INTRODUCTION
Influenza virus infection causes an acute respiratory tract disease with a global annual mortality of 250 000–500 000.1,2 In addition to seasonal strains, humans face the persistent threat of novel strains that can emerge and spread quickly in a naive population, potentially resulting in a pandemic.3,4 Seasonal influenza often affects individuals with weakened immune systems including very old, very young and immunocompromised populations.5–7 Conversely, certain highly pathogenic influenza viruses such as the 1918 H1N1 strain confer greater mortality in otherwise healthy individuals through a combination of enhanced inflammation leading to excessive tissue damage and secondary bacterial infection.8,9 To develop effective management strategies to combat both seasonal and pandemic influenza infection, a detailed understanding of the cellular and molecular mechanisms that regulate the quality and magnitude of the host immune response is essential.

Following influenza infection, a rapid yet nonspecific innate immune response is activated in an attempt to control initial viral replication and is followed by the mobilization of professional antigen-presenting cells including dendritic cells (DCs) and macrophages. Activated antigen-presenting cells express class I or class II major histocompatibility complex (MHC) molecules as well as co-stimulatory markers such as the cluster of differentiation (CD) molecules CD40, CD80 and CD86.10 Expression of these markers, particularly by DCs, is required for the priming and activation of naive T and B cells in the lung-associated lymph nodes (LALNs), leading to the formation of an adaptive immune response.11–15 Activated T and B cells are recruited to the site of primary infection to mediate antigen-specific immunity through cell-mediated control of viral replication and the production of specific antibodies that provide lasting protection against the infecting strain, respectively.10

Initial recognition of the influenza virus and subsequent activation of innate immunity occurs through host pattern recognition receptors. Two of the best-studied pattern recognition receptors for influenza recognition are the endosomal Toll-like receptors (TLR)3, TLR7 and TLR9, which are implicated in the clearance that coincided with increased tissue pathology during infection. Taken together, these findings establish a role for Unc93b1 and endosomal TLRs in the activation of both myeloid and lymphoid cells during the innate immune response to influenza.
Despite conflicting reports as to whether a true double-stranded RNA replication intermediate forms during influenza virus replication, TLR3 expression is upregulated in human alveolar and bronchial epithelial cells as well as mouse lungs following influenza infection, suggesting that it contributes to the host immune response.\textsuperscript{7,19,28,29} TLR3 has been specifically linked to the early production of proinflammatory mediators such as IL-6, IL-8 and CCL5 in human bronchial cells during influenza infection.\textsuperscript{17,19} Compared with wild-type mice, TLR3-deficient mice have diminished production of IL-6 and IL-12 p40/p70, elevated expression of IL-9, IL-10 and IFN-γ, and fewer macrophages and CD8\(^+\) T cells in the airways.\textsuperscript{28} Somewhat unexpectedly, these mice had an improved rate of survival despite a higher lung viral load, suggesting that the TLR3-mediated host response during influenza infection is deleterious.\textsuperscript{28} On the other hand, subsequent studies of TRIF-deficient mice that also lack TLR3 signaling did not reveal a significant role for this adaptor molecule following influenza infection.\textsuperscript{25,26} Finally, analysis of a single patient with influenza-associated encephalopathy revealed a loss-of-function missense mutation in TLR3, suggesting that this pattern recognition receptor may have a crucial role in protection rather than disease pathogenesis.\textsuperscript{30} Although the discordant experimental findings on the role of these TLRs and adaptor molecules in influenza infection may be attributable to differences in the strain, dose and volume of virus administered to mice with distinct genetic backgrounds, complementary human studies have failed thus far to identify the genes that are required for protection of the host.\textsuperscript{31} Thus, on the basis of current knowledge, it is not possible to draw firm conclusions about the contributions of the TLR7-MyD88 and TLR3–TRIF signaling pathways to innate immunity against influenza infection.

Studies of inbred mouse strains as well as gene-deficient mice have been essential for elucidating key features of the host response to viral infection that are relevant to human immunity.\textsuperscript{22,23} Owing to the limited number of available inbred and knockout strains, alternative approaches, such as the creation of random heritable mutations using the chemical N-ethyl-N-nitrosourea (ENU), have evolved in recent years.\textsuperscript{32} Rather than abrogating gene expression, ENU mutagenesis can create unique point mutations that alter protein function.\textsuperscript{34} Such mutations more closely mirror human genetic variation and can reveal specific roles for individual protein domains.\textsuperscript{34} This forward genetic approach has been successfully used to study molecules and pathways that are crucial to host defense against various pathogens.\textsuperscript{35} Here we report the creation of an ENU-induced point mutation in the mouse Unc93b1 gene that results in the complete deletion of exon 4, which encodes the third and fourth transmembrane domains of the full-length protein. We have named this mutation \textit{Letr} for ‘loss of endosomal TLR response’ as it confers a lack of responsiveness to stimulation with nucleic acid structures that represent pathogen-associated molecular patterns (PAMPs) known to activate the TLR3, TLR7 and TLR9 signaling pathways.

Based on the previously demonstrated function of UNC93B1 as a chaperone for endosomal TLRs,\textsuperscript{21–23} as well as its contribution to the immune response against various parasites (Toxoplasma gondii, Trypanosoma cruzi and Leishmania major),\textsuperscript{36–40} DNA viruses (mouse cytomegalovirus (MCMV) and Herpes simplex virus-1)\textsuperscript{41–43} and neuroadapted Sindbis virus (a positive-sense single-stranded RNA virus),\textsuperscript{44} we hypothesized that defective UNC93B1 function would impair the innate immune response to influenza A/PR/8/34 (H1N1), a negative-sense single-stranded RNA virus that targets the respiratory mucosa. Using our ENU-induced mutant mice that lack endosomal TLR signaling, we found that \textit{Unc93b1} contributes to the expression of type I IFN (IFN-α/β) and type II IFN (IFN-γ), recruitment of activated exudate macrophages (ExMs), and activation of T cells in the lung during the early phase of influenza infection. Importantly, despite a significant delay in viral clearance that coincided with increased tissue inflammation and epithelial reaction, the failure of \textit{Unc93b1}-mediated innate immune activation did not significantly alter mortality, suggesting that alternative pathways can compensate for the lack of endosomal TLR function in this model. Collectively, these results establish a distinct role for \textit{Unc93b1} in the early activation of the innate immune response to influenza and demonstrate the importance of redundant mechanisms that protect the host against lethal infection.

\section*{RESULTS}

ENU mutagenesis induced a mutation that confers defective \textit{in vitro} and \textit{in vivo} responses to TLR3, TLR7 and TLR9 ligands to identify novel genes or novel roles for known genes in TLR-specific immune signaling pathways, we injected male 12951 G0 mice with ENU and screened cells from G3 mutagenized mice for defective responses to polyinosinic-polycytidylic acid (polyIC), lipopolysaccharide (LPS), imiquimod and unmethylated CpG dinucleotides (CpG DNA). These structures were chosen as they are known to activate TLR3, TLR4, TLR7 and TLR9 signaling pathways, respectively.\textsuperscript{45–48} The cytokines, cell types and time points selected for analysis following \textit{in vitro} and \textit{in vivo} PAMP stimulation were based on data from the initial publications that identified these TLR ligands.\textsuperscript{45–48} Mutagenized G0 mice were bred to C57BL/6 female mice and the G1 progeny from independent G0 crosses were intercrossed to increase the number of recessive mutations available for screening at the G3 generation (Figure 1a).

