# IL-17 contributes to neutrophil recruitment but not to control of viral replication during acute mouse adenovirus type 1 respiratory infection

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## ARTICLE INFO

## ABSTRACT

IL-17-producing CD4$^+$ helper T cells (Th17 cells) promote inflammatory responses to many pathogens. We used mouse adenovirus type 1 (MAV-1) to determine contributions of IL-17 to adenovirus pathogenesis. MAV-1 infection of C57BL/6 mice upregulated lung expression of IL-17 and the Th17-associated factors IL-23 and RORγt. Only CD4$^+$T cells were associated with virus-specific IL-17 production. Fewer neutrophils were recruited to airways of IL-17$^{-/-}$ mice following MAV-1 infection, but there were no other differences in pulmonary inflammation between IL-17$^{+/+}$ and IL-17$^{-/-}$ mice. Mice depleted of neutrophils using anti-Gr-1 antibody had greater lung viral loads than controls. Despite impaired neutrophil recruitment, there were no differences between IL-17$^{+/+}$ and IL-17$^{-/-}$ mice in peak lung viral loads, clearance of virus from the lungs, or establishment of protective immunity. We demonstrate robust Th17 responses during MAV-1 respiratory infection, but these responses are not essential for control of virus infection or for virus-induced pulmonary inflammation.

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## Introduction

The human adenoviruses (HAdV) are common causes of acute respiratory infection (Stempel et al., 2009). Adenovirus respiratory infections can present with a wide range of clinical syndromes ranging from mild upper respiratory tract infections to more severe manifestations such as necrotizing pneumonitis and bronchiolitis obliterans (Horwitz, 2001). Immunocompromised patients, such as those who have undergone bone marrow transplantation, are at risk for greatly increased morbidity and mortality from adenovirus infection (Hale et al., 1999; Walls et al., 2003).

The strict species specificity of the adenoviruses complicates animal studies with a HAdV. We have established mouse adenovirus type 1 (MAV-1) as a model to study the pathogenesis of adenovirus respiratory infection in the natural host of the virus. Using this model, we have shown that acute MAV-1 respiratory infection is associated with cellular inflammation and increased production of multiple cytokines and chemokines in the lungs (Anderson et al., 2009; Procario et al., 2012; Weinberg et al., 2005). In particular, the marked induction of IFN-γ expression without induction of IL-4 production (Procario et al., 2012) is consistent with T helper type 1 (Th1) polarization in the lungs following MAV-1 infection. IFN-γ deficiency is associated with small increases in MAV-1 lung viral loads (Procario et al., 2012), while Th2 polarization using cockroach antigen sensitization in an allergic airways disease model has no effect on MAV-1 replication in the lungs (Anderson et al., 2009).

Th1 CD4$^+$ T cells are classically characterized by their production of IFN-γ, and Th2 CD4$^+$ T cells are characterized by production of cytokines such as IL-4, IL-5, and IL-13 (Mosmann and Coffman, 1989). An additional lineage of T cells, Th17 T cells, is defined by the production by cytokines such as IL-17A and IL-17F (Harrington et al., 2005; Park et al., 2005). Th17 cell differentiation from naïve CD4$^+$ T cells is promoted by IL-1β, IL-6, TGF-β and IL-23 and occurs under the control of the transcriptional regulators RORγt and RORα, while it is negatively regulated by IFN-γ, IL-4, and IL-13 (Korn et al., 2009). Th17-associated cytokines have been implicated in a variety of inflammatory and autoimmune diseases, and animals deficient in IL-17 or its receptor, IL-17RA, are more susceptible to a variety of bacterial and fungal infections (Korn et al., 2009).

Less information is available regarding the role of Th17 immune function in the pathogenesis of respiratory viral infections. IL-17 expression in the lungs is induced by infection with viruses such

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as respiratory syncytial virus (RSV), influenza virus, and pneumonia virus of mice, an RNA virus in the same family (*Paramyxoviridae*) and genus (*Pneumovirus*) as RSV (Crowe et al., 2009; Li et al., 2012; Mukherjee et al., 2011; Spolski et al., 2012). IL-17 promotes pulmonary inflammation and viral replication during RSV infection and negatively regulates the generation of RSV-specific CD8^{+} T cells (Mukherjee et al., 2011). Signaling through IL-17RA is necessary for weight loss and neutrophil migration to the lungs following influenza infection, although IL-17RA signaling is not necessary for recruitment of virus-specific CD8^{+} T cells or viral clearance (Crowe et al., 2009). Th17 function may play a role in an effective memory response to influenza, as antibody neutralization of IL-17 decreased protection from challenge with a different subtype of influenza virus (Hamada et al., 2009). Importantly, Th17 responses to adenovirus respiratory infection have not been characterized. In this study, we used MAV-1 to characterize Th17 responses to adenovirus respiratory infection and to test the hypothesis that IL-17 is required for control of MAV-1 replication during acute respiratory infection.

## Results

### MAV-1 induces IL-17 production in lungs of infected mice

Acute MAV-1 respiratory infection increases production of the Th1 cytokine IFN-$\gamma$ in the lungs but not Th2 cytokines such as IL-4 and IL-13 (Anderson et al., 2009; McCarthy et al., 2013; Procario et al., 2012). To investigate whether MAV-1 respiratory infection induces lung IL-17 production, C57BL/6 mice were infected intranasally (i.n.) with MAV-1 and bronchoalveolar lavage fluid (BALF) and lung tissue were harvested at times corresponding to early infection (4 days post infection, dpi), the peak of viral replication at 7 dpi (Procario et al., 2012; Weinberg et al., 2005), and a later time corresponding to clearance of virus from the lungs (14 dpi). Reverse transcriptase quantitative real-time PCR (RT-qPCR) was used to measure IL-17A mRNA levels following MAV-1 infection

(Fig. 1A). IL-17A mRNA levels were increased in the lungs of infected mice compared to mock infected controls. IL-17A expression peaked at 7 dpi and then decreased by 14 dpi. The concentration of IL-17A protein (hereafter referred to as IL-17) was also increased in BALF at 7 dpi (Fig. 1B), the time corresponding to the peak of IL-17A mRNA levels and the typical peak of MAV-1 replication in the lungs (McCarthy et al., 2013; Procario et al., 2012; Weinberg et al., 2005). Likewise, the protein concentration of IL-23 was significantly increased in BALF at 7 dpi (Fig. 1C). At the same time, the mRNA level of ROR$\gamma$t, a key regulator of Th17 differentiation (Xu and Cao, 2010), was significantly greater in lungs of infected mice than in mock infected controls (Fig. 1D).

