

Toll-like receptor 7 gene deficiency and early-life *Pneumovirus* infection interact to predispose toward the development of asthma-like pathology in mice

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Background: Respiratory tract viruses are a major environmental risk factor for both the inception and exacerbations of asthma. Genetic defects in Toll-like receptor (TLR) 7-mediated signaling, impaired type I interferon responses, or both have been reported in asthmatic patients, although their contribution to the onset and exacerbation of asthma remains poorly understood.

Objective: We sought to determine whether *Pneumovirus* infection in the absence of TLR7 predisposes to bronchiolitis and the inception of asthma.

Methods: Wild-type and TLR7-deficient (*TLR7*^{-/-}) mice were inoculated with the rodent-specific pathogen pneumonia virus of mice at 1 (primary), 7 (secondary), and 13 (tertiary) weeks of age, and pathologic features of bronchiolitis or asthma were assessed. In some experiments infected mice were exposed to low-dose cockroach antigen.

Results: TLR7 deficiency increased viral load in the airway epithelium, which became sloughed and necrotic, and promoted an IFN- α/β ^{low}, IL-12p70^{low}, IL-1 β ^{high}, IL-25^{high}, and IL-33^{high} cytokine microenvironment that was associated with the recruitment of type 2 innate lymphoid cells/nuocytes and increased T_H2-type cytokine production. Viral challenge of *TLR7*^{-/-} mice induced all of the cardinal pathophysiologic features of asthma, including tissue eosinophilia, mast cell hyperplasia, IgE production, airway smooth muscle alterations,

and airways hyperreactivity in a memory CD4⁺ T cell-dependent manner. Importantly, infections with pneumonia virus of mice promoted allergic sensitization to inhaled cockroach antigen in the absence but not the presence of TLR7. **Conclusion:** *TLR7* gene defects and *Pneumovirus* infection interact to establish an aberrant adaptive response that might underlie virus-induced asthma exacerbations in later life. (J Allergy Clin Immunol 2013;■■■■:■■■■-■■■■.)

Key words: Toll-like receptor 7, Pneumovirus, infection, type 2 innate lymphoid cell, nuocyte, asthma, IL-13, exacerbation, respiratory syncytial virus, bronchiolitis

Asthma is a chronic inflammatory disease of the lungs that is typically dominated by an aberrant CD4⁺ T_H2 immune response.¹ In addition to classical allergens, this T_H2 response can be induced under certain conditions in response to respiratory tract viruses, such as rhinovirus or respiratory syncytial virus (RSV).^{2,3} These RNA viruses are causally linked to the majority of acute asthma exacerbations in both children and adults⁴⁻⁷ and cause prolonged and heightened chest symptoms (wheeze and dyspnea),⁸ leading to significant morbidity and health care expenditure. After experimental rhinovirus infection in asthmatic patients, these symptoms were found to positively correlate with a greater viral load and CD4⁺ T_H2 cell cytokine expression,³ yet the mechanistic processes that underlie the development of the aberrant virus-specific T_H2 response remain poorly understood.

Several prospective birth cohort studies have shown that the severity or frequency of wheezy lower respiratory tract (LRT) viral infections in infancy are a major risk factor for the development of asthma in later childhood.^{6,9-12} Thus the development of virus-specific T_H2 responses might be established in early life during initial encounter with a viral pathogen. Although virtually all human subjects have been infected with RSV by age 3 years, the population at large is not asthmatic, suggesting that an interaction between the virus and a susceptibility allele or alleles might predispose to both bronchiolitis and asthma. The diminished production of type I (α/β) and type III interferons by airway epithelial cells (AECs)^{13,14} and hematopoietic cells of asthmatic patients^{13,15-17} has advanced the notion that antiviral innate host defense is impaired in these patients. Pattern recognition receptors (PRRs) play a pivotal role in sensing structural elements of pathogens. The activation of these pathways not only initiates the immediate innate inflammatory response but also provides the instructive cues that shape the development of the T_H response, which in turn coordinates the appropriate “module” of adaptive

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Abbreviations used

AEC:	Airway epithelial cell
APC:	Allophycocyanin
BAL:	Bronchoalveolar lavage
cDC:	Conventional dendritic cell
DLN:	Draining lymph node
dpi:	Days after inoculation
ILC:	Innate lymphoid cell
LRT:	Lower respiratory tract
pDC:	Plasmacytoid dendritic cell
PE:	Phycoerythrin
PFU:	Plaque-forming unit
PRR:	Pattern recognition receptor
PVM:	Pneumonia virus of mice
RSV:	Respiratory syncytial virus
TLR7:	Toll-like receptor 7
TSLP:	Thymic stromal lymphopoietin

immunity for pathogen elimination.¹⁸ Hence genetic abnormalities of PRRs or downstream signaling components might adversely alter the immediate host response to primary infection, as well as establish pathogenic memory T_H responses that are recalled to secondary exposures. Viruses are largely sensed by PRRs that detect viral nucleic acids, namely the retinoic acid-inducible gene I (RIG-I)-like receptors and the Toll-like receptors (TLRs; TLR3, TLR7, TLR8, and TLR9). Genome-wide association studies have revealed strong linkage between susceptibility to asthma and the sex chromosome Xp22, which harbors the *TLR7* and *TLR8* loci.^{19,20} Additionally, *TLR7* single nucleotide polymorphisms have been associated with asthma risk,^{21,22} whereas PBMCs from adolescent asthmatic patients produce significantly lower antiviral responses to TLR7, but not TLR3, stimuli,²³ raising the possibility that viral infection of *TLR7* polymorphic subjects might be a central gene-environment interaction that underlies the inception of asthma.

TLR7 is highly expressed by plasmacytoid dendritic cells (pDCs), which provide the host's immediate source of type I interferon in response to infection.^{24,25} In asthmatic patients circulating pDC numbers are reduced,^{26,27} and they produce less type I interferon in response to influenza virus.²⁸ Moreover, their numbers in the first year of life have been inversely associated with the incidence of severe LRT infections in infancy and a diagnosis of asthma at 5 years of age.²⁹ Thus pDCs and TLR7 play a central role in the antiviral response and can be impaired in asthmatic patients, although whether these defects contribute to the onset of asthma remains to be formally proved. The natural mouse pathogen pneumonia virus of mice (PVM) shows great similarity to human RSV both in terms of genomic phylogeny and viral pathogenesis in the relevant host.³⁰ We have previously used PVM to demonstrate that TLR7-mediated *Pneumovirus* recognition by pDCs alone is sufficient to elicit a robust antiviral response.³¹ In the current study we sought to determine whether the absence of TLR7 predisposes to severe viral bronchiolitis and a virus-induced T_H2 response that underlies acute episodes of asthma on reinfection in later life.

METHODS

For more detailed information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Mice, PVM, and treatment regimens

Specific pathogen-free wild-type and *TLR7*-deficient (*TLR7*^{-/-}) BALB/c (and C57BL/6) mice were intranasally inoculated at 7 days of age with 5 plaque-forming units (PFU) of PVM (J3666 strain).³¹ Adult BALB/c (and C57BL/6) mice were reinoculated with 200 PFU of PVM in 40 μ L of vehicle at 42 days after primary infection (days after inoculation [dpi]) and again at 84 dpi, where indicated. Control mice received vehicle alone. Mice were killed 6 days after secondary and tertiary infection. In some experiments mice were inoculated with PVM and then exposed to inhaled cockroach antigen (1 μ g; Greer Laboratories, Lenoir, NC) at 3, 10, 17, and 31 dpi and killed at 34 dpi. All studies were approved by the University of Newcastle or the University of Queensland Animal Care and Ethics Committees.

Real-time quantitative PCR

Lungs were excised and frozen in RNAlater solution (Ambion, Carlsbad, Calif). Total RNA was isolated with TriReagent solution (Ambion), according to the manufacturer's instructions. Standard RT-PCR reaction conditions were used. Primer sequences are presented in [Table E1](#) in this article's Online Repository at www.jacionline.org.

For further information on measurements of airway function, histology, and immunohistochemistry, see the [Methods](#) section in this article's Online Repository.

Draining lymph node assays

Single-cell suspensions of lymph node cells were cultured at 5×10^5 cells per well per 200 μ L of growth medium for 3 days. Supernatants were analyzed by means of ELISA.

Flow cytometry

Isolated single-cell suspensions of lung cells were phenotyped by using multicolorimetric analysis. Antibodies are listed in the [Methods](#) section in this article's Online Repository. Type 2 innate lymphoid cells (ILCs) were identified as lineage-negative, CD45⁺, CD44⁺, ST2⁺, Sca-1^{+/lo}, c-kit^{+/lo}. Cells were analyzed on a FACSCanto or FACS LSR II (BD Biosciences, San Jose, Calif).

Assessment of airway function

Airways hyperreactivity was measured, as previously described.³²

For further information on statistical analyses, see the [Methods](#) section in this article's Online Repository.

