

Interleukin-17 Drives Pulmonary Eosinophilia following Repeated Exposure to *Aspergillus fumigatus* Conidia

Benjamin J. Murdock, Nicole R. Falkowski, Andrew B. Shreiner, Amir A. Sadighi Akha, Roderick A. McDonald, Eric S. White, Galen B. Toews and Gary B. Huffnagle
Infect. Immun. 2012, 80(4):1424. DOI: 10.1128/IAI.05529-11.
Published Ahead of Print 17 January 2012.

Updated information and services can be found at:
<http://iai.asm.org/content/80/4/1424>

These include:

REFERENCES

This article cites 72 articles, 42 of which can be accessed free at: <http://iai.asm.org/content/80/4/1424#ref-list-1>

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), [more»](#)

Information about commercial reprint orders: <http://iai.asm.org/site/misc/reprints.xhtml>
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

Interleukin-17 Drives Pulmonary Eosinophilia following Repeated Exposure to *Aspergillus fumigatus* Conidia

Benjamin J. Murdock,^a Nicole R. Falkowski,^a Andrew B. Shreiner,^a Amir A. Sadighi Akha,^a Roderick A. McDonald,^a Eric S. White,^a Galen B. Toews,^a and Gary B. Huffnagle^{a,b}

Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine,^a and Department of Microbiology and Immunology,^b University of Michigan Medical School, Ann Arbor, Michigan, USA

Previous research in our laboratory has demonstrated that repeated intranasal exposure to *Aspergillus fumigatus* conidia in C57BL/6 mice results in a chronic pulmonary inflammatory response that reaches its maximal level after four challenges. The inflammatory response is characterized by eosinophilia, goblet cell metaplasia, and T helper T_{H2} cytokine production, which is accompanied by sustained interleukin-17 (IL-17) expression that persists even after the T_{H2} response has begun to resolve. T_{H17} cells could develop in mice deficient in gamma interferon (IFN- γ), IL-4, or IL-10. In the lungs of IL-17 knockout mice repeatedly challenged with *A. fumigatus* conidia, inflammation was attenuated (with the most significant decrease occurring in eosinophils), conidial clearance was enhanced, and the early transient peak of CD4⁺ CD25⁺ FoxP3⁺ cells blunted. IL-17 appeared to play only a minor role in eosinophil differentiation in the bone marrow but a central role in eosinophil extravasation from the blood into the lungs. These observations point to an expanded role for IL-17 in driving T_{H2}-type inflammation to repeated inhalation of fungal conidia.

It has been proposed that interleukin-17A (IL-17A) (IL-17) and the T helper T_{H17} response can function as a defense against extracellular pathogens, as a protective mechanism for mucosal surfaces, or as a bridge between the innate and adaptive immune systems (23, 31, 57, 61). Though its precise role is unclear, the T_{H17} response can be reciprocally regulated by the T_{H1}, T_{H2}, and regulatory arms of adaptive immunity (10, 12, 24, 26, 43, 47). While the targets of the T_{H17} response include a wide array of microbes including fungi (14, 17, 25), T_{H17} responses are implicated in a number of inflammatory diseases that were once thought to be T_{H1} mediated, including Crohn's disease, rheumatoid arthritis, and multiple sclerosis (64).

For years, IL-17 has been implicated in T_{H2} allergic reactions as well. Allergic airway diseases, such as asthma, inflammatory lung disease, and airway hypersensitivity, have all been linked to IL-17 (3, 7, 28, 33, 39, 59). Asthma is generally considered a T_{H2} response (9) and is a disease whose severity is correlated with IL-17 levels in human patients (5). Previous studies using mouse models of allergic inflammation have found that IL-17 depletion in ovalbumin (OVA)-sensitized mice attenuates the inflammatory response and that IL-17 is particularly important in driving neutrophilia (2, 18). However, multiple studies have found that IL-17 is protective against the allergic response. By altering the timing of the anti-IL-17 treatment, Schnyder-Candrian et al. were able to exacerbate eosinophil influx and mucous secretion, and by adding IL-17, they were able to attenuate cellular influx into the lungs following challenges with ovalbumin (54). Using a similar model, Hellings et al. also found that IL-17 depletion exacerbated pulmonary eosinophilia (18). These observations indicate that IL-17 plays a more complex role in T_{H2} responses than simply driving inflammation.

Aspergillus fumigatus is a ubiquitous airborne fungus that is constantly inhaled into terminal airways, and large doses of conidia are not uncommon (16, 27). We have previously shown that repeated pulmonary exposure to *A. fumigatus* conidia generates an aggressive inflammatory response consistent with a T_{H2}

allergic reaction (34). Peak inflammation following four exposures is characterized by eosinophilia, goblet cell metaplasia, and cytokine production consistent with a T_{H2} response. This inflammation is accompanied by a mixed adaptive response, with increased T_{H1}, T_{H2}, T_{H17}, and regulatory T (T_{reg}) cells all present during peak inflammation. In particular, we observed that IL-17 expression was sustained following repeated exposures even as inflammation and other immune responses began to wane. Zymosan, a β -glucan extract from fungal cell walls, induces dendritic cell expression of IL-23 (15), and many microbes, including *A. fumigatus*, are known to induce a T_{H17} response (4). Moreover, since IL-17 has frequently been implicated in allergic airway responses, we wished to elucidate the role of IL-17 in driving the pulmonary inflammatory response, particularly the role of IL-17 in driving eosinophilia in the lungs. We show here a central role for IL-17 in driving pulmonary inflammation during the allergic inflammatory response, and our data demonstrate a novel role for IL-17 in driving eosinophilia and suggest a possible role for IL-17 in the initiation of the inflammatory response.

MATERIALS AND METHODS

Mice. Wild-type (WT) (C57BL/6J) mice obtained from Jackson Laboratory (Bar Harbor, ME) were housed under pathogen-free conditions in enclosed filter-topped cages. IL-17 knockout mice were obtained from a breeding colony at the University of Michigan. The IL-17 knockout breeders were kindly provided by Yoichiro Iwakura (Tokyo University) and have been described previously (35). Clean food and water were given

Received 16 June 2011 Returned for modification 29 June 2011

Accepted 27 December 2011

Published ahead of print 17 January 2012

Editor: G. S. Deepe, Jr.

Address correspondence to Gary B. Huffnagle, ghuff@umich.edu.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.05529-11

to mice *ad libitum*. The mice were handled and maintained using microisolator techniques, with daily monitoring by a veterinarian. All studies involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan.

***Aspergillus fumigatus*.** *Aspergillus fumigatus* strain ATCC 13073 was grown on Sabouraud dextrose agar (SDA) (Difco) for 14 days. Conidia were harvested by washing the plates with sterile phosphate-buffered saline (pH 7.4) with 0.1% Tween 80 (PBS-Tween), followed by filtration of the suspension through two layers of sterile gauze to remove hyphae. The conidia were washed in PBS-Tween, counted with a hemocytometer, diluted to 10^8 spores/ml in sterile PBS-Tween, and stored at 4°C.

Intranasal challenge. To sedate the mice, the mice were injected intraperitoneally with 0.4 mg/ml xylazine (Lloyd Laboratories, Shenandoah, IA) and 10 mg/ml ketamine (Fort Dodge, Fort Dodge, IA) in sterile saline (Hospira Inc., Lake Forest, IL) based on weight. Following sedation, 20 μ l of *Aspergillus fumigatus* suspension was administered intranasally for a total of 2×10^6 conidia per mouse per challenge.

Lung histology. The lungs were fixed by inflation with 10% neutral buffered formalin (Sigma). After paraffin embedding, 5- μ m sections were cut and stained with hematoxylin and eosin (H&E) for histological analysis, periodic acid-Schiff (PAS) to detect mucus and goblet cell metaplasia, Masson's trichrome stain to detect collagen deposition, or Grocott's methenamine silver (GMS) stain to detect conidia and hyphae (McClinchey Histology Lab, Stockbridge, MI).

Lung digestion for whole-lung leukocyte enrichment. The lungs from each mouse were excised, washed in PBS, minced, and digested enzymatically for 30 min in 15 ml/lung of digestion buffer (RPMI, 5% fetal calf serum, 1 mg/ml collagenase [Boehringer Mannheim Biochemical, Chicago, IL], and 30 μ g/ml DNase [Sigma Chemical Co., St. Louis, MO]) as previously described (37). After erythrocyte lysis using NH_4Cl buffer (0.83% NH_4Cl , 0.1% KHCO_3 , 0.037% Na_2EDTA [pH 7.4]), the cells were washed, resuspended in RPMI with 5% fetal calf serum and 20% Percoll (Sigma), and centrifuged for 30 min at $2,000 \times g$ to separate leukocytes from cell debris and epithelial cells. The total number of lung leukocytes were counted in the presence of trypan blue using a hemocytometer.

***In vivo* quantification of viable conidia.** Following digestion of the lung, an aliquot was taken for analysis prior to centrifugation and erythrocyte lysis. The sample was serially diluted and plated on SDA in duplicate. Individual mycelial colonies were counted to determine the number of CFU per dilution and then multiplied back to yield the total number of viable conidia in the lungs of each mouse 24 h after inoculation.

Flow cytometry. Cells were washed and resuspended at a concentration of 10^6 cells/25 μ l FA buffer (Difco) plus 0.1% NaN_3 . Fc receptors were blocked by the addition of unlabeled anti-CD16/32 (Fc block; BD Pharmingen, San Diego, CA). After Fc receptor blocking, 0.5×10^6 to 1×10^6 cells were stained in a final volume of 50 μ l in 96-well round-bottom plates (Corning Incorporated, Corning, NY) for 30 min at 4°C. The cells were washed twice with FA buffer, resuspended in 120 μ l of 4% formalin (Sigma), and transferred to polystyrene tubes (12 by 75 mm²) (Becton Dickinson, Franklin Lakes, NJ). A minimum of 100,000 events were acquired on a FACSCanto flow cytometer (BD Pharmingen) using CellQuest software (BD Pharmingen). The data acquired were analyzed with FlowJo software (Tree Star, Stanford, CA). Fluorochrome-conjugated antibodies directed against the following antigens were obtained from the following vendors: CD45 (BioLegend, San Diego, CA), CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, CD31, CD44, CD49b, CD69, CD80, CD86, Gr1, siglec F, Fc ϵ receptor I (Fc ϵ RI), gamma interferon (IFN- γ), IL-4, IL-10, IL-17, Ly6C, chemokine (C-C motif) receptor 3 (CCR3), IL-5 receptor (IL-5R) (BD Pharmingen), and Foxp3 (eBioscience, San Diego, CA).