### Th17 cells and $\gamma\delta$ T cells are the main contributors to IL-17A production following MAV-1 infection

To determine the source of IL-17 production in the lung, we isolated lung lymphocytes at 7 dpi and used intracellular cytokine staining to define the extent of Th1, Th2, and Th17 polarization following MAV-1 infection. Consistent with cytokine induction in the lungs (Fig. 1), the percentage of IL-17^{+}CD4^{+} T cells was significantly increased in the lungs of infected mice compared to mock infected controls (Fig. 2A). As we have previously demonstrated (Procario et al., 2012), the percentage of IFN-$\gamma$[+]CD4^{+} T cells was also significantly increased (Fig. 2B), whereas we detected a corresponding very small but statistically significant decrease in the percentage of IL-4^{+}CD4^{+} T cells (Fig. 2C). When stimulated *ex vivo* with anti-CD3 antibody, lymphocytes isolated from the lungs of infected mice produced significantly more IL-17 (Fig. 2D) and IFN-$\gamma$ (Fig. 2E) than cells isolated from mock infected controls. T cells isolated from the MLN of mock infected and infected mice produced equivalent amounts of IL-17 (Fig. 2F), although T cells from MLN of infected mice produced more IFN-$\gamma$ than cells isolated from mock infected controls (Fig. 2G).

Next, we isolated lymphocytes from the lungs of mice at 7 dpi and used intracellular cytokine staining following stimulation with
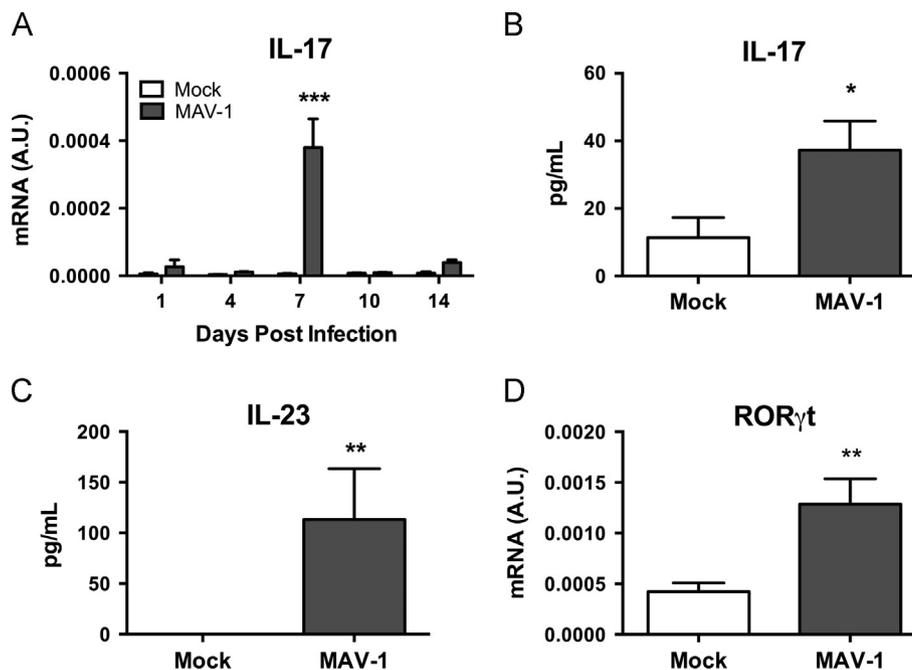


**Fig. 1.** MAV-1-induced IL-17 responses in the lung. Mice were infected i.n. with MAV-1 or mock infected with conditioned media. (A) RNA was extracted from lungs harvested at the indicated time points and RT-qPCR was used to quantify IL-17A mRNA, which is expressed in arbitrary units. (B–C) ELISA was used to quantify IL-17 and IL-23 concentrations in BALF from mice at 7 dpi. (D) RT-qPCR was used to quantify lung ROR$\gamma$t expression at 7 dpi. Combined data from $n = 3–13$ (A), $n = 10$ (B and D) and $n = 5$ (C) mice per group are presented as means ± S.E.M. Statistical comparisons were made using two-way ANOVA followed by Bonferroni's multiple comparison tests (A) or Mann–Whitney test (B–D). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, comparing mock to MAV-1.
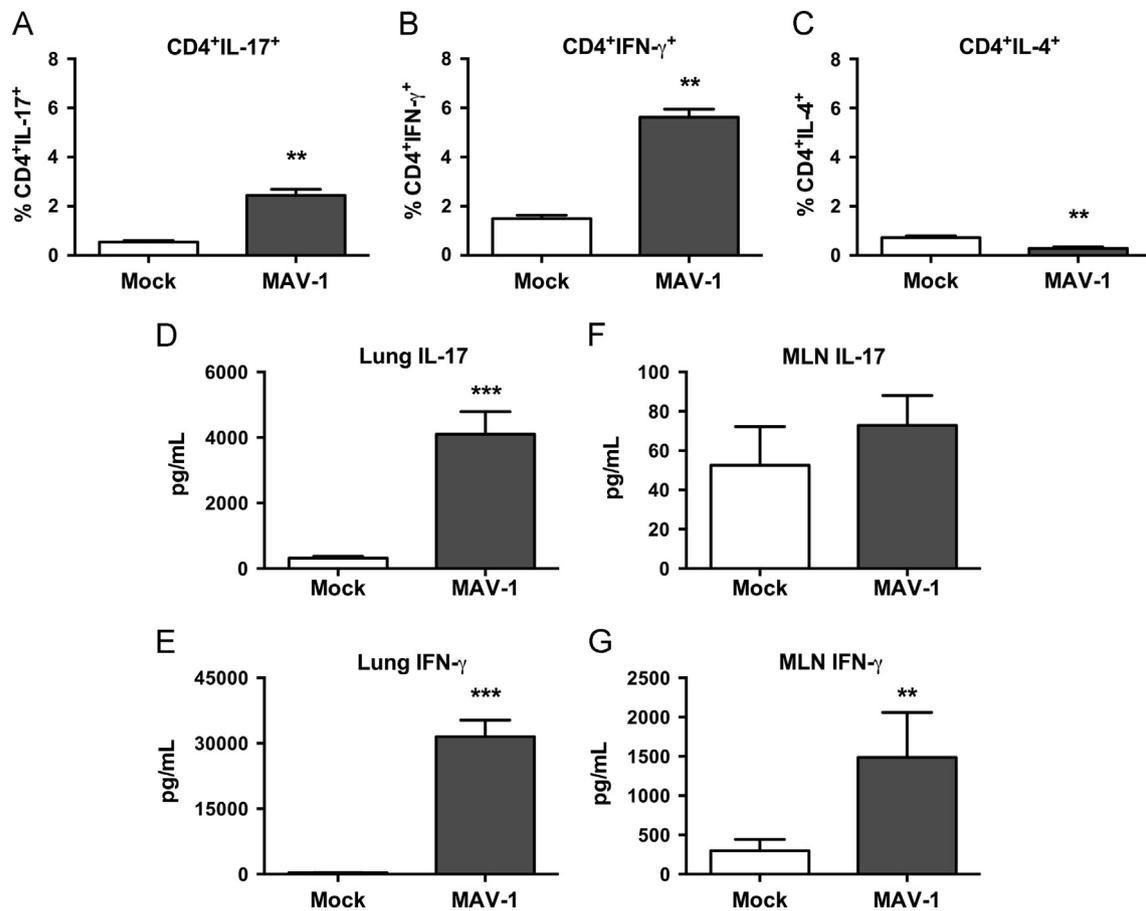
**Fig. 2.** T cell polarization in lungs after MAV-1 infection. Mice were infected i.n. with MAV-1 or mock infected with conditioned media and lung lymphocytes were isolated at 7 dpi. (A–C) Intracellular cytokine staining was used to quantify the percentage of CD4$^+$ T cells that were IL-17$^+$, IFN-$\gamma^+$, or IL-4$^+$. (D–G) Lung leukocytes or mediastinal lymph node cells were stimulated overnight with anti-CD3 antibody and ELISA was used to measure IL-17 or IFN-$\gamma$ production in the supernatant. Combined data from $n=5$ (A–C) and $n=10$ (D–G) mice per group are presented as means $\pm$ S.E.M. Statistical comparisons were made using Mann–Whitney test. $^{**}P < 0.01$ and $^{***}P < 0.001$, comparing mock to MAV-1.

