Antiviral Research 92 (2011) 346–355

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

FimH, a TLR4 ligand, induces innate antiviral responses in the lung leading to protection against lethal influenza infection in mice

Mohamed F. Abdul-Careem [a,1,2], M. Firoz Mian [a,2], Amy E. Gillgrass [a], Meghan J. Chenoweth [a], Nicole G. Barra [a], Tiffany Chan [a], Amal A. Al-Garawi [a], Marianne V. Chew [a], Geoffry Yue [a], Nico van Roojen [c], Zhou Xing [a,b], Ali A. Ashkar [a,b,*]

[a] Center for Gene Therapeutics, Department of Pathology and Molecular Medicine, McMaster University Health Sciences Center, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5
[b] Michael DeGroote Institute for Infectious Disease Research, McMaster University Health Sciences Center, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5
[c] Department of Molecular Cell Biology, Free University Medical Center, Amsterdam 1081 BT, The Netherlands

ARTICLE INFO

Article history:
Received 21 December 2010
Revised 3 September 2011
Accepted 7 September 2011
Available online 14 September 2011

Keywords:
Influenza
TLR4
FimH
Innate immunity
Lung pathology

ABSTRACT

Fimbriae H protein (FimH) is a novel TLR4 ligand that has been shown to stimulate the innate immune system and elicits protective responses against bacterial and viral infections. Here, we evaluated the protective role of local delivery of FimH against influenza A infection in a mouse model. We show that intranasal delivery of FimH prior to lethal challenge with influenza A virus, resulted in decreased morbidity and mortality in wild-type, but not TLR4$^{-/-}$, mice. Importantly, FimH was able to reduce the early viral burden in the lung leading to minimal cell infiltration into the airway lumen and reduced pulmonary pathology following infection in wild type mice compared to TLR4$^{-/-}$ mice. Local delivery of FimH to C57BL/6, not TLR4$^{-/-}$, mice in a prophylactic manner increased the IL-12 and RANTES responses as well as neutrophil recruitment into the airway lumen. These effects correlate to the course of influenza infection. The FimH-mediated antiviral response against influenza virus appears to be partially dependent on alveolar macrophages. The antiviral effects are likely mediated by the innate mediators (TNF-α, IL-12 or RANTES) and/or by activation of a feedback inhibition loop to curtail the pulmonary inflammation possibly be the potential mechanisms involved in FimH-mediated protection. FimH thus holds promise to be a possible prophylactic mean of control against influenza viral infection.

1. Introduction

Influenza A virus belongs to the Orthomyxoviridae family of RNA viruses and is the causative agent of major health issues around the world. In humans, influenza virus replication is limited to the respiratory tract and the associated morbidity and mortality have been linked to excessive pulmonary inflammation, viral replication (Grebe et al., 2010; Lin et al., 2008; Marsolais et al., 2009; Nakamura et al., 2010) or secondary bacterial infections (Jamieson et al., 2010; Shahangian et al., 2009; Small et al., 2010; Taubenberger and Morens, 2008). The control of influenza A virus infection in humans is based on vaccination and the vaccine induced immunity is specific for the influenza subtype. The influenza A virus has been classified into different subtypes according to their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). These two proteins show a high frequency of antigenic variation which may be driven by immune responses generated by infection or vaccination (Bush et al., 1999). As such, influenza outbreaks are commonly recorded in immunized populations. Although heterosubtypic partial protection has been evoked by vaccination (Corti et al., 2010) and broadly effective subtype specific DNA vaccination strategies have been shown to be promising (Wei et al., 2010), designing novel control strategies requires further investigations.

Stimulation of the innate immune system via a crude mixture of bacterial products containing bacterial lysates or bacterial toxins has been investigated as alternative or adjunct control measures against many viral infections, including influenza (Norton et al., 2010; Tuvim et al., 2009; Williams et al., 2004). The basis of these studies is that bacterial products contain pathogen associated molecular patterns (PAMPs) that act as ligands for germ-line encoded Pattern Recognition Receptors (PRRs) such as Toll-like

* Corresponding author at: Department of Pathology and Molecular Medicine, Centre for Gene Therapeutics, Room: MDCL-4074, McMaster University Health Sciences Center, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5. Tel.: +1 905 525 9140x22311; fax: +1 905 522 6750.
E-mail address: ashkara@mcmaster.ca (A.A. Ashkar).
[1] Present address: Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Health Research Innovation Center 2C64, 3330 Hospital Drive NW, Calgary, Canada AB T2N 4N1.
[2] These authors contributed equally to this work.

0166-3542/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2011.09.004

receptors (TLRs). Triggering these receptors elicits protective responses through the activation of signaling molecules that induce anti-viral host mechanisms which eventually drives the adaptive arm of the immune system to generate long lasting antigen specific immunity (Williams et al., 2004). It has been shown previously that TLR4 signaling is important for protection against a number of infectious diseases when TLR4 ligands are delivered prophylactically (Cluff et al., 2005; Errea et al., 2010; Lembo et al., 2008). Synthetic TLR4 ligands such as aminoalkyl glucosaminide phosphate (AGP) have also been shown to be protective against influenza infection (Cluff et al., 2005).

A novel TLR4 ligand, FimH (Mossman et al., 2008) has been shown to be protective against bacterial infections such as uropathogenic *Escherichia coli* and viral infections such as herpes simplex virus (HSV)-2 infections (Ashkar et al., 2008; Mian et al., 2010). FimH protein (279 amino acids) is a minor component of type 1 fimbriae encoded by uropathogenic *E. coli* and serve as an adhesion component (Krogfelt et al., 1990). The three dimensional structure of FimH consists of two immunoglobulin-like domains jointed by a short linker (Choudhury et al., 1999).

In the present study, we investigated whether the prophylactic use of intranasally delivered TLR4 ligand, FimH could stimulate mucosal immune responses and protect against influenza infection in a mouse model. Our data demonstrate that this novel TLR4 ligand protects mice against lethal influenza infection by reducing the viral burdens in the lung. The protection against influenza infection correlated with significant reduction in the inflammatory mediators in the air way lumen. The FimH mediated protection against influenza virus infection is partially dependent on alveolar macrophages. These results demonstrate the protective effect of FimH against influenza virus infection and indicate that it may have a potential in a clinical setting as a prophylactic measure.

2. Materials and methods

2.1. Animals and virus

C57BL/6 mice (Charles River Laboratory, Quebec, Canada) were purchased and TLR4$^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME) were bred and maintained in specific-pathogen-free conditions at the Central Animal Facility, McMaster University (Hamilton, Ontario). H1N1 influenza viral strain (A/PR/8/34) was kindly provided by Adolfo Garsia-Sastre, Mount Sinai School of Medicine, NY, USA.

2.2. Isolation and purification of FimH

The isolation and purification of FimH has been described previously (Mian et al., 2010; Mossman et al., 2008). Briefly, the fimH gene from *E. coli* strain EC99 (O78) was cloned into pQE-30 and expressed in BL-21-competent *E. coli*. The isolated recombinant FimH protein was purified through nickel-column and gel filtration FPLC. The protein concentration was determined and stored at −80 °C. The endotoxin concentration in purified FimH as tested by *Limulus* ameobocyte lysate endotoxin detection kit was too minimal (4–7 pg per μg of FimH) to induce any activity, therefore the stimulatory activity of FimH was unlikely due to contamination with endotoxins (Mossman et al., 2008).

2.3. Local delivery of TLR4 ligands and infection of mice

Male C57BL/6 and TLR4$^{-/-}$ mice were anesthetized and treated intranasally with 30 μg/mouse FimH or PBS. Twenty-four hours later, the mice were anesthetized and infected with 5×10^3 PFU of the PR-8 strain of influenza virus intranasally. The challenge of mice 24 h post-treatment was based on our previous observations with TLR-3 ligand, Poly I:C in mouse model of herpes simplex virus-2 infection (Ashkar et al., 2004). It also has been shown that TLR-3 ligand, Poly IC and other innate immune stimulants such as bacterial lysate are effective against influenza viral challenge when delivered prophylactically 24 h before the challenge (Wong et al., 1995; Tuvim et al., 2009). The mice in the survival study were weighed daily for 21 days post-infection and the end points were determined based on loss of ⩾20% bodyweights according to institutional animal care guidelines. For the purpose of viral titration, collection of bronchoalveolar-lavage fluid (BALF) and histological examination of the lungs the mice were euthanized at 3 and 7 days post-infection (dpi). For the evaluation of the effects of FimH delivery in the lung, a group of treated and control mice were necropsied 24 h post-treatment and lungs and BALF were collected for the evaluation of histological changes and cytokine and chemokine responses.

2.4. Alveolar macrophage depletion

Clodronate (dichloromethylene bisphosphonate, Cl_2MBP), a gift from Roche Diagnostics, was packaged into liposomes as previously described (van Rooijen and Hendrikx, 2010). Empty liposomes were prepared in the same conditions in phosphate-buffered saline (PBS) without clodronate. Liposome–Cl_2MBP (50 μl) was delivered intratracheally for two consecutive days along with controls that received liposome–PBS and treated with FimH intranasally 1 day after the last liposome–Cl_2MBP delivery. One day following the TLR ligand treatment the mice were necropsied to collect BALF. For the survival study, the mice were anesthetized and infected with 5×10^3 PFU of PR-8 strain of influenza virus intranasally 24 h post-treatment. The mice were observed for bodyweights up to 21 dpi to determine the end point.

