Published in final edited form as:
Eur J Immunol. 2009 February ; 39(2): 426–438. doi:10.1002/eji.200838726.

A Novel IL-17 Dependent Mechanism of Cross Protection: Respiratory Infection with Mycoplasma Protects Against a Secondary Listeria Infection

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Summary

Immune responses to pathogens occur within the context of current and previous infections. Cross protection refers to the phenomena where infection with a particular pathogen provides enhanced resistance to a subsequent unrelated pathogen in an antigen independent manner. Proposed mechanisms of antigen-independent cross protection have involved the secretion of IFN-γ, which activates macrophages thus providing enhanced innate immunity against the secondary viral or bacterial pathogen. Here we provide evidence that a primary infection with the chronic respiratory pathogen, *Mycoplasma pulmonis*, provides a novel form of cross protection against a secondary infection with *Listeria monocytogenes* that is not mediated by IFN-γ, but instead relies upon IL-17 and mobilization of neutrophils. Mice infected with *M. pulmonis* have enhanced clearance of *L. monocytogenes* from the spleen and liver which is associated with increased numbers of Gr-1+CD11b+ cells and higher levels of IL-17. This enhanced clearance of *L. monocytogenes* was absent in mice depleted of Gr-1+ cells or in mice deficient in the IL-17 receptor. Additionally, both the IL-17 receptor and neutrophils were essential for optimal clearance of *M. pulmonis*. Thus, a natural component of the immune response directed against *M. pulmonis* was able to enhance clearance of *L. monocytogenes*.

Keywords

Bacterial infection; cytokine; cytokine receptor; neutrophil

Introduction

Innate and adaptive immune responses against pathogens are extremely complex, involving multiple cell types and effector molecules. Innate immune responses are initiated by bacterial, viral, fungal or parasitic elements that are conserved across organisms, while adaptive immune responses develop against specific antigenic epitopes contained within these pathogens. Activation of innate and adaptive immune cell populations is transiently

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Conflict of Interest
The authors declare no financial or commercial conflict of interest.

induced through interactions with the specific pathogen. Thus, immune responses are generally self-regulating and specifically directed against the particular pathogen encountered. However, in certain circumstances, immune responses against a given pathogen can also act on a completely unrelated pathogen. These “cross protective” responses can enhance the clearance of pathogens in an antigen non-specific fashion, but can in some situations involve adaptive immune cell populations.

Previously described mechanisms of cross protection involve the production of IFN-γ which in turn can activate macrophages, generating a heightened state of innate immunity to a secondary pathogen. *Classical* cross protection is offered by IFN-γ secreting effector lymphocytes responding to a primary pathogen. Early studies suggest that macrophages activated during a primary viral or bacterial infection could respond to an unrelated viral or bacterial pathogen, thus providing increased protection compared to naive animals [1;2]. This form of cross protection is effective while the primary pathogen persists, and then subsides when the effector lymphocytes differentiate into memory cells [1–5]. In contrast, *latency-induced* cross protection involves the secretion of IFN-γ in response to latent viral proteins which can provide protection for up to three months following the initial encounter with the primary viral pathogen [3]. Macrophage-dependent control of the secondary bacterial infection was shown to be the mechanism of this enhanced protection. A third method of cross protection describes a mechanism by which memory T cell populations can provide protection against a new secondary pathogen, long after the primary pathogen has been cleared from the system. In this *memory-induced* model of cross protection, memory CD8+ T cells secrete IFN-γ in an antigen-independent manner in response to IL-12 and IL-18 which are innately secreted during the initial immune response to a secondary infection [6;7]. In this scenario, the T cells that developed into memory cells during the adaptive immune response to a primary viral pathogen now function to heighten the innate immune response to a secondary bacterial pathogen. All of these methods of cross protection involve an increase in IFN-γ production which can lead to macrophage activation. Here, we propose a novel mechanism of cross protection that instead is dependent on IL-17 production and granulocyte mobilization.

Production of IL-17 occurs in a wide variety of chronic inflammatory conditions, and mice lacking or showing reduced IL-17 production have decreased susceptibility to the development of autoimmune diseases [8–11]. Furthermore, IL-17 is produced during a number of bacterial, viral, and fungal infections [12–19]. The necessity of IL-17 in the clearance of multiple pathogens was determined using different experimental approaches that negate IL-17 production or signaling [12;13;15;20;21]. The ability of IL-17 to mobilize neutrophils is thought to be the principal reason this cytokine is important for protection against infectious pathogens. IL-17 is known to induce the mobilization of neutrophils through the production of cytokines and chemokines such as IL-6, G-CSF, GM-CSF, CXCL1, CXCL2, and CXCL8 [9]. To our knowledge, it is yet to be shown that this immune pathway is utilized as a mechanism of cross protection.

Mycoplasma pulmonis is an atypical bacterium, due to the absence of a cell wall, that induces a chronic respiratory infection in rodents that is characterized by neutrophilic inflammation and lymphocyte infiltration. This rodent model is similar to the human disease induced by *Mycoplasma pneumoniae* [22;23], which is the leading cause of pneumonia worldwide [24–26]. Multiple cell types, cytokines, and chemokines are involved in the immune response to mycoplasma infections along the respiratory tract. We recently found increased IL-17 mRNA expression in the lungs of *M. pulmonis* infected mice [27]. Consistent with this, another report found that IL-17 protein and mRNA levels were increased during *M. pneumoniae* infection in mice [28]. Furthermore, IL-17 levels were associated with neutrophil recruitment during *M. pneumoniae* infection. Multiple studies

report increased numbers of neutrophils in the lungs and bronchoalveolar lavage during both *M. pulmonis* [29–32] and *M. pneumoniae* infection [28;33;34]. Collectively, these data suggest that the production of IL-17 during mycoplasma infections may induce the mobilization of neutrophils.

Listeria monocytogenes is a gram-positive intracellular bacterium that is a common source of contamination in both raw and processed foods that can lead to septicemia and death in susceptible individuals [35]. *L. monocytogenes* primarily replicates within the spleen and liver following intravenous or oral inoculation. While the sterilizing clearance of *L. monocytogenes* is dependent on T cells, the initial immune response that limits bacterial numbers involves macrophages and neutrophils. Depletion of neutrophils, with an anti-Gr-1 antibody, resulted in impaired clearance of *L. monocytogenes* [36–38]. Likewise, depletion of macrophages [39;40] or prevention of accumulation of CD11b expressing cells [41–43] both increase susceptibility to *L. monocytogenes* infection. While IFN-γ is an essential cytokine for the clearance of *L. monocytogenes* during natural infection [44;45], IL-17 production has not yet been determined to be a natural requirement of the immune response against *Listeria monocytogenes*. However, mice deficient in leukocyte function-associated antigen-1 (LFA-1, or CD11a/CD18) have decreased susceptibility to *L. monocytogenes* that was associated with increased serum levels of IL-17 and G-CSF [46]. This increase in IL-17 was associated with enhanced neutrophil recruitment to the liver. Thus, the production of IL-17 during *L. monocytogenes* infection appears to increase resistance to *L. monocytogenes* by amplifying neutrophil mobilization.

The current study investigates the impact of a chronic respiratory infection with *M. pulmonis* on a subsequent *L. monocytogenes* infection. The investigation of immune responses to pathogens in the context of other infections is an important topic of research. Individuals are likely to simultaneously encounter multiple pathogens, and the interactions between the immune responses against those different pathogens may be a determining factor in susceptibility to the infections. Furthermore, subclinical chronic diseases that may go undetected in the population may impact subsequent acute infections. The pre-existing immune environment during chronic infections with pathogens such as *M. pulmonis* and *M. pneumoniae* could influence the developing immune response to infection with an unrelated pathogen. In this study, we provide evidence that chronic infection with the pulmonary pathogen, *M. pulmonis*, provides a yet undiscovered method of cross protection against *L. monocytogenes* infection that is dependent on the IL-17 receptor and granulocytes. Furthermore, we show that the IL-17 receptor and neutrophils are required for optimal clearance of *M. pulmonis*.

Results

Chronic infection with M. pulmonis facilitates clearance of L. monocytogenes

To investigate the impact of chronic *M. pulmonis* infection on the course of a subsequent, unrelated, bacterial infection, C57BL/6 mice were first inoculated with *M. pulmonis* or mycoplasma broth medium (sham). Seventeen days later, half of the broth control and *M. pulmonis* inoculated mice were innoculated with *L. monocytogenes* or phosphate buffered saline (PBS). Bacterial CFU counts were enumerated in the spleen and liver at day 3 post-infection (p.i.) with *L. monocytogenes*. As seen in Figure 1C, prior infection with *M. pulmonis* (Myco) significantly decreased *L. monocytogenes* (LM) CFU counts, independent of the organ assayed, $p \leq 0.05$. While C57BL/6 mice are known to have a greater resistance to *M. pulmonis* infection[47], this phenomena was also observed in a strain of mice that is highly susceptible to *M. pulmonis* infection: BALB/c mice displayed the same pattern of prior infection with *M. pulmonis* reducing *L. monocytogenes* CFU counts (data not shown). Thus, a primary infection with *M. pulmonis* provided protection against a secondary

infection *with L. monocytogenes* in two strains of mice that vary in their susceptibility to *M. pulmonis* infection.

