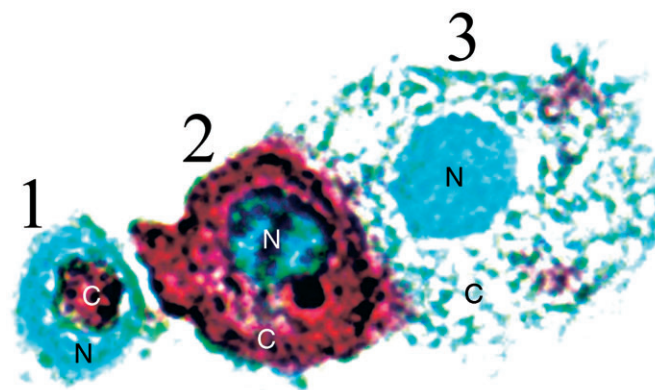


Figure 3. Photograph of ICC staining for Fas antigen in BALF cytoplasm ($\times 1,000$). Red staining indicates Fas antigen. The number 1 indicates a Fas⁺ eosinophil, 2 indicates a Fas⁺ macrophage, 3 indicates a Fas⁻ macrophage, N indicates cell nucleus, and C indicates cell cytoplasm.

factor (M-CSF) ($n = 5$, 55 ± 2 versus 57 ± 2 pg/ml, $P = 0.5$), or MCP-1 ($n = 12-13$, 11 ± 3 versus 34 ± 15 pg/ml, $P = 0.2$) for mice pretreated with a-IL-17 versus the isotype control antibody, respectively. GM-CSF was not detectable in any of the BALF samples from either treatment group ($n = 12-13$).

Blood and BM

Pretreatment with a-IL-17 did not lead to any statistically significant changes in total or differential cell counts in blood (Figure 6A) or BM (Figure 6B).

Chemotaxis of Blood Monocytes

Recombinant mouse MCP-1 induced migration of blood monocytes from OVA-challenged but not from PBS-exposed animals ($P = 0.0053$ and $P = 0.67$, respectively; Figure 7). Compared with the positive control MCP-1, recombinant mouse IL-17 protein induced substantial migration of blood monocytes harvested from the OVA-challenged animals ($P = 0.0125$; Figure 7).

Culture of BALF Macrophages

Stimulation with recombinant mouse IL-17 protein prolonged the survival of BALF macrophages *in vitro* from both OVA- and PBS-exposed mice in a concentration-dependent and statistically

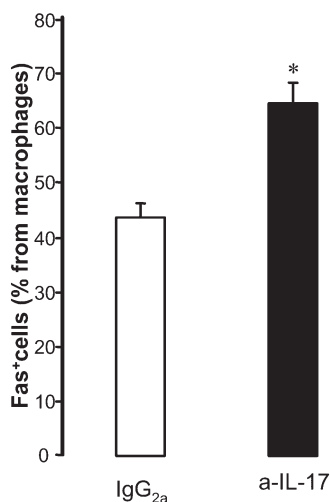


Figure 2. Relative number of Fas-positive BALF macrophages in OVA-sensitized and -challenged BALB/c mice. Mice received systemic pretreatment with a-IL-17 or IgG_{2a}. Data are shown as mean with SEM. * $P < 0.05$, $n = 12-13$.

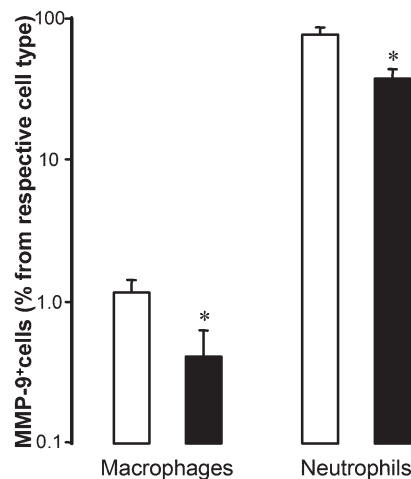


Figure 4. Relative number of MMP-9-positive BALF macrophages and neutrophils in OVA-sensitized and -challenged BALB/c mice. Mice received systemic pretreatment with a-IL-17 (filled columns) or IgG_{2a} (open columns). Data are shown as mean with SEM. * $P < 0.05$, $n = 6$.

