IL-17A produced by αβ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction

Makoto Kudo1,2,6, Andrew C Melton1,6, Chun Chen1, Mary B Engler3, Katherine E Huang1, Xin Ren1, Yanli Wang1, Xin Bernstein1, John T Li1,4, Kamran Atabai1,5, Xiaozhu Huang1,6 & Dean Sheppard1,6

Emerging evidence suggests that the T helper 17 (Th17) subset of αβ T cells contributes to the development of allergic asthma. In this study, we found that mice lacking the αvβ8 integrin on dendritic cells did not generate Th17 cells in the lung and were protected from airway hyper-responsiveness in response to house dust mite and ovalbumin sensitization and challenge. Because loss of Th17 cells inhibited airway narrowing without any obvious effects on airway inflammation or epithelial morphology, we examined the direct effects of Th17 cytokines on mouse and human airway smooth muscle function. Interleukin-17A (IL-17A), but not IL-17F or IL-22, enhanced contractile force generation of airway smooth muscle through an IL-17 receptor A (IL-17RA)–IL-17RC, nuclear factor κ light-chain enhancer of activated B cells (NF-κB)–ras homolog gene family, member A (RhoA)–Rho-associated coiled-coil containing protein kinase 2 (ROCK2) signaling cascade. Mice lacking integrin αvβ8 on dendritic cells showed impaired activation of this pathway after ovalbumin sensitization and challenge, and the diminished contraction of the tracheal rings in these mice was reversed by IL-17A. These data indicate that the IL-17A produced by Th17 cells contributes to allergen-induced airway hyper-responsiveness through direct effects on airway smooth muscle.

Tth2 cells are classically thought to drive the development of asthma through the production of IL-4, IL-5, IL-9 and IL-13 (refs 1,2), but recent evidence suggests that the Tth17-derived cytokine, IL-17A, may also have a key role1-5. Tth17 cells make up a pro-inflammatory subset of T helper cells that is defined by its production of IL-17A, IL-17F and IL-22, and Tth17 cells are thought to have evolved to aid in host defense against bacteria and fungi6. Elevated concentrations of IL-17A have been found in the serum, sputum and bronchoalveolar lavage fluids (BAL) of patients with asthma, and the IL-17A concentration found at these sites positively correlates with asthma severity in these patients7,8. Furthermore, genetic association studies in patients with asthma have identified variations in several genes that are key for Tth17 cell differentiation, including those encoding transforming growth factor β1 (TGF-β1)9-11, IL-1β12 and IL-6 (ref. 13). Although Tth17 cells seem to have a role in the development of allergic asthma, the mechanisms controlling their development in the lung are less clear.

Initial studies of Tth17 differentiation showed that IL-6 and TGF-β together are sufficient for the conversion of naive T cells into Tth17 cells14,15. The combination of T cell receptor (TCR) stimulation and the presence of TGF-β induces the synthesis of the retinoic-acid–related orphan receptor γ (RORγt) transcription factor, which is specific for the Tth17 lineage among the T cell subsets16. IL-6 signaling triggers binding of signal transducer and activator of transcription 3 (STAT3) to the RORγt locus, resulting in a positive feedback loop that drives T cell differentiation towards the Tth17 lineage17. More recent studies have shown that other stimuli, such as IL-1β, IL-21, IL-23, commensal-bacteria–derived ATP, segmented filamentous bacterium and activators of the aryl hydrocarbon receptor, also promote differentiation of Tth17 cells or expand Tth17 populations18-22. However, TGF-β is a required factor for Tth17 cell development in both humans and mice, and its regulation is probably a crucial mechanism that controls the differentiation of Tth17 cells22,23. TGF-β is produced as part of a latent complex and needs to be activated to bind TGF-β receptors24. We recently reported that integrin αvβ8 expressed on dendritic cells activates latent TGF-β and mediates the differentiation of both adaptive regulatory T cells and Tth17 cells in the colon25,26. However, the relevance of this pathway for T cell differentiation in the lung and in the development of allergic airway disease has not been previously investigated.