Thioglycollate-elicited peritoneal macrophages or splenocytes from G3 progeny of G2 brother–sister matings were tested for \textit{in vitro} expression of IL-6 in response to PAMP stimulation. One pedigree (B28W6) showed a deviant phenotype in response to stimulation with polyIC, imiquimod and CpG DNA but had normal expression following LPS stimulation (Figure 1b). Heterozygous G2 fathers from this pedigree were outcrossed to C57BL/6 female mice and the resulting F1 female progeny were backcrossed to create N2 progeny (Figure 1a). N2 mice were intercrossed and deviant lines carrying a homozygous mutation were identified by survival splenectomy and repeat \textit{in vitro} phenotyping. Cells from heterozygous mice responded normally to CpG stimulation, confirming that the mutation has a recessive mode of inheritance (Figure 1b). To analyze the phenotype \textit{in vivo}, progeny of deviant and normal N2 mice were injected intraperitoneally with LPS, polyIC, imiquimod or CpG DNA and serum was collected 3 h after injection. Compared with the progeny of normal N2 mice, those from deviant N2 mice had significantly decreased expression of serum IL-12/IL-23 p40 in response to stimulation with polyIC, imiquimod or CpG DNA but retained similar responses to LPS (Figure 1c).

An ENU-induced single-nucleotide transversion in \textit{Unc93b1} results in the loss of exon 4 due to alternative splicing.

To map the gene responsible for the \textit{Letr} phenotype, we performed a low-density genome scan using 375 single-nucleotide polymorphisms among 54 mice derived from a \textit{Lett} × \textit{Lett} backcross. The \textit{Lett} mutation was localized to a 5.4 Mb region on chromosome 19 between markers rs31277487 and rs30935927. To test the heritability of the \textit{Lett} mutation, an additional 281 mice from the same backcross were screened for IL-6 expression following \textit{in vitro} stimulation of splenocytes with CpG DNA. Of these mice, 139 (49.5\%) showed a normal IL-6 response, while the other 142 (50.5\%) mice lacked IL-6 expression with perfect concordance between the mutant phenotype and genotype for a recessive mutation.

Based on a previous ENU mutagenesis study that created a mouse, termed \textit{3d}, with a similar hyporesponsive phenotype caused by a point mutation in \textit{Unc93b1} on chromosome 19,\textsuperscript{23}
we took a targeted approach to identify the Letr mutation. Nucleotide sequencing of Unc93b1 genomic DNA from normal and deviant (Letr) mice identified a single T to A transversion at the 5' splice donor site in the intron between exons 4 and 5 of Unc93b1 that rendered the site ineffective (Figure 2a, non-coding strand shown). Sequencing of complementary DNA (cDNA) from both Unc93b1+/+ and Unc93b1Letr/Letr mice determined that the mutation resulted in the loss of exon 4 in the 11-exon mRNA transcript (Figure 2c).

To confirm that the ENU-induced Letr mutation caused alternative splicing, spleen RNA from Unc93b1Letr/Letr and Unc93b1+/+ mice as well as C57BL/6, C3H/HeN and 129S1 inbred strains was reverse transcribed and a 918-base pair (bp) region of Unc93b1 cDNA that included exon 4 was amplified and visualized by gel electrophoresis. All three inbred strains presented a single band at the predicted size, while Unc93b1Letr/Letr cDNA presented a smaller band that is compatible with the 162-bp deletion of exon 4 (Figure 2b). Analysis of heterozygous Unc93b1+/Letr cDNA showed both wild-type and mutant Unc93b1 transcripts.

To evaluate the relative importance of exon 4 to the integrity of the UNC93B1 protein, the degree of amino-acid sequence conservation was compared among diverse species. Alignment of UNC93B1 amino-acid sequences encompassing exon 4 demonstrated a 54-amino-acid deletion in Unc93b1Letr/Letr mice (Figure 2d). These missing amino acids were identical among all species examined, with the exception of Xenopus tropicalis that was distinguished by the substitution of nine amino-acid residues. Protein modeling software was used to confirm that the wild-type UNC93B1 amino-acid sequence forms a 12-helix transport protein (Figure 2e).23 By mapping the amino acids encoded by exon 4 to the wild-type model, the splicing defect caused by the Letr mutation is predicted to eliminate the third and fourth transmembrane domains of the functional UNC93B1 protein and is distinct from the 3d mutation, which causes a single-amino-acid missense mutation (H412R) in the ninth transmembrane domain (Figure 2e).23

Allelic complementation does not rescue the hyporesponsive phenotype in Unc93b1Letr/Letr mice
To confirm that the Letr mutation was in the Unc93b1 gene, we performed an allelic complementation study by generating Unc93b1Letr/3d F1 hybrids from Unc93b1Letr/Letr and Unc93b13d/3d F1 hybrids.
mice. If the \textit{Letr} mutation were elsewhere in the genome, the lack of response to PAMP stimulation would be rescued in the compound heterozygotes. All genotypes were tested for IL-6 expression following \textit{in vitro} stimulation with PAMPs, as done during the initial screen. Both thioglycollate-elicited peritoneal macrophages and splenocytes stimulated with media alone produced undetectable levels of IL-6 (data not shown). Thioglycollate-elicited peritoneal macrophages from \textit{Unc93b1} \textit{Letr/Letr}, \textit{Unc93b1} \textit{3d/3d} and \textit{Unc93b1} \textit{Letr/3d} strains had a comparable IL-6 response to \textit{Unc93b1} \textit{þ/þ} mice following LPS stimulation (Figure 3a). Conversely, the \textit{Unc93b1} \textit{Letr/Letr}, \textit{Unc93b1} \textit{3d/3d} and \textit{Unc93b1} \textit{Letr/3d} strains had significantly decreased or undetectable IL-6 expression compared with \textit{Unc93b1} \textit{þ/þ} mice following stimulation with polyI:C, imiquimod or CpG DNA (Figures 3b–d).

Unc93b1 \textit{Letr/Letr} mice have fewer activated ExMs in the lungs following influenza infection

Unc93B1 functions as a chaperone for TLR3, TLR7 and TLR9 and has a role in host defense against various pathogens including the DNA viruses MCMV and Herpes simplex virus-1 and the RNA virus neuroadapted Sindbis virus.\textsuperscript{21–23,41–44} To determine if Unc93b1 also mediates innate immunity against RNA viral infection of the respiratory mucosa, we characterized the response of \textit{Unc93b1} \textit{Letr/Letr} mice to experimental challenge with influenza A/PR/8/34 (H1N1). TLR3, TLR7 and the MyD88 adaptor protein have been implicated in the variable response to influenza infection, albeit with variable outcomes.\textsuperscript{16–18,20,25,26,28} \textit{Unc93b1} \textit{Letr/Letr} mice provide a unique opportunity to determine the combined consequence of defective endosomal TLR3 and TLR7 activation in the presence of a functional MyD88 molecule that also participates in IL-1R- and IL-18R-mediated signaling.\textsuperscript{50}

As a role for Unc93b1 has been linked to antigen presentation during \textit{T. gondii} infection,\textsuperscript{38} we sought to determine whether the loss of \textit{Unc93b1} function would affect the activation of lung macrophages and DCs during influenza infection.\textsuperscript{51} Based on the literature, day 3 was selected as a representative time point for characterization of early immune differences following experimental influenza challenge.\textsuperscript{27,28,52} Initially, CD11c and MHCII were used to distinguish macrophages (CD11c\textsuperscript{þ}MHCII\textsuperscript{int}) from DCs (CD11c\textsuperscript{þ}MHCII\textsuperscript{hi}) and CD11b expression was then used to separate the former population into resident alveolar macrophages (AMs) (CD11b\textsuperscript{−/−}) and monocyte-derived ExMs (CD11b\textsuperscript{þ}) (Figure 4a). CD80 expression was used as a marker of cell activation (Figure 4a). Following influenza infection, the absolute number of DCs, macrophages and ExMs was not significantly different between \textit{Unc93b1} \textit{þ/þ} and \textit{Letr} (yellow) mice in the lungs of \textit{Unc93b1} \textit{Letr/Letr} compared with.
Unc93b1+/+ mice at this time point (Figure 4c). Simultaneous analysis of the LALNs showed there was no significant difference in the number of CD80+ DCs, while the number of CD80− ExMfs was too low for reliable detection (Supplementary Figure 1D), suggesting that the differential activation seen in the Unc93b1Letr/Letr mice was restricted to the site of infection.