The reduced bacterial burden observed in the spleen and liver could have been a result of decreased bacterial spread of *L. monocytogenes* to these organs. Due to the upregulation of neutrophils and macrophages during *M. pulmonis* infection [28;31;32;48], enhanced phagocytosis of *L. monocytogenes* immediately following i.v. innoculation could have occurred in the blood and lungs of mice with the chronic respiratory *M. pulmonis* infection, prior to *L. monocytogenes* reaching the spleen or liver. To test this hypothesis, *L. monocytogenes* CFU counts were assayed in the blood, lung, spleen, and liver at earlier time points p.i. with *L. monocytogenes*. In order to observe detectable levels of *L. monocytogenes* in the blood and lungs at these earlier time points, higher inoculation doses of *L. monocytogenes* were used. Increases in *L. monocytogenes* CFU counts in the blood and lungs were not observed at either 3 hrs p.i. or 24 hrs p.i. with *L. monocytogenes* (Figures 1A and 1B), indicating that *L. monocytogenes* was not being engulfed by cells in either of these tissues to a greater degree in *M. pulmonis* infected mice, $p \geq 0.05$. Furthermore, chronic *M. pulmonis* infection did not facilitate the clearance of *L. monocytogenes* in the spleen and liver at these early time points, $p \geq 0.05$.

If the reduction of CFU counts in the spleen and liver at day 3 p.i. with *L. monocytogenes* was mediated by a decreased bacterial spread to these organs, there should be reduced CFU counts in these organs from the onset of infection. However, this was not observed at either 3 or 24 hrs p.i. with higher inoculation doses. To determine if the early time point or the higher inoculation dose led to the lack of cross-protection observed, CFU counts were enumerated at 3, 24, and 72 hrs p.i. using the same inoculation dose of *L. monocytogenes* (Figures 1D, 1E, and 1F). The enhanced clearance of *L. monocytogenes* offered by *M. pulmonis* was again only evident at 72 hrs p.i. with *L. monocytogenes* (Figure 1F), $p \leq 0.05$. Thus, *M. pulmonis* infection facilitated clearance of *L. monocytogenes* is mediated by a more complex immune mechanism than an increase in phagocytosis and trapping of *L. monocytogenes* in the lungs or peripheral blood of mice with a pulmonary mycoplasma infection. Enhanced protection against *L. monocytogenes* appears to occur following bacterial spread to the spleen and liver. The pre-existing immune environment induced by a chronic respiratory infection determined the subsequent development of the immune response against *L. monocytogenes*. Therefore, an interaction between the long-lasting immune response to *M. pulmonis* and the immune response being mounted against an acute *L. monocytogenes* infection was necessary to reduce the bacterial burden in *L. monocytogenes* infected mice.

M. pulmonis and L. monocytogenes co-infection increases Gr-1+CD11b+ cells

Flow cytometric analysis of the immune cell populations in the spleen was performed to investigate which cell types might be mediating the chronic *M. pulmonis* infection induced facilitated clearance of *L. monocytogenes*. Two-way ANOVAs found no detectable differences between *L. monocytogenes* infected mice and mice infected with *M. pulmonis* followed by *L. monocytogenes* in the percentage of CD4+ T cells, CD8+ T cells, NK cells, NK-T cells, or macrophages/monocytes (data not shown). However, there was a significant increase in the percentage of granulocytes (Gr-1+) in the spleens of mice co-infected with *M. pulmonis* and *L. monocytogenes* (Figure 2A), $p \leq 0.05$. Given that CD11b is expressed on activated granulocytes [46;49], the cell surface expression of Gr-1 and CD11b was analyzed in splenocytes from 1) 1°broth/ 2°PBS, 2) 1°broth/ 2°*L. monocytogenes*, 3) 1°*M. pulmonis*/ 2°PBS, and 4) 1°*M. pulmonis*/ 2°*L. monocytogenes* infected C57BL/6 mice. As shown in Figures 2B and 2C, while both *M. pulmonis* and *L. monocytogenes* infection alone induced an increase in the percentage of Gr-1+CD11b+ cells in the spleen, the interaction between the immune responses against these pathogens led to a substantially greater

increase in this cell population, $p \leq 0.05$. These data suggest that neutrophils may be involved in the cross protection offered by *M. pulmonis* against *L. monocytogenes*.

IL-17, but not IFN-γ, production is increased during chronic M. pulmonis infection

While IFN-γ is known to be essential for the clearance of *L. monocytogenes* [44;45], in certain circumstances IL-17 has also been shown to increase resistance to this pathogen [46]. IL-17 has not yet been shown to be required for resistance to *L. monocytogenes* [50], but increased production of IL-17 in LFA deficient mice has been found to amplify the neutrophil recruitment leading to facilitated clearance of this pathogen [46]. Therefore, the possibility existed that either IFN-γ or IL-17 could be mediating the *M. pulmonis* induced enhanced clearance of *L. monocytogenes*. To investigate this, we performed experiments to measure both of the cytokines in this co-infection model. At day 17 p.i. with *M. pulmonis* or broth, C57BL/6 mice were infected with *L. monocytogenes* or PBS. At day 3 p.i. with *L. monocytogenes*, whole lung homogenates and sera were collected to analyze IL-17 with the Luminex assay. As seen in Figure 3A, *M. pulmonis* infection significantly increased IL-17 levels in the lungs of *L. monocytogenes* infected and uninfected mice, $p \leq 0.05$. A two-way ANOVA detected no significant differences in the level of IL-17 in the serum (data not shown), $p \geq 0.05$. Thus, while a local increase in IL-17 was observed in the lung, a systemic increase in IL-17 in response to *M. pulmonis* infection was not detected.

Intracellular cytokine staining and flow cytometry were used to evaluate the production of IL-17 from splenocytes and cervical lymph node cells. At day 17 p.i. with *M. pulmonis* or broth, C57BL/6 mice were infected with *L. monocytogenes* or PBS. At day 3 p.i. with *L. monocytogenes*, splenocytes and cervical lymph node cells from uninfected, singly infected, or co-infected mice were harvested and cultured overnight with or without *M. pulmonis* antigen. Cells were subsequently stained with antibodies directed against CD4, CD8, γδ T cell receptor, and intracellular IL-17. Two-way ANOVAs determined that *M. pulmonis* infection increased the percentage of CD4+ T cells that produced IL-17 in *L. monocytogenes* infected and uninfected mice in both the spleen and cervical lymph node, $p \leq 0.05$. However, CD4+ T cells from mice infected with *L. monocytogenes* alone did not produce IL-17 above background levels seen in uninfected mice in the spleen or cervical lymph node, $p \geq 0.05$. Furthermore, the greatest degree of IL-17 production was seen in cells isolated from *M. pulmonis* mice that were stimulated with *M. pulmonis* antigen (Figures 3B and 3C). In unstimulated cultures, IL-17 producing cells from *M. pulmonis* infected mice could also be detected, but far fewer existed than with *M. pulmonis* antigen stimulation (data not shown). Appreciable percentages of CD8+ T cells and γδ+ T cells that produced IL-17 were not detected in the spleen (data not shown). However, in the cervical lymph node, *M. pulmonis* infection also increased the percentage of γδ+ T cells that produced IL-17, $p \leq 0.05$ (Figure 3D). Collectively, these data suggest that IL-17 is being specifically produced in response to *M. pulmonis* infection by CD4+ and γδ+ T cells.

In order to quantify the amount of IL-17 being produced by the cervical lymph node cells, supernatants from these cultures were analyzed using an enzyme-linked immunosorbent assay (ELISA). To this end, cervical lymph node cells from uninfected, singly infected, or co-infected mice were harvested and cultured for two days with or without *M. pulmonis* antigen or heat-killed *L. monocytogenes*. There were no detectable levels of IL-17 from any unstimulated cultures or from cultures stimulated with heat-killed *L. monocytogenes* (data not shown). However, *M. pulmonis* antigen stimulated the secretion of IL-17 in cell cultures from *M. pulmonis* infected mice (1°Myco/2°Sham and 1°Myco/2°LM groups), $p \leq 0.05$ (Figure 3E). No IL-17 was detected in cultures of cells isolated from mice not infected with *M. pulmonis*. Thus, IL-17 production was dependent on *M. pulmonis* infection, and was observed only in response to *M. pulmonis* antigen stimulation.