Tth17-derived cytokines promote inflammation by a variety of mechanisms. IL-17A enhances neutrophil recruitment by stimulating bone marrow stromal cells to produce granulocyte colony-stimulating factor (G-CSF), which augments granulopoiesis and the conversion of neutrophil progenitors from CD34+ cells27. IL-17A also induces neutrophil recruitment by stimulating the expression of chemokine

1Lung Biology Center, Department of Medicine, University of California, San Francisco, California, USA. 2Department of Internal Medicine and Clinical Immunology, Yokohama City University, Graduate School of Medicine, Kanazawa, Yokohama, Japan. 3Department of Physiological Nursing, University of California, San Francisco, California, USA. 4Department of Pediatrics, University of California, San Francisco, California, USA. 5Cardiovascular Research Institute, University of California, San Francisco, California, USA. 6These authors contributed equally to this work. Correspondence should be addressed to D.S. (dean.sheppard@ucsf.edu).

Received 29 September 2011; accepted 20 January 2012; published online 4 March 2012; doi:10.1038/nm.2684
OVA-treated control and P samples were not statistically significant (NS) compared to saline-treated from the lungs. The top and middle rows show CD4 + samples. The amounts of each type of cell in both of the lial cells28. IL-17A promotes antibody isotype class switching in B cells near-complete absence of T H17 cells in the lung. We therefore tested lung inflammation and found that this protection correlated with a from airway hyper-responsiveness (AHR) without any changes in ease. Notably, we observed that the mice in this model were protected expressed on dendritic cells in a mouse model of allergic airway dis - derived cytokines in the disease remain largely unexplored.

IL-17A promotes antibody isotype class switching in B cells and stimulates T H17 cells in allergic asthma and show that IL-17A released from these cells con -tributes to airway hyper-responsiveness by directly increasing the contractility of ASM.

**RESULTS**

**Igfbp3lox/lox, CD11c-Cre mice are protected from AHR**

To determine the role of integrin αβ8 on dendritic cells in allergic airway disease, we sensitized and challenged control mice or mice lacking integrin αβ8 on their dendritic cells (Igfbp3lox/lox, CD11c-Cre mice), as we previously described25,26. OVA sensitization and challenge increased AHR in response to the bronchoconstrictor acetylcholine in control mice, but Igfbp3lox/lox, CD11c-Cre mice were significantly protected from allergen-induced AHR (Fig. 1a).

To determine the mechanism for the diminished AHR seen in Igfbp3lox/lox, CD11c-Cre mice, we characterized mucus production and leukocyte accumulation in the lungs of these mice after OVA sensitization and challenge. Control mice and Igfbp3lox/lox, CD11c-Cre
mice had similar numbers of mucus-secreting cells, as determined by periodic acid-Schiff (PAS) staining, as well as similar numbers of macrophages, eosinophils, lymphocytes and polymorphonuclear cells in the BAL fluid (Fig. 1b–d). We also examined whether IL-17A and CD11c-Cre mice were protected from AHR in the house dust mite (HDM) asthma model. Il17bflx/flx, CD11c-Cre mice had significantly (P < 0.05) reduced AHR after HDM sensitization and challenge when compared to control mice (Supplementary Fig. 1a). Intranasal delivery of IL-13 is known to provoke AHR, mucus production and leukocyte accumulation in the lungs of mice.31 Therefore, we tested whether Il17bflx/flx, CD11c-Cre mice were capable of developing AHR after intranasal administration of IL-13 to their lungs. Intranasal IL-13 increased similar amounts of AHR in both Il17bflx/flx, CD11c-Cre and control mice, indicating that Il17bflx/flx, CD11c-Cre mice have the capacity to develop AHR (Supplementary Fig. 1b).

Because AHR in response to OVA or HDM requires an adaptive immune response, and our previous work implicated integrin αvβ8 on dendritic cells in the regulation of CD4+ T-cell differentiation, we tested the recall response of lymphocytes to OVA and characterized the CD4+ T-cell subsets in the lungs of the mice from the two groups. Control and Il17bflx/flx, CD11c-Cre mice had similar lymphocyte recall responses to OVA (Fig. 1e). T cells, identified by intracellular flow cytometry for IL-13, were increased similarly in response to OVA in control and Il17bflx/flx, CD11c-Cre mice (Fig. 1f,g). We found less Foxp3+ regulatory T cells in the lungs of Il17bflx/flx, CD11c-Cre mice compared to control mice before OVA sensitization and challenge, but there was no significant difference in the percentage of regulatory T-cells in the lungs of Il17bflx/flx, CD11c-Cre mice compared to control mice induced in response to OVA (Fig. 1f,g). OVA sensitization and challenge increased the percentage of Treg cells, marked by expression of IL-17A and CD44, in the lungs of control mice, but Il17bflx/flx, CD11c-Cre mice had a near absence of Treg cells before and after OVA sensitization and challenge. Notably, the inability to generate IL-17A–producing T cells in Il17bflx/flx, CD11c-Cre mice was restricted to the Treg lineage, as the number of IL-17A–producing γδ T cells in these mice was comparable to that seen in the control mice (Fig. 1f,g). This finding suggests that Il17bflx/flx, CD11c-Cre mice may be protected from AHR through a specific defect in the generation of Treg cells.

