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IL-17 is required for Th1 immunity and host resistance to the intracellular pathogen *Francisella tularensis LVS*

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Summary

The importance of T helper type 1(Th1) immunity in host resistance to the intracellular bacterium *Francisella tularensis* is well established. However, the relative roles of Interleukin (IL)-12/Th1 and IL-23/T helper type 17(Th17) responses in immunity to *F.tularensis* have not been studied. The IL-23/Th17 pathway is critical for protective immunity against extracellular bacterial infections. In contrast, the IL-23/Th17 pathway is dispensable for protection against intracellular pathogens such as *Mycobacteria*. Our data show that the IL-23/Th17 pathway regulates the IL-12/Th1 pathway and is required for protective immunity against *F.tularensis Live Vaccine Strain* (LVS). We show that IL-17, but not IL-17F or IL-22 induces IL-12 production in dendritic cells and mediates Th1 responses. Furthermore, we show that IL-17 also induces IL-12 and IFNγ production in macrophages and mediates bacterial killing. Together, these findings illustrate a novel biological function for IL-17 in regulating IL-12/Th1 immunity and host responses to an intracellular pathogen.

Keywords

IL-23; IL-17; lung; tularemia; intracellular pathogens

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Introduction

Francisella tularensis, the causative agent of tularemia is a facultative intracellular bacterium, classified by the US Center for Disease Control as a Category A agent of bioterrorism (Dennis et al., 2001). Routes of infection include contact, ingestion or inhalation, with inhalation considered the most likely route of bioterrorism due to the severe disease caused by low doses of airborne bacteria (<10 colony forming units, CFU)(Dennis et al., 2001). A *F.tularensis Live Vaccine Strain (LVS)* has been developed from a *F. tularensis B* strain and has potential for use as a vaccine, but is currently not licensed for use in humans. *LVS* causes disease in mice that is similar to human tularemia and is used to study the immune components required for protection against tularemia (Duckett et al., 2005).

The importance of Interferon gamma (IFNγ) and T helper type 1 (Th1) responses in immunity to *F.tularensis* infection is well established (Anthony et al., 1989; Duckett et al., 2005). IL-12, made up of IL-12p35 and IL-12p40 subunits is critical for the induction of IFNγ production from T and Natural Killer (NK) T cells (Trinchieri et al., 2003). IL-12p35−/− mice are susceptible to respiratory *LVS* infection and exogenous IL-12 treatment increases survival (Duckett et al., 2005). IL-23 is also comprised of the p40 subunit, and p19, a four-α helix molecule (Oppmann et al., 2000) and is required for maintenance of T helper type 17 (Th17) responses (Dong, 2006). Th17 cells produce IL-17A (IL-17) and the related cytokine IL-17F, and IL-21 and IL-22 (Ouyang et al., 2008) and are involved in the induction of inflammation and tissue destruction associated with models of autoimmune diseases (Langrish et al., 2005). Conversely, IL-23-dependent IL-17 responses are important for protective immunity against extracellular bacterial infections for optimal induction of chemokines, recruitment of neutrophils and bacterial killing (Happel et al., 2005; Ye et al., 2001). However, Th17 cytokines appear to be dispensable for protection against most intracellular infections examined so far. For example, mice deficient in the IL-23/Th17 pathway are resistant to *Mycobacteria* (Khader et al., 2005; Umemura et al., 2007; Aujla et al., 2008;), *Listeria* (Aujla et al., 2008) and *Salmonella* infections (Schulz et al., 2008). In vitro infection of human monocytes with the virulent strain of *F. tularensis SCHU S4* induces IL-23 (Butchar et al., 2007). Further, pulmonary infection with *LVS* induces Th17 cells in the lung (Woolard et al., 2008), suggesting that Th17 cells may play a role in immunity to *F.tularensis*.

In light of these data, the goal of this study was to define the relative roles of IL-12/Th1 and IL-23/Th17 pathways in immunity to pulmonary *LVS* infection. Our data provide evidence that, unlike immunity to other intracellular pathogen infections (Khader et al., 2005; Aujla et al., 2008), the IL-23/Th17 pathway plays a unique role in protection against *LVS* pulmonary infection. We show that IL-17, but not IL-17F or IL-22, can induce IL-12 production in DCs and mediate protective Th1 responses. Further, we also show that IL-17 induces IL-12 and IFNγ in macrophages and enhances clearance of *LVS*. Our data therefore suggest that the IL-23/Th17 pathway is critical for optimal induction of Th1 responses and protection against *LVS*.

Results

IL-12p40 cytokine members are induced in the lung in response to *LVS* infection

To determine whether IL-12p40 cytokines were induced in the lung during pulmonary tularemia, we infected wild type C57BL/6 (B6) mice intratracheally with *LVS* and found that IL-23p19 mRNA was induced at day 2, with lower induction between days 4-6 (Figure 1A). However, induction of IL-12p35 and IL-12p40 mRNA was detected at day 2 and maintained through days 4-6 (Figure 1A). Intracellular killing of *LVS* by activated macrophages is dependent on IFNγ-dependent inducible nitric oxide synthase (iNOS) (Lindgren et al., 2005). We found induction of IFNγ and IL-17 mRNA between days 2-4, and coincided with induction of iNOS mRNA (Figure 1B). These data indicate that *LVS* induces IL-12p40, IL-12 and IL-23

in the infected lung and implicate both the IL-23/Th17 and IL-12/Th1 pathway in host responses to *LVS*.

IL-12-dependent IFN-γ is critical for protection following pulmonary infection with *LVS*

To determine whether there was a protective role for IL-12p40-dependent cytokines in control of *LVS* infection, we infected B6 mice, mice lacking all IL-12p40 cytokines (IL-12p40−/−) (Figure 1C), IL-12RB1−/− mice, and mice lacking both IL-12p35 and IL-23p19 subunits (Figure 1D) with *LVS* and determined CFU in infected lungs. IL-12p40−/−, IL-12RB1−/− and IL-12p35−/− IL-23p19−/− mice had significantly higher CFU when compared to B6 mice (Figure 1C, D) and did not survive past day 8, whereas all B6 infected mice survived (Figure S1 and data not shown). Further, we found that both IFNγ−/− and IL-12p35−/− mice also had significantly higher lung CFU compared to B6 mice (Figure 1E). Interestingly, all IFNγ−/− mice did not survive past day 6, while IL-12p35−/− mice survived until day 8 post infection (Figure S1). Also IL-12p35−/− mice had significantly lower lung CFU when compared to IFNγ−/− mice, suggesting other IL-12p40-dependent IL-12-independent mechanisms of protection. IFNγ protein was induced in infected B6 lungs, but the absence of IL-12p40, IL-12RB1 and IL-12/IL-23 cytokines resulted in a profound defect in the induction of lung IFNγ levels (Figure 1F). IL-12p35−/− mice displayed a similar reduction in IFNγ production, indicating that of the IL-12p40 cytokine members, IL-12 is critical for generation of Th1 immunity during *LVS* infection. Further, MHC Class II expression on lung CD11c+ cells was up regulated in B6 infected mice, but was significantly ablated in gene-deficient infected lungs (Figure S2A,B). Also, absence of IFNγ ablated the induction of inflammatory molecules including TNF-α, IL-1α, IL-β and MIP-1α in infected IFNγ−/− lungs (Figure S2C). These data demonstrate that IL-12 is required for induction of IFNγ responses and that IFNγ responses coincide with activation of lung CD11c+ cells and control of pulmonary *LVS* infection.

IL-17 is induced in the lungs of *LVS*-infected mice and is negatively regulated by IFNγ

Since IL-17 mRNA (Figure 1B) and IL-17 protein was induced in infected B6 lungs (Figure 1G), we determined if this was dependent on IL-12p40 cytokines. As expected, the IL-17 levels were ablated in the absence of IL-12p40 and IL-12RB1, and significantly higher in the absence of IL-12 and IFNγ (Figure 1G). Further, the major producers of IL-17 in the lungs of *LVS*-infected mice were antigen-dependent (Figure S3A). However, we also detected IL-17-producing cells in the wells that did not receive antigen (Figure S3A), suggesting other cellular sources, such as gamma delta (γδ) T cells (Umemura et al., 2007) and NK T cells (Lee et al., 2008), as likely producers of IL-17. Although IL-12p40 and IL-12RB1 were required for the induction of IL-17-producing cells in infected mice, there was no significant increase in the total number of IL-17-producing cells detected in the IFNγ−/− and IL-12−/− infected lungs when compared to B6 lungs (Figure S3A). These data suggest that the higher levels of lung IL-17 protein in IFNγ−/− and IL-12p35−/− mice (Figure 1G) were likely due to a higher production of IL-17 on a per cell basis.

