Allergic Sensitization through the Airway Primes Th17-dependent Neutrophilia and Airway Hyperresponsiveness

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***Rationale*: In humans, immune responses to inhaled aeroallergens develop in the lung and draining lymph nodes. Many animal models of asthma bypass this route and instead use intraperitoneal injections of allergen using aluminum hydroxide as an adjuvant.**

***Objectives*: We investigated whether allergic sensitization through the airway elicits immune responses qualitatively different than those arising in the peritoneum.**

***Methods*: Mice were sensitized to allergen through the airway using low-dose LPS as an adjuvant, or through the peritoneum using aluminum hydroxide as an adjuvant. After a single allergen challenge, ELISA and flow cytometry were used to measure cytokines and leukocyte subsets. Invasive measurements of airway resistance were used to measure allergen-induced airway hyperreactivity (AHR).**

***Measurements and Main Results*: Sensitization through the peritoneum primed strong Th2 responses and eosinophilia, but not AHR, after a single allergen challenge. By contrast, allergic sensitization through the airway primed only modest Th2 responses, but strong Th17 responses. Th17 cells homed to the lung and released IL-17 into the airway on subsequent encounter with inhaled allergen. As a result, these mice developed IL-17–dependent airway neutrophilia and AHR. This AHR was neutrophil-dependent because it was abrogated in CXCR2-deficient mice and also in wild-type mice receiving a neutrophil-depleting antibody. Individually, neither IL-17 nor ongoing Th2 responses were sufficient to confer AHR, but together they acted synergistically to promote neutrophil recruitment, eosinophil recruitment and AHR.**

***Conclusions*: Allergic sensitization through the airway primes modest Th2 responses but strong Th17 responses that promote airway neutrophilia and acute AHR. These findings support a causal role for neutrophils in severe asthma.**

Keywords: asthma; lung; immunity

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

In humans, immune responses to inhaled aeroallergens develop in the lung and draining lymph nodes. Many animal models of asthma bypass this route, and instead use intraperitoneal injections of allergen using aluminum hydroxide as an adjuvant.

What This Study Adds to the Field

Allergic sensitization through the airway, but not through the peritoneum, primes robust Th17 immunity. A subsequent single allergen challenge provokes airway hyperreactivity, which is dependent on airway neutrophilia and Th2 responses. Thus, neutrophils might also have a causal role in human asthma.

The most widely used mouse model of asthma involves allergic sensitization by intraperitoneal injections of ovalbumin (OVA) complexed with the Th2 adjuvant, aluminum hydroxide (alum) (8). These animals are typically challenged by intranasal instillation, or an aerosol, of OVA. Variations of this model have been used for many years and have been valuable for studying Th2-mediated responses in allergic pulmonary inflammation. However, some features of this model are inconsistent with human asthma. For example, the level of airway eosinophils can reach 80% in this mouse model (8), whereas in human patients it is rarely greater than 5% (9). Also, in this model, neutrophil recruitment to the airway is transient (10) and dispensable for airway hyperreactivity (AHR) (11). Therefore the alum-mediated model is not useful for studying the function of neutrophils in asthma. We hypothesized that some differences between this mouse model and human asthma might reflect the impact of the unique lung environment on developing immune responses. For example, alveolar macrophages and airway epithelial cells produce the regulatory cytokine, TGF-β (12, 13), which is required for the induction of both regulatory T cells (Treg) and Th17 cells. The extent to which unique features of the lung selectively affect developing immune responses to inhaled antigens is not currently known.

Here, we demonstrate that allergic sensitization through the airway induces a profoundly different immune response than alum-mediated sensitization through the peritoneum. Unlike sensitization through the peritoneum, LPS-mediated sensitization through the airway selectively primes Th17 cells that home to the lung and release IL-17 into the airway on allergen challenge. Signaling responses to IL-17 promote robust and prolonged neutrophilia and AHR. Moreover, blockade of neutrophil recruitment to the airway is sufficient to prevent this AHR. These findings suggest that neutrophils might also have a causal role in human asthma and establish a new approach to the study of Th17 immune responses in the lung.

METHODS

Mice

C57BL/6, BALB/c, *Stat6*$^{-/-}$, and *Cxcr2*$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). *IL-17ra*$^{-/-}$ mice were

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obtained from Taconic Farms, Inc. (Germantown, NY) and used with permission from Amgen Inc. (Thousand Oaks, CA). The generation of these mice has been previously described (14). All mice were used between 6 and 12 weeks of age, and all experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the National Institute of Environmental Health Sciences.

Ovalbumin Sensitizations and Challenges

Mice were sensitized to OVA on Days 0 and 6. For airway sensitizations using low-dose LPS, mice received oropharyngeal applications of 100 μg of low-endotoxin OVA (endotoxin concentration <1 EU/mg OVA) (Profos AG, Regensburg, Germany), supplemented with 0.1 μg LPS prepared from *Escherichia coli* 0111:B4 (Sigma, St.Louis, MO) with PBS as a vehicle, in a total volume of 50 μl. Animals were anesthetized with isoflurane and vertically suspended by their teeth with a rubber band. The tongue was gently grasped with forceps and held to one side to prevent swallowing, and 50 μl of the OVA solution deposited at the back of the oral cavity. Th1 sensitizations were done in the same manner, except that 15 μg LPS were added to the OVA, and not 100 μg LPS, as has been previously described by others for this purpose (15). In our hands, both 15 and 100 μg of LPS are sufficient to induce Th1 responses, but the lower dose is more easily tolerated by the mice. For intraperitoneal sensitizations, and unless stated otherwise, mice were injected with 100 μg ovalbumin (Sigma) complexed in 50% aluminum hydroxide (Pierce, Rockford, IL) in a total volume of 200 μl. Where indicated, LPS was occasionally used as an adjuvant for intraperitoneal injections. In all experiments, mice were challenged on a single occasion (Day 13) for 1 hour with an aerosol of 1% OVA (Sigma) in saline. The animals were harvested immediately (0 h), or at the indicated times post challenge.

Treatment of Mice with Cytokines or Antibodies

For CXCL1 and CXCL5 instillations, mice were lightly anesthetized with isoflurane and given 0.35 μg of recombinant protein CXCL1/KC or CXCL5/LIX (R&D, Inc, Minneapolis, MN) in sterile phosphate-buffered saline (PBS; Sigma) in a total volume of 50 μl, 4 hours post OVA challenge. Where indicated, 100 μg of anti-mouse Ly6G (Gr-1) antibody (clone RB6–8C5) or the isotype control rat IgG2b (clone eB149/10H5, eBioscience, San Diego, CA) was diluted in 200 μl of PBS and injected intraperitoneally 6 hours before OVA challenge. Recombinant mouse IL-17A or IL-17F (R&D, Inc.) at a dose of 1.5 μg per mouse in 50 μl of PBS was delivered via oropharyngeal instillation immediately after aerosol challenge. According to the manufacturer, endotoxin contamination in all chemokine and cytokine preparations is less than 1 EU/μg protein.

Analysis of Airway Inflammation and Cytokines

Whole-lung lavage was performed and cell differentials determined as previously described (16). Concentrations of IL-4, IL-5, IL-13, IL-17, and IFN-γ in bronchoalveolar lavage (BAL) fluid were determined using a commercial multiplexed fluorescent bead-based immunoassay (Bio-Rad Laboratories, Hercules, CA) according to the manufacture's instructions. CXCL1 and CXCL5 protein concentrations were detected using ELISA kits from R&D Systems.