Loss of Unc93b1 function reduces CD4+ and CD8+ T-cell activation in the lungs following influenza infection

As Unc93b1 also has a role in the activation of CD4+ and CD8+ T cells during T. gondii infection,38 we analyzed the number and activation status of these lymphoid cell subsets in our model. Activated CD4+ and CD8+ T cells produce an antigen-specific response that contributes to control of lung viral load. Specifically, CD8+ T cells function primarily through direct lysis of virally infected cells, while CD4+ T cells are classically known as helper cells that enhance CD8+ T-cell and B-cell activation and, more recently, have been shown to have a direct cytolytic function as well.53–55

Flow cytometry analysis at days 0, 3 and 7 post infection did not reveal a difference in the total number of CD4+ or CD8+ T cells observed in the LALNs or lungs between Unc93b1+/+ and Unc93b1Letr/Letr mice (Figures 5a and b and Supplementary Figure 2G). To determine whether the loss of Unc93b1 altered T-cell activation during influenza infection, the surface expression of an early activation marker (CD69) as well as markers of a naive (CD62L) and effector (CD44) T-cell phenotype were evaluated at days 3 and 7 post infection. Representative expression plots for T-cell activation markers are displayed as histograms (Figures 5c–e, left panel). The basal level of all CD4+ and CD8+ T-cell activation markers examined was similar between Unc93b1+/+ and Unc93b1Letr/Letr mice (Figures 5c–e, right panel). At day 3, post-infection challenge both CD4+ and CD8+ T cells in the lungs of Unc93b1Letr/Letr mice had significantly less expression of CD69 and significantly increased expression of CD62L compared with Unc93b1+/+ mice (Figures 5c and d). No significant differences in the expression of CD44 on CD4+ and CD8+ T cells derived from Unc93b1+/+ or Unc93b1Letr/Letr mice were observed at day 3 post infection (Figure 5e). Finally, the difference in lung CD4+ and CD8+ T-cell activation between Unc93b1+/+ and Unc93b1Letr/Letr strains was not observed at day 7 in the lungs (Supplementary Figures 2A–C) nor was there a difference in the activation of CD4+ and CD8+ T cells in the LALNs at days 3 or 7 post infection (Supplementary Figures 2D–F).

Unc93b1Letr/Letr mice have a selective decrease in type I/II IFN and CXCL10 expression during influenza infection

Following influenza infection, the lungs produce a variety of soluble mediators that are involved in the recruitment and activation of innate and adaptive immune cells as well as the destruction of virally infected host cells.10 To determine whether the diminished cellular activation in Unc93b1Letr/Letr mice was associated with an altered pattern of inflammatory mediator secretion in the airway or lungs, the expression of representative cytokines and chemokines that have been implicated in the host response to influenza was determined at serial time points. At day 3 post infection, the expression of type I IFN was significantly lower in the airways of Unc93b1Letr/Letr mice compared with the Unc93b1+/+ strain; however, at day 7 post infection a comparable increase was observed in both strains (Figure 6a). Significantly lower expression of CXCL10 (Figure 6b) and IFN-γ (Figure 6c) was also observed at day 3 post infection in the airways and lungs of Unc93b1Letr/Letr mutants compared with the Unc93b1+/+ strain. The expression of other proinflammatory mediators in the airway, including the cytokines IL-6 and tumor necrosis factor-α, and the chemokines CCL2, CCL3, CXCL1 and CXCL2 was not significantly different at days 3 or 7 post infection (Supplementary Figure 3). These findings demonstrate that soluble inflammatory mediator production at the site of infection was selectively altered by the loss of Unc93b1 function.

Unc93b1Letr/Letr mice have delayed viral clearance and increased tissue inflammation following influenza infection

To determine if the significant differences in immune cell activation and inflammatory mediator expression caused by the loss of Unc93b1 function had an impact on host outcome following influenza infection, a comparative analysis of lung viral load, inflammation and mortality was conducted between Unc93b1+/+ and Unc93b1Letr/Letr strains after intranasal challenge with 400 p.f.u. of the virulent influenza A/PR/8/34 (H1N1) strain. In both genotypes, the peak lung viral load was observed at day 3 post infection and decreased thereafter (Figure 7a).28,56 Notably, at...
day 7 post infection, the lung viral load in Unc93b1Letr/Letr mice was significantly higher compared with Unc93b1+/+ mice (Figure 7a).

In response to influenza challenge, both mouse strains showed a similar pattern of weight loss with a nadir at day 9 followed by recovery of initial weight in surviving animals by day 21 post infection (Figure 7c). Under these experimental conditions, Unc93b1+/+ mice had a median survival time of 14 days and an overall mortality of 60%, while Unc93b1Letr/Letr mice had a median survival time of 11.5 days and an overall mortality of 70% (Figure 7b). These data suggest that the loss of Unc93b1 may be associated with an earlier onset of death but does not significantly affect the overall survival rate following severe influenza infection. Finally, analysis of lung tissue sections stained with hematoxylin and eosin at day 7 post infection demonstrated that Unc93b1Letr/Letr mice had increased inflammation and signs of epithelial reactivity compared with Unc93b1+/+ mice (Figures 7f and g). The differential tissue pathology was coincident with a significantly higher viral load in the Unc93b1Letr/Letr lungs (Figure 7a), suggesting that inflammation in the Unc93b1Letr/Letr lungs may reflect a heightened response to increased viral replication. Analysis of lung tissue sections from uninfected mice did not show any difference between genotypes (Figures 7d and e). Taken together, these data indicate that impaired Unc93b1 function predisposes the host to a more severe initial disease course following influenza challenge; however, this difference does not significantly alter the ability of the Unc93b1Letr/Letr mice to survive the infection.

