

# Interleukin-17 is a negative regulator of established allergic asthma

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**T helper (Th)17 cells producing interleukin (IL)-17 play a role in autoimmune and allergic inflammation. Here, we show that IL-23 induces IL-17 in the lung and IL-17 is required during antigen sensitization to develop allergic asthma, as shown in IL-17R-deficient mice. Since IL-17 expression increased further upon antigen challenge, we addressed its function in the effector phase. Most strikingly, neutralization of IL-17 augmented the allergic response in sensitized mice. Conversely, exogenous IL-17 reduced pulmonary eosinophil recruitment and bronchial hyperreactivity, demonstrating a novel regulatory role of IL-17. Mechanistically, IL-17 down modulated eosinophil-chemokine eotaxin (CCL11) and thymus- and activation-regulated chemokine/CCL17 (TARC) in lungs in vivo and ex vivo upon antigen restimulation. In vitro, IL-17 reduced TARC production in dendritic cells (DCs)—the major source of TARC—and antigen uptake by DCs and IL-5 and IL-13 production in regional lymph nodes. Furthermore, IL-17 is regulated in an IL-4-dependent manner since mice deficient for IL-4R $\alpha$  signaling showed a marked increase in IL-17 concentration with inhibited eosinophil recruitment. Therefore, endogenous IL-17 is controlled by IL-4 and has a dual role. Although it is essential during antigen sensitization to establish allergic asthma, in sensitized mice IL-17 attenuates the allergic response by inhibiting DCs and chemokine synthesis.**

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Abbreviations used: BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; Penh, enhanced respiratory pause; TARC, thymus- and activation-regulated chemokine/CCL17; Th cell, T helper cell; T reg cell, T regulatory.

Allergic asthma is a chronic inflammatory disorder of the lung with a prevailing T helper (Th)2 immune response to inhaled allergens leading to bronchial hyperreactivity, recruitment of eosinophils, mast cells, and lymphocytes, and hyperplasia of smooth muscle and goblet cells, often associated with increased serum IgE concentrations (1). Central to the pathogenesis of allergic airway disease are antigen-specific memory T cell responses. Th2 cells are recruited along concentration gradients of the thymus- and activation-regulated chemokine/CCL17 (TARC) (2) and produce the proallergic cytokines IL-4, IL-5, and IL-13. Th cells play an important role both in the initiation and challenge phases of allergic asthma, and understanding the mechanisms by which these cells respond to allergen challenges could lead to novel therapeutic approaches (3).

IL-17 (IL-17A), originally discovered as a close homologue of a protein of the T cell tropic Herpesvirus *saimiri*, is produced by the

memory T cells termed Th17 (4–9), a Th cell lineage distinct from Th1 and Th2 cells, which is negatively regulated by IFN- $\gamma$  and IL-4 (10, 11). IL-17 is critical in the clearance of several pathogens (12–14), and in vivo expression of IL-17 leads to expansion of hemopoietic progenitors and neutrophilia (15), which, if uncontrolled, may lead to chronic and autoimmune inflammation such as encephalomyelitis, type II collagen-induced arthritis, and chronic enterocolitis (16–22).

Elevated IL-17 concentrations were found in the lung and blood of allergic asthma patients and linked to severity of asthma (23–28). However, the differentiation of Th17 cells is induced by T regulator (T reg) cytokine TGF- $\beta$ 1, a regulator of the Th2 response (1, 29), together with proinflammatory cytokines (IL-6, IL-1, TNF, and IL-23) (30). In vitro investigations revealed that IL-17 inhibited CD4<sup>+</sup> T cell activation (31, 32), the expression of the chemokines Rantes (CCL5), Fractalkine (CX3CL1),

and CTACK (CCL27), and vascular cell adhesion molecule-1 in TNF-activated mesenchymal cells, interfering with the IFN-responsive factor-1 transcription factor and NF-κB activities (33–36). Based on our findings that IL-17 conveys dual effects (stimulation of proinflammatory cytokines and down-regulation of chemokines) in fibroblasts with the inhibitory IL-17 effect prevailing at low concentrations (36), we hypothesized a regulatory role of IL-17 in vivo and investigated the potential of IL-17 for regulating the Th2 response to inhaled allergens.

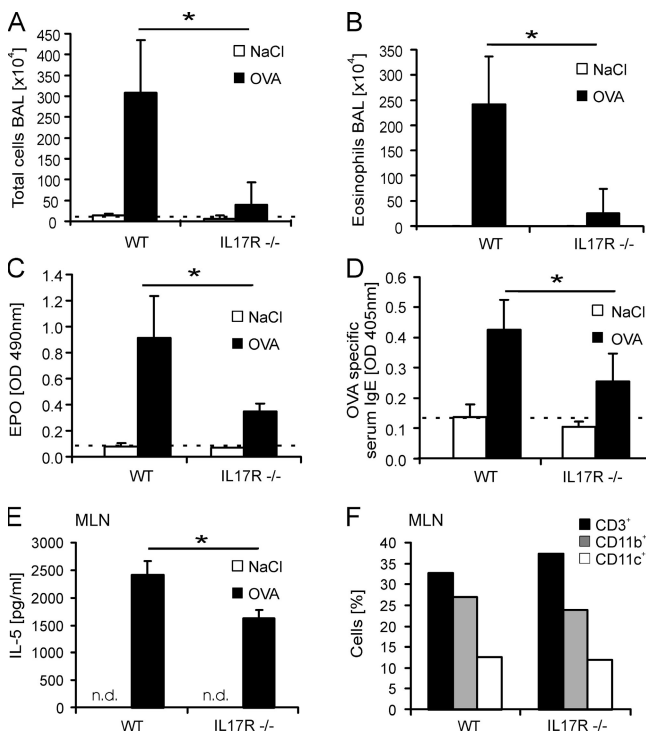
We document here a dual effect of IL-17 in allergic asthma: it is required for induction but negatively regulates established asthma. We show in vitro for the first time that IL-17 inhibits chemokine TARC production by DCs, which play a key role in the Th2 response and therefore in allergic asthma (37). Mucosal IL-17 administration inhibited asthma by reducing the pulmonary production of IL-5 and the chemokines TARC and eotaxin, which control eosinophil recruitment and asthma. Neutralizing IL-17 antibodies increased

asthma, further demonstrating that IL-17 is present during the allergic response as a negative regulator of asthma.