RESULTS**TLR7 protects against severe infection and pathology during neonatal exposure to respiratory *Pneumovirus***

To determine whether the absence of TLR7 would increase the severity of disease and promote necrotic airway epithelial sloughing,³³ we inoculated neonatal (7-day-old) wild-type (*TLR7*^{+/+}) and *TLR7* gene-deleted (*TLR7*^{-/-}) mice with 5 PFU of PVM. The absence of TLR7 significantly increased mortality, primarily between 7 and 10 days post infection (dpi; [Fig 1, A](#)) and increased viral burden greater than 5-fold at 7 dpi ([Fig 1, B](#)). Lung sections probed with anti-PVM revealed that the virus was predominantly localized to the airway epithelium but spread to the underlying mucosa in the absence of TLR7 ([Fig 1, C](#)). *TLR7*^{-/-} mice presented with parenchymal consolidation (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org) and enhanced epithelial denudation of the airway ([Fig 1, D](#)). Extending our previous findings,³¹ the absence of TLR7 was associated with an attenuated type I IFN- α and IFN- β response (see [Fig E1, B](#)).

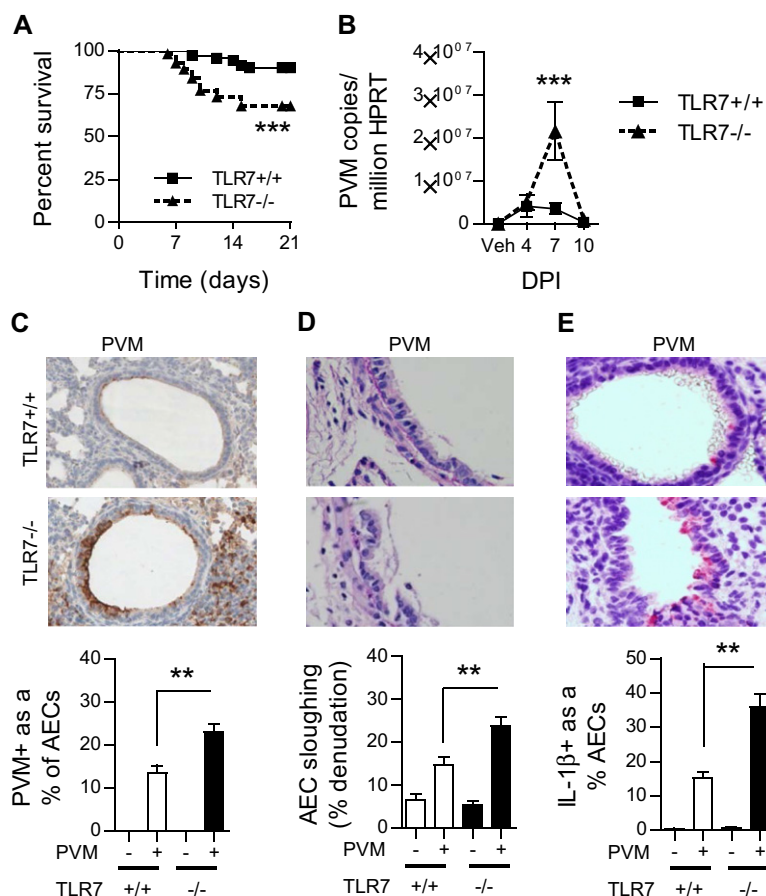


FIG 1. Reduced viral clearance and enhanced pathology occurs in the absence of TLR7. **A**, Survival. **B**, Viral load in the lungs determined by means of quantitative PCR. *HPRT*, Hypoxanthine-guanine phosphoribosyl-transferase. **C**, Micrographs (×400 magnification) of lung sections probed with anti-PVM (brown) and quantification of AEC expression (7 dpi). **D**, Micrographs (×1000 magnification) of epithelial shedding and quantification of denudation at 7 dpi. **E**, Micrographs (×1000 magnification) of lung sections probed with anti-IL-1β (red) and quantification of AEC expression at 4 dpi. ***P* < .01 and ****P* < .001 compared with PVM-infected TLR7^{+/+} mice. Data are presented as means ± SEMs for one experiment representative of 2 experiments, each with 4 to 7 mice per group.

and a 2- to 3-day delay in the expression of the T_H1 cytokine IFN- γ (see Fig E1, C). Febrile LRT infections are associated with asthma risk.³⁴ Because type I interferons cross-regulate inflammasome activation and hence maturation of the potent pyrogen IL-1 β ,³⁵ we hypothesized that IL-1 β expression would be increased in TLR7^{-/-} mice. IL-1 β production and caspase-1 activity increased in response to PVM at 4 dpi in both strains, although the concentration of IL-1 β was significantly greater in the absence of TLR7 (see Fig E1, D-F). Lung sections probed with anti-caspase-1 and anti-IL-1 β revealed that the airway epithelium was the primary source of both mediators (data not shown and Fig 1, E).

Altered dendritic cell subset recruitment and a T_H2 response to neonatal *Pneumovirus* infection is promoted in the absence of TLR7

We next sought to determine whether enhanced epithelial damage would lead to the recruitment of type 2 ILCs (alternatively named nuocytes), which were recently shown to mediate epithelial

repair during influenza virus infection through the provision of the epidermal growth factor receptor ligand amphiregulin.³⁶ In response to PVM infection, the numbers of type 2 ILCs in the lungs increased markedly at 7 dpi in both TLR7^{+/+} and TLR7^{-/-} mice; however, at 10 dpi, type 2 ILC numbers were significantly greater in the absence of TLR7 (Fig 2, A). Of note, the increased concentration of amphiregulin in lung homogenates (Fig 2, B) in the absence of TLR7 was associated with increased AEC hyperplasia (Fig 2, C). In addition to secreting amphiregulin, type 2 ILCs are major producers of the T_H2 -type cytokine IL-13.³⁶⁻³⁸ In the absence of TLR7, levels of IL-13 mRNA in the lung and protein in bronchoalveolar lavage (BAL) fluid were significantly increased at 7 and 10 dpi, respectively (Fig 2, D). IL-4 gene expression was similarly increased in the lungs of TLR7^{-/-} mice (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Cytokine production in the draining lymph nodes (DLNs) at 10 dpi revealed significantly greater IL-5 production in TLR7^{-/-} mice than in TLR7^{+/+} mice, whereas IFN- γ production was equivalent (see Fig E2, B), consistent with IFN- γ gene expression in the lung at 10 dpi (see Fig E1, C). IL-17A mRNA (data not shown) and protein

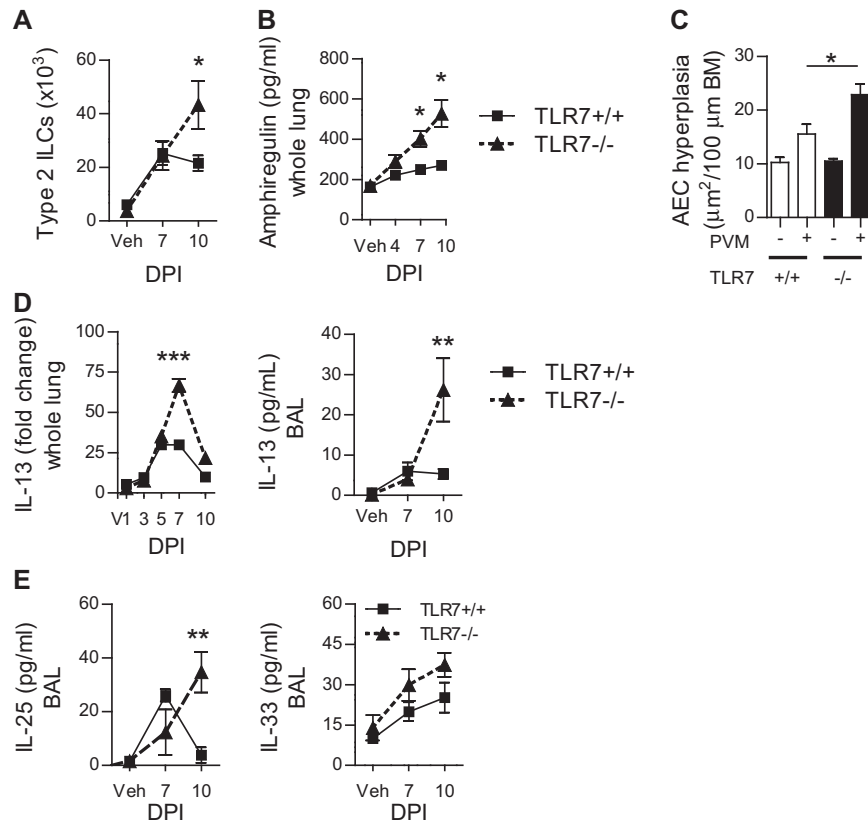


FIG 2. Pneumoviral infection in the absence of TLR7 promotes T_H2 immunity. **A**, Type 2 ILCs in the lungs. **B**, Amphiregulin protein in lung homogenates. **C**, AEC hyperplasia at 10 dpi. **D**, IL-13 gene and protein expression in the lung and BAL fluid. **E**, IL-25 and IL-33 protein expression in BAL fluid. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with PVM-infected samples. Data are presented as means $\pm$ SEMs of one experiment representative of 2 experiments, each with 5 to 7 mice per group.

expression (Fig E2, B) was not altered by the absence of TLR7. Consistent with a T_H2 -type response, expression of the eosinophil chemoattractant eotaxin-2/CCL24 (see Fig E2, C) and other T_H2 -associated chemokines (CCL5/RANTES, eotaxin-1/CCL11, and CCL22; data not shown) was increased in the absence of TLR7 and associated with mild tissue eosinophilia (see Fig E2, D).