phorbol-12-myristate-13-acetate (PMA) and ionomycin to more specifically determine the lymphocyte type(s) responsible for IL-17 production. The percentages of IL-17$^+$CD4$^+$ T cells and IL-17$^+$ $\gamma\delta$ T cells were significantly increased in the lungs of infected mice compared to mock infected controls (Fig. 3A). In contrast, we observed no difference in IL-17$^+$ (NK), natural killer T (NKT), or CD8 T cell populations obtained from mock infected and infected mice. In order to determine whether the IL-17-producing cell populations were antigen-specific, we stimulated lung lymphocytes with antigen presenting cells (APCs) exposed to MAV-1. We detected significantly more virus-specific IL-17$^+$CD4$^+$ T cells in lymphocytes obtained from infected mice compared to mock infected controls (Fig. 3B). Virus-specific IL-17 production was not noted in other lymphocyte populations, suggesting that only the classical Th17 cells (IL-17$^+$CD4$^+$ T cells) are antigen-specific. In contrast, virus-specific IFN-$\gamma$ production was observed in both CD4$^+$ and CD8$^+$ T cells (Fig. 3C). Together, these data demonstrate that both CD4$^+$ and $\gamma\delta$ T cells are major contributors to IL-17 production in the lungs following MAV-1 infection, but only IL-17 production by CD4$^+$ T cells is virus-specific.

*IL-17 contributes to neutrophil recruitment to the airways of mice infected with MAV-1*

To investigate contributions of IL-17 to MAV-1 pathogenesis, we infected IL-17$^{+/+}$ and IL-17$^{-/-}$ mice with MAV-1. No MAV-1-associated mortality occurred in IL-17$^{+/+}$ or IL-17$^{-/-}$ animals (data not shown). Acute MAV-1 respiratory infection induced

histological changes in the lungs of both IL-17$^{+/+}$ and IL-17$^{-/-}$ mice at 7 dpi that were characterized by bronchopneumonia and interstitial infiltrates (Fig. 4A). Minimal residual inflammation was present in the lungs of IL-17$^{+/+}$ and IL-17$^{-/-}$ mice at 21 dpi. Next, we isolated cells from airways of IL-17$^{+/+}$ and IL-17$^{-/-}$ mice by BAL. Following infection, fewer cells overall were recruited to the airways of IL-17$^{-/-}$ mice than IL-17$^{+/+}$ mice at 7 dpi, the peak of histologically apparent inflammation (Fig. 4B). Differential counting revealed that fewer neutrophils were recruited to the airways of IL-17$^{-/-}$ mice compared to IL-17$^{+/+}$ mice, with a corresponding increase in the percentage of macrophages that were recruited to airways of IL-17$^{+/+}$ mice (Fig. 4C).

*IL-17 deficiency has little effect on T cell polarization in the lungs of MAV-1-infected mice*

To determine whether IL-17 deficiency altered T cell polarization in the lungs following MAV-1 infection, we isolated lung lymphocytes from IL-17$^{+/+}$ and IL-17$^{-/-}$ mice at various times following infection and measured cytokine production following *ex vivo* stimulation with anti-CD3 antibody. As expected, T cells harvested from the lungs of IL-17$^{-/-}$ mice did not produce IL-17 (Fig. 5A). IFN-$\gamma$ was produced in equivalent amounts by lung T cells harvested from IL-17$^{+/+}$ and IL-17$^{-/-}$ mice at 7 dpi (Fig. 5B), while very little IL-4 was produced by stimulated T cells from IL-17$^{+/+}$ or IL-17$^{-/-}$ mice at any time point (Fig. 5C). We detected no IL-17A mRNA in the lungs or IL-17 protein in the airways of IL-17$^{-/-}$ mice following MAV-1 infection (data not shown). In addition, we did not observe increases in mRNA
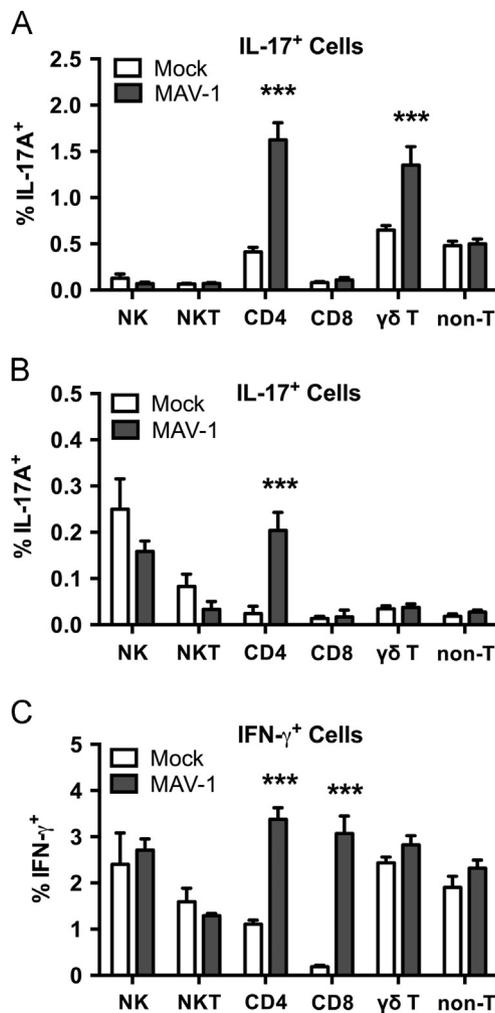
**Fig. 3.** Cell types contributing to IL-17 and IFN-γ production after MAV-1 infection. Mice were infected i.n. with MAV-1 or mock infected with conditioned media and lung lymphocytes were isolated at 7 dpi. (A) Lung leukocytes were stimulated with PMA/ionomycin and intracellular cytokine staining was used to quantify the percentage of each cell type that was IL-17$^+$. (B) Lung leukocytes were stimulated overnight with MAV-1-infected APCs and intracellular cytokine staining was used to quantify the percent of each cell type that was IL-17$^+$. (C) Lung leukocytes were stimulated with PMA/ionomycin and intracellular cytokine staining was used to quantify the percentage of each cell type that was IFN-γ$^+$. Combined data from $n=5$ mice per group are presented as means $\pm$ S.E.M. Statistical comparisons were made using two-way ANOVA followed by Bonferroni's multiple comparison tests. ***$P < 0.001$, comparing mock to MAV-1.

