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IL-21 Promotes the Pathologic Immune Response to Pneumovirus Infection

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Abstract

IL-21 is a cytokine with pleiotropic actions, promoting terminal differentiation of B cells, increased immunoglobulin production, and the development of Th17 and T follicular helper cells. IL-21 is also implicated in the development of autoimmune disease and has anti-tumor activity. Here we investigated the role of IL-21 in host-defense to pneumonia virus of mice (PVM), which initiates an infection in mice resembling that of respiratory syncytial virus disease in humans. We found that PVM-infected mice expressed IL-21 in lung $CD4^+$ T cells. Following infection, *Il21r*$^{-/-}$ mice exhibited less lung infiltration by neutrophils than did WT mice and correspondingly had lower levels of the chemokine CXCL1 in bronchoalveolar lavage fluid and lung parenchyma. $CD8^+$, $CD4^+$, and γδ T-cell numbers were also lower in the lungs of PVM-infected *Il21r*$^{-/-}$ mice than in infected WT mice, with normal Th17 cytokines but diminished IL-6 production in PVM-infected *Il21r*$^{-/-}$ mice. Strikingly, *Il21r*$^{-/-}$ mice had enhanced survival following PVM-infection, and moreover, treatment of WT mice with soluble IL-21R-Fc fusion protein enhanced their survival. These data reveal that IL-21 promotes the pathogenic inflammatory effect of PVM and indicate that manipulating IL-21 signaling may represent an immunomodulatory strategy for controlling PVM and potentially other respiratory virus infections.

Introduction

Interleukin-21 (IL-21) is a pleiotropic four α-helical bundle type I cytokine that is produced primarily by $CD4^+$ T cell populations, including T follicular helper cells, Th17 populations, and NKT cells, and acts on a broad range of target cells including B cells, T cells, natural killer cells, dendritic cells, macrophages, and epithelial cells (1). The functional IL-21 receptor is a heterodimer of an IL-21-specific protein, IL-21R, and the common cytokine receptor γ chain, γ_c, which is shared by the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (2), and is encoded by the gene that is mutated in humans with X-linked severe combined immunodeficiency (3). IL-21 functions as a T cell co-mitogen and can also cooperate with IL-7 and IL-15 to drive expansion of $CD8^+$ T cells (4). Within the B-cell lineage, it is a critical regulator of immunoglobulin (Ig) production, contributes to Ig class switch, and drives terminal B cell differentiation to plasma cells via its regulation of the *PRDM1* gene,

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which encodes BLIMP1 (5, 6). Interestingly, IL-21 is also pro-apoptotic for B cells (5, 7, 8) and NK cells (9) and can suppress dendritic cell function (10). IL-21 plays a role in both $CD8^+$ T cell and NK cell function related to anti-tumor responses, and IL-21 exhibits potent anti-cancer activity in a number of animal models (1). Although IL-21 exerts positive effects on differentiation and function, it also has inhibitory effects on immune responses based on its role in B cell apoptosis, its induction of IL-10 in T and B cells leading to immunosuppression (11), and its suppressive effects on dendritic cell function (10). IL-21 also promotes the development and expansion of the inflammatory Th17 subset of $CD4^+$ T cells (12-14) as well as T follicular helper cells (15).

IL-21 can promote inflammation in a variety of disease-related settings. For example, in the NOD mouse model of type1 diabetes, IL-21 signaling promotes pancreatic islet inflammation and destruction, as shown by the lack of disease when mice are crossed to the *Il21r*$^{-/-}$ background (16, 17). Analogously, IL-21 also promotes autoimmune systemic lupus erythematosus (SLE) related to its role in T cell activation, with diminished disease development in two different mouse models when IL-21 signaling is blocked (18, 19). Moreover, in the experimental autoimmune encephalitis (EAE) mouse model of multiple sclerosis, IL-21 promotes the differentiation and expansion of IL-17-producing $CD4^+$ T cells, and the absence of IL-21 signaling has been associated with reduced severity of disease (12, 13).

Despite the large body of information known related to the biology of IL-21, relatively limited information is available regarding its role following viral infection. In human immunodeficiency virus (HIV)-infected individuals, IL-21-expressing $CD4^+$ T cells were present, and these numbers decreased as viral levels declined (20). Additionally, IL-21 producing HIV-specific $CD8^+$ T cells were induced following HIV infection, and interestingly, the presence of these cells correlated better with HIV-1 control than did IL-21-producing $CD4^+$ T cells (21). However, these findings were only correlative, and a full understanding of the role of IL-21 in host defense to HIV requires additional investigation. IL-21 is also elevated during lymphochoriomeningitis virus (LCMV) infection of mice, and experiments with *Il21r*$^{-/-}$ mice revealed a role for IL-21 in controlling $CD8^+$ T cell function during this infection (22-24). However, the role of IL-21 in the pathogenesis of acute respiratory viruses has not yet been explored. In this study, we focus on Pneumonia virus of mice (PVM), a negative strand RNA virus that is in the same family (*Paramyxoviridae*) and genus (*Pneumovirus*) as human respiratory syncytial virus (RSV) (25, 26). PVM infection can result in disease in mice similar to the more severe forms of RSV infection, with viral replication in respiratory epithelial cells that is associated with the infiltration of neutrophils and lymphocytes, and the production of IFN-γ and the chemokines CCL2, CCL3, CXCL1, and CXCL10. Here, we examined the role of IL-21 in both the innate and adaptive responses to PVM infection and unexpectedly found that mice lacking *Il21r*$^{-/-}$ exhibited prolonged survival to PVM. Moreover, treatment of wild type mice with a soluble IL-21R-Fc fusion protein was protective, indicating a role for IL-21 in promoting the pathogenic effects of this respiratory virus.

MATERIALS AND METHODS

Mice

Wild type C57Bl/6 mice were obtained from the Jackson Laboratory. *Il21r*$^{-/-}$ mice (6) were analyzed at 8-12 weeks of age. Mice expressing the *Il2-EmGFP/Il21-mCherry* recombineered Bac reporter transgene have been described (27) Transgenic mice expressing the human IL-21 cDNA under the control of the $H\text{-}2K^b$ promoter and IgM enhancer have been described (5). All experiments were performed under protocols approved by the

NHLBI and NIAID Animal Care and Use Committees, and followed NIH guidelines for use of animals in intramural research.

Virus inoculation

Virus stocks (PVM strain J3666) were prepared as previously described (28). Mice were anesthetized briefly via inhalation of 20% halothane and were inoculated intranasally with 12-60 PFU of PVM in 50-100 μl of PBS. IL-21R/Fc fusion protein was obtained from R&D Systems. Human Fc Cγ1 control protein was obtained from BioXCell.

Histology

Lungs were inflated before excision, fixed in 10% formalin, embedded in paraffin, 5 μm thick sections were cut and slides were stained with hematoxylin and eosin. Clinical scores for morphology and inflammation and edema were evaluated.

Bronchoalveolar lavage fluid and lung cell preparation

Lungs were inflated intratracheally with 1 ml cold 0.1% BSA in PBS and recovered fluid was used for ELISA and to prepare BAL cells. Lung tissue was minced into small pieces using a razor blade and digested in a solution containing 0.5 mg/ml Liberase (Roche) and 0.5 mg/ml DNase I (Sigma-Aldrich) in serum free RPMI for 30 min at 37°C. Digested tissue was then pushed through a cell strainer with a syringe. Cells were centrifuged, and RBCs were lysed with ACK, followed by two washes with complete RPMI.

Flow cytometric analysis

Single cell suspensions from BAL fluid or lung tissue were surface stained in FACS buffer (PBS containing 0.5% BSA and 0.02% azide) using antibodies from BD Biosciences. For intracellular staining of cytokines, cells were activated with PMA (10 ng/ml) and ionomycin (1 μM) (Sigma-Aldrich) for 4 h in the presence of Golgi Plug (BD Biosciences). Cells were first surface-stained with anti-CD8, fixed, and permeabilized with CytoFix/Cytoperm (BD Biosciences). Data were acquired with either a BD FACS Canto II or an LSR II and were analyzed with FlowJo software.