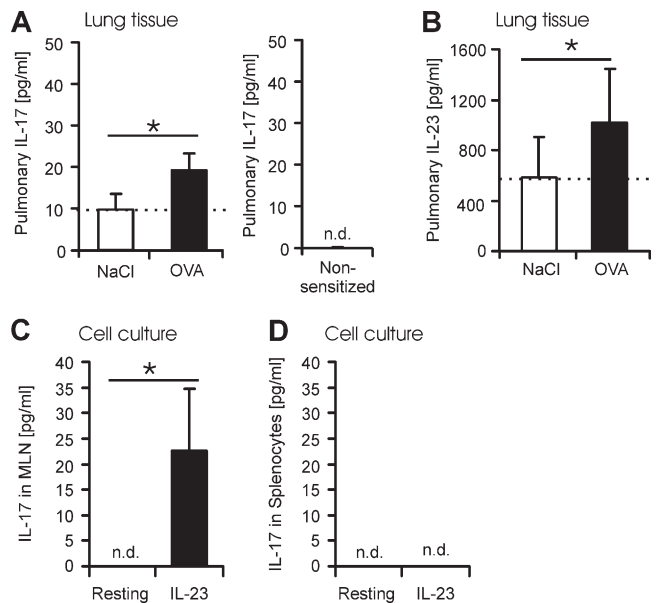
**RESULTS**

**IL-17 is required in the initiation of allergic asthma**

To investigate the role of IL-17 in allergic asthma we first used receptor IL-17R gene-deficient (IL-17R KO) mice in an OVA-induced model of asthma. Reduced lung and airway recruitment of granulocytes, predominantly eosinophils, were found in IL-17R KO mice compared with WT mice (Fig. 1, A and B). Furthermore, eosinophil peroxidase (EPO) activity in lung tissues (Fig. 1 C) and OVA-specific serum IgE concentrations were reduced (Fig. 1 D) in the absence of IL-17R signaling. The reduced asthma like sequelae of antigen challenge in IL-17R KO compared with WT mice correlates with a reduced regional Th2 cytokine production. Regional lymph nodes from antigen-sensitized and intranasally challenged mice were restimulated ex vivo (in cell culture), resulting in reduced IL-5 production in cells from IL-17R KO compared with WT mice (Fig. 1 E). The composition of CD3<sup>+</sup>, CD11b<sup>+</sup>, and CD11c<sup>+</sup> lymph node cells was similar in IL-17R KO and WT mice (Fig. 1 F), indicating that reduced antigen-induced cell activation rather than recruitment of immune cells to the lymph nodes of IL-17R KO mice causes attenuated Th2 responses. Therefore, signaling through IL-17R is critically required to develop allergic asthma.



**Figure 1. Role of IL-17 in development of allergic inflammation, as assessed in receptor IL-17R KO mice.** OVA/alum-sensitized C57BL/6 mice (WT) and IL-17R KO (IL-17R<sup>-/-</sup>) mice were challenged three times intranasally with OVA. 48 h after the third challenge, the BAL cells and eosinophils were counted (A and B), and lung tissue activities of EPO (C) and OVA-specific serum IgE concentrations (D) were determined. Mediastinal lymph nodes (MLNs) were removed from antigen-sensitized and challenged mice, restimulated in vitro with 50 μg/ml OVA, and analyzed for IL-5 production (E). In F, the cell composition of freshly excised MLNs was determined by FACS analysis. The percentages of CD3<sup>+</sup>, CD11b<sup>+</sup> and CD11c<sup>+</sup> positive cells are shown. The bars represent the mean ± SD (n = 8 animals per group). \*, P ≤ 0.05; dotted line indicates basal levels.



**Figure 2. IL-17 is induced in the lung upon allergen challenge.** Mice were sensitized and challenged with OVA, and IL-17 and IL-23 were determined by ELISA in the lung homogenate (A and B). In naive, non-sensitized mice, no IL-17 was detected (A). In C and D, cells from mediastinal lymph nodes (MLNs) or splenocytes of sensitized and challenged mice were restimulated in vitro with 300 ng/ml IL-23. The bars represent the mean ± SD (n = 8 animals per group). n.d., not detected. \*, P ≤ 0.05; dotted line indicates basal levels.

### IL-17 is induced in the lung upon local allergen challenge

Pulmonary IL-17 was induced in allergen-sensitized compared with nonsensitized mice and further increased upon intranasal challenge (Fig. 2 A). IL-17 production correlated with pulmonary IL-23 induction (Fig. 2 B). In cell cultures derived from allergen-sensitized and challenged mice, IL-23 was able to induce IL-17 in mediastinal lymph node (Fig. 2 C) but not in splenocyte cultures (Fig. 2 D), suggesting a local pulmonary role of IL-17. Therefore, we show in a model of allergic asthma that IL-17 is locally augmented by allergen challenge and induced by IL-23.

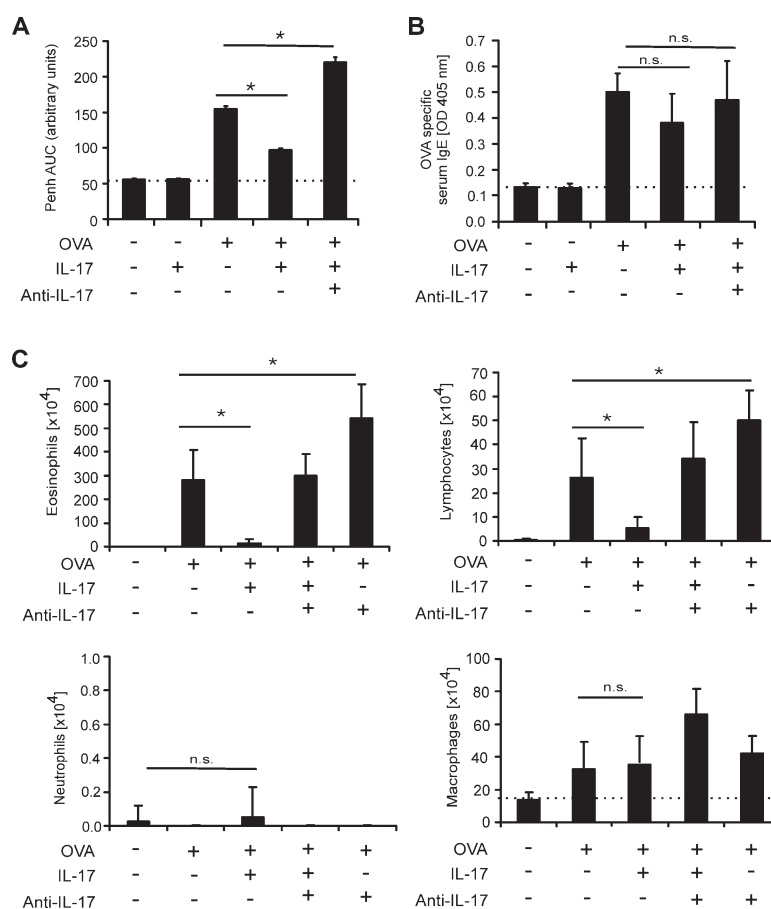
### Exogenous IL-17 reduces methacholine response upon antigen challenge

OVA-sensitized C57BL/6 mice challenged intranasally with OVA, but not NaCl or IL-17 alone, developed a robust response to aerosolized methacholine given in enhanced respiratory pause (Penh) values (Fig. 3 A) (38). Penh values measured provide an estimate for airway obstruction and may indicate

airways hyperreactivity and inflammation. Recombinant murine IL-17 (0.1  $\mu$ g) given together with the OVA challenge inhibited methacholine response by  $58 \pm 18\%$  ( $P < 0.03$ ; Fig. 3 A), and this effect was similar at high IL-17 dose (10  $\mu$ g). Moreover, the response of mice sensitized with OVA plus adjuvant aluminium hydroxide and challenged intranasally with OVA was also reduced by IL-17 (unpublished data).