In order to determine if a previous infection with *M. pulmonis* induced increased IFN-γ production upon infection with *L. monocytogenes*, IFN-γ levels were measured. At day 17 p.i. with *M. pulmonis* or broth, C57BL/6 mice were infected with *L. monocytogenes* or PBS. At day 3 p.i. with *L. monocytogenes*, sera and whole spleen homogenates were collected to analyze IFN-γ with the Luminex assay. *M. pulmonis* infection alone did not lead to production of IFN-γ, which is not unexpected at this late time point in the C57BL/6 strain of mice [27]. Importantly, a two-way ANOVA determined that mice infected with *L. monocytogenes* or co-infected with *M. pulmonis* and *L. monocytogenes* did not differ in the amount of IFN-γ present in the serum or spleen (Figures 3F and 3G), $p \geq 0.05$. Levels of IFN-γ in the lung were not detected above background (data not shown). In addition, flow cytometry data analyzing IFN-γ production in the cervical lymph nodes did not show a significant increase of IFN-γ secreting CD8 T or CD4 T cells under any condition measured (Figure 3H and data not shown). IFN-γ producing γδ+ T cells were not detectable in the CLN (data not shown).

Thus, while prior infection with *M. pulmonis* did not induce changes in IFN-γ production during a subsequent *L. monocytogenes* infection, it did increase the level of IL-17 that was produced during *L. monocytogenes* infection. Collectively, these data suggest that heightened levels of IL-17 during *M. pulmonis* infection could be responsible for increased neutrophil mobilization and the higher numbers of Gr-1+CD11b+ cells observed during the secondary *L. monocytogenes* infection.

Gr-1 depletion eliminates co-infection facilitated clearance of L. monocytogenes

Infiltration of granulocytes into infected spleen and liver is essential for early anti-*Listeria* defense [37;38;41]. Thus, the necessity of this increased granulocyte population in the facilitated clearance of *L. monocytogenes* induced by *M. pulmonis* infection was investigated using a Gr-1 depleting antibody. At day 16 p.i. with *M. pulmonis* or broth, C57BL/6 mice were injected with anti-Gr-1 or control antibody. Mice were infected with 1×10^4 CFUs of *L. monocytogenes* 24 hrs later, and the *L. monocytogenes* CFU counts in the spleen and liver were enumerated at day 3 p.i. with *L. monocytogenes*. A lower inoculation dose of *L. monocytogenes* was used in this experiment because previous studies have shown that depletion of granulocytes results in increased susceptibility to *L. monocytogenes* [36–38]. As seen in Figure 4, depletion of Gr-1+ cells completely abrogated the *M. pulmonis* induced facilitated clearance of *L. monocytogenes* in the spleen and significantly reduced it in the liver, $p \leq 0.05$. Therefore, the presence of granulocytes appears to mediate the cross protection that *M. pulmonis* infection offers against *L. monocytogenes* infection.

The IL-17 receptor mediates co-infection induced CFU decrease and Gr-1+CD11b+ cell increase

IL-17 is known to induce the mobilization of neutrophils through the production of cytokines and chemokines [9], and mice deficient in the IL-17 receptor have a reduced granulocyte response during respiratory infection [13]. Upregulation of IL-17 occurs in the lungs and bronchoalveolar lavage of mice infected with *M. pulmonis* and *M. pneuomoniae* [27;28]. To investigate whether the ability to respond to IL-17 produced during chronic *M. pulmonis* infection underlies the increases in granulocytes and the enhanced clearance of *L. monocytogenes*, IL-17 receptor deficient mice were utilized. As seen in Figure 5A and 5B, the enhanced clearance of *L. monocytogenes* induced by *M. pulmonis* infection is completely absent in IL-17 receptor deficient mice as compared to wild type C57BL/6 mice infected with *L. monocytogenes*. Furthermore, the increased number (Figure 5C) and percent (Figure 5D) of Gr-1+CD11b+ cells in the spleens of mice co-infected with *M. pulmonis* and *L. monocytogenes* is markedly reduced in the IL-17 receptor deficient mice. Wild type C57BL/6 mice have a significantly greater number and percentage of Gr-1+CD11b+ cells in the

spleen when co-infected with *M. pulmonis* and *L. monocytogenes* as compared to uninfected, *M. pulmonis* infected, or *L. monocytogenes* infected mice, $p \leq 0.05$. While the co-infection induced increase in this cell population is still observable in the IL-17 receptor deficient mice, it is significantly reduced as compared to wild type C57BL/6 mice, $p \leq 0.05$. Thus, the IL-17 receptor mediated not only the cross protection offered by *M. pulmonis* against a secondary *L. monocytogenes* infection, but also the increased Gr-1+CD11b+ population of cells observed in the co-infected mice. This data suggests that IL-17 produced during *M. pulmonis* infection was able to amplify the neutrophil mobilization during *L. monocytogenes* infection, thus facilitating the clearance of *L. monocytogenes*.

Gr-1+ cells are involved in the clearance of M. pulmonis

Neutrophils numbers are known to increase during *M. pulmonis* infection [29–32]. Here we depleted granulocytes from days 16–20 p.i. with *M. pulmonis*. This depletion was found to eliminate the facilitated clearance of *L. monocytogenes* induced by *M. pulmonis*. However, it was unknown whether granulocytes were involved in the clearance of *M. pulmonis* at this stage of infection. Indeed, Gr-1 depleted mice had higher *M. pulmonis* CFU counts in the lungs and nasal passages as compared to mice that had received the control antibody (Figure 6), $p \leq 0.05$. Depletion of granulocytes impaired the clearance of *M. pulmonis*. Therefore, the upregulation of granulocytes represents an element of a normal immune response against chronic *M. pulmonis* infection that is able to offer cross protection against an additional pathogen.

IL-17 receptor deficient mice are more susceptible to M. pulmonis infection

Expression of IL-17 was shown to be increased during *M. pulmonis* and *M. pneumoniae* infection [27;28], however the role that IL-17 might play in the clearance of *M. pulmonis* had not been investigated. To determine if the IL-17 receptor is an essential component of the immune response against chronic *M. pulmonis* infection, IL-17 receptor deficient mice or wild type C57BL/6 mice were inoculated intranasally with *M. pulmonis* or mycoplasma broth medium. At day 20 p.i. with *M. pulmonis*, CFU counts were enumerated in the lungs and nasal passage washes. As seen in Figure 7, IL-17 receptor deficient mice were less capable of clearing *M. pulmonis* from these tissues, $p \leq 0.05$. Thus, the IL-17 receptor is necessary to mount an optimal immune response against *M. pulmonis*. This optimal immune response against *M. pulmonis* is then able to confer protection against a second pathogen, *L. monocytogenes*.

Discussion

Our data shows that a chronic infection with the respiratory pathogen *M. pulmonis* facilitated the clearance of an acute *L. monocytogenes* infection. The enhanced clearance of *L. monocytogenes* observed at day 3 p.i. in the spleen and liver, however, was not seen at earlier time-points p.i. with *L. monocytogenes*. This indicates that the reduced CFU counts were not attributable to decreased spread of *L. monocytogenes* to the spleen and liver. Likewise, the cross protection offered by *M. pulmonis* was not mediated by enhanced phagocytosis of *L. monocytogenes* in the peripheral blood or lungs of *M. pulmonis* infected mice, in that no increase in *L. monocytogenes* CFU counts existed in these tissues. Alternatively, an interaction between the established immune responses against *M. pulmonis* and a developing immune response against *L. monocytogenes* was required to facilitate the clearance of *L. monocytogenes*. Analysis of the cell types involved in this cross protection revealed that there was an increase in the percentage of granulocytes in the spleens of co-infected mice. Further studies using the Gr-1 depleting antibody found that the *M. pulmonis* induced enhanced clearance of *L. monocytogenes* was dependent on this granulocyte population. An increase in both IL-17 protein levels and the percentage of IL-17 producing

cells was found in the *M. pulmonis* infected mice, while IFN-γ protein levels and IFN-γ secreting cells were not impacted by the co-infection with *M. pulmonis*. A deficiency in the IL-17 receptor eliminated the cross protection offered by *M. pulmonis* against *L. monocytogenes* and reduced the increase in Gr-1+CD11b+ cells observed during the co-infection. Taken together, these data show that a chronic respiratory infection with *M. pulmonis* facilitates clearance of systemic *L. monocytogenes*, and that this cross protection is dependent on the IL-17 receptor and granulocytes.