IL-17A enhances ASM contraction

IL-13 and tumor necrosis factor α (TNF-α) have been reported to act directly on ASM to enhance AHR, and we wondered whether IL-17 cytokines might also act in this way.32,33 We detected mRNA for both IL-17 receptor A (IL-17RA) and IL-17RC in cultured primary ASM cells from wild-type C57BL/6 mice (Fig. 2a). We then determined the effects of IL-17A on the contractile force generated by mouse tracheal rings stimulated with MCh or depolarization with KCl and on the degree of airway narrowing induced by MCh in agarose-filled lung slices. IL-17A treatment alone had no effects on contraction when applied for 15 min directly to tracheal rings in the organ bath (data not shown). However, incubation with IL-17A for 12 h significantly enhanced both MCh- and KCl-induced tracheal ring contraction (Fig. 2b) and MCh-induced airway narrowing in the lung slices (Fig. 2c,d). To determine whether IL-17A was acting on the smooth muscle or the epithelium, we denuded the epithelium from the tracheal rings and then measured the effects of IL-17A on their contraction. As others have reported, epithelial removal enhanced the amount of force generated in response to MCh and KCl (Supplementary Fig. 2).34,35 In tracheal rings completely denuded of epithelium, IL-17A increased the contraction to a similar degree as it did in intact tracheal rings, indicating that IL-17A acts directly on smooth muscle to enhance contraction. We next determined whether IL-17A also enhanced ASM contraction in human tissue. In isolated human bronchi, a 12-h pretreatment with IL-17A significantly increased the contraction of human bronchi in response to MCh and KCl (Fig. 2e). These data suggest that IL-17A acts on ASM in mice and humans to enhance contractile responses.

To test whether IL-17A was interacting directly or indirectly with the ASM, we added atropine, a muscarinic receptor antagonist, to tracheal rings from wild-type C57BL/6 mice to determine whether IL-17A augmented contractile responses through modulation of MCh signaling. Atropine completely prevented contractile responses triggered by MCh in tracheal rings pretreated with IL-17A but had no effect on the contractile responses stimulated by KCl (Fig. 3a). We then confirmed that enhanced contraction was a result of IL-17A and not of other contaminants in our IL-17A preparation. Concurrent incubation with IL-17A and antibodies that block IL-17A activity prevented the enhanced contraction seen with IL-17A alone in response to MCh and KCl (Fig. 3b). TNF-α is known to enhance ASM
Figure 3  IL-17A–mediated enhanced ASM contraction is not caused by contaminants or TNF-α release. (a-d) Contractile force measurements from tracheal rings stimulated with MCh or KCl after treatment with or without IL-17A in the presence of atropine (a), IL-17–neutralizing antibodies (anti–IL-17) or isotype control antibodies (b), TNF–α–neutralizing antibodies (anti–TNF) or isotype control antibodies (c), and TNF–α in the presence of TNF–α–neutralizing antibodies or isotype control antibodies (d). *P < 0.05, **P < 0.01, ***P < 0.001 in IL-17A– or TNF–α–treated samples compared to control samples. Data are means ± s.e.m. of at least five tracheal rings per group. Student’s t test was used to calculate statistical significance throughout this figure.