Flow Cytometric Analysis

Lungs were extracted, minced, and digested with collagenase A and XI, hyaluronidase, and DNase for 1 hour at 37°C, and the reaction was stopped with ethylenediaminetetraacetic acid. Single cell suspensions were enriched on a discontinuous density gradient using Histopaque (Sigma). Washed cells were diluted to 20 million cells per ml and incubated with a blocking cocktail of purified rat anti-mouse CD16/CD32 (BD Pharmingen, San Jose, CA), normal mouse, and normal rat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 20 minutes. For staining of surface antigens, cells were labeled with antibodies against mouse CD4 (clone GK1.5, eBioscience, or clone GK1.5, BD Pharmingen), TCR β (clone H57–597, BD Pharmingen), TCR γδ (clone GL3, BD Pharmingen), or the appropriate isotype control antibodies. CD1d-restriced natural killer T (NKT) cells were identified using PBS57-loaded, CD1d tetramers or empty CD1d tetramers as a negative control (NIH Tetramer Core Facility at Emory, Atlanta, GA). For intracellular staining, cells were stimulated with 50 ng/ml phorbol myristate acetate and 500 ng/ml ionomycin (Sigma) or with antibodies against CD3 and CD28 for 4 hours before staining and incubated with GolgiStop (BD Pharmingen) during the last 3 hours of stimulation. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and labeled with antibodies against IL-17A (clone eBio17B7, eBioscience). $CD4^+$ lymphocytes were identified as non-autofluorescent cells within a lymphocyte gate based on forward and side scatter. Cells were collected using a BD LSR II cytometer (BD Biosciences) and data were analyzed using FlowJo 7.2.2 software (Tree Star, Inc., Ashland, OR).

Histology

Lungs were fixed in 10% formalin and embedded in paraffin. Left lobe longitudinal sections 5 to 7 μm thick were stained with either Alcian Blue alone or together with periodic acid-Schiff.

Airway Physiology

Airway responses to aerosolized methacholine were measured on anesthetized mice as previously described (17), using the Flexivent mechanical ventilator system (Scireq, Montreal, PQ, Canada). The single compartment model was used to assess total respiratory system resistance (R) after administration of increasing doses of methacholine (0–50 mg/ml). Individual peak responses were determined at each dose per mouse. Results are represented as fold increases of R(cm H_2O·s/ml) above baseline, calculated as follows: [R(response) − R(baseline)]/R(baseline).

Statistics

Data are expressed as mean ± SEM. Statistical differences between groups were calculated using Student *t* test. A two-tailed *P* value of less than 0.05 was considered statistically significant.

RESULTS

Physiologic and Biologic Responses to Allergen Challenge Depend on Route of Sensitization

We first investigated the impact of the route of sensitization on immune responses by comparing physiologic responses to subsequent allergen challenge. C57BL/6 mice were sensitized by airway delivery of OVA together with low doses of LPS (15), or by the more conventional method of intraperitoneal injections of OVA complexed with alum. These animals were challenged on a single occasion with aerosolized OVA and analyzed 48 hours later. Microscopic analysis of lung sections revealed that OVA-challenged mice had similar levels of mucus-producing cells lining the airway, regardless of whether the animals had been sensitized to OVA through the airway or by intraperitoneal injections (Figure 1A). Therefore, both sensitization methods can prime immune responses required for mucus production in the airway.

We next used invasive measurements of airway resistance to study the impact of different sensitization procedures on the development of AHR, another cardinal feature of allergic asthma. As expected, unsensitized C57BL/6 mice challenged with OVA on a single occasion did not develop AHR (Figure 1B). Similarly, mice sensitized by intraperitoneal injections and challenged once with OVA also failed to develop AHR. By contrast, mice sensitized through the airway displayed robust AHR after the single OVA challenge. This response was not unique to C57BL/6 mice because airway-sensitized BALB/c mice also developed AHR after a single challenge (*see* Figure E1A in the online supplement). Thus, LPS-mediated sensitization through the airway triggers events in the lung or draining lymph nodes that lead to AHR on subsequent exposure to the sensitizing antigen, whereas these same events do not occur after alum-mediated sensitization through the peritoneum.

AHR is generally associated with extensive pulmonary inflammation, although there are several exceptions to this trend (18–21). Analysis of total cells in the airway of C57BL/6 mice after BAL revealed that both intraperitoneally and airway-