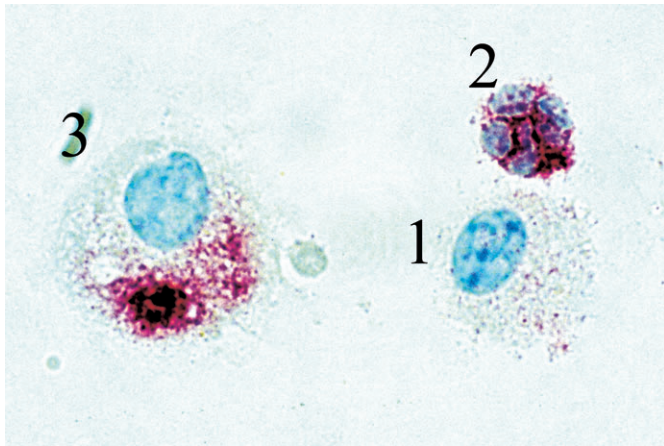


Figure 5. Photograph of ICC staining for MMP-9 in BALF cytospin ($\times 1,000$). Red staining indicates MMP-9. The number 1 indicates a MMP-9⁻ macrophage, 2 indicates a MMP-9⁺ neutrophil, and 3 indicates a MMP-9⁺ macrophage.

significant manner ($R_s = 0.8$, $P < 0.0001$ and $R_s = 0.6$, $P = 0.02$, respectively; Figure 8). The IL-17-induced (1 ng/ml) increase in survival was higher for macrophages from animals challenged with OVA compared with those from PBS-exposed mice (33.8 versus 20.1%, respectively, $P = 0.0495$).

Stimulation of BALF macrophages recovered from OVA-challenged mice with recombinant IL-17 protein did not markedly change the concentration of GM-CSF, MIP-2, or MCP-1 protein in the conditioned medium ($n = 3-6$, data not shown).

DISCUSSION

Our study shows that intravenous pre-treatment with neutralizing α -IL-17 decreases the number of BALF macrophages and neutrophils after allergen challenge in sensitized mice *in vivo*. This effect is paralleled by an upregulation of Fas antigen expression on BALF macrophages, but not on other airway cells *in vivo*. In addition, recombinant IL-17 protein is chemotactic for blood monocytes and it prolongs the survival of BALF macrophages *in vitro*. Finally, intravenous pretreatment with α -IL-17 decreases the number of MMP-9-positive macrophages and neutrophils in BALF *in vivo*.

Even though it is now well established that the expression of IL-17 is increased in the airway of patients with asthma (22, 23), the pathogenetic role of IL-17 in allergic inflammation is poorly understood. Indeed, there is only one published study on the effect of systemic blockade of IL-17 during allergic airway inflammation in mice (24). The referred study indicated that systemic blockade of IL-17 reduces neutrophil number and increases eosinophil number after allergen exposure in sensitized airway, and suggested alterations in BM granulocytopenesis and chemotaxis of mature granulocytes as plausible mechanisms of action. In our study, we now confirm a corresponding role of endogenous IL-17 in allergen-induced accumulation of neutrophils in the airway. Moreover, our study expands the role of IL-17 in allergic inflammation by showing that systemic blockade of IL-17 also attenuates the allergen-induced accumulation of macrophages in sensitized airway. This effect of blocking IL-17 occurs without any substantial changes in the cell counts in blood or BM in our study. It seems likely that the observed discrepancies in IL-17 blockade-caused alterations in number of airway macrophages and eosinophils for our study and the study by Hellings and coworkers are due to differences between the re-