Dendritic cell isolation

Splenic dendritic cells were isolated by collagenase digestion followed by positive selection with pan-DC microbeads (Miltenyi Biotec). Splenic DCs were seeded at 2×10^6/sample in 24-well plates, rested at 37°C for 1 h, and stimulated with cytokines. Supernatants were collected, and IL-6 levels were measured by ELISA (BD Biosciences).

RNA preparation and real time PCR

RNA was extracted from lung tissue by homogenization in Trizol (Invitrogen) followed by RNA cleanup with the RNeasy kit (Qiagen). RNA was reverse-transcribed using Omniscript (Qiagen). PCR reactions to quantitatively detect cytokine and chemokine RNAs used probe sets from Applied Biosystems and the 7900HT Sequence Detection System. Relative levels of PVM *SH* gene expression (29) were determined by quantitative RT-PCR, with normalization to *Rpl7* expression.

ELISAs

Lung tissue was homogenized in cold PBS and tissue debris was removed by centrifugation (14,000 rpm for 10 min). Levels of CXCL1 in equal volumes of BAL fluid and equal amounts of protein from lung homogenates were measured using a kit (Antigenix).

Statistical analysis

Two-tailed paired t-tests were performed using Prism 4.0 (GraphPad software). In the figures, statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

IL-21 expression is induced following infection with PVM

To determine whether IL-21 is expressed in the lung in response to PVM infection, wild type (WT) mice were inoculated intra-nasally with PVM virions, and IL-21 mRNA expression was measured in the lung at time points during the 10 days following infection. Expression of the PVM *SH* gene as an indicator of virus burden (29) was greatest at day 6 after inoculation **(Fig. 1A)**. As expected, infected mice developed pneumonia and began to succumb to infection starting at day 7 and SH gene expression declined, with it no longer being detected among survivors at day 10 (Fig. 1A). *Il21* mRNA was detected as early as day 5, with a peak at day 6, and levels declined thereafter (**Fig. 1B**). Interestingly, at day 10, virus was no longer detected in lung tissue, but *Il21* mRNA could be detected in the surviving mice.

To identify IL-21-producing cells, we used transgenic reporter mice in which the mCherry reporter is inserted at the translation start site of the *Il21* gene in a bacterial artificial chromosome (BAC) clone by recombineering technology. These mice were inoculated with PVM, and single cell suspensions from lung tissue and lymphoid organs were examined *ex vivo* by flow cytometry. mCherry expression was detected in approximately 5% of $CD4^+$ T cells in lung tissue of uninfected mice, with expression increasing to approximately 10-15% at day 6 after inoculation **(Fig. 1C and 1D)**, which corresponded to peak IL-21 mRNA expression **(Fig. 1B)**, whereas mCherry expression was not detected in $NK1.1^+$ cells (NK cells or NKT cells), γδ T cells, or $CD8^+$ T cells in the lung **(Suppl. Fig. 1)**. In addition to this acute response in the lung, a systemic immune response also developed, as indicated by the increase in mCherry expression from approximately 1.9% of splenic $CD4^+$ T cells in uninfected reporter mice (TG Ctrl) to approximately 5.9% at day 6 (TG PVM) (**Fig. 1D**). $CD4^+$ T cells expressing IL-21 were enriched for expression of the ICOS/ CXCR5 surface markers characteristic of T follicular helper cells (Tfh) (Fig. 1E and 1F) compared to $CD4^+$ T cells that did not express IL-21, with a particularly high percentage of Tfh cells in mediastinal lymph node IL-21 expressors.

Reduced lung inflammation in response to PVM infection in *Il21r*$^{-/-}$ mice

The increased numbers of IL-21-producing $CD4^+$ T cells in the lung after PVM infection suggested that this cytokine might participate in antiviral host defense and/or viral pathogenesis. To investigate this possibility, WT and *Il21r*$^{-/-}$ mice were infected with PVM and cellular inflammatory responses were evaluated. As observed previously (30, 31), lung tissue from PVM-infected WT mice exhibited progressive, severe inflammation diffusely through the lung at days 5 and 6, whereas lungs from *Il21r*$^{-/-}$ mice had only mild and focal perivascular inflammation at these time points **(Fig. 2A)**. Infiltration of both neutrophils (arrowheads) and lymphocytes (arrows) were evident in the WT mice (**Fig. 2B**). We also examined bronchoalveolar lavage (BAL) fluid from PVM-infected mice and found significantly fewer cells in the BAL fluid from the *Il21r*$^{-/-}$ mice than from WT mice on days 5, 6 and 9 post-inoculation **(Fig. 3A)**, as well as fewer cells in lung parenchyma at days 6 and 9 **(Fig. 3B)**. Granulocytic infiltration is a hallmark of the early inflammatory response to PVM (28), and there were fewer $Ly6G^+CD11b^+$ neutrophils in the BAL fluid at days 5, 6 and 9 **(Fig. 3C)** and lung parenchyma **(Fig. 3D)** at day 6 in the *Il21r*$^{-/-}$ than WT mice inoculated with PVM. Consistent with this, at day 6 post-inoculation, *Il21r*$^{-/-}$ mice also had significantly decreased levels of mRNAs encoding matrix metalloprotease-8 (MMP8) and

S100A8 **(Fig. 3E and 3F)**, two neutrophil-derived proteins involved in mediating the inflammatory/lung remodeling response (32, 33).

Because T cells were reported to be important for the resolution of sub-lethal PVM infection (34), we assessed the presence of $CD4^+$ T cells, $CD8^+$ T cells, $\gamma\delta^+$ T cells, and NK cells in the lung during the course of PVM infection in WT and *Il21r*$^{-/-}$ mice **(Fig. 4)**. $CD4^+$ T cell infiltration was apparent by day 6 after inoculation, but to a greater degree in WT mice than in *Il21r*$^{-/-}$ mice, with elevated $CD4^+$ T cell numbers persisting through day 9 post-infection **(Fig. 4A)**. Increased numbers of $CD8^+$ T cells were observed in the lungs of WT mice as early as 3 days after PVM inoculation but then declined, whereas fewer $CD8^+$ T cells were observed in the lungs of infected *Il21r*$^{-/-}$ mice **(Fig. 4B)**. Although $\gamma\delta^+$ T cells infiltrated both WT and *Il21r*$^{-/-}$lungs equivalently starting on day 5, fewer $\gamma\delta^+$ T cells were seen in the *Il21r*$^{-/-}$ lungs at days 6 and 9 **(Fig. 4C)**. NK cell numbers were also significantly lower in the lung parenchyma in the *Il21r*$^{-/-}$ mice (**Fig. 4D**). Thus, the recruitment of $CD4^+$, $CD8^+$, and γδ T cells, as well as NK cells was diminished in the absence of IL-21 signaling.

$CD8^+$ T cell function was previously reported to be suppressed in PVM-infected lungs (35). Interestingly, the percentage of lung $CD8^+$ T cells producing IFNγ increased during the course of infection in both WT and *Il21r*$^{-/-}$ mice **(Fig. 4E)**, but because of the reduced number of $CD8^+$ T cells in the lungs of *Il21r*$^{-/-}$ mice **(Fig. 4B)**, the total number of IFNγ-producing $CD8^+$ T cells was significantly diminished **(Fig. 4F)**. PVM peptides that allow the detection of viral-specific $CD8^+$ T cells have not been identified; thus, these $CD8^+$ T cells may include both PVM-specific cytotoxic cells as well as bystander cells activated by the inflammatory environment.