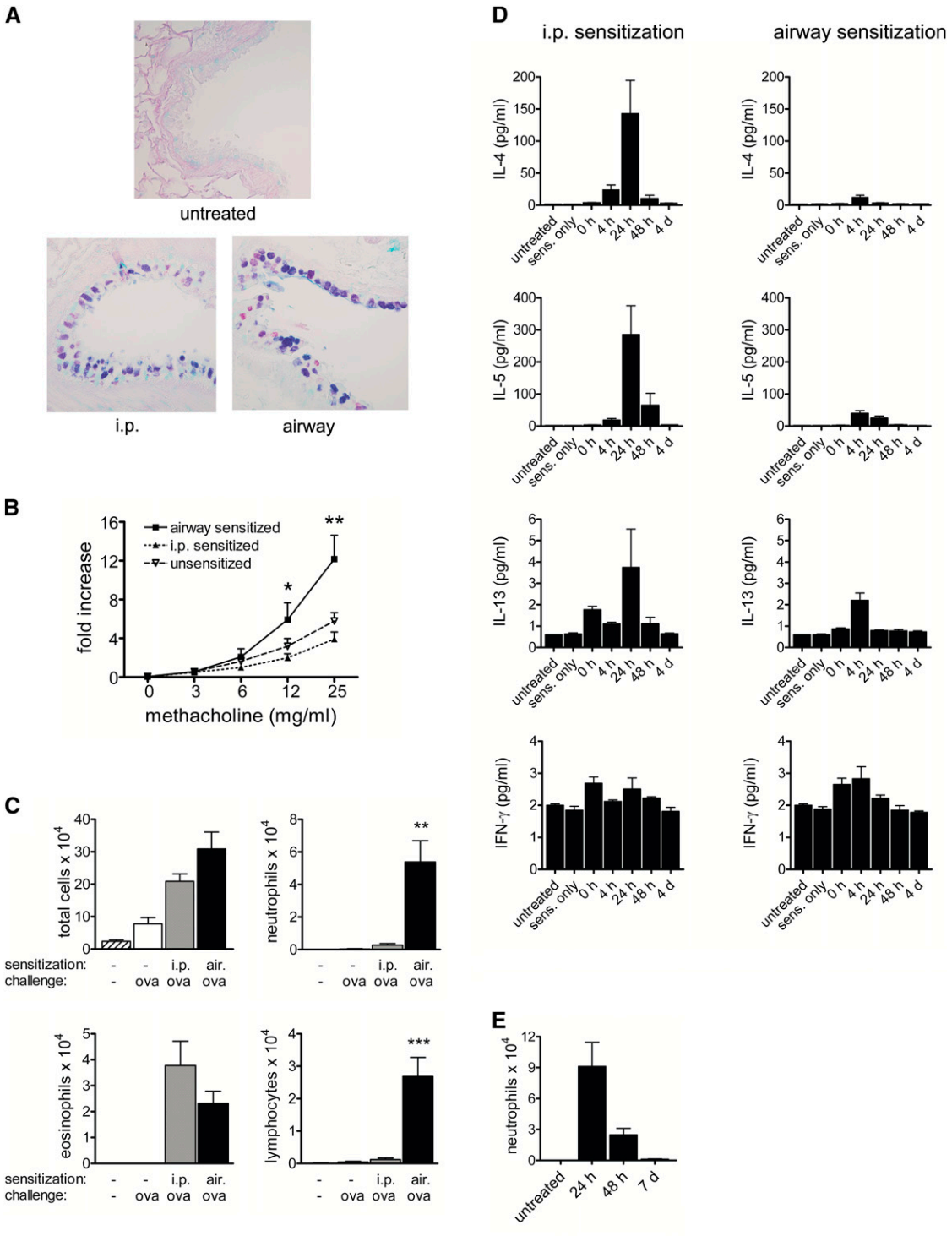

Figure 1. Method of allergen sensitization affects biologic and pathophysiologic responses to allergen challenge. C57BL/6 mice were sensitized to ovalbumin (OVA) through the peritoneum using aluminum hydroxide as an adjuvant, or through the airway using LPS as an adjuvant. All animals were challenged on a single occasion with aerosolized OVA and analyzed 48 hours post challenge. (*A*) Alcian blue staining of mucus-producing cells. (*B*) Invasive measurements of methacholine-induced airway resistance. (*C*) Comparison of total leukocytes and individual leukocyte subsets in the bronchoalveolar lavage (BAL) of airway-sensitized and intraperitoneally sensitized mice. (*D*) Cytokine levels in the BAL at various times post challenge. (*E*) Time course of neutrophil recruitment to the airway after sensitization only. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$ denote significance between intraperitoneally sensitized and airway-sensitized groups of mice (n = 6–13 mice per group).

sensitized animals developed significant airway inflammation after OVA challenge (Figure 1C). Levels of eosinophils after OVA challenge were generally lower in airway-sensitized mice than in those sensitized through the peritoneum, although this difference was not always statistically significant. The robust eosinophilic responses in intraperitoneally sensitized mice suggested that their lack of AHR after OVA challenge is not simply due to delayed priming in these animals. Levels of neutrophils and lymphocytes in the BAL were both significantly increased in airway-sensitized mice compared with intraperitoneally sensitized mice. Similar findings were obtained when BALB/c mice were used, although levels of eosinophils were much higher in this strain than in C57BL/6 animals, especially after intraperitoneal sensitization and challenge (Figure E1B).

Having found that the method of sensitization affects the profile of leukocytes recruited to the airway after OVA challenge, we compared levels of Th2 cytokines in the BAL fluid at various times post challenge. This analysis revealed that both airway-sensitized and intraperitoneally sensitized mice had increased levels of the Th2 cytokines IL-4 and IL-5 compared with untreated mice or sensitized mice that had not been challenged. However, levels of these Th2 cytokines were highest in challenged mice that had been sensitized through the peritoneum (Figure 1D), again suggesting that the lack of AHR in these

animals is not due to insufficient or delayed priming of Th2 cells. Levels of IL-13 were also higher in the intraperitoneally sensitized mice than in airway-sensitized animals, although this difference did not reach statistical significance. The Th1-associated cytokine IFN-γ was present at relatively low levels and was similar in all groups, suggesting that the observed differences in AHR between airway-sensitized and intraperitoneally sensitized mice do not result from differences in Th1 responses.

LPS is a potent proinflammatory molecule and its introduction to the airway induces a rapid influx of neutrophils. We therefore considered the possibility that the observed neutrophilia seen in airway-sensitized and challenged mice resulted from the sensitization procedure itself, rather than from the OVA challenge. Analysis of leukocytes in the BAL at various times post sensitization revealed that this procedure did induce neutrophil recruitment to the airway. However, this response was short-lived, and there were virtually no neutrophils in the airway at 1 week post sensitization, the time at which the animals are normally challenged in this model (Figure 1E). Therefore, the accumulation of neutrophils and eosinophils to the airway after the OVA challenge likely results from an adaptive immune response to OVA challenge, and not simply from a prolonged, innate immune response to the sensitization procedure itself.

The two methods used here to achieve allergic sensitization used different routes of allergen administration and different adjuvants. Therefore, the differences in immune and physiologic responses elicited by these methods might have resulted solely from the different adjuvant used or from the different routes of sensitization. To resolve this, we compared responses of mice that had been sensitized through the airway, or through the peritoneum, using different doses of LPS as an adjuvant (Figure 2). Mice receiving airway administrations of low-dose LPS together with OVA again developed modest eosinophilia and robust neutrophilia on challenge, whereas mice sensitized through the peritoneum with these same reagents did not display responses to OVA challenge. Mice sensitized through the airway with 15 μg of LPS together with OVA developed robust neutrophilia after challenge, but no eosinophils were seen, in agreement with previous reports that high doses of LPS induce Th1 responses in the airway (15). By contrast, mice sensitized through the peritoneum with OVA plus 15 μg of LPS developed only very low levels of eosinophils, and some neutrophils, on challenge. For efficient sensitization through the airway, both OVA and LPS were necessary because mice receiving OVA alone, or LPS alone, failed to respond to subsequent OVA challenge. These data suggest that LPS has unique properties as an adjuvant in the airway.

Th17 Cells Are Selectively Induced by Sensitization through the Airway

In addition to the well-characterized lineages of Th1 and Th2 cells, a third T helper cell subset that produces IL-17 has been recently described (22, 23). Th17 cells develop in the presence of IL-6 and TGF-β and are further amplified by IL-23. IL-17, also known as IL-17A, and its related family member IL-17F, have been recently linked to neutrophil recruitment in various tissues, including the lung (24). IL-17 and IL-17F do not act directly on neutrophils, but can nonetheless promote their recruitment by inducing the production of neutrophil-attracting chemokines, such as CXCL1 (KC) and CXCL5 (25). We reasoned that allergic sensitization through the airway might selectively prime OVA-specific Th17 cells that would in turn promote airway neutrophilia on subsequent OVA challenge. To test this, we performed intracellular staining for IL-17 on $CD4^+$ T cells prepared from lungs of mice that had been sensitized, but not challenged. Flow cytometric analysis of these cells revealed that airway sensitization, but not intraperitoneal sensitization, led to dramatic increases in the number of IL-17–producing $CD4^+$ T cells in the lung (Figures 3A and 3B). Similar results were found in mice that had been sensitized and also challenged (Figure E2A).

Conventional αβ TCR^+ Th17 cells are perhaps the best-known source of IL-17, but this cytokine can also be produced in the lung by γδ TCR T cells (26) and by CD1d-restricted, invariant NKT (iNKT) cells (27). To better characterize the IL-17–containing cells in the lungs of mice sensitized through the airway, we performed additional experiments. The percentage of IL-17^+ lymphocytes displaying the αβ TCR was 10-fold greater than that of cells displaying the γδ TCR (Figure 3C). Experiments using CD1d tetramers revealed that iNKT cells were present in the lung, but were not an abundant source of IL-17 (Figure E2B). Taken together, these findings suggest that neither γδ TCR^+ T cells nor iNKT cells are a primary source of IL-17 in airway-sensitized mice, and that conventional Th17 cells displaying the αβ TCR^+ compose the majority of IL-17–producing cells in the lung. To determine if systemic Th17 responses were also increased by sensitization through the airway, we analyzed IL-17^+ $CD4^+$ T cells in the spleen. In contrast to our findings in the lung, splenic IL-17^+ $CD4^+$ T cells were almost undetectable (Figure 3D). This result suggests that allergic sensitization through the airway uniquely affects immune responses in the lung by selectively priming Th17 cells that subsequently home back to that organ.