IL-17 is known to induce chemokines such as Granulocyte stimulating factor (G-CSF) and Granulocyte-Macrophage stimulating factor (GM-CSF)(Ouyang et al., 2008), and we found that G-CSF, GM-CSF, Keratinocyte chemoattractant (KC) and Monocyte Chemoattractant Protein (MCP)-1 levels were all significantly higher in IFNγ−/− infected lungs when compared to B6 mice (Figure S3B). These data demonstrate that IL-17 is induced in the lung following *LVS* infection and that IFNγ negatively regulates IL-17 responses during pulmonary tularemia. However, high levels of IL-17 in the absence of Th1 immunity fails to confer protection against *LVS* infection.

IL-23 dependent IL-17 is required for protection against pulmonary tularemia

The IL-23/IL-17 axis is dispensable for protection against studied intracellular pathogens (Khader et al., 2005; Umemura et al., 2007; Aujla et al., 2008). Interestingly, when challenged with *LVS*, the IL-23p19−/− mice had significantly higher lung CFU (Figure 2A), which correlated with decreased lung IL-17 levels (Figure 2B) and number of lung IL-17-producing cells (Figure 2C). Further, both IL-17−/− and IL-17R−/− mice also had significantly higher lung CFU compared to B6 mice (Figure 2D), and did not survive past day 10 post infection (Figure S1). In addition, neutralization of IL-17 in *LVS*–infected B6 mice also increased lung CFU when compared to isotype control-treated B6 mice (Figure 2E).

IL-22, another cytokine produced by Th17 cells, induces production of anti-microbial peptides for host defense against extracellular bacteria (Aujla et al., 2008; Zheng et al., 2008). Surprisingly, IL-22−/− mice were not more susceptible than B6 mice and had comparable lung CFU to B6 mice (Figure 2F). γδ T lymphocytes are one of the major producers of IL-17 in response to intracellular pathogen infections (Umemura et al., 2007). Following infection with *LVS*, γδ−/− mice had significantly higher CFU (Figure 2F), and decreased induction of lung IL-17 when compared to B6 mice (B6 mice 55.8+/−14.5, γδ −/− mice 35.8+/− 7.9, p value= 0.050). These data demonstrate that IL-17 but not IL-22, is critical for protection against pulmonary tularemia and that both conventional T cells, and γδ T cells are likely cellular sources of IL-17.

IL-17 is required for induction of IFNγ responses during pulmonary tularemia

A role for IL-17 in the induction of G-CSF and recruitment of neutrophils is well documented (Ye et al., 2001). Accordingly, G-CSF levels were significantly decreased in IL-17−/− and IL-17R−/− infected lungs (Figure 3A) which correlated with a decrease in the percentage of neutrophils (Figure 3B, S4A) and cellular infiltrates (Figure S4B,C). Nonetheless, IL-17−/− and IL-17R−/− mice still recruited significant percentages of neutrophils to the lungs when compared to uninfected lungs (Figure 3B). Therefore, we next determined whether the absence of IL-17 or IL-17R impacted induction of protective molecules such as TNFα (Leiby et al., 1992) and IFNγ (Duckett et al., 2005) in infected lungs. Although induction of TNFα was comparable (Figure 3C), IFNγ levels were significantly reduced in the lungs of infected IL-17 −/− and IL-17R−/− mice (Figure 3D). As expected, IL-17 levels were elevated in the IL-17R −/− infected lungs (Smith et al., 2008) (Figure 3E). Although IFNγ and IL-12p35 mRNA are induced in the infected B6 lungs, significantly reduced induction of these transcripts was detected in the infected IL-17−/− lungs (Figure 3F). These data suggest that following *LVS* infection, IL-17 is important for the induction of G-CSF, recruitment of neutrophils to the lung, and generation of protective IL-12-dependent IFNγ responses.

IL-17 but not IL-17F or IL-22 induces IL-12 from DCs

Our in vivo data suggests that IL-17 is required for induction of IL-12 and IFNγ responses following pulmonary *LVS* infection. DCs are one of the likely producers of IL-12 following stimulation with *LVS* (Hong et al., 2007). Therefore, we stimulated Bone Marrow derived Dendritic Cells (BMDCs) with *LVS* alone, or with IL-17, and measured IL-12 levels in supernatants. IL-17 treatment of *LVS*-stimulated BMDCs resulted in 10-fold induction of IL-12 in comparison to *LVS* alone treatment (Figure 4A). However, IL-17 treatment did not impact production of other Th17 polarizing cytokines such as IL-6 by *LVS*-stimulated BMDCs (Figure 4B), suggesting that *LVS* by itself was a strong inducer of IL-6. Interestingly, IL-17 treatment alone induced IL-12 production (Figure 4C) and up regulated reporter IL-12p40 expression in BMDCs generated from *yet40* mice (Reinhardt et al., 2006) (Figure S5A). In contrast, IL-22 treatment did not impact IL-12 production by *LVS*-stimulated BMDCs (Figure 4C).

IL-17 and IL-17F share many biological activities, but also have unique biological functions (Chang and Dong, 2009), and we found that, unlike the addition of IL-17, the addition of IL-17F to BMDCs did not induce IL-12 production (Figure 4D). Moreover, IL-17 but not IL-17F induced the cytokines, IFNγ and IL-6, as well as the chemokines, MIP-1α and KC in BMDCs, but did not induce $^{TNF-\alpha}$ (Figure 4D). Furthermore, IL-17 induced IL-12 and IFNγ in culture supernatants within one hour of treatment (Figure S5B). Thus, IL-17 rather than IL-17F, is the primary mediator of IL-12 and IFNγ expression in DCs.

IL-17R comprised of IL-17RA subunit was initially identified as the receptor for IL-17 (Yao et al., 1995), but recently IL-17RA has been shown to partner with IL-17RC (Toy et al., 2006). Co-treatment of BMDCs with soluble IL-17RA but not IL-17RC significantly reduced the ability of IL-17 to induce IL-12, IFNγ and IL-6 (Figure 4E), as well as chemokines (data not shown). Therefore our data suggests that IL-17, but not IL-17F or IL-22 can induce the production of IL-12 and IFNγ in BMDCs and that this induction is independent of pathogen stimulation but is dependent on IL-17RA. Induction of IL-12 in macrophages is dependent on IFNγ (Flesch et al., 1995; Skeen et al., 1996), but DCs may not be subject to similar regulatory effects (Ma et al., 1997; Schulz et al., 2000). In support of this, we found that the absence of IFNγ did not impact the ability of IL-17 to induce IL-12 (Figure S5C). Further, we also found that sorted lung CD11c+ cells from B6 mice co-treated with *LVS* and IL-17, but not IL-17F, induced IL-12 and IFNγ (Figure 4F). Also, IL-17 induced IL-12 and IFNγ in the absence of pathogen stimulation in lung CD11c+ cells (Figure 4F).

IL-17 activates NF-κB, a transcription factor involved with inflammation (Yao et al., 1995) and we show that BMDCs treated with IL-17 resulted in activation of NF-κB (Figure 4G). Interestingly, addition of a pharmacological inhibitor of NF-κB, pyrrolidine derivative of dithiocarbamate (PTDC), during IL-17 treatment of BMDCs resulted in significant reduction in IFNγ and IL-6 but did not impact the induction of IL-12 levels (Figure S5). These data suggest that IL-17 treatment activates NF-κB in BMDCs, and that some, but not all downstream events are mediated via NF-κB signaling.