In light of the T_H2 -type response, we next measured the expression of the innate T_H2 -inducing cytokines thymic stromal lymphopoietin (TSLP), IL-25, and IL-33. Gene expression of IL-25 and IL-33 (but not TSLP) was significantly upregulated at 4 dpi in the absence of TLR7 (see Fig E2, E) at a time when viral load did not differ (Fig 1, B). In BAL fluid the concentration of IL-25 (Fig 2, E) but not TSLP (data not shown) was markedly greater at 10 dpi in the $TLR7^{-/-}$ mice compared with the $TLR7^{+/+}$ mice. Similarly, there was a trend toward increased IL-33 levels in the BAL fluid, although this trend was not statistically significant (Fig 2, E). It has been reported that pDCs are protective, whereas conventional dendritic cells (cDCs) are necessary for the development of T_H2 immunity.³⁹ Of note, cDC numbers did not fluctuate in $TLR7^{+/+}$ mice but increased 3-fold in the lungs of $TLR7^{-/-}$ mice (see Fig E2, F). Because we have previously demonstrated that PVM-associated infiltration of pDCs does not occur in $TLR7^{-/-}$ mice,³¹ infection in the absence of TLR7 dramatically skewed the dendritic cell pool from antiviral pDCs toward proinflammatory cDCs.

Re-exposure to *Pneumovirus* in later life induces an asthmatic phenotype

In both adults and children, the majority of acute asthmatic episodes are precipitated by a viral infection. Thus we reinfected $TLR7^{-/-}$ mice in later life (see Fig 3, A, for the study design) to determine whether this would recall the T_H2 response primed in infancy, initiate T_H2 -type airway inflammation, and induce airway hyperreactivity. Of note, the absence of TLR7 did not affect the concentration of PVM-specific IgG in the serum, either after reinfection with PVM (Fig 3, B) or before reinfection at 42 dpi (data not shown). In contrast to our findings in the primary infection (Fig 1, B), we were unable to detect viral replication (Fig 3, C) or type I interferon gene expression (data not shown) in either $TLR7^{+/+}$ or $TLR7^{-/-}$ mice at 6 days after secondary infection.

Despite the ability of both strains to effectively clear the virus, the immunologic profile was markedly different. Gene expression of IL-5 and IL-13 in the lung was a log higher in $TLR7^{-/-}$ mice compared with that seen in $TLR7^{+/+}$ mice (Fig 3, D). IL-4, IFN- γ , and eotaxin-1 also showed increased expression in the absence of TLR7. Muc5AC transcripts (Fig 3, D) and numbers of mucus-producing cells (see Fig E3, A, in this article's Online Repository at www.jacionline.org) were similarly increased in both $TLR7^{+/+}$ and $TLR7^{-/-}$ mice. In the DLNs IL-5, but not IFN- γ or IL-17A, production was increased (Fig 3, E), as was the number of tissue eosinophils (Fig 3, F). Importantly, airways hyperreactivity

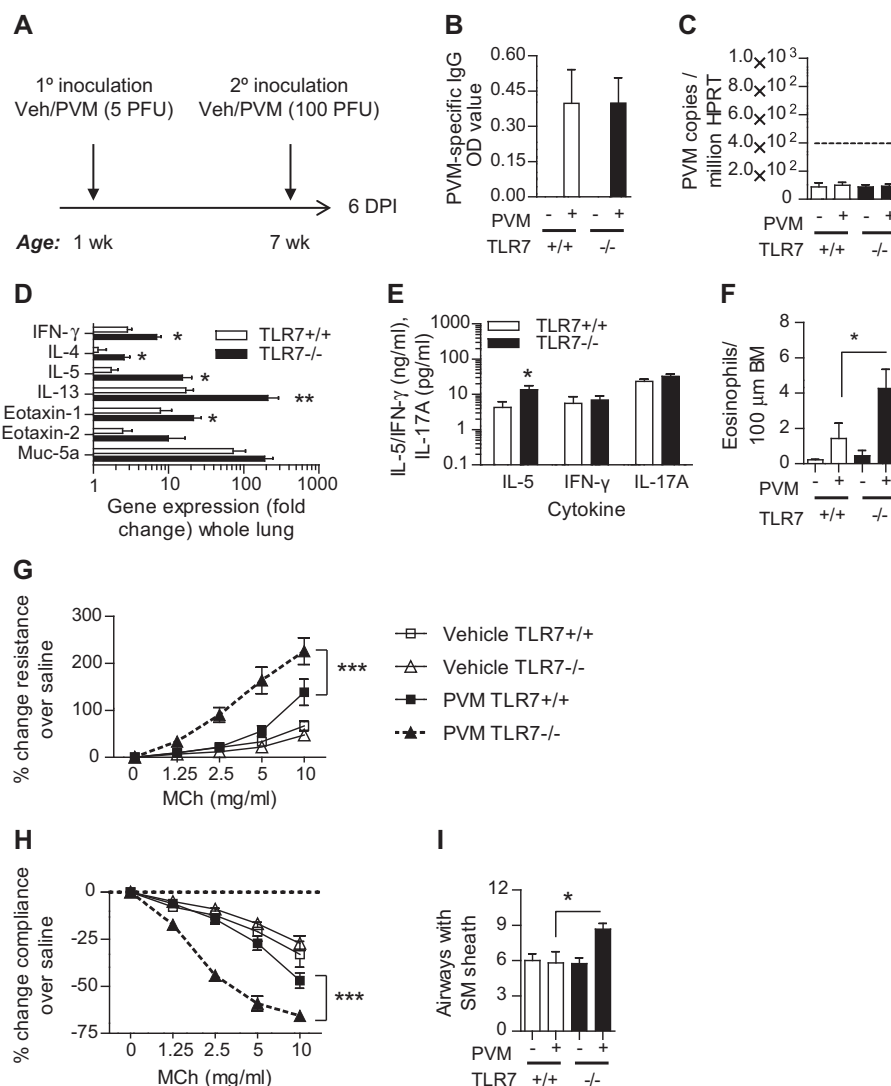


FIG 3. Reinfection with virus in later life leads to T_H2 responses and airways hyperreactivity in the absence of TLR7. **A**, Study design. **B**, Serum PVM-specific IgG at 6 days after secondary infection. **C**, Viral load in the lungs. The dotted line indicates limit of detection. **D**, Gene expression in the lung. **E**, Cytokine release from DLN cells. **F**, Tissue eosinophils. **BM**, Basement membrane. **G** and **H**, Airways resistance and airways compliance. **I**, Number of airways with a contiguous surrounding layer of smooth muscle (SM) sheath. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with PVM-infected TLR7^{+/+} mice. Data are presented as means $\pm$ SEMs for one experiment representative of 2 experiments, each with 4 to 7 mice per group.

(airways resistance and dynamic compliance) was significantly increased in response to increasing doses of aerosolized methacholine in TLR7^{-/-} mice but not TLR7^{+/+} mice (Fig 3, G and H). Remarkably, the number of medium- to large-sized airways surrounded by a contiguous layer of smooth muscle had increased by almost 50% in TLR7^{-/-} mice reinfected with PVM compared with that seen in vehicle-inoculated controls (Fig 3, I, and see Fig E3, B). This phenotype was not apparent in TLR7^{+/+} mice. The T_H2 phenotype was not restricted to mice on a BALB/c (often associated with T_H2 -skewed immunity) background because we observed increased eotaxin-2 protein in the lung, eosinophilia in BAL fluid, and IL-13 protein in BAL fluid and DLN cultures after PVM reinfection in TLR7^{-/-} mice on a C57BL/6 background (associated with T_H1 -skewed immunity, see Fig E4 in this article's Online Repository at www.jacionline.org).

TLR7 protects against the outgrowth of mast cells and IgE after repeated *Pneumovirus* infection

The frequency of wheezy LRT viral infections in early life alone and more so with concurrent allergic sensitization is a major risk factor for the development of childhood asthma.^{9,40,41} In the absence of TLR7, each sequential PVM infection led to an increase in the concentration of serum IgE (Fig 4, A). Concomitantly, there was a significant increase in the numbers of mast cells in the airway mucosa proximal to the smooth muscle layer on each subsequent infection in the absence but not the presence of TLR7 (Fig 4, B, and see Fig E5 in this article's Online Repository at www.jacionline.org).