We next investigated whether the Th17 cells observed in the lung after airway sensitization constitutively release IL-17 into the airway or are triggered to do so after allergen challenge. Despite the high levels of intracellular IL-17 within T cells of mice that had been sensitized through the airway, almost no IL-17 was found in the BAL fluid before OVA challenge. However, within 4 hours of challenge, a strong increase was seen in airway levels of IL-17 (Figure 3E). No such increase was seen in intraperitoneally sensitized mice. Thus, the Th17 cells residing in the lung release IL-17 on exposure to inhaled allergens.

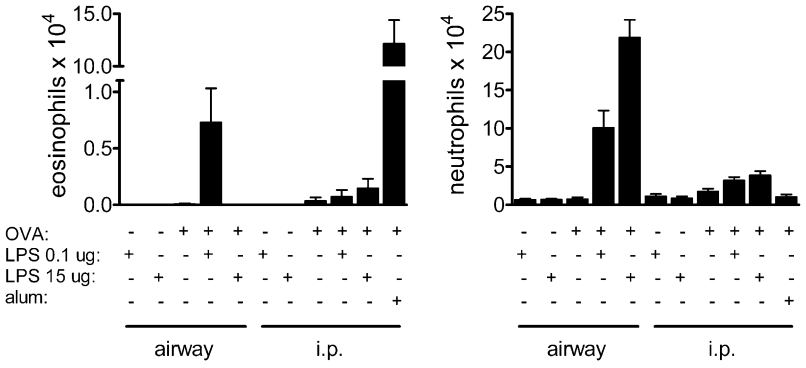

Figure 2. Impact of LPS on ovalbumin (OVA) sensitization through the airway and peritoneum. C57BL/6 mice were sensitized through the airway or peritoneum using the indicated levels of LPS as adjuvant. In addition to mice receiving OVA together with LPS, control groups include mice receiving OVA alone or LPS alone. For intraperitoneal injections, one group received aluminum hydroxide as a control adjuvant. All animals were challenged on a single occasion with aerosolized OVA and analyzed 48 hours post challenge (n = 7 mice per group).

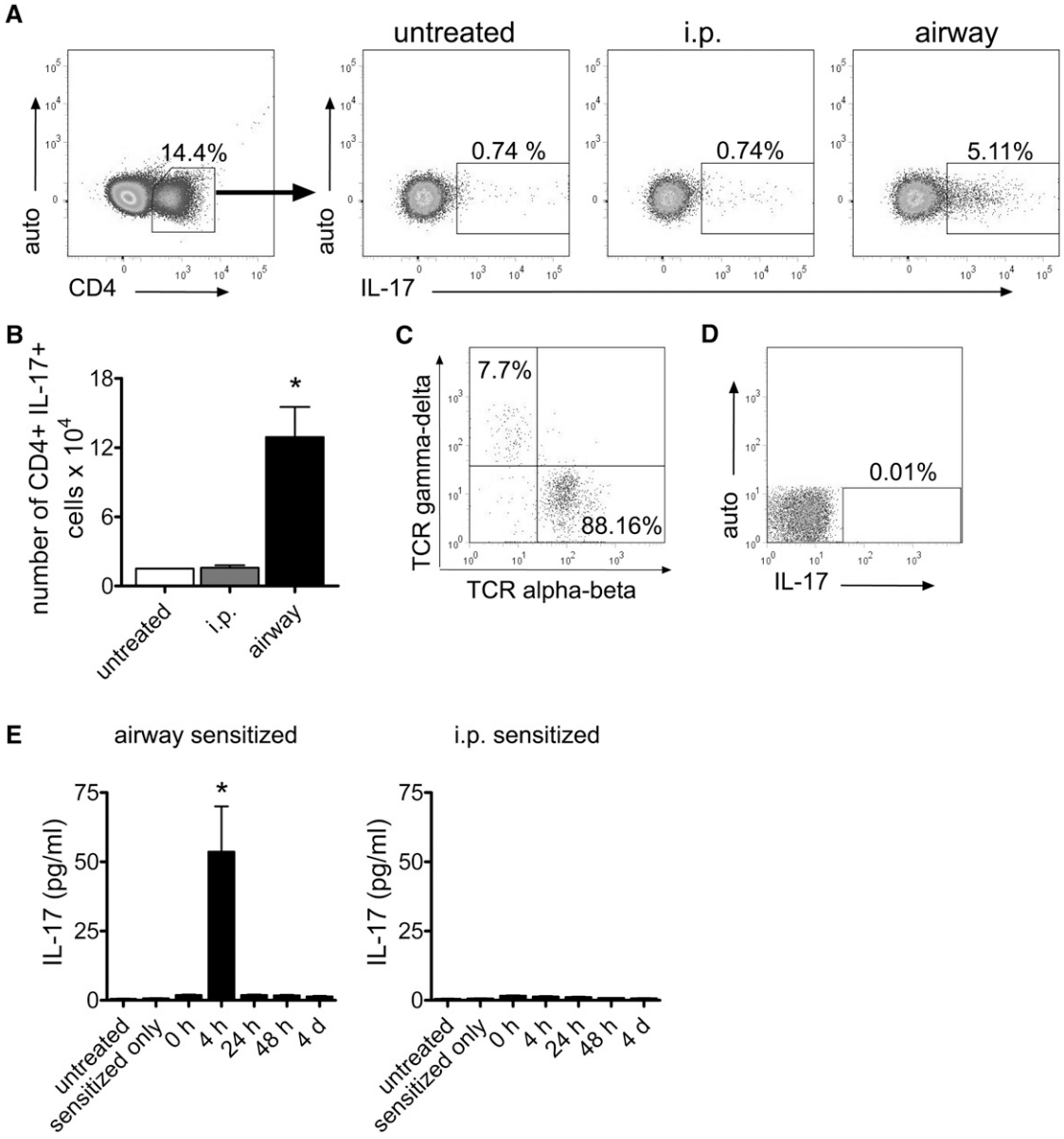

Figure 3. Sensitization through the airway selectively primes IL-17 cells. Mice were sensitized through the airway or peritoneum and harvested 1 week after the second sensitization. Leukocytes were prepared from lungs of these mice, as well as from untreated control animals, and analyzed by flow cytometry. (*A*) Representative individual flow plots and gating strategies are shown for $CD4^+$ T cells, as well as for IL-17^+ cells within the $CD4^+$ gate. (*B*) Bar histograms depict total number of $CD4^+$ IL-17^+ T cells of untreated, and of airway-sensitized and intraperitoneally sensitized mice. (*C*) $\alpha\beta$ and $\delta\gamma$ TCR staining of cells within IL-17^+ cell gate of airway-sensitized mice. (*D*) Analysis of IL-17^+ T cells in the spleen of airway-sensitized mice. (*E*) Levels of IL-17 in the airway at various times post ovalbumin challenge. $^*P \leq 0.05$ denotes significance between intraperitoneally sensitized and airway-sensitized groups, each having three pools of mice with three mice in each pool. Data shown represent the results of one of two similar experiments.

Signaling Responses to IL-17 Are Required for Neutrophil Recruitment and AHR in Mice Sensitized through the Airway

To determine whether the IL-17 released after allergen challenge is responsible for the neutrophilia and AHR seen in airway-sensitized mice, we performed a series of experiments with mice lacking the IL-17 receptor A (IL-17RA). This receptor signals in response to IL-17 and IL-17F (28). On airway sensitization and challenge, wild-type (WT) and *Il-17ra*$^{-/-}$ mice developed similar overall levels of airway inflammation, as judged by the number of total leukocytes in the BAL (Figure 4A). However, differential staining of these cells revealed that eosinophils were more abundant in *Il-17ra*$^{-/-}$ mice than in similarly treated WT mice (Figure 4A), consistent with a previous report showing that IL-17F can negatively regulate Th2 responses (29). In contrast to this increase in eosinophils, the accumulation of neutrophils seen at 48 hours post challenge in WT mice was virtually abolished in *Il-17ra*$^{-/-}$ mice (Figure 4A). Therefore, signaling responses to IL-17 are absolutely required for the airway neutrophilia seen in airway-sensitized mice at 48 hours post challenge.