IL-17 polarizes IFNγ-producing T cells during *LVS* infection

To address whether exogenous IL-17 could impact polarization of naïve T cells into IFNγ-producing cells, we cultured BMDCs with *LVS* and either IL-17, IL-12 or IL-22 and determined the ability of naïve CD4+ OT-II TCR Transgenic (Tg) T cells to differentiate into IFNγ or IL-17-producing T cells. *LVS*-stimulated BMDCs significantly increased the number of IFNγ and IL-17 producing cells when compared to uninfected BMDC cultures (Figure 5A). As expected, addition of IL-12 to *LVS*-stimulated BMDC cultures significantly increased IFNγ-producing cells and reduced IL-17-producing cells when compared to *LVS* alone treated cultures. Importantly, addition of IL-17, but not IL-22, to the *LVS*-stimulated BMDCs significantly increased IFNγ-producing cells (Figure 5A), when compared to *LVS* alone treated cultures. However, neither IL-17 nor IL-22 influenced the generation of IL-17 producing cells relative to *LVS* alone stimulated cultures. These data demonstrate that IL-17 can impact Th1 immunity during intracellular infections by priming of naïve CD4 T cells into IFNγ-producing cells.

To address whether IL-17 could induce Th1 responses in a pathogen–free system, we cultured naïve OT-II Tg CD4+ cells with BMDCs, antigen and IL-17 or IL-12. Addition of IL-12 to B6 BMDCs resulted in a significant increase in IFNγ-producing cells compared to untreated wells (Figure 5C). Importantly, we show that IL-17 treatment also significantly increased the number of IFNγ–producing T cells when compared to untreated cultures (Figure 5C). We then addressed whether differentiation of IFNγ-producing cells in cultures treated with IL-17 was mediated via DC-expressed IL-12 and IL-17R. As expected, exogenous IL-12 resulted in

significant induction of IFNγ-producing T cells even when IL-12p40−/− or IL-17R−/− BMDCs were used as APCs (Figure 5D, E). Importantly, addition of IL-17 to IL-12p40−/− or IL-17R −/− BMDCs resulted in significantly lower induction of IFNγ–producing cells when compared to B6 cultures (Figure 5D,E,G). Although the absence of IFNγ in BMDCs did not impact the IL-17-dependent IL-12 induction in BMDC cultures (Figure S5C), we found that IFNγ−/− BMDCs co-cultured with naïve CD4+ OT-II Tg cells and IL-17, resulted in lower induction of IFN-γ-producing cells when compared to B6 cultures (Figure 5F,G). These data demonstrate that IL-17 mediates the induction of Th1 immunity by priming of naïve CD4 T cells into IFNγ-producing cells and that APC-expressed IL-12, IL-17R and IFNγ, are required for optimal induction of IFN-γ producing cells.

IL-17 induces IL-12 and IFNγ in macrophages and enhances bacterial clearance

IL-17 was recently shown to induce IL-12 in peritoneal macrophages (Ishigame et al., 2009). We found that *LVS* treatment induced low levels of IL-12 in bone marrow derived macrophages (BMDMs)(Figure 6A), but addition of IL-17 to *LVS*-stimulated BMDMs resulted in induction of 10-fold more IL-12 compared to *LVS* alone-stimulated cultures (Figure 6A). Furthermore, IL-17 treatment in the absence of pathogen could also induce IL-12 production in BMDM supernatants (Figure 6A). In addition, IL-17 could also induce IFNγ and chemokines MIP-1α and KC (Figure 6A), but not TNF-α in BMDMs (Figure 6A). In contrast to the effect of IL-17 on BMDCs, co-treatment with both IL-17 and *LVS* resulted in synergistic induction of IL-12, IFNγ, IL-6, MIP-1α and KC in BMDMs. Further, addition of soluble IL-17RA but not IL-17RC, resulted in significant inhibition of cytokine and chemokine production (Figure 6A), suggesting that IL-17-mediated induction of these molecules in macrophages is also mediated by IL-17RA.

IFNγ is documented to enhance the ability of macrophages to produce IL-12 (Flesch et al., 1995; Skeen et al., 1996). In support of this, we found that IFNγ−/− BMDMs induced significantly lower levels of IL-12 following IL-17 treatment (Figure 6B). Since IL-17 induces IFNγ production in BMDMs, we next determined whether addition of IL-17 to BMDMs infected with live *LVS* would impact intracellular bacterial clearance. As expected, addition of IFNγ to *LVS*-infected BMDM cultures resulted in enhanced bacterial clearance (Figure 6C). Importantly, addition of IL-17 to *LVS*-infected BMDMs also resulted in enhanced bacterial clearance when compared to untreated *LVS*-infected BMDM cultures (Figure 6C) and this response was abrogated when IFNγ−/− BMDMs were used (Figure 6D). These data demonstrate that IL-17 can induce IFNγ and IL-12 production in macrophages and enhance bacterial clearance. Alveolar macrophages are amongst the first innate cells that are infected following *LVS* pulmonary infection (Hall et al., 2008). Co-treatment of LVS-stimulated alveolar macrophages with IL-17, but not IL-17F, resulted in induction of IL-12 and IFNγ in supernatants when compared to LVS-alone treatment (Figure 6E). These data demonstrate that in addition to lung CD11C+ cells, lung alveolar macrophages can also induce the production of IL-12 and IFNγ in response to IL-17.

Cellular sources of IL-17 in the lung following *F.tularensis LVS* infection

To determine the cellular sources of IL-17 in the lungs of day-6 infected B6 mice, we performed intra-cellular staining and flow cytometry. We found that one of the major producers of IL-17 in the lungs of *LVS*-infected mice was TCRβ+ T cells, of which the majority were CD3+ CD4 + T cells, with some IL-17–producing CD8+ T cells. Moreover, γδ T cells also produced IL-17 in B6 infected lungs when compared to uninfected lungs (Figure 7A). Furthermore, purified populations of these cells from infected B6 lungs showed significant induction of IL-17 mRNA (Figure 7B). These data suggest that innate as well as adaptive immune cells in the lung can produce IL-17 in response to *LVS* infection.

Since myeloid cells can respond to IL-17, we next determined the expression of IL-17RA and RC mRNA in BMDCs, BMDMs and purified lung cell populations. We found high levels of IL-17RA mRNA in myeloid cells such as DCs, macrophages and neutrophils, whereas we found lower levels of IL-17RA mRNA in γδ, CD4, CD8 T cells as well as NK1.1 cells (Figure 7C). Expression of IL-17RC mRNA was lower but detectable in both myeloid and lymphoid populations (Figure 7C).These data suggest that BMDCs, BMDMs and lung CD11c+ and alveolar macrophages express IL-17RA and IL-17RC and therefore can respond to IL-17 in vivo.

Our in vitro data shows that IL-17 could induce IL-12 and IFNγ production in innate cells such as DCs and macrophages and elicit IL-12-dependent Th1 responses in CD4 T cells (Figures 4-6). However, the reduced IFNγ protein seen in the IL-17R−/− infected lungs could reflect reduced IFNγ production in several cell populations. We found that CD4, CD8 T cells and NK1.1 cells produced IFNγ in the B6 infected lungs, and IFNγ production in these populations was significantly reduced in the lungs of infected IL-17R−/− mice (Figure 7D). These data support our findings that IL-17 is required for the induction of IFNγ in CD4 T cells and also suggests that IL-17 could play a critical role in eliciting IFNγ expression in other cell types, such as CD8 and NK1.1 cells. Furthermore, CD11c+ cells and neutrophils isolated from IL-17R−/− infected lungs expressed lower levels of IL-12p35 mRNA when compared to cells sorted from B6 infected lungs (Figure 7E). Since IFNγ was reduced in the lungs of infected IL-17−/− mice when compared to infected B6 lungs (Figure 3D), we determined whether exogenous delivery of IL-17 would rescue IFNγ responses in vivo in IL-23p19−/− and IL-17−/− mice. We found that intratracheal delivery of rIL-17 into infected IL-23−/− and IL-17−/− mice resulted in a significant increase of IFNγ but not TNFα, when compared to PBS treatment (Figure 7F). These data suggest that IL-17 is required in vivo for the induction of IFNγ from several cell populations and exogenous delivery of IL-17 can rescue IFNγ levels in infected lungs.