2.5. Bronchoalveolar lavage

BALF was collected in order to characterize cell and cytokine responses in the airway as previously described (Al-Garawi et al., 2009). Briefly, the mice were first anesthetized by inhalation of isoflurane, sacrificed, and bled thoroughly, while the lungs, heart, and trachea were then dissected out carefully. The trachea was intubated with a sterile 0.965/0.58 (outer/inner) mm diameter polyethylene tube attached to a 23-gauge needle. Two volumes of PBS, first 250 μl and then 200 μl, were delivered into the lung through the needle attached to a tube and BALF was then collected following a gentle massage of the lung. An average 70% of the PBS was recovered. The total leukocytes in the BALF were counted using a hemocytometer following a 1:4 dilution in TURK's solution (Merk, Gibbstown, NJ). For differential counts, cells were spun onto slides at 300 rpm for 2 min and stained with a Hema 3 staining kit protocol (Fisher Scientific). A total of 300 cells were identified and counted per slide as neutrophils and mononuclear cells. Total mononuclear cells and neutrophils were calculated using the total cell counts and differential leukocyte counts.

2.6. Ex vivo viral replication and cytokine production of BAL cells

BAL cells (alveolar macrophages) isolated from naïve C57BL/6 mice and were cultured ex vivo (1.5×10^6 cells/ml) either alone or with 10 μg/ml of FimH for 20 h. Cell free supernatants were harvested for cytokine (TNF-α and IL-12) measurements using ELISA. Cells were then washed with serum free MEM-F11 medium and infected with PR-8 strain of Influenza virus at MOI of 0.2 and 2.0 for 24 h at 37 °C. Cells were then harvested, homogenized and stored at −80 °C. To assess the replication of virus in BAL cells, Influenza

viral plaque assay was performed on fully confluent monolayers of MDCK cells in 6-well culture plates using 10-fold serial dilutions of cell lysates following the protocol as outlined below.

2.7. Flowcytometry

The BAL cells were isolated from FimH treated (30 μg/mouse) or PBS treated C57BL/6 mice 24 h post-treatment and cell surface staining was done using anti mouse CD11b-PE (eBioscience), anti mouse F4/80-APC (eBioscience) and anti mouse Gr-1-Alexafluor 700 (eBioscience). Flow cytometry data were collected on the FACSCanto (BD Biosciences) and analyzed using FlowJo version 7.2.5. To obtain macrophages, BAL cells were gated for F4/80 + CD11b + Gr-1–population and to obtain neutrophils, BAL cells were gated for CD11b-F4/80 + Gr-1 + population.

2.8. ELISA

BALF collected at each experiment was assayed for IFN-λ, TNF-α, IL-12 and regulated upon activation, normal T-cell expressed, and secreted (RANTES) concentrations by the DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. The production of IFN-β was assayed using PBL Biomedical kits according to the protocol described by the manufacturer (Piscatway, NJ). The plates were read using the Sapphire ELISA plate reader at 450 nm wave length.

2.9. Influenza virus titration

A portion of the lung (left lobe) was collected (Crowe et al., 2009; Norton et al., 2010) in 1 ml of PBS, homogenized, centrifuged at 5000 rpm for 10 min at 4 °C, and the supernatant was aliquoted and stored at −80 °C until titrated. Viral titers were determined by plaque assay on monolayer of Madin-Darby Canine Kidney (MDCK) epithelial cells as previously described with modifications (Gaush and Smith, 1968). Briefly, the lung homogenates were serially diluted in cold PBS and added to confluent six-well plated MDCK cells. Following the incubation of plates at 37 °C under 5% CO_2 for 30 min, 3 ml of overlay (1% agar, serum free 2× MEM-F-11, 5 μg/ml trypsin) per well was added. The overlay was allowed to solidify at room temperature and incubated at 37 °C under 5% CO_2 for 48 h. The plates were fixed with Carnoy's solution (25% acetic acid + 75% methanol) and washed to remove the overlay for subsequent counting of plaques under a light microscope.

2.10. Lung histology

The right lobe of the lung was inflated via the trachea, fixed in 2% paraformaldehyde for 48 h, trimmed, and submitted in 70% ethanol to the Histology core laboratory of the Center for Gene Therapeutics, McMaster University, Hamilton for sectioning and hematoxylin and eosin (H&E) staining to screen for inflammatory changes. The histological changes in the lung sections were scored following haematoxylin and eosin (H&E) staining as described previously (Crowe et al., 2009). A score of 0, 1, 2, 3 and 4 was assigned for no lesion, perivascular cuff of inflammatory cells, mild inflammation (<25% of the area of the lung was affected), moderate inflammation (25–50% of the lung area was affected) and severe inflammation (>50% of the lung is affected), respectively.

2.11. Data analysis

All data except survival proportions were analyzed by Student's *t*-test to identify differences between groups using the statistical package, MINITAB® release 14 (Minitab Inc., State College, PA, USA). Differences in survival between FimH and PBS delivered mice as well as between macrophage depleted and control mice were analyzed by χ^2 test using GraphPad Prism 4 (GraphPad Prism Software, La Jolla, CA). Comparisons were considered significant at $P \leqslant 0.05$.

3. Results

3.1. Intranasal delivery of FimH protects C57BL/6 mice against influenza infection

Intranasal delivery of Poly I:C and CPG oligonucleotide, TLR 3 and TLR 9, agonists, respectively, has been shown to be protective against influenza infection (Wong et al., 2009; Wong et al., 1995). Innate antiviral effects of FimH against genital HSV-2 infection have been demonstrated (Ashkar et al., 2008). We hypothesized that intranasal delivery of FimH may elicit protective effects against influenza infection. Notably, intranasal delivery of FimH 1 day before a lethal influenza infection prevented loss of significant bodyweight in C57BL/6 mice (Fig. 1a, $P = 0.048$, 0.038 and 0.029 on 6, 7 and 8 dpi). Also, the delivery of FimH led to 80% protection (Fig. 1b, $P = 0.0046$). The C57BL/6 mice that received PBS 1 day before the infection were not protected and reached the end point (Fig. 1b). The protection observed among the FimH treated mice was associated with a trend of reduced inflammation (Fig. 1c and d). Histologically, loss of integrity of epithelial cells lining the bronchial lumen and increased cell infiltration in the lung of PBS treated and influenza infected mice was observed when compared to that of FimH treated and influenza infected mice.

3.2. Intranasal delivery of FimH induces cellular and cytokine responses in the airway lumen

Since FimH treatment resulted in protection against lethal influenza infection, we hypothesized that cellular and cytokine responses in the airway lumen are different between FimH- and PBS treated mice at the time of influenza challenge. C57BL/6 and $TLR4^{-/-}$ mice were treated intranasally with either FimH or PBS and 1 day after treatment, the unifected mice were necropsied to collect BALF and lung sections. BALFs were analyzed for cell types and cytokines, and the lung sections for histopathology. Interestingly, FimH treated lung sections (C57BL/6) showed mild perivascular cuffs of cells compared to changes observed in PBS treated lungs (Fig. 2a). However, there was minimal change in the integrity of epithelium lining the bronchial lumen and the air exchange areas with mild cell infiltration in FimH treated mice when compared to PBS treated mice. In general, cells collected from BALF of PBS and FimH treated C57BL/6 mice predominantly had mononuclear cells (Fig. 2b). In contrast to the PBS treated C57BL/6 and $TLR4^{-/-}$ and FimH treated $TLR4^{-/-}$, FimH treated C57BL/6 mice displayed significantly higher neutrophil counts in the BALF (Fig. 2e; $P = 0.002$ and 0.0015). Flowcytometry analysis of BAL cells confirmed our observation of increased neutrophils as compared to the controls following FimH treatment in C57BL/6 mice (Fig. 2c). No differences in airway luminal total and mononuclear cell counts were found between PBS and FimH treated animals (Fig. 2d, $P > 0.05$; data not shown for total mononuclear cells). Of the cytokines and chemokines examined, IFN-β and IFN-λ expressions in the airway lumen were below the detection limits of the respective ELISA (data not shown). Although the expression of TNF-α in BALF of FimH treated mice was significantly higher than the PBS treated mice, the concentrations were considerably low (data not shown, $P = 0.0115$). Further evidence that the expression of RANTES (Fig. 2f, $P = 0.0001$ and $P = 0.005$) and IL-12 (Fig. 2g, $P = 0.0003$