Reduced levels of IL-6 in lungs of PVM-infected *Il21r*$^{-/-}$ mice

The inflammatory response to PVM in WT mice includes rapid infiltration of both neutrophils and lymphocytes into the lung, and, as noted above, these responses were reduced in *Il21r*$^{-/-}$ mice. Neutrophil infiltration in PVM infection is coordinated by multiple cytokines and proinflammatory chemokines (28, 30, 31). In other settings, IL-17 has been shown to promote neutrophil responses, leading to the induction of cytokines and chemokines that augment the levels of neutrophil progenitors and the subsequent expansion of peripheral neutrophil populations (36). Because IL-21 promotes the differentiation of IL-17-producing cells (12-14), we measured the levels of cytokines associated with Th17 and Tc17 responses after PVM infection. Levels of both *Il17a* (**Fig. 5A**) and *Il22* (**Fig. 5B**) mRNAs in lung tissue were not significantly different in WT and *Il21r*$^{-/-}$ PVM-infected mice. *Tnfa* (**Fig. 5C**) and *Ifng* (**Fig. 5D**) mRNAs tended to be slightly lower in the *Il21r*$^{-/-}$ mice at day 6 after inoculation, but the differences were not statistically significant. *Il1b* mRNA was significantly decreased at days 6 and 9 (Fig. 5E), although differences in IL-1β protein were not observed (data not shown). However, *Il6* mRNA was significantly decreased at days 5, 6, and 9 (**Fig. 5F**), with a corresponding decrease in IL-6 protein at day 6 in both BAL fluid and lung tissue (**Fig. 5G&H**). These results suggest that IL-21 signaling may directly or indirectly control the production of IL-6 during PVM infection.

Diminished levels of CXCL1 in PVM infected *Il21r*$^{-/-}$ mice

Because the accumulation of neutrophils in the lungs was diminished in PVM-infected *Il21r*$^{-/-}$ mice (**Fig. 3**), we examined the expression of several chemokines known to promote neutrophil recruitment during PVM infection (37). Interestingly, *Cxcl1* mRNA was less potently induced in lung tissue of *Il21r*$^{-/-}$ mice than in WT mice during PVM infection (**Fig. 6A**), whereas mRNA encoding CXCL10, a chemokine involved in lymphocyte recruitment, was induced with slightly more rapid kinetics in WT mice **(Fig. 6B)**, but by day 6 the levels were not significantly different in WT vs the *Il21r*$^{-/-}$ mice. We observed no significant

difference in the expression of the chemokine CCL3 (also known as macrophage inflammatory protein-1α, MIP-1α), which has been implicated in neutrophil recruitment and immunomodulatory protection in PVM infection (28) **(Fig. 6C)**, and although lower *Cxcl2* mRNA expression was observed in *Il21r*$^{-/-}$ mice at day 6 (**Fig. 6D**), there was no significant difference in CXCL2 protein expression in the lung (data now shown). Corresponding to the diminished levels of *Cxcl1* mRNA (**Fig. 6A**), CXCL1 protein levels were significantly lower in BAL fluid (**Fig. 6E**) and lung homogenate (**Fig. 6F**) at days 5 and 6 after PVM infection of the *Il21r*$^{-/-}$ mice, correlating with the reduced neutrophil infiltration in the lungs of these mice.

Similar immune responses in lung draining lymph nodes from PVM-infected *Il21r*$^{-/-}$ mice

Because of the roles that IL-21 plays in the development of cellular immune responses, it was of interest to determine whether changes in cellularity, chemokines, and cytokines were specific to the lung response or whether they reflected an overall deficiency of the immune response in *Il21r*$^{-/-}$ mice. We thus examined lymphoid populations in the mediastinal lymph nodes (MLN) at days 0 and 6 after PVM infection and found that both WT and *Il21r*$^{-/-}$ MLN undergo T cell expansion (Fig.7A), including both CD4^{+} (Fig. 7B) and CD8^{+} (Fig. 7C) in response to infection, although this was reduced in the *Il21r*$^{-/-}$ MLN (Fig. 7A, 7B, and 7C). Similar levels of *Ifng*, *Tnfa*, and *Il6* mRNAs were measured in the MLN, although *Il17a* and *Il1b* mRNA levels were lower in the *Il21r*$^{-/-}$ MLN **(Fig. 7D-7H)**. These data indicate that although lymphoid expansion was lower in the *Il21r*$^{-/-}$ MLN, most inflammatory cytokine responses were similar, suggesting that the cellular immune response was not completely defective in the KO mice.

Constitutive expression of IL-21 leads to increased cellular infiltration to the lung and increased IL-6 production

The production of IL-21 by CD4^{+} T cells in the normal lung suggested that IL-21 may play a role in normal lung homeostasis as well as in the development of the inflammatory response to PVM infection. To investigate this possibility, we examined lung cellularity in transgenic mice that constitutively express IL-21 in immune cells (TG21) (5). We observed increased cellularity in both the BAL fluid (Fig. 8A) and the lung (Fig. 8B), with an elevated percentage of neutrophils in both BAL and lung in the uninfected TG21 mice (Fig. 8C). Levels of *Cxcl1* mRNA (Fig. 8D) and *Il6* mRNA (Fig. 8E) were both increased in the TG21 lungs. Interestingly, however, levels of the neutrophil-expressed *Mmp8* mRNA were similar in WT and TG21 lungs (Fig. 8F), suggesting that the enhanced recruitment of neutrophils to the lung occurred without an associated inflammatory response. In order to determine whether IL-21 could directly induce the production of IL-6, we purified dendritic cell populations and found that IL-21 significantly induced both IL-6 mRNA and protein (Fig. 8G & 8H) in these cells.

Prolonged survival of PVM-infected *Il21r*$^{-/-}$ mice

Above, we observed an increase in IL-21 production in response to PVM infection as well as decreased neutrophil and lymphocyte accumulation and diminished production of IL-6 in *Il21r*$^{-/-}$ *mice*. It was unclear, however, whether IL-21 mediated the PVM pathogenic response or instead promoted host-defense. To clarify the role of IL-21, we inoculated WT and *Il21r*$^{-/-}$ mice intra-nasally with a dose of PVM previously shown to be sufficient to kill WT mice, and determined their survival **(Fig. 7A)**. As anticipated, 60% of the WT mice died on day 7 and all were dead by day 10 after PVM inoculation. In contrast, 90% of the *Il21r*$^{-/-}$ mice were still alive at day 7, and 30% of the *Il21r*$^{-/-}$ mice survived beyond day 10, with 5% (1 of 19 mice) surviving through day 21. Thus, the absence of IL-21 signaling conferred a survival advantage ($p < 0.0001$), even though there was no significant difference in PVM *SH* gene expression, as an indicator of virus copy number, in WT versus *Il21r*$^{-/-}$ lungs (**Fig. 7B**).

These results suggest that IL-21 does not have a significant effect on viral replication or clearance but that it promotes the inflammatory response following infection with PVM, with earlier and augmented mortality in response to PVM infection in WT mice.

To determine whether the enhanced survival of the *Il21r*$^{-/-}$ mice was a direct result of the effects of IL-21 in the lung during the response to PVM infection, we next used an IL-21R/Fc fusion protein to block IL-21 activity. When WT mice were intratracheally treated with the IL-21R/Fc fusion protein one day prior and 2 days after PVM inoculation, there was significantly higher survival than in mice treated with an Fc control protein (**Fig. 7C**), even though no significant differences in virus replication, based on *SH* gene expression, were detected between these two groups at day 6 of infection (**Fig. 7D**). Moreover, when we inoculated mice with a lower dose of PVM, treatment with the IL-21R/Fc fusion protein conferred complete protection (**Fig. 7E**), and again there were no differences in viral burden (**Fig. 7F**). These data indicate that the survival advantage seen in *Il21r*$^{-/-}$ mice did not result from developmental differences but rather resulted from the lack of IL-21 signaling.