Similar to the secondary infection, tertiary exposure to PVM elicited a T_H2 response and asthma-like pathology. The expression of IL-4 and IL-5 (but not IFN- γ) was once again significantly

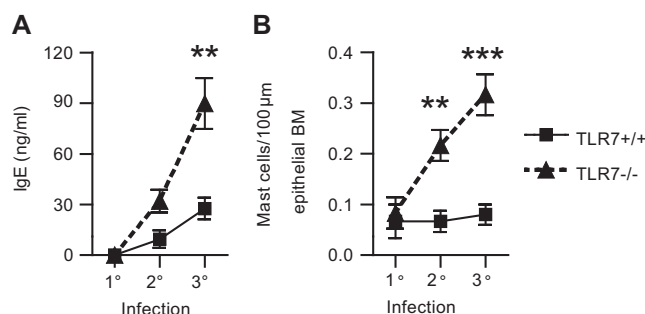


FIG 4. Mast cell numbers and IgE levels increase with each subsequent viral infection. Measurements were conducted 7, 6, and 6 days after the first, second, and third PVM infection, respectively. **A**, Concentration of serum total IgE. **B**, Toluidine blue-positive mast cells. BM, Basement membrane. $**P < .01$ and $***P < .001$ compared with PVM-infected $TLR7^{+/+}$ mice. Data are presented as means $\pm$ SEMs for one experiment representative of 2 experiments, each with 4 to 7 mice per group.

increased in the lungs of $TLR7^{-/-}$ mice over that seen in $TLR7^{+/+}$ mice (see Fig E6, A, in this article's Online Repository at www.jacionline.org). Lymph node cell IL-5/IFN- γ ratio, tissue eosinophilia, airway smooth muscle changes, and airways hyperreactivity were all significantly increased in $TLR7^{-/-}$ mice compared with that seen in $TLR7^{+/+}$ mice after tertiary infection (see Fig E6, B-F).

Virus-induced allergic airways disease is dependent on memory CD4 T cells

A CD4⁺ cell-depleting antibody was administered to $TLR7^{-/-}$ mice at days 21, 23, and 25 after infection to determine whether the virus-induced asthma-like pathology was mediated by type 2 ILCs or conventional CD4⁺ T_H2 cells primed during the primary viral infection. Importantly, this strategy depleted both naive and memory (virus-specific and nonspecific) CD4⁺ T cells from the lungs (8% to <0.5%) and lymph nodes (26% to <0.5%) after inflammation had resolved but allowed for the complete regeneration of the naive CD4⁺ T-cell pool before reinfection (see Fig E7 in this article's Online Repository at www.jacionline.org). Thus we were able to selectively examine whether the memory CD4⁺ T-cell compartment was required for the induction of a virus-induced exacerbation. Virus presence was not detectable by means of quantitative PCR at 6 dpi in either group (data not shown), suggesting that memory CD4⁺ T cells were unlikely to play a major role in mediating protective immunity against secondary infection. However, virus-induced expression of the T_H2-associated cytokines/chemokines, including IL-4, IL-5, IL-13, eotaxin-1, and eotaxin-2, was significantly diminished, whereas the expression of IFN- γ remained unchanged (Fig 5, A). Muc5a gene expression was decreased, although not significantly (Fig 5, A). The production of IL-5 and IFN- γ , but not IL-17A, by DLN cells was reduced after memory CD4⁺ T-cell depletion (Fig 5, B), as was the concentration of serum IgE (data not shown). Importantly, the depletion of memory

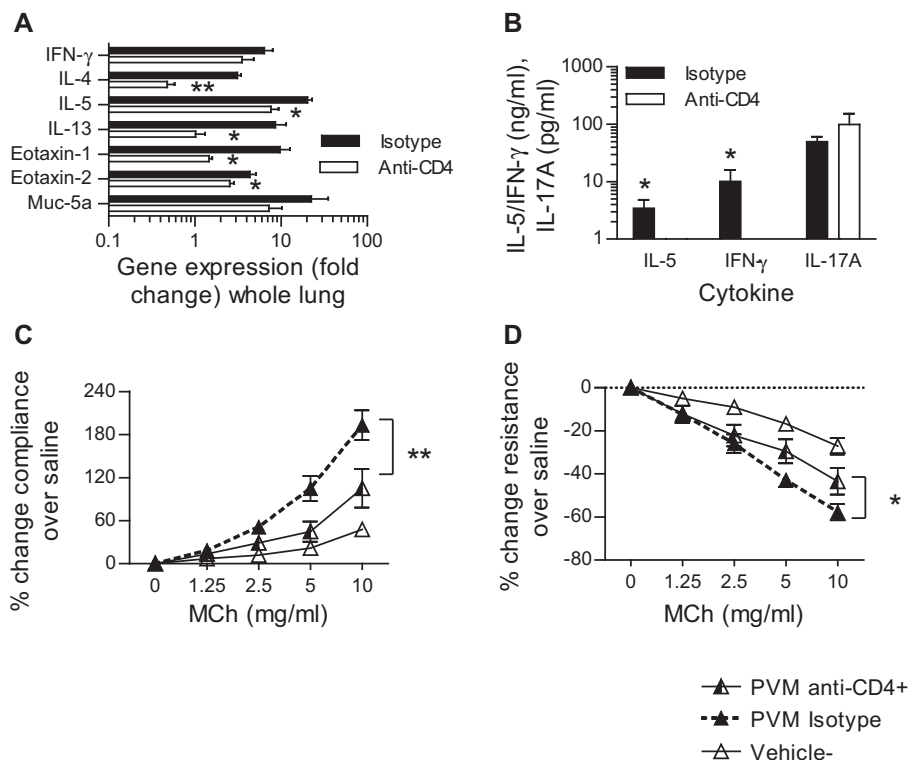


FIG 5. Virus-induced airway inflammation and airway hyperreactivity is dependent on memory CD4 T cells. **A**, Gene expression in the lung. **B**, Cytokine release by DLN cells. **C** and **D**, Airways resistance and airways compliance. MCh, Methacholine. Vehicle represents the PVM vehicle control/noninfected $TLR7^{-/-}$ mice. $*P < .05$ and $**P < .01$ compared with isotype-treated mice. Data are presented as means $\pm$ SEMs for 4 to 7 mice per group.

CD4⁺ T cells significantly ameliorated virus-induced alterations in airway resistance and compliance in *TLR7*^{-/-} mice (Fig 5, C and D). However, the absence of memory CD4⁺ T cells did not completely ablate airway hyperreactivity (to baseline vehicle control levels), suggesting a nonmemory CD4⁺ T cell–driven mechanism might also contribute.

***Pneumovirus* infection in the absence of TLR7 promotes allergic sensitization to inhaled cockroach antigen**

To examine whether early-life pneumoviral infection in the absence of TLR7 predisposed to allergic sensitization and T_H2 lung inflammation, mice were inoculated with PVM and then exposed to inhaled cockroach antigen per the study design (Fig 6, A). T_H2 (but not T_H1 or T_H17)–associated gene (IL-5, IL-13, and eotaxin-2) and protein (IL-5) expression was significantly increased in PVM/cockroach antigen–exposed *TLR7*^{-/-} mice compared with that seen in *TLR7*^{+/+} mice (Fig 6, B, and see Fig E8 in this article's Online Repository at www.jacionline.org). This T_H2 profile was associated with increased numbers of mucus-secreting cells, together with increased numbers of eosinophils and mast cells in the airway submucosa (Fig 6, C–E). These data suggest that the absence of TLR7 predisposes to pneumoviral infection–associated allergic sensitization in early life.

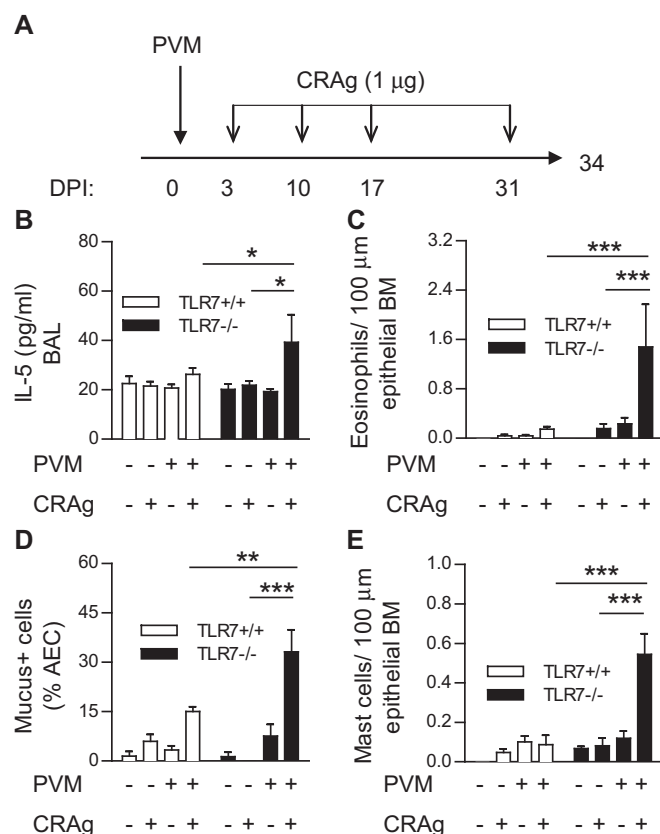


FIG 6. Pneumoviral infection in early life in the absence of TLR7 increases sensitization to cockroach antigen (CRAg) and promotes T_H2 lung inflammation. **A**, Study design. **B**, IL-5 in BAL fluid. **C**, Tissue eosinophils. **BM**, Basement membrane. **D**, Mucus-secreting cells. **E**, Toluidine blue–positive mast cells. **P* < .05, ***P* < .01, and ****P* < .001 compared with PVM + CRAg TLR7^{+/+} mice or CRAg TLR7^{-/-} mice, as indicated. Data are presented as means ± SEMs for 4 to 6 mice per group.