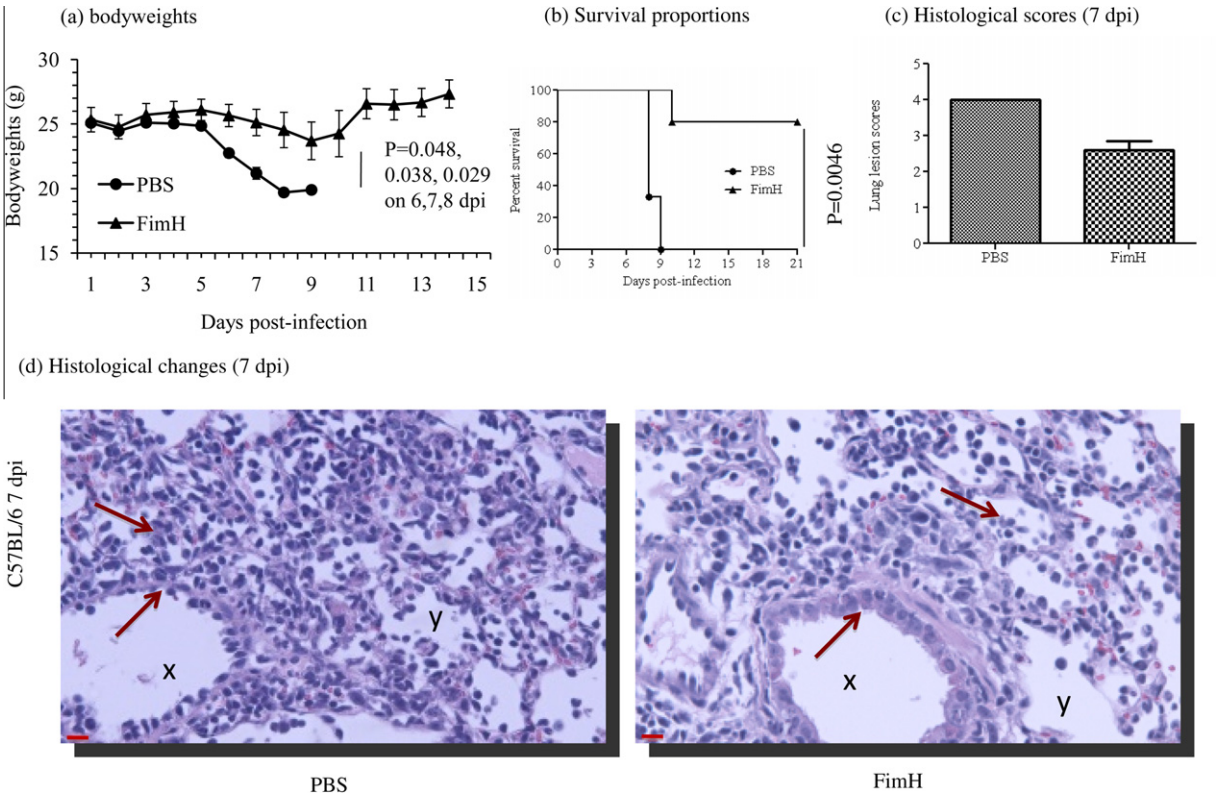

Fig. 1. FimH treatment protects C57BL/6 mice against influenza-induced morbidity and mortality. Male C57BL/6 mice were treated intranasally either with FimH (30 μg) or PBS and 24 h later, mice were infected with 5×10^3 PFU of PR-8 strain of influenza A virus intranasally ($n = 10$ per group). A portion of the mice were observed up to 21 dpi for endpoints. ($n = 5$ per group) and the rest were necropsied at 7 dpi to determine histological changes in the lung ($n = 5$ per group). The endpoints were based on ⩾20% loss of bodyweights. (a) bodyweight, (b) survival proportions, (c) lung lesion scores (7 dpi), (d) histological changes in lung (7 dpi). *X* and *Y* in figure c represent bronchial lumen and air exchange area, respectively. The arrow in the bronchial lumen points towards the epithelium covering the bronchial lumen and outside points infiltration of air exchange areas. The bar in figure c represents 100 μm. The data represent results from two independent experiments.

and $P = 0.001$) in the airway lumen of FimH treated C57BL/6 mice were significantly higher than in the PBS treated C57BL/6 and TLR4$^{-/-}$ and FimH treated TLR4$^{-/-}$ mice. Together, these data clearly demonstrate that intranasal delivery of FimH induces marked cellular and cytokine responses with minimal histolopathological changes in the airway mucosal surfaces.

3.3. FimH-mediated protection against morbidity and mortality from influenza infection is abrogated in TLR4 deficient mice

A number of recent evidence suggests that FimH binds to TLR4 and activates innate signaling systems (Ashkar et al., 2008; Mossman et al., 2008). To evaluate the requirement of TLR4 signaling for FimH-mediated protection against influenza infection, C57BL/6 and TLR4$^{-/-}$ mice were treated either with PBS or FimH and then infected with a lethal dose of intranasal influenza A virus along with uninfected controls (only PBS and only FimH). The mice were observed for bodyweight changes to determine the end point. Importantly, FimH treatment failed to protect TLR4$^{-/-}$ mice, in contrast, provided complete protection in C57BL/6 mice against intransal influenza infection (Fig. 3a, $P = 0.0069$). It has been well established that influenza virus replication reaches to the peak levels 3 days after infection in mouse model of influenza infection (Smith et al., 2011). As expected, FimH treatments of TLR4$^{-/-}$ mice failed to arrest viral replication as evidenced by the detection of high viral titers at 3 dpi in the lung comparable to the PBS treated controls (Fig. 3c, $P > 0.05$). By contrast, FimH treated C57BL/6 mice at 3 and 7 dpi had significantly diminished influenza viral titers in the lung compared to the PBS treated mice (Fig. 3b, $P = 0.0293$ and $P = 0.0475$, respectively). FimH treated C57BL/6 mice had significantly lower viral titers when compared to that observed in FimH treated TLR4$^{-/-}$ mice ($P = 0.0293$). Lung histology data show that the C57BL/6 and TLR4$^{-/-}$ mice had various degrees of peri-vascular and -bronchiolar cuffs of cells and inflammatory cell infiltration in the air exchange areas at 3 dpi (Fig. 3c and d). Of the note, the difference in the degree of pulmonary inflammation in TLR4$^{-/-}$ mice following PBS or FimH treatment was minimal. C57BL/6 mice after FimH treatments exhibited minimal pulmonary inflammatory changes compared with the PBS treated controls. The inflammatory changes in the lung of TLR4$^{-/-}$ mice consisted of loss of integrity of the epithelial lining the bronchial lumen and increased cell infiltration into the alveoli of the lung. The histological features of the lungs correlated with the BAL cells recovered from the airway lumen at 3 dpi. In addition, we observed significantly lower total cells ($P = 0.003$), neutrophils ($P = 0.048$) and mononuclear cells ($P = 0.010$) counts in BALF from FimH treated C57BL/6 mice than the PBS treated mice (Fig. 3e–g). In contrast, BALF cells from both FimH and PBS treated TLR4$^{-/-}$ mice displayed identical populations of total cells, neutrophils and mononuclear cell counts at 3 dpi (Fig. 3h–j; $P > 0.05$). We further measured the cytokines and chemokine concentrations in the BALF collected at 3 dpi from all groups of experimental mice. Since the concentrations of TNF-α, RANTES and IL-12 in uninfected controls were below the detection limit, only data from infected animals are shown. Remarkably, we detected comparable levels of RANTES and IL-12 both from FimH and PBS treated C57BL/6 mice (Fig. 3l and m, respectively; $P > 0.05$), but TNF-α, level was below the detection limit (Fig. 3k). TLR4$^{-/-}$ mice, on the other hand, displayed significantly elevated concentrations of RANTES and TNF-α, in BALF from FimH treated mice compared to the PBS controls (Fig. 3n and o,