DISCUSSION

In this study, we have shown that IL-21 signaling plays a critical role in promoting the lung inflammatory response to acute pneumovirus infection. IL-21 expression in the lung in response to acute or chronic viral infections in general and to PVM in particular has not previously been reported. Our studies revealed that IL-21 mRNA levels are significantly elevated in lung tissue after PVM infection and that this induction parallels increased lung inflammation. Correspondingly, studies with *Il21-mCherry* reporter mice showed that enhanced IL-21 expression in the lung after PVM infection is specific for CD4$^+$ T cells. Interestingly, uninfected lungs also contained a small but detectable population of CD4$^+$ T cells expressing mCherry, suggesting a potential role for IL-21 in basal lung homeostatic responses to environmental antigens.

To determine if the production of IL-21 promoted or diminished PVM-induced disease, we examined PVM-inoculated *Il21r*$^{-/-}$ mice and found they had significantly reduced lung pathology, with decreased infiltration of both neutrophils and lymphocytes. Previous studies showed that CCL3/MIP-1α, a chemokine produced by lung epithelium in response to RSV infection (38), was critical for neutrophil accumulation in the lungs and survival after PVM infection(39). Interestingly, CCL3 levels in the *Il21r*$^{-/-}$ mice were at most modestly diminished, although neutrophil infiltration was significantly reduced. In contrast, the level of CXCL1, which also mediates neutrophil chemotaxis (40), was much less potently induced in the BAL fluid and lungs of *Il21r*$^{-/-}$ mice than in WT mice; thus, the diminished CXCL1 may at least in part explain the diminished neutrophil accumulation in *Il21r*$^{-/-}$ mice.

Our results reveal a potential role for IL-21 in normal lung homeostasis. Uninfected IL-21 transgenic mice had increased lung cellularity, including neutrophilia. Additionally, lungs from TG21 mice had increased levels of IL-6 and CXCL1, both involved in mediating inflammatory pathology. Interestingly, IL-21 could directly induce IL-6 expression in dendritic cells, providing a direct link between IL-21 and the inflammatory process. Previously IL-6 was shown to induce IL-21 expression in CD4$^+$ T cells (14, 41). Our results suggest that IL-21 can potentially amplify its own induction via the induction of IL-6.

T cell responses significantly contribute to the host response to sublethal PVM infection, with both CD4$^+$ and CD8$^+$ T cell subsets contributing to viral clearance (34). Although both CD4$^+$ and CD8$^+$ T cell numbers were decreased in the *Il21r*$^{-/-}$ mice, there was no apparent change in viral replication or clearance. Th17 cytokines are known to mediate inflammation and IL-21 serves to promote Th17 differentiation (12-14); however, there was no apparent

decrease in Th17 cells or of IL-17A or IL-22 production in the lungs of PVM-infected *Il21r*$^{-/-}$ mice. Thus, Th17 differentiation does not appear to play a substantial role in PVM infection.

It was previously reported that most PVM-specific CD8^{+} T cells produce diminished levels of cytokines upon antigenic stimulation (35). In our studies, however, there was no difference in the percentage of CD8^{+} T cells that could produce IFNγ in WT and *Il21r*$^{-/-}$ mice. However, the total number of CD8^{+} T cells was greatly decreased, consistent with the known role of IL-21 for CD8^{+} T cell expansion and differentiation (4, 42), and thus the absolute number of IFNγ^{+} CD8^{+} T cells was also greatly reduced in the *Il21r*$^{-/-}$ lungs. It is possible that although the absence of IL-21 did not affect viral clearance in the primary response to PVM, CD8^{+} T cells primed in the absence of IL-21 might have defective memory responses to PVM upon secondary challenge.

In summary, we have demonstrated that IL-21 plays a previously unappreciated role in the host response to PVM, promoting an unanticipated pathogenic inflammatory response to the virus that is associated with reduced host survival, which is in marked contrast to the protective role played by IL-21 in chronic LCMV infections and its correlation with control of HIV. Importantly, we also found that pre-treatment of WT animals with a soluble IL-21R-Fc fusion protein was protective, an observation with therapeutic implications for PVM and potentially for other viral infections as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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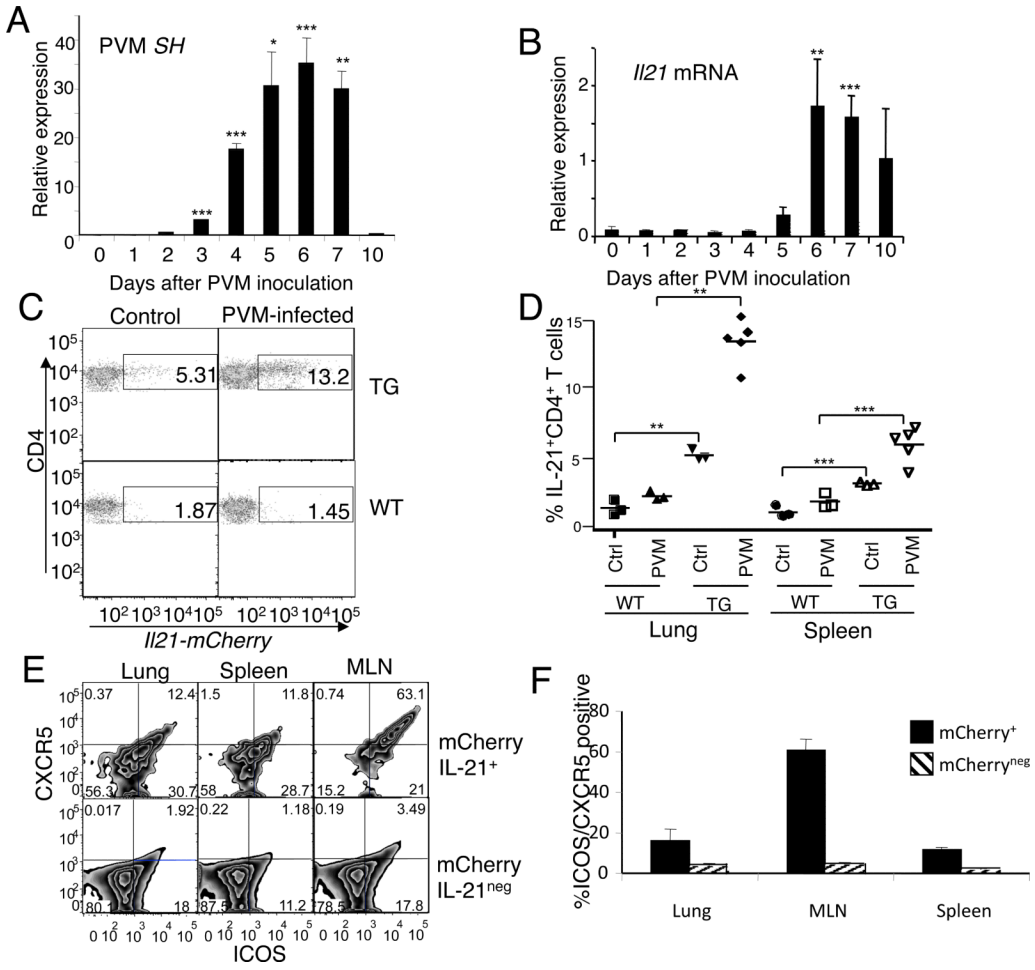

Figure 1. IL-21 is expressed by lung CD4$^+$ T cells following PVM infection

(A and B) C57BL/6 WT mice were inoculated with PVM, sacrificed at indicated time points, lung RNA isolated from lung tissue, and RT-PCR analysis of the PVM *SH* (A) and *Il21* (B) genes was performed. Shown are values ± S.E.M. relative to *Rpl7* expression (n = 5 for each time point). (C-F) *Il21-mCherry* transgenic reporter mice (TG) or WT littermates were infected with PVM, lung cells were isolated 6 days later, and IL-21 expression measured by flow cytometry after surface staining with anti-CD4 and anti-TCRβ. A representative experiment is shown in (C), (D) and (E); the experiment was performed 3 times with similar results. (F) Summary of 3 experiments.