For all genes and proteins measured in this study, basal expression of vehicle control mice did not differ between strains.

DISCUSSION

Asthma is a chronic inflammatory airway disease caused by environmental insults to genetically predisposed subjects. Frequent febrile LRT viral infections in early life are a major risk factor for the later development of childhood asthma,^{6,9-12} whereas genetic studies have linked polymorphisms in the viral sensor TLR7 to asthma risk.¹⁹⁻²² Thus we postulated that this gene-environment interaction might underpin the association between severe bronchiolitis, attenuated interferon responses, and inception of asthma. We show here that *Pneumovirus* infection in *TLR7*^{-/-} mice predisposed toward a bronchiolitis-like disease and promoted the outgrowth of T_H2 immunity both to virus alone or bystander allergen. Central to both phenotypes was the defect in antiviral cytokine production and its sequelae: a markedly greater viral load in the mucosal epithelium, which became sloughed and necrotic, and increased expression of the damage-associated cytokine IL-33 and IL-25. This was associated with the recruitment of type 2 ILCs, T_H2-type cytokine expression (IL-4, IL-5, IL-13, and amphiregulin), and AEC hyperplasia, which is consistent with a role for T_H2 immunity in promoting tissue repair and homeostasis after damage.^{36,42} Virus-induced T_H2 cells were reactivated on subsequent infectious exposures in later life to induce an asthma-like pathology and airways hyperreactivity. Thus our data demonstrate how a clinically relevant gene-environment interaction might underlie the association between severe/frequent viral LRT infections and the inception of asthma and provide a mechanistic understanding to explain why later viral infections might cause exacerbations of asthma.

We have previously shown that PVM is first recognized by TLR7 on pDCs.³¹ Here we found that the absence of TLR7 increased viral load in the airway epithelium, which became necrotic and sloughed. This damaged phenotype was associated with increased production of the T_H2-instructive cytokines IL-25 and IL-33. Of note, IL-33 is an alarmin that is released by necrotic cells, while we have previously shown that RSV infection promotes the expression of epithelial IL-25 in the absence of innate IFN-γ production.⁴³ IL-1β production was also induced in the damaged epithelium, possibly to promote a wound-healing response.⁴⁴ Thus the failure to mount a TLR7-mediated antiviral response to PVM infection led to an IFN-α/β^{low}, IL-12p70^{low}, IL-1β^{high}, IL-25^{high}, IL-33^{high} cytokine milieu that was associated with a T_H2-type response. In addition to conventional CD4⁺ T_H2 cells, it is now appreciated that innate cells, such as macrophages, natural killer T cells, type 2 ILCs, and others, are important sources of IL-13.^{37,45,46} Here we report for the first time that type 2 ILCs are recruited to the lung in response to *Pneumovirus* infection and that this response is greater in the absence of TLR7. We speculate that in the absence of a protective TLR7-mediated interferon response, the wounded epithelium might produce IL-25/IL-33 to induce the recruitment and activation of type 2 ILCs, which can promote epithelial homeostasis and tissue repair through the production of amphiregulin and profibrotic T_H2 cytokines.³⁶

In the absence of TLR7, a secondary and tertiary infection was associated with many of the hallmark pathophysiologic features of asthma, including increased T_H2 cytokine levels, increased

numbers of tissue eosinophils and mast cells, a mild degree of IgE production, and airway hyperreactivity. Importantly, this phenotype appeared to be independent of the genetic background strain of the mice. Uniquely, we found that a pneumoviral infection in the absence of TLR7 increased the numbers of airways with a contiguous sheath of smooth muscle, a classical tissue tropism of asthma that the majority of experimental models of asthma fail to elicit. Intriguingly, these changes might have been initiated in response to the primary infection in early life, when the lung is developing most rapidly. Tertiary PVM infection of *TLR7*^{-/-} mice again triggered an acute episode of airway disease characterized by airway hyperreactivity, T_H2 inflammation, tissue eosinophilia, and further increased mast cell numbers. In addition, the serum IgE concentration showed a mild increase with each subsequent viral exposure. Moreover, the absence of TLR7 dramatically increased PVM-associated sensitization to cockroach antigen and T_H2 lung inflammation, mirroring the greater incidence of asthma in those children who are both sensitized and experience frequent wheezy LRT viral infections in early life.^{9,40,41} Importantly, in our model the depletion of memory CD4⁺ T cells before secondary infection ameliorated the virus-induced exacerbation, which is consistent with the view that virus-specific CD4⁺ T_H2 cells are primed in early life and are asthmagenic.⁴⁷ Thus damage to the mucosal airway during primary infection might establish a cytokine environment that favors the priming of pathogenic CD4⁺ T_H2 cells. Notably, because PVM replicates in mice, we were able to use a low dose of virus and demonstrate that the absence of TLR7 heightens this process by (1) delaying the induction of antiviral cytokines (which can counterregulate T_H2 responses⁴⁸⁻⁵⁰) and (2) increasing host susceptibility to tissue damage with the resultant production of T_H2-instructive cytokines. Hence the model might provide a unifying hypothesis to explain the association between severe viral respiratory tract infections in early life, attenuated interferon responses in asthmatic patients, heightened damage/repair processes at the mucosal epithelium, airway remodeling, and the outgrowth of T_H2 immunity, and as such will provide an opportunity to further unravel the cellular and molecular processes that induce the onset of asthma.

Others have shown, using sterile models of experimental asthma, that the induction or direct administration of type I interferons can ameliorate disease through the suppression of CD4⁺ T_H2 responses. Moreover, attempts to correct the impaired innate immune responses in asthmatic patients and patients with allergic rhinitis are already underway and show some promise,^{51,52} although these strategies do not target the cause of disease, which will require a greater understanding of the processes that give rise to virus-specific T_H2 responses.

In conclusion, our data demonstrate a clinically relevant gene-environment interaction that increases host susceptibility to severe virus-associated bronchiolitis and favors the development of T_H2 immunity. These long-lived virus-induced T_H2 cells might be subsequently reactivated in response to repeat (seasonal) viral exposures to trigger virus-induced exacerbations of asthma or predispose to inhaled allergen sensitization early in life. The model presented herein reflects many of the epidemiologic observations that have been linked to asthma risk while presenting all of the clinical hallmark pathologic features of asthma. We anticipate that this model will advance the understanding of the complex processes that predispose toward the inception of asthma in early life.

We thank Elisha Wybacz for providing technical assistance to test lung function.

Key messages

- *TLR7* gene deficiency and early-life *Pneumovirus* infection leads to a bronchiolitis-like pathology and severe infection in neonatal mice.
- *TLR7* gene deficiency (genetic factor) and early-life *Pneumovirus* infection (environmental factor) interaction promotes an aberrant CD4 T-cell memory response that contributes to virus-induced exacerbations of asthma on secondary infection in later life and also enhanced sensitization to an inhaled allergen.

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METHODS

Real-time quantitative PCR

Lungs were excised and frozen in RNAlater solution (Ambion). Total RNA was isolated with TriReagent solution (Ambion), and DNase digestion was performed with Turbo DNase (Ambion), both of which were conducted according to the manufacturer's instructions. Standard RT-PCR reaction conditions were used, and primer sequences are presented in Table E1. PVM titers were quantified by using plasmid standards containing either the SH domain of the PVM genome or the housekeeping gene *hprt* at a known copy number.

Lymph node assays

Single-cell suspensions of lymph node cells were cultured at 5×10^5 cells per well per 200 μ L of growth medium (RPMI 1640, 5×10^{-5} mol/L 2-mercaptoethanol, 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 20 mmol/L HEPES, 100 μ g/mL penicillin, 100 μ g/mL streptomycin, and 0.1 mmol/L sodium pyruvate). Supernatants were collected and frozen at -80°C for ELISA.