Discussion

IL-23/Th17 pathway is critical for protective immunity against extracellular bacterial infections (Happel et al., 2005; Ye et al., 2001). However, the IL-23/Th17 axis is thought to be dispensable for protection against intracellular pathogens (Khader et al., 2005; Schulz et al., 2008; Aujla et al., 2008). We show that absence of IL-23 and IL-17 but not IL-22 results in increased susceptibility to pulmonary *LVS* infection. We also show that IL-17 can induce IL-12 and IFNγ from DCs and mediate Th1 responses, as well as induce IL-12 and IFNγ from macrophages and mediate bacterial clearance. Thus, we demonstrate a novel biological function for IL-17 in regulating IL-12/Th1 immunity in protection against an intracellular pathogen.

Our data shows that *LVS* effectively induces the production of IL-12p40, IL-12 and IL-23 in the lungs of infected mice. Similar to other pulmonary infection models (Happel et al., 2005), early induction of IL-23p19 and the more sustained production of IL-12p35 in the *LVS*-infected lungs suggests that IL-23 functions during the early immune response. Induction of IL-23 and IL-17 between days 2-4 may contribute to the sustained IL-12 mRNA levels detected in infected B6 lungs. Both Th1 and Th17 responses are induced in the lungs of *LVS*-infected mice, and are dependent on IL-12p40 cytokines. Specifically, IL-12 is required for the generation of Th1 responses while IL-23 is required for the generation of Th17 responses. Further, IL-12 and IFNγ negatively regulate Th17 responses in vivo during *LVS* infection. However, the failure of high levels of IL-17 or its inducible chemokines to protect in the absence of IL-12/Th1 axis suggests that Th17 pathway alone cannot confer protection against pulmonary tularemia.

Our data shows that the IL-17 response is antigen-driven (Figure S3A) and a majority of the IL-17-producers were TCRB+ CD3+ and CD4+ T cells, with a smaller population of CD8 cells (Figure 7A,B). A proportion of IL-17-producing cells were detected in wells that did not receive antigen (Figure S3A) and are produced by γδ T cells in *LVS* infected lungs (Figure7A,B). Furthermore, that γδ−/− mice have reduced levels of IL-17 and are susceptible to pulmonary tularemia suggests that IL-17 produced by innate cells may serve as a defense mechanism until the adaptive immune cells are recruited to control the infection.

Although depletion of neutrophils resulted in reduced protection against systemic *Francisella* infection (Sjostedt et al., 1994), *Francisella* can evade neutrophil-killing (Allen and McCaffrey, 2007). Therefore, although decreased recruitment of neutrophils in the lungs of infected IL-17/IL-17R−/− mice may impact protective immunity against *LVS*, there is nonetheless significant recruitment of neutrophils in absence of the IL-17/IL-17R pathway. This suggests that the increased susceptibility seen in mice lacking IL-17/IL-17R is likely due to the markedly reduced Th1 responses. We show that IL-17, but not IL-17F or IL-22 can enhance the ability of *LVS*-stimulated DCs to produce IL-12. This extends the recent finding that IL-17 can induce the IL-12 production in peritoneal macrophages (Ishigame et al., 2009), to include BMDCs, BMDMs, lung alveolar macrophages and lung CD11C+ cells. Importantly, since IL-17 can induce the production of IL-12 and IFN-γ within one hour of treatment, we conclude that IL-17 dependent induction of IL-12 in APCs is a rapid event that can impact downstream T cell events. This pathway may provide the basis for the plasticity of conversion of Th17 to Th1 responses seen in vivo (Lee et al., 2009), where it is possible that IL-17 produced by Th17 cells can impact the induction of IL-12 in APCs and induce the conversion of Th17 to Th1 cells.

Until recently it was thought that the primary responses to IL-17 occurred in non-hematopoietic cells such as fibroblast and epithelial cells (Shen and Gaffen, 2008). However, our data, as well as data using peritoneal macrophages (Ishigame et al., 2009), suggest that myeloid cells such as DCs and macrophages express significant levels of IL-17RA mRNA and respond to IL-17 by production of cytokines and chemokines. That this response is mediated primarily through IL-17RA but not IL-17RC, is consistent with studies that show that murine IL-17RC can only bind to murine IL-17F but not IL-17, whereas human IL-17RC can bind to both human IL-17 and IL-17F (Kuestner et al., 2007). IL-17 is known to activate NF-κβ and our studies show that IL-17 treatment of BMDCs results in activation of NF-κβ. However, co-treatment with an NF-κβ inhibitor results in loss of some IL-17-induced cytokines such as IFNγ and IL-6, but not others such as IL-12. That NF-κβ inhibitor PTDC did not completely repress TLR driven IL-12 production in DCs (Bohnenkamp et al., 2007) suggests that other pathways such as mitogen activated protein kinases (MAPK) are involved. Additional experiments using pathway specific inhibitors will delineate the signaling pathways involved in IL-17 mediated responses in APCs.

An important facet of our findings is that IL-17 can induce IL-12 and IFNγ production and enhance *LVS* bacterial clearance in macrophages. In a *Bordetella pertussis* model, it was shown that IL-17 treatment of macrophages enhanced bacterial clearance (Higgins et al., 2006) and was thought to be mediated by direct macrophage activation. Our data suggest that IL-17-dependent activation of macrophages and bacterial killing is mediated through induction of IFNγ, since IFNγ−/− BMDMs could not mediate IL-17-dependent control of bacteria. This suggests that IL-17 can modulate the innate responses and contribute to immunity and bacterial clearance until the arrival of adaptive immune cells to the site of infection.

Given that IL-17 can induce IL-12 and IFNγ production from APCs in the absence of pathogen stimulation suggests that IL-17 acts downstream of the initial pathogen-APC interaction. Although it is the Th1 response that controls intracellular bacteria, the IL-17 pathway provides

critical “help” for induction of the Th1 pathway. This is evident from the reduced IFNγ levels in the lungs of infected IL-17−/− and IL-17R−/− mice and the increased IFNγ responses in infected IL-23p19−/− and IL-17−/− mice treated with exogenous IL-17. Further proof for IL-17 in generating Th1 responses is reflected by the decreased frequencies of IFNγ-producing cells and decreased induction of IL-12p35 mRNA in CD11c+ cells and neutrophils in IL-17R−/− infected lungs. That IFNγ production in CD8 T cells and NK 1.1 cells is also decreased in IL-17R−/− infected lungs, suggests an even broader role for IL-17 in maintenance of Th1 immune responses in the lung. Recent work shows that IL-17 inhibits T-bet expression and Th1 differentiation in anti-CD3 anti-CD28 stimulated Th1 cultures (O’Connor et al., 2009). However, our data shows that Th cultures generated in the presence of APC and IL-17 can induce Th1 responses via induction of IL-12, suggesting that differential outcomes can be expected depending on whether the Th1 differentiation is mediated in the presence or absence of DCs.

Absence of the IL-23/Th17 axis did not impact IFNγ responses during *M.tuberculosis* (Khader et al., 2005) or *L.monocytogenes* (Aujla et al., 2008) infections, but resulted in reduced IFNγ responses following *M.bovis BCG* infection (Umemura et al., 2007). The unique requirement for the IL-23/Th17 pathway in induction of Th1 responses during *LVS* and *M. bovis BCG*, but not other intracellular infections is intriguing. Induction of IL-12 by *LVS* infection is dependent on TLR-2 signaling (Hong et al., 2007), while induction of IL-12p40 by a heat shock protein of *Francisella* is dependent on TLR-4 (Ashtekar et al., 2008). *M.bovis BCG* lipomannans induce IL-12p40 through TLR2 (Quesniaux et al., 2004). In contrast, IL-12 production by DCs following *M.tuberculosis* infection can take place in the absence of either TLR-2 and TLR-4 (Jang et al., 2004) and requires signals from both TLR2 and TLR9 (Bafica et al., 2005).This suggests that different intracellular bacteria may stimulate differential TLRs on APCs and produce distinct polarizing cytokines that impact host immune response to infection. It is likely that some intracellular bacterial infections can effectively induce IL-12/IFNγ responses in the host, while other pathogens require the IL-23/IL-17 pathway for effective induction of host IL-12/IFNγ responses for pathogen control.