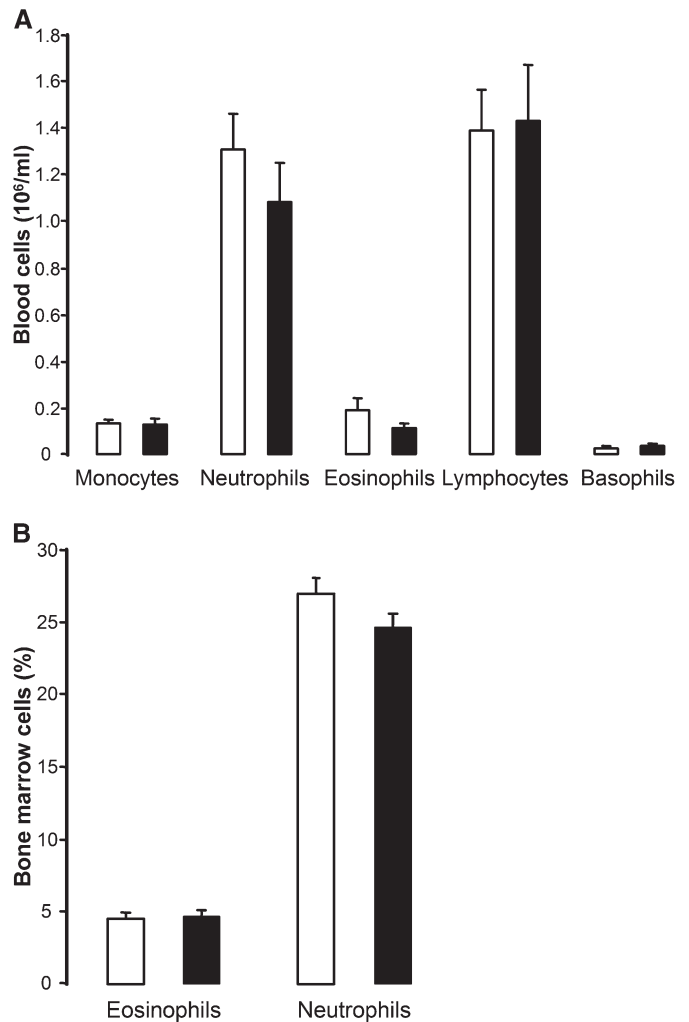


Figure 6. (A) Blood differential cell counts and (B) relative number of BM eosinophils and neutrophils in OVA-sensitized and -challenged BALB/c mice. Mice received systemic pre-treatment with α -IL-17 (filled columns) or IgG_{2a} (open columns). Data are shown as mean with SEM ($n = 12-13$).

spective experimental models. These differences include the protocol for allergen exposure, the dose of neutralizing anti-IL-17 antibody, the route and timing of anti-IL-17 antibody administration, and the timing of sample harvest. In agreement with previous observations on the role of IL-17 in endotoxin-induced airway inflammation (31), our data imply that endogenous IL-17 primarily exerts an effect on airway neutrophils and macrophages via local rather than systemic mechanisms.

One of the plausible mechanisms behind α -IL-17-caused reduction in the number of airway macrophages after allergen challenge is the inhibition of cell recruitment into the airway. To assess this hypothesis, we first evaluated whether recombinant IL-17 protein has an effect on the migration of blood monocytes harvested from allergen-challenged or vehicle-exposed mice and subsequently cultured *in vitro*. Our study shows that recombinant IL-17 protein directly induces the migration (i.e., chemotaxis) of blood monocytes from allergen-challenged mice. Moreover, the chemotactic effect of IL-17 was of similar magnitude as that of positive control—MCP-1. Because, in the case of neutrophils, the accumulating effect of IL-17 is believed to be indirect, mediated mainly via secondary mediators (21), we

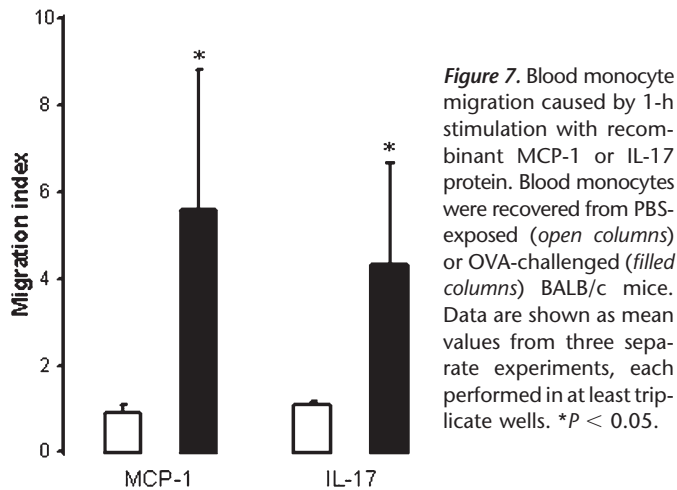


Figure 7. Blood monocyte migration caused by 1-h stimulation with recombinant MCP-1 or IL-17 protein. Blood monocytes were recovered from PBS-exposed (open columns) or OVA-challenged (filled columns) BALB/c mice. Data are shown as mean values from three separate experiments, each performed in at least triplicate wells. * $P < 0.05$.