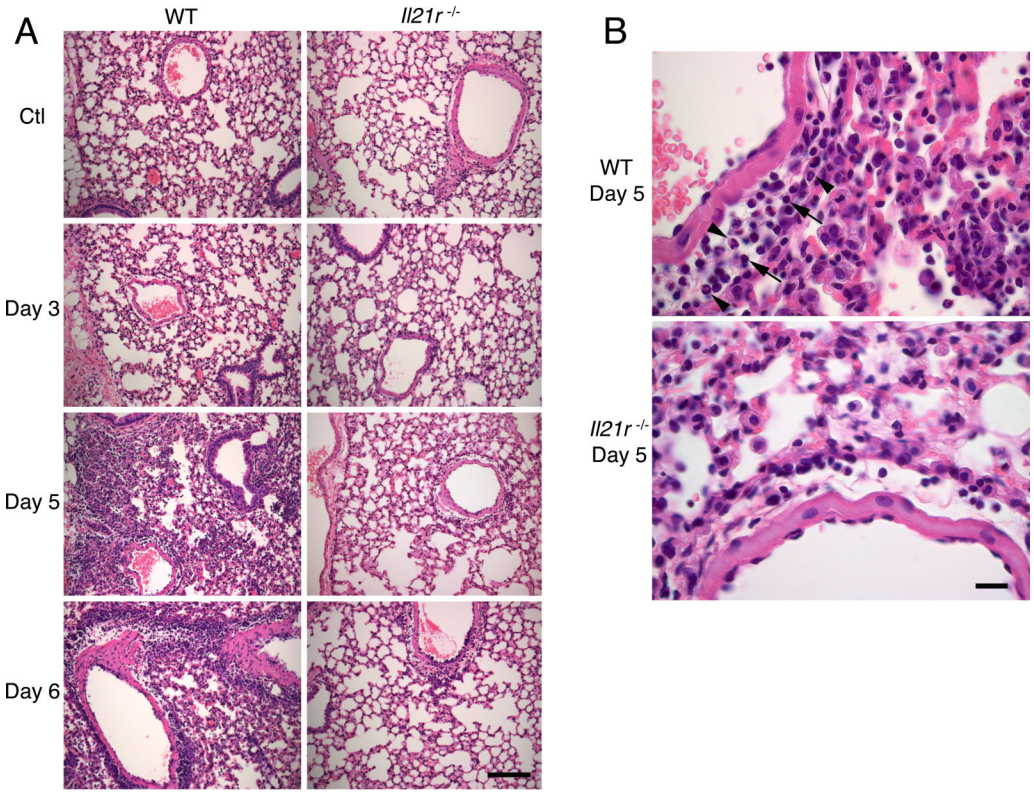

Figure 2. Reduced lung inflammatory pathology in PVM-infected *Il21r*$^{-/-}$ mice

Representative lung sections (stained with H & E) from either uninfected (CTL) or PVM-infected WT and *Il21r*$^{-/-}$ mice. (A) Compared to the *Il21r*$^{-/-}$ mice, WT lungs showing severe cell infiltration at days 5 and 6 after PVM inoculation. Scale bar, 100 μm for all panels. (B) Higher magnification views shows the inflammatory cells include lymphocytes (arrows) and neutrophils (arrowhead). Scale bar = 20 μm.

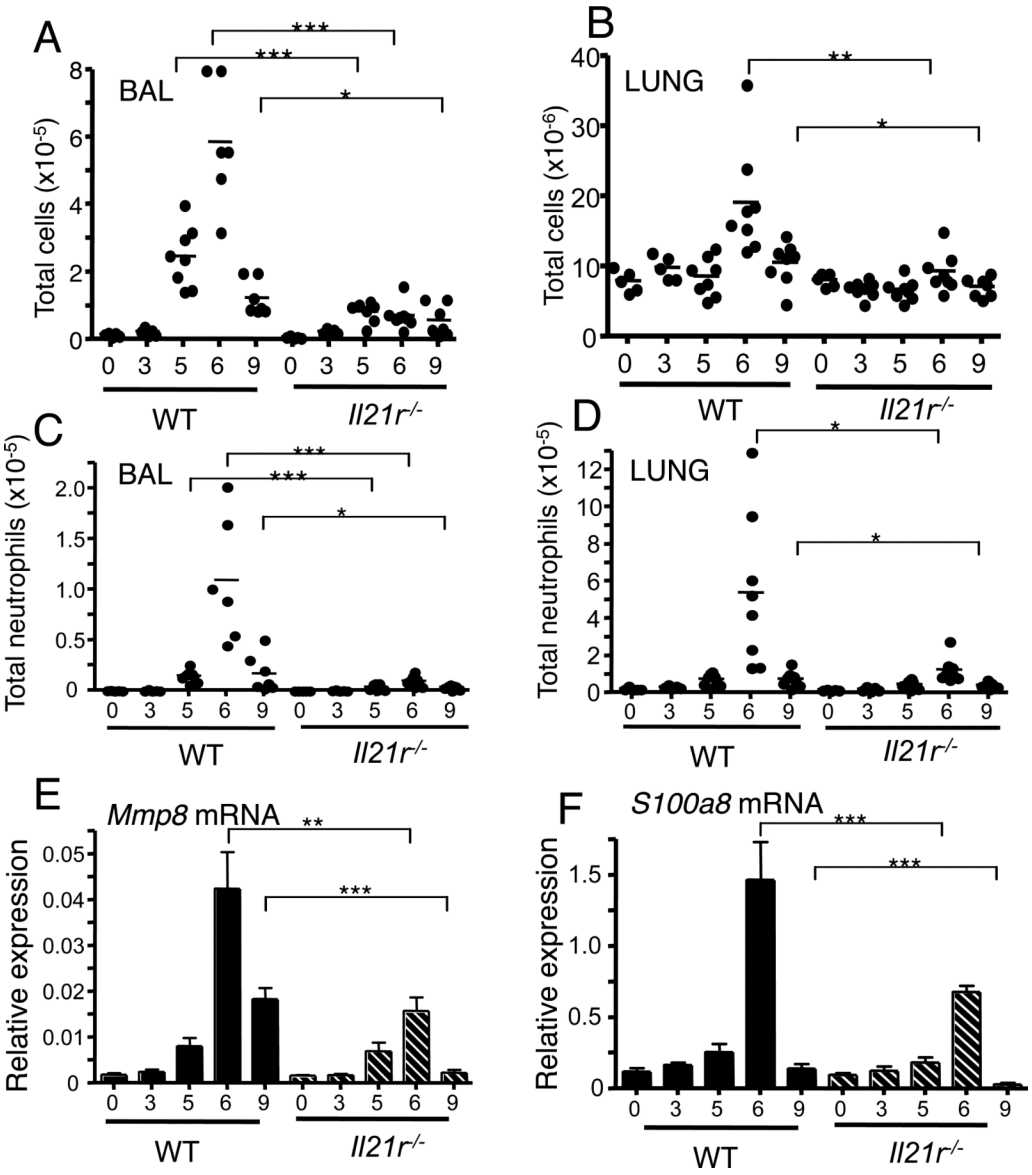

Figure 3. Reduced cellular infiltration in lungs of *Il21r*$^{-/-}$ mice in response to PVM infection

Total number of cells in BAL fluid (A) or of cells isolated from one lobe of the lung (B) were measured at the indicated time points after PVM infection of WT or *Il21r*$^{-/-}$ mice. The total number of neutrophils was calculated after analyzing the percent Ly6G^{+}CD11b^{+} cells in either the BAL fluid (C) or lung (D). (E and F) RNA was isolated from the other lung lobe in these mice and RT-PCR was used to evaluate the relative levels of MMP8 or S100A8 mRNA in the lungs of these PVM-infected mice. Shown are the means ± S.E.M from one of three experiments with similar results (n = 5-8 mice per group).