IL-17-induced inhibition of methacholine response was fully reversed by neutralizing IL-17 antibody treatment (Fig. 3 A), confirming the specificity of exogenous IL-17 effect. IL-17 antibody administration augmented the methacholine response above controls ( $P < 0.01$ ) (Fig. 3 A, bar 5 vs. 3), suggesting that endogenous IL-17 was also neutralized and contributed to methacholine response.

IL-17 did not inhibit systemic OVA-specific serum IgE concentrations, which reflect intact T cell-B cell interactions (Fig. 3 B). Therefore, the data suggest that IL-17 given with antigen challenge has an inhibitory effect on methacholine response.



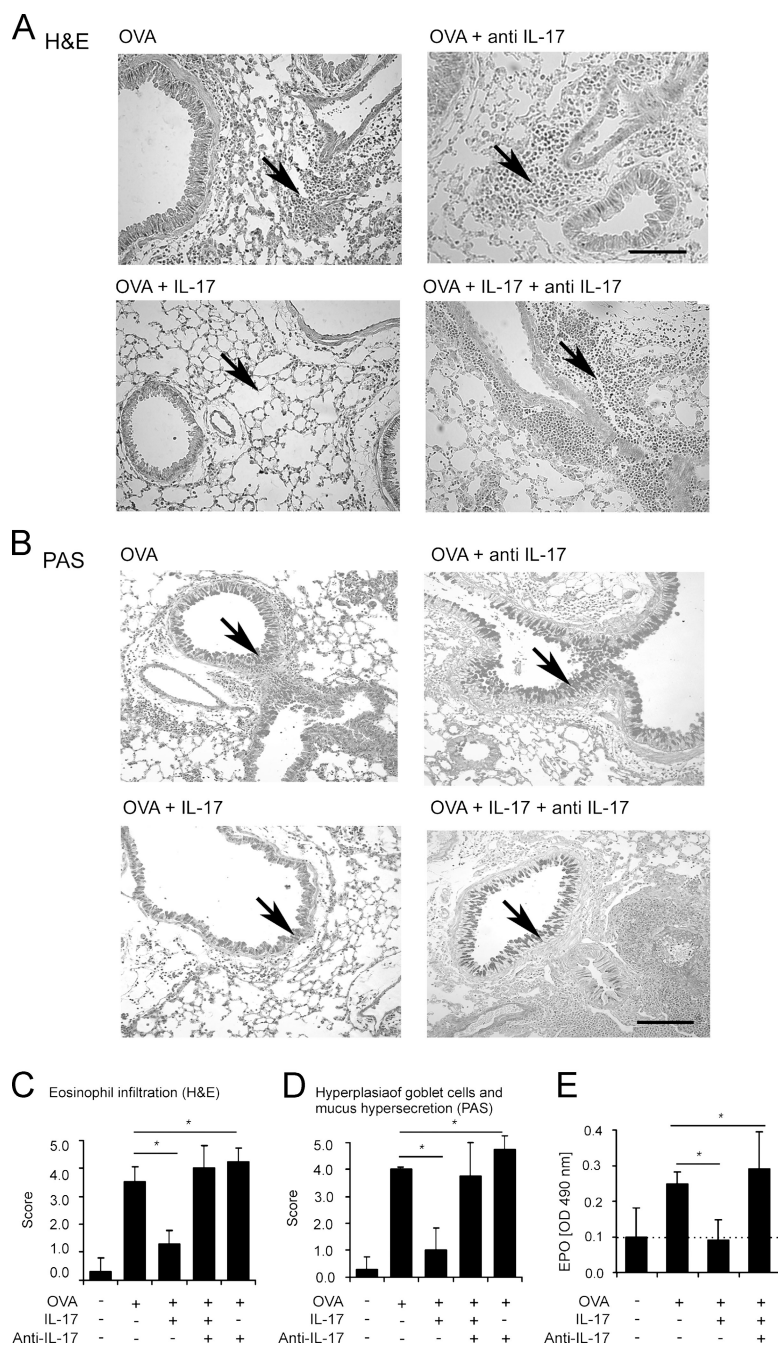
**Figure 3. Exogenous IL-17 inhibits methacholine response upon allergen challenge.** OVA-sensitized C57BL/6 mice were challenged intranasally with either saline, OVA alone, IL-17 alone, OVA with IL-17, or OVA with IL-17 plus neutralizing IL-17 antibodies. 24 h after the challenges, the methacholine response was measured using whole-body plethysmography. The intensity is measured in Penh arbitrary units, and the calculated area under the Penh time-curves (AUC) is shown. 48 h after the

third challenge, OVA-specific serum IgE concentrations were determined, given in absorbance (OD 405 nm) values (B), and the BAL cells were counted. Eosinophil, lymphocyte, macrophage, and neutrophil counts (C) are presented. The same effect of IL-17 was found in mice sensitized with OVA/alum (unpublished data). The results represent the mean  $\pm$  SD of  $n = 8$  animals per group. \*,  $P \leq 0.05$ ; dotted line indicates basal levels.

**IL-17 decreases lung eosinophil recruitment**

We then investigated the regulatory role of IL-17 in antigen-induced lung inflammation. 24 h after methacholine response analysis, inflammatory cell recruitment into the lung tissue

and the alveolar space was analyzed (Fig. 3 C). The total bronchoalveolar lavage (BAL) cell numbers increased up to 24-fold in OVA-challenged compared to saline-treated control mice ( $15 \times 10^4$  to  $354 \times 10^4$  cells/BAL). The



**Figure 4. IL-17 inhibits cell recruitment to the lungs and mucus hypersecretion.** Lung sections of OVA-sensitized C57BL/6 mice killed 2 d after the third challenge with either saline, OVA, OVA with IL-17 antibodies, OVA with IL-17, or OVA with IL-17 plus IL-17 antibodies are shown. The formalin-fixed tissue sections were stained with hematoxylin and eosin to visualize cell recruitment (A) and with periodic acid Schiff reagent (PAS) to visualize mucus (B), as shown by the arrows. Bars, 200  $\mu$ m.