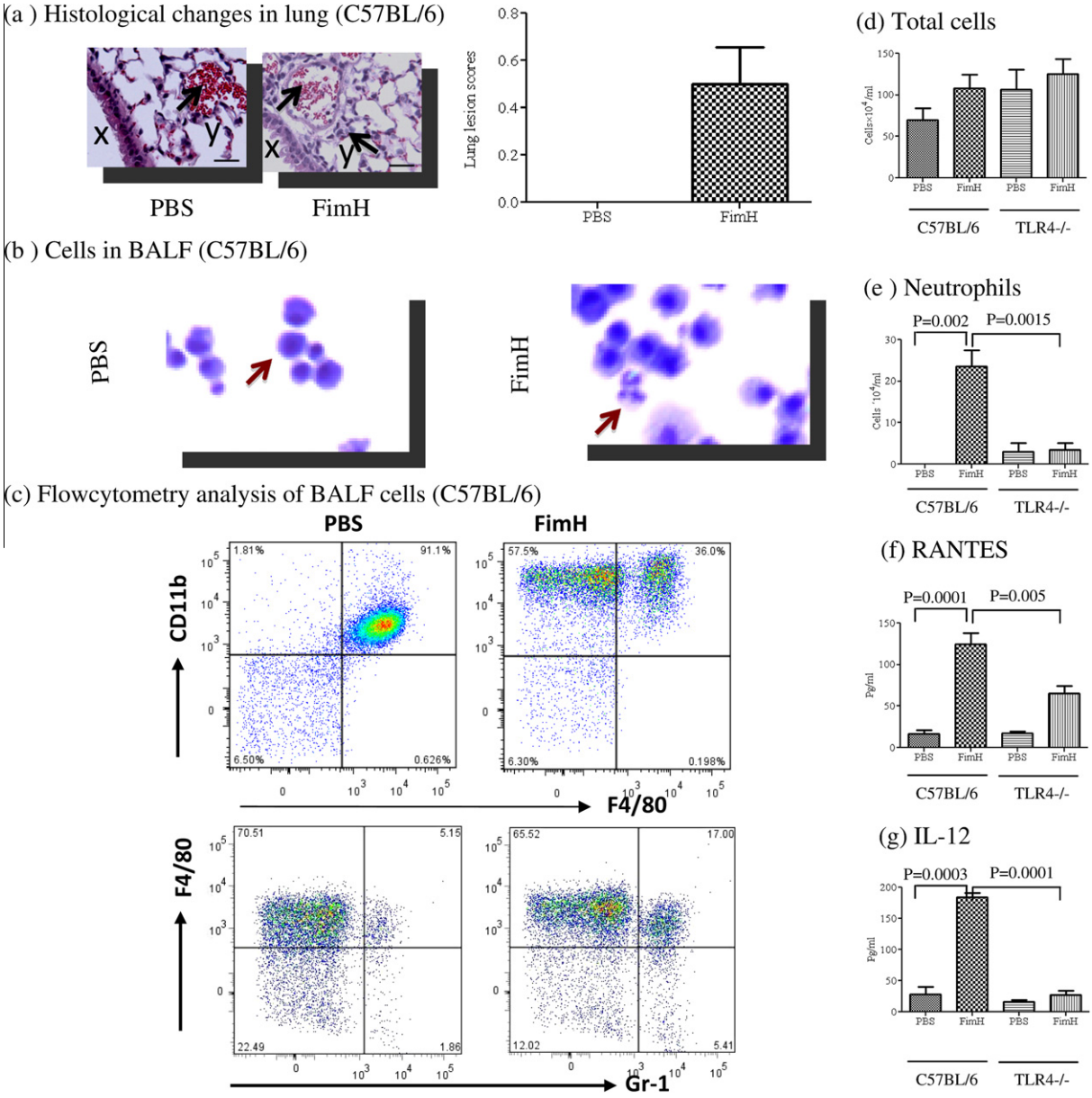

Fig. 2. Intranasal delivery of FimH in naive C57BL/6 mice induces recruitment of cells and release of cytokines and chemokine in airway lumen with minimal histopathology in the lung. Male naïve uninfected C57BL/6 and TLR4$^{-/-}$ mice were delivered FimH (30 μg/mouse) or PBS (control) intranasally. BALF from five mice of each treatment group were collected at 24 h post-treatment. In addition to total and differential cell counts, the concentrations of TNF-α, RANTES, IL-12, IFNλ and IFNβ in cell free supernatants were measured by ELISA. Following BALF collection the ½ of the lungs (right) from C57BL/6 were inflated with 2% PFA and processed for routine H&E staining. (a) photomicrographs represent sections of lung tissue from C57BL/6 mice treated with FimH, or PBS and lung lesion scores. (b) Representative photomicrograph of cells in BALF of C57BL/6, (c) Flow cytometry analysis of BAL cells of C57BL/6 stained with CD11b-PE, F4/80-APC and Gr-1-Alexa fluor 700 to determine the macrophages (F4/80 + Cd11b + Gr-1−) and neutrophils (CD11b-F4/80 + Gr-1+) populations, (d) total cell counts in BALF, (e) total neutrophil counts in BALF. The detection of IFN-λ, IFN-β and TNF-α was below or just above the detection limit in BALF (data not shown); however, FimH delivery stimulates production of RANTES (f) and IL-12 (g) in BALF. *X* and *Y* in figure a represent bronchial lumen and air exchange area, respectively. In the figure a (PBS group) the arrow points towards red blood cells within a capillary. In the figure a (FimH group) the arrows point towards perivascular cuff of cells and red blood cells within a capillary. The arrow shows mononuclear cells (PBS) and neutrophils (FimH) in figure b. The bar in figure a represents 100 μm. The data represent results from two independent experiments.

respectively; $P = 0.0061$ and 0.0013, respectively), while IL-12 levels were comparable in either group (Fig. 3p, $P > 0.05$).

3.4. Role of alveolar macrophages in FimH-mediated protection against influenza infection

As discussed in the previous section, FimH stimulates elevated levels of IL-12 and RANTES in the BALF after 24 h of treatment. Accumulating evidence suggests that these innate mediators can be produced by antigen presenting cells including macrophages (Devergne et al., 1994; Marques et al., 1999; Trinchieri, 1995). We, therefore hypothesized that alveolar macrophages may play an essential role in FimH-induced protection against influenza A virus infection. To define the role of alveolar macrophages in protection mediated by FimH against influenza infection, we depleted macrophages by delivering liposome–CL_2MBP intranasally for two consecutive days, while liposome–PBS served as controls. Mice were then treated with FimH intranasally 1 day after the last liposome–CL_2MBP or -PBS delivery. On the day of FimH treatment, a group of mice were necropsied and BALF was collected to assess

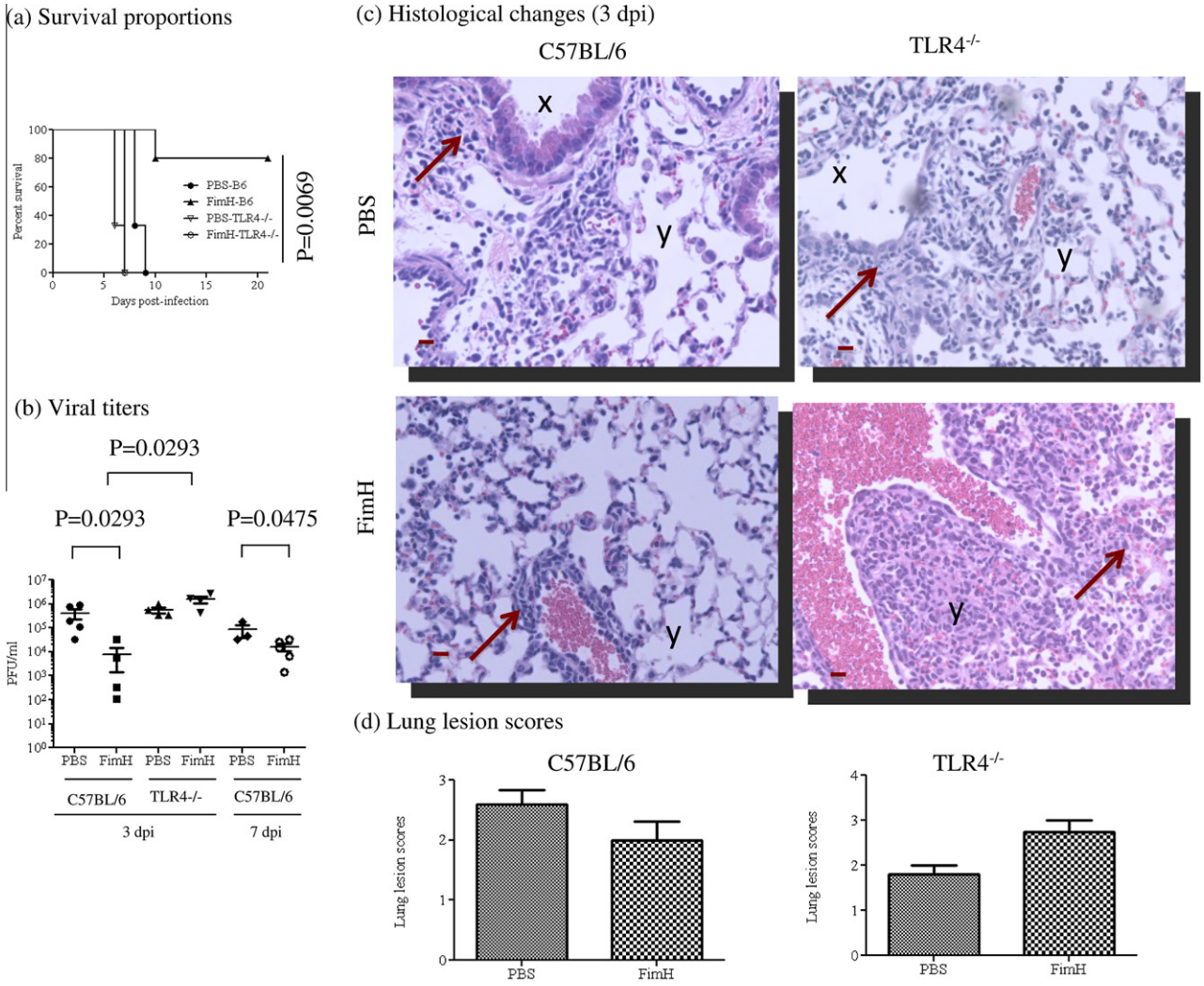

Fig. 3. The FimH-induced protection against influenza virus caused morbidity and mortality is abrogated in TLR4$^{-/-}$ mice. C57BL/6 and TLR4$^{-/-}$ mice were delivered FimH (30 μg) or PBS intranasally and were infected with 5×10^3 PFU of PR-8 influenza A virus intranasally 24 h post-treatment along with unifected controls (only PBS and only FimH groups). Some of the mice were observed up to 21 dpi for bodyweights to determine the end points (⩾20% loss of bodyweights) (n = 5 per group). The rest of the mice were necropsised 3 dpi to collect BALF for total and differential cell counts and measuring TNF-α, RANTES and IL-12 (n = 3–5 per group). Following BALF collection, the ½ of the lungs were inflated with 2% PFA and processed for routine H&E staining for histological evaluation (right) or collected for viral titration (left). (a) Survival proportions, (b) influenza viral titers in lung of C57BL/6 (3 and 7 dpi) and TLR4$^{-/-}$ (3 dpi) mice, respectively (n = 3–5 per group), (c) histological changes in lung, (d) lung lesion scores, (e and h) total cell counts, (f and i) total neutrophil counts, (g and j) total mononuclear cells, (k and n) TNF-α, (l and o)-RANTES, (m and p)-IL-12 in the BALF of C57BL/6 and TLR4$^{-/-}$ mice, respectively. The concentrations of TNF-α, RANTES and IL-12 in unifected animals were below the detection limit (data not shown). *X* and *Y* in figure c represent bronchial lumen and air exchange area, respectively. The arrow in figure c of C57BL/6 PBS and FimH treated lung represents peribronchiolar and peri vascular cell infiltration whereas that in TLR$^{-/-}$ PBS and FimH represents peribronchiolar cell infiltration and infiltration of air exchange areas, respectively. The bar in figure c represents 100 μm. The data represent results from two independent experiments.