In summary, we show that IL-23 dependent IL-17 is induced during *LVS* infection and is required for induction of IL-12, optimal induction of Th1 responses and host resistance to infection. Further studies to understand the unique requirement for IL-17 in protection against *LVS* will advance our understanding of the fundamental requirements for protective immunity to intracellular pathogens.

Experimental Procedures

Animals

C57BL/6 (B6), IL-12p40−/−, IL-12p35−/−, IFNγ−/−, γδ−/−, *yet40* reporter mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-12RB1−/−(Piccotti et al., 1999), IL-23p19−/− (Ghilardi et al., 2004), IL-12p35−/− IL-23p19−/− (Khader et al., 2005), IL-17−/− (Nakae et al., 2002), IL-17R−/− (Ye et al., 2001), IL-22−/− (Zheng et al., 2008) on the B6 background were used in accordance with University of Pittsburgh IACUC guidelines.

Experimental infection, cytokine and antibody treatment

LVS(BEI Resources) was grown in Mueller–Hinton (MH) broth or agar (Duckett et al., 2005). For pulmonary infections, mice were infected intratracheally with 1000 CFU *LVS*. Heat inactivated *LVS* stocks were prepared by incubating bacteria at 60° C for 1 hour. Some mice received recombinant IL-17(R and D Systems) intratracheally (1.5 μg) on day 3 post infection. Some B6 mice received 100 μg of isotype control antibody (501040) or anti-IL-17 (54447, R

and D Systems) intraperitoneally on days 2 and 4 post infection. Serial dilutions of homogenized infected lungs were plated on day 6 to determine lung CFUs.

Cell Preparation

Lung cell suspensions were prepared as described before (Khader et al., 2005) and used for ELISpot, flow cytometric analyses or for sorting purified populations.

Detection of cytokine producing cells by ELISpot assay

Detection of antigen-specific IFNγ- and IL-17-producing cells was carried out using an ELISpot assay (Khader et al., 2005). *LVS* heat inactivated antigen (0.5μg/ml, BEI Resources) or $OVA_{323-339}$ peptide (5μM) was used to stimulate cells from LVS-infected or OT-II αβTCR Tg mice respectively.

Generation and stimulation of BMDMs and BMDCs

BMDMs and BMDCs were generated from the bone marrow cells (Khader et al., 2005) by culturing in cDMEM containing GM-CSF (PeproTech). On day 7, nonadherent cells were used as BMDCs, while adherent cells were used as BMDMs. Lung alveolar macrophages were obtained by bronchoalveolar lavage. Cells were stimulated with heat inactivated *LVS* (MOI 1:100) alone or in combination with IL-17, IL-17F or IL-22 (100 ng/ml each) and either soluble IL-17RA Fc chimera or soluble IL-17RC (1000ng/ml each; R and D Systems). In some experiments, the BMDCs were pretreated with a NF-κβ inhibitor PTDC (100uM) for 45 mins, followed by co-treatment with IL-17 for 45 mins. Culture supernatants were analyzed by luminex assays. For macrophage killing assays, BMDMs were infected with live *LVS* (MOI 1:10) for 24 hours in the presence of either IFNγ or IL-17 (both at 100 ng/ml) in antibiotic-free cDMEM. BMDMs were washed extensively, lysed with sterile water, serially diluted and CFU was determined.

Determination of protein levels

IL-17, IL-12, IFNγ, $^{\mathrm{TNF}-\alpha}$. IL-6, G-CSF, GM-CSF, KC, MIP-1α, MCP-1, IL-1α, IL-1β protein levels were measured in lung homogenates and cell culture supernatants using a 22-plex mouse Luminex assay (Linco/Millipore).

Naive $CD4^+$ T cell isolation and in vitro effector generation

Naïve $CD4^+$ T cells were isolated from OT-II αβTCR Tg mice using magnetic $CD4^+$ beads (GK1.5) (Miltenyi Biotec) and purified based on CD62L high and CD25 low expression on a BD FACSAria (> 98%). Naive OT-II $CD4^+$ T cells (1×10^6 cells/ml) were cultured with *LVS*-stimulated or unstimulated BMDCs (1×10^6 cells/ml), $OVA_{323–339}$ peptide (5 μM) for 6 days. In some wells IL-12, IL-17 or IL-22 (100 ng/ml) were added. At the end of the culture period, the numbers of IFNγ- or IL-17-producing $CD4^+$ T cells was determined by ELISpot assay or intracellular staining.

Flow cytometry and cell sorting

Single cell suspensions were stained with fluorochrome-labeled antibodies specific for CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), TCRβ (H57-597), TCRγδ(GL3), NK1.1(PK136), Gr1 (RB6-8C5), CD11b (M1/70), CD11c (HL3), and MHC class II $I\text{-}A^b$ (AF6-120.1). For intracellular analyses of cells, cells stimulated with Phorbol myristate acetate (50ng/ml), ionomycin (750 ng/ml; Sigma Aldrich) and Golgistop (BD Pharmingen), were surface stained, permeabilized with Cytofix/Cytoperm solution (BD Pharmingen) and stained with anti-IFNγ (XMG1.2) and anti-IL-17(TC11-18H10). To determine activation of NF-κB using the Phospho-NF-kB p65 (3H1, Cell signaling), cells were stained according to manufacturer's

instructions. To sort for purified lung population, stained cells were sorted on BD FACS Aria flow cytometer as CD3+ CD4+ (>94%), CD3+ CD8+ (>96%) and CD11b+Gr1+ neutrophils (>95%). For other sorts, CD11C+ (> 91%), γδ cells (>84%) and NK1.1 (>93%) cells were sorted based on respective staining. For analysis, FlowJo (Tree Star Inc, CA) was used.

Real Time PCR (RT-PCR)

RNA was extracted (Khader et al., 2005), reverse transcribed and amplified using FAM-labeled probe and primers on the ABI Prism 7700 detection system. Fold increase in signal over that derived from uninfected samples was determined using the ΔΔct calculation. The primer and probes sequences have been previously published (Khader et al., 2005) or were commercially purchased (ABI Biosystems).

Statistical Analysis

Differences between the means of groups were analyzed using the two tailed Student's *t*-test in GraphPad Prism 4 (La Jolla, CA). Inherently logarithmic data from bacterial growth and RT-PCR were transformed for statistical analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Th, T (helper)
(LVS), Live vaccine strain
(BMDC), Bone-marrow Dendritic Cells
(BMDMs), Bone marrow derived macrophages
(CFU), Colony Forming units
(IL), Interleukin
(IFNγ), Interferon gamma
(NK), Natural Killer
(IL-12RB1), Interleukin-12 Receptor beta 1
(IL-17R), Interleukin 17 Receptor
(TNFα), Tumor Necrosis Factor alpha
(IL-1α), Interleukin 1 alpha
(IL-1β), Interleukin 1 beta
(MIP-1α), Macrophage inflammatory protein 1 alpha
(G-CSF), Granulocyte Colony stimulating factor
(GM-CSF), Granulocyte-Macrophage Colony stimulating factor
(KC), Keratinocyte chemoattractant
(MCP-1), Monocyte Chemoattractant Protein 1

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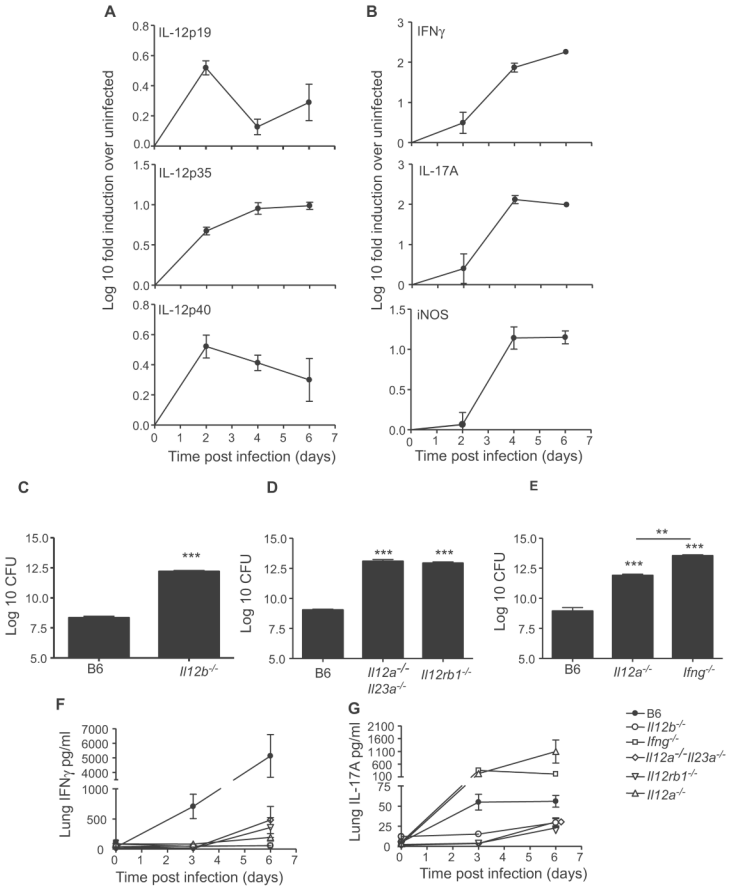