In C and D, the histology sections were quantitated for eosinophil recruitment and mucus hypersecretion, respectively. A score scale from 0 to 5 is given on the y axis. The EPO in the lung tissue homogenates was determined 48 h after the last challenge, given in OD values (OD 490 nm) (E). The results represent the mean  $\pm$  SD of  $n = 8$  animals per group. n.d., not detected. \*,  $P \leq 0.05$ ; dotted line indicates basal levels.

higher cell number was mainly caused by eosinophil recruitment, increasing more than 700-fold ( $P < 0.01$ ; Fig. 3 C).

IL-17 administration at 0.1  $\mu\text{g}$  significantly reduced the total BAL cells by 91% ( $P < 0.01$ ), the eosinophil counts by 95% ( $P < 0.01$ ; Fig. 3 C), and lymphocyte counts by 84% ( $P < 0.01$ ; Fig. 3 C). These regulatory IL-17 effects were fully reversed by the administration of neutralizing IL-17 antibody (Fig. 3 C, bar 4). IL-17 antibody administration augmented eosinophil counts above controls ( $P < 0.01$ ) (Fig. 3 C, bar 5 vs. 2), suggesting that endogenous IL-17 was also neutralized, which contributed to eosinophil recruitment. Application of isotype-matched control antibody with the OVA challenge had no effect on OVA-induced response (unpublished data).

Microscopically the lung tissue of OVA-challenged mice showed peribronchial cell recruitment together with hyperplasia of bronchial smooth muscle. IL-17 reduced peribronchial eosinophil infiltration and the mucus hypersecretion and hyperplasia of goblet cells (Fig. 4, A and B) from 4 to 1 using scoring scale from 0 to 5 (Fig. 4, C and D). Reduction of eosinophil infiltration correlated with reduced EPO activity in the lung tissue of IL-17-treated animals (Fig. 4 E). These effects were reversed by neutralizing IL-17 antibodies. Neutralizing IL-17 antibodies given alone to OVA-challenged mice augmented eosinophil infiltration and mucus secretion above OVA-challenged controls (Fig. 4, C and D, bar 5 vs. 2), suggesting that endogenous IL-17 was also neutralized, which contributed to pathology.

### IL-17 inhibits pulmonary CC-chemokine expression in vivo

CC-chemokines, eotaxin, Rantes, and TARC, contribute to allergic inflammation, as demonstrated in neutralization experiments and gene-deficient mice (39–41). IL-17 reportedly inhibits Rantes expression in TNF-stimulated lung fibroblasts and in colonic myofibroblasts (33, 36). We tested whether IL-17 inhibits the mononuclear cell-chemokine production in allergic lungs.

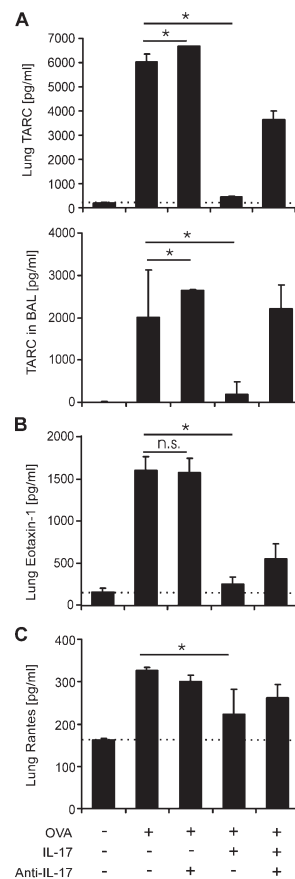
OVA-sensitized and intranasally challenged mice showed increased TARC, eotaxin, and Rantes production in the lung tissue (Fig. 5). Exogenous IL-17 abolished TARC and eotaxin expression completely and reduced Rantes induction by 50% (Fig. 5, A–C). As expected, the inhibition was largely reversed by neutralizing IL-17 antibody treatment (Fig. 5), demonstrating the specificity of the IL-17 effects. Neutralizing IL-17 antibodies given alone to OVA-challenged mice augmented TARC expression above OVA-challenged controls (Fig. 5, A and B, bar 2 vs. 3), suggesting that endogenous IL-17 also affects TARC production.

In the allergen-inflamed BAL, TARC (Fig. 5 A), but neither eotaxin nor Rantes (unpublished data), were detected. Reduced TARC production correlated with reduced lymphocyte and eosinophil counts. Our findings suggest that IL-17 attenuates antigen-induced asthma by inhibiting the production of CC-chemokines, TARC, eotaxin, and Rantes.

### IL-17 decreases CC-chemokine expression in lung explant cell culture and inhibits DC function

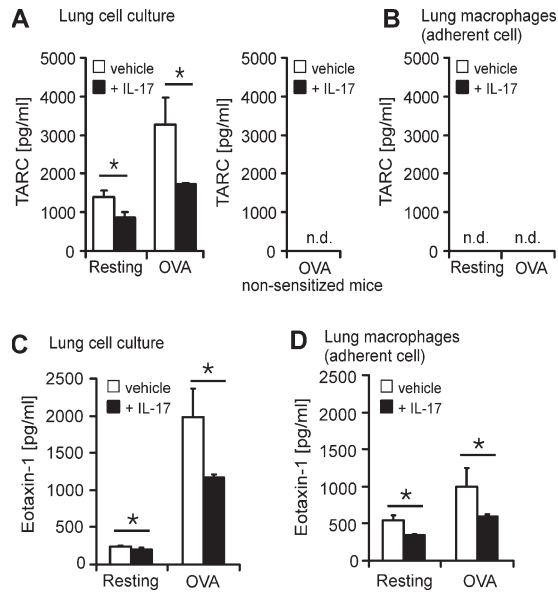
IL-17-mediated inhibition of the chemokines TARC and eotaxin observed in vivo was consolidated in cell cultures. Mice were sensitized, intranasally challenged with OVA, and 2 d after the last challenge lung explant cell cultures, mediastinal lymph node cells, and splenocyte cultures thereof were restimulated ex vivo with 50  $\mu\text{g}/\text{ml}$  OVA in the presence or absence of IL-17 (Fig. 6). In lung explant cell cultures, IL-17 reduced spontaneous and OVA-induced expression of eotaxin and TARC (Fig. 6, A and C). In lung macrophages, specifically eotaxin (Fig. 6 D), but not TARC (Fig. 6 B), was expressed, implying that TARC may be expressed by nonadherent leukocytes, likely DCs, a major source of TARC.