also evaluated whether endogenous IL-17 exerts an effect on the macrophage chemotactic factors after allergen challenge. However, systemic pretreatment with α -IL-17 did not cause any substantial changes in the concentration of either MCP-1, M-CSF, or GM-CSF proteins in BALF. Thus, our results indicate that endogenous IL-17 constitutes a recruitment factor with direct chemotactic effect on the precursor cells for airway macrophages in allergen-induced airway inflammation.

Another plausible mechanism behind the effect of endogenous IL-17 on the number of airway macrophages is the enhancement of cell viability via a decrease in cell apoptosis. The Fas–Fas ligand system is recognized as a major extrinsic pathway of apoptosis (13). Fas antigen belongs to the TNF receptor family, is expressed in macrophages, and its binding to ligand induces apoptosis in Fas-bearing cells (12, 13). It is therefore of particular interest that, in our study, pretreatment with α -IL-17 caused an increase in the expression of Fas antigen selectively in airway macrophages in sensitized and allergen-challenged mice. This implicates that endogenous IL-17 promotes macrophage survival via downregulation of programmed cell death mediated by the Fas–Fas ligand system. In support of these *in vivo* observations, stimulation of BALF macrophages with recombinant IL-17 protein *in vitro* prolonged their survival in concentration-dependent manner. It is noteworthy that macrophages from allergen-challenged mice were more susceptible to IL-17 than were macrophages from vehicle-exposed mice. Taken together, these data forward endogenous IL-17 as a local survival factor for airway macrophages during allergic airway inflammation, acting in part through the inhibition of Fas-mediated cell apoptosis.

MMP-9 is the most abundant MMP present in BALF from patients with asthma (32). Importantly, the MMP-9 concentration is increased in subjects with asthma compared with healthy subjects (32). In our study, we found that pretreatment with α -IL-17 reduces the proportion of MMP-9-positive macrophages and neutrophils in the airway of allergen-sensitized and -challenged mice. The potential pathogenetic relevance of this finding is indicated by the fact that in asthma, the immunoreactivity for MMP-9 in airway tissue is linked to disease severity, including impaired lung function and increased numbers of tissue neutrophils and macrophages (33). Because MMP-9 exerts a substantial tissue-degrading collagenolytic and elastolytic activity (34), our findings prompt for further investigation of the pathogenetic significance of IL-17 in controlling proteolytic load in the airway during allergic airway inflammation.

In conclusion, our study on mice reveals a previously unrecognized

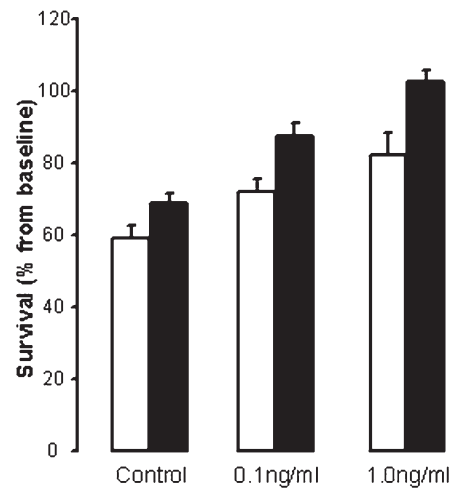


Figure 8. The effect of 20 h stimulation with recombinant IL-17 protein on the survival of BALF macrophages, recovered from PBS-exposed (open columns) or OVA-challenged (filled columns) BALB/c mice. Data are shown as mean values from three separate experiments, each performed in at least triplicate wells.

role of endogenous IL-17 in allergic airway inflammation: IL-17 contributes to the local accumulation of macrophages in bronchoalveolar space by recruiting macrophage precursor cells and by increasing the survival of macrophages within the airway. In addition, IL-17 may indirectly control the local load of potentially tissue-destructing enzymes such as MMP-9. IL-17 therefore bears the potential to play a role in tissue destruction and remodeling in the airway, which is fully compatible with recent data on IL-17 in the airway of patients with asthma (22, 23).

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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