We next tested whether IL-17RA is also required for the physiologic responses to allergen challenge in airway-sensitized mice. Similar levels of mucus-producing cells were seen in WT and *Il-17ra*$^{-/-}$ mice (Figure 4B). However, dramatic differences between these two strains were observed when AHR was assessed. Although WT mice developed robust AHR, *Il-17ra*$^{-/-}$ mice were virtually unresponsive in this assay (Figure 4C). Interestingly, the phenotype of these airway-sensitized *Il-17ra*$^{-/-}$ mice strongly resembled that of intraperitoneally sensitized WT mice; both groups of mice displayed high levels of airway eosinophils and mucus-producing cells, but had few neutrophils and no AHR. Taken together, these observations suggest that the unique phenotypic features of mice sensitized through the airway result from the selective induction and actions of Th17 cells.

Neutrophilia Is Required for AHR in Airway-sensitized Mice

Neutrophils are associated with severe asthma in humans (5, 6), but it is not known if these cells contribute directly to disease severity or whether they are simply a consequence of dysregu-

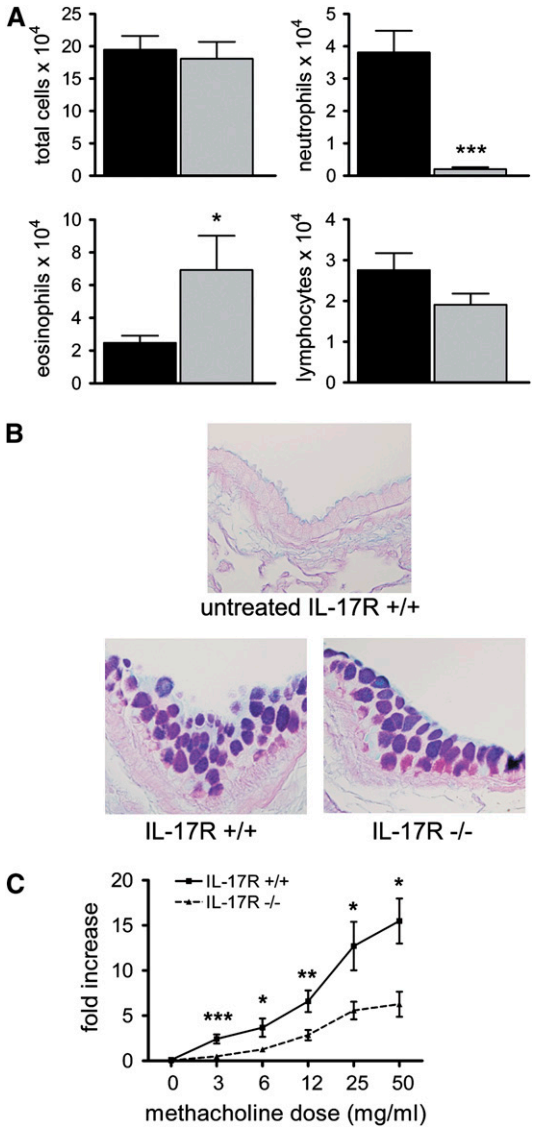

Figure 4. Airway neutrophilia and airway hyperreactivity is dependent on signaling responses to IL-17. Mice were sensitized twice through the airway and analyzed 48 hours after a single ovalbumin (OVA) challenge. (*A*) Total leukocytes and individual subsets in the bronchoalveolar lavage of wild-type (WT) and *Il-17ra*$^{-/-}$ mice. Data shown are compiled from three similar experiments (n = 24 mice per group). *Solid columns* represent IL-17R$^{+/+}$, *shaded columns* represent IL-17R$^{-/-}$. (*B*) Alcian blue/periodic acid-Schiff staining of mucus-producing cells. (*C*) Invasive measurements of lung resistance 48 hours post OVA challenge in WT and *IL-17ra*$^{-/-}$ mice (n = 16 mice per group). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. The data shown are compiled from two similar experiments yielding similar results.

lated pulmonary inflammation. To directly test whether neutrophils are required for the development of AHR, we blocked the recruitment of these cells to the airway with an anti–Gr-1 (Ly6-C and G) antibody before allergen challenge. Analysis of leukocytes in the BAL 48 hours post challenge confirmed that this anti–Gr-1 antibody, but not an isotype control antibody, abolished neutrophil recruitment to the airway (Figure 5A), whereas eosinophil recruitment was not significantly diminished. Measurements of lung resistance revealed that injections of the anti–Gr-1 antibody were also sufficient to block AHR (Figure 5A). Thus, preventing neutrophil recruitment to the airway also prevented the development of AHR and suggested that at least in this model neutrophils are required for the development of AHR.

To gain greater insight into neutrophil recruitment to the airway, we measured levels of the neutrophilic chemokines CXCL1 and CXCL5. Immediately after challenge (0 h), CXCL1 was found in the airways of both intraperitoneally and airway-sensitized WT mice (Figure 5B), and surprisingly, was even higher in the former group than in the latter. This finding might explain the previously described, transient neutrophil recruitment seen after OVA challenge in these animals, which has been previously shown to be dispensable for AHR (11). In contrast to CXCL1, CXCL5 was produced at much higher levels in airway-sensitized WT mice than in intraperitoneally sensitized WT mice (Figure 5B). CXCL5 also remained at elevated levels for several days after OVA challenge of airway-sensitized mice, reflecting the prolonged presence of neutrophils in the airways of WT mice, but not *Il-17ra*$^{-/-}$ mice (Figure 5C). To determine whether IL-17RA was required for this chemokine production, we performed a similar experiment that included *Il-17ra*$^{-/-}$ mice (Figure 5C). The transient increase in CXCL1 seen in WT mice was also seen *Il-17ra*$^{-/-}$ mice, whereas the prolonged increase of CXCL5 was largely absent in the latter strain. Together, these findings suggest that IL-17–dependent production of CXCL5 is responsible for the airway neutrophilia seen in airway-sensitized and challenged WT mice.

The receptor for both CXCL1 and CXCL5 is CXCR2. We reasoned that this receptor might mediate neutrophil recruitment to the airway, and if so, would provide an alternative means to test whether this recruitment is required for the development of AHR. On airway sensitization and challenge, *Cxcr2*$^{-/-}$ mice had significantly fewer neutrophils than their WT counterparts, whereas eosinophils were present at similar levels (Figure 5D). It is unlikely that the reduced levels of neutrophils were due to a requirement for Th17 induction because CXCL1 and CXCL5 were produced at even higher levels in *Cxcr2*$^{-/-}$ mice than in WT mice (Figure E3). Rather, CXCR2 is likely required to direct neutrophils toward high concentrations of its ligands in the airway. We next carried out AHR experiments with airway-sensitized and challenged *Cxcr2*$^{-/-}$ mice. As seen earlier, WT mice developed robust AHR. However, this response was significantly diminished in *Cxcr2*$^{-/-}$ mice (Figure 5D). These findings confirm that neutrophil recruitment to the airway is required for the development of AHR and further shows that this recruitment is directed by CXCR2.