Figure 4  IL-17A activates NF-κB, RhoA and ROCK2. Contractile force in ASM is generated through actin–myosin cross-bridging and is regulated by myosin light chain (MLC) phosphorylation. To determine whether IL-17A enhances ASM contraction through regulation of MLC phosphorylation, we measured MLC phosphorylation with an antibody specific to phosphorylated Ser19 of MLC. IL-17A markedly increased MCh-induced Ser19 phosphorylation of MLC (Fig. 4a,b). IL-17A has been reported to activate of the NF-κB pathway. To determine whether IL-17A enhanced ASM contraction through activation of NF-κB, we measured NF-κB activation in tracheal ASM from wild-type C57BL/6 mice after treatment with 100 ng ml⁻¹ IL-17A. IL-17A rapidly decreased the cytosolic concentration of NF-κB and induced the nuclear translocation of NF-κB, indicating that IL-17A activates NF-κB in tracheal rings at a concentration that also promotes enhanced contraction (Fig. 4c,d). To determine whether IL-17A–induced activation of NF-κB is required for this enhanced contraction, we treated tracheal rings with the NF-κB small-molecule inhibitor BAY 11-7082. BAY 11-7082 dose-dependently reduced the IL-17A–mediated enhanced contractile response to both MCh and KCl.
Figure 5 IL-17F and IL-22 do not enhance ASM contraction, and IL-17A signals through IL-17RC. (a) Contractile force measurements from tracheal rings treated with or without IL-17F or IL-22 and stimulated with MCh or KCl. *P < 0.05 in IL-17A–treated samples compared to control-treated, IL-17F–treated or IL-22–treated samples. (c) Western blot analysis of p-MLC and MLC in tracheal smooth muscle samples treated with IL-17A, IL-17F or IL-22 and MCh. (d) Contractile force measurements from tracheal rings isolated from control or Il17rc−/− mice treated with or without IL-17A and stimulated with increasing doses of MCh or KCl. *P < 0.05, **P < 0.01 in IL-17A–treated control samples compared to Il17rc−/− samples. Data are means ± s.e.m of at least five tracheal rings per group. (e) Pulmonary resistance measurements after administration of acetylcholine in control and Il17rc−/− mice immunized and intranasally challenged with OVA or saline. *P < 0.05 in OVA-treated control mice compared to OVA-treated Il17rc−/− mice (n = 9). Student’s t-test was used to calculate statistical significance throughout this figure, except for the analysis of pulmonary resistance measurements, in which a t-test with Bonferroni adjustment was used.

MCh and KCl (Fig. 4e). NF-κB is known to regulate the expression of the small GTPase RhoA and its effector kinases ROCK1 and ROCK2 (refs. 39,40). The RhoA-ROCK signaling pathway is a key mediator of ASM contraction. Treatment of tracheal rings with IL-17A increased the expression of RhoA and ROCK2 but not ROCK1 (Fig. 4f,g). ROCK2 regulates MLC phosphorylation through phosphorylation of the myosin-binding subunit of myosin phosphatase (MYPT1), which inhibits the activity of myosin phosphatase. IL-17A treatment of ASM stimulated MYPT1 phosphorylation (Fig. 4f,g).

To determine whether the RhoA-ROCK2-MYPT1 pathway is required for IL-17A–mediated enhanced contraction, we blocked ROCK activity with the small-molecule inhibitor Y-27632. Y-27632 inhibited the IL-17A–mediated enhancement of tracheal ring contraction in a dose-dependent manner (Fig. 4h). To test whether stimulation of RhoA and ROCK2 expression was mediated through NF-κB, we also measured the expression of these proteins after treatment of tracheal rings with BAY 11-7082. Pretreatment of tracheal rings with BAY 11-7082 inhibited the effects of IL-17A on the expression of RhoA and decreased the phosphorylation of MYPT1 and MLC after stimulation with MCh (Fig. 4i). Treatment with Y-27632 under similar conditions had no effect on nuclear NF-κB translocation or RhoA expression but did block the phosphorylation of MYPT1 and MLC (Fig. 4i). We also determined whether IL-17A regulated ASM contraction through modulation of calcium signaling. Treatment of lung slices with IL-17A had no effect on MCh-induced calcium-wave oscillation amplitude or frequency (Supplementary Fig. 3). Collectively, these data indicate that IL-17A enhances the contractile responses of ASM through the activation of NF-κB and the subsequent induction of RhoA and ROCK2 expression.