the efficiency of macrophage depletion (Fig. 4a). In agreement with previous observations (Leemans et al., 2001), we observed 80% depletion of macrophages while assessed the airway lumen cells. Another subgroup of mice was necropsied 1 day after the TLR ligand treatment to collect BALF. The total cells, neutrophils and mononuclear cell populations were counted from the BALF as illustrated in Fig. 4b–d. Depletion of alveolar macrophages resulted in significantly diminished mononuclear cell counts in BALF from FimH treated group (P = 0.0154). These reduced mononuclear cell counts following macrophage depletion in the FimH treated mice (P = 0.0256) markedly reflected the trend of lower total cell counts compared to the non-depleted (macrophage) control mice. We then sought to determine if this depletion of alveolar macrophages has any impact on conferring protection by FimH against morbidity and mortality from influenza virus infection. As illustrated in Fig. 4e, although a decline in the survival of macrophage depleted and PBS control mice was observed, however, no significant reduction in the survival rate was observed among mice that were alveolar macrophage depleted but FimH treated (P > 0.05). All the FimH untreated control mice reached end point by 9 days post infection. It has been shown previously that macrophage depletion does not alter the course of pathogenesis of PR-8 influenza infection hence survival rate in mice (Tate et al., 2010). We also have not seen consistent effect of macrophage depletion on 3 dpi viral titers (only 3 out of five macrophage depleted mice had lower viral titers) (Fig. 4e, P > 0.05), lung pathology (data not shown) and RANTES or TNF-α production (data not shown). Since the macrophage depletion may lead to complex consequences in vivo, we then extended our experimental approach in vitro to confirm the specific role of alveolar macrophages in providing protection by FimH against influenza infection. To achieve this, alveolar macrophages were collected from BALF of naïve C57BL/6 mice, cultured ex vivo and stimulated with FimH. Cell free supernatant showed robust production of TNF-α and IL-12 following FimH stimulation

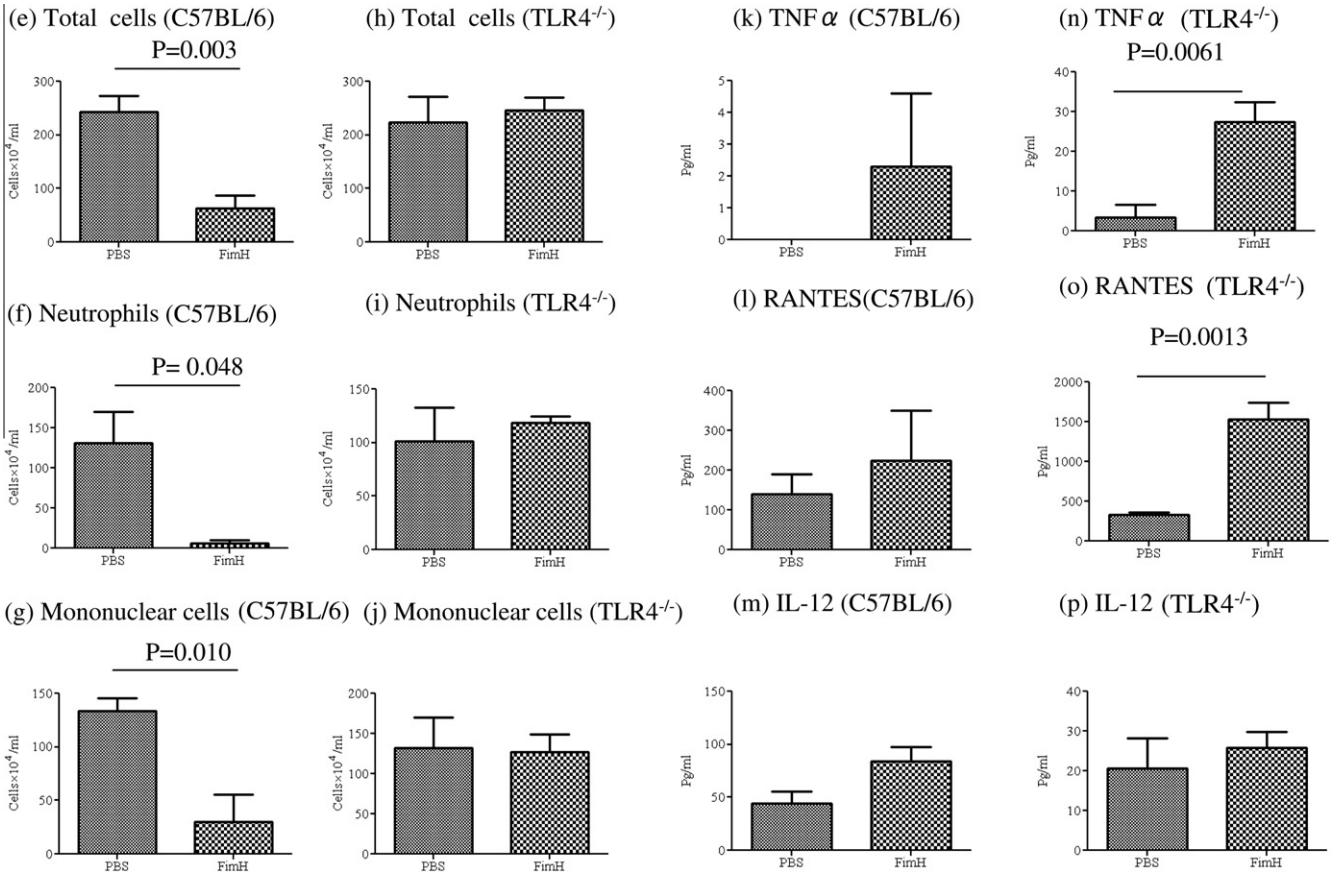

Fig. 3 (*continued*)

in vitro (Fig. 4h and i, $P = 0.000297$ and 0.001432, respectively). In addition, ex vivo stimulation of alveolar macrophages by FimH profoundly prevented the replication of influenza virus in alveolar macrophages compared to the untreated control macrophages (Fig. 4g, $P = 0.0194$). Collectively, these in vivo and in vitro data provide evidence that alveolar macrophages play a role in part, if not critical, to confer protection mediated by TLR4 ligand, FimH against influenza infection.

4. Discussion

The studies described in this manuscript led to three major findings. First, FimH elicits protective responses against lethal influenza infection in mice, and this protection was dependent on TLR4 signaling. The protection was associated with a reduction in influenza viral replication in the lung at an early stage of infection in the FimH delivered mice. Further, the protection correlated with a significant reduction of inflammatory cells in the airway lumen and pulmonary pathology in the FimH treated mice on 3 and 7 dpi. Secondly, the local delivery of FimH stimulated a significant expansion of cells such as neutrophils with minimal histological changes in the lung. In addition, it also significantly increased the concentrations of cytokines such as IL-12 and RANTES in the airway lumen at a time point when optimal influenza replication occurs. Finally, the protection mediated by FimH against influenza infection was not critically, but in part, dependent on the alveolar macrophages.

Synthetic TLR4 ligands and LPS have been shown to be protective against pneumonic tularemia and *Bordetella pertussis* infections, respectively (Errea et al., 2010; Lembo et al., 2008), as such, also protective against viral infections including influenza virus (Cluff et al., 2005). Vaginal delivery of FimH has been shown to elicit protective responses against genital HSV-2 infection (Ashkar et al., 2008). In agreement with these observations, we observed that local delivery of FimH could protect mice against lethal influenza infection.

We next investigated the factors that mediate protection in FimH treated mice by evaluating the cellular and cytokine profiles in the airway lumen following influenza infection. The total alveolar cells and neutrophil populations were increased following FimH treatment in the airway lumen with minimal alterations in the lung histolopathology. Since the mononuclear cell counts were not altered significantly following FimH delivery, the total cell counts reflected the elevated neutrophil populations in the airway lumen. These neutrophils may have captured and processed influenza virus infected cells that undergo apoptosis in the respiratory mucosa (Hashimoto et al., 2007). As such, they can play a protective role along with other immune mediators reducing the viral replication during the influenza infection (Fujisawa, 2008). This may be a plausible explanation as to why the FimH-treated mice showed significant reduction in viral burdens in the lung following infection.