While multiple mechanisms of antigen-independent cross protection have been proposed, each of them has relied on the production of IFN-γ which in turn can activate macrophages [1–7]. The current findings suggest that a heightened state of immunity induced by IL-17 production and neutrophil mobilization can also provide an antigen-independent form of cross protection. One implication of this data is that infections which induce IL-17 production, and thus the proliferation, activation, and recruitment of neutrophils, will be able to provide cross protection against secondary infections in which neutrophils play a key role in susceptibility. Neutrophils are a primary innate immune cell population key to the initial inflammatory response and control of most pathogens. IL-17 induced inflammatory responses are utilized during infection, as well as autoimmunity. The production of IL-17 was shown to be key to the progression of multiple autoimmune conditions including Multiple Sclerosis, Rheumatoid Arthritis, psoriasis, and Crohn's disease (for reviews see [8–11]). Thus, IL-17 inducing infections may influence the development of such autoimmune diseases, hastening the onset of symptoms or worsening the severity of autoimmune conditions. IL-17 is produced during a wide variety of infectious diseases: bacterial pathogens such as *Klebsiella pneumonia*e, *Pseudomonas aeruginosa*, *Bacteroides fragilis*, *Citrobacter rodentium*, *Escherichia coli*, *Bordetella pertussis*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and *Mycoplasma pneumoniae*; fungal pathogens such as *Candida albicans*, *Cryptococcus neoformans*, and *Pneumocystis carinii*; parasitic pathogens such as *Toxoplasma gondii*; viral infections such as vaccinia virus (for review see[17]). Infections with these pathogens may not only worsen autoimmune conditions, but also have the possibility of leading to cross protection against subsequent infections with an unrelated pathogen via IL-17 induced neutrophil proliferation, activation, and recruitment. To our knowledge, this is the first time that a novel mechanism of cross protection mediated by IL-17 secretion and neutrophil mobilization has been shown.

The current study also found that granulocytes and the IL-17 receptor are involved in the clearance of *M. pulmonis* from the lungs and nasal passages. Mice depleted of neutrophils with the Gr-1 antibody at days 16–20 p.i. with *M. pulmonis* had increased *M. pulmonis* CFU counts in the lungs and nasal passages. The necessity of granulocytes for the clearance of *M. pulmonis* had not previously been determined at this stage of infection. Neutrophils have been reported to be increased in the lungs and bronchoalveolar lavage fluids during *M. pulmonis* infection [29–32]. Depletion of neutrophils using cyclophosphamide was shown to increase *M. pulmonis* CFUs in the lungs at day 3 p.i. [31], however the impact of neutrophils on *M. pulmonis* clearance at later stages during infection had not been determined. In our co-infection model, we provide evidence that the mobilization of neutrophils is related to the production of IL-17. IL-17 levels in the lung were found to be increased at day 20 p.i. with *M. pulmonis* (Figure 3). This is consistent with previous reports that found increased IL-17 mRNA in the lungs at days 14 and 28 p.i. with *M. pulmonis* [27], increased IL-17 levels in the bronchoalveolar lavage at days 1, 3, and 7 p.i. with *M. pneumoniae*, and increased IL-17 mRNA in the lungs at 24 hrs p.i. with *M. pneumoniae* [28]. We extend these findings to show the necessity of the IL-17 receptor in the clearance of *M. pulmonis*. IL-17RKO mice had increased CFU counts in the lungs and nasal passages at day 20 p.i. with *M. pulmonis*. This is the first data to show that a deficiency in IL-17 signaling leads to increased susceptibility to *M. pulmonis*. Taken together, these data suggest that IL-17 induced

neutrophil activation, proliferation or recruitment is essential to the clearance of *M. pulmonis* from the upper and lower respiratory tracts.

According to the data presented in this paper as well as a recently published report, IL-17RKO mice do not have impaired clearance of *L. monocytogenes* as compared to wild type mice at days 3 (Figure 5) or 4 p.i. [50]. However, under certain circumstances IL-17 production is capable of influencing *L. monocytogenes* infection. Evidence for this comes from another study: mice deficient in LFA-1 (LFA-$1^{-/-}$) have increased clearance of *L. monocytogenes* concurrent with increased IL-17 production [46]. Furthermore, the current study confirms that IL-17 production can increase resistance to *L. monocytogenes*. Not only was IL-17 production increased in the animals with enhanced resistance to *L. monocytogenes*, but more importantly, deficiency in the IL-17R abrogated this effect. Given that IL-17 induces the secretion of multiple cytokines and chemokines that are known to induce the mobilization, proliferation, activation, and recruitment of neutrophils [9], it is not surprising that IL-17 is able to provide protection against *L. monocytogenes*. Neutrophils are essential for resistance to *L. monocytogenes* and neutrophil mobilization during *L. monocytogenes* infection can be amplified by the presence of IL-17 [36–38;46]. Indeed, the heightened resistance to *L. monocytogenes* observed during *M. pulmonis* infection was associated not only with higher levels of IL-17 but also greater neutrophil numbers. While IL-17 independent mechanisms of neutrophil recruitment are also utilized during *L. monocytogenes* infection, the cross-protection offered by *M. pulmonis* and the heightened granulocyte numbers during co-infection were dependent on IL-17. This is consistent with previous findings showing that LFA-$1^{-/-}$ mice had increased levels of IL-17, neutrophilia, and increased resistance to *L. monocytogenes* [46]. In the current study, we show that IL-17 production is able to induce neutrophilia that facilitates the clearance of *L. monocytogenes* in wild-type mice.

It is interesting to note that in the current study, a respiratory pathogen was able to enhance clearance of a secondary unrelated pathogen that resides within the spleen and liver. The chronic respiratory infection with *M. pulmonis* was associated with increases in IL-17 in not only the lungs and draining lymph nodes, but also the spleen. Furthermore, *M. pulmonis* infection worked synergistically with *L. monocytogenes* infection to substantially increase granulocyte numbers in the spleen. There are two potential explanations for how a respiratory pathogen such as *M. pulmonis* is able to modulate the immune response to *L. monocytogenes* within the spleen and liver. One hypothesis is that *M. pulmonis* disseminates to the spleen and liver, thus providing localized IL-17 and neutrophil responses to *M. pulmonis*, which are also able to influence the *L. monocytogenes* infection that is within the vicinity. As evidence against this hypothesis, a previous report [51], as well as our unpublished data at day 20 p.i. with 2×10^5 CFUs of *M. pulmonis*, found no detectable levels of *M. pulmonis* in the spleen. Thus, it does not appear that the IL-17 and granulocyte response within the spleen is a result of localized *M. pulmonis* infection within the spleen. Our preliminary data suggest low, but inconsistent levels of *M. pulmonis* may be recovered from the liver at day 20 p.i. with 2×10^5 CFUs of *M. pulmonis* (unpublished data), indicating that a potential local interaction between *M. pulmonis* specific immune responses and the clearance of *L. monocytogenes* is possible within this organ. Alternatively, localized *M. pulmonis* infection, and the subsequent *M. pulmonis* directed immune responses, may not be required within the spleen and liver to provide cross protection against *L. monocytogenes* in these tissues. Another possible mechanism is that *M. pulmonis* infection within the lungs and nasal passages may be influencing the development of IL-17 secreting T cells within the draining lymph nodes. These IL-17 secreting T cells could traffic into not only the lung and nasal passage tissues, but also the spleen and liver. IL-17 is known to induce the production of IL-6, G-CSF, GM-CSF, CXCL1, CXCL2, and CXCL8 [9]. Production of these cytokines and chemokines within the spleen, lymph nodes and lungs in response to IL-17, could

subsequently induce the systemic mobilization of neutrophils, which would be poised and ready to respond to any infectious microorganism that they encounter. In this scenario a localized infection within the lungs would potentially be able to provide cross protection against an unrelated pathogen within other tissues in the body. IL-17 induced mobilization of neutrophils may therefore be a wide-reaching mechanism of cross protection.

Materials and Methods

Mice

C57BL/6 and BALB/c mice, tested to be virus- and mycoplasma-free, were obtained from Harlan Sprague-Dawley (Indianapolis, IN) or Taconic (Germantown, NY). IL-17 receptor knockout mice (IL-17RKO) backcrossed on a C57BL/6 genetic background have previously been described [13]. Female mice between 6 to 12 weeks of age were housed with food and water ad libitum in sterile microisolator cages with sterile bedding at the University of North Texas Health Science Center AAALAC accredited animal facility. All animal studies were performed under the approval of the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center.

Pathogens and Infections

The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described [52], in mycoplasma broth medium and frozen at −80°C. For innoculation, thawed aliquots were diluted to 2×10^5 CFU/ 20 μl. Before infection, mice were anesthetized with an intramuscular injection of ketamine-xylazine, and nasal-pulmonary innoculations of 20 μl of diluted mycoplasma in mycoplasma broth medium were given for experimental infections. Control mice were inoculated with 20 μl sterile mycoplasma broth medium.