DISCUSSION

The intricate balance between host immunity and pathogen virulence determines the outcome of an infection. ENU mutagenesis is an advantageous strategy for interrogation of the immune response to infection because it efficiently creates random heritable point mutations throughout the genome that may not occur through natural variation in inbred strains.32 ENU can also cause distinct single-nucleotide alterations within a gene and create mutations that may closely resemble human conditions.32 Using this technique, we screened for a recessive defect in TLR signaling in vitro to target immune processes that have an important role in microbial infection.35 We discovered an alternative splicing mutation in Unc93b1, a gene that has been implicated in host immunity to diverse pathogens, and have identified a specific role for this gene in the innate immune response to influenza. Our findings demonstrate that Unc93b1 contributes to the early activation of ExMs and CD4+ and CD8+ T cells in the lungs as well as expression of type I IFN, type II IFN and CXCL10 in the airways. Mutation of Unc93b1 also delayed lung viral clearance and promoted tissue inflammation during infection, although it did not significantly alter survival. These findings establish the specific contribution of Unc93b1-dependent endosomal TLR activation during the initial host inflammatory response and highlight the role of complementary immune mechanisms that mediate survival and disease resolution following severe influenza infection.
In contrast to the ENU-induced splicing mutation of the Unc93b1 gene that results in the deletion of exon 4 in the spliced mRNA transcript. The Letr mutation encodes a predicted protein that lacks the third and fourth transmembrane domains of the full-length UNC93B1. The 54-amino-acid residues eliminated by the Letr mutation are highly conserved between Mus musculus, Homo sapiens and other diverse organisms, suggesting that the two missing transmembrane helices encode important protein domains. Functional analysis using in vitro and in vivo PAMP stimulation clearly demonstrates that the ENU-induced Letr mutation is recessive and confers a complete loss of endosomal TLR function with no detectable pattern of codominance. Residual IL-6 production following polyI:C stimulation in mice or cells carrying the Letr mutation is most likely attributable to recognition by the cytosolic melanoma differentiation-associated protein 5 pathway.57

Owing to the inherently random nature of ENU mutagenesis, one cannot control the genes, or sites within a gene, which are altered. A major advantage of this hypothesis-free approach is that screening for a particular phenotype can lead to the discovery of previously unknown genes as well as novel functions for known genes.32 An earlier large-scale ENU mutagenesis study also identified a loss-of-function mutation, termed 3d, in the Unc93b1 gene and clearly established its role in immune responsiveness.23 In contrast to the ENU-induced splicing mutation of Unc93b1 that we describe here, the 3d mutation causes a missense mutation in exon 9 that leads to a single-amino-acid substitution (H412R) in the ninth transmembrane domain of UNC93B1. Despite the different location and unique consequences of these two ENU-induced mutations, both produce a loss-of-function phenotype that confirms the essential contribution of the affected transmembrane-spanning domains to protein integrity. In vitro studies have shown that the 3d mutation inhibits UNC93B1 binding and trafficking of endosomal TLRs from the ER to the endosome following PAMP stimulation.21,22 A similar defect in stimulus-dependent trafficking to the plasma membrane was also observed with naturally occurring deletions of multiple transmembrane domains of the SLC7A7 transport protein.58 Although we have not formally demonstrated that TLR binding and translocation is inhibited by the Letr mutation, in light of the predicted deletion of two transmembrane domains of the UNC93B1 protein, it is tempting to speculate that a related mechanism occurs as a result of this ENU-induced allele. Alternatively, deletion of two transmembrane domains of UNC93B1 could also cause a severe structural defect that abrogates protein production, as observed following the loss of a single transmembrane span from the 10-transmembrane domain glucose-6-phosphate protein.59

Studies using Unc93b13d/3d mice have demonstrated an enhanced pathogen load and increased susceptibility to T. gondii, T. cruzi, L. major and MCMV infection in association with reduced expression of inflammatory mediators including IFN-γ (T. cruzi, MCMV, L. major), IFN-α (MCMV) and IL-12p40 (T. gondii, T. cruzi).23,37,38,40,42 The current report using Unc93b1Letr/Letr mice extends the role of endosomal TLR-mediated inflammation to early host defense against influenza. Thus, the Unc93b1Letr/Letr and Unc93b13d/3d mice represent alternative models for the study of diverse host–pathogen interactions. In contrast, Fukui et al. have shown that a point mutation in the cytoplasmic amino-terminus of UNC93B1 (D34A) causes lethal inflammation.
cell activation following infection\textsuperscript{39} and is consistent with a previous study in which a lack of endosomal TLR signaling did not affect co-stimulatory molecule expression on DCs.\textsuperscript{40}

Residual antigen-presenting cell activation in our model can be attributed to alternative pathogen recognition mechanisms such as the nucleotide-binding domain and leucine-rich repeat containing receptor family, pyrin domain containing 3 (NLRP3) inflammasome in macrophages and DCs\textsuperscript{67} or the cytosolic retinoic acid-inducible gene 1 (RIG-I) pathway in DCs.\textsuperscript{68,69} Recognition of viral infection in conventional DCs preferentially utilizes RIG-I signaling,\textsuperscript{66,68} while plasmacytoid DCs rely on TLR7,16,20. As plasmacytoid DCs are the main producers of type I IFN, it is plausible that the delayed expression of type I IFN in the lungs of Unc93b\textsuperscript{I/Let} mice is due to defective TLR7-mediated activation of this specialized cell type. Conversely, the fact that conventional DCs produce type I IFN through a RIG-I-dependent mechanism\textsuperscript{68,70} may explain why the type I IFN response was diminished but not completely abolished in Unc93b\textsuperscript{I/Let} mice. Only the combined loss of MyD88 and mitochondrial antiviral signaling protein, the adaptor molecule for RIG-I and melanoma differentiation-associated protein 5, has been shown to completely abolish type I IFN signaling following influenza infection in vivo.\textsuperscript{18}

At days 2 or 3 post infection with influenza, T cells in the LALNs downregulate CD62L and CCR7, proliferate, and subsequently traffic to the site of infection where they express various activation markers and acquire the capability to secrete IFN-g.\textsuperscript{56,71} Consistent with an impaired activation state, CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the lungs of Unc93b\textsuperscript{I/Let} mice showed a decrease in CD69 and a concomitant increase in CD62L expression at day 3 post infection. Importantly, TRls have previously been shown to directly activate T cells.\textsuperscript{72} Tlr1, Tlr2, Tlr3, Tlr6 and Tlr7 are expressed on both human peripheral blood and mouse C57BL/6 T cells\textsuperscript{73,74} and direct stimulation with TLR3 or TLR7 ligands promotes T-cell survival, CD38 and CD69 expression, and IFN-γ production.\textsuperscript{72,73,77,78} Studies in murine lymphocytic choriomeningitis virus and T. gondii infection have demonstrated that the selective loss of MyD88 in T cells leads to decreased survival and IFN-γ production\textsuperscript{79,80} and, in HIV-infected patients, direct TLR7 activation of CD8\textsuperscript{+} T cells in vitro increased T-cell proliferation and IFN-γ production.\textsuperscript{81} As DC activation in Unc93b\textsuperscript{I/Let} mice appeared to be normal, the delayed lymphocyte activation phenotype in our model may represent an intrinsic defect in CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell activation caused by impaired Unc93b1 function. Nevertheless, at day 7 post infection, the CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell activation profile in Unc93b\textsuperscript{I/Let} lungs was comparable to the wild-type strain and most likely reflects increased infiltration of primed CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from the LALNs.\textsuperscript{66,71} Indeed, the formation of antigen-specific CD8\textsuperscript{+} T cells and IFN-γ - CD4\textsuperscript{+} T cells at later time points post infection can occur in the absence of TLR7 activation.\textsuperscript{78,82} Studies in MyD88-deficient mice have also demonstrated that signaling through this adaptor protein is not required for antigen-specific CD8\textsuperscript{+} T-cell activation later in the infection, while evidence for the importance of MyD88 in CD4\textsuperscript{+} T-cell activation is variable.\textsuperscript{78,26}

Immune activation and cell migration following influenza infection is mediated by the expression of a large number of proinflammatory cytokines and chemokines.\textsuperscript{10} In our model, the loss of Unc93b1 function led to a reduction in the expression of IFN-γ, CXCL10 and type I IFN at day 3 post infection. Natural killer cells can produce IFN-γ in response to IFN-α production by macrophages.\textsuperscript{83} Accordingly, the diminished IFN-γ response in the Unc93b1\textsuperscript{I/Let} mice at this early time point may be attributable to fewer activated ExMs in the lung. At day 7 post infection, the comparable level of IFN-γ between Unc93b1\textsuperscript{I/Let} and Unc93b1\textsuperscript{I/Let} mice is most likely attributable to production by antigen-specific T cells.\textsuperscript{56,71} CXCL10 primarily attracts lymphocytes to the lungs and can be produced in response to

Specifically, Unc93b\textsuperscript{D34A/D34A} mice show preferential and constitutive transport of TLR7 over TLR9, resulting in a severe autoimmune phenotype in the absence of external stimulation. A comparison of the 3d, Letr and D34A mutations provides crucial insights into the structure–function relationship of UNC93B1 and demonstrates that unique point mutations of the Unc93b1 gene confer a deficient or overexuberant immune phenotype.