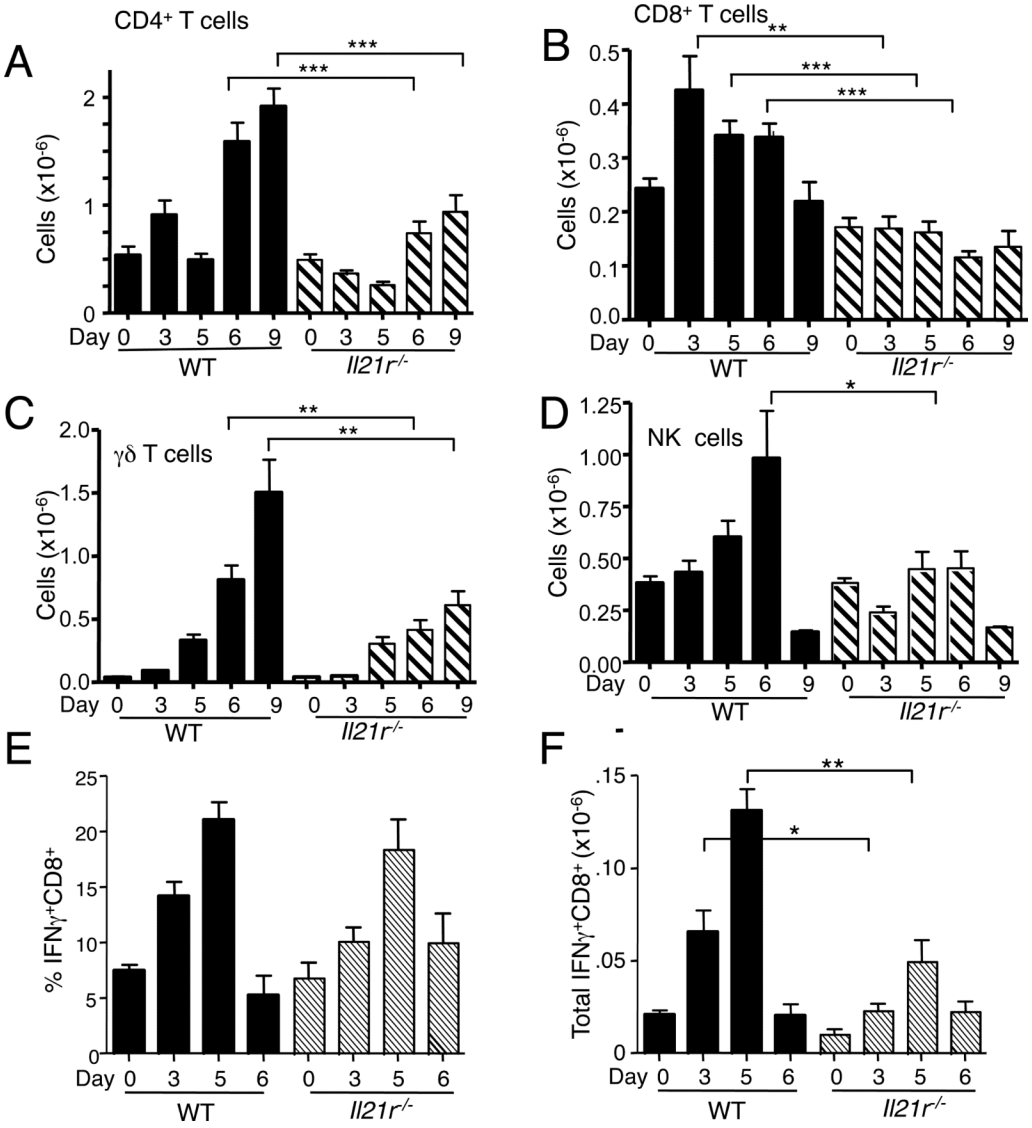

Figure 4. Lymphocyte infiltration in response to PVM infection is diminished in *Il21r*$^{-/-}$ mice

Flow cytometry was used to assess the total number of CD4$^+$ T cells (A), CD8$^+$ T cells (B), γδ T cells (C), and NK cells (D) in lungs of WT and *Il21r*$^{-/-}$ mice. Total cells were calculated from the % of each population and the total cell number isolated from one lung lobe. IFNγ-producing cells were stained after PMA/ionomycin/Golgi Plug incubation for 4 hr after isolation. The % of IFNγ$^+$CD8$^+$ T cells is shown in (E) and the calculated value of total IFNγ$^+$CD8$^+$ T cells is displayed in (F). Shown are the means ± S.E.M. for one of three experiments with similar results (n = 5-8 mice per group).

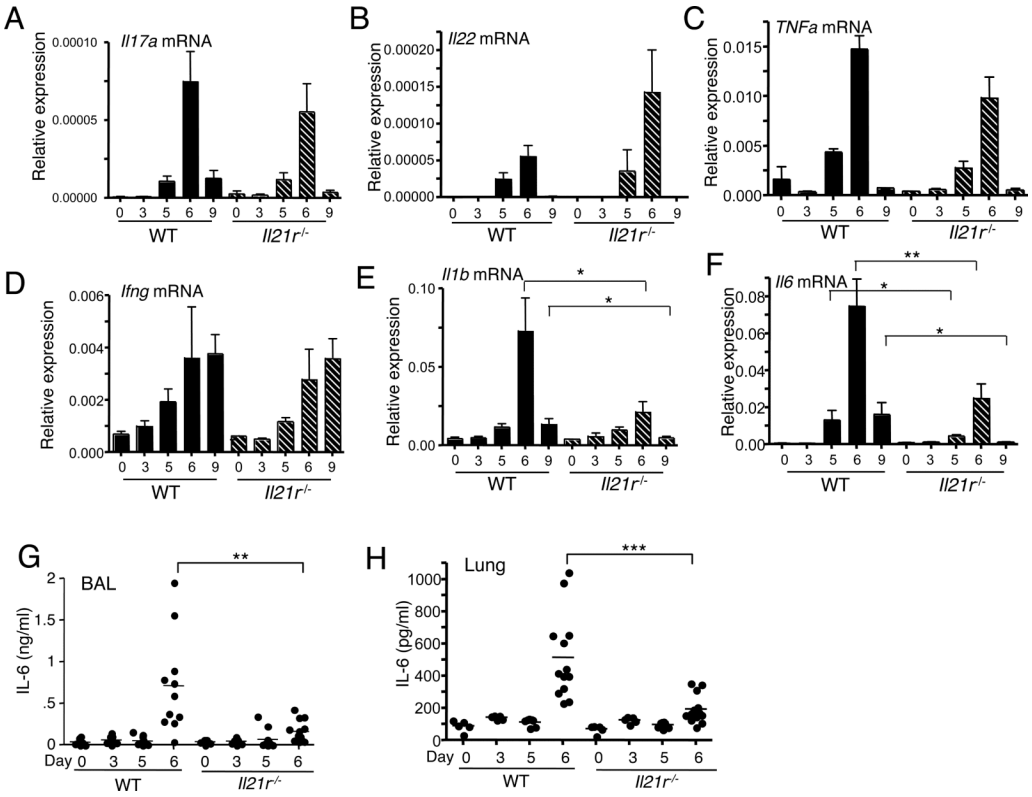

Figure 5. Levels of Th17 cytokines are similar but IL-6 levels are reduced in lung tissue of PVM-infected *Il21r*$^{-/-}$ mice

(A-F) Lung RNA was isolated from WT and *Il21r*$^{-/-}$ mice after PVM infection and relative levels of mRNA encoding IL-17A (A), IL-22 (B), TNFα (C), IFNγ (D), IL-1β (E), and IL-6 (F) were quantitated by RT-PCR. IL-6 protein levels in BAL fluid (G) or lung homogenate (H) were measured by ELISA. Shown are the means ± S.E.M. from one of three experiments with similar results (n = 5-8 mice per group).