L. monocytogenes 10403 serotype 1 was grown on brain-heart infusion (BHI) agar plates (BD Bacto, Sparks, MD), and virulent stocks were maintained by repeated passage through C57BL/6 mice. For infection of mice, log-phase cultures of *L. monocytogenes* grown in BHI broth were washed twice and diluted in phosphate buffered saline (PBS) to the desired concentration. *L. monocytogenes* or PBS was injected i.v. into the lateral tail vein at day 17 p.i. with *M. pulmonis*. Unless otherwise indicated, mice were innoculated with approximately 2.5×10^4 *L. monocytogenes*.

Neutrophil Depletion

Neutrophils were depleted using a Gr-1 depleting antibody (RB6-8C5, BioXCell, West Lebanon, NH) one day prior to infection with *L. monocytogenes*. At day 16 p.i. with *M. pulmonis* or broth, mice were injected with 200 μg anti-Gr-1 or isotype control antibody. Mice were infected with *L. monocytogenes* 24 hrs later, and sacrificed at day 3 p.i. with *L. monocytogenes*. Successful depletion of the Gr-1+ cells was confirmed using flow cytometry in the spleen and peripheral blood on the day of sacrifice (data not shown).

Quantification of Bacterial Growth In Vivo

The number of *M. pulmonis* CFUs in the lungs and nasal passages were determined as previously described [53]. Briefly, lungs were minced and placed in mycoplasma broth medium. Nasal passages were flushed with 1 ml of mycoplasma broth medium. The samples were sonicated (Vibra cell sonicator; Sonics & Materials/Vibro Cell, Newtown, CT) for 1 min at 50 amplitudes without pulsing. After sonication, serial dilutions (1:10) were prepared, and 20 μl of each dilution was plated onto mycoplasma agar medium. After 7 days of incubation at 37°C, colonies were counted, and the *M. pulmonis* CFUs recovered from each tissue were calculated.

To determine *L. monocytogenes* CFUs, the spleen, liver, and lungs from infected mice were homogenized in sterile ddH_2O. Whole blood was collected into tubes containing Hank's balanced salt solution (HBSS) supplemented with 5% FCS and 0.67 mg/ml heparin and was centrifuged at 12,000 rpm for 3 min. Following the removal of the supernatant, the pellet was resuspended in sterile ddH_2O. Serial dilutions (1:10) of all of the tissues were prepared, and 50 μl of each dilution was plated on BHI agar plates. After overnight incubation at 37°C, colonies were counted, and the *L. monocytogenes* CFUs recovered from each tissue were calculated.

Preparation of M. pulmonis Antigen

A crude preparation of *M. pulmonis* membranes was used for in vitro stimulation and was prepared as previously described [54]. Briefly, *M. pulmonis* was grown at 37°C in mycoplasma broth medium. Cells were then centrifuged at 10,000 rpm for 20 min, and the pellets were washed and suspended in 2M glycerol. For the subsequent cell lysis, the suspension of cells was sonicated and then forced through a 27-gauge needle into cold sterile distilled water. The unlysed organisms were removed by another round of high speed centrifugation. Supernatants were then centrifuged at 20,000 rpm for 1 hr to obtain mycoplasma membrane preparation. Protein concentration of the membrane preparation, resuspended in sterile PBS, was determined by Bradford assay assay (Bio-Rad, Hercules, CA), and the membranes were stored at −80°C until further use. A final concentration of 5 μg/ml of *M. pulmonis* membrane preparation was used for *in vitro* stimulation.

In Vitro Antigen Stimulation

Cervical lymph nodes and spleens were ground between glass slides and red blood cells were subsequently lysed with Tris ammonium chloride (pH 7.2). The resulting cells were then cultured overnight in complete RPMI medium supplemented with 10% FCS (Atlanta Biologicals, Nocross, GA), L-glutamine, vitamins, penicillin/streptomycin, non-essential amino acids, and sodium pyruvate. All supplements were from Invitrogen-Gibco (Carlsbad, CA). Cell culture was performed at 37°C in humidified air containing 5% CO_2. Cell cultures were either stimulated overnight with or without *M. pulmonis* membrane antigen, with 50 ng/ml Phorbol 12-Myristate 13- Acetate (PMA; Sigma-Aldrich, St. Louis, MO), and 500 ng/ml Ionomycin (EMD, Gibbstown, NJ) for the last 5 hrs of the culture and analyzed with flow cytometry, or were stimulated with *M. pulmonis* membrane antigen or heat-killed *L. monocytogenes* (MOI 50:1) for two days and the filtered culture supernatants were analyzed using ELISA.

Antibodies and Cell Staining

For cell staining, the following antibodies from BD PharMingen (San Diego, CA) were used: anti-Gr-1 FITC (RB6-8C5), anti-CD11b PE-Cy7 (M1/70), anti-CD3e FITC (145-2C11), anti-CD4 PE-Cy7 (RM4-5), anti-CD8α FITC (53-6.7), anti-NK1.1 PE (PK136), anti-γδ T cell receptor (GL3) FITC, anti-CD16/CD32 (2.4G2), anti-IFN-γ APC (XMG1.2), and anti-IL-17 PE (TC11-18H10). To accomplish intracellular cytokine staining, GolgiPlug containing Brefeldin A (BFA) (BD PharMingen), was added 4 hrs prior to the harvest of the cell cultures. Staining of the cells involved incubation at 4°C for 15 min in staining buffer (PBS + 2% FCS + 0.1% sodium azide) with saturating amounts of the cell-surface antibodies and anti-CD16/CD32 to block Fc receptors. To measure intracellular cytokines, cells were fixed and permeabilized at 4°C for 20 min using the intracellular cytokine staining kit from BD PharMingen. After washing in permeabilization wash buffer, the cells were incubated in saturating amounts of anti-IFN-γ and anti-IL-17 at 4°C for 20 min. Data was acquired and analyzed using a Beckman Coulter Cytomics FC500 (Fullerton, CA).

Luminex Suspension Array

Levels of IL-17 and IFN-γ in the lungs, spleen, and serum were measured using a LINCOplex™ mouse cytokine kit (LINCO Research, Inc., Saint Charles, MO). Lungs and spleen were homogenized using a PRO200 homogenizer (PRO Scientific, Oxford, CT) in PBS containing a protease inhibitor cocktail (Roche, Switzerland). Serum was obtained by removing the supernatant from whole blood following centrifugation at 12,000 rpm for 30 min. IL-17 and IFN-γ concentrations were assessed in the samples according to the protocol provided in the LINCOplex™ kit. Samples were read using a Bio-Plex 100 system (Bio-Rad, Hercules, CA). Cytokine levels were determined by comparison with standard curves generated from the murine recombinant cytokines provided in the LINCOplex™ kit and analyzed using a Bio-Plex Manager software (Bio-Rad).

ELISA

An ELISA was conducted on cell culture supernatants using plate bound purified anti-IL-17 (BD Pharmingen, clone TC11-18H10, 2 μg/ml) capture antibody and biotinylated anti-IL-17 (BD Pharmingen, clone TC11-8H4.1, 2 μg/ml) detection antibody, with quantification by reference to rIL-17 standard (R&D Systems). IL-17 concentrations were measured at 450nm on an EL808 instrument (BioTek).

Statistical Analyses

Analyses of variances (ANOVAs) were conducted on the normally distributed data. Bonferroni t-tests and Tukey-Kramer analyses were used for post-hoc analyses. Kruskal-Wallis tests with Dunn's multiple comparison post-hoc tests were used to analyze nonparametric data. *M. pulmonis* CFU data was log transformed prior to analysis, and is represented as such in the figures. A p value of 0.05 or less was considered significant in all cases.

Abbreviations

p.i.	post-infection
LM	*Listeria monocytogenes*
Myco	*Mycoplasma pulmonis*
BFA	Brefeldin A
CLN	cervical lymph node
NP	nasal passage wash

Acknowledgments

The authors would like to thank Joshua Balch and Nicole Dobbs for their excellent technical assistance and Dena Johnson for her critical review of the manuscript. Flow cytometry was performed in the Flow Cytometry and Laser Capture Microdissection Core Facility at The University of North Texas Health Science Center. This research was funded by NIH AI064592 (to R.E.B.) and NIH AI042075 (to J.W.S.).

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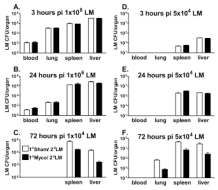

Figure 1.

Prior Infection with *M. Pulmonis* Confers Resistance Against *L. Monocytogenes* Infection. C57BL/6 mice were i.v. infected with 1×10^8 (A), 1×10^6 (B), 1×10^4 (C), or 5×10^4 (D, E, F) CFUs of *L. monocytogenes* (LM) 17 days after nasal-pulmonary inoculation with mycoplasma broth medium (1°Sham/2°LM) or *M. pulmonis* (1°Myco/2°LM). At 3 hrs (A & D), 24 hrs (B & E), or 72 hrs (C & F) p.i., *L. monocytogenes* CFU counts were assessed in the indicated tissues. A two-way ANOVA detected a significant effect of *M. pulmonis* infection, such that *M. pulmonis* infection decreased *L. monocytogenes* CFU counts independent of organ assayed at 72 hrs p.i., $\underline{p} \leq 0.05$ (C &F). However, two-way ANOVAs did not detect any significant differences in any tissue assayed at 3 and 24 hrs p.i., $\underline{p} > 0.05$ (A, B, D, E). These data are representative of 3 independent experiments. All data are expressed as the mean ± SEM (n = 4/group).