Figure 6 Impaired activation of an IL-17A signaling pathway in Itgb8flox/flox; Cd11c-Cre mice. (a) Western blots were prepared as described in Figure 3 with tracheal smooth muscle samples isolated from control and Itgb8flox/flox; Cd11c-Cre mice immunized with saline or OVA. Lysates from five individual mice in the saline-immunized groups and lysates from six individual mice in the OVA-immunized groups are shown. (b) Contractile force measurements from tracheal rings isolated from control and Itgb8flox/flox; Cd11c-Cre mice immunized with saline or OVA and treated with or without IL-17A followed by stimulation with MCh or KCl. *P < 0.05, **P < 0.01, ***P < 0.001 in IL-17A–treated samples from control mice compared to saline-treated samples (above left and right graphs), or IL-17A–treated samples from Itgb8flox/flox; Cd11c-Cre mice compared to control samples (below left and right graphs). (c) Contractile force measurements from IL-17A–treated samples from OVA-immunized control and Itgb8flox/flox; Cd11c-Cre mice and treated with the indicated concentrations of IL-17A followed by stimulation with MCh or KCl. *P < 0.05, **P < 0.01 in IL-17A–treated samples from control mice compared to IL-17A–treated samples from Itgb8flox/flox; Cd11c-Cre mice. t-test with Bonferroni adjustment was used to calculate statistical significance in b. Student’s t-test was used to calculate significance in c.
We next explored whether other cytokines produced by $T_{H17}$ cells promote ASM contraction. Incubation of tracheal rings from wild-type C57BL/6 mice with IL-17F or IL-22 for 12 h at a range of doses (30–300 ng ml$^{-1}$) had no significant effect on MCh- or KCl-induced contractile responses (Fig. 5a,b). In contrast, incubation with IL-17A dose-dependently increased MCh- and KCl-induced force generation (Fig. 5b). Treatment with 100 ng ml$^{-1}$ IL-17F or 100 ng ml$^{-1}$ IL-22 followed by stimulation with MCh also had no effect on MLC phosphorylation, whereas IL-17A at this dose triggered a substantial increase in MLC phosphorylation (Fig. 5c). IL-17A and IL-17F both bind to the IL-17RA–IL-17RC heterodimer$^{43}$. Despite this, administration of 300 ng ml$^{-1}$ IL-17F had no effect on ASM contractile responses. Therefore, we confirmed that IL-17A enhanced ASM contraction through binding to IL-17RA and IL-17RC. IL-17A did not enhance the contractile responses in tracheal rings isolated from mice deficient in IL-17RC after stimulation with MCh and KCl (Fig. 5d). Mice deficient in IL-17RC were also protected from AHR in the OVA sensitization and challenge model of allergic asthma, which provides further evidence that IL-17A promotes the development of AHR in this model (Fig. 5e).

To determine whether mice lacking integrin $\alpha$V$\beta$8 on their dendritic cells were protected from AHR through impaired activation of the NF-$\kappa$B–RhoA–ROCK2 signaling cascade in response to IL-17A, we studied the activation of this pathway in OVA-challenged control and $\text{Itgb8}^{\text{flox/flox}}$; $\text{CD11c-Cre}$ mice. OVA sensitization and challenge induced a robust increased the amount of nuclear NF-$\kappa$B, RhoA, ROCK2, phosphorylated MYPT1 (p-MYPT1) and p-MLC in ASM dissected from control mice but had substantially reduced effects on each of these endpoints in ASM from $\text{Itgb8}^{\text{flox/flox}}$; $\text{CD11c-Cre}$ mice (Fig. 6a). Tracheal rings from OVA-sensitized and OVA-challenged $\text{Itgb8}^{\text{flox/flox}}$; $\text{CD11c-Cre}$ mice also had impaired contractile responses to both MCh and KCl (Fig. 6b). To test whether a lack of IL-17A was responsible for these impaired contractile responses, we incubated tracheal rings from OVA-sensitized and OVA-challenged control and $\text{Itgb8}^{\text{flox/flox}}$; $\text{CD11c-Cre}$ mice with IL-17A. Preincubation of tracheal rings from OVA-sensitized and OVA-challenged $\text{Itgb8}^{\text{flox/flox}}$; $\text{CD11c-Cre}$ mice with IL-17A enhanced MCh-induced force generation in a dose-dependent manner to the amounts seen in control mice (Fig. 6c). IL-17A preincubation of tracheal rings from OVA-sensitized and OVA-challenged control mice had minimal effects on contractility, possibly because these tissues had already been exposed to IL-17A in vivo. These data suggest that mice lacking integrin $\alpha$V$\beta$8 on their dendritic cells are protected from AHR as a consequence of reduced exposure of ASM to IL-17A.