Of the cytokines and chemokines evaluated, IFN-β amount was below the detection level in all the BALF samples examined. Using IFN-α/β receptor$^{-/-}$ mice, it has been shown that type 1 interferons are critical for the prevention of influenza induced morbidity and mortality (Szretter et al., 2009). Our data showing a lack of IFN-β production in the respiratory mucosa following the delivery of FimH does not contradict the findings of Szretter and coworkers (Szretter et al., 2009) as the production of IFN-β in the respiratory mucosa was not observed in their study. The lack of IFN-β response following FimH treatment in the airway lumen could be supported

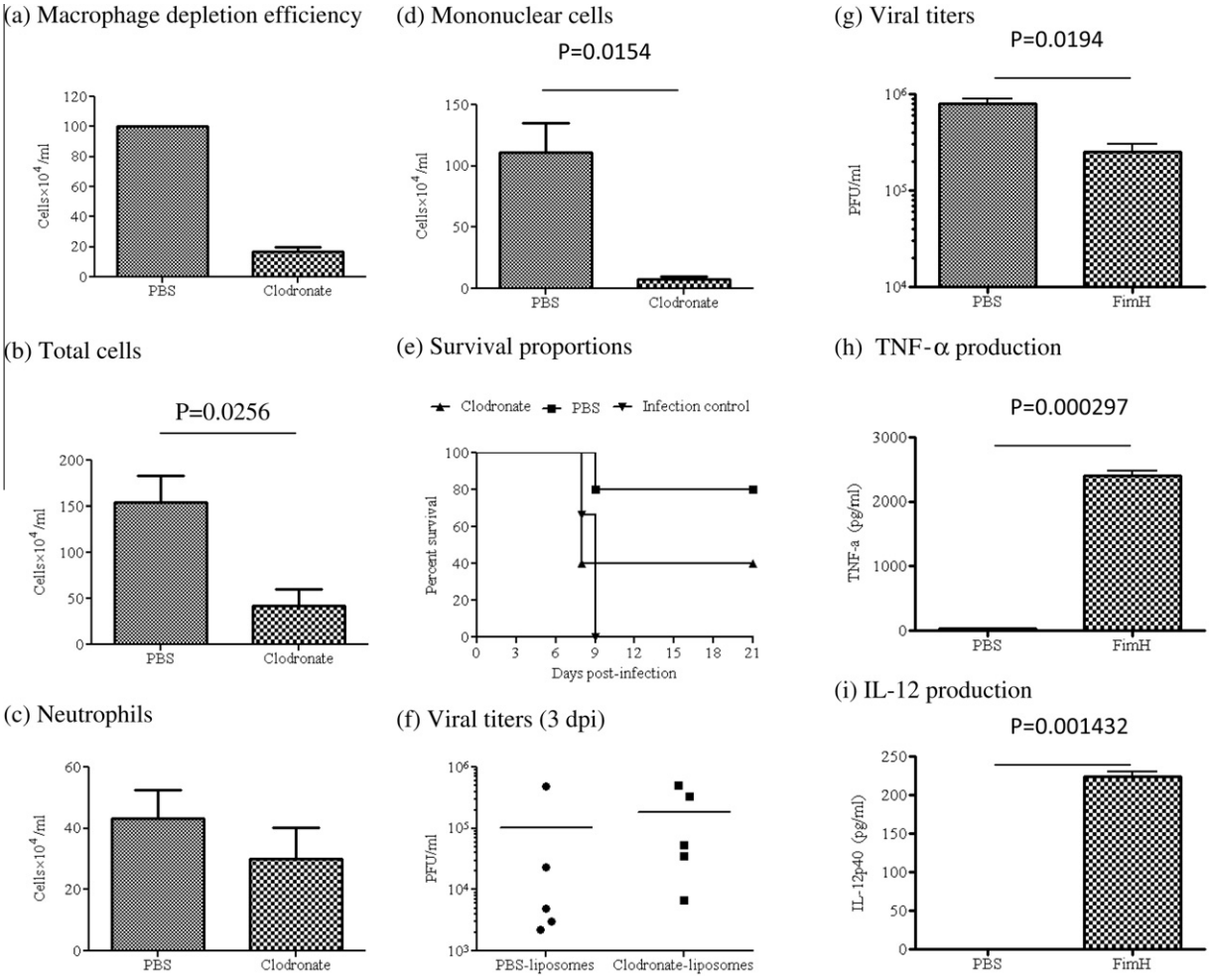

Fig. 4. Role of alveolar macrophages in TLR4 ligand-induced innate protection against influenza infection. Male C57BL/6 mice were treated either with clodronate-containing liposome (50 μl), PBS liposome or PBS (50 μl) intratracheally twice at 24 h interval. The efficiency of macrophage depletion was evaluated 24 h after the last treatment using a subset of mice. Each of the remaining mice in macrophage depleted and control groups were then treated intranasally with FimH (30 μg/mouse). BALF from five mice of each treatment group were collected after 24 h of FimH treatment and cells counted for total and differential populations. Some of the treated mice (n = 3–5 per group) were infected 24 h post treatment with 5×10^3 PFU of PR-8 influenza A virus intranasally and monitored up to 21 dpi for endpoints. (a) Impact of macrophage depletion by intratracheal administration of clodronate containing liposome in untreated mice, (b–d) represent total-, mononuclear- and neutrophil-cell counts in BALF, (e) survival propotion following viral infection and (f) 3 dpi viral titers. The BAL cells isolated from naïve C57BL/6 mice were cultured ex vivo either alone or with 10 μg/ml of FimH for 20 h. Cell free supernatants were harvested for TNF-α (h) and IL-12 (i) measurements. Cells were then washed with serum free MEM-F11 medium and infected with PR-8 strain of Influenza virus at MOI of 0.2 and 2.0 for 24 h at 37 °C. Cells were then harvested by scrapping, homogenized and stored at −80 °C. To assess the replication of virus in BAL cells, Influenza viral plaque assay was performed and results are depicted in g. The data represent results from two separate experiments.

by a number of observations. Firstly, it has been shown that local delivery of TLR4 ligands in the vaginal mucosa does not lead to detectable IFN-β production (Gill et al., 2006). In agreement with this observation but in a different context, Hutchens et al. (2008) have also reported a lack of IFN-β response in lungs following vaccinia virus infection. Further evidence supports that TLR4 activation does not lead to IFN-β production in murine alveolar macrophages, even though alveolar macrophages express TLR4 (Punturieri et al., 2004) and LPS induces TLR4 activation in human neutrophils (Tamassia et al., 2007). Furthermore, bronchial epithelial cells have been shown to be poor producers of type 1 interferons, IFN-α and IFN-β (Khaitov et al., 2009). An explanation for these observations may be the potential lack of the TRIF dependent pathway of TLR signaling in alveolar macrophages, neutrophils and respiratory epithelial cells. Evidence suggests that some cells (i.e. astrocytes, human neutrophils) lack the TRIF dependent pathway of TLR4 signaling, therefore, lack IFN-β response following LPS stimulation (Krasowska-Zoladek et al., 2007; Tamassia et al., 2007).

We demonstrated that FimH treatment of mice induced significant production of RANTES that correlated to the degree of protection against influenza infection. Alveolar macrophages are thought to be the main source of RANTES following TLR ligand stimulation (Miller et al., 2004; Punturieri et al., 2004). RANTES is a chemokine with pleiotropic activities (Levy, 2009), including attracting a variety of leukocytes to the site of inflammation and antiviral activities against HIV (Cocchi et al., 1995) and RSV (Culley et al., 2006). Significant increases in TNF-α levels in the airway lumen was also observed following local delivery of FimH. The main cellular source of TNF-α is macrophages (Becker et al., 1991; Powe and Castleman, 2009). The importance of TNF-α in influenza infection could be many fold. First, TNF-α has a direct cytolytic effect against influenza infected cells (Kuwano et al., 1993; Seo and Webster, 2002). Secondly, TNF-α can elicit an antiviral state in uninfected cells, which prevents further viral infection (Neuzil et al., 1996; Ruggiero et al., 1986). We also observed a significant elevation of IL-12 in the airway lumen following local delivery of FimH. Since FimH is a bacterial product, it is possible that alveolar macrophages were the main cellular source of IL-12 (Trinchieri, 1995). Indirectly, IL-12 could activate phagocytic cells through the activation of IFN-γ producing NK and T cells. This, in turn,

may promote the adaptive arm of the immune system for the generation of the antigen specific immune responses (Trinchieri, 1995).

We then investigated whether TLR4 signaling is required for the FimH mediated protection against influenza infection. Importantly, FimH treatment was unable to protect mice against influenza infection when delivered locally to TLR4$^{-/-}$ mice. This confirms the previously reported observation demonstrating FimH signals through TLR4 (Ashkar et al., 2008; Mossman et al., 2008). In the FimH treated wild type mice, the protection against influenza infection correlated with a significant reduction in the lung viral burdens at an early stage of infection, also to the reduction of inflammatory cells or unaltered cytokine and chemokine profiles in the airway lumen and lung parenchyma. This suggests two potential mechanisms might drive the protection against influenza infection following FimH treatment. One of the mechanisms may be the induction of antiviral effects by recruitment of inflammatory cells and their secreted innate mediators following the delivery of FimH. Our observation that FimH stimulations resulted in increased RANTES and IL-12 responses as well as neutrophil recruitment in the airway lumen that possibly elicit potent antiviral activity against influenza infection (Cocchi et al., 1995; Culley et al., 2006; Fujisawa, 2008; Hashimoto et al., 2007; Kuwano et al., 1993; Neuzil et al., 1996; Ruggiero et al., 1986). Secondly, the cells and other immune mediators brought into the airway lumen following TLR4 ligand stimulations could in turn activate a feedback inhibition loop diminishing the pulmonary inflammation characterized by the reduction of total cells, mononuclear cells, neutrophils, cytokines and chemokines to the baseline or even lower levels. These phenomena we have strikingly observed in influenza protected mice. Attenuation of pulmonary inflammatory responses could have resulted from several mechanisms. For example, IL-12 can induce production of anti-inflammatory cytokines such as IL-10 (Durrant and Metzger, 2010; Morris et al., 1994) and lead to feedback inhibition of proinflammatory mediators (D'Andrea et al., 1993) reducing pulmonary inflammation. Neutrophils could also have reduced the pulmonary damage and inflammation (Tate et al., 2009). It is also a possibility that engagement of TLR4 by their ligands up-regulate the activating transcription factor 3 (ATF3), which in turn, could attenuate proinflammatory cytokine responses by acting as a negative regulator of TLR4 signaling (Gilchrist et al., 2006; Whitmore et al., 2007). Altogether, these alveolar cells and innate mediators might have played a regulatory role in the attenuation of immune response in the lung.