Differential analysis. Cells from whole-lung digestion were analyzed as follows. First, lung leukocytes were identified by CD45 expression. The following leukocyte subsets were then identified within this gate (56). Neutrophils were identified using a CD11c versus Gr1 plot as cells ex-

pressing little CD11c but large amounts of Gr1. (ii) Mature eosinophils were identified as cells expressing moderate amounts of CD11c and Gr1 and further expressing large amounts of siglec F. (iii) Macrophages and dendritic cells (DC) were identified as cells expressing high levels of CD11c and moderate amounts of Gr1, with macrophages having high levels of autofluorescence and DC having low autofluorescence. (iv) Lymphocytes were identified within the population of cells displaying low forward scatter (FSC^{low}) and low side scatter (SSC^{low}) and then subdivided into CD4, CD8, or B cells (CD19), based on cell surface staining. Basophils were identified as FSC^{low} SSC^{low} cells which were Gr1⁺ CD11c⁺ CD3⁺ CD19⁺ CD49b⁺ Fc ϵ RI⁺ cells as described previously described (38).

Intracellular flow staining. Prior to intracellular cytokine staining, the cells were stimulated *in vitro* for 6 h with phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (1 μ g/ml) in the presence of brefeldin A (BD Pharmingen) to promote the intracellular accumulation of cytokines. After stimulation, the cells were washed twice prior to surface molecule staining. Subsequently, intracellular molecules were stained using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen).

Bone marrow collection. Bone marrow was flushed from femurs and tibias using complete medium in a 10-ml syringe (BD Pharmingen) tipped with a 25-gauge needle (BD Pharmingen). The cells were drawn into a 10-ml syringe and expressed through a 21-gauge needle to disperse the cells. After erythrocyte lysis using NH_4Cl buffer, the cells were washed and resuspended in complete medium prior to analysis.

RNA isolation and cDNA generation. Following lung leukocyte enrichment or bone marrow isolation, the cells were resuspended in 2 ml of TRIzol (Invitrogen). The cells were incubated for 5 min at room temperature then combined with 600 μ l of chloroform and shaken for 1 min. Following a 3-min incubation at room temperature (RT), samples were spun for 15 min at 13,000 rpm at 4°C, and the resultant supernatants were transferred to DNase/RNase-free tubes (Corning). Equal volumes of isopropanol were added, and the samples were mixed gently prior to a 20-min incubation at RT. The samples were spun for 15 min at 13,000 rpm at 4°C, the supernatant was decanted, 1 ml of 80% ethanol was added to each sample, and the samples were spun again for 10 min at 13,000 rpm at 4°C. The supernatant was again decanted, and the pellet was allowed to air dry. Diethyl pyrocarbonate (DEPC) water (100 to 600 μ l) was added to each pellet, depending on the size of the pellet, and contains 1 μ l/ml RNasin. A primer cocktail (Promega AccessRT kit) was then added to 10 μ l of RNA samples at 0.1 μ g/ μ l (1 μ g of total RNA per reaction) and run on a thermocycler (Applied Biosystems) per the manufacturer's instructions.

Quantification of mRNA levels using multiplex quantitative PCR (qPCR) arrays. RNA was isolated from each tissue using the TRIzol method (Ambion). Following the ethanol precipitation step in the TRIzol isolation protocol, RNA was cleaned up using the RNeasy minikit (Qiagen). A Nanodrop instrument (Thermo Scientific) was used to determine the concentration of each RNA isolate. cDNA was generated from each RNA sample using the RT2 First Strand kit (Qiagen). Expression levels of genes under study were determined using Mouse RT2Profiler PCR Arrays, custom-made to contain replicate sets of 48 primer pairs (Qiagen). Each well of the replicate sets was loaded with 2.5 ng of cDNA reaction product. Each card was run on a LightCycler 480 real-time PCR system (Roche). The relative RNA expression levels were inferred from the threshold cycle (C_T) values. β -Actin levels were used as the control.

qPCR. cDNA was mixed with SYBR (Applied Biosystems) and GATA-1 primers (Integrated DNA Technologies) per the manufacturer's instructions. The GATA-1 primers were FWD (FWD stands for forward) (5'-CCTGCCATTGGCCCTTGT-3') and REV (REV stands for reverse) (5'-CCTGTCCTGTCCTCCGCCA-3'). The samples were run on a 7300 real-time system (Applied Biosystems) and analyzed using 7300 system SDS software (Applied Biosystems). Quantitation was by the $2^{-\Delta\Delta C_T}$ method.

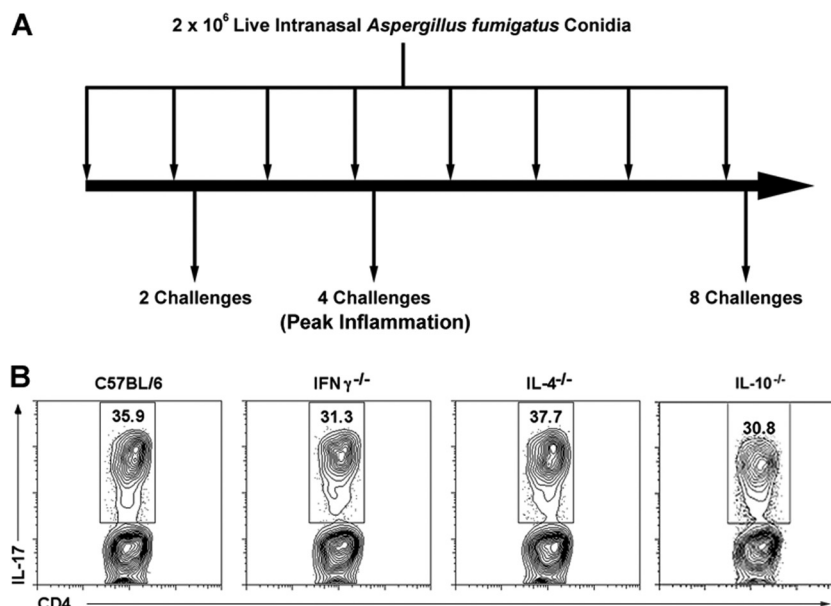


FIG 1 *Aspergillus fumigatus* exposure and IL-17 production in the absence of IFN- γ , IL-4, or IL-10. (A) C57BL/6 mice were challenged intranasally with 2×10^6 *A. fumigatus* conidia once a week for up to eight challenges. Cellular infiltrates and responses were analyzed 24 h after the second, fourth, and eighth challenges. (B) Leukocytes were collected from the airways of mice using BAL during peak inflammation (four challenges). Intracellular CD4 T cell IL-17 cytokine production was examined using flow cytometry in IFN- γ ^{-/-}, IL-4^{-/-}, and IL-10^{-/-} mice on a C57BL/6 background. Flow gates indicate the percentage of CD4 T cells producing IL-17 and are a representative flow plot (2 experiments, 3 mice per group).

Blood collection and serum separation. Blood samples were collected by retro-orbital vein bleed at the time of harvest. Serum samples were collected after centrifugation for 2 min at 6,000 rpm in Microtainer tubes (BD Pharmingen). The remaining blood was collected in K2E tubes (BD Pharmingen), the volume of blood was measured, and the tubes were centrifuged for 10 min at 10,000 rpm. After erythrocyte lysis using NH₄Cl buffer, the cells were washed, resuspended in complete medium, and counted with a hemocytometer prior to analysis.

Statistical analyses. At least four separate experiments (Fig. 2, 4, and 8) or two separate experiments (Fig. 5, 7, and 11) with three mice per group per experiment were performed. The quantitative data shown in each graph are from the cumulative analysis across multiple experiments (i.e., the graphs do not show data from a single representative data set). All values are reported as mean $\pm$ standard error of the mean. Differences between wild-type and IL-17^{-/-} mice at each time point were evaluated using a Student's *t* test with Bonferroni's correction for multiple comparisons; a *P* value of <0.05 was considered statistically significant.

RESULTS

Roles of IFN- γ , IL-4, and IL-10 in the development of T_H17 cells.

We have previously reported that repeated exposure to *A. fumigatus* conidia results in inflammation that is accompanied by a mixed adaptive response. Briefly, C57BL/6 mice are challenged weekly with 2×10^6 live *A. fumigatus* conidia, and lung leukocytes are harvested 24 h after the second, fourth, and eighth challenges with peak inflammation occurring after four challenges (Fig. 1A). Increased T_H1, T_H2, T_H17, and T_{reg} cells are present during peak inflammation (34). We have also found that IL-10 knockout mice have a defective T_H2 response to challenge with *A. fumigatus* conidia (56). Since IFN- γ can also regulate the development of T_H17 cells (11), we examined whether IL-10, IFN- γ , or IL-4 play a role in regulating the development of T_H17 cells in the lungs of mice exposed to *A. fumigatus* conidia, using cytokine-specific knockout mice. T_H17 cells developed in the lungs after ex-

posures to conidia, even in the absence of IFN- γ , IL-4, or IL-10 (Fig. 1B). Thus, development of T_H17 cells in the lungs after exposure to *A. fumigatus* conidia occurs via a pathway independent of IL-10, IFN- γ , and IL-4.

Role of IL-17 in clearance of conidia from the lungs. To analyze the clearance of conidia in the presence and absence of IL-17, the lungs from *A. fumigatus*-challenged mice were enzymatically dispersed, and aliquots were serially diluted on SDA plates to determine the number of viable conidia remaining in the lungs 24 h after the final challenge. Mice capable of producing IL-17 have relatively consistent numbers of viable conidia in the lung, with no significant difference in the conidial load 24 h after challenge following two, four, or eight challenges. Thus, repeated *A. fumigatus* conidial challenges do not improve clearance, even after eight consecutive weeks of exposure and the engagement of the adaptive immune system. In contrast, there is a significant reduction in the conidial load following four challenges in the absence of IL-17 (Fig. 2). Following eight challenges, there is a reduction in fungal load though the difference does not reach significance. Therefore, production of IL-17 appears to impair conidial clearance following repeated *A. fumigatus* challenges.