**DISCUSSION**

A growing body of evidence implicates $T_{H17}$ cells and their associated cytokines in allergic asthma, especially in severe cases in which inflammation is predominantly driven by neutrophils$^{3,4}$. The data from this study indicate a previously unidentified mechanism for $T_{H17}$ cells in asthma pathogenesis. We show that IL-17A enhances the contractile response of ASM to MCh and KCl. The IL-17 cytokine family signals through heterodimeric complexes of IL-17RA and IL-17RE, and IL-17A and IL-17F are known to bind the IL-17RA–IL-17RC heterodimer in other tissues$^{28}$. It was somewhat surprising to us that IL-17F, which, like IL-17A, can ligate IL-17RA–IL-17RC heterodimers, did not affect ASM contractility over a range of concentrations that were sufficient to produce the effects of IL-17A. We suspect that this result reflects the lower affinity of IL-17F for these receptors$^{43}$. The NF-$\kappa$B transcription factor is a major target of pro-inflammatory cytokines, and we found here that IL-17A causes a robust activation of NF-$\kappa$B after stimulation of ASM by MCh. Because the primary phenotype we observed after IL-17A treatment was enhanced contraction, we looked for NF-$\kappa$B target genes that would modulate ASM contraction. IL-17A treatment increased RhoA and ROCK2 expression, which are two prominent regulators of MLC phosphorylation and subsequent smooth muscle contraction$^{36}$. Several groups have reported in studies of smooth muscle that activation of RhoA stimulates ROCK2 to phosphorylate MYPT1$^{44–46}$. This pathway was also stimulated in ASM of OVA-sensitized and OVA-challenged mice and was impaired in mice lacking integrin $\alpha$V$\beta$8 on their dendritic cells. Further, reduced tracheal ring contraction in OVA-sensitized and OVA-challenged mice lacking integrin $\alpha$V$\beta$8 on their dendritic cells results from impaired IL-17A signaling. Because these mice had a near absence of pulmonary IL-17A–producing $\gamma\delta$ T cells (T $\gamma$$\delta$), but had similar numbers of IL-17A–producing $\gamma\delta$ T cells as control mice before and after OVA sensitization and challenge, our data suggest a model in which the IL-17A produced by T $\gamma$$\delta$ cells acts on ASM to induce AHR.

$T_{H17}$ cells and IL-17A have been implicated in several mouse models of asthma, although some of the data from these models are conflicting. Adoptive transfer of antigen-specific TCR transgenic T cells polarized in vitro to the $T_{H17}$ phenotype into antigen-challenged mice enhances AHR, as does pulmonary administration of an IL-17A–overexpressing adenovirus$^{47}$. Further, as we observed in this study with IL-17RC–deficient mice, IL-17RA–deficient mice have been reported to show substantial protection from AHR in an OVA sensitization and challenge model of allergic asthma$^{48}$. However, that same previous study found that neutralization of IL-17A at the time of OVA challenge exacerbated AHR and that local administration of IL-17A reduced eosinophilia and AHR. Another study using a similar model showed impaired induction of AHR in IL-17A–deficient mice crossed with OT-II TCR transgenic mice$^{49}$. In several other studies, neutralization of IL-17A with antibodies or administration of exogenous IL-17A yielded mixed effects on allergen-induced AHR, but the preponderance of evidence supports a key role for IL-17A in allergic airway inflammation$^{50–53}$. Notably, the relative contributions of various cells and cytokines to models of allergic asthma can differ among inbred strains of mice, which might be one explanation for the apparently conflicting reports about the contributions of IL-17 to allergic asthma. Because we performed our study described here in C57BL/6 mice, we cannot be certain that the same effects would be seen in other strains (for example, BALB/c mice) in which T $\gamma$$\delta$ cells have more potent effects. IL-17A was recently shown to act synergistically with IL-13 to enhance AHR in the A/J mouse strain, whereas the C3H/HeJ mouse strain is naturally protected from AHR through decreased production of IL-17A$^{5}$. Indeed, we and others have previously reported that inhibition of IL-13 can potently inhibit AHR in models of allergic asthma and that overexpression or administration of IL-13 is sufficient to induce AHR, in part through direct effects of IL-13 on airway epithelial cells. Based on the results of this study, we suspect that transgenic or intranasal administration of IL-13 results in higher concentrations of IL-13 than those released in the context of allergen challenge and that IL-17 may not be required for the development of AHR under these circumstances. Although inhibitor studies have confirmed that secreted IL-13 contributes to the development of allergen-induced AHR$^{54,55}$, our results suggest that the small amounts
of IL-13 released in response to allergen challenge are not sufficient to induce AHR but, rather, require the additional effect of IL-17.