Figure 1. IL-12 dependent IFN-γ is critical for protection following pulmonary tularemia

mRNA induction for IL-23p19, IL-12p35 and IL-12p40 (A) and IFNγ, IL-17 and iNOS (B) in LVS infected B6 lungs vs. uninfected lungs was determined by RT-PCR. B6 mice and IL-12p40−/− (C), B6, IL-12p35−/−IL-23p19−/−, IL-12RB1−/− (D) and B6, IL-12p35−/− and IFNγ−/− (E) were infected with *LVS* and the lung CFU determined and lung homogenates were assayed for IFNγ (F) or IL-17 (G) levels. Data points represent mean (±SD) from 4-5 mice (A-G). **, $p \leq 0.005$, ***, $p \leq 0.0005$. 1 experiment representative of 2 or more.

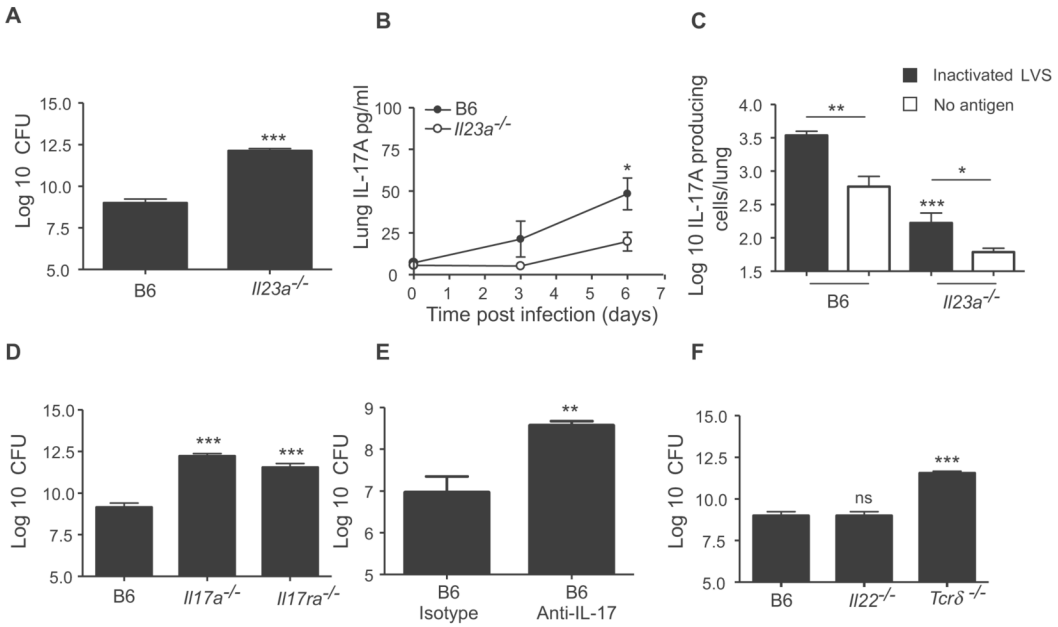

Figure 2. IL-23 dependent IL-17 is required for protection against pulmonary tularemia

B6 and IL-23p19−/− were infected with *LVS* and the lung CFU determined (A). Lung IL-17 levels (B) and the number of lung IL-17 producing cells from day 4-infected B6 and IL-23p19 −/− mice was determined (C). B6, IL-17−/, IL-17R−/− were infected with *LVS* and lung CFU determined (D). *LVS* infected-B6 mice were treated with either control or anti-IL-17 neutralizing antibody and lung CFU determined (E). B6, IL-22−/− and γδ−/− mice (F) were infected with *LVS* and lung CFU determined. Data points represent mean (±SD) from 4-5 mice (A-F). *, $p \leq 0.05$. **, $p \leq 0.005$, ***, $p \leq 0.0005$. ns-not significant. 1 experiment representative of 2 or more.

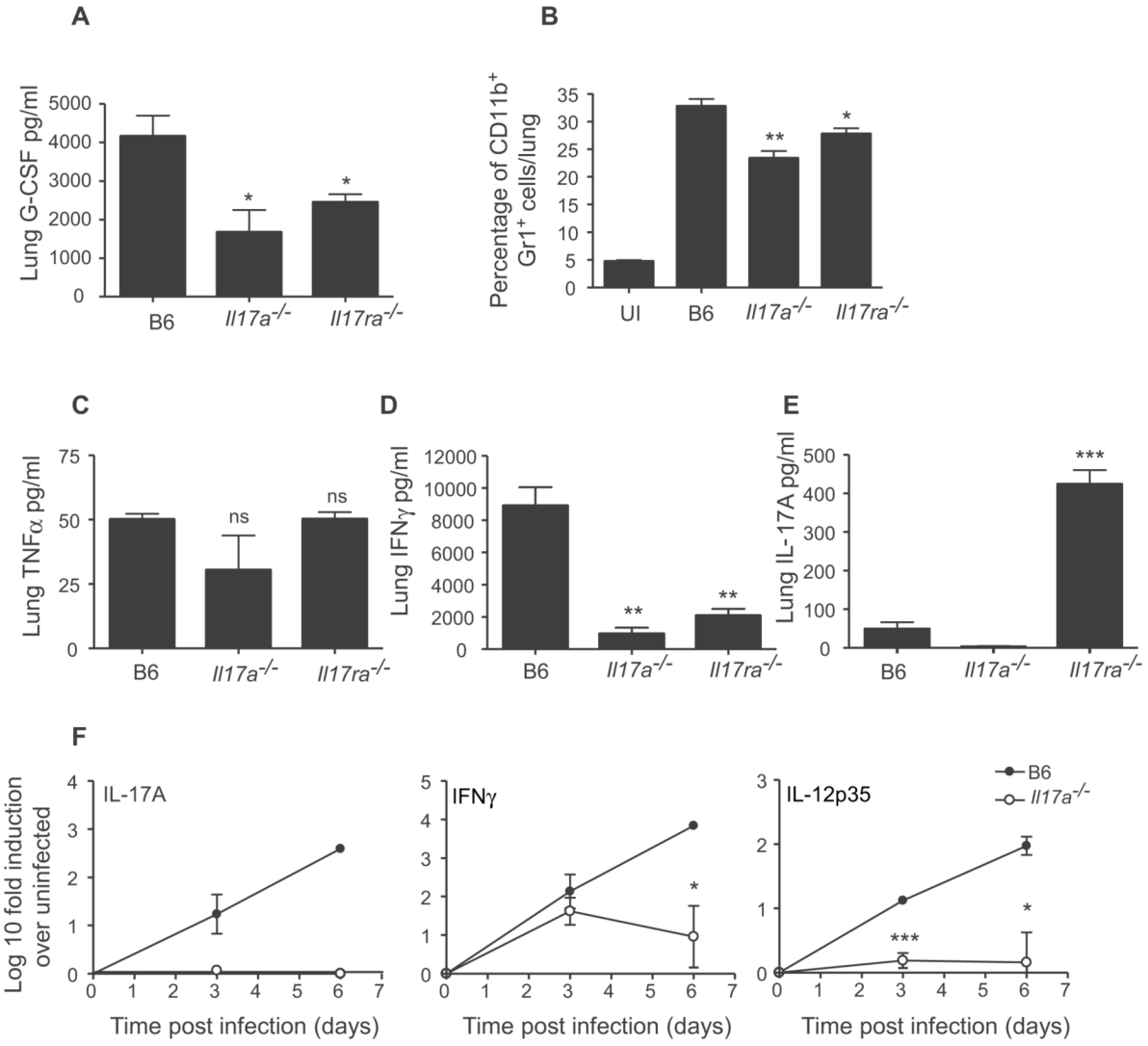

Figure 3. IL-17 is required for induction of IFNγ responses during pulmonary tularemia

B6, IL-17−/− and IL-17R−/− mice were infected with *LVS* and lung G-CSF levels determined (A). Percentage of lung neutrophils in uninfected (UI) or infected B6, IL-17−/− and IL-17R−/− mice was determined (B). Lung homogenates from day 6-infected B6, IL-17−/− and IL-17R−/− mice were assayed for TNF-α (C), IFNγ (D) and IL-17 (E). The induction of specific mRNA was determined by RT-PCR in infected B6 and IL-17−/− lungs (F). *, $p \leq 0.05$. **, $p \leq 0.005$. ***, $p \leq 0.0005$. 1 experiment representative of 2 or more.