Following influenza challenge, macrophages and DCs are among the first cells recruited to the respiratory tract.\textsuperscript{62,63} Pulmonary macrophages may be classified into two types: AMs that reside in naive lungs and elicit an early response along with respiratory epithelial cells, and monocyte-derived ExMs that are recruited from the bloodstream in a CCR2-dependent manner and differentiate into effector phagocytes that can lyse infected cells.\textsuperscript{53,64} Inflammatory DC subsets are also recruited from the bloodstream, take up antigen at the site of infection and traffic in a CCR7-dependent manner to the LALNs to activate naive T cells.\textsuperscript{10} Both ExMs and inflammatory DCs express CD80; however, ExMs are relatively poor activators of naive T cells.\textsuperscript{53,60} At an early time point after influenza infection, we detected fewer activated CD80\textsuperscript{+} ExMs, but not CD80\textsuperscript{+} DCs, in the lungs of Unc93b\textsuperscript{I/Let} mice. This data reaffirm a role for Unc93b1 in antigen-presenting

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**Figure 6.** Defective Unc93b1-dependent signaling leads to reduced expression of type I IFN and CXCL10. (a) Relative type I IFN expression in the airways of Unc93b\textsuperscript{I/+/}, Unc93b\textsuperscript{I/+}, and Unc93b\textsuperscript{I/Let} mice at days 3 and 7 following influenza infection. Data are expressed as fold change compared with uninfected samples. Airway and lung expression of (b) CXCL10 and (c) IFN-γ from Unc93b\textsuperscript{I/+/} and Unc93b\textsuperscript{I/Let} mice at days 3 and 7 post infection. Data are pooled from two independent experiments ($n \geq 9$ per group). *$P<0.05$; **$P<0.01$; ***$P<0.001$ determined by two-tailed unpaired t-test.
IFN-γ and by cells of both the innate and adaptive immune response, including ExMs. The reduced expression of CXCL10 in Unc93b1Letr/Letr mice at day 3 post infection may also be linked to fewer activated ExMs with comparable induction at day 7 post infection mediated by infiltrating CD4+ and CD8+ T cells. The selective defect in proinflammatory mediator expression by Unc93b1Letr/Letr mice following influenza infection is similar to observations in Unc93b1Letr/Letr mice following T. gondii infection as

**Figure 7.** Unc93b1 has a role in mediating viral clearance and tissue inflammation but does not influence survival following influenza A/PR/8/34 (H1N1) infection. (a) Lung viral load in Unc93b1+/+ (gray bars) and Unc93b1Letr/Letr (white bars) mice at days 3, 7 and 10 following influenza infection. (b) Survival and (c) weight loss of Unc93b1+/+ (solid line) and Unc93b1Letr/Letr (dashed line) mice over a 21-day period following influenza infection. Representative lung sections from (d, e) uninfected or (f, g) infected lungs at day 7 post infection of (d, f) Unc93b1+/+ and (e, g) Unc93b1Letr/Letr mice. Day 7 post infection is visualized at ×4 and ×10 magnification. Data are pooled from independent experiments. (a) n=10 per group, (b) n=34 per group, (c) n=27 per group. **P<0.01 determined by (a) two-tailed unpaired t-test or (b) log-rank test. **
well as patients with Herpes simplex virus-1 encephalitis and natural UNC93B1 mutations. 38,41

In contrast to studies of Unc93b1Letr/Letr mice that have shown increased mortality following infection with MCMV, neuroadapted Sindbis virus, T. cruzi and T. gondii, 33,37,38,44 the Unc93b1Letr/Letr mice and their wild-type counterparts had a comparable rate of survival following influenza infection. Nonetheless, during the innate immune response to influenza, Unc93b1Letr/Letr mice exhibited several early immune defects including reduced myeloid cell recruitment, lymphoid cell activation and soluble inflammatory mediator production that collectively led to impaired viral clearance as well as increased tissue inflammation and epithelial reactivity during the course of infection. The different disease outcome in previous studies of Unc93b1 and the current report indicates that there is a limited requirement for Unc93b1 in the generation of protective immunity against influenza. Indeed, previous studies have shown that influenza activates a series of inflammatory pathways that could provide compensatory immunity in Unc93b1Letr/Letr mice. For example, the cytosolic RIG-I signaling pathway, while not essential for survival, has been implicated in type I IFN expression from myeloid DCs, fibroblasts and human lung epithelial cells following influenza infection. 38,37,48 NLPR3-dependent activation of caspase-1 is also essential for survival of an influenza infection and the lack of either component leads to decreased recruitment of monocytes and neutrophils to the airways and lower expression of IL-1β, IL-18, CXCL1 and CXCL2 at day 3 post-infection. 37,52 Remarkably, in the absence of pattern recognition through TLR7 and mitochondrial antiviral signaling, IL-1R signaling in DCs was shown to be necessary and sufficient for the generation of virus-specific CD8+ T-cell immunity against influenza. 82 Finally, our data highlights the differences in host response following systemic challenge with a purified PAMP that potently activates a single TLR and mucosal differences in host response following systemic challenge with a single-cell suspension that was created by myeloid cell depletions were harvest for initial phenotypic screening. G2 fathers of G3 mice that displayed a deviant phenotype were outcrossed to C57BL/6 females and the resulting progeny were backcrossed to their G2 fathers. All backcross progeny were re-phenotyped using survival splenectomy and deviant male and female animals were selected for creation of homozygous lines. Deviant animals were outcrossed to C3H/HeN mice and the F1 progeny were backcrossed to the deviant parent to generate S4 segregating mice used to map the genetic location of the mutation.