Flow cytometry

Isolated single-cell suspensions of lung cells were washed in PBS/2% FCS and incubated with anti-mouse CD16/32 Fc receptor block at 20 μ g/mL per 10^6 cells for 15 minutes on ice. Cells were incubated with antibody on ice for 20 minutes in the dark. Antibodies included anti-mouse CD4-allophycocyanin (APC; RM4-5), Gr-1-APC (RB6-8C5), CD11c-fluorescein isothiocyanate (HL3), CD11b-peridinin-chlorophyll-protein complex (M1/70), MHC class II-phycoerythrin (PE; M5/114.15.2), CD117 (c-kit)-peridinin-chlorophyll-protein complex (2B8), CD45R-AF488 (RA36B2), CD3-fluorescein isothiocyanate (145-2C11, all from BD Biosciences), Ly6A/E (SCA-1)-PE (D7), CD44-BV570 (1M7), CD45-BV421 (30-F11; BioLegend, San Diego, Calif), ST2/IL-1R4-APC (245707; R&D Systems, Minneapolis, Minn). Type 2 ILCs were identified as lineage-negative, CD45⁺, CD44⁺, ST2⁺, Sca-1⁺, c-kit⁺. All isotype control antibodies were obtained from BD Biosciences. Cells were washed 3 times, fixed in 1% formaldehyde (Polysciences, Warrington, Pa)/PBS, and analyzed on a FACSCanto or FACS LSR II (BD Biosciences).

ELISA

The concentrations of serum IgE, the cell-culture supernatant IL-5, IFN- γ (BD Biosciences), and IL-17A (eBioscience, San Diego, Calif) and lung homogenate or BAL fluid IL-1 β , eotaxin-2/CCL24, TSLP, IL-25, IL-33, amphiregulin or IFN- α (R&D Systems) were quantified by means of ELISA. PVM-specific IgG titers were detected in mouse sera by using a SMART-spot ELISA kit, according to the manufacturer's instructions (Biotech Trading Partners, Encinitas, Calif).

Caspase-1 activity

Caspase-1 activity was measured in lung homogenates by using a fluorescent substrate cleavage assay, according to the manufacturer's instructions (Promega, Madison, Wis).

Assessment of airway function

Airways hyperreactivity was measured, as previously described.^{E1} Briefly, airway responsiveness was determined by means of direct measurements of airway resistance and compliance in anesthetized (0.2 mg/10 g xylazine and 0.4 mg/10 g ketamine) and tracheostomized mice in response to nebulized methacholine (Sigma, St Louis, Mo) at concentrations of 1.25 to 20 mg/mL

for 1 minute. The percentage increase over baseline (saline) in response to increasing doses of methacholine was calculated.

Histology

AEC sloughing was calculated by measuring the length of denuded airway epithelial basement membrane/total perimeter of the airway and expressed as a percentage. AEC hyperplasia was calculated by measuring the total area of the airway epithelium/total perimeter of the airway. Both analyses were conducted with Aperio Image Scope software (Aperio, Vista, Calif). Lung tissue sections were stained with Chromotrope 2R, Toluidine blue, or periodic acid-Schiff to enumerate eosinophils, mast cells, and mucus-secreting cells, respectively. Toluidine blue-positive mast cells and Chromotrope 2R-positive eosinophils were enumerated around the airways and expressed as cells per 100 μ m of epithelial basement membrane. Periodic acid-Schiff-positive mucus-secreting cells were counted and expressed as a percentage of all AECs. All medium and large airways within the lung section of each mouse were scored. Images were captured at $\times 40$ or $\times 100$ magnification with an Olympus microscope (model BX51), a digital camera (Olympus DP70), and DP software (Olympus, Center Valley, Pa).

Immunohistochemistry

Paraffin-embedded sections were prepared, as previously described.^{E2} For immunofluorescent staining of smooth muscle actin, sections were blocked with 25% normal goat serum for 30 minutes and washed in PBS/Tween 20. Sections were then incubated with PE-conjugated anti-smooth muscle actin (Dako, Carpinteria, Calif) for 1 hour at 37°C , washed 3 times, and mounted with fluorescent mounting medium. For PVM and IL-1 β detection, endogenous peroxidase was blocked before the addition of 10% normal goat serum. Primary antibodies against PVM (kindly provided by Dr Ulla Buchholz, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases) and anti-mouse IL-1 β (Santa Cruz Biotechnology, Santa Cruz, Calif) were incubated at 1:8000 and 1:250 dilution overnight at 4°C . Sections were washed 3 times in PBS/Tween 20 before 30 minutes of incubation with either 100 μ L of biotinylated anti-mouse polyclonal antibody (Invitrogen, Carlsbad, Calif) for PVM detection or goat anti-rabbit IgG-alkaline phosphatase (Sigma-Aldrich) for IL-1 β detection. Sections stained for PVM detection were washed and incubated with streptavidin-peroxidase conjugate for 10 minutes before washing, and color was developed with diaminobenzidine or Fast Red substrate (Sigma), as appropriate. Sections probed with anti-IL-1 β underwent color development with Fast Red substrate (Sigma). Sections were counterstained with hematoxylin before mounting with Glycer-gel (Dako). The number of PVM- or IL-1 β -positive AECs was quantified and described as a percentage of AECs around a minimum of 5 airways.

Statistical analyses

Statistical analyses were performed with GraphPad Prism version 5.0 software (GraphPad Software, La Jolla, Calif), with the appropriate parametric or nonparametric test applied based on normal or nonnormal distribution. The Gehan-Breslow-Wilcoxon test was used for survival. A *P* value of less than .05 was considered statistically significant.

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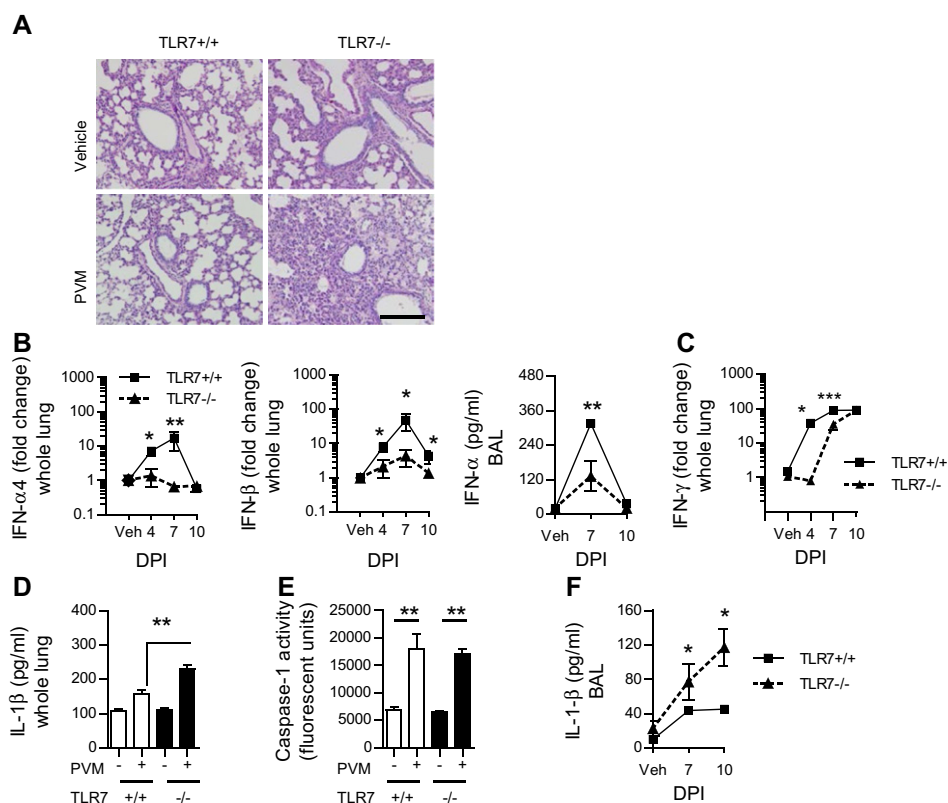


FIG E1. The absence of TLR7 attenuates *Pneumovirus*-induced interferon responses and increases inflammasome activation. **A**, Hematoxylin-stained lung sections demonstrate parenchymal consolidation. **B**, IFN- α and IFN- β gene and IFN- α protein expression in BAL fluid. **C**, IFN- γ lung gene expression. **D**, IL-1 β protein in lung homogenate. **E**, Caspase-1 activity in lung homogenate. **F**, IL-1 β in the BAL fluid. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with PVM-infected TLR7^{+/+} mice or ** $P < .01$ compared with vehicle control mice (caspase-1 activity). Data are presented as means $\pm$ SEMs for one experiment representative of 2 experiments, each with 4 to 7 mice per group.