It has been shown that in vivo macrophage depletion does not alter the survival following influenza infection using PR-8 strain (Tate et al., 2010). In our study, macrophage depletion followed by TLR ligand delivery resulted in decreased macrophage counts and a trend of reduction in neutrophil counts in the airway lumen. Yet, depletion of macrophages did not significantly alter the protection mediated by FimH in terms of viral titers, lung pathology and inflammatory mediators in the air way lumen. It is possible that, due to the complexity of in vivo system, macrophage depletion experiment may not have clarified the role of macrophages. It is also possible that TLR4 may be expressed in other cells in the respiratory mucosa other than alveolar macrophages (Muzio et al., 2000; Raoust et al., 2009) and they may have played a role in FimH-mediated signaling. Indeed, the data of in vitro experiment showed that FimH signal through alveolar macrophages and increases the production of cytokines and reduces influenza viral replication. When our in vivo viral titer data were individually analyzed, it is clear that three out of five PBS–liposome and FimH treated mice had much lower titers. Altogether, our in vivo and in vitro data suggest that alveolar macrophages may play a role during FimH-induced protection against influenza infection, where in, the in vivo macrophage depletion data should be interpreted cautiously since in the absence of macrophages, FimH may signal through other cells that express its receptor. The later view need to be substantiated by further studies.

Nevertheless, the work should be extended to address following issues. Only one dose was tested in the experiments described in this manuscript and the efficacy and adverse effects of higher and lower doses should be investigated in future studies. Secondly, some of the TLR ligands have been shown to be species specific (Werling et al., 2009), further studies need to be conducted to explore the applicability of our data in human influenza control. In case our data is applicable for human, FimH can be useful only for the purpose of prevention of spread of influenza infection among susceptible contacts and not as a post-exposure treatment option.

In conclusion, we have shown that prophylactic local delivery of TLR4 ligand, FimH, leads to protection against lethal influenza infection. Protection against influenza infection correlates with the reduced viral burden in the lung and cellular and cytokine changes at the airway lumen induced by the local delivery of the TLR4 ligand. Pulmonary inflammatory changes were minimal following influenza infection in the mice that received FimH treatment. The FimH mediated protection against influenza infection is partially but not critically dependent on alveolar macrophages in vivo. Our study provides insights into the use of FimH as a strategy for the prevention of respiratory viral infections, particularly influenza infection.

Acknowledgements

This work was supported in part by a CIHR grant to A.A.A. M.F. Abdul-Careem is a recipient of a postdoctoral fellowship from Canadian Institutes of Health Research (CIHR). A.A.A. is a recipient of career award in health sciences for RxD/CIHR.

Reference

Al-Garawi, A.A., Fattouh, R., Walker, T.D., Jamula, E.B., Botelho, F., Goncharova, S., Reed, J., Stampfli, M.R., O'Byrne, P.M., Coyle, A.J., Jordana, M., 2009. Acute, but not resolved, influenza A infection enhances susceptibility to house dust mite-induced allergic disease. J. Immunol. 182, 3095–3104.

Ashkar, A.A., Mossman, K.L., Coombes, B.K., Gyles, C.L., Mackenzie, R., 2008. FimH adhesin of type 1 fimbriae is a potent inducer of innate antimicrobial responses which requires TLR4 and type 1 interferon signalling. PLoS Pathog. 4, e1000233.

Ashkar, A.A., Yao, X.D., Gill, N., Sajic, D., Patrick, A.J., Rosenthal, K.L., 2004. Toll-like receptor (TLR)-3, but not TLR4, agonist protects against genital herpes infection in the absence of inflammation seen with CpG DNA. J. Infect. Dis. 190, 1841–1849.

Becker, S., Quay, J., Soukup, J., 1991. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. J. Immunol. 147, 4307–4312.

Bush, R.M., Fitch, W.M., Bender, C.A., Cox, N.J., 1999. Positive selection on the H3 hemagglutinin gene of human influenza virus A. Mol. Biol. Evol. 16, 1457–1465.

Choudhury, D., Thompson, A., Stojanoff, V., Langermann, S., Pinkner, J., Hultgren, S.J., Knight, S.D., 1999. X-ray structure of the FimC–FimH chaperone-adhesin complex from uropathogenic *Escherichia coli*. Science 285, 1061–1066.

Cluff, C.W., Baldridge, J.R., Stover, A.G., Evans, J.T., Johnson, D.A., Lacy, M.J., Clawson, V.G., Yorgensen, V.M., Johnson, C.L., Livesay, M.T., Hershberg, R.M., Persing, D.H., 2005. Synthetic toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. Infect. Immun. 73, 3044–3052.

Cocchi, F., DeVico, A.L., Garzino-Demo, A., Arya, S.K., Gallo, R.C., Lusso, P., 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 270, 1811–1815.

Corti, D., Suguitan Jr., A.L., Pinna, D., Silacci, C., Fernandez-Rodriguez, B.M., Vanzetta, F., Santos, C., Luke, C.J., Torres-Velez, F.J., Temperton, N.J., Weiss, R.A., Sallusto, F., Subbarao, K., Lanzavecchia, A., 2010. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J. Clin. Invest. 120, 1663–1673.

Crowe, C.R., Chen, K., Pociask, D.A., Alcorn, J.F., Krivich, C., Enelow, R.I., Ross, T.M., Witztum, J.L., Kolls, J.K., 2009. Critical role of IL-17RA in immunopathology of influenza infection. J. Immunol. 183, 5301–5310.

Culley, F.J., Pennycook, A.M., Tregoning, J.S., Dodd, J.S., Walzl, G., Wells, T.N., Hussell, T., Openshaw, P.J., 2006. Role of CCL5 (RANTES) in viral lung disease. J. Virol. 80, 8151–8157.

D'Andrea, A., Aste-Amezaga, M., Valiante, N.M., Ma, X., Kubin, M., Trinchieri, G., 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J. Exp. Med. 178, 1041–1048.

Devergne, O., Marfaing-Koka, A., Schall, T.J., Leger-Ravet, M.B., Sadick, M., Peuchmaur, M., Crevon, M.C., Kim, K.J., Schall, T.T., Kim, T., et al., 1994. Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. J. Exp. Med. 179, 1689–1694.

Durrant, D.M., Metzger, D.W., 2010. IL-12 can alleviate Th17-mediated allergic lung inflammation through induction of pulmonary IL-10 expression. Mucosal Immunol. 3, 301–311.

Errea, A., Moreno, G., Sisti, F., Fernandez, J., Rumbo, M., Hozbor, D.F., 2010. Mucosal innate response stimulation induced by lipopolysaccharide protects against *Bordetella pertussis* colonization. Med. Microbiol. Immunol. 199, 103–108.

Fujisawa, H., 2008. Neutrophils play an essential role in cooperation with antibody in both protection against and recovery from pulmonary infection with influenza virus in mice. J. Virol. 82, 2772–2783.

Gaush, C.R., Smith, T.F., 1968. Replication and plaque assay of influenza virus in an established line of canine kidney cells. Appl. Microbiol. 16, 588–594.

Gilchrist, M., Thorsson, V., Li, B., Rust, A.G., Korb, M., Roach, J.C., Kennedy, K., Hai, T., Bolouri, H., Aderem, A., 2006. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. Nature 441, 173–178.

Gill, N., Deacon, P.M., Lichty, B., Mossman, K.L., Ashkar, A.A., 2006. Induction of innate immunity against herpes simplex virus type 2 infection via local delivery of Toll-like receptor ligands correlates with beta interferon production. J. Virol. 80, 9943–9950.

Grebe, K.M., Takeda, K., Hickman, H.D., Bailey, A.L., Embry, A.C., Bennink, J.R., Yewdell, J.W., 2010. Cutting edge: sympathetic nervous system increases proinflammatory cytokines and exacerbates influenza A virus pathogenesis. J. Immunol. 184, 540–544.

Hashimoto, Y., Moki, T., Takizawa, T., Shiratsuchi, A., Nakanishi, Y., 2007. Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice. J. Immunol. 178, 2448–2457.

Hutchens, M.A., Luker, K.E., Sonstein, J., Nunez, G., Curtis, J.L., Luker, G.D., 2008. Protective effect of Toll-like receptor 4 in pulmonary vaccinia infection. PLoS Pathog. 4, e1000153.

Jamieson, A.M., Yu, S., Annicelli, C.H., Medzhitov, R., 2010. Influenza virus-induced glucocorticoids compromise innate host defense against a secondary bacterial infection. Cell Host Microbe 7, 103–114.

Khaitov, M.R., Laza-Stanca, V., Edwards, M.R., Walton, R.P., Rohde, G., Contoli, M., Papi, A., Stanciu, L.A., Kotenko, S.V., Johnston, S.L., 2009. Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. Allergy 64, 375–386.