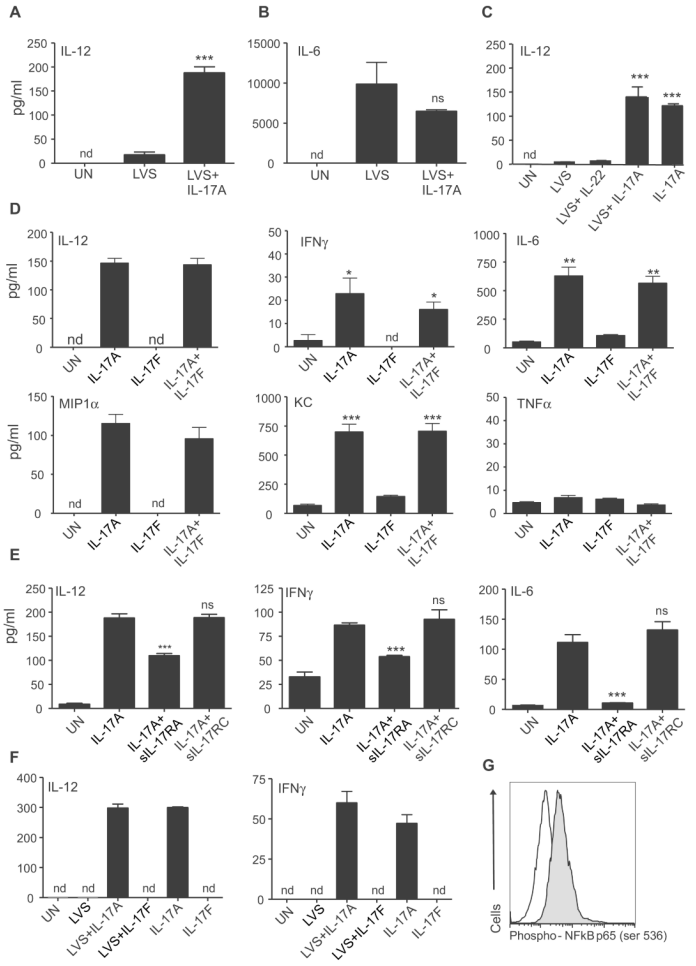

Figure 4. IL-17 but not IL-17F or IL-22 can induce IL-12 from BMDCs

B6 BMDCs were left untreated (UN), stimulated with *LVS* alone or with IL-17 (100 ng/ml) and supernatants assayed for IL-12 (A) or IL-6 (B). B6 BMDCs were treated with *LVS* alone or with IL-22 or IL-17 (100 ng/ml), or IL-17 (100 ng/ml) alone and IL-12 levels determined (C). B6 BMDCs were left untreated or treated with IL-17 (100 ng/ml), IL-17F (100 ng/ml), or both IL-17 and IL-17F (100 ng/ml each) and cytokine levels determined in supernatants (D). B6 BMDCs were left untreated or treated with IL-17 (100 ng/ml) alone or with soluble IL-17RA or IL-17RC (1000 ng/ml each) and cytokine levels determined in supernatants (E). Lung CD11c + cells from B6 mice were left untreated or treated with *LVS* alone or with IL-17(100 ng/ml) or IL-17F (100 ng/ml). IL-17 and IL-17F alone treated cultures were also included and supernatants assayed for IL-12 and IFNγ levels (F). BMDCs were left untreated (white histogram) or treated with IL-17 (grey histogram) and expression of Phospho-NF-κβ p65 determined. Samples were treated in triplicates (A-G). nd-not detectable, *, $p \leq 0.05$. **, $p \leq 0.005$. ***, $p \leq 0.0005$, 1 experiment representative of 2 or more.

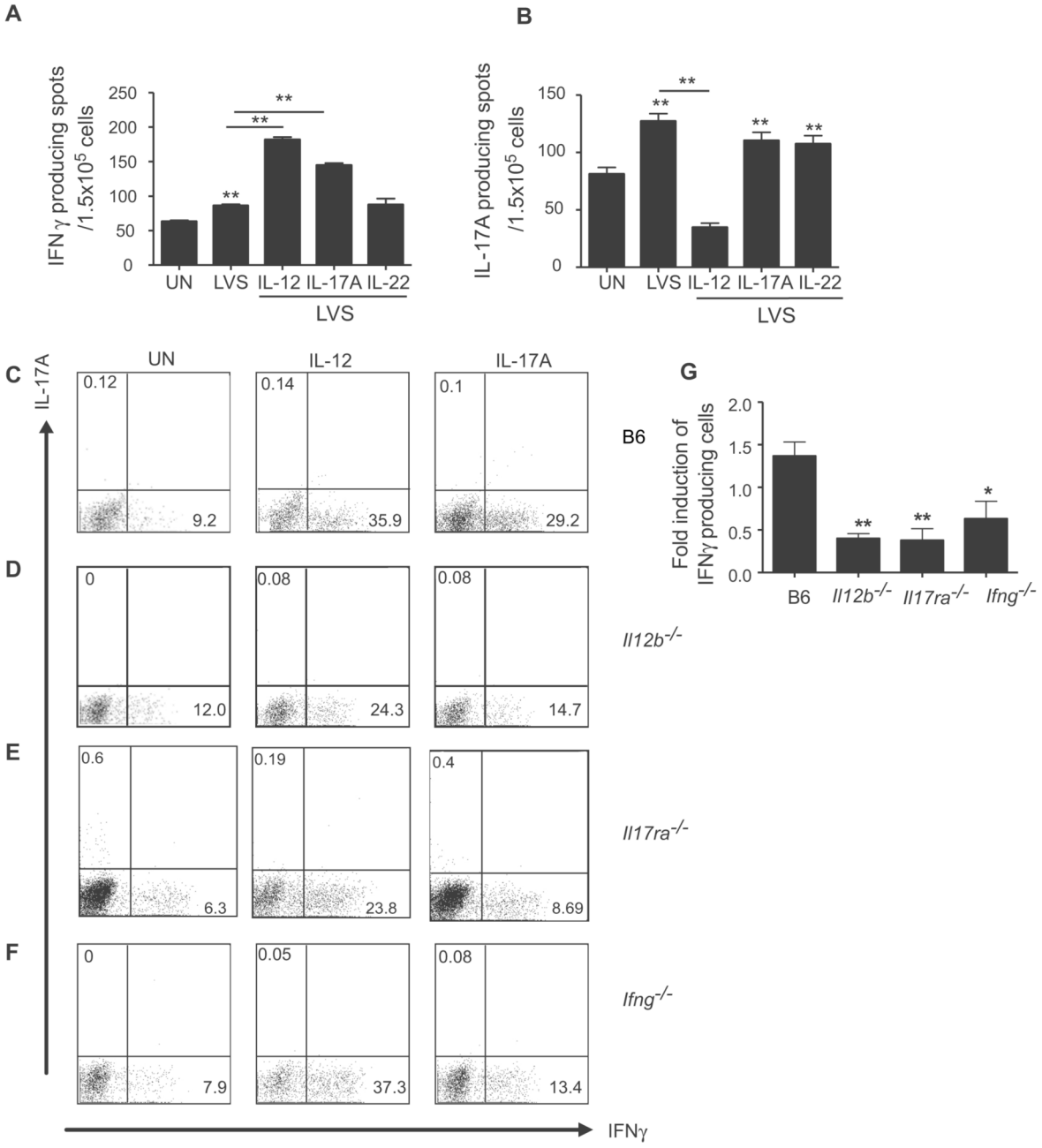

Figure 5. IL-17 can induce the polarization of naïve T cells into IFNγ-producing T cells