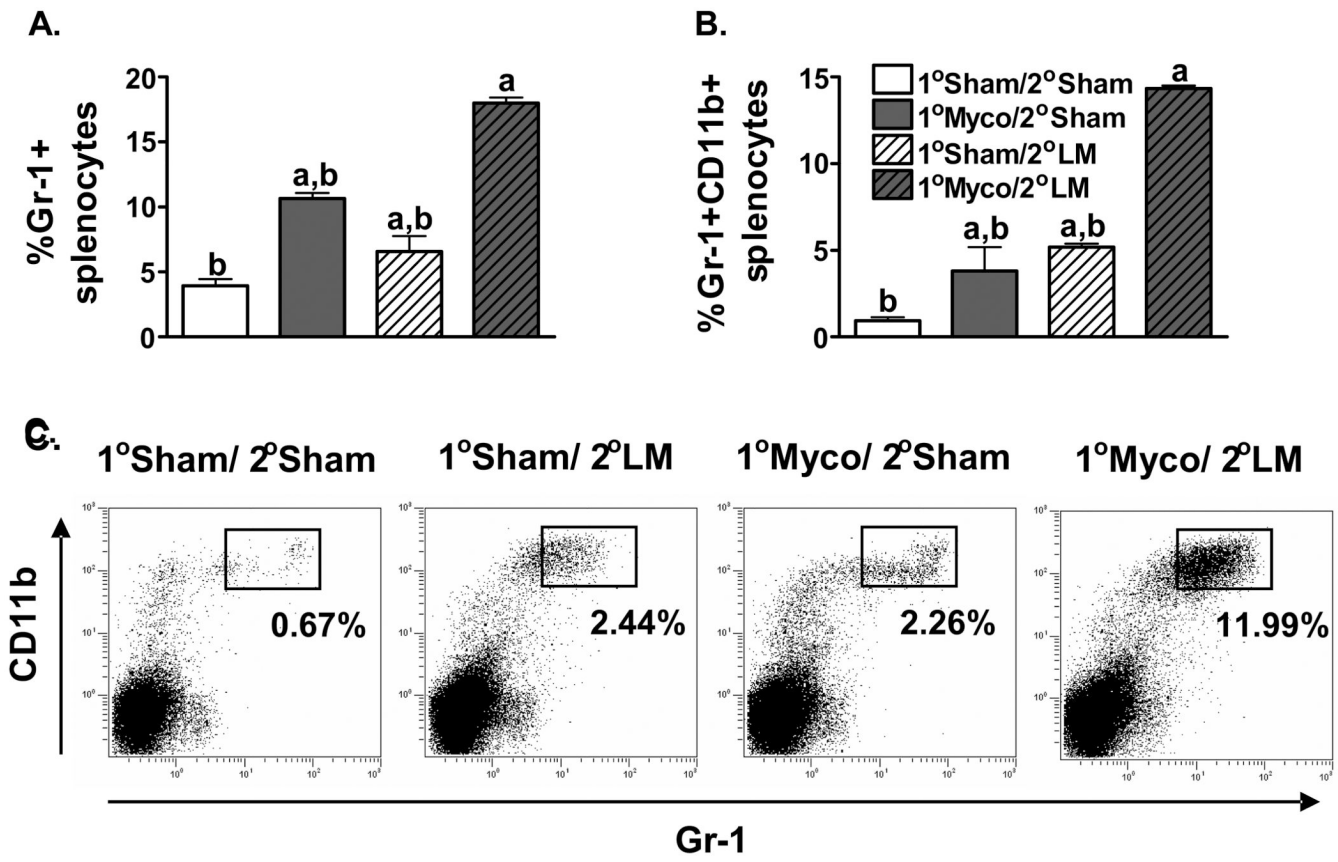

Figure 2.

Gr-1+CD11b+ Cells Increase During Co-Infection with *L. Monocytogenes* and *M. Pulmonis*. At day 17 p.i. with *M. pulmonis*, C57BL/6 mice were inoculated with 5×10^4 CFUs of *L. monocytogenes* or PBS. At day 3 p.i. with *L. monocytogenes*, splenocytes were analyzed for expression of Gr-1 and CD11b in broth/PBS (1°Sham/2°Sham), *M. pulmonis*/PBS (1° Myco/2°Sham), broth/*L. monocytogenes* (1°Sham/2°LM), and *M. pulmonis*/*L. monocytogenes* (1° Myco/2°LM) inoculated mice. A two-way ANOVA detected a significant increase in the percentage of both Gr-1+ (A) and Gr-1+CD11b+ (B) splenocytes in response to both *L. monocytogenes* and *M. pulmonis* infection. Furthermore, co-infection with *M. pulmonis* and *L. monocytogenes* induced a greater increase in the percentage of these cell populations than infection with either pathogen alone (A, B, C). a, denotes a significant difference from the 1°Sham/ 2°Sham group, $p \leq 0.05$. b, denotes a significant difference from the 1°Myco/ 2°LM group, $p \leq 0.05$. These data are representative of 3 independent experiments. All data are expressed as the mean ± SEM (n = 4/group). Representative flow cytometry dot plots of Gr-1 and CD11b expression are presented (C).

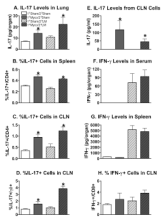

Figure 3.

Infection with *M. Pulmonis* Increases IL-17, but not IFN-γ, Levels in *L. Monocytogenes* Infected Mice. At day 17 p.i. with *M. pulmonis* or broth, C57BL/6 mice were infected with *L. monocytogenes* or PBS. The concentration of IL-17 in lung homogenates from day 3 p.i. with *L. monocytogenes* was analyzed using a Luminex suspension array (A). A two-way ANOVA detected a significant effect of *M. pulmonis* infection, such that prior infection with *M. pulmonis* increased IL-17 levels in the lungs. Intracellular cytokine staining was used to determine the percentage of IL-17 producing CD4+ T cells in the spleen (B) and cervical lymph nodes (CLN) (C) and the percentage of IL-17 producing γδ+ T cells (D) in the CLN of uninfected (1°Sham/ 2°Sham), singly infected (1°Myco/ 2°Sham and 1°Sham/2°LM), and co-infected (1°Myco/ 2°LM) mice. Again, a two-way ANOVA detected a significant effect of *M. pulmonis* infection, such that prior infection with *M. pulmonis* increased the percentage of IL-17 producing cells (B, C, D). Supernatants from CLN cells cultured for two days with *M. pulmonis* membrane antigen were analyzed using ELISA for the concentration of IL-17 produced (E). A Kruskal-Wallis test detected that both the 1°Myco/2°Sham and 1°Myco/2°LM had increased IL-17 levels ($\underline{p} \leq 0.05$), and no other differences were significant, $\underline{p} \geq 0.05$. The concentration of IFN-γ in serum (F) and spleen homogenates (G) from day 3 p.i. with *L. monocytogenes* was analyzed using a Luminex suspension array. A two-way ANOVA determined that *L. monocytogenes* increased IFN-γ levels in both tissues, but *M. pulmonis* infection did not impact levels of this cytokine during *L. monocytogenes* infection. Likewise, two-way ANOVAs conducted on flow cytometry data did not detect a significant effect of *M. pulmonis* infection on the percentage of IFN-γ producing cells during *L. monocytogenes* infection (H), $\underline{p} \geq 0.05$. These data are representative of 2 independent experiments. All data are expressed as the mean ± SEM (n = 3–4/group). An * denotes the 1° Myco groups differ from the 1° Sham groups ($\underline{p} \leq 0.05$).