In vitro and in vivo phenotyping using PAMPs

Whole spleens were removed, placed in 4 ml of RPMI (Gibco, Burlington, ON, Canada) supplemented with 1% l-glutamine (Gibco), 1% penicillin/streptomycin (Gibco) and 10% fetal bovine serum (Gibco) and mechanically disrupted using the frosted end of a microscope slide (Fisher Scientific, Toronto, ON, Canada). A single-cell suspension was created by multiple passages through 1200 G3 Na2EDTA, pH 7.4), and collection by centrifugation, all cells were enumerated with a Coulter Z 1 Particle Counter (Becton Coulter, Mississauga, ON, Canada). Peritoneal cells were incubated for 24 h in RPMI supplemented with 1% l-glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum to allow for macrophage adherence, washed with sterile phosphate-buffered saline (PBS) (Wisent, St-Bruno, QC, Canada) and stimulated with 10 ml of complete RPMI. Following treatment with ACK lysis buffer (0.82% NH4Cl, 0.1% KHCO3, 0.0038% Na2EDTA, pH 7.4), and collection by centrifugation, all cells were enumerated with a Coulter Z 1 Particle Counter (Becton Coulter, Mississauga, ON, Canada) and 2 × 106 cells were plated on 96-well tissue culture plates (Sarstedt, Montréal, QC, Canada). Peritoneal cells were inoculated for 45 h in RPMI supplemented with 1% l-glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum to allow for macrophage adherence, washed with sterile phosphate-buffered saline (PBS) (Wisent, St-Bruno, QC, Canada) and supplemented with 100 ng ml−1 of LPS from Escherichia coli 055:B5 82 (Sigma-Aldrich, Oakville, ON, Canada). Splenocytes were stimulated immediately after plating with 1 μM unmethylation CpG oligodeoxynucleotide 85 (AlphaDNA, Montréal, QC, Canada), 5 μg ml−1 imiquimod 86 (InvivoGen) or 100 ng ml−1 polyI:C (InvivoGen). Cell supernatants were harvested 24 or 48 h after stimulation. For in vivo characterization, mice were injected intraperitoneally with 0.5 mg ml−1 of LPS from Escherichia coli 055:B5 48 (150 μg ml−1 imiquimod, 2.5 μg ml−1 polyI:C in 20 mg ml−1 D-(−)-Galactosamine hydrochloride (DGalN) 85 (Sigma-Aldrich) or 50 nmol ml−1 CpG oligodeoxynucleotide in 20 mg ml−1 DGaIN 86 delivered in 1 ml of sterile 0.9% saline. Mice were killed 3 h after injection, blood was collected by cardiac puncture and serum was separated using the Z-Gel microtubes (Becton Dickinson, Franklin Lakes, NJ) and stored at −80°C. Cell pellets were thawed, washed with PBS and cell counts were performed on a Coulter Z1 Particle Counter (Becton Coulter, Mississauga, ON, Canada). Cell pellets were then resuspended in 0.5 ml of RPMI 1640 media (Gibco, Burlington, ON, Canada), supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) and seeded onto a 96-well plate at a density of 2 × 105 cells per well (Becton Coulter, Mississauga, ON, Canada). At 24 h post incubation, medium was removed and 100 μl of AlamarBlue 87 (InvivoGen) was added per well and plates were incubated for 4 h before reading at 570 nm and 600 nm. SEAP activity was measured by adding 40 μl of KinaseGlow 87 (PerkinElmer, Waltham, MA) to the supernatants and reading at 450 nm at 0, 1, 2, 4 and 8 h post infection.

Genotyping

Genomic DNA from a panel of F1 (Letr/Letr × C3H/HeN) F1 × Letr/Letr mice was submitted for typing of 375 informative single-nucleotide polymorphisms using a low-density Illumina genotyping platform and the Unc93b1 gene was sequenced using genomic DNA and cDNA derived from normal and deviant mice (The Centre for Applied Genomics, Toronto, ON, Canada). The primers used for genomic DNA sequencing were: EseqF: 5'-GTAGTTGTAAGAATGTTGAGCA-3', EseqR: 5'-GCTAATGTTGAGCAAAAAGA-3', GseqF: 5'-CAATGCGCCACCACCAAGG3', GseqR: 5'-CCATGCGACCCAGAATG-3'. The primers used for cDNA sequencing were: UncP28 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP29 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP30 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP31 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP32 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP33 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP34 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP35 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP36 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP37 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); UncP38 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse); and UncP39 (forward), 5'-CCACCTGGGTTGCACTGTA3'-UncP28 (reverse). The uncorrected genetic maps were generated using Cervus 1.0 software 88 and compared with the mouse map generated by the Mouse and Human Genome Sequencing Center at the Lawrence Livermore National Laboratory 89.
Unc93b1 mutation impairs influenza innate immunity

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RNA was extracted from spleens using the RNeasy Mini Kit (Qiagen, Toronto, ON, Canada) with DNase digestion using the RNa-free DNase Set (Qiagen) according to the manufacturer’s instructions. Reverse transcription was carried out using the High Capacity cDNA Archive Kit (Applied Biosystems, Burlington, ON, Canada). PCR was performed on 20 ng of cDNA with 10 μl × Herculase Buffer and 0.5 μl Herculase Polynucleotides (Agilent, Mississauga, ON, Canada), 5 μl dNTP (Invitrogen, Burlington, ON, Canada) and 100 ng of each primer (forward: 5’-GGTGCCCAGAATCTGAG-3’; reverse: 5’-CCATGAGACGCTCTCTACA-3’) in a final volume of 50 μl. The PCR cycling conditions were 94°C for 10 min, 35 cycles of 94°C for 30 s/52°C for 30 s/72°C for 1 min and 72°C for 7 min. The PCR product was visualized on a 1% agarose gel in TAE buffer containing 0.5 μg ml⁻¹ of ethidium bromide.

Analysis of Unc93b1 structure and protein sequence

The relative size of the Unc93b1 introns and exons was determined from the Ensemble database (ENSMMUST00000162708). Unc93b1 amino-acid sequences were derived from the NCBI nucleotide database for the following species Mus musculus (NM_0194492.2), Homo sapiens (NM_030930.2), Pan troglodytes (XM_003313185.1), Canis familiaris (XM_548013.3), Felis catus (XM_003997571.1), Bos taurus (NM_001193147.1), Equus caballus (XM_001916968.8), Ovis aries (XM_004032404.1) and Xenopus tropicalis (NM_001159053.1). The amino-acid sequences were aligned using Clustal Omega software, version 1.2 (European Molecular Biology Laboratory, Heidelberg, Germany). To determine the predicted three-dimensional protein structure, the wild-type Unc93b1 amino-acid sequence was entered into the Protein Homology/analogy Recognition Engine (Phyre), version 2.0 (Structural Bioinformatics Group, Imperial College, London, UK). The predicted model, including the location of the 3d and Letr mutations, was visualized using the PyMol Molecular Graphics System, version 1.7 (Schrödinger, LLC, Portland, OR, USA).

In vivo influenza infection

Six-week-old Unc93b1+/− and Unc93b1−/− F2 mice were anesthetized by intraperitoneal injection of 150 mg kg⁻¹ of ketamine (Bioniche, Pointe-Claire, QC, Canada) and 10 mg kg⁻¹ of xylazine (Bayer, Toronto, ON, Canada) in sterile PBS and injected intranasally with 400 p.f.u. of influenza A/PR/8/34 (H1N1). Infected mice were monitored daily for signs of sickness.

Flow cytometry

Lungs were extracted in 5 ml of incomplete Dulbecco’s modified Eagle’s medium, filtered through a 100 μm nylon strainer (BD Biosciences) and centrifuged by collection. BAL cells were prepared as described for splenocytes. Red blood cells were lysed with ACK buffer and cells were counted using a hemocytometer. Cells were stained with Fixable Cyanin (clone DX5), anti-mouse CD44-PE (clone IM7) (eBioscience). Data were collected on the LSRIIIsorta using FacsDiv software version 6.2 (BD Biosciences) and analyzed using FlowJo, version 9.1 (Tree Star Inc., Ashland, OR, USA) with fluorescence-minus-one gating controls.