Pulmonary inflammation following repeated pulmonary exposure to *Aspergillus fumigatus* conidia. We wished to elucidate the role of IL-17 in driving the expansion of specific leukocyte populations following consecutive intranasal challenges with *A. fumigatus* conidia. We began by examining cellular infiltrate and goblet cell metaplasia in mice challenged intranasally with 2×10^6 viable resting *A. fumigatus* conidia at weekly intervals in the presence or absence of IL-17. Histological sections taken from mice not challenged with *A. fumigatus* conidia or in mice challenged two, four, and eight times with *A. fumigatus* conidia were stained with H&E and periodic acid-Schiff (PAS). In the presence of IL-

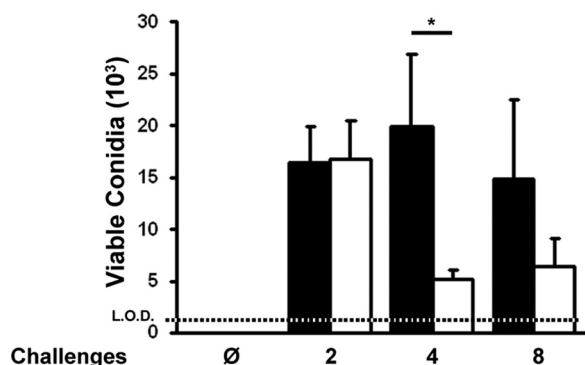
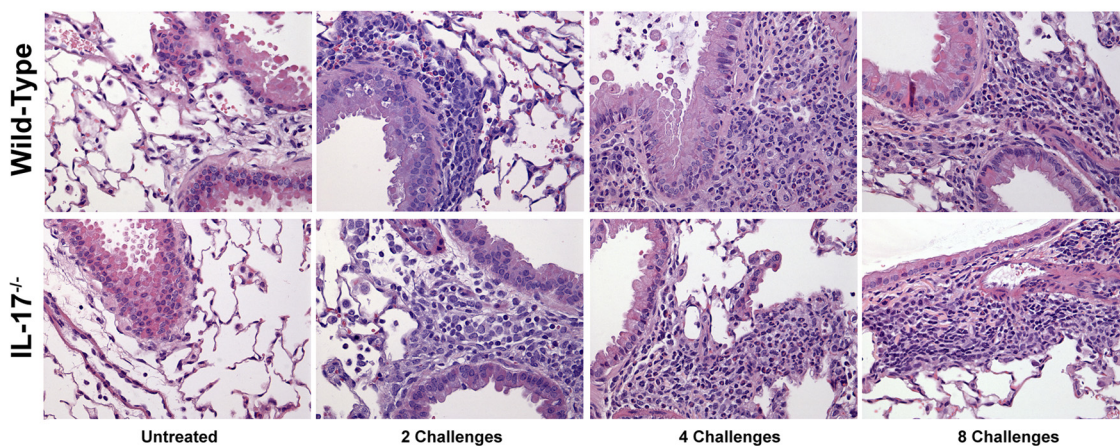


FIG 2 Fungal clearance of conidia following intranasal challenges. Twenty-four hours after the final challenge, lungs were digested, an aliquot of the lung digestion product was serially diluted and plated on SDA medium, and mycelial colonies were counted. The average viable conidia per lung detected 24 h after zero, two, four, and eight challenges in WT mice (black bars) and IL-17^{-/-} mice (white bars) are shown. Values are means plus standard errors of the means (SEM) (error bars) (3 mice per group per time point) from 4 separate experiments. The limit of detection (L.O.D.) is indicated by the broken line. Values that are statistically significantly different ($P < 0.05$) are indicated by a short horizontal black bar and asterisk above the black and white bars.

17, mice challenged four times with conidia displayed the characteristic cellular infiltrate previously shown during peak inflammation including accumulation of eosinophils and neutrophils around the airways (34). Conversely, a lack of IL-17 attenuates cellular infiltration into the lungs following four challenges (Fig. 3A). Cellular infiltrate could still be observed around the airways, but the intensity of cellular infiltrate was greatly reduced in mice lacking IL-17. Differences in cellular infiltrate following two and eight challenges were not observed, however. PAS was then used to observe goblet cell metaplasia within the airways. Goblet cell metaplasia, a hallmark of T_H2 responses, is seen in mice challenged repeatedly with *A. fumigatus* conidia. Interestingly, PAS staining revealed that goblet cell metaplasia could still be detected in the airways of mice lacking IL-17 (Fig. 3B) despite reduced cellular infiltrate. IL-17 therefore appears to play a role in driving inflammation following repeated conidial challenges but is dispensable with regard to goblet cell metaplasia.

We next analyzed baseline cellular populations within the leukocyte infiltrate and after two, four, and eight conidial challenges. We have previously observed that mice repeatedly challenged with *A. fumigatus* conidia demonstrate a significant increase in the

A H&E



B PAS

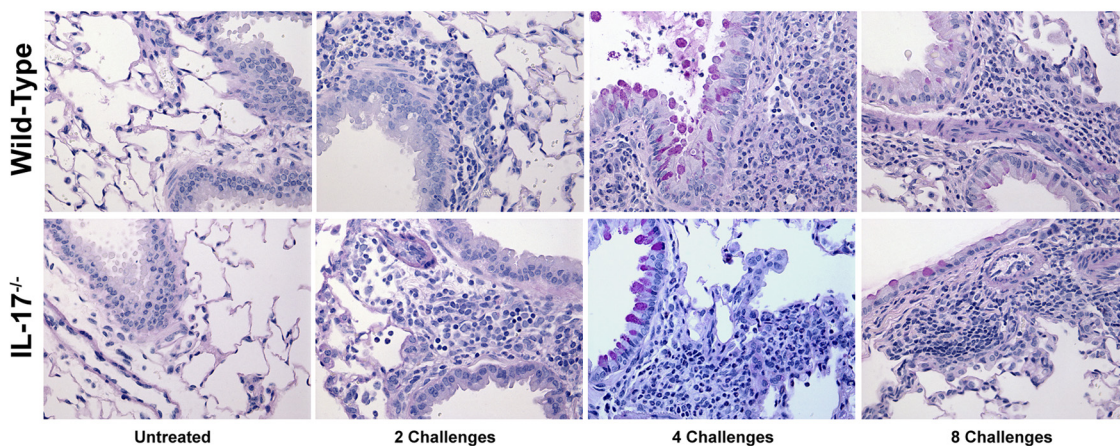


FIG 3 Cellular infiltrate around the airways and goblet cell metaplasia following repeated intranasal exposure to *Aspergillus fumigatus* conidia in the presence and absence of IL-17 production. Lungs from WT and IL-17^{-/-} mice challenged zero, two, four, or eight times were fixed in formalin and embedded in paraffin blocks. Histological slices were then stained with H&E (A) or PAS (B) and examined at a magnification of $\times 400$.

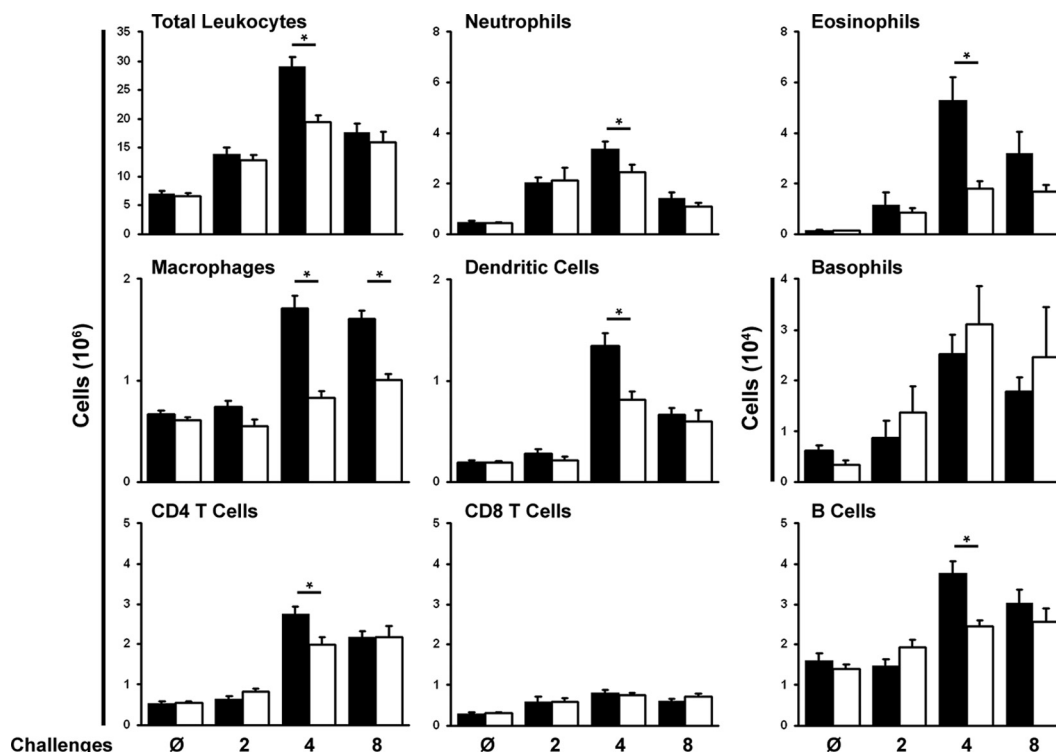


FIG 4 Pulmonary cellular infiltrate in response to repeated conidial challenge. WT mice (white bars) and IL-17^{-/-} mice (black bars) were challenged intranasally zero, two, four, and eight times with *A. fumigatus* conidia. Following enzymatic dispersion of the lungs, individual cell populations were identified using flow cytometry as detailed in Materials and Methods. Values shown are the means plus SEM (3 mice per group per time point) from 4 to 8 separate experiments. Values that are statistically significantly different ($P < 0.05$) are indicated by a short horizontal black bar and asterisk.

number of total leukocytes in the lung between two and four challenges (34), and in mice capable of producing IL-17, we once again observed heightened inflammation following four challenges. Consistent with histological examination, there was a significant reduction in the total leukocyte number in the absence of IL-17 following four challenges (Fig. 4), but there was no difference in the total leukocyte number in the basal condition and following two or eight challenges. Thus, IL-17 is necessary for the induction of peak inflammation following four challenges but unnecessary for general inflammation in response to *A. fumigatus* conidia.