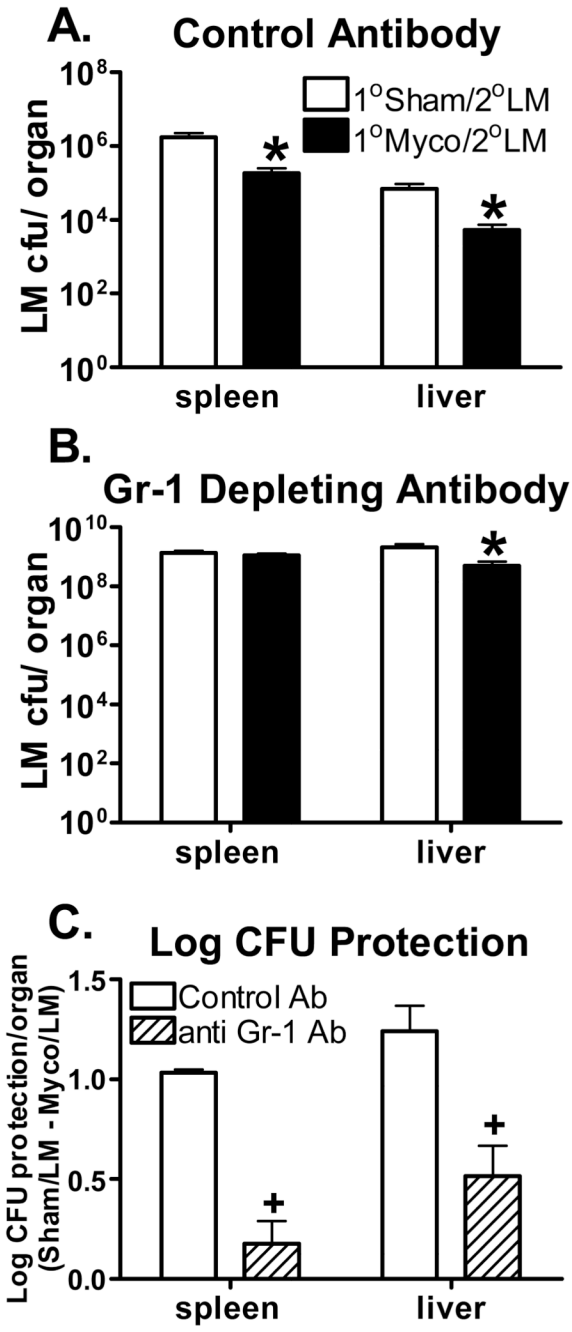

Figure 4.

Granulocyte Depletion Diminishes Resistance to *L. Monocytogenes* Conferred by *M. Pulmonis*. At day 16 p.i. with *M. pulmonis* or broth, mice were injected with the control antibody (A) or Gr-1 depleting antibody (B). Twenty-four hrs following depletion, mice were infected with 1×10^4 CFUs of *L. monocytogenes*. *L. monocytogenes* CFU counts were enumerated in the spleen and liver at day 3 p.i. with *L. monocytogenes*. All data are expressed as the mean ± SEM (n = 6/group). A two-way ANOVA detected a significant effect of *M. pulmonis* infection in the spleen and liver of mice that received the control antibody, such that *M. pulmonis* infection decreased *L. monocytogenes* CFU counts. While a significant effect of *M. pulmonis* was still detected in the liver of Gr-1 depleted mice, there

was no decrease in CFUs observed in the spleen. * , denotes 1°Sham/2°LM differ from 1°Myco/2°LM ($p \leq 0.05$). Log CFU protection conferred by *M. pulmonis* infection against *L. monocytogenes* was calculated by subtracting the average log bacterial burden in the spleen and livers of *M. pulmonis* infected mice from the average bacterial burden in the same organs of broth inoculated mice (C). Data presented are derived from two independent experiments with four to six mice in each group per experiment. A two-way ANOVA detected a significant effect of Gr-1 depletion, such that Gr-1 depleted mice had decreased log CFU protection in both the spleen and liver as compared to mice that received the control antibody. +, denotes the control antibody differs from the anti Gr-1 antibody ($p \leq 0.05$).

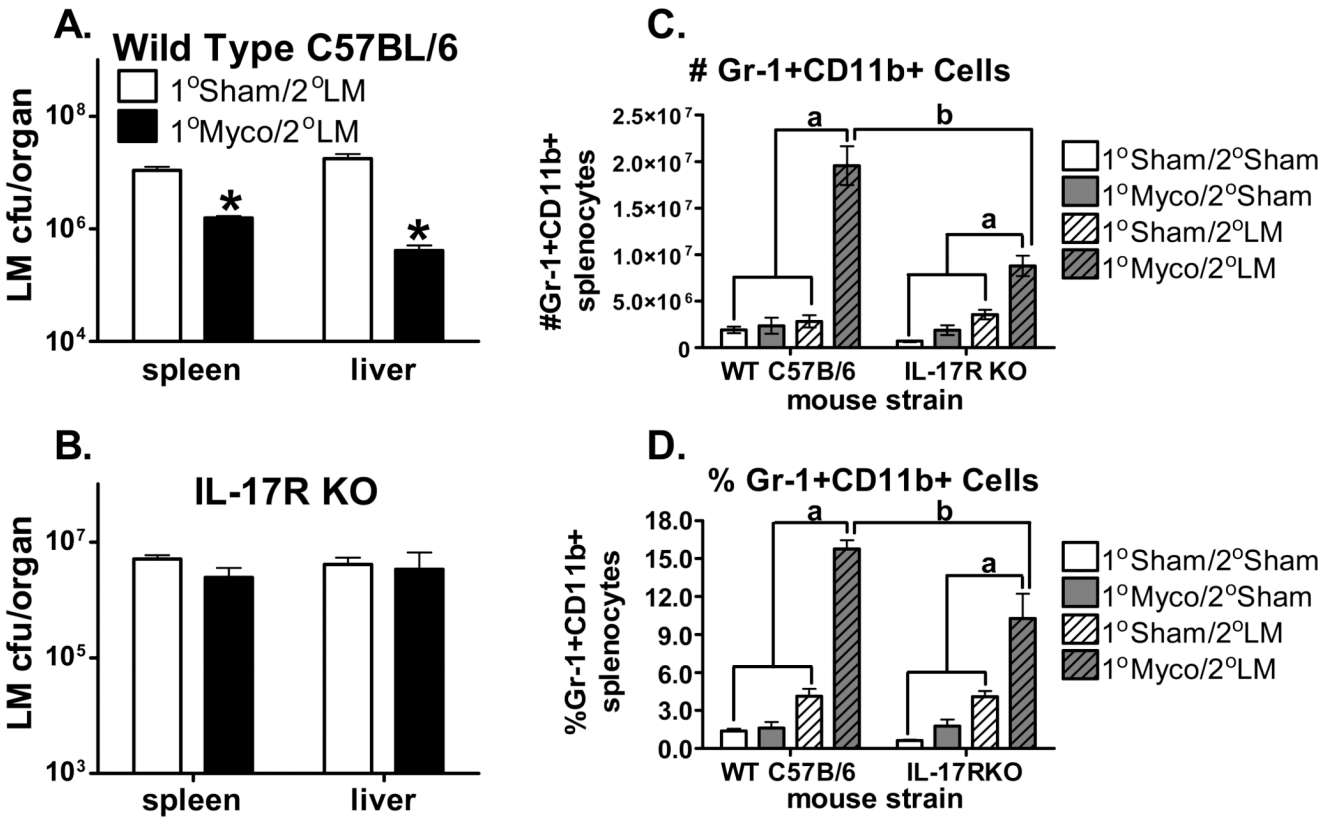

Figure 5.

In IL-17 Receptor Deficient Mice, *M. Pulmonis* Infection Does Not Facilitate Clearance of *L. Monocytogenes* or Increase Gr-1+CD11b+ Cells. At day 17 p.i. with *M. pulmonis* or broth, C57BL/6 and IL-17 receptor deficient mice were infected with *L. monocytogenes* or PBS. *L. monocytogenes* CFU counts were enumerated in the spleen and liver at day 3 p.i. with *L. monocytogenes*. A two-way ANOVA detected a significant effect of *M. pulmonis*, such that *M. pulmonis* decreased *L. monocytogenes* CFU counts in the spleen and liver of wild-type C57BL/6 mice (A). No significant effect of *M. pulmonis* was detected in the IL-17 receptor deficient mice (B). *, denotes that the 1°Sham/2°LM group differs from the 1°Myco/2°LM group ($p \leq 0.05$). All data are expressed as the mean ± SEM and are representative of two independent experiments with four mice per group. Splenocytes from broth/ PBS (1° Sham/2° Sham), *M. pulmonis*/ PBS (1° Myco/2° Sham), broth/ *L. monocytogenes* (1° Sham/2° LM), and *M. pulmonis*/ *L. monocytogenes* (1° Myco/2° LM) mice were stained for expression of Gr-1 and CD11b. Two-way ANOVAs detected a significant effect of infection, such that mice co-infected with *M. pulmonis* and *L. monocytogenes* had an increase in the number (C) and percentage (D) of Gr-1+CD11b+ cells as compared to all other groups. While this effect was evident in wild-type C57BL/6 (WT C57B/6) and IL-17 receptor deficient (IL-17RKO) mice, it was significantly diminished in IL-17 receptor deficient mice as compared to wild type mice. a, denotes that all groups differ from 1°Myco/2°LM group ($p < 0.05$). b, denotes WT C57B/6 mice differ from IL-17RKO mice ($p \leq 0.05$).