Therefore, we analyzed whether IL-17 reduces DC functions. TARC was produced in unstimulated and TNF-activated



**Figure 5. IL-17 inhibits pulmonary contents of the mononuclear cell attracting chemokines TARC, eotaxin and Rantes.** OVA-sensitized C57BL/6 mice were challenged three times intranasally with either saline, OVA alone, OVA plus IL-17-neutralizing antibodies, OVA with IL-17, or OVA with IL-17 plus IL-17 antibodies. 48 h after the third challenge, BAL was performed and the lung tissues were excised. The chemokine contents in the liquid phase of the lung homogenate and in the BAL were measured by ELISA specific for TARC (A), eotaxin (B), and Rantes (C). The results represent the mean  $\pm$  SD ( $n = 8$  animals per group). \*,  $P \leq 0.05$ ; dotted line indicates basal levels.





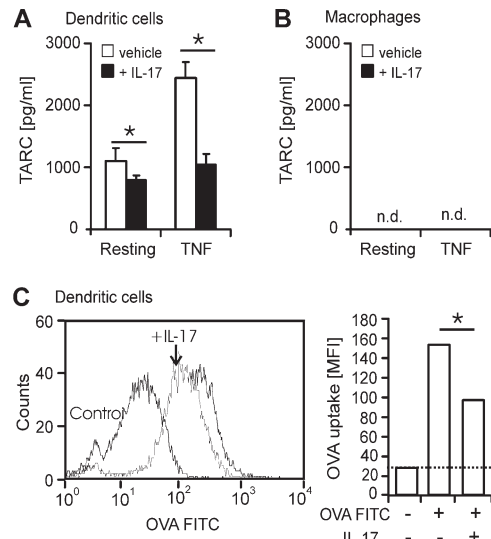
**Figure 6. IL-17 inhibits TARC and eotaxin production ex vivo in lung explants.** Lung explants (A and C) and lung adherent cells (B and D) were prepared from OVA-sensitized and challenged animals. Cells were treated with saline, 50  $\mu$ g/ml OVA alone, IL-17 alone, or OVA plus 30 ng/ml IL-17 for 24 h. The concentrations of TARC (A and B) and eotaxin (C and D) were measured by ELISA. In lung cell cultures of naive, nonsensitized mice no TARC was detected (A). The results represent the mean  $\pm$  SD of 8 animals per group. n.d., not detected. \*,  $P \leq 0.05$ .

bone marrow–derived DCs (CD11c<sup>+</sup>) (Fig. 7 A) but not in bone marrow–derived macrophages (CD11c<sup>-</sup>) (Fig. 7 B). IL-17 reduced spontaneous and TNF-induced expression of TARC (Fig. 7 A). Furthermore, dendritic antigen uptake of fluorescence-labeled OVA was reduced by IL-17 by 46% (Fig. 7 C).

Therefore, the data show that IL-17 reduces TARC and eotaxin expression directly in lung cells, providing a key effector mechanism of how IL-17 inhibits allergy in vivo. The absence of TARC production upon OVA stimulation in regional lymph node and splenocyte cultures (Fig. 8) supports the relevance of TARC in lungs rather than lymphoid organs.

### IL-17 reduces Th2 cytokine production

Since IL-17 reduces functions of the APCs, the DCs, we addressed whether IL-17 reduces antigen-induced Th2 cytokine production in vivo and in cell cultures. In vivo, IL-17 reduced allergen-induced IL-5 production in lung tissues by  $72 \pm 6\%$  ( $P < 0.05$ ) and in the BAL fluid by 100% from  $54.3 \pm 29.8$  pg/ml to below detection limit (1 pg/ml). BAL IL-4 concentrations were reduced by  $98 \pm 0.5\%$  in the presence of IL-17, from  $13.0 \pm 3.3$  pg/ml to  $0.3 \pm 0.5$  pg/ml. The Th2 cytokine expression of IL-5 and IL-13 in restimulated regional lymph node cell cultures was also inhibited by IL-17 by  $>50\%$  (Fig. 8, A and B) but not in splenocyte cultures (Fig. 8, E and F). Similarly, IL-17 reduced OVA-induced Rantes expression by regional lymph node cells (Fig. 8 D) but not splenocyte cultures (Fig. 8 H). Therefore, the data



**Figure 7. IL-17 inhibits TARC production and OVA uptake in DCs.** DCs (A and C) and macrophages (B) were differentiated in vitro from naive bone marrow–derived cells. Cells were treated with saline, IL-17 alone, 3 ng/ml TNF, or TNF with 30 ng/ml IL-17 for 24 h. The concentrations of TARC (A and B) were measured by ELISA. The results represent the mean  $\pm$  SD of 8 animals per group. In C, uptake of 100  $\mu$ g/ml OVA-FITC by DCs in the absence and presence of IL-17 (+IL-17) was analyzed by FACS. A histogram is shown in the left panel. The mean fluorescence intensity (MFI) thereof is given in the right hand panel. n.d., not detected. \*,  $P \leq 0.05$ .

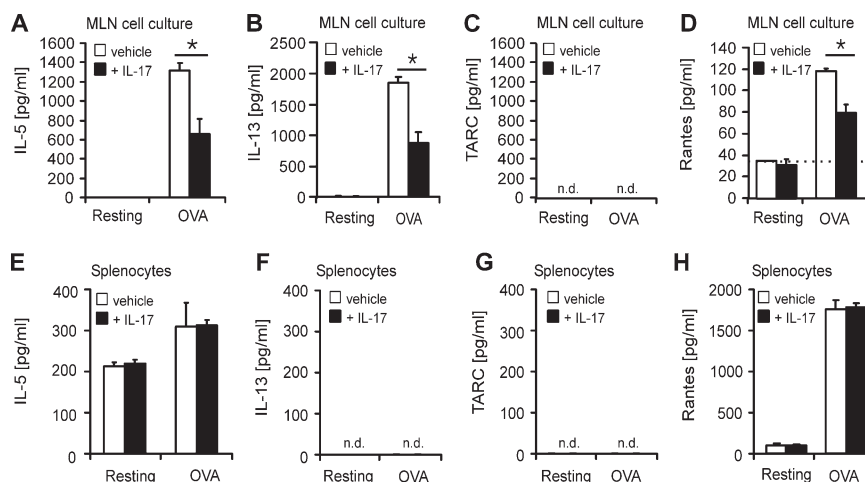
show that IL-17 markedly inhibits antigen-stimulated lung and regional lymph node cells, providing a key target mechanism of how IL-17 inhibits allergy.

### IL-17 was augmented in IL-4R $\alpha$ KO mice and reduced allergic asthma

Since IL-4 signaling has a major effect on the allergic response (42) and has been implicated in vitro to act on Th17 development (10, 11), we addressed the question of whether IL-4R $\alpha$  KO mice have altered IL-17 expression. Allergen challenge in IL-4R $\alpha$  KO mice increased IL-17 concentrations from  $20 \pm 4$  pg/ml in WT mice to  $32 \pm 7$  pg/ml (Fig. 9 A). Pulmonary IL-23 concentrations upon OVA challenge were equal in IL-4R $\alpha$  KO and WT mice, indicating that IL-4 acts directly on IL-17 production (unpublished data).