We next identified and quantified individual populations of leukocytes using flow cytometry. With IL-17 present, most cell populations are elevated following four conidial challenges and then decrease by the eighth challenge. In mice lacking IL-17 production, however, mice challenged four times had reduced numbers of neutrophils, eosinophils, macrophages, dendritic cells, CD4⁺ T cells, and B cells (Fig. 4). Lack of IL-17 produced no differences after two challenges, and the only population that showed a significant difference after eight challenges was the macrophage populations which remained high over the course of exposure in wild-type mice but not in the absence of IL-17. We also examined the number of basophils present, as basophils have recently been shown to play a central role in driving the T_H2 response (58). Basophil levels were nearly identical in the presence and absence of IL-17 following two, four, and eight challenges. Therefore, the data indicate that IL-17 plays a central role in driving the inflammatory response toward *A. fumigatus* conidia, and it does not significantly alter the recruitment of basophils despite the

attenuation of eosinophilia and reduced accumulation of other leukocytes following four conidial challenges.

Cytokine expression, CD25⁺ Foxp3⁺ expression, and CD4⁺ T cell activation in mice following repeated *A. fumigatus* challenge in the presence and absence of IL-17. Our next objective was to determine whether a lack of IL-17 production altered the immune response via a change in CD4⁺ T cell function. Hence, CD4⁺ T cells isolated from the lung were examined by intracellular flow cytometry for the baseline expression of IFN- γ , IL-4, IL-10, and IL-17 and expression after two, four, and eight conidial challenges. In mice with sufficient IL-17 (WT mice), repeated intranasal conidial challenges resulted in elevated CD4⁺ T cell expression of IL-10 following two challenges, IL-4 following four challenges, and IL-17 over the course of exposure (Fig. 5A). A lack of IL-17 production yielded very little difference in CD4⁺ T cell cytokine expression following four and eight challenges, and the percentage of CD4⁺ T cells expressing IFN- γ or IL-4 showed no significant differences at any time point. An unexpected observation, however, was the reduced number of IL-10-expressing CD4⁺ T cells following two challenges in the absence of IL-17. These data suggest that while IL-17 production may alter the early immune response to inhaled conidia, it does not significantly affect the development of T_H1 and T_H2 cells.

Consistent with the increased number of IL-10-expressing CD4⁺ T cells, there was a significant increase in the total percentage of CD4⁺ T cells displaying a regulatory phenotype (CD25⁺ Foxp3⁺). This increase was noticeably absent when IL-17 was not present (Fig. 5B). We next examined the CD44 and CD69 surface

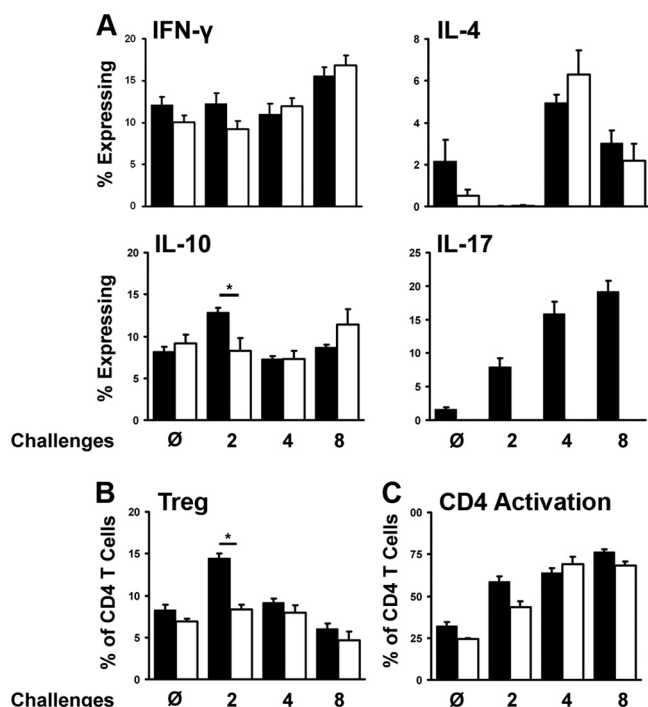


FIG 5 CD4 T cell response to repeated conidial challenge in the presence and absence of IL-17. Using flow cytometry, CD4 T cells were examined for cytokine production (A), regulatory phenotype (CD25⁺ Foxp3⁺) (B), or activation (CD44^{high} CD69⁺) (C). The percentage of CD4 cells expressing the cytokine or phenotype of interest in WT mice (black bars) and IL-17^{-/-} mice (white bars) is shown. Values are means plus SEM (3 mice per group per time point) from 2 or 3 experiments. Values that are statistically significantly different ($P < 0.05$) are indicated by a short horizontal black bar and asterisk.

expression of CD4 T cells to determine whether IL-17 plays a role in T cell activation. CD4⁺ T cells that were CD44^{high} and CD69⁺ were counted as activated. In the presence of IL-17, there is an increase in CD4⁺ activation following conidial challenge that is maintained over the course of exposure. A similar pattern is seen in the absence of IL-17, and there is no significant difference in CD4⁺ T cell activation following two, four, and eight challenges whether or not IL-17 is present (Fig. 5C). Thus, while IL-17 production plays a role in initiating the IL-10⁺ CD4⁺ and CD25⁺ Foxp3⁺ CD4⁺ T cell responses following two challenges, it has no role in skewing these responses or T cell activation at later time points.

Role of IL-17 in pulmonary arterial remodeling. We have recently demonstrated that long-term repeated exposure to *A. fumigatus* conidia results in the development of progressive remodeling of small-to-medium-sized pulmonary arteries with concomitant luminal narrowing (56). The process was partially mediated by CD4⁺ T cells and by IL-4 production, did not require eosinophils, and was independent of IFN- γ . We evaluated the development of pulmonary arterial remodeling in IL-17 knockout mice after eight conidial exposures. Similar to wild-type mice, IL-17 knockout mice developed pulmonary arterial remodeling with luminal narrowing after repeated exposure, indicating that the Th17 response and the eosinophil component mediated by the IL-17 response are not required in this process (Fig. 6).

Bone marrow production of eosinophils in response to IL-17. There are several other possible explanations for the difference

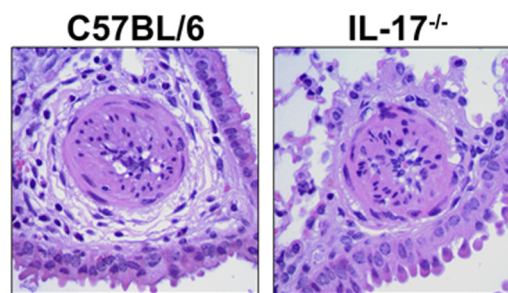


FIG 6 Pulmonary arterial remodeling in response to repeated challenge by *A. fumigatus* conidia. Lungs from WT and IL-17^{-/-} mice challenged eight times were fixed in formalin and embedded in paraffin blocks. Histological slices were then stained with H&E and examined at $\times 1,000$ magnification.

in pulmonary eosinophil levels seen during the inflammation response in the absence of IL-17. Hellings et al. demonstrated that IL-17 depletion can increase eosinophil production in bone marrow (18). Since IL-17 has an effect on bone marrow eosinophil production, we wished to determine whether the absence of IL-17 was altering eosinophil differentiation during repeated exposure to *A. fumigatus* conidia. Thus, we isolated bone marrow cells from mice that were either left untreated or were challenged four times, and then we analyzed the cell surface expression of Ly6C and CD31—two markers that can be used to observe the expansion precursor cell populations in bone marrow (36, 65). Cells in the resulting flow plot fall within six distinct gates (Fig. 5A), with granulocytes, including eosinophils, found within gate V. We found that cellular distribution of precursor cells is slightly different in untreated mice with and without IL-17, but during peak inflammation (four challenges), the cellular populations are indistinguishable regardless of IL-17 production (Fig. 7A). Quantitatively, we found that in the presence and absence of IL-17, there was no significant difference in the percentage of cells within gate V during peak inflammation (Fig. 7B). To further determine eosinophil differentiation in bone marrow, we analyzed the mRNA expression of GATA-1, a transcription factor previously linked with eosinophil differentiation (32, 69). Interestingly, mRNA expression of GATA-1 was unchanged in WT mice during the inflammatory response, indicating that repeated exposure to conidia is not driving excess production of eosinophils in relation to other cell types. However, mice with no IL-17 production showed a significant decrease in GATA-1 mRNA production relative to wild-type mice following four challenges (Fig. 7C). Coupled with the flow cytometry data on cellular differentiation, GATA-1 mRNA expression indicates that IL-17 is likely playing a minor role in eosinophil production in bone marrow.