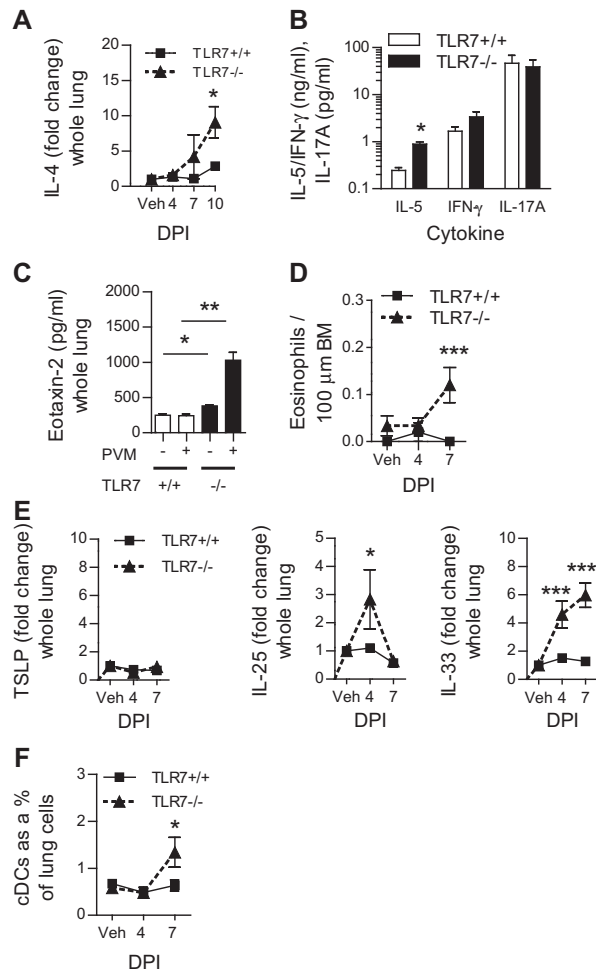


FIG E2. Pneumoviral infection in the absence of TLR7 promotes T_H2 responses. **A**, IL-4 lung gene expression. **B**, Cytokine release from DLNs. **C**, Eotaxin-2 protein in lung homogenates at 7 dpi. **D**, Tissue eosinophils. **BM**, Basement membrane. **E**, TSLP, IL-25, and IL-33 gene expression in the lung. **F**, CD11c⁺CD11b⁺ MHC class II-positive conventional DCs (cDCs) in the lungs. * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with PVM-infected TLR7^{+/+} mice or vehicle control TLR7^{+/+} mice. Data are presented as means $\pm$ SEMs of one experiment representative of 2 experiments, each with 5 to 7 mice per group.

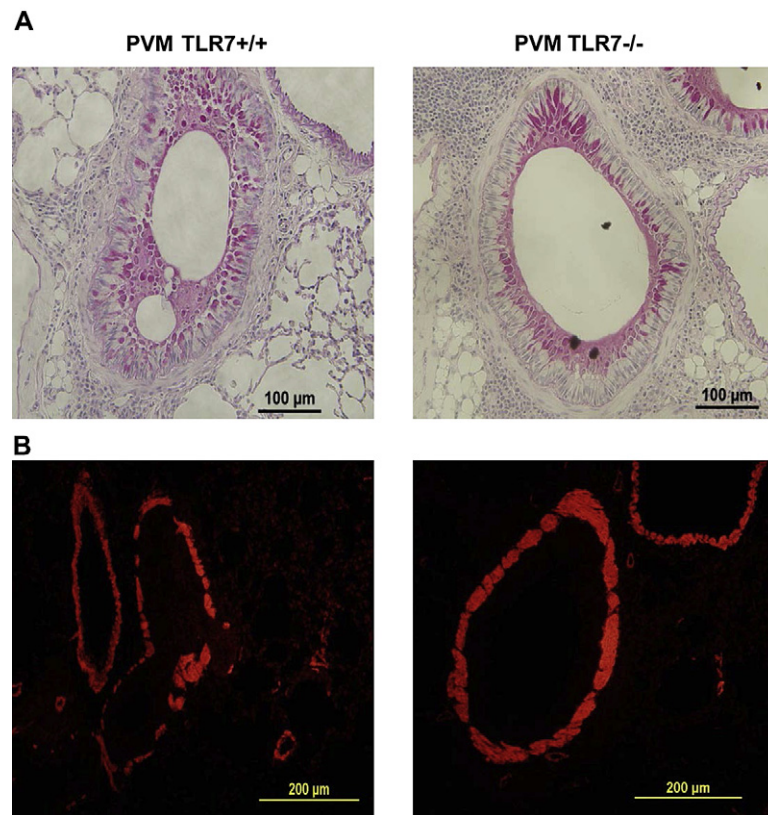


FIG E3. Pneumoviral infection induces profuse airway mucus production independent of TLR7. **A,** Lung histologic sections were stained for hematoxylin and periodic acid-Schiff and assessed for mucus-secreting cells. **B,** Lung histologic sections were stained for α -smooth muscle actin and assessed for smooth muscle banding.

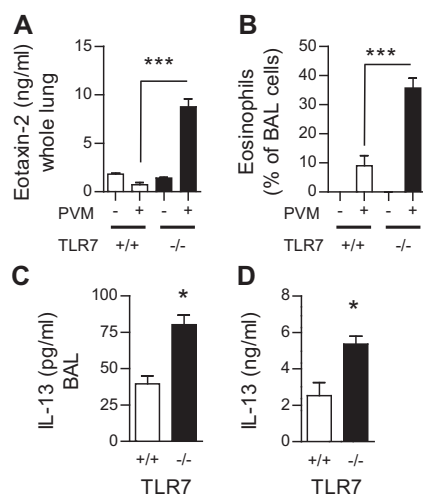


FIG E4. Reinfection of TLR7^{-/-} mice on a C57BL/6 background is associated with T_H2 lung inflammation. **A**, Eotaxin-2 protein in lung homogenates. **B**, Airway eosinophils in BAL fluid. **C**, IL-13 protein in BAL fluid. **D**, IL-13 release by DLN cells. **P* < .05 and ****P* < .001 compared with PVM-infected TLR7^{+/+} mice. Data are presented as means ± SEMs, with 4 to 7 mice per group.

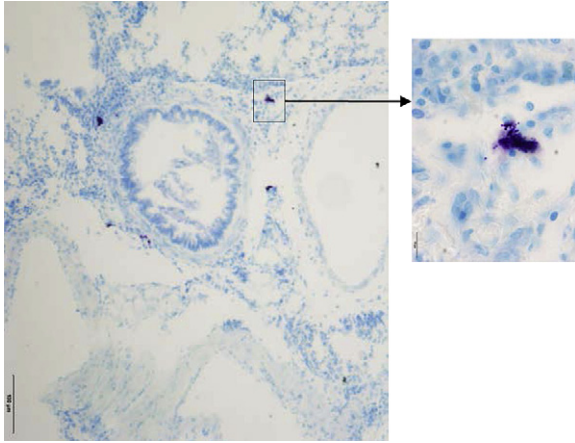


FIG E5. Mast cells accumulate in the airway submucosa of TLR7^{-/-} mice after successive pneumoviral infections. Micrographs at $\times 400$ and $\times 1000$ magnification show mast cells (*deep blue*) around the airways of TLR7^{-/-} mice.

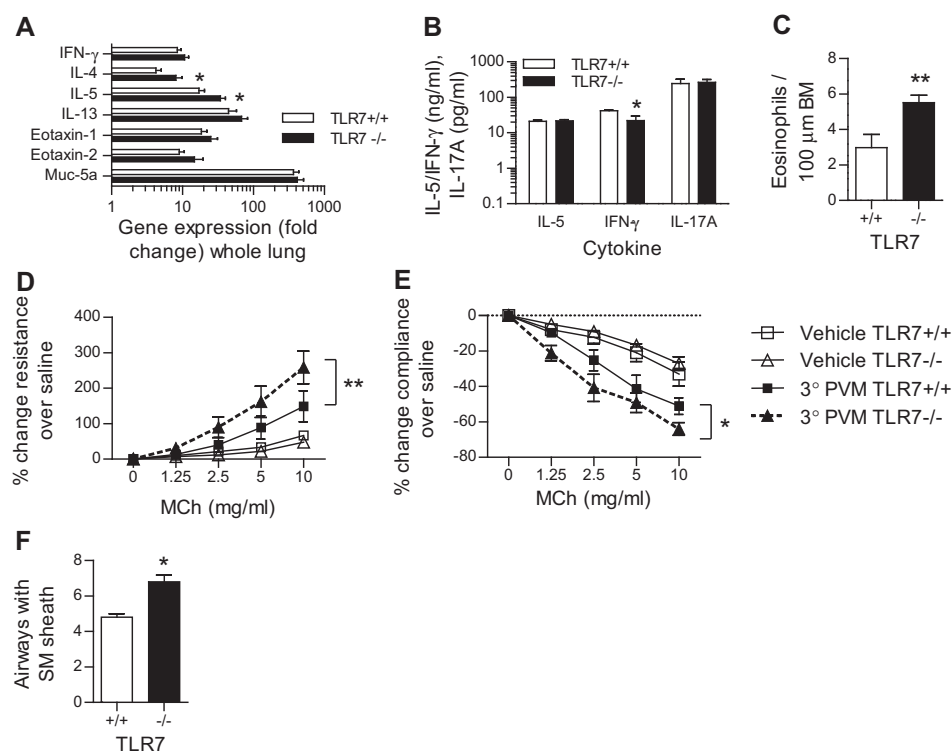


FIG E6. Tertiary infection in the absence of TLR7 leads to exacerbation of T_H2 responses and airways hyper-reactivity. **A**, Gene expression in the lung. **B**, Cytokine release from DLN cells. **C**, Tissue eosinophils. **BM**, Basement membrane. **D** and **E**, Airways resistance and airways compliance. **F**, The number of airways with a contiguous surrounding layer of smooth muscle (*SM*) sheath. * $P < .05$ and ** $P < .01$ compared with PVM-infected TLR7^{+/+} mice. Data are presented as means $\pm$ SEMs for one experiment representative of 2 experiments, each with 4 to 7 mice per group.