IL-4R $\alpha$  KO mice had reduced chemokine TARC production and allergic inflammation upon OVA challenge. Blocking of endogenous IL-17 by neutralizing antibodies reversed the reduced pulmonary TARC production, eosinophil recruitment to the airways, and EPO activity in the lungs in IL-4R $\alpha$  KO mice (Fig. 9, B–D). This indicates that the inhibition of asthma in IL-4R $\alpha$  KO mice is partially caused by endogenous IL-17. This marked effect of endogenous IL-17 in IL-4R $\alpha$  KO confirms the effect found on TARC expression of endogenous IL-17 in WT mice shown in Fig. 5.

In parallel, IL-17 neutralization reduced neutrophil recruitment to the airways, myeloid peroxidase activity in the lungs, and neutrophil chemokine KC production. This



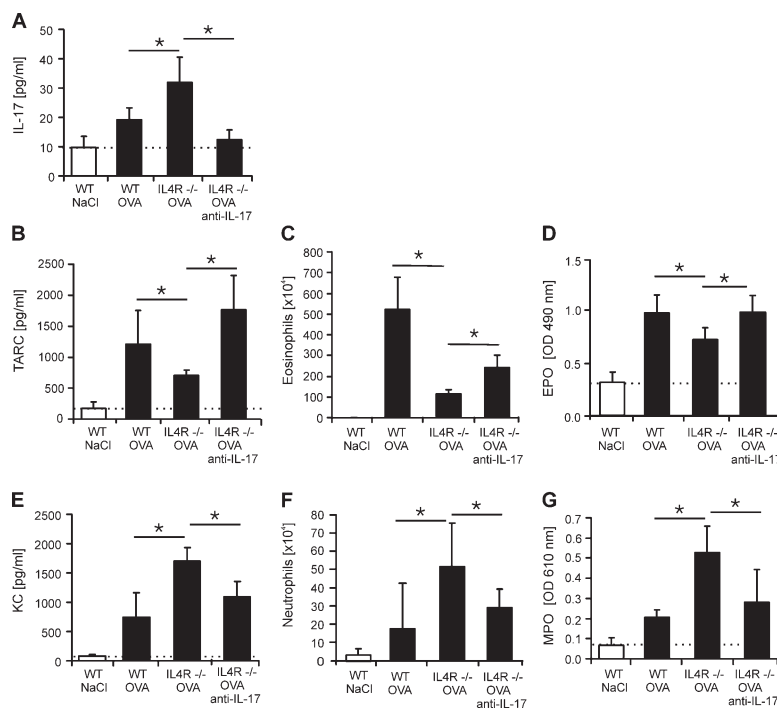
**Figure 8.** IL-17 inhibits IL-5, IL-13, and Rantes production in mediastinal lymph nodes (MLNs). MLN (A–D) and splenocyte (E–H) cultures were prepared from OVA-sensitized and challenged animals. Cells were treated with saline, 50  $\mu$ g/ml OVA alone, IL-17 alone, or OVA plus

30 ng/ml IL-17 for 24 h. The concentrations of IL-5, IL-13, TARC, and Rantes were measured by ELISA. The results represent the mean  $\pm$  SD of 8 animals per group. n.d., not detected. \*,  $P \leq 0.05$ ; dotted line indicates basal levels.

demonstrates that augmented neutrophil recruitment in IL-4R $\alpha$  KO mice is partially caused by endogenous IL-17 (Fig. 9, E–G). These data suggest a novel down-regulatory role of endogenous IL-17 in the effector phase of allergic asthma, which may, however, be accompanied by increased neutrophil accumulation in the absence of IL-4 signaling.

## DISCUSSION

We demonstrate a novel, negative regulatory function of IL-17 in allergic asthma in mice. During local antigen challenge, pulmonary IL-17 production was enhanced and reduced the hallmarks of allergic asthma, lung inflammation and dysfunction, and epithelial mucus hypersecretion. Mechanistically,



**Figure 9.** IL-4 signaling suppresses pulmonary IL-17 production. OVA/alum-sensitized BALB/c mice (WT) and IL-4R $\alpha$  KO (IL4R $^{-/-}$ ) mice were challenged twice intranasally with either saline or OVA alone. In parallel, the IL-4R $\alpha$  KO mice were challenged with OVA plus neutralizing anti-IL-17 antibodies. 48 h after the last challenge, IL-17, TARC, and KC

concentrations in the lung homogenates were measured by ELISAs (A, B, and E), and the cells infiltrating the BAL were counted (C and F). Lung tissue activities of EPO and myeloperoxidase (D and G). The results represent the mean  $\pm$  SD of 8 animals per group.

IL-17 administration reduced allergen-induced production of the Th2 lymphocyte-attracting chemokine TARC, eosinophil-attracting chemokine eotaxin, and IL-5, which related to reduced lymphocyte and eosinophil recruitment. Inhibition of chemokines TARC, eotaxin, and Rantes reportedly reduce allergic asthma, as demonstrated in experimental neutralization or gene-deficient mice (39–41). Therefore, these data suggest that IL-17 is a negative regulator of the effector phase of asthma.

T reg cells promote under proinflammatory conditions and in presence of IL-23 the development of Th17 cells and production of IL-17 (30). Here we show that IL-17 is a downmodulator of the DC-derived Th2 chemoattractant TARC, providing evidence for a novel feedback mechanism by which T reg cells may control a Th2 response in the effector phase of allergic asthma.

IL-17 had an inhibitory effect on the local production of the Th2 cytokines IL-4, IL-13, and IL-5 in the lung and regional lymph nodes, but not in splenocyte cultures. Together with the finding that IL-17 inhibited TARC production of DCs, a Th2 chemoattractant (2), we conclude that IL-17 acts on DC activation and antigen uptake, which leads to reduced activation of T cells and reduced IL-4, IL-13, and IL-5 production, resulting in reduced allergic response (37).

Although we describe a novel function of IL-17 in the resolution of allergic asthma, we also confirm its previously reported role in initiating allergic asthma (43) and inducing neutrophil recruitment. High neutrophil recruitment and KC expression was particularly seen in IL-4R $\alpha$  KO mice that express markedly high concentration of IL-17. These proallergic and inflammatory roles could be explained by IL-17-inducing hematopoietic colony-stimulating factors (12), antigen activation of T cells (44), and production of neutrophil-chemokines KC and IL-8 (CXC) (33–36).