Role of IL-17 in eosinophil maturation following repeated intranasal *A. fumigatus* challenges. We next wished to determine whether IL-17 drives eosinophil maturation at the site of inflammation. Since eosinophils in the lungs were detected by their siglec F expression, a maturation marker on eosinophils, we wished to see whether differences in eosinophil maturation were responsible for the differences in total eosinophil numbers seen in the lung in the presence and absence of IL-17. To test this, we first used flow cytometry to isolate eosinophils from the total leukocyte population. CD45⁺ cells were first separated from the pool of total lung cells, and then eosinophils were further isolated from the leukocyte population by gating for FSC^{low} cells with a SSC^{mid-high} profile

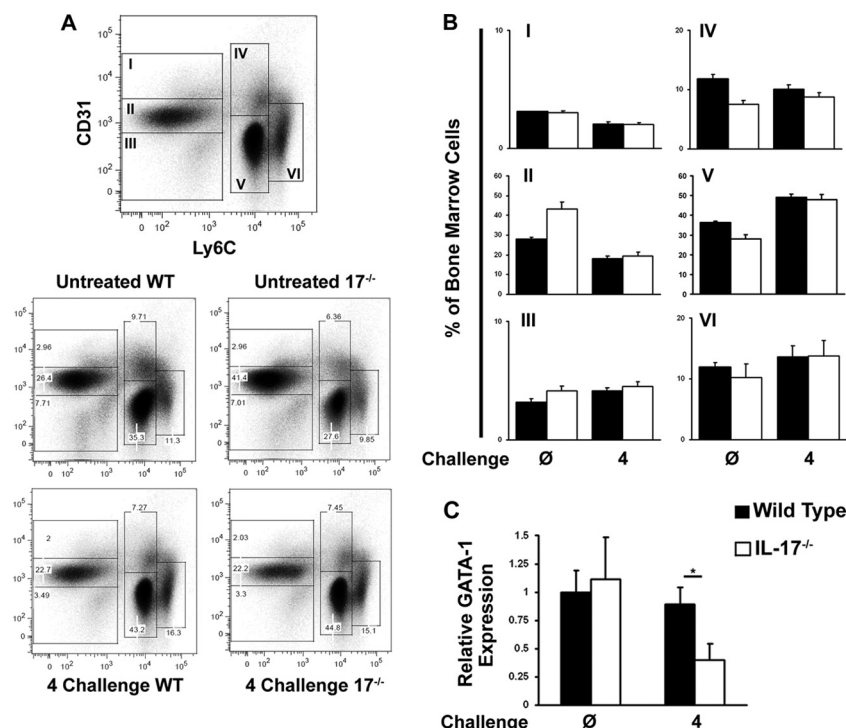


FIG 7 Role of IL-17 in eosinophil bone marrow differentiation during peak inflammation. (A) Bone marrow samples from WT and IL-17^{-/-} mice were examined using flow cytometry. CD45⁺ cells were segregated into six distinct groups (groups I to VI) based on their expression of Ly6C and CD31. Group V is composed of granulocytes. (B) Percentage of cells within each gate in mice not challenged with *A. fumigatus* conidia (untreated mice) and mice challenged four times with *A. fumigatus* conidia. Values for WT mice (black bars) and IL-17^{-/-} mice (white bars) are shown. (C) mRNA was isolated from bone marrow cells from mice challenged zero or four times with *A. fumigatus* conidia. Following conversion to cDNA, qPCR was used to measure relative expression of GATA-1. Values are means plus SEM (3 mice per group per time point) from 2 to 4 experiments. Values that are statistically significantly different ($P < 0.05$) are indicated by a short horizontal black bar and asterisk.

(Fig. 8A). The resulting population was examined for expression of CCR3, a receptor necessary for eosinophil trafficking to the lungs during allergic responses (20, 45), and the IL-5 receptor (IL-5R) (53). The eosinophil population was then analyzed by looking at expression of IL-5R. We found that eosinophils in the lung could be subdivided into three distinct subgroups based on their IL-5R expression; these groups were named IL-5R^{high}, IL-5R^{mid}, and IL-5R^{low} groups (Fig. 8A). Consistent with previous studies, expression of siglec F (sigF) in each group showed that cells with high IL-5R expression had almost no sigF expression (Fig. 9), while those that were IL-5R^{low} almost universally expressed sigF, indicating that IL-5R^{low} eosinophils represent the activated population (29). Using this staining protocol, we could therefore differentiate between eosinophils in various stages of maturation.

We then compared cellular distribution within these three gates during the inflammatory response in the presence and absence of IL-17 production. For a positive control, we performed identical staining on cells isolated from wild-type C57BL/6 mice infected with *Cryptococcus neoformans*, which results in a strong T_H2 response and extreme eosinophilia (19). Untreated mice expressed similar amounts of surface IL-5R regardless of IL-17 production, and similarly, there were no differences seen following four *A. fumigatus* challenges whether IL-17 was present or absent (Fig. 8B). Significant differences could be seen between untreated mice and those that had been challenged four times with *A. fumigatus* conidia. In agreement with previous data suggesting that

low IL-5R expression is associated with maturation (29), mice that were untreated had the highest percentage of IL-5R^{high} cells, while those infected with *C. neoformans* had nearly 90% of their eosinophils in the IL-5R^{low} gate.

Quantitatively, mice challenged four times with *A. fumigatus* conidia had a significantly higher percentage of eosinophils within the IL-5R^{low} gate than untreated mice did, but the absence of IL-17 had no effect on eosinophil maturation at any time point (Fig. 8C). Within each of the three IL-5R gates, there was no difference in surface expression of sigF whether the mice were untreated, challenged four times, or infected with *C. neoformans*: eosinophils that expressed high, moderate, or low levels of IL-5R had nearly identical expression patterns of sigF regardless of the number of *A. fumigatus* exposures or exposure to *C. neoformans* (Fig. 9). Similarly, IL-17 had no effect on the expression pattern of sigF within the IL-5R gates. Taken together, these data indicate that IL-17 does not play a direct role in driving eosinophil maturation following intranasal *A. fumigatus* challenge and that siglec F expression is dependent on the maturation of eosinophils regardless of antigen challenge or IL-17 expression.

Cellular composition of blood leukocytes during peak inflammation. One final possibility for the role of IL-17 in pulmonary eosinophilia is leukocyte trafficking from the blood to the lungs. CD11b is more highly expressed on macrophage and dendritic cells that have recently arrived in the lungs (41), and we observed that the percentage of macrophages expressing CD11b following four conidial challenges was higher in the presence of

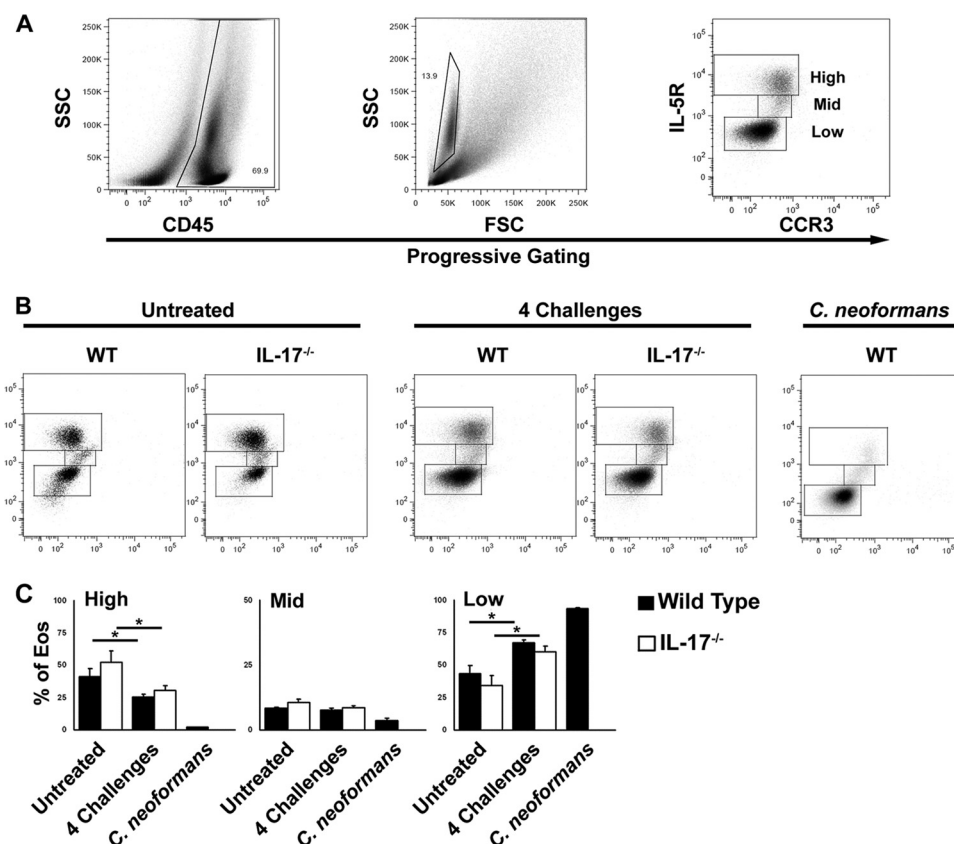


FIG 8 Pulmonary eosinophil maturation during inflammation in the presence and absence of IL-17. (A) Mature and immature eosinophils were examined by focusing on FSC^{low} SSC^{high} cells (FSC stands for forward scatter and SSC stands for side scatter) within the CD45⁺ leukocyte population isolated from the lung. IL-5 receptor and CCR3 expression was subsequently examined. Eosinophils fell within three distinct gates: IL-5R^{high}, IL-5R^{mid}, and IL-5R^{low}. (B) IL-5R expression patterns are shown for WT and IL-17^{-/-} mice that were untreated or challenged four times with *A. fumigatus* conidia. As a positive control, eosinophils from WT mice infected for 2 weeks with *Cryptococcus neoformans* were also studied. (C) The percentage of eosinophils falling within each gate is shown for WT mice and IL-17^{-/-} mice that were untreated, challenged four times, or infected with *C. neoformans* (WT only). Values are means plus SEM (3 mice per group per time point) from 2 to 8 experiments. Values that are statistically significantly different ($P < 0.05$) are indicated by a short horizontal black bar and asterisk.

IL-17 production (Fig. 10). Moreover, siglec F, which is highly expressed on alveolar but not exudate macrophages (60), is more highly expressed in the absence of IL-17. This suggests that IL-17 plays a role in recruiting new cells to the lung during peak inflammation.

Therefore, to see whether altered trafficking to the lung was responsible for the relatively decreased levels of eosinophils observed following four conidial challenges in the absence of IL-17, we analyzed the total number of leukocytes and cellular composition of whole blood of mice challenged four times. Leukocytes were analyzed using CD45 staining, and CD11b⁺ cells were broken down into two groups: eosinophils (SSC^{high}) and all other myeloid cells (FSC^{low} SSC^{low}) (neutrophils and monocytes) (Fig. 11A). In uninfected mice, there was no significant difference seen between the concentration of total leukocytes in blood in the presence or absence of IL-17. Likewise, the concentrations of eosinophils and monocytes/neutrophils were not significantly different. However, there was a striking and significant difference between the leukocyte concentrations in WT mice that had been challenged four times and those that lacked IL-17 production (Fig. 11B). Moreover, there were significantly higher concentrations of eosinophils and monocytes/neutrophils in blood in the absence of

IL-17. The data therefore show that in the absence of IL-17, there are fewer new cells arriving in the lung during peak inflammation, and at the same time, there is an accumulation of myeloid cells, including eosinophils, in the blood of IL-17^{-/-} mice following four conidial challenges.