levels of other members of the IL-17 family, such as IL-17C, IL-17D or IL-17F, in IL-17$^{-/-}$ mice after infection that would suggest compensatory expression of these IL-17 subtypes (data not shown). IFN-γ concentrations were similar in BALF of IL-17$^{+/+}$ and IL-17$^{-/-}$ mice at 7 and 14 dpi (Fig. 5D).

*IL-17 is not essential for control of viral replication*

IL-17 deficiency was associated with decreased neutrophil recruitment to the airways during acute MAV-1 respiratory infection. To determine whether IL-17 deficiency and the associated defect in neutrophil recruitment could have an effect on control of viral replication in the lungs, we first depleted neutrophils from C57BL/6 mice using an anti-Gr-1 antibody. Antibody depletion decreased the total number of cells recruited to the airways of infected mice (Fig. 6A), and no neutrophils were detected in the airways of depleted mice (Fig. 6B). It is important to consider that the anti-Gr-1 antibody is also capable of depleting other types of

cells including dendritic cells and monocytes (Egan et al., 2008), although we did not observe a significant decrease in the absolute number of monocytes recruited to the airways in anti-Gr-1-treated mice (Fig. 6B). All anti-Gr-1-treated and control animals survived infection (data not shown). Lung viral loads were significantly higher in the lungs of anti-Gr-1-treated mice than in control mice at 7 dpi, the peak of viral replication in the lungs (Fig. 6C). This difference suggests that neutrophils contribute to the control of viral replication to some extent. In contrast, there were no statistically significant differences between lung viral loads measured in IL-17$^{+/+}$ and IL-17$^{-/-}$ mice at any time point (Fig. 7A).

To assess whether IL-17 affects the development of an adaptive immune response, we infected IL-17$^{-/-}$ and IL-17$^{+/+}$ mice with MAV-1 and rechallenged with MAV-1 28 days after the first infection. In both IL-17$^{+/+}$ and IL-17$^{-/-}$ mice, lung viral loads were substantially lower at 7 days following rechallenge (Fig. 7B) than they were at 7 days following primary infection (Fig. 7A), suggesting that protective immunity developed following primary infection. Lung viral loads did not differ between IL-17$^{-/-}$ and IL-17$^{+/+}$ mice 7 days following rechallenge (Fig. 7B). Thus, while MAV-1-induced IL-17 production in the lungs promotes recruitment of neutrophils to the lungs during acute MAV-1 respiratory infection, our data demonstrate that IL-17 is not essential for the control of viral replication in the lungs or for clearance of virus from the lungs during acute respiratory infection. In addition, IL-17 is not essential for the establishment of protective immunity following primary infection.

**Discussion**

IL-17 and related components of Th17 immune function are increasingly identified as contributors to the pathogenesis of many infections, including respiratory infections caused by viruses. Induction of IL-17 expression has been described in studies using recombinant HAdV-based vectors in mice or rats (Hou et al., 2013; Vavrincova-Yaghi et al., 2011), but no data exist that describe IL-17 induction in the context of respiratory infection by an adenovirus in its natural host. Our results clearly demonstrate robust induction of IL-17A mRNA and protein in the lungs of mice infected with MAV-1.