Increased IL-17 concentrations in allergic asthma, chronic bronchitis, chronic obstructive pulmonary disease, cystic fibrosis, and acute respiratory distress syndrome (for review see references 9, 26), but also rheumatoid arthritis, were linked to the pathology of the diseases (18). IL-17 neutralization inhibits experimental murine arthritis (17, 18) and is a potential alternative therapy to TNF neutralization in rheumatoid arthritis. Our data indicate, however, that such a therapy bears the potential risk of exacerbating allergic inflammation. On the other hand, neutralization of Th2 cytokines is a therapeutic strategy to reduce allergic asthma, for example, by using neutralizing IL-4 and IL-13 antibodies or soluble IL-4R $\alpha$  molecules (45, 46). Here we show that allergic asthma is reduced in mice lacking the IL-4 signal transduction mechanisms through receptor IL-4R $\alpha$ , as also shown in mice defective for transcription factor Stat6 (47, 48), but that this is associated with increased pulmonary neutrophil recruitment and IL-17 production. Inhibition of the neutrophil infiltration by neutralizing IL-17 antibodies was associated with elevated eosinophil infiltration in the allergic response *in vivo*. Therefore, the regulation of IL-17 directly influences the nature of the cellular recruitment at the site of inflammation,

and IL-17 represents an effector molecule regulating neutrophil and eosinophil infiltration. Here, we provide a mechanistic understanding of the proallergic effects mediated by IL-4R $\alpha$ , through down-regulation of IL-17, which we identify as a negative regulator of asthma.

In contrast to the control of eosinophil infiltration, antigen-specific IgE production was not affected by IL-17 administered during the antigen challenge, which indicates intact T and B cell interactions and adaptive immunity to the allergen. IgE elevations reportedly correlate with and contribute to allergic reactions (49), although they are not sufficient for the development of allergy. Intact antigen-specific IgE concentrations in the current study may explain why IL-17 did not completely block but rather reduced allergy.

Th17 cell-derived IL-17 expression is further induced in an established allergic response together with IL-23 and controlled by IL-4R $\alpha$  signals. Although neutralizing IL-17 antibodies augmented an established allergic response, IL-17 reduced the response by inhibiting dendritic functions, TARC, IL-4, and IL-5 expression. Therefore, the data demonstrate that IL-17 is a novel endogenous negative regulator of allergic asthma, and IL-17 or Th17 cells may represent an interesting therapeutic target in asthma.

## MATERIALS AND METHODS

**Materials.** O-phenylenediamine, 3-amino-1,2,4-triazole, horseradish peroxidase, and bovine serum albumin grade V were obtained from Sigma Chemical Company. Mouse recombinant IL-17 and anti-IL-17 neutralizing monoclonal antibody were provided by Schering Plough and R&D Systems. The antibodies used for FACS analysis, PE-anti-CD3 (clone 145-2C11), APC-anti-CD11b (clone M1/70), FITC-anti-CD11c (HL3), isotype-matched control antibodies for FACS analysis, and isotype-matched control antibody IgG1a for *in vivo* application were purchased from BD Biosciences. Human IL-23 was provided by Di Padova (Novartis, Basel, Switzerland). FITC-conjugated OVA was purchased from Molecular Probes.

**Mice.** C57BL/6, BALB/c mice WT, and receptor IL-4R $\alpha$  KO mice were bred in our specific pathogen-free animal facility at CNRS. Receptor IL-4R $\alpha$  KO mice were on BALB/c genetic background as described previously (46). Receptor IL-17R KO mice were on C57BL/6 genetic background as described previously (46) and provided by Amgen. Mice were maintained in a temperature-controlled (23°C) facility with a strict 12-h light–dark cycle and were given free access to food and water. The experiments were performed with gender-matched mice aged 6–8 wk. All protocols complied with the French Government's ethical and animal experiment regulations.

**Experimental protocol.** Mice were sensitized *s.c.* twice at weekly intervals with 0.4 ml saline containing 10  $\mu$ g OVA with 1.6 mg aluminium hydroxide. Mice were also sensitized with 10  $\mu$ g OVA without adjuvant aluminium hydroxide, yielding the same results as in the presence of aluminium hydroxide. 1 wk after the second immunization, C57BL/6 mice were challenged three times (at days 14, 16, and 18) and BALB/c mice were challenged twice (at days 14 and 16) as follows. Animals were held under light *i.v.* Ketamine/Xylazine anesthesia and administered intranasally with 40  $\mu$ l saline (0.9%) containing 10  $\mu$ g OVA alone, or 10 OVA with 0.1  $\mu$ g IL-17, or OVA with 5  $\mu$ g anti-IL-17 antibodies, or OVA with 0.1  $\mu$ g IL-17 and 5  $\mu$ g anti-IL-17 antibodies. Control mice were challenged with saline alone, or IL-17 alone, given in 40  $\mu$ l saline solution. The doses of exogenous IL-17 applied were estimated from a previous *in vivo* study in rat neuritis (50). 1 d after the last challenge, plethysmography analysis was performed.



1 and 2 d after the last challenge, respectively, BALB/c and C57BL/6 mice were killed and the lungs were analyzed.

Mice were given a high dose of Ketamine/Xylazine i.p. and bled out. Via a tracheal canula, the lungs were washed four times with 0.5 ml ice-cold PBS (see BAL section). After BAL, the lung was perfused via heart puncture with ISOTON II Acid free balanced electrolyte solution (Beckman Coulter). Half of the lung was stored at  $-80^{\circ}\text{C}$  for EPO enzyme analysis, and the other half was fixed overnight in buffered 4% formaldehyde solution for histology analysis. BAL fluid was analyzed for cell composition and cytokine concentrations. Experiments were performed at least twice using groups of four animals.

**FACS.** Mediastinal lymph node cells, derived from antigen-sensitized and challenged WT or IL-17R KO mice, were saturated with normal mouse serum before staining with fluorescence-conjugated antibodies for 60 min. Cells were washed three times in PBS/1% BSA and analyzed using a FACS-Calibur-Flow cytometer and CellQuest Software (Becton Dickinson). The markers for the monovariant histogram (unpublished data) were set based on the negative staining control using isotype-matched control antibodies.

**Plethysmography.** Response to aerosolized methacholine was investigated at 24 h after the last OVA challenge. Unrestrained conscious mice were placed in whole-body plethysmography chambers (Buxco Electronic). C57BL/6 mice were exposed for 50 s to increasing methacholine concentrations (15, 50, and 150 mM), with 5-min intervals between the nebulizations. BALB/c mice were exposed for 50 s to 100 mM methacholine. The response was measured for 15 min after nebulization.

Penh measurement can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves; increased phase shift correlates with increased respiratory system resistance. Penh is calculated by the formula  $\text{Penh} = (\text{Te}/\text{RT}-1) \times \text{PEF}/\text{PIF}$ , where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow. For the graphics, the Penh mean values are given for  $-3$  to  $-1$  min (base line) and 14 points or minutes after the methacholine nebulization. The area under the curve was calculated as the sum of all Penh values minus the average of the base line values.