Krasowska-Zoladek, A., Banaszewska, M., Kraszpulski, M., Konat, G.W., 2007. Kinetics of inflammatory response of astrocytes induced by TLR 3 and TLR4 ligation. J. Neurosci. Res. 85, 205–212.

Krogfelt, K.A., Bergmans, H., Klemm, P., 1990. Direct evidence that the FimH protein is the mannose-specific adhesin of Escherichia coli type 1 fimbriae. Infect. Immun. 58, 1995–1998.

Kuwano, K., Kawashima, T., Arai, S., 1993. Antiviral effect of TNF-alpha and IFN-gamma secreted from a CD8+ influenza virus-specific CTL clone. Viral Immunol. 6, 1–11.

Leemans, J.C., Juffermans, N.P., Florquin, S., van Rooijen, N., Vervoordeldonk, M.J., Verbon, A., van Deventer, S.J., van der Poll, T., 2001. Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. J. Immunol. 166, 4604–4611.

Lembo, A., Pelletier, M., Iyer, R., Timko, M., Dudda, J.C., West, T.E., Wilson, C.B., Hajjar, A.M., Skerrett, S.J., 2008. Administration of a synthetic TLR4 agonist protects mice from pneumonic tularemia. J. Immunol. 180, 7574–7581.

Levy, J.A., 2009. The unexpected pleiotropic activities of RANTES. J. Immunol. 182, 3945–3946.

Lin, K.L., Suzuki, Y., Nakano, H., Ramsburg, E., Gunn, M.D., 2008. CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. J. Immunol. 180, 2562–2572.

Marques, L.J., Zheng, L., Poulakis, N., Guzman, J., Costabel, U., 1999. Pentoxifylline inhibits TNF-alpha production from human alveolar macrophages. Am. J. Respir. Crit. Care Med. 159, 508–511.

Marsolais, D., Hahm, B., Walsh, K.B., Edelmann, K.H., McGavern, D., Hatta, Y., Kawaoka, Y., Rosen, H., Oldstone, M.B., 2009. A critical role for the sphingosine analog AAL-R in dampening the cytokine response during influenza virus infection. Proc. Natl. Acad. Sci. USA 106, 1560–1565.

Mian, M.F., Lauzon, N.M., Andrews, D.W., Lichty, B.D., Ashkar, A.A., 2010. FimH can directly activate human and murine natural killer cells via TLR4. Mol. Ther. 18, 1379–1388.

Miller, A.L., Bowlin, T.L., Lukacs, N.W., 2004. Respiratory syncytial virus-induced chemokine production: linking viral replication to chemokine production in vitro and in vivo. J. Infect. Dis. 189, 1419–1430.

Morris, S.C., Madden, K.B., Adamovicz, J.J., Gause, W.C., Hubbard, B.R., Gately, M.K., Finkelman, F.D., 1994. Effects of IL-12 on in vivo cytokine gene expression and Ig isotype selection. J. Immunol. 152, 1047–1056.

Mossman, K.L., Mian, M.F., Lauzon, N.M., Gyles, C.L., Lichty, B., Mackenzie, R., Gill, N., Ashkar, A.A., 2008. Cutting edge: FimH adhesin of type 1 fimbriae is a novel TLR4 ligand. J. Immunol. 181, 6702–6706.

Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L.P., Allavena, P., Mantovani, A., 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J. Immunol. 164, 5998–6004.

Nakamura, R., Maeda, N., Shibata, K., Yamada, H., Kase, T., Yoshikai, Y., 2010. Interleukin-15 is critical in the pathogenesis of influenza a virus-induced acute lung injury. J. Virol. 84, 5574–5582.

Neuzil, K.M., Tang, Y.W., Graham, B.S., 1996. Protective role of TNF-alpha in respiratory syncytial virus infection in vitro and in vivo. Am. J. Med. Sci. 311, 201–204.

Norton, E.B., Clements, J.D., Voss, T.G., Cardenas-Freytag, L., 2010. Prophylactic administration of bacterially derived immunomodulators improves the outcome of influenza virus infection in a murine model. J. Virol. 84, 2983–2995.

Powe, J.R., Castleman, W.L., 2009. Canine influenza virus replicates in alveolar macrophages and induces TNF-alpha. Vet. Pathol. 46, 1187–1196.

Punturieri, A., Alviani, R.S., Polak, T., Copper, P., Sonstein, J., Curtis, J.L., 2004. Specific engagement of TLR4 or TLR3 does not lead to IFN-beta-mediated innate signal amplification and STAT1 phosphorylation in resident murine alveolar macrophages. J. Immunol. 173, 1033–1042.

Raoust, E., Balloy, V., Garcia-Verdugo, I., Touqui, L., Ramphal, R., Chignard, M., 2009. *Pseudomonas aeruginosa* LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells. PLoS ONE 4, e7259.

Ruggiero, V., Tavernier, J., Fiers, W., Baglioni, C., 1986. Induction of the synthesis of tumor necrosis factor receptors by interferon-gamma. J. Immunol. 136, 2445–2450.

Seo, S.H., Webster, R.G., 2002. Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells. J. Virol. 76, 1071–1076.

Shahangian, A., Chow, E.K., Tian, X., Kang, J.R., Ghaffari, A., Liu, S.Y., Belperio, J.A., Cheng, G., Deng, J.C., 2009. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J. Clin. Invest. 119, 1910–1920.

Small, C.L., Shaler, C.R., McCormick, S., Jeyanathan, M., Damjanovic, D., Brown, E.G., Arck, P., Jordana, M., Kaushic, C., Ashkar, A.A., Xing, Z., 2010. Influenza infection leads to increased susceptibility to subsequent bacterial superinfection by impairing NK cell responses in the lung. J. Immunol. 184, 2048–2056.

Smith, A.M., Adler, F.R., McAuley, J.L., Gutenkunst, R.N., Ribeiro, R.M., McCullers, J.A., Perelson, A.S., 2011. Effect of 1918 PB1–F2 expression on influenza A virus infection kinetics. PLoS Comput. Biol. 7, e1001081.

Szretter, K.J., Gangappa, S., Belser, J.A., Zeng, H., Chen, H., Matsuoka, Y., Sambhara, S., Swayne, D.E., Tumpey, T.M., Katz, J.M., 2009. Early control of H5N1 influenza virus replication by the type I interferon response in mice. J. Virol. 83, 5825–5834.

Tamassia, N., Le Moigne, V., Calzetti, F., Donini, M., Gasperini, S., Ear, T., Cloutier, A., Martinez, F.O., Fabbri, M., Locati, M., Mantovani, A., McDonald, P.P., Cassatella, M.A., 2007. The MyD88-independent pathway is not mobilized in human neutrophils stimulated via TLR4. J. Immunol. 178, 7344–7356.

Tate, M.D., Deng, Y.M., Jones, J.E., Anderson, G.P., Brooks, A.G., Reading, P.C., 2009. Neutrophils ameliorate lung injury and the development of severe disease during influenza infection. J. Immunol. 183, 7441–7450.

Tate, M.D., Pickett, D.L., van Rooijen, N., Brooks, A.G., Reading, P.C., 2010. Critical role of airway macrophages in modulating disease severity during influenza virus infection of mice. J. Virol. 84, 7569–7580.

Taubenberger, J.K., Morens, D.M., 2008. The pathology of influenza virus infections. Annu. Rev. Pathol. 3, 499–522.

Trinchieri, G., 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. 13, 251–276.

Tuvim, M.J., Evans, S.E., Clement, C.G., Dickey, B.F., Gilbert, B.E., 2009. Augmented lung inflammation protects against influenza A pneumonia. PLoS ONE 4, e4176.

van Rooijen, N., Hendrikx, E., 2010. Liposomes for specific depletion of macrophages from organs and tissues. Methods Mol. Biol. 605, 189–203.

Wei, C.J., Boyington, J.C., McTamney, P.M., Kong, W.P., Pearce, M.B., Xu, L., Andersen, H., Rao, S., Tumpey, T.M., Yang, Z.Y., Nabel, G.J., 2010. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. Science 329, 1060–1064.

Werling, D., Jann, O.C., Offord, V., Glass, E.J., Coffey, T.J., 2009. Variation matters: TLR structure and species-specific pathogen recognition. Trends Immunol. 30, 124–130.

Whitmore, M.M., Iparraguirre, A., Kubelka, L., Weninger, W., Hai, T., Williams, B.R., 2007. Negative regulation of TLR-signaling pathways by activating transcription factor-3. J. Immunol. 179, 3622–3630.

Williams, A.E., Edwards, L., Humphreys, I.R., Snelgrove, R., Rae, A., Rappuoli, R., Hussell, T., 2004. Innate imprinting by the modified heat-labile toxin of *Escherichia coli* (LTK63) provides generic protection against lung infectious disease. J. Immunol. 173, 7435–7443.

Wong, J.P., Christopher, M.E., Viswanathan, S., Karpoff, N., Dai, X., Das, D., Sun, L.Q., Wang, M., Salazar, A.M., 2009. Activation of toll-like receptor signaling pathway for protection against influenza virus infection. Vaccine 27, 3481–3483.

Wong, J.P., Saravolac, E.G., Sabuda, D., Levy, H.B., Kende, M., 1995. Prophylactic and therapeutic efficacies of poly(IC.LC) against respiratory influenza A virus infection in mice. Antimicrob. Agents Chemother. 39, 2574–2576.