Although αβ$^+$ CD4$^+$ T cells are often considered to be the primary source of IL-17 production, other cell types are also capable of producing IL-17 (Xu and Cao, 2010). For instance, γδ T cells have been identified as the source of early, non-antigen specific IL-17 production in some circumstances (Martin et al., 2009; Shibata et al., 2007). IL-17 production by CD8$^+$ T cells, NKT cells, neutrophils and macrophages has also been described (Xu and Cao, 2010). In this study, we detected IL-17 production by CD4$^+$ T cells and γδ T cells but not in CD8$^+$ T cells, NK cells, or NKT cells isolated from lungs of MAV-1 infected mice (Fig. 3A). Only CD4$^+$ T cell production of IL-17 was virus-specific (Fig. 3B), consistent with nonspecific IL-17 production by γδ T cells observed in other studies (Martin et al., 2009). IL-17-producing CD8$^+$ (Tc17) T cells can be detected in the lung as early as 4 days following influenza infection (Hamada et al., 2009). We did not detect IL-17 production by CD8$^+$ T cells at 7 days following MAV-1 infection, suggesting that the development of Tc17 responses may not be a universal feature of respiratory viral infections. We may have missed Tc17 responses that develop later during the course of MAV-1 infection. However, we detected typical Th1 and Th17 responses in the lungs of MAV-1-infected mice at 7 dpi, and Tc17 responses develop with kinetics similar to those of Th1 and Th17 responses following influenza infection (Hamada et al., 2009). It therefore seems unlikely that Tc17 responses are induced by MAV-1 respiratory infection.
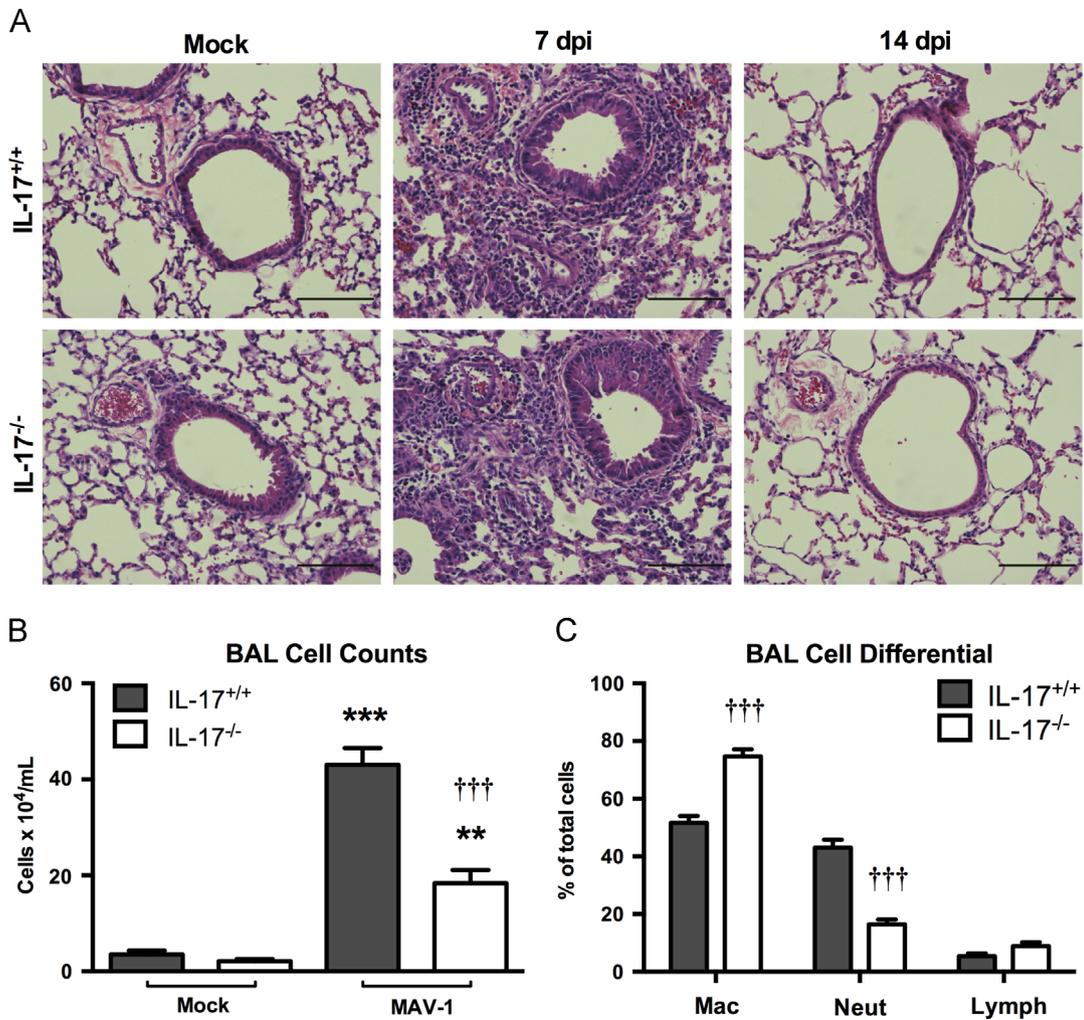
**Fig. 4.** Effect of IL-17 deficiency on MAV-1-induced inflammation. Wild type (IL-17$^{+/+}$) and IL-17$^{-/-}$ mice were infected i.n. with MAV-1 or mock infected with conditioned media and lung tissue was harvested at 7 and 21 dpi. (A) Hematoxylin and eosin-stained sections were prepared from paraffin-embedded sections. Scale bars, 100 μm. (B) Total numbers of inflammatory cells in BALF at 7 dpi were quantified using a hemocytometer. (C) Differential counting of macrophages/monocytes (Mac), neutrophils (Neut) and lymphocytes (Lymph) was performed on cytospin preparations of BALF cells obtained at 7 dpi. Combined data from $n = 8$–14 (B), and $n = 12$–13 (C) mice per group are presented as means $\pm$ S.E.M. Statistical comparisons were made using two-way ANOVA followed by Bonferroni's multiple comparison tests. **$P < 0.01$ and ***$P < 0.001$, comparing mock to MAV-1. †††$P < 0.001$, comparing IL-17$^{+/+}$ to IL-17$^{-/-}$ mice.

IL-17 stimulates the production of growth factors such as G-CSF (Fossiez et al., 1996; Laan et al., 2003) and chemokines such as CXCL1 (Witowski et al., 2000; Ye et al., 2001), which in turn lead to increased neutrophil production and recruitment of neutrophils to sites of inflammation. Consistent with these functions of IL-17, we detected neutrophil recruitment to the airways of MAV-1-infected IL-17$^{+/+}$ mice that was significantly less in IL-17$^{-/-}$ mice (Fig. 4). Similar effects of IL-17 deficiency or IL-17 blockade on virus-induced neutrophil recruitment have been reported in studies of influenza and RSV infection (Crowe et al., 2009; Mukherjee et al., 2011). In addition, IL-17 deficiency is associated with overall decreases in pulmonary inflammation and lung injury following infection with RSV (Mukherjee et al., 2011) or influenza (Li et al., 2012). Other than decreased neutrophil recruitment to the airways, IL-17 deficiency had no substantial effect on the extent of MAV-1-induced pulmonary inflammation at its peak, 7 dpi, or on the resolution of pulmonary inflammation by 21 dpi. This suggests that MAV-1 induces a variety of other proinflammatory cytokines and chemokines not affected by IL-17 that are more important contributors to pulmonary inflammation in our model.

Because neutrophil recruitment was impaired in IL-17$^{-/-}$ mice, we sought to characterize the effects of neutrophils on MAV-1