Cytokine expression in the lungs of mice during peak inflammation. We measured the expression of IL-17E (IL-25) and IL-17F in untreated mice and mice challenged four times with *A. fumigatus*. Consistent with our other observations, there was a significant increase in the expression of IL-17E, a T_H2 cytokine (63), during peak inflammation in WT mice (Fig. 12). However, there was no significant difference in IL-17E expression in IL-17^{-/-} mice not challenged with *A. fumigatus* or mice challenged four times with *A. fumigatus*. A major difference in IL-17E production was seen during peak inflammation in WT and IL-17^{-/-} mice, but this difference did not reach statistical significance ($P = 0.063$). Surprisingly, the greatest difference in IL-17F expression was seen between the baseline (untreated) values for WT and IL-17^{-/-} mice. While IL-17F expression was significantly higher in IL-17^{-/-} mice than in WT mice during peak inflammation, there was a significant decrease in IL-17F expression in IL-17^{-/-} mice following conidial exposure. We did not see significant differences

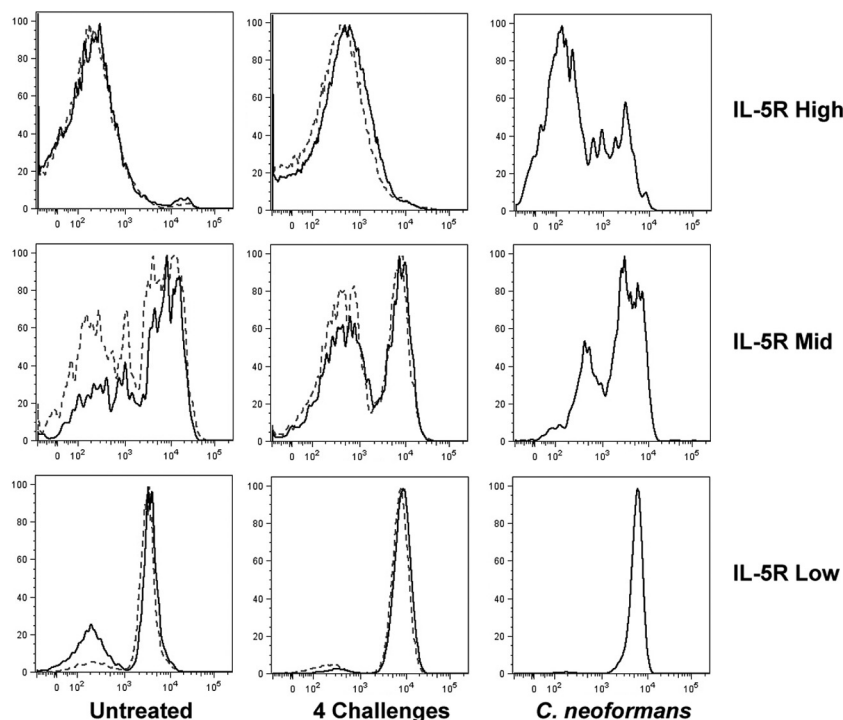


FIG 9 Siglec F expression within IL-5R^{high}, IL-5R^{mid}, and IL-5R^{low} eosinophil gates during exposure and the role of IL-17. Histograms showing eosinophil siglec F expression within each of the three IL-5R gates were generated for mice that were untreated, challenged four times, or infected with *C. neoformans* (WT only). Values for WT mice (solid black lines) and IL-17^{-/-} mice (dashed lines) are shown.

in the levels of expression of IL-4, IL-5, IL-13, CCL2, CCL3, CCL4, CCL5, or granulocyte-macrophage colony-stimulating factor (GM-CSF) in the whole lungs of WT and IL-17^{-/-} mice after four challenges (data not shown).

DISCUSSION

The current study was designed to determine the role of IL-17 in a developing T_H2 inflammatory response following repeated pulmonary exposure to *Aspergillus fumigatus* conidia. We have previously demonstrated that repeated conidial challenges generate a robust T_H2 response accompanied by increasing IL-17 expression, suggesting a role for IL-17 in the development of a T_H2 response. We therefore wished to determine

whether manipulations of IL-17 production would alter fungal clearance, cellular infiltrate, mucous secretion, or CD4 T cell cytokine production at the site of inflammation. To do this, we initially examined cellular levels in the lungs using histology and flow cytometry following repeated intranasal exposure to *A. fumigatus* conidia in the presence and absence of IL-17. We found that a lack of IL-17 correlated with reduced overall cellular levels in the lungs and enhanced clearance of conidia following four challenges with *A. fumigatus* conidia: during the T_H2 inflammatory response, the numbers of CD4 T cells, B cells, neutrophils, macrophages, and dendritic cells were reduced during peak inflammation (four challenges) in the ab-

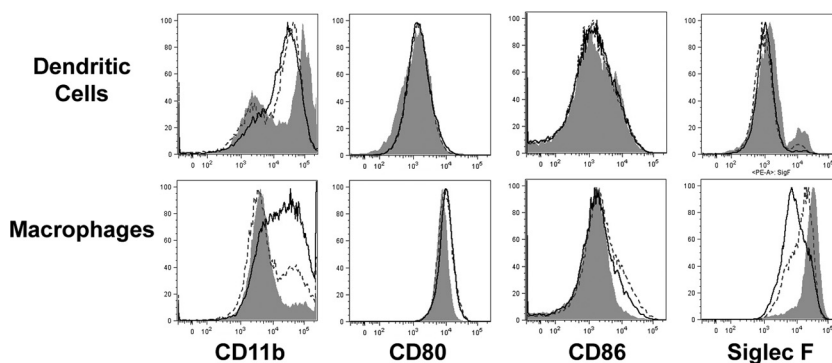


FIG 10 Surface expression of pulmonary dendritic cells and macrophages during peak inflammation in the presence and absence of IL-17. Histograms were generated showing surface expression of CD11b, CD80, CD86, and siglec F. Values for untreated mice (gray background peaks), WT mice challenged four times with conidia (solid black lines), and IL-17^{-/-} mice challenged four times with conidia (dashed lines) are shown. Histograms were generated by pooling data from three separate mice from each group.

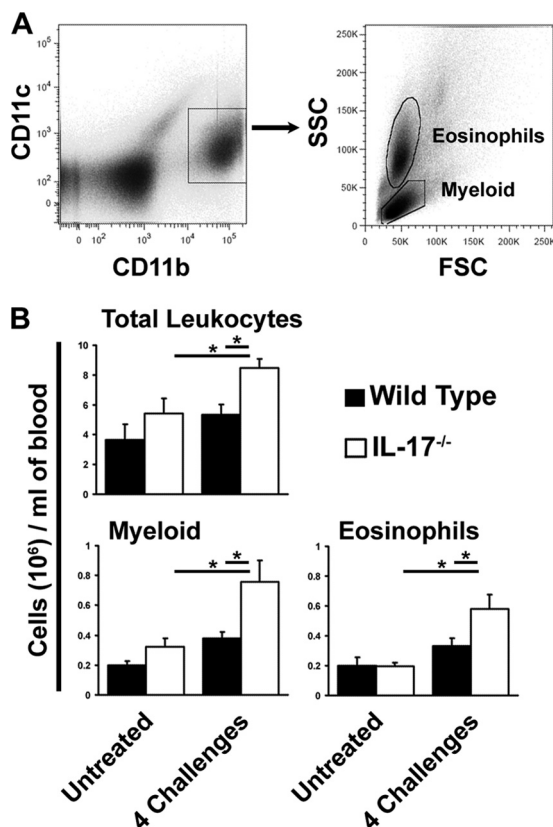


FIG 11 Eosinophil levels in blood during peak inflammation. Blood from WT and IL-17^{-/-} mice was collected from untreated animals and those challenged four times with conidia. (A) Using flow cytometry, trafficking myeloid cells were segregated from the general CD45⁺ leukocyte population based on CD11b expression. CD11b⁺ leukocytes were then further separated into eosinophils and small myeloid cells based on their forward- and side-scatter profiles. (B) The concentrations of total leukocytes, CD11b⁺ noneosinophil myeloid cells, and eosinophils in blood in untreated mice and mice challenged four times are shown. Values are means plus SEM (3 mice per group per time point) from 2 to 4 experiments. Values that are statistically significantly different ($P < 0.05$) are indicated by a horizontal black bar and asterisk.

sence of IL-17. These observations are consistent with those of the study by Zelante et al., in which the absence of IL-17 production paradoxically resulted in enhanced *A. fumigatus* clearance despite reduced cellular inflammation (71). Subsequent

studies demonstrated that this was due to enhanced indoleamine 2,3-dioxygenase expression (51, 52, 70, 71). Similarly, we have been able to demonstrate that IL-17 participates in the acute allergic inflammatory response (following four challenges) but is not required for chronic inflammation (eight challenges) or conidial clearance.

Additionally, lack of IL-17 resulted in attenuation of eosinophilia in the lungs during peak inflammation. These data demonstrate a central role for IL-17 in driving eosinophilia in T_H2-mediated airway inflammation. Eosinophil levels are reduced in IL-17 receptor (IL-17R) knockout mice (54), but this is likely due to the loss of IL-17RA receptor chain, which is also used in IL-25 signaling (49). IL-25 plays a central role in amplifying the T_H2 response (2, 42, 62, 63), and knockout of the IL-25 adaptor protein CIKS attenuates allergic airway inflammation (8). Thus, abrogation of eosinophilia in these models is most likely due to reduced T_H2 cytokine signaling. Other groups have demonstrated a more direct role for IL-17 in exacerbating eosinophilia. Eosinophil levels were decreased in traditional OVA models of allergic inflammation using IL-17 depleting antibody (44), IL-23 depleting antibody (67), or increased using adoptive transfer of T_H17 CD4 T cells (67). Similarly, eosinophilia induced by OVA in bronchoalveolar lavage (BAL) fluid and low levels of *A. fumigatus* antigen were attenuated in IL-17 knockout mice (68). Our observations are consistent with these previous studies in identifying a role for IL-17 in allergic pulmonary eosinophilia.