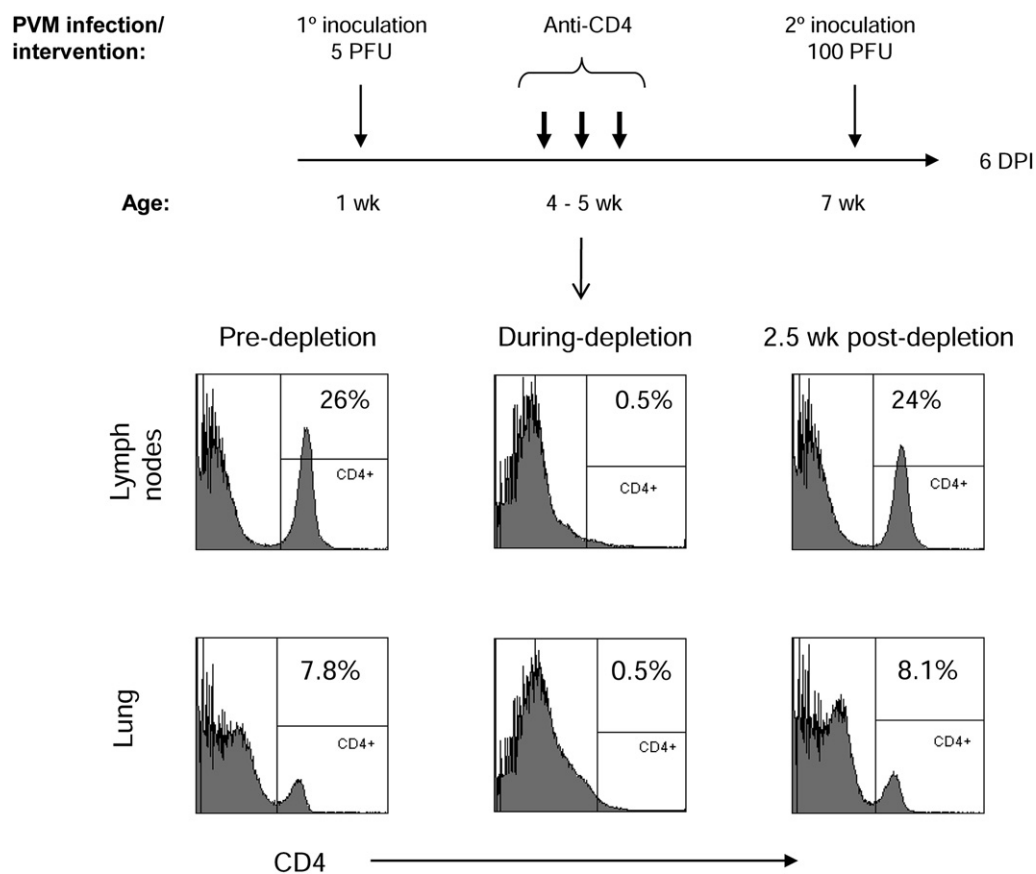


FIG E7. Study design and confirmation of depletion of CD4⁺ T cells. Neonatal (7-day-old) mice were inoculated with PVM at day 0 and then treated with an anti-CD4 depleting antibody (GK1.5) at days 21, 23, and 25. At day 42, mice were reinoculated with PVM. Fluorescence-activated cell sorting histograms demonstrate the percentage of CD4 T cells (*numbers in top right-hand quadrant*) in the lungs and lymph nodes before depletion, during depletion, and at day 42 (2.5 weeks after depletion).

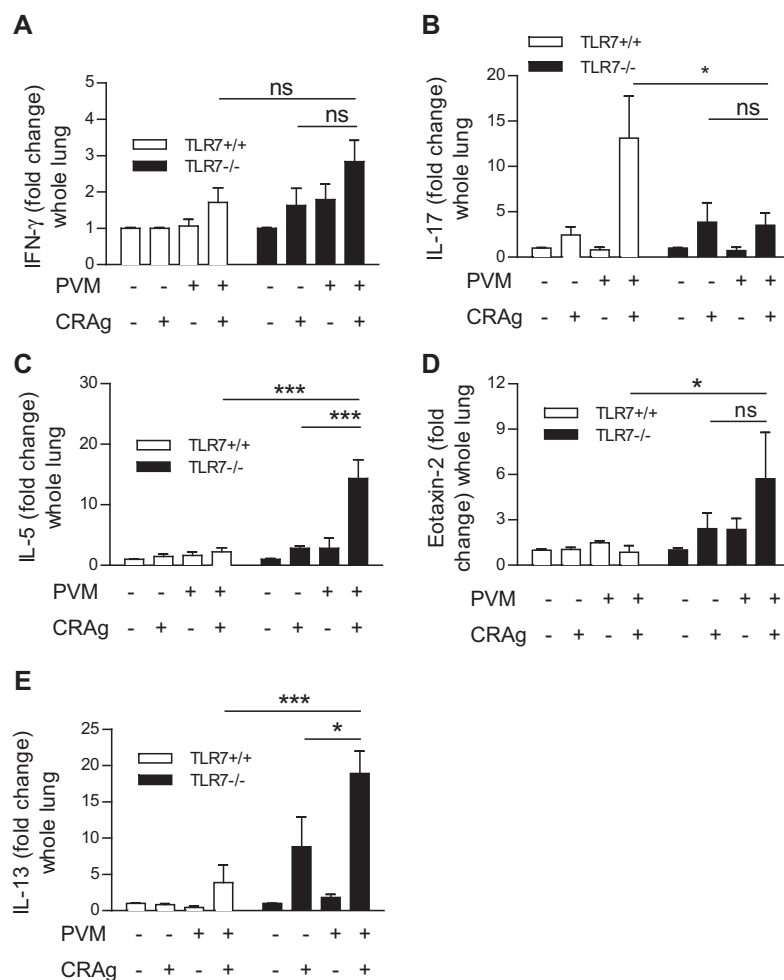


FIG E8. Pneumoviral infection in early life in the absence of TLR7 increases T_H2 gene expression in response to inhaled cockroach antigen (CRAg). Gene expression in the lung was analyzed for IFN- γ (A), IL-17A (B), IL-5 (C), eotaxin-2 (D), and IL-13 (E). * $P < .05$ and *** $P < .001$ compared with PVM + CRAg TLR7^{+/+} mice or CRAg TLR7^{-/-} mice, as indicated. Data are presented as means $\pm$ SEMs for 4 to 6 mice per group.

TABLE E1. Primer/probe sequences

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Murine <i>IL4</i>	ttgagagagatcatcggcattttg	tcaagcatggagtttcccatgt
Murine <i>IL5</i>	tgttgacaagcaatgagacgatga	aatagcatttcacagtaccccca
Murine <i>IL13</i>	agctgagcaacatcacacaagacc	tgggctacttcgattttggtatcg
Murine <i>IL25</i>	ctcaacagcagggccatctctc	ctgtctgtaggctgacgcagtgtg
Murine <i>IL33</i>	ctggcctcaccataagaaggaga	agggaggcaggagactgtgtfaaa
Murine <i>CCL11</i>	cccaacacactactgaagagctacaa	tttgcccaacctggtcttg
Murine <i>CCL24</i>	tagcctgcgcgtgttgcatcttcc	taaacctcgggtgctattgccacgg
Murine <i>MUC5AC</i>	gcagttgtgtcaccatcatctgtg	ggggcagtccttgactaacctctt
Murine <i>IFNG</i>	tcttgaaagacaatcaggccatcc	gaatcagcagcgactcctttcc
Murine <i>IFNA4</i>	accaacagatccagaaggctcaag	agtccttcctgggtcagaggagggt
Murine <i>IFNB1</i>	agagttacactgcctttgccatcc	ccacgtcaatcttctcttgcctt
Murine <i>TSLP</i>	cagatctttcggccagacaacttt	tctggaaactcctctcggatcttg
Murine <i>Areg</i>	ggcgaatgcagatacatcgagaa	ccacaccgttcaccaaagtaatctt
Murine <i>HPRT</i>	aggccagactttgttgatttgaa	caacttgcgctcatcttaggcttt
PVM SH domain	gcctgcatcaacacagtgtgt	gcctgatgtggcagtgcctt

HPRT, Hypoxanthine-guanine phosphoribosyltransferase.