Viral quantification by plaque assay

Lungs were extracted from mice at multiple time points post infection and placed in incomplete Dulbecco’s modified Eagle’s medium (Wisent) at a 20% weight/volume ratio. Lungs were homogenized using a rotor–stator homogenizer and supernatants were collected by centrifugation. Madin–Darby canine kidney cells were cultured using standard techniques in Dulbecco’s modified Eagle’s medium supplemented with 1% l-glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum. Three to six cell passages were conducted before one million cells were plated per well on a six-well plate and incubated overnight at 37°C in 5% CO₂. Plaque assays were performed in a similar manner to a previous protocol. Briefly, cells were washed twice with sterile PBS and 100 μl of serially diluted lung homogenate in incomplete Dulbecco’s modified Eagle’s medium was added. Following a 30-min incubation at 37°C in 5% CO₂, sample was removed and cells were covered with 3 ml of a 1:1 mixture of 1.6% agarose and 2 × minimum essential medium (Gibco) supplemented with 6% of a 7.5% NACO₂ solution (BioShop Canada Inc., Burlington, ON, Canada), 1% l-glutamine, 2% penicillin/streptomycin and 0.1% TPCK-trypsin (Sigma-Aldrich). After a 48-h incubation at 37°C in 5% CO₂, 3 ml of a 3:1 methanol (Sigma-Aldrich) and acetic acid (Sigma–Aldrich) solution (Sigma-Aldrich) was added. After 4 h, the solution and agarose plugs were removed and plates were allowed to dry for 24 h. Plaques were visualized by staining with a 0.2% Crystal Violet solution in 20% ethanol (Sigma-Aldrich).

Statistical analysis

Survival curve analysis was performed using the log-rank test. For comparison between multiple columns a one-way analysis of variance with post-hoc test was used. A two-tailed unpaired t-test was conducted for all other pairwise comparisons. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.
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REFERENCES

1 Fauci AS. Seasonal and pandemic influenza preparedness: science and countermeasures. J Infect Dis 2006; 194(Suppl 2): 573–576.
2 WHO. Influenza Fact Sheet No.211. In 2009.
3 Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD et al. Pandemic potential of a strain of influenza A (H1N1): early findings.

Science 2009; 324: 1557–1561.
4 Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 2009; 459: 931–939.
5 Barker WH, Mullooly JP. Impact of epidemic type A influenza in a defined adult population. Am J Epidemol 1980; 112: 798–811.
6 Toapanta FR, Ross TM. Impaired immune responses in the lungs of aged mice following influenza infection. Respir Res 2009; 10: 112.
7 Webster RG. Immunity to the influenza in the elderly. Vaccine 2000; 18: 1686–1689.
8 Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis 2008; 198: 96–99.
9 Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog 2008; 4: e1000115.
10 Kohlmeier JE, Woodland DL. Immunity to respiratory viruses. Annu Rev Immunol 2009; 27: 61–86.
11 Ho AW, Prabhu N, Betts RJ, Ge MQ, Dai X, Hutchinson PE et al. Lung CD103+ dendritic cells efficiently transport influenza virus to the lymph node and load viral antigen onto MHC class I for presentation to CD8 T cells. J Immunol 2011; 187: 6011–6021.
12 Ingulli E, Funatake C, Jacovetty EL, Zanetti M. Cutting edge: antigen presentation

and encoding an unc-93-like protein. PLoS Pathog 2008; 4: e4204.
13 Kim TS, Braciale TJ. Respiratory dendritic cell subsets differ in their capacity to

to CD8 T cells after influenza A virus infection. J Immunol 2011; 187: 798–811.
14 Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory

dendritic cells rapidly transport antigen from the airways to the thoracic lymph

nodes. J Immunol 2011; 187: 2003–2013.
15 Wraithman J, Mintem JD. Dendritic cells and influenza A virus infection. Virulence 2012; 3: 603–608.
16 Diebold SS, Kariko K, Tsubouchi H. Innate immune responses to influenza A virus infection in the brain. Annu Rev Pathol 2012; 7: 559–563.
17 Guillot L, Le Goffic R, Broux O, Escouriol G, Eychenne B, Hameyrot A et al. Toll-like receptor 7 in the induction of IFN-alpha in the brain. J Neurovirol 2004; 10: 320–327.
18 Oseimi M, Matsumoto M, Funami K, Akazawa T, Taya Y. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 2003; 4: 161–167.
19 Le Goffic R, Balloy V, Lagrandrie M, Alexopoulos L, Escouriol G, Flavell R et al. Deterrent contribution of the Toll-like receptor (TLR3) to influenza A virus-induced acute pneumonia. PLoS Pathog 2006; 2: e53.
20 Weber F, Wagner V, Rasmussen SB, Hartmann SR. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol 2006; 80: 5059–5064.
21 Hidaka F, Matsuo S, Muta T, Takeshige K, Mizukami T, Nunoi H. A missense mutation of the Toll-like receptor 3 gene in a patient with influenza-associated encephalopathy. Clin Immunol 2006; 119: 188–194.
22 Horby P, Nguyen NY, Dunstan SJ, Baillie JK. The role of host genetics in suscepti-

bility to influenza: a systematic review. PLoS ONE 2012; 7: e33180.
23 Acevedo-Arozena A, Wells S, Potter P, Kelly M, Cox RD, Brown SD. ENU mutagenesis, a way forward to understand gene function. Annu Rev Genomics Hum Genet 2008; 9: 49–69.
24 Buer J, Balling R, Mice, microbes and models of infection. Nat Rev Genet 2003; 4: 195–200.
25 Justice MJ, Noveroske JK, Weber JS, Zheng B, Bradley A. Mouse ENU mutagenesis. Hum Mol Genet 1999; 8: 1955–1963.
26 Richer E, Qureshi ST, Vidal SM, Malo D. Chemical mutagenesis: a new strategy against the global threat of infectious diseases. Mamm Genome 2008; 19: 309–317.
27 Andrade WA, Souza Mdo C, Ramos-Martinez E, Nagal K, Dutra MS, Melo MB et al. Combined action of nucleic acid-sensing Toll-like receptors and TLR11/TLR12 heterodimers regulates interferon and Th1 responses. PLoS Pathog 2010; 6: e1001071.
28 Pfifer R, Benson A, Sturge CR, Yarowsky F. TLR3 is essential for TLR7 activation and IL-12-dependent host resistance to Toxoplasma gondii. J Biol Chem 2012; 287: 3307–3314.
29 Schambacher BL, Petritis PM, Kaetano BC, Martinez ER, Okuda K, Golenbock D et al. TLR3 and TLR4 stimulate Toll-like receptor-mediated host defense against the intracellular pathogen Leishmania major. J Biol Chem 2013; 288: 7127–7136.
30 Casrouge A, Zhang SY, Edenschenk C, Jouanguy E, Puel A, Yang K et al. Herpes simplex virus encephalitis in humans. Science 2006; 314: 308–312.
31 Crane MJ, Gaddi PJ, Salazar-Mather TP. TLR3 mediates innate inflammation and antiviral defense in the liver during acute murine cytomegalovirus infection. PLoS ONE 2012; 7: e39161.
32 Wang JP, Bowen GN, Zhou S, Cerny A, Zacharia A, Knipe DM et al. Role of specific innate immune responses in herpes simplex virus infection of the central nervous system. J Virol 2012; 86: 2273–2281.
33 Essen N, Blakely PK, Rainey-Barger EK, Irani DN. Complexity of the microglial activation pathways that drive innate host responses during lethal alphavirus infection. Annu Rev Neurosci 2012; 35: 197–217.
34 Alexopoulos L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of TLR3. Nat Immunol 2001; 2: 739–747.
35 Hemmi H, Kasai T, Takeuchi O, Sato A, Sanjo H, Hoshino K et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 2002; 3: 196–200.
36 Hemmi H, Takeuchi O, Kawai T, Sanjo H, Sato A et al. TLR7 and TLR8 recognize single-stranded RNA in the human immune system. J Biol Chem 2004; 279: 740–745.
37 Takeuchi O, Hoshino K, Kawai T, Sanjo H, Akira S et al. Toll-like receptors (TLRs) 1-9 are required for TLR signaling. J Biol Chem 2001; 276: 5598–5603.
38 Kikuchi A, Nakauchi H, Takeda H, Akira S et al. TLR4 deficiencies lead to a systemic hyper-responsiveness to LPS. Nature 2000; 408: 740–745.
39 Chen C, Oshiro K, Kawanohara R, Hoshino K et al. TLR4-mediated host defense against Gram-negative bacteria. Nature 2000; 408: 740–745.
40 Takeuchi O, Hoshino K, Kawai T, Sanjo H, Akira S et al. TLR4-mediated host defense against Gram-negative bacteria. Nature 2000; 408: 740–745.
41 Lafferty et al. Unc93b1 mutation impairs influenza innate immunity

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50. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 1998; 9: 143–150.