There are several possible reasons for the diminished numbers of eosinophils observed in the lungs of IL-17-deficient mice. For instance, IL-17 could play a role in eosinophil development in the bone marrow, could drive eosinophil trafficking to the lungs, could enhance eosinophil maturation in the lungs, or could alter T_H2 cytokine production at the site of infection. Therefore, to determine the reason for the attenuated eosinophilia found in the absence of IL-17, we examined eosinophils in bone marrow, blood, and lungs. We examined eosinophil maturation in the lungs using IL-5R expression as assessed by flow cytometry. Eosinophils in uninfected mice express relatively high levels of IL-5R; during peak inflammation (four challenges) and *Cryptococcus* infection, eosinophils mature and express lower levels of IL-5R. This observation is consistent with those of Liu et al. who found that as bronchoalveolar eosinophils mature, they shed their IL-5R and become unresponsive to further IL-5 stimulation (29). However,

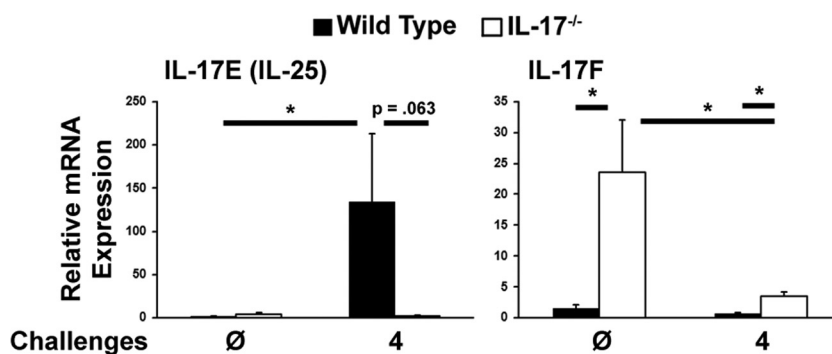


FIG 12 IL-17E (IL-25) and IL-17F expression in the absence of IL-17. WT and IL-17^{-/-} mice were not challenged (zero) or were challenged intranasally four times with *A. fumigatus* conidia. Lung leukocytes were collected following enzymatic digestion and resuspended in TRIzol. Following conversion to cDNA, qPCR arrays were used to measure relative expression of IL-17E and IL-17F. Values are means plus SEM (3 mice per group per time point) from 2 experiments. Values that are statistically significantly different ($P < 0.05$) are indicated by a horizontal black bar and asterisk.

the reduced IL-5R expression seen in WT mice during inflammation is also observed in the absence of IL-17, and there is no significant difference between the two mouse strains in terms of maturation in the presence or absence of *A. fumigatus* challenge. Surface expression of siglec F was similarly unaffected. We therefore conclude that IL-17 does not play a role in driving eosinophil maturation in the lungs during inflammation.

In bone marrow, initial analysis of differentiation using flow cytometry showed that bone marrow cells in the presence and absence of IL-17 had identical expression patterns of CD31 and Ly6C following four *A. fumigatus* challenges. However, the bone marrow granulocyte population of both WT and IL-17^{-/-} mice expanded in response to exposure to *A. fumigatus* conidia, indicating that the lack of IL-17 was not significantly inhibiting the differentiation of granulocytes during repeated fungal exposure. However, when using qPCR to detect eosinophil differentiation via GATA-1 expression (32, 69), we observed that relative GATA-1 mRNA expression was reduced during peak inflammation in the absence of IL-17. This indicates that IL-17 plays a minor but significant role in the differentiation of eosinophils in bone marrow.

Along with the minor role in bone marrow differentiation, we found that IL-17 plays an important role in eosinophil trafficking from the blood to the lungs during inflammation. Blood samples from WT mice and from mice incapable of producing IL-17 were analyzed for the presence of leukocytes during peak inflammation. In the absence of IL-17, there was a significant increase in the number of total leukocytes, eosinophils, and noneosinophil myeloid cells in blood following four conidial challenges. These data are consistent with previous studies that have indirectly implicated IL-17 in eosinophil trafficking (46, 72), and at least one report has shown that IL-17 is capable of inducing eotaxin-1 expression in human airway smooth muscle cells *in vitro* (48). However, numerous studies have shown that using IL-17 depletion or IL-17^{-/-} mice with traditional OVA models does not result in attenuation of eosinophilia (39).

How then can we explain the lack of eosinophilia seen here compared with previous studies? Differences in sensitization protocols have been shown to drastically alter eosinophilia in other investigations (20, 30). Most of these studies of allergic airway responses have primarily utilized OVA sensitization and subsequent OVA challenge to yield a robust T_H2 response accompanied by neutrophilia and eosinophilia. Conversely, our method of fungal challenge uses viable *A. fumigatus* conidia to simulate conditions that are more analogous to those that could be experienced on a daily basis. This yields a highly reproducible T_H2 response that is nevertheless mild in comparison with traditional OVA-based models and also utilizes other branches of the adaptive immune response, including T_H17 and T_{reg} responses and, to a lesser extent, the T_H1 response. It is likely then that under physiological allergic conditions, there is a more complex balance between various aspects of the immune system than seen in traditional OVA-based models, one in which the T_H17 response plays a more prominent role.

In our studies, a lack of IL-17 production resulted in decreased macrophage levels following four and eight challenges, but previous studies by other researchers examining IL-17 using OVA models have not reported similar decreases in macrophage or monocyte levels in IL-17-depleted mice (18, 54) despite the direct role for IL-17 in monocyte recruitment (55). Airway macrophages

have been shown to be responsible for recruiting eosinophils to the sites of inflammation through their production of eotaxin-2 (13, 40), and it is possible that IL-17 is not driving eosinophilia through direct production of eosinophil-specific chemokines but rather through the accumulation of macrophages that are in turn responsible for the recruitment of eosinophils.

Finally, though IL-17 production did not alter cytokine expression during later time points, we found that the presence of IL-17 resulted in early CD4 T cell IL-10 production and an accumulation of CD25⁺ Foxp3⁺ CD4 T cells following two challenges with *A. fumigatus* conidia. This suggests that despite the otherwise gradual accumulation of T_H17 CD4 T cells following repeated conidial challenges in WT mice, IL-17 may play an early role in shaping the immune response to *Aspergillus fumigatus* exposure. More than driving cellular accumulation, IL-17 may modulate the cytokine response during the initial adaptive immune reaction to *A. fumigatus* inhalation. While early T_H1 and T_H2 numbers are unchanged, there is a significant reduction in the percentage of CD4 T cells expressing IL-10 following two challenges in IL-17^{-/-} mice. Similarly, there is a significant reduction in the percentage of CD4⁺ CD25⁺ FoxP3⁺ T cells and a lower rate of CD4 T cell activation. We have previously shown that IL-10^{-/-} mice develop far less inflammation following four challenges than WT mice and do not develop pulmonary eosinophilia or T_H2 cells (56). It is therefore possible that IL-17 plays not only a direct role in promoting pulmonary eosinophilia and cellular accumulation during the allergic response but has a central, albeit subtle, role in shaping the initial immune response itself.

Similar to IL-17, expression of other members of the IL-17 cytokine family were altered during the inflammatory response. IL-17E (IL-25) was upregulated during peak inflammation in WT mice, an observation that is consistent with the role of IL-17E in T_H2 responses (50). Given the relatively similar induction patterns of T_H2 cells in WT and IL-17^{-/-} mice, IL-17E expression may ultimately be responsible for the differences in inflammation seen in the presence and absence of IL-17. Whereas there was a small but significant difference in IL-17F expression during peak inflammation in WT mice compared to IL-17^{-/-} mice, untreated IL-17^{-/-} mice expressed IL-17F mRNA at a rate nearly 25-fold higher than that of their WT counterparts. This high baseline level of IL-17F in the absence of IL-17 expression is consistent with previous reports (66). The role of IL-17F in driving pulmonary inflammation is not yet clear. Most studies have found strong evidence that IL-17F exacerbates T_H2 inflammation. IL-17F is upregulated in individuals with asthma (21), it correlates with disease severity (1, 21), the addition of nonfunctional IL-17F in model systems protects against T_H2 inflammation (22), and IL-17F induces the production of cytokines and chemokines by eosinophils (6). However, at least one study has demonstrated using IL-17F knockout mice that IL-17F protects against eosinophilia and plays a role in modulating other branches of the adaptive immune response (68). Therefore, like IL-17, IL-17F likely plays a more complex role than simply driving inflammation. This is supported by our observations where IL-17F expression was higher in the absence of IL-17 but reduced overall during inflammation. It is likely that similar to IL-17, the timing of IL-17F expression plays a central role in its pro-T_H2 or anti-T_H2 inflammatory activities.

ACKNOWLEDGMENTS

We thank members of the Young lab for feedback regarding data. We also thank John Osterholzer and Gwo-Hsiao Chen at the University of Michigan and Veterans Affairs Healthcare System for help identifying cellular populations. We also thank Vincent Young, John Erb-Downward, and John Kao for helpful discussions on this project.

This work was supported in part by grants R21AI083473 and R01AI064479 from the National Institute of Allergy and Infectious Diseases, grant R01HL085083 by the National Heart, Lung, and Blood Institute, and funding from the Drews Sarcoidosis Research Fund at the University of Michigan. We have no conflicting financial interests in this project.