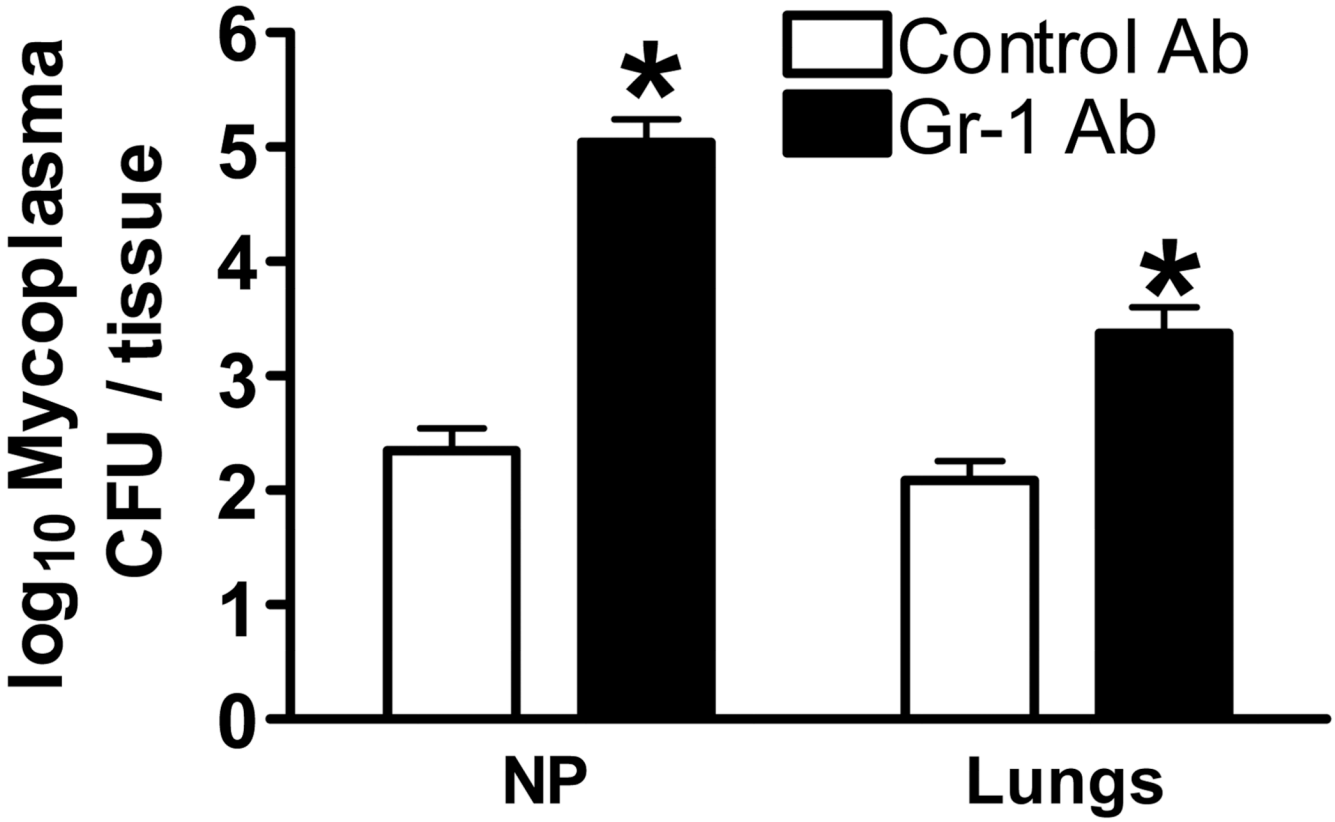

Figure 6.

Granulocytes are Required for Optimal Clearance of *M. Pulmonis* From the Lungs and Nasal Passages. C57BL/6 mice were depleted of granulocytes using a Gr-1 depletion antibody from days 16 to 20 p.i. with *M. pulmonis*. At day 20 p.i. with *M. pulmonis*, CFU counts were assessed in the lungs and nasal passage wash (NP). A two-way ANOVA detected a significant effect of granulocyte depletion, such that mice given the Gr-1 depleting antibody had increased *M. pulmonis* CFU counts in the spleen and NP as compared to mice that received the control antibody. The means of log transformed data ± SEM are presented and are representative of two independent experiments with four mice per group. *, denotes the control antibody differs from the anti Gr-1 antibody ($\underline{p} \leq 0.05$).

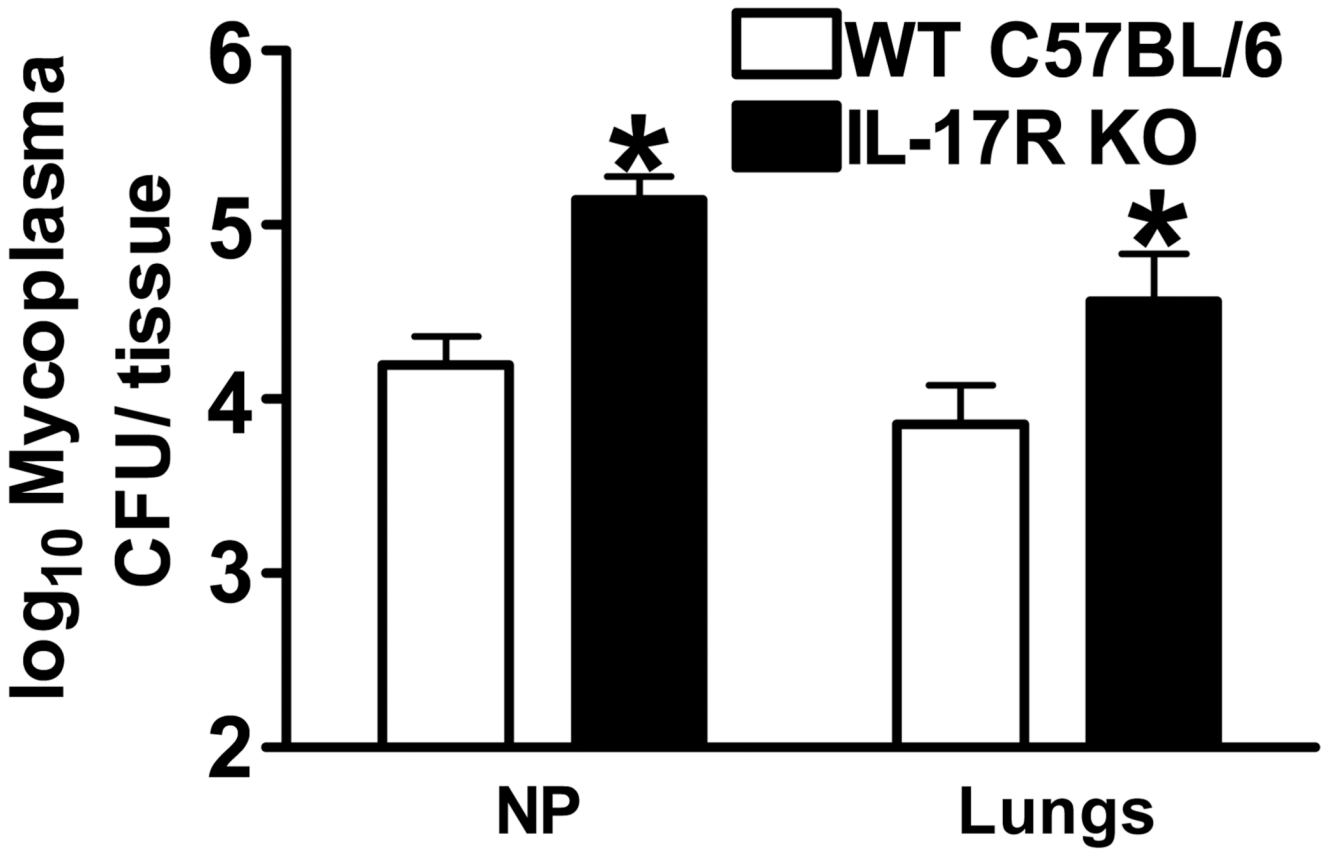

Figure 7.

IL-17 Receptor Deficient Mice Have Increased Susceptibility to *M. Pulmonis* Infection. Wild type C57BL/6 and IL-17 receptor deficient mice were inoculated with *M. pulmonis*. At day 20 p.i. with *M. pulmonis*, CFU counts were enumerated in the lungs and NP. A two-way ANOVA detected a significant effect of mouse strain, such that IL-17 receptor deficient mice had higher *M. pulmonis* CFU counts in the lungs and NP as compared to wild type mice. The means of log transformed data ± SEM are presented and are representative of three independent experiments with four mice per group. *, denotes WT C57B/6 mice differ from IL-17R KO mice ($p \leq 0.05$).