The results of this study provide several new insights into how T_{H}17 cells and T_{H}17 cytokines contribute to the development of allergic asthma and, thus, identify a number of new potential therapeutic targets. Our results identify integrin αβ8 as a crucial upstream activator that is essential for T_{H}17 cell generation in allergic airway disease. We also show that loss of this integrin on dendritic cells markedly inhibits T_{H}17 cell generation without having any effects on the IL-17A-producing γδ T cells, suggesting that it might be possible to inhibit this T_{H}17 induction without eliminating the key role of IL-17A-producing γδ T cells in the pulmonary defense against bacterial and fungal pathogens. Furthermore, our findings are the first, to our knowledge, to show that IL-17A, but not IL-17F or IL-17RA, are major determinants of AHR.

The results of this study provide several new insights into how TH17 cells contribute to the development of allergic airway disease. The TH17 lymphocyte subset is characterized by the production of IL-17A, IL-17F, and IL-17RA cytokines. A critical role for IL-17A in the development of allergic airway disease is established by the findings that mice deficient in IL-17A (Tgat deficient) are protected from allergic airway disease induced by allergen challenge. This protective effect is mediated by the IL-17RA receptor, which is expressed on T cells and macrophages. These findings suggest that targeting IL-17A might be a novel therapeutic approach for the treatment of allergic airway disease.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health (NIH) grants HL64353, HL33949, HD083950, AI024674 and U19 AI077439 (to D.S.), a NIH Ruth L. Kirschstein National Research Service Award HL095314 (to A.C.M.), funds from the University of California, San Francisco (UCSF) Strategic Asthma Basic Research Center. Identified human lung was kindly provided by M. Mathay (UCSF) and P. Wolters (UCSF). IL-17RC knockout mice were kindly provided by M. Matthay (UCSF) and P. Wolters (UCSF). IL-17RC knockout mice were kindly provided by M. Matthay (UCSF) and P. Wolters (UCSF).

AUTHOR CONTRIBUTIONS

M.K. designed the study, generated all of the tracheal ring contraction and western blot data, performed analyses and wrote the manuscript. A.C.M. designed the study, generated all of the flow cytometry and recall data, performed analyses and wrote the manuscript. C.C. generated all of the lung slice data, performed analyses and wrote the manuscript. A.C.M. designed and constructed the muscle bath and provided expertise in the methodology and modifications for the analysis of mouse tracheal ring contraction. I.L. and K.A. provided substantial intellectual contribution. X.H. and D.S. oversaw the design and interpretation of all studies described and oversaw the writing of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
40. Shimada, H. & Rajagopalan, L.E. Rho kinase-2 activation in human endothelial cells drives lysophosphatidic acid-mediated expression of cell adhesion molecules via NF-κB p65. *J. Biol. Chem.* **285**, 12536–12542 (2010).

41. Kimura, K., Choudhry, N., Stevenson, A.S., Somlyo, A.V. & Eto, M. Phosphorylation-dependent autoinhibition of myosin light chain phosphatase accounts for Ca^{2+}-sensitization force of smooth muscle contraction. *J. Biol. Chem.* **284**, 21569–21579 (2009).

42. Eck, K., Fischer, S. & Garcia, K.C. Structural basis of receptor sharing by interleukin 17 cytokines. *Nat. Immunol.* **10**, 1245–1251 (2009).

43. Patil, S.B. & Bitar, K.N. RhoA- and PKC-α-mediated phosphorylation of MYPT and its association with HSP27 in colonic smooth muscle cells. *An. J. Physiol. Gastrointest. Liver Physiol.* **290**, G83–G95 (2006).

44. Ohama, T., Hori, M., Sato, K., Ozaki, H. & Karaki, H. Chronic treatment with interleukin-1β attenuates contractions by decreasing the activities of CPI-17 and MYPT-1 in intestinal smooth muscle. *J. Biol. Chem.* **278**, 48794–48804 (2003).