REFERENCES

- Al-Ramli W, et al. 2009. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J. Allergy Clin. Immunol.* 123:1185–1187.
- Angkasekwinai P, et al. 2007. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J. Exp. Med.* 204:1509–1517.
- Barczyk A, Pierzchala W, Sozanska E. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir. Med.* 97:726–733.
- Bettelli E, Korn T, Oukka M, Kuchroo VK. 2008. Induction and effector functions of T(H)17 cells. *Nature* 453:1051–1057.
- Chakir J, et al. 2003. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J. Allergy Clin. Immunol.* 111:1293–1298.
- Cheung PF, Wong CK, Lam CW. 2008. Molecular mechanisms of cytokine and chemokine release from eosinophils activated by IL-17A, IL-17F, and IL-23: implication for Th17 lymphocytes-mediated allergic inflammation. *J. Immunol.* 180:5625–5635.
- Ciprandi G, et al. 2009. Serum interleukin-17 levels are related to clinical severity in allergic rhinitis. *Allergy* 64:1375–1378.
- Claudio E, et al. 2009. The adaptor protein CIKS/Act1 is essential for IL-25-mediated allergic airway inflammation. *J. Immunol.* 182:1617–1630.
- Cohn L, Elias JA, Chupp GL. 2004. Asthma: mechanisms of disease persistence and progression. *Annu. Rev. Immunol.* 22:789–815.
- Cruz A, et al. 2006. IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. *J. Immunol.* 177:1416–1420.
- El-behi M, Rostami A, Ciric B. 2010. Current views on the roles of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *J. Neuro-immune Pharmacol.* 5:189–197.
- Feng G, et al. 2008. Exogenous IFN-gamma ex vivo shapes the alloreactive T-cell repertoire by inhibition of Th17 responses and generation of functional Foxp3+ regulatory T cells. *Eur. J. Immunol.* 38:2512–2527.
- Fulkerson PC, Fischetti CA, Rothenberg ME. 2006. Eosinophils and CCR3 regulate interleukin-13 transgene-induced pulmonary remodeling. *Am. J. Pathol.* 169:2117–2126.
- Gafa V, et al. 2006. Human dendritic cells following *Aspergillus fumigatus* infection express the CCR7 receptor and a differential pattern of interleukin-12 (IL-12), IL-23, and IL-27 cytokines, which lead to a Th1 response. *Infect. Immun.* 74:1480–1489.
- Gerosa F, et al. 2008. Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. *J. Exp. Med.* 205:1447–1461.
- Gouldley JM, Clayton YM, Hay RJ. 1994. Environmental sampling for aspergilli during building construction on a hospital site. *J. Hosp. Infect.* 26:27–35.
- Happel KI, et al. 2005. Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J. Exp. Med.* 202:761–769.
- Hellings PW, et al. 2003. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 28:42–50.
- Huffnagle GB, Boyd MB, Street NE, Lipscomb MF. 1998. IL-5 is required for eosinophil recruitment, crystal deposition, and mononuclear cell recruitment during a pulmonary *Cryptococcus neoformans* infection in genetically susceptible mice (C57BL/6). *J. Immunol.* 160:2393–2400.
- Humbles AA, et al. 2002. The murine CCR3 receptor regulates both the role of eosinophils and mast cells in allergen-induced airway inflammation and hyperresponsiveness. *Proc. Natl. Acad. Sci. U. S. A.* 99:1479–1484.
- Kawaguchi M, et al. 2001. Identification of a novel cytokine, ML-1, and its expression in subjects with asthma. *J. Immunol.* 167:4430–4435.
- Kawaguchi M, et al. 2006. IL-17F sequence variant (His161Arg) is associated with protection against asthma and antagonizes wild-type IL-17F activity. *J. Allergy Clin. Immunol.* 117:795–801.
- Khader SA, et al. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat. Immunol.* 8:369–377.
- Kitani A, Xu L. 2008. Regulatory T cells and the induction of IL-17. *Mucosal Immunol.* 1(Suppl. 1):S43–S46.
- Kleinschek MA, et al. 2006. IL-23 enhances the inflammatory cell response in *Cryptococcus neoformans* infection and induces a cytokine pattern distinct from IL-12. *J. Immunol.* 176:1098–1106.
- Kleinschek MA, et al. 2007. IL-25 regulates Th17 function in autoimmune inflammation. *J. Exp. Med.* 204:161–170.
- Latge JP. 1999. *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12:310–350.
- Linden A, Laan M, Anderson GP. 2005. Neutrophils, interleukin-17A and lung disease. *Eur. Respir. J.* 25:159–172.
- Liu LY, et al. 2002. Decreased expression of membrane IL-5 receptor alpha on human eosinophils. II. IL-5 down-modulates its receptor via a proteinase-mediated process. *J. Immunol.* 169:6459–6466.
- Ma W, et al. 2002. CCR3 is essential for skin eosinophilia and airway hyperresponsiveness in a murine model of allergic skin inflammation. *J. Clin. Invest.* 109:621–628.
- Mangan PR, et al. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441:231–234.
- McNagny K, Graf T. 2002. Making eosinophils through subtle shifts in transcription factor expression. *J. Exp. Med.* 195:F43–F47.
- Molet S, et al. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J. Allergy Clin. Immunol.* 108:430–438.
- Murdock BJ, et al. 2011. Coevolution of TH1, TH2, and TH17 responses during repeated pulmonary exposure to *Aspergillus fumigatus* conidia. *Infect. Immun.* 79:125–135.
- Nakae S, et al. 2002. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 17:375–387.
- Nikolic T, de Bruijn MF, Lutz MB, Leenen PJ. 2003. Developmental stages of myeloid dendritic cells in mouse bone marrow. *Int. Immunol.* 15:515–524.
- Noverr MC, Cox GM, Perfect JR, Huffnagle GB. 2003. Role of PLB1 in pulmonary inflammation and cryptococcal eicosanoid production. *Infect. Immun.* 71:1538–1547.
- Obata K, et al. 2007. Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* 110:913–920.
- Oboki K, Ohno T, Saito H, Nakae S. 2008. Th17 and allergy. *Allergol. Int.* 57:121–134.
- Ohnmacht C, Pullner A, van Rooijen N, Voehringer D. 2007. Analysis of eosinophil turnover in vivo reveals their active recruitment to and prolonged survival in the peritoneal cavity. *J. Immunol.* 179:4766–4774.
- Osterholzer JJ, et al. 2009. Accumulation of CD11b+ lung dendritic cells in response to fungal infection results from the CCR2-mediated recruitment and differentiation of Ly-6C^{high} monocytes. *J. Immunol.* 183:8044–8053.
- Owyang AM, et al. 2006. Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J. Exp. Med.* 203:843–849.
- Park H, et al. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6:1133–1141.
- Park SJ, et al. 2009. Peroxisome proliferator-activated receptor gamma agonist down-regulates IL-17 expression in a murine model of allergic airway inflammation. *J. Immunol.* 183:3259–3267.
- Ponath PD, et al. 1996. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J. Exp. Med.* 183:2437–2448.
- Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME. 2005. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J. Immunol.* 175:5341–5350.
- Radhakrishnan S, et al. 2008. Reprogrammed FoxP3+ T regulatory cells

- become IL-17+ antigen-specific autoimmune effectors in vitro and in vivo. *J. Immunol.* **181**:3137–3147.
48. Rahman MS, et al. 2006. IL-17A induces eotaxin-1/CC chemokine ligand 11 expression in human airway smooth muscle cells: role of MAPK (Erk1/2, JNK, and p38) pathways. *J. Immunol.* **177**:4064–4071.
 49. Rickel EA, et al. 2008. Identification of functional roles for both IL-17RB and IL-17RA in mediating IL-25-induced activities. *J. Immunol.* **181**:4299–4310.
 50. Robinson DS. 2010. The role of the T cell in asthma. *J. Allergy Clin. Immunol.* **126**:1081–1093.
 51. Romani L, et al. 2009. Indoleamine 2,3-dioxygenase (IDO) in inflammation and allergy to *Aspergillus*. *Med. Mycol.* **47**(Suppl. 1):S154–S161.
 52. Romani L, Zelante T, De Luca A, Fallarino F, Puccetti P. 2008. IL-17 and therapeutic kynurenines in pathogenic inflammation to fungi. *J. Immunol.* **180**:5157–5162.
 53. Sanderson CJ. 1992. Interleukin-5, eosinophils, and disease. *Blood* **79**:3101–3109.
 54. Schnyder-Candrian S, et al. 2006. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* **203**:2715–2725.
 55. Shahrara S, Pickens SR, Dorfleutner A, Pope RM. 2009. IL-17 induces monocyte migration in rheumatoid arthritis. *J. Immunol.* **182**:3884–3891.
 56. Shreiner AB, et al. 2012. Repeated exposure to *Aspergillus fumigatus* conidia results in CD4+ T cell-dependent and -independent pulmonary arterial remodeling in a mixed Th1/Th2/Th17 microenvironment that requires interleukin-4 (IL-4) and IL-10. *Infect. Immun.* **80**:388–397.
 57. Sivick KE, Schaller MA, Smith SN, Mobley HL. 2010. The innate immune response to uropathogenic *Escherichia coli* involves IL-17A in a murine model of urinary tract infection. *J. Immunol.* **184**:2065–2075.
 58. Sokol CL, Barton GM, Farr AG, Medzhitov R. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* **9**:310–318.
 59. Song C, et al. 2008. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J. Immunol.* **181**:6117–6124.
 60. Stevens WW, Kim TS, Pujanauski LM, Hao X, Braciale TJ. 2007. Detection and quantitation of eosinophils in the murine respiratory tract by flow cytometry. *J. Immunol. Methods* **327**:63–74.
 61. Stockinger B, Veldhoen M, Martin B. 2007. Th17 T cells: linking innate and adaptive immunity. *Semin. Immunol.* **19**:353–361.
 62. Tamachi T, Maezawa Y, Ikeda K, Iwamoto I, Nakajima H. 2006. Interleukin 25 in allergic airway inflammation. *Int. Arch. Allergy Immunol.* **140**(Suppl. 1):59–62.
 63. Tamachi T, et al. 2006. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. *J. Allergy Clin. Immunol.* **118**:606–614.
 64. Tesmer LA, Lundy SK, Sarkar S, Fox DA. 2008. Th17 cells in human disease. *Immunol. Rev.* **223**:87–113.
 65. van der Loo JC, Slieker WA, Kieboom D, Ploemacher RE. 1995. Identification of hematopoietic stem cell subsets on the basis of their primitiveness using antibody ER-MP12. *Blood* **85**:952–962.
 66. von Vietinghoff S, Ley K. 2009. IL-17A controls IL-17F production and maintains blood neutrophil counts in mice. *J. Immunol.* **183**:865–873.
 67. Wakashin H, et al. 2008. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit. Care Med.* **178**:1023–1032.
 68. Yang XO, et al. 2008. Regulation of inflammatory responses by IL-17F. *J. Exp. Med.* **205**:1063–1075.
 69. Yu C, et al. 2002. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. *J. Exp. Med.* **195**:1387–1395.
 70. Zelante T, et al. 2009. Th17 cells in the setting of *Aspergillus* infection and pathology. *Med. Mycol.* **47**(Suppl. 1):S162–S169.
 71. Zelante T, et al. 2007. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur. J. Immunol.* **37**:2695–2706.
 72. Zimmermann N, Hershey GK, Foster PS, Rothenberg ME. 2003. Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J. Allergy Clin. Immunol.* **111**:227–242.