infection by depleting neutrophils with anti-Gr-1 antibody. Our results suggest that neutrophils do contribute to the control of viral replication in the lungs, although we are unable to rule out the possibility that depletion of other cell types by the anti-Gr-1 antibody, which recognizes Ly6G on peripheral neutrophils but also on dendritic cell and monocyte populations (Egan et al., 2008), may have been responsible for higher viral loads detected in animals treated with anti-Gr-1 antibody. Even though we detected fewer neutrophils in the airways of IL-17$^{-/-}$ mice following infection, there were no differences in lung viral loads between IL-17$^{+/+}$ and IL-17$^{-/-}$ mice at any time point. Neutrophil recruitment was impaired but not absent in IL-17$^{-/-}$ mice, potentially mitigating the effect in IL-17$^{-/-}$ mice compared to mice treated with anti-Gr-1 antibody, in which neutrophils were almost completely absent. It is also likely that IL-17 deficiency does not have a substantial effect on other host factors that are important for control of viral replication in our model. For instance, IFN-γ has some suppressive effect on MAV-1 replication in vitro (Kajon and Spindler, 2000), and we have shown that lung viral loads are higher in IFN-γ-deficient mice on a BALB/c background (Procario et al., 2012). IFN-γ production in response to MAV-1 infection did not differ between IL-17$^{+/+}$ and IL-17$^{-/-}$ mice (Fig. 5), suggesting that IFN-γ may continue to provide a protective
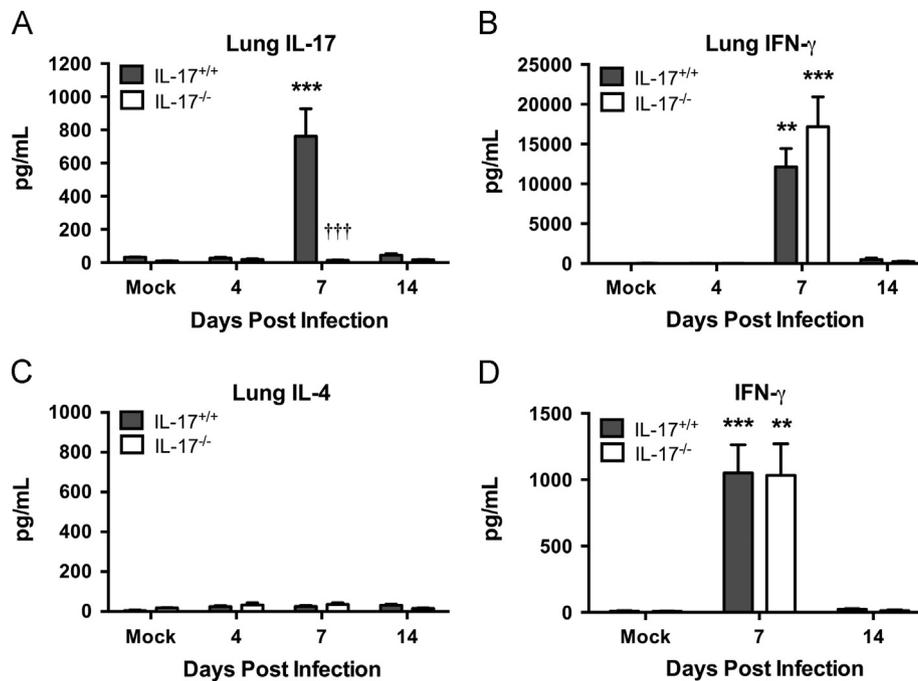
**Fig. 5.** Effect of IL-17 deficiency on MAV-1-induced Th1, Th2, and Th17 cytokine production. Wild type (IL-17$^{+/+}$) and IL-17$^{-/-}$ mice were infected i.n. with MAV-1 or mock infected with conditioned media. (A–C) Lung leukocytes isolated from mice at the indicated time points were stimulated overnight with anti-CD3 antibody and ELISA was used to measure IL-17, IFN-γ, and IL-4 production. (D) ELISA was used to quantify IFN-γ concentrations in BALF at the indicated time points. Combined data from $n=3$–5 (A–C) and $n=8$–21 (D) mice per group are presented as means ± S.E.M. Statistical comparisons were made using two-way ANOVA followed by Bonferroni's multiple comparison tests. $^{**}P < 0.01$ and $^{***}P < 0.001$, comparing mock to MAV-1. $^{†††}P < 0.001$, comparing IL-17$^{+/+}$ to IL-17$^{-/-}$ mice.

effect even in the absence of IL-17 production. IL-17 signaling through IL-17RA is also dispensable for control of influenza replication during acute infection (Crowe et al., 2009), and decreased instead of increased RSV viral loads were noted in mice treated with anti-IL-17 antibody (Mukherjee et al., 2011). Using MAV-1, we therefore provide additional evidence that IL-17 is not crucial for control of viral replication in the lungs during acute infection.

IL-17 may be important for the establishment of effective adaptive immune responses to respiratory viruses. IL-17-secreting CD4$^+$ and CD8$^+$ effector T cells can be detected in the lung in response to influenza infection, antibody-mediated neutralization of IL-17 diminishes the protection resulting from priming with heterosubtypic influenza virus, and transfer of *ex vivo*-generated IL-17$^+$ CD8$^+$ T cells (Tc17 effectors) to naïve mice protects against subsequent lethal challenge with influenza, (Hamada et al., 2009). Interestingly, the protective effect of Tc17 cells was associated with early recruitment of neutrophils to the lungs of mice challenged with influenza, again providing some evidence that neutrophils could contribute to control of respiratory virus infection. This does not seem to be a universal response to respiratory virus infection, as we did not detect IL-17$^+$ CD8$^+$ T cells in the lungs of mice infected with MAV-1 (Fig. 3). However, IL-17$^{-/-}$ mice cleared virus from the lungs by 21 dpi as effectively as IL-17$^{+/+}$ mice (Fig. 7A), suggesting that adaptive immune responses to MAV-1 are likely to be preserved in the absence of IL-17. Consistent with this, IL-17$^{-/-}$ mice controlled viral replication just as well as IL-17$^{+/+}$ mice after MAV-1 rechallenge (Fig. 7B).

In summary, acute MAV-1 respiratory infection induces the production of IL-17 in the lungs. CD4$^+$ T cells are the major source of IL-17 production in the lungs and MLN of MAV-1-infected mice. Although IL-17 facilitates the recruitment of neutrophils to the airways, and neutrophils may contribute to control of MAV-1 replication, IL-17 is not essential for the control of viral MAV-1 replication in the lungs or for clearance of MAV-1 from the lungs during acute infection. In addition, IL-17 is not essential for the establishment of protective immunity following primary infection. Immunomodulation by targeting IL-17 may prove to be an appealing therapeutic target in situations in which IL-17-mediated inflammation plays a substantial role in the pathogenesis of virus-associated lung injury, as it may with pathogens such as influenza and RSV. Our results suggest that IL-17 neutralization would have a small impact on adenovirus-induced lung injury, although it would likely not have a negative impact on host control of adenovirus replication in the lung.

## Materials and methods

### Mice

All animal studies were approved by the University of Michigan Committee on Use and Care of Animals. C57BL/6J mice were obtained from The Jackson Laboratory. IL-17$^{-/-}$ mice on a C57BL/6 background (Nakae et al., 2002) were obtained from Bethany Moore (University of Michigan) with the permission of Yoichiro Iwakura (Tokyo University of Science, Japan) and bred at the University of Michigan. All mice were maintained under specific-pathogen-free conditions.