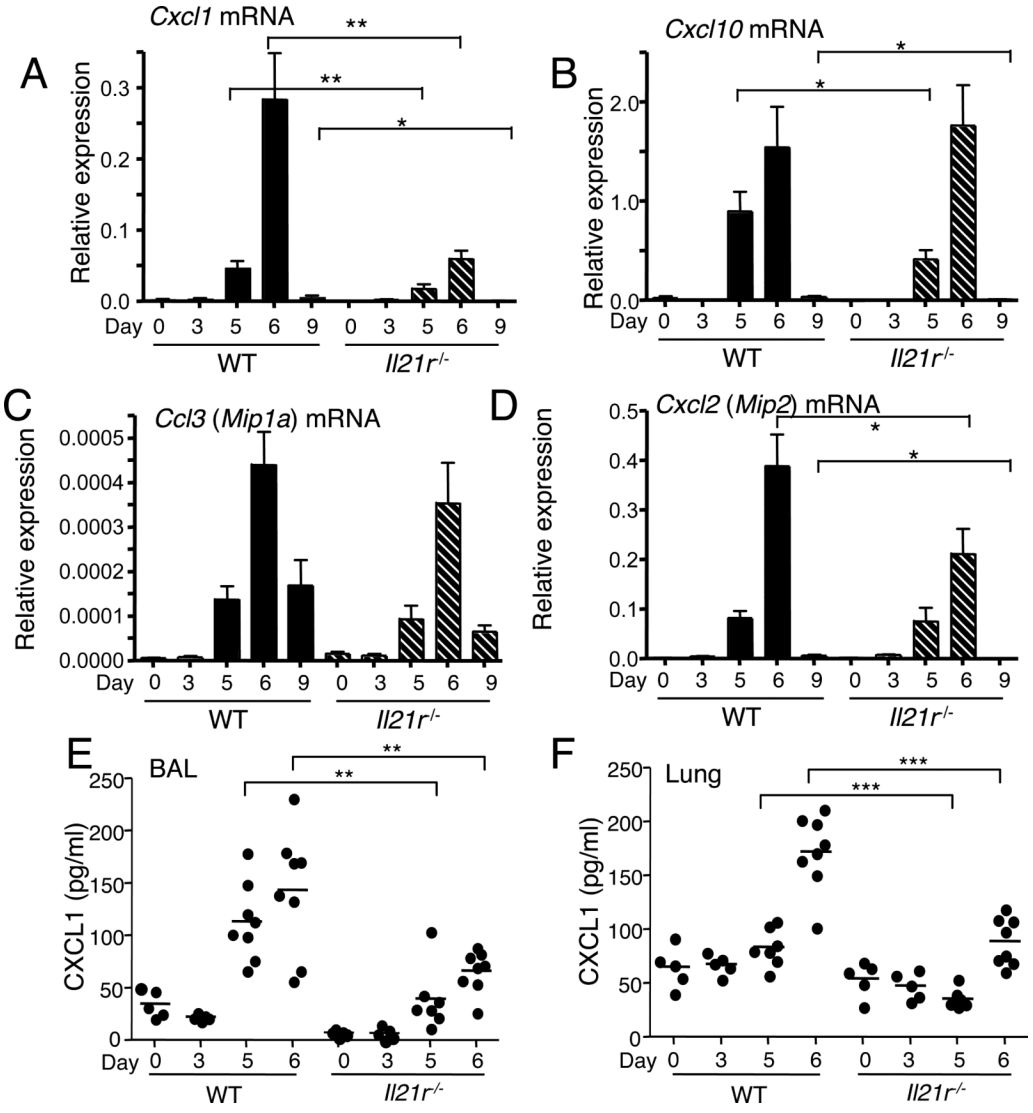

Figure 6. Induction of CXCL1 mRNA and protein levels in response to PVM infection are diminished in lung tissue from *Il21r*$^{-/-}$ mice

(A-D) Lung RNA was isolated from WT and *Il21r*$^{-/-}$ mice after PVM infection and relative levels of mRNA encoding the chemokines CXCL1 (A), CXCL10 (B), CCL3 (C), and CXCL2 (D) were quantitated by RT-PCR. (E and F) CXCL1 protein levels were measured by ELISA in BAL fluid (E) or in equal amounts (25ug) of protein from lung homogenates (F) of WT and *Il21r*$^{-/-}$ mice. Shown are the means ± S.E.M. from one of three experiments with similar results (n = 5-8 mice per group).

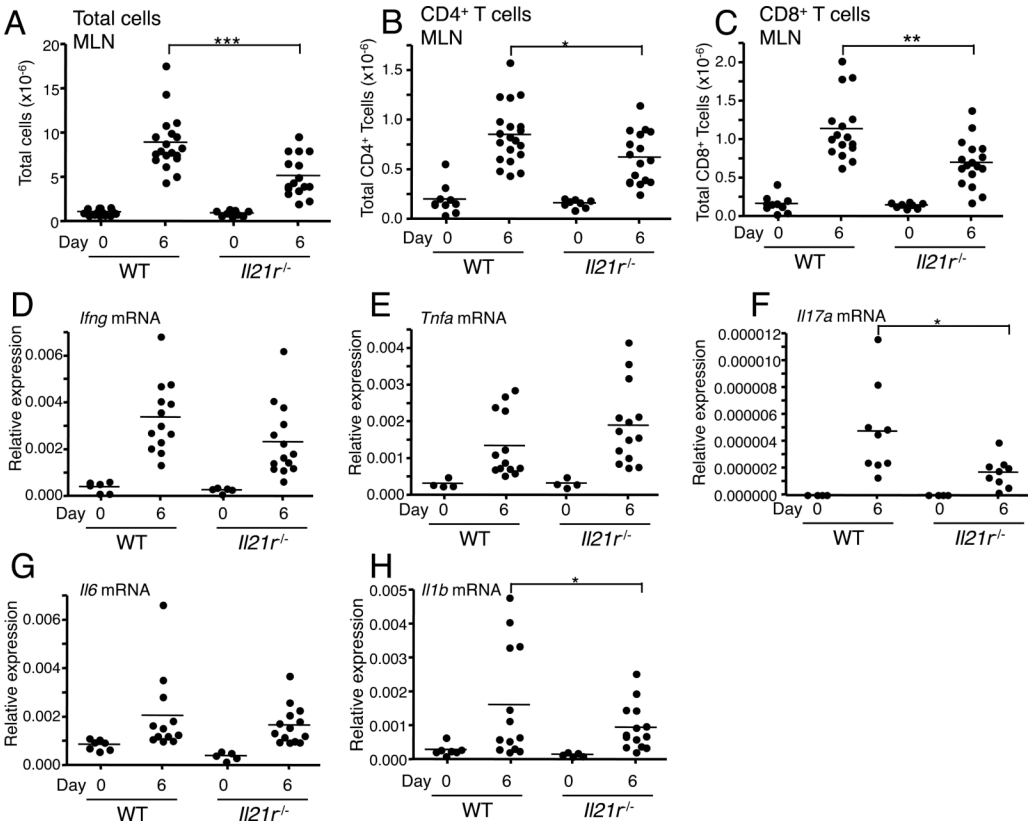

Figure 7. Cellular infiltration and cytokine production in lung-draining lymph nodes after PVM infection of WT and *Il21r*$^{-/-}$ mice

(A) Total cellularity was measured in mediastinal lymph nodes either before or at day 6 after PVM infection. **(B and C)** Flow cytometry was used to assess the total number of $CD4^+$ (B) or $CD8^+$ (C) T cells based on the % of each population and the total cell number. **(D-H)** MLN RNA was isolated and relative levels of the indicated cytokines were measured by RT-PCR. Shown are pooled data from 3 independent experiments.