Airway Neutrophilia Is Not Sufficient for AHR

Having established that airway neutrophilia is required for AHR using two different approaches, we next investigated whether the neutrophil recruitment seen in airway-sensitized and challenged mice is sufficient for this physiologic response. Unlike low doses of LPS, which induce Th2 responses, high doses of LPS are reported to induce Th1 responses (15). After using the latter procedure to sensitize mice, we found that as before (Figure 2), these mice accumulated even higher levels of neutrophils in the airway after OVA challenge than mice sensitized using low doses of LPS, which induce Th2 and Th17 responses (Figure E4). However, despite having high levels of neutrophils, Th1-responding mice fail to develop AHR. Therefore, airway neutrophilia cannot be sufficient to trigger AHR.

We next tested whether the combination of airway neutrophilia together with ongoing Th2 responses in the lung are sufficient to provoke AHR. To induce Th2 responses, we sensitized mice by intraperitoneal injections of OVA/alum and challenged with aerosolized OVA. To mimic conditions that gave rise to neutrophil recruitment in airway-sensitized mice, we administered exogenous CXCL5 directly to the airway of intraperitoneally

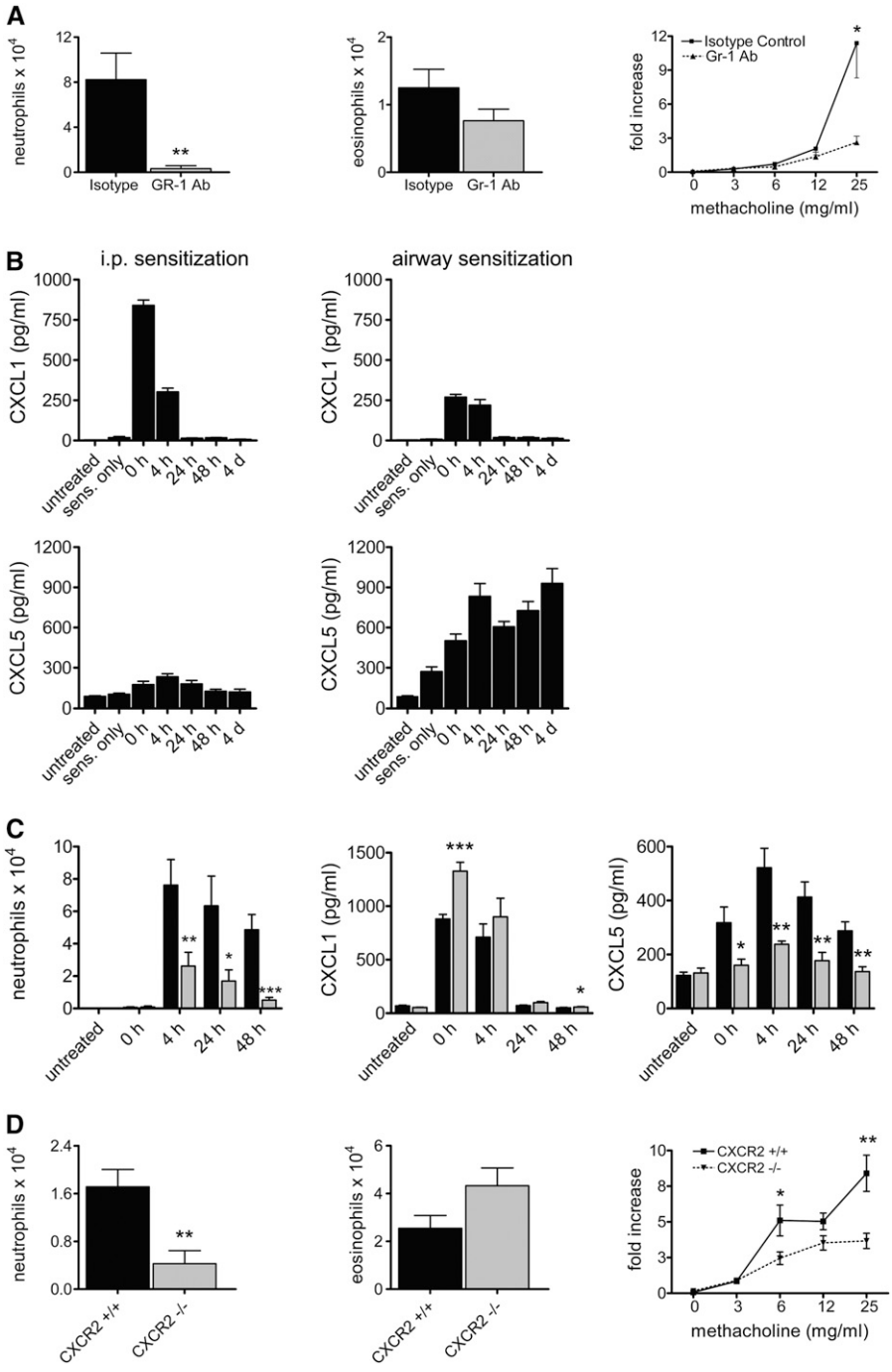

Figure 5. Neutrophil recruitment to the airway is required for airway hyperreactivity (AHR). (*A*) Mice were sensitized twice through the airway and given anti–GR-1 antibody or isotype control antibody 6 hours before ovalbumin (OVA) challenge. The accumulation of neutrophils and eosinophils in the airway is shown, as well as AHR (n = 8 mice per group). (*B*) C57BL/6 mice were sensitized twice through the airway or peritoneum as indicated and harvested at various times post OVA challenge. CXCL1 and CXCL5 levels in the bronchoalveolar lavage (BAL) were measured by ELISA. (*C*) C57BL/6 and *Il-17ra*$^{-/-}$ mice were sensitized through the airway and harvested at various times post challenge. CXCL1 and CXCL5 levels in the BAL were measured by ELISA. *Solid columns* represent IL-17R$^{+/+}$; *shaded columns* represent IL-17R$^{-/-}$. (*D*) Wild-type BALB/c and genetically matched *Cxcr2*$^{-/-}$ mice were sensitized twice through the airway. Levels of neutrophils, eosinophils, and AHR were measured at 48 hours post OVA challenge. (n = 21 mice per group) *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

sensitized mice after their challenge with OVA. Administration of this chemokine successfully induced levels of airway neutrophilia that were at least as high as those seen in airway-sensitized and challenged mice (Figure 6). However, these animals failed to develop AHR. In a similar experiment, administration of CXCL1 also induced high levels of neutrophilia, but not AHR, in intraperitoneally sensitized mice. Therefore, although required for AHR, airway neutrophilia alone is not sufficient for this response, even in the setting of ongoing Th2 responses.

Th2 and Th17 Responses Act Synergistically to Promote Neutrophilia and AHR

We next tested whether exogenous IL-17 or IL-17F is sufficient to induce neutrophilia and AHR, or if the impact of these cytokines depends on ongoing Th2 responses in recipient mice. Exogenous IL-17 was delivered to the airway of mice after intraperitoneal sensitization only, or to mice undergoing Th2 responses after both intraperitoneal sensitization and OVA challenge. In mice that had been sensitized but not challenged, IL-17 induced only moderate levels of neutrophilia (Figure 7A). However, significantly increased levels of neutrophilia were seen when this cytokine was given to allergic mice undergoing Th2 responses after both sensitization and challenge. IL-17F also elicited greater neutrophilia in sensitized and challenged mice than in mice that had been sensitized only, but it was less effective than IL-17. Neither IL-17 nor IL-17F promoted eosinophil recruitment in unchallenged or challenged mice. These findings demonstrate that IL-17 and Th2 responses act synergistically to promote airway neutrophilia.