### Virus and infections

MAV-1 was grown and passaged in NIH 3T6 fibroblasts, and titers of viral stocks were determined by plaque assay on 3T6 cells as previously described (Korn et al., 2009). Mice (4–6 weeks of age) were anesthetized with ketamine and xylazine and infected i.n. with 10$^5$ plaque-forming units (PFU) of MAV-1 in 40 μl of sterile phosphate-buffered saline (PBS). Control mice were mock infected i.n. with conditioned medium at an equivalent dilution in sterile PBS. To assess protective immunity, a subset of mice was rechallenged i.n. with 10$^5$ pfu of MAV-1 28 days following primary infection. Mice were euthanized by pentobarbital overdose at the indicated time points. Lungs were harvested, snap frozen in dry ice, and stored at −80 °C until processed further.
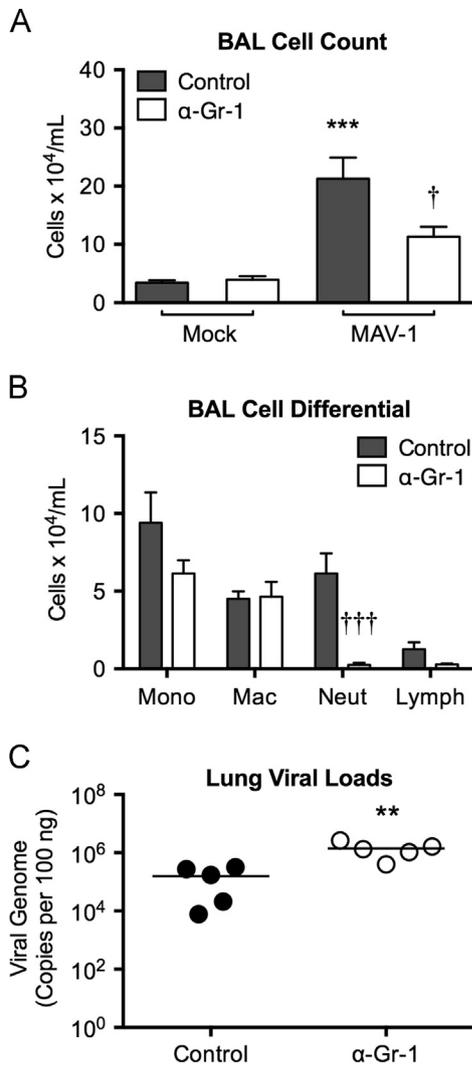
**Fig. 6.** Effect of neutrophil depletion on acute MAV-1 infection. Wild type mice were infected i.n. with MAV-1 or mock infected with conditioned media. Mice were treated every other day with anti-Gr-1 antibody (100 μg/dose given i.p.) or control serum until samples were harvested at 7 dpi. (A) Total numbers of inflammatory cells in BALF were quantified using a hemocytometer. (B) Differential counting of monocytes (Mono), macrophages (Mac), neutrophils (Neut) and lymphocytes (Lymph) was performed on cytospin preparations of BALF cells. (C) DNA was extracted from lungs and qPCR was used to quantify MAV-1 genome copy number. Lung DNA viral loads are expressed as copies of MAV-1 genome per 100 ng of input DNA. Individual circles represent values for individual mice and horizontal bars represent means for each group. Combined data from $n=3$–5 (A–B) mice per group are presented as means $\pm$ S.E.M. Statistical comparisons were made using two-way ANOVA followed by Bonferroni's multiple comparison tests (A–B) or Mann–Whitney test (C). **$P < 0.01$ and ****$P < 0.001$, comparing mock to MAV-1. †††$P < 0.001$, comparing Control to α-Gr-1.

*Histology*

Lungs were harvested from a subset of mice and fixed in 10% formalin. Prior to fixation, lungs were gently inflated with PBS via the trachea to maintain lung architecture. After fixation, organs were embedded in paraffin, and 5 μm sections were obtained for histopathology. Sections were stained with hematoxylin and eosin to evaluate cellular infiltrates. All sectioning and staining was performed by the Pathology Cores for Animal Research in the University of Michigan Unit for Laboratory Management. Slides were viewed through an Olympus BX41 microscope and digital images were processed using Olympus DP Manager software. Final images were assembled using Adobe Illustrator (Adobe Systems).

Adjustments to the color balance of digital images were applied in Adobe Illustrator equally to all experimental and control images.

*Isolation of RNA and DNA*

DNA was extracted from the middle lobe of the right lung using the DNeasy Tissue Kit (Qiagen Inc.). Total RNA was extracted from lungs using TRIzol (Invitrogen) as previously described (Nguyen et al., 2008).

*Analysis of viral loads*

MAV-1 viral loads were measured in organs using quantitative real-time polymerase chain reaction (qPCR) as previously described (Nguyen et al., 2008; Procario et al., 2012). Primers and probe used in this assay to detect a 59 bp region of the MAV-1 E1A gene are described in Table 1. Results were standardized to the nanogram (ng) amount of input DNA.

*Analysis of host gene expression*

Cytokine gene expression was quantified using RT-qPCR. First, 2.5 μg of RNA were reverse transcribed using MMLV reverse transcriptase (Invitrogen) in 20 μl reactions according to manufacturer's instructions. Water was added to the cDNA product to bring the total volume to 50 μl. Primers used to detect IL-17A, RORγt, and IFN-γ are described in Table 1. For these measurements, 5 μl of cDNA were added to reactions containing Power SYBR Green PCR Mix (Applied Biosystems) and forward and reverse primers (each at 200 nM final concentration) in a 25 μl reaction volume. Separate reactions were prepared with primers for mouse GAPDH (Table 1, used at 200 nM each). In all cases, RT-qPCR analysis consisted of 40 cycles of 15 s at 90 °C and 60 s at 60 °C. Quantification of target gene mRNA was normalized to GAPDH and expressed in arbitrary units as $2^{-\Delta Ct}$, where Ct is the threshold cycle and $\Delta Ct = Ct(target) - Ct(GAPDH)$.

*Analysis of inflammatory cells in bronchoalveolar lavage fluid*

Mice were euthanized via pentobarbital overdose at the indicated time points. Lungs were lavaged three times with the same aliquot of 1 mL sterile PBS containing protease inhibitor (complete, Mini, EDTA-free tablets; Roche Applied Science). Cells in BALF were counted using a hemocytometer. Samples were centrifuged at 13,500 rpm for 10 min at 4 °C, after which the supernatant was removed and the cell pellet was resuspended in 125 μL sterile PBS containing protease inhibitor. The remaining BALF was stored at −80 °C. The cells were centrifuged in a Shandon Cytospin (Shandon Elliot) and differential cell counting was performed after staining with Hema 3 Stain Set (Fisher Scientific).