**BAL.** BAL fluids were prepared by washing the lungs four times with 0.5 ml ice-cold PBS. The cells were sedimented by centrifugation at 400 g for 10 min at  $4^{\circ}\text{C}$ . The supernatant (cell-free BAL fluid) of the first lavage was stored at  $-70^{\circ}\text{C}$  for cytokine analysis. An aliquot of the cell pellets was stained with Turk's solution, counted, and 200,000 cells were centrifuged on microscopic slides (cytospin at 1,000 rpm for 10 min, at RT). Air-dried preparations were fixed and stained with Diff-Quik (Merz & Dade A.G.). Differential counts were made under oil immersion microscopy. 100 cells were counted twice for the determination of the relative percentage of each cell type present in the BAL.

**Lung histology.** The organs were fixed in 4% buffered formaldehyde overnight and embedded in paraffin as described previously (51). Lung sections of 3  $\mu\text{m}$  were stained with hematoxylin and eosin or with periodic acid Schiff reagent and examined with a Leica microscope ( $\times 40$  and  $\times 100$  magnification). Peribronchial infiltrates and smooth muscle hyperplasia were assessed by a semiquantitative score (0–5) by two observers independently.

**Pulmonary EPO activity.** EPO activity was determined to estimate the recruitment of eosinophils to the lung parenchyme. After BAL lavage and perfusion (see Experimental protocol), the lungs were excised, homogenized for 30 s in 1 ml of 0.05 M Tris-HCl buffer (pH 8.0) using a Polytron (Kinematic AG), and 0.1% (vol/vol) Triton X-100 added. The homogenate was centrifuged for 15 min at  $4^{\circ}\text{C}$  at 10,000 g. EPO activity in the supernatant was determined as estimated from the oxidation of *O*-phenylenediamine by EPO in the presence of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) using the protocol by Van Oosterhout et al. (52). The substrate solution consisted of 10 mM *O*-phenylenediamine in 0.05 M Tris-buffer

(pH 8.0) and 4 mM  $\text{H}_2\text{O}_2$  (BDH). Substrate solution was added to samples in a 96-well microplate and incubated at room temperature for 30 min before stopping the reaction by addition of 4 M sulfuric acid. Duplicate incubations were performed in the absence and presence of the EPO inhibitor 3-amino-1,2,4-triazole (2 mmol/l). The absorbance was then measured at 492 nm (Flow Labs). Results are expressed as OD 492 nm and were corrected for the activity of other peroxidases, which were not inhibited by 3-amino-1,2,4-triazole.

**Quantification of IL-17, IL-23, IL-5, IL-13, KC, Rantes, eotaxin, and TARC.** Cytokine and chemokine concentrations in BAL, lung homogenate, or cell culture supernatants were determined by ELISA using commercial kits from R&D Systems. The lungs were homogenized for 1 min using a Polytron (Kinematic AG), and the cell debris were eliminated by centrifugation at 10,000 g for 10 min. The cytokines were determined in the supernatants. The cytokine detection limit was 1 pg/ml.

**Splenocytes and draining lymph node cultures.** Murine spleens or draining lymph nodes (mediastinal) were taken into suspension by passing them through 100  $\mu\text{m}$  mesh. The cells were washed once with PBS by centrifugation for 10 min at 1,300 rpm. Erythrocytes were lysed by a short hypotonic shock with water in the case of splenocytes but not of lymph nodes. The cells were washed twice with cold PBS, counted, and the cell pellet was resuspended at  $10^6$  cells per ml in culture medium consisting of RPMI supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. The cells were cultivated in 24-well plates, 1-ml cell suspension per well, with the indicated stimuli for 24 h at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ .

**Lung explant cultures.** Lungs were excised, minced, and digested for 2 h in a HBSS solution containing 200 U/ml of collagenase II (GIBCO BRL), 50 U/ml DNase I, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (53). The homogenate was passed through 100  $\mu\text{m}$  pore size filters, and the cell suspension was washed three times with PBS supplemented with 2% FCS, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin, and centrifuged at 200 g. The pellet was resuspended at  $10^6$  cells per ml in culture medium consisting of DMEM supplemented with 10% FCS and 100 U/ml penicillin/streptomycin. The cells were cultured in 24-well plates, in 1 ml cell suspension per well with the stimuli indicated, for 24 h at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ .

Adherent lung macrophages were obtained by adhering cells of lung explants for 2 h. Nonadherent cells were removed by washing three times with medium.  $5 \times 10^5$  cells per well were cultured in 24-well plates in culture medium consisting of DMEM supplemented with 10% FCS and 100 U/ml penicillin/streptomycin.

**Bone marrow-derived DCs and macrophages.** Murine bone marrow cells were isolated from femurs and differentiated into myeloid DC by culturing at  $2 \times 10^5$  cells/ml for 10 d in RPMI medium supplemented with 10% FCS, nonessential amino acids, 0.05  $\mu\text{g}/\text{ml}$  asparagine, MEM vitamins, sodium pyruvate, gentamycin 50  $\mu\text{g}/\text{ml}$ , 10,000 U/ml penicillin, 10 mg/ml streptomycin, 50  $\mu\text{M}$  2-mercaptoethanol, and 4% J558L cell-conditioned medium as a source of GM-CSF (change on days 3, 6, and 8) as described previously (54). For the differentiation into macrophages,  $10^6$  cells/ml were cultured for 10 d in DMEM (Sigma-Aldrich) supplemented with 20% horse serum and 30% L929 cell-conditioned medium as a source of M-CSF. After 7 d, cells were washed and recultured in fresh medium for another 3 d. The cell preparation contained a homogenous population of macrophages.

For the experiment, the cells were resuspended in medium containing 0.5% FCS and plated in 96-well microtiter plates (at  $10^5$  cells/well) and stimulated upon adherence with TNF. Cell supernatants were harvested after 24 h and analyzed directly for cytokine quantification or stored frozen at  $-80^{\circ}\text{C}$ .

**DC uptake of OVA-FITC.** 100  $\mu\text{g}/\text{ml}$  OVA-FITC was added to DC cultures at day 12. Cells were incubated for 2 h at  $37^{\circ}\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$ . Cells were collected, washed three times with cold

PBS containing 1% BSA, and analyzed using a FACS-Calibur-Flow cytometer and CellQuest Software (Becton Dickinson). The markers for the monovariant histogram (unpublished data) were set based on the negative staining control, which was provided by incubating DCs with OVA-FITC at 4°C.

**Statistical analysis.** The data are presented as the mean  $\pm$  SD with  $n = 8$  animals per condition. The significance of differences between two groups was determined by Student *t* test (two-tailed). Statistical significance was reported if  $P < 0.05$  was achieved.

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