51. Short KR, Brooks AG, Reading PC, Londrigan SL. The fate of influenza A virus after infection of human macrophages and dendritic cells. *J Gen Virol* 2012; **93** (Pt 11): 2315–2325.

52. Thomas PG, Dash P, Aldridge Jr. JR, Ellebedy AH, Reynolds C, Funk AJ et al. The intracellular sensor NLPR3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 2009; **30**: 566–575.

53. Brown DM, Dízler AM, Meents DL, Swain SL. CD4 T cell-mediated protection from influenza infection: perforin and antibody-mediated mechanisms give a one-two punch. *J Immunol* 2006; **177**: 2888–2898.

54. Brown DM, Lee S, García-Hernández Mde L, Swain SL. Multifunctional CD4 cells expressing gamma interferon and perforin mediate protection against lethal influenza virus infection. *J Virol* 2012; **86**: 6792–6803.

55. Hufford MM, Kim TS, Sun J, Bricaire TJ. Antiviral CD8+ T cell effector activities in situ are regulated by target cell type. *J Exp Med* 2011; **208**: 167–180.

56. Roman E, Miller E, Harmsen A, Wiley J, Von Andrian UH, Huston G et al. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J Exp Med* 2002; **196**: 957–968.

57. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K et al. Differential roles of MD2 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006; **441**: 101–105.

58. Mykkänen J, Torrents D, Pineda M, Camps M, Yoldi ME, Horelli-Kuitunen N et al. Functional analysis of novel mutations in y+/-: LAT-1 amino acid transporter gene causing lysinuric protein intolerance (LPI). *Hum Mol Genet* 2000; **9**: 431–438.

59. Chen LY, Lin B, Pan CJ, Hiraiwa H, Chou JY. Structural requirements for the stability of myxovirus infection. *Hum Mol Genet* 2000; **275**: 3420–3426.

60. Fukui R, Saitoh S, Kanno A, Onji M, Shibata T, Ito A et al. MyD88-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Immunity* 2006; **25**: 246–253.

61. Thomas PG, Dash P, Aldridge Jr. JR, Ellebedy AH, Reynolds C, Funk AJ et al. The intracellular sensor NLPR3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 2009; **30**: 566–575.

62. Short KR, Brooks AG, Reading PC, Londrigan SL. The fate of influenza A virus after infection of human macrophages and dendritic cells. *J Gen Virol* 2012; **93** (Pt 11): 2315–2325.

63. Lin KL, Suzuki Y, Nakano H, Ramsburg E, Gunn MD. CCR2 receptor ligands induce human T cell activation and death, a model for HIV pathogenesis. *PLoS ONE* 2008; **3**: e1915.

64. Tabiasco J, Devvive E, Rufer N, Salaun B, Cerottini JC, Speiser D et al. Human effector CD8+ T lymphocytes express TL1R3 as a functional co-receptor. *J Immunol* 2006; **177**: 8708–8713.

65. LaRosa DF, Stumhofer JS, Gelman AE, Rahman AH, Taylor DK, Hunter CA et al. T cell expression of MyD88 is required for resistance to Toxoplasma gondii. *Proc Natl Acad Sci USA* 2008; **105**: 3865–3860.

66. Rahman AH, Cui W, Larosa DF, Taylor DK, Zhang J, Goldstein DR et al. MyD88 plays a critical T cell-intrinsic role in supporting CDB T cell expansion during acute lymphocytic choriomeningitis virus infection. *J Immunol* 2008; **181**: 3804–3810.

67. Kulkarni R, Behboudi S, Sharif S. Insights into the role of Toll-like receptors in modulation of T cell responses. *Cell Tissue Res* 2011; **343**: 141–152.

68. Funderburg N, Luciano AA, Wang W, Rodriguez B, Siegf LD, Lernmark MM. Toll-like receptor ligands induce human T cell activation and death, a model for HIV pathogenesis. *PLoS ONE* 2008; **3**: e1915.

69. Tabiasco J, Devvive E, Rufer N, Salaun B, Cerottini JC, Speiser D et al. Human effector CD8+ T lymphocytes express TL1R3 as a functional co-receptor. *J Immunol* 2006; **177**: 8708–8713.

70. LaRosa DF, Stumhofer JS, Gelman AE, Rahman AH, Taylor DK, Hunter CA et al. T cell expression of MyD88 is required for resistance to Toxoplasma gondii. *Proc Natl Acad Sci USA* 2008; **105**: 3865–3860.

71. Richer E, Prendergast C, Zhang DE, Qureshi ST, Vidal SM, Malo D. N-ethyl-N-nitrosourea-induced mutation in ubiquitin-specific peptidase 18 causes hyper-activity of TLR7 in CD8+ T cells leads to TLR7-mediated activation and accessory cell-dependent IFN-gamma production in HIV type 1 infection. *AIDS Res Hum Retroviruses* 2009; **25**: 1287–1295.

72. Pask I, Ichinohe T, Inakawa A. IL-1R signaling in dendritic cells replaces pattern-recognition receptors in promoting CD8+ T cell responses to influenza A virus. *Nat Immunol* 2013; **14**: 246–253.

73. Matikainen S, Paananen A, Miettinen M, Kurimoto M, Timonen T, Julkunen I et al. IFN-alpha and IL-18 synergistically enhance IFN-gamma production in human NK cells: differential regulation of Stat4 activation and IFN-gamma gene expression by IFN-alpha and IL-12. *J Immunol* 2001; **165**: 2236–2245.

74. Zhou J, Law HK, Cheung CY, Ng IH, Peiris JS, Lau YL. Differential expression of chemokines and their receptors in adult and neonatal macrophages infected with human or avian influenza viruses. *J Infect Dis* 2006; **194**: 61–70.

75. Tiege RM, Liang J, Liu N, Jung Y, Jiang D, Gunn MD et al. Recruited exudative macrophages selectively produce CXCL10 after noninfectious lung injury. *Am J Respir Cell Mol Biol* 2011; **45**: 781–788.

76. Testa F, Wilm A, Dineen D, Gibson T, Karplus K, Li W et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* 2011; **7**: 539.

77. Kelley LA, Sternberg MJ. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc* 2009; **4**: 363–371.

78. The PyMol Molecular Graphics System. version 1.7: Schrödinger, LLC.

79. Brown JG. Increased virulence of a mouse-adapted variant of influenza A/FM/1/47 virus is controlled by mutations in genome segments 4, 5, 7, and 8. *J Virol* 1990; **64**: 4523–4533.

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