45. Chen, X.Y., Dun, J.N., Miao, Q.F. & Zhang, Y.J. Fasudil hydrochloride hydrate, a Rho-kinase inhibitor, suppresses 5-hydroxytryptamine–induced pulmonary artery smooth muscle cell proliferation via JNK and ERK1/2 pathway. *Pharmacology* **83**, 67–79 (2009).

47. McKinley, L. et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* **181**, 4089–4097 (2008).

48. Schnyder-Candrian, S. et al. Interleukin-17 is a negative regulator of established allergic asthma. *J. Exp. Med.* **203**, 2715–2725 (2006).

49. Nakae, S. et al. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* **17**, 375–387 (2002).

50. Wilson, R.H. et al. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* **180**, 720–730 (2009).

51. Oda, N. et al. Interleukin-17F induces pulmonary neutrophilia and amplifies antigen-induced allergic response. *Am. J. Respir. Crit. Care Med.* **171**, 12–18 (2005).

52. Hellings, P.W. et al. 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 (2003).

53. Song, C. et al. IL-17–producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J. Immunol.* **181**, 6117–6124 (2008).

54. Willis-Karp, M. et al. Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258–2261 (1998).

55. Grünig, G. et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**, 2261–2263 (1998).
ONLINE METHODS

Mice. We generated mice lacking integrin αβ8 on their dendritic cells (αβ8fl/fl, CD11c-Cre) and mice deficient in IL-17RC (Il17rc−/−), as previously described25,56. All mice were on the C57BL/6 background, and experiments were approved by the Institutional Animal Care and Use Committee of UCSF.

Allergen challenge models. We sensitized equal numbers of male and female 6- to 8-week-old mice on days 0, 7, 14 and 18 by intraperitoneal injection of 50 µg of OVA (Sigma-Aldrich) emulsified in 1 mg of aluminum potassium sulfate. One week after the last sensitization, mice were intranasally challenged on 3 consecutive days with 100 µg OVA in 40 µl saline. Alternatively, 40 µl dust mite fecal pellet preparation (2.5 mg/ml; Greer Laboratories) or saline was administered intranasally on days 0, 7, 14 and 21. Twenty-four hours after the last OVA challenge and 72 h after the last HDM challenge, mice were attached with 1.0 ml of 2% agarose (37 °C, low-melt preparative agarose) through a tracheal catheter. Air (0.2 ml) was injected to flush the agarose out of the airways. Lungs were cooled to 4 °C, and serial 140-µm sections were cut with a vibratome (model EMS-4000, EMS). Slices were maintained in DMEM supplemented with antibiotics at 37 °C and 10% CO2 for up to 3 d. Images of whole-lung slices were acquired using the area scan function and 5× objective on a temperature-controlled Leica DM6000 inverted microscope at 37 °C.

For measurement of calcium oscillations, lung slices were loaded with Oregon green 488 BAPTA-AM (20 µM in Hank’s Balanced Salt Solution (HBSS) containing 0.1% Pluronic F-127 and 100 µM sulfobromophthalein) for 45 min at 30 °C and de-esterified for 30 min at 30 °C in HBSS containing 100 µM sulfobromophthalein. Fluorescence imaging was performed with a Nikon spinning disk confocal microscope at 20 frames per second. Changes in fluorescence intensity from selected regions of interest (5 × 5 pixels) were analyzed with ImageJ software.

Western blots. Smooth muscle was dissected from control or αβ8fl/fl, CD11c-Cre mouse tracheas and homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P40, 10 mM NaF, 1 mM Na3VO4) and complete Mini protease inhibitor cocktail (Roche). TKM buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl2) with 0.25 M sucrose was used for the nuclear extracts. Samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked for 1 h in 5% skim milk or BSA, incubated for 2 h with primary antibodies, incubated for 1 h with peroxidase-conjugated secondary antibody and developed with ECL reagent (PerkinElmer).

Statistical analyses. The statistical significance of the differences between paired groups was calculated with a two-tailed Student’s t test. One-way analysis of variance was used for comparisons of multiple groups using SigmaStat 3.11, and when differences were statistically significant (P ≤ 0.05), this was followed with a Bonferroni t test for subsequent pairwise analysis.

56. Zheng, Y. et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat. Med. 14, 282–289 (2008).
57. Bai, Y. & Sanderson, M.J. Modulation of the Ca2+ sensitivity of airway smooth muscle cells in murine lung slices. Am. J. Physiol. Lung Cell. Mol. Physiol. 291, L208–L221 (2006).