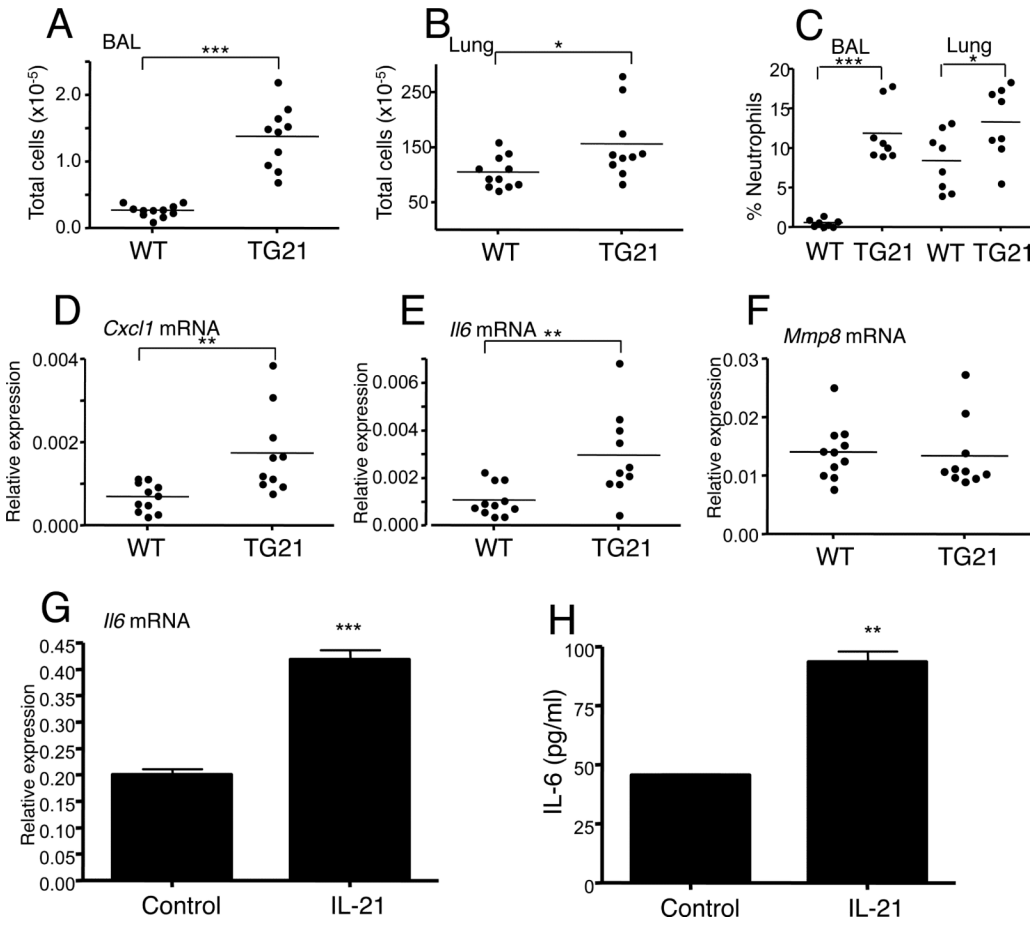

Figure 8. IL-21 increases lung cellularity and induces IL-6 production

(A-C) Total cellularity was measured in BAL fluid (A) and lungs (B) of WT and IL-21 transgenic mice (TG21). The % neutrophils was determined by flow cytometry of $Ly6G^{+}CD11b^{+}$ cells (C). **(D-F)** Lung RNA was isolated and relative levels of *Cxcl1* (D), *Il6* (E) and *Mmp8* (F) mRNA were measured by RT-PCR. **(G, H)** Splenic dendritic cells were isolated and stimulated in vitro with IL-21 for 5 h, at which time *Il6* mRNA was measured by RT-PCR (G) and IL-6 protein was measured at 16 h by ELISA (H).

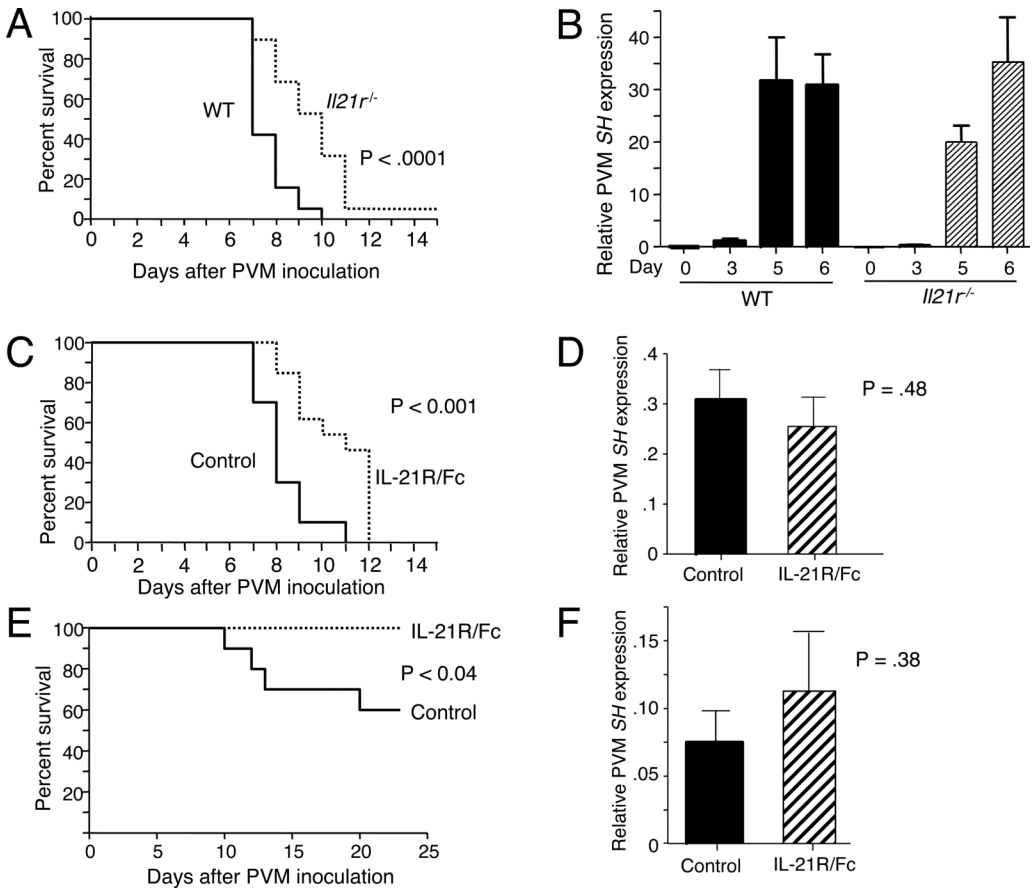

Figure 9. Prolonged survival after PVM infection in *Il21r*$^{-/-}$ mice or in WT mice treated with an IL-21R/Fc fusion protein

(A) WT and *Il21r*$^{-/-}$ mice were infected with PVM and their survival was monitored daily over the next 3 weeks. Infection of the long-term survivor was documented by confirming sero-conversion to PVM antigens (SMART-M12, El Cerrito, CA; data not shown). Statistical significance was evaluated using Kaplan-Meier survival curve (GraphPad Prism). n= 19 mice per group. (B) RT-PCR evaluation of the PVM *SH* gene in total lung RNA. Shown are the means ± S.E.M. from one of three experiments with similar results (n = 5-8 mice per group). (C and E) WT mice received 50 μg of either IL-21R/Fc or control Fc intratracheally one day prior to and 2 days post-inoculation with PVM and their survival was monitored. Mice in (C) and (E) received 60 pfu and 12 pfu, respectively, of PVM intranasally. In (C), p = .001 and in (E) p = .039, with 10 mice per group in each panel. (D and F) PVM *SH* expression was determined by RT-PCR of total lung RNA at day 6 after PVM infection. (D) corresponds to the experiment in (C) and (F) corresponds to the experiment in (E). Shown are the means ± S.E.M. from 5 mice in each group.