Measurements of lung resistance in IL-17–treated mice revealed that the impact of IL-17 on AHR also depended on the immune and inflammatory status of recipient mice. Thus, in mice that had been intraperitoneally sensitized, but not challenged, IL-17 failed to significantly increase airway resistance (Figure 7B). By contrast, IL-17 triggered robust AHR in mice undergoing Th2 responses after intraperitoneal sensitization and OVA challenge. This finding shows that the ability of IL-17 to induce AHR is markedly enhanced in the context of ongoing Th2 inflammation. IL-17F failed to induce significant AHR, even in sensitized and challenged mice undergoing Th2 responses (Figure 7B). Thus, in this model of airway sensitization and challenge, IL-17, but not IL-17F, acts synergistically with ongoing Th2 responses to promote AHR.

To confirm that Th2 responses are required for IL-17–dependent AHR in airway-sensitized mice, we performed loss-of-function experiments using *Stat6*$^{-/-}$ animals. Stat6 is a transcription factor required for signaling responses to IL-4 and IL-13, and *Stat6*$^{-/-}$ mice do not develop antigen-specific IgE or AHR after intraperitoneal sensitization and multiple OVA challenges (30, 31). However, responses to IL-5 are not dependent on Stat6, and intraperitoneal injections of OVA/alum can promote Th2 responses in *Stat6*$^{-/-}$ mice (32), including eosinophil recruitment to the airway on subsequent challenge (31). The requirement of Stat6 for eosinophil recruitment and AHR in mice sensitized through the airway using LPS as an adjuvant has not been reported. As seen in our previous experiments, airway-sensitized WT mice developed both eosinophilia and neutrophilia after a single OVA challenge (Figure 7C). However, similarly-treated *Stat6*$^{-/-}$ mice were devoid of airway eosinophils, showing that, unlike intraperitoneally sensitized mice, airway-sensitized mice are completely dependent on Stat6 for eosinophilia. The *Stat6*$^{-/-}$ mice did develop modest airway neutrophilia, consistent with a previous report that Th17 responses can develop normally in these mice (22). However, levels of neutrophils in these mice were lower than those in WT mice. This result is in agreement with our gain-of-function experiments showing that Th2 responses are required for maximum IL-17–dependent neutrophil recruitment to the airways (Figure 7A). Analysis of airway resistance showed that AHR was also abolished in airway-sensitized and challenged *Stat6*$^{-/-}$ mice (Figure 7C). Therefore, both Th17 and Th2 responses are required for the development of acute AHR.

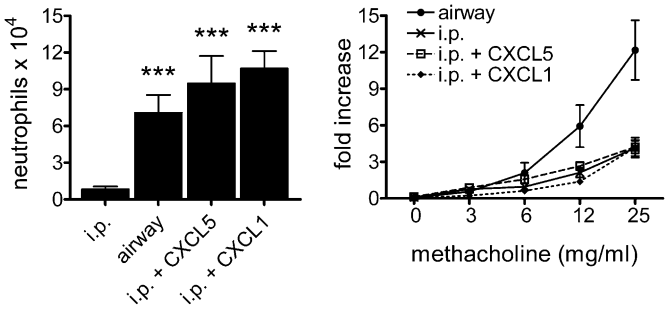

Figure 6. Airway administration of chemokines induces neutrophilia, but not airway hyperreactivity. Mice were sensitized through the airway or peritoneum and challenged on a single occasion with aerosolized ovalbumin (OVA). Where indicated, intraperitoneally sensitized mice received airway delivery of either CXCL5 or CXCL1 at 4 hours post OVA challenge. All animals were harvested 48 hours later for levels of bronchoalveolar lavage neutrophils and airway hyperreactivity. ***$P \leqslant 0.001$.

DISCUSSION

Allergic sensitization through the airway is likely the first in a cascade of events that ultimately leads to allergic asthma. To fully understand the mechanisms underlying the initiation of allergic asthma, it is essential to study early events in the lung and draining lymph nodes that determine the commitment to either immunotolerance or allergic sensitization. Sensitization through the peritoneum using alum as an adjuvant has been used for many years, and has been very useful for studying Th2-mediated responses to allergen challenge. However, the unique features of the lung suggest that inhaled allergens might provoke immune responses that are qualitatively different from those arising in the peritoneum. Accordingly, we have adopted a model in which mice are sensitized to OVA through the airway using LPS as an adjuvant. Previous descriptions of this model have shown that LPS-mediated sensitization is dependent on TLR4 and MyD88, whereas alum-mediated sensitization through the peritoneum is not (15, 33). Here, we show that immune responses arising from these two methods are also substantially different. Whereas the latter are characterized by very strong Th2 responses and sustained inflammation, the former display only modest Th2 responses but very strong Th17 responses. It is likely that the different adjuvants used in these two approaches are at least partially responsible for these immunologic differences. However, our data suggest that the route of sensitization itself also affects immune responses. This question is difficult to fully address experimentally because alum would cause asphyxiation if administered to the airways of mice. However, in the converse experiment, we found that neither low nor high doses of LPS were effective adjuvants in the peritoneum. It is possible that the lung is particularly prone to develop Th17 responses because one of the cytokines required for the development of these cells, TGF-β, is constitutively produced by alveolar macrophages (13). On its own, TGF-β primes the development of Treg cells. However, in the presence of IL-6, TGF-β supports the differentiation of Th17 cells. By inducing the production of IL-6, LPS might convert the lung from a tolerogenic environment to one that is particularly supportive of Th17 development.

We cannot rule out an involvement of IL-17–producing cells other than Th17 cells in the model used here. For example, macrophages, γδ TCR T cells, and CD1d-restricted (i)NKT cells have previously been shown to be important sources of IL-17 in the lung (26, 27, 34). However, intracellular staining for IL-17 revealed that these cell types were represented at very low frequencies compared with IL-17–containing αβ TCR T cells. Moreover, airway-sensitized CD1d-deficient mice developed neutrophilia and AHR on a single OVA challenge, indicating that iNKT cells are not required for AHR in this model (Wilson, personal communication). Thus, LPS-mediated sensitization through the airway appears to efficiently prime conventional Th17 cells that home back to the lung and are poised to release IL-17 into the airway on subsequent encounter with the sensitizing allergen. It seems possible, therefore, that inhaled aeroallergens might also provoke similar Th17-dominated immune responses in humans.

Regardless of the relative impacts of the different adjuvants and routes of sensitizations in the two models used here, comparisons between the elicited responses themselves allow several conclusions to be drawn regarding the molecular and cellular requirements for AHR. The high levels of IL-4, IL-5, and IL-13 and the robust eosinophil accumulation in the airways of intraperitoneally sensitized mice after a single challenge indicate that efficient priming had occurred. This interpretation is consistent with the previous finding that mediastinal lymph nodes, which drain the lung, also drain the peritoneal cavity and contain antigen-responsive T cells within 2 days of intraperito-