B6 BMDCs were stimulated with *LVS* alone or with IL-12 (100 ng/ml), IL-22 (100 ng/ml) or IL-17 (100 ng/ml) and naive OT-II TCR-Tg $CD4^+$ T cells. The frequency of IFNγ (A) and IL-17 producing cells (B) were determined by ELISpot assay. BMDCs generated from B6 mice (C), IL-12p40−/− (D), IL-17R−/−(E) or IFNγ−/− (F) were cultured with naïve OT-II TCR Tg CD4+ T cells alone or with IL-12 (100 ng/ml) or IL-17(100 ng/ml) in triplicates and IL-17 and IFNγ levels determined by flow cytometry; fold induction relative to untreated control group was determined (G). *, $p \leq 0.05$. **, $p \leq 0.005$. ***, $p \leq 0.0005$.

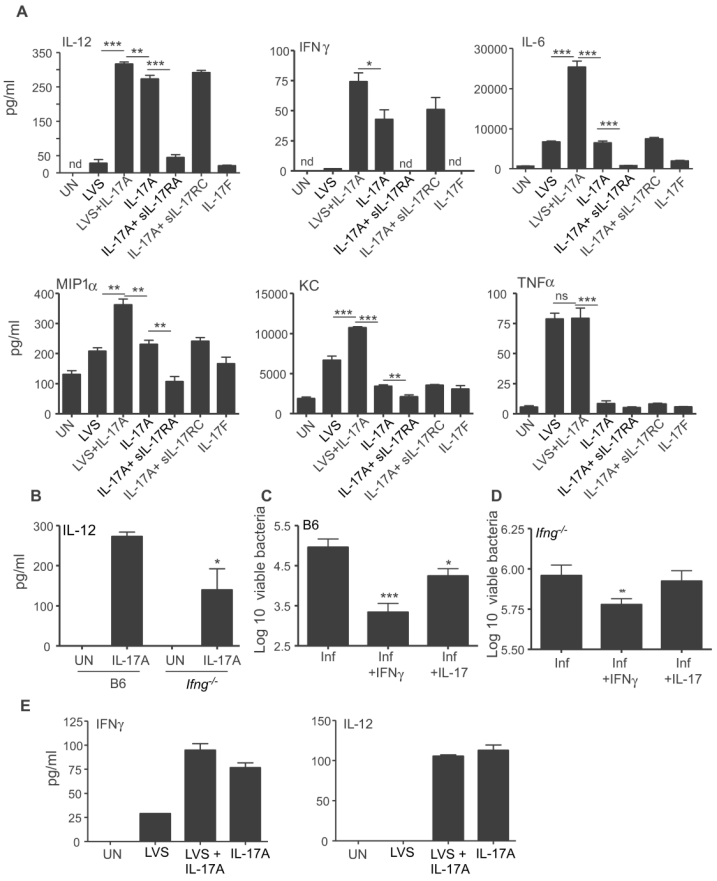

Figure 6. IL-17 induces IFNγ and IL-12 from macrophages and enhances bacterial clearance

BMDMs from B6 mice were either left untreated (UN) or treated with *LVS* alone, IL-17 (100 ng/ml) alone, IL-17F alone (100 ng/ml) or *LVS* and IL-17 (100 ng/ml). Some wells received IL-17(100 ng/ml) and soluble IL-17RA or IL-17RC (1000ng/ml each) and protein levels in supernatants determined (A). BMDMs from B6 or IFNγ−/− mice were left untreated or treated with IL-17 (100 ng/ml) and IL-12 levels determined (B). BMDMs from B6 mice (C) or IFNγ −/− (D) were infected with live *LVS* alone, or with IFN-γ (100 ng/ml) or IL-17 (100 ng/ml) for 24 hours and intracellular CFU determined. Lung alveolar macrophages were treated with *LVS* alone or with IL-17, IL-17 (100 ng/ml) alone or IL-17F alone (100 ng/ml) and cytokine levels determined (E). Samples were treated in triplicates (A-E). nd-not detectable, *, $p \leq 0.05$. **, $p \leq 0.005$. ***, $p \leq 0.0005$.

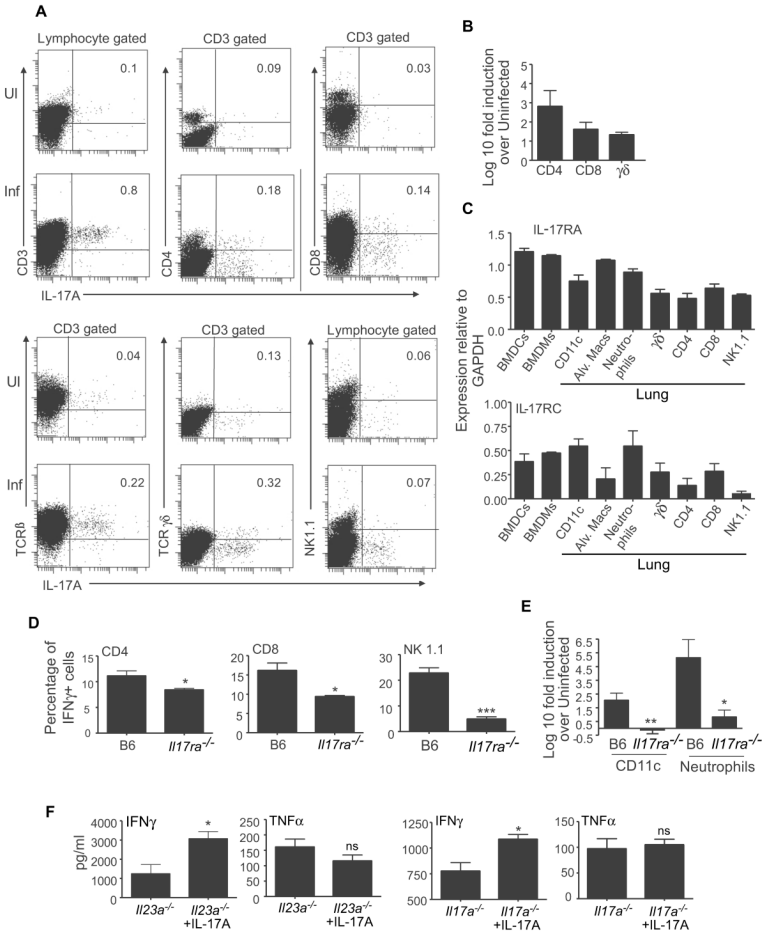

Figure 7. Cellular sources of IL-17 in the lung following *LVS* infection

Uninfected or LVS-infected B6 (A) mice were assayed for cellular sources of IL-17 producing cells in day-6 infected lungs by flow cytometry. The percentage of cells expressing IL-17 within gated populations is shown (A). Log_{10} fold induction of IL-17 mRNA in sorted lung cell populations from day-6 infected B6 lungs vs. uninfected lungs was determined by RT-PCR (B). mRNA expression of IL-17RA or IL-17RC relative to GAPDH levels was determined by RT-PCR on sorted cell populations (C). The percentage of IFNγ-producing cells within each population in B6 and IL-17−/− infected lungs is shown (D). Log_{10} fold induction of IL-12p35 mRNA in sorted cells from infected vs. uninfected lungs was determined by RT-PCR (E). LVS-infected IL-23p19−/− or IL-17−/− mice were treated with PBS or rIL-17 and day6-infected lung homogenates assayed for IFNγ and TNF-α (F). The data points represent the mean (±SD) of values from 3-5 mice (A-F). *, $p \leq 0.05$. **, $p \leq 0.005$. ***, $p \leq 0.0005$.