*Isolation of cells from lungs and mediastinal lymph nodes*

In some experiments, left lungs were excised and cut into small pieces before digestion for 30 min at 37 °C in a 1 mg/ml solution of collagenase A (Sigma). The digested tissue was then pushed through a syringe with a 1.5-in 22-gauge needle and pelleted at 3000 rpm (402$g$) for 5 min. After lysis of red blood cells in 1× lysing buffer (BD PharMingen) for 3 min, tissue debris was removed by a brief spin (~5–10 s) at 1000 rpm (45$g$). The remaining cells were pelleted at 1200 rpm (64$g$) for 6 min prior to staining.

Single cell suspensions from mediastinal lymph nodes were prepared by grinding the tissue gently between glass slides and passing cells through a 64 μm cell strainer. The cells were pelleted at 1200 rpm (64$g$) for 6 min prior to staining.
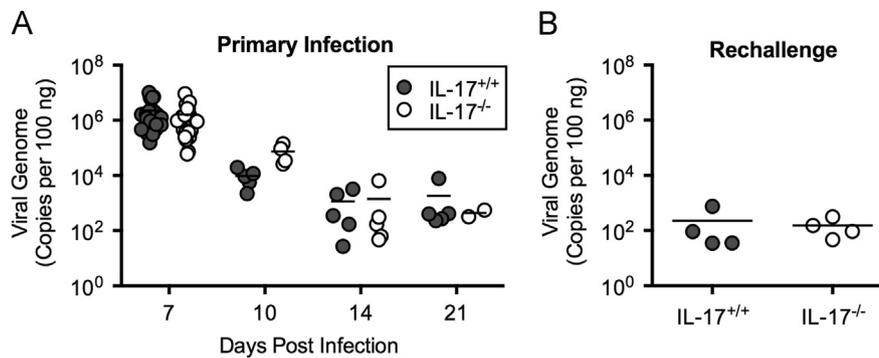
**Fig. 7.** Effect of IL-17 deficiency on control of MAV-1 replication in lungs. (A) Wild type (IL-17$^{+/+}$) and IL-17$^{-/-}$ mice were infected i.n. with MAV-1 or mock infected with conditioned media and lungs were harvested at the indicated time points. (B) IL-17$^{+/+}$ and IL-17$^{-/-}$ mice were infected i.n. with MAV-1 and then rechallenged with MAV-1 28 days after the primary infection. Lungs were harvested at 7 days post rechallenge. DNA was extracted from lungs and qPCR was used to quantify MAV-1 genome copy number. DNA viral loads are expressed as copies of MAV-1 genome per 100 ng of input DNA. Individual circles represent values for individual mice and horizontal bars represent means for each group. Statistical comparisons were made using two-way ANOVA followed by Bonferroni's multiple comparison tests.

**Table 1**
Primers and probes used for real-time PCR analysis.

| Target | Oligonucleotide | Sequence (5′ to 3′) |
|---|---|---|
| MAV-1 E1A | Forward primer | GCACTCCATGGCAGGATTCT |
| | Reverse primer | GGTCGAAGCAGACGGTTCTTC |
| | Probe | TACTGCCACTTCTGC |
| IL-17A | Forward primer | GGGTCTTCATTGCGGTGG |
| | Reverse primer | CTCCAGAAGGCCCTCAGACTAC |
| RORγt | Forward primer | CCGCTGAGAGGGCTTCAC |
| | Reverse primer | TGCAGGAGTAGGCCACATTACA |
| IFN-γ | Forward primer | AAAGAGATAATCTGGCTCTGC |
| | Reverse primer | GCTCTGAGACAATGAACGCT |
| GAPDH | Forward primer | TGCACCACCAACTGCTTAG |
| | Reverse primer | GGATGCAGGGATGATGTTC |

*Intracellular cytokine staining*

Cells isolated from lungs or MLN were plated at $10^6$ cells/ml and stimulated with 50 ng/ml PMA and 1.5 μM ionomycin (Calbiochem) for 5 h at 37 °C. For antigen presenting cell (APC) stimulation, $5 \times 10^5$ cells were cocultured overnight in 96-well plates with MAV-1-pulsed APCs ($10^6$ cells/well). APCs were prepared by depleting T cells from single cell suspensions of splenocytes using anti-CD3 microbeads (Miltenyi Biotech). Prior to coculture with cells isolated from lungs or MLN, APCs were exposed to MAV-1 at a multiplicity of infection of 5 for 48 h and then irradiated with 3000 rads. Monensin (Sigma) was added at 3 μM during the last 3 h of coculture. Cells were preincubated with anti-FcγR mAb 2.4G2 to block nonspecific binding before they were stained with the following Per-CP-, APC-, FITC-, biotin- and PE-Cy7-conjugated antibodies: CD4 (L3T4), CD8 (53–6.7), TCR-β (H57-597), TCR-γδ (GL3), and NK1.1 (PK136) antibodies (BD Biosciences). Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.2% saponin (Sigma). Finally, cells were stained with APC-Cy7- and PE-labeled IL17A (TC11-18H10) and IFN-γ (XMG1.2) antibodies (BD Biosciences) and analyzed by flow cytometry. Events were acquired on a FACSCanto (BD) flow cytometer, and data were analyzed with FlowJo software (Tree Star). Cells were classified as CD4$^+$ T cells (TCRβ$^+$CD4$^+$), CD8$^+$ T cells (TCRβ$^+$CD8$^+$), NK cells (NK1.1$^+$TCRβ$^-$), NKT cells (NK1.1$^+$TCRβ$^+$), γδ T cells (TCRδ$^+$TCRβ$^-$), and non-T cells (TCRβ$^-$$^-$TCRδ–NK1.1$^-$).

*Lymphocyte stimulation*

Lymphocytes were seeded at a concentration of $3 \times 10^5$ cells/well in 96-well plates coated with anti-CD3 antibody (BioLegend,

5 μg/ml) and incubated for 24 h. Supernatant was then collected for ELISA.

*Analysis of cytokine protein*

Cytokine protein concentrations in BALF and cell culture supernatant were determined by ELISA (Duoset Kits, R&D Systems) according to manufacturer's protocol.

*Neutrophil depletion*

Mice were pretreated intraperitoneally (i.p.) with 100 μg of anti-Gr-1 antibody (clone RB6-8C5), a generous gift from Dr. Gary Huffnagle, beginning 24 h before MAV-1 infection and then every other day until day 6. The control group received an equivalent amount of pre-immune mouse serum (Sigma) in sterile PBS.

*Statistics*

Analysis of data for statistical significance was conducted using Prism 6 for Macintosh (GraphPad Software, Incorporated). Differences between groups at multiple time points were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests. Comparisons between two groups at a single time point were made using the Mann–Whitney rank sum test. $P$ values less than 0.05 were considered statistically significant.

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