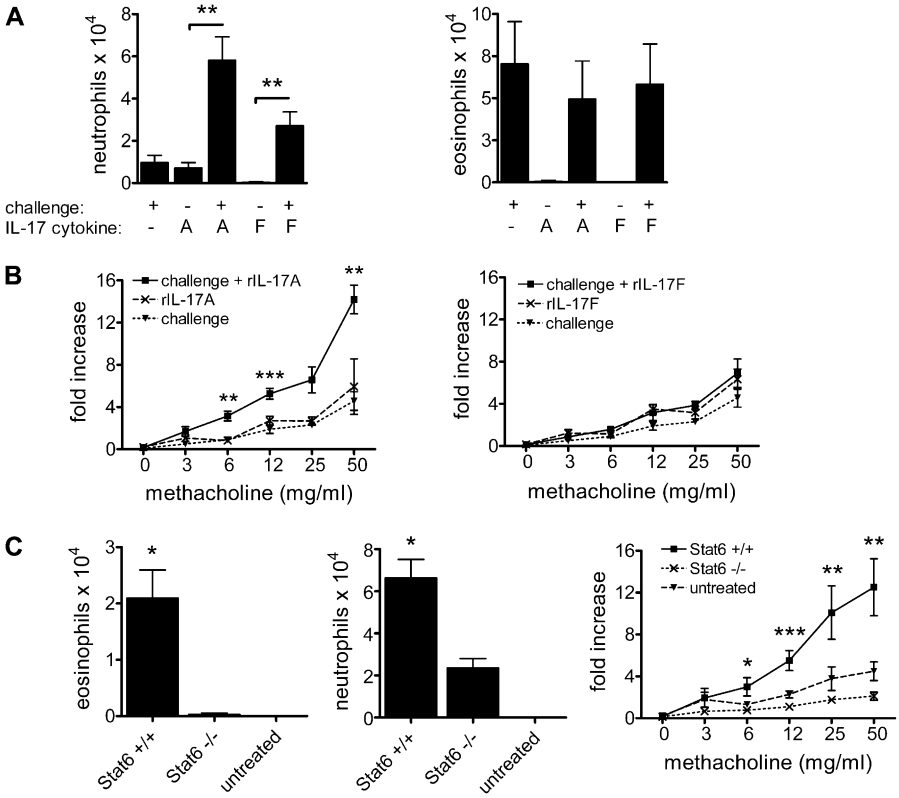

Figure 7. IL-17 and Th2 responses act synergistically to promote airway neutrophilia and airway hyperreactivity (AHR). All groups of C57BL/6 mice received intraperitoneal sensitizations to prime Th2 immunity, and some of these mice were also challenged with aerosolized ovalbumin (OVA). Where indicated, mice also received airway delivery of exogenous IL-17 or IL-17F. (*A*) Neutrophil and eosinophil recruitment to the airway 48 hours post OVA challenge. (*B*) Methacholine-induced airway resistance in mice receiving IL-17 (*left panel*) or IL-17F (*right panel*). Data shown represent the results of three similar experiments. (*C*) Wild-type and *Stat6*$^{-/-}$ mice were sensitized through the airway, challenged with OVA, and assessed for eosinophil and neutrophil accumulation in the airway, and for methacholine-induced airway hyperreactivity at 48 hours post challenge. Data shown represent the results of two similar experiments (n = 10 mice per group). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

neal sensitization (35). However, the absence of AHR in intraperitoneally sensitized mice challenged on a single occasion, despite their relatively robust Th2 responses, suggests that these Th2 responses are not sufficient for the development of AHR. The presence of AHR in airway-sensitized mice, which display only modest Th2 responses but strong Th17 responses, suggested that both Th2 and Th17 responses are required for AHR. This was confirmed by the absence of AHR in airway-sensitized *IL-17ra*$^{-/-}$ mice, which cannot respond to IL-17, and with airway-sensitized *Stat6*$^{-/-}$ mice, which cannot respond to IL-4 or IL-13. Moreover, airway delivery of exogenous IL-17 was sufficient to provoke AHR in intraperitoneally sensitized mice undergoing Th2 responses, but not in unchallenged mice.

An increasing body of evidence has revealed that neutrophils are associated with severe asthma, although the functional relevance of these cells to disease progression remains unclear (5–7). To date, animal models have not been particularly helpful in this regard because although mice sensitized through the peritoneum undergo a very transient influx of neutrophils within 8 hours of intranasal allergen challenge, these cells are no longer evident at 48 hours post challenge (10). Here, we confirm and extend these previous findings. The transient neutrophilia seen in intraperitoneally sensitized mice was associated with a similarly transient production of CXCL1, which was not dependent on IL-17RA. In contrast, the more prolonged neutrophilia seen in airway-sensitized mice after a single challenge was associated with a sustained increase in a different chemokine, CXCL5. This production of CXCL5 was in turn dependent on IL-17RA. Therefore, the transient neutrophil recruitment to the airway in intraperitoneally sensitized mice and more prolonged accumulation of these cells in airway-sensitized mice result from different signaling pathways. Moreover, blockade of neutrophil recruitment to the airway with neutrophil-depleting antibodies, or through genetic deletion of *Cxcr2*, prevented the development of AHR in airway-sensitized mice. However, neutrophil accumulation in the airway cannot be sufficient for AHR, even in the presence of eosinophils, because intraperitoneally sensitized mice that received CXCL1 or CXCL5 developed robust neutrophilia, but not AHR. This finding suggests that AHR requires the activation of neutrophils, in addition to their recruitment to the lung.

The phenotype of airway-sensitized mice appears to have been shaped by the interactions of both Th2 and Th17 immune responses. IL-17 has been previously reported to inhibit Th2 responses. Consistent with these previous observations, we found that eosinophil accumulation in the lung was increased in *Il17ra*$^{-/-}$ mice compared with WT mice. In light of this result, it was surprising that IL-17 synergized with ongoing Th2 responses to promote airway neutrophilia and AHR. This synergistic interaction was seen in both gain-of-function experiments, in which IL-17 promoted increased neutrophilia and AHR in the setting of ongoing Th2 inflammation, and in loss-of-function experiments, in which neutrophilia and AHR was reduced and abolished, respectively, in *Stat6*$^{-/-}$ mice. It is unlikely that the reduced neutrophilia seen in *Stat6*$^{-/-}$ mice is due to impaired Th17 development because these mice are reported to have normal Th17 responses (22). The absence of IL-13 signaling might account for the absence of AHR in *Stat6*$^{-/-}$ mice, but the reduction in neutrophilia seen in these mice is more difficult to explain. One possibility is that ongoing Th2 responses promote neutrophil recruitment indirectly. For example, these responses might alter the physical properties of the airway, thereby making it more accessible to neutrophils. Alternatively, one or more cytokines present during ongoing Th2 responses might act directly or indirectly on neutrophils to promote their recruitment.

It should be noted that allergic asthma is a chronic disease, whereas in the experiments here the mice were challenged on a single occasion. Thus, we do not yet know whether the model

used here will also be useful for studying chronic responses to allergen challenge. Our preliminary evidence suggests that unlike the heightened inflammation and AHR seen with continued challenges of intraperitoneally sensitized mice, continued OVA challenges suppress the acute responses seen after a single challenge of airway-sensitized mice. However, if these allergen challenges were temporarily discontinued and then resumed, robust inflammatory responses develop once again (Whitehead, personal communication). This aspect of our findings might be particularly relevant to individuals who experience infrequent, but severe, exacerbations. It is likely that in these patients, the actions of effector T cells are usually constrained, perhaps by regulatory T cells (Tregs). Interestingly, in addition to its well-described ability to induce innate immune responses, inhaled LPS can also lead to a delayed accumulation of Treg cells in the lung (Landon King, personal communication). Thus, in our model of LPS-mediated allergic sensitization and prolonged OVA challenge, Tregs might suppress the actions of Th2 and Th17 cells. The extent to which inhaled LPS determines the balance between Th17 and Treg responses in humans is not known. However, LPS is ubiquitous in the environment and it is possible that a propensity to develop Th17 responses rather than Treg responses after exposure to LPS might be one factor that contributes to asthma susceptibility. If so, this model might provide insight into the mechanisms that give rise to exacerbations in individuals whose asthma is normally asymptomatic.

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

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