Detrimental Contribution of the Toll-Like Receptor (TLR)3 to Influenza A Virus–Induced Acute Pneumonia

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Influenza A virus (IAV) is the etiological agent of a highly contagious acute respiratory disease that causes epidemics and considerable mortality annually. Recently, we demonstrated, using an in vitro approach, that the pattern recognition Toll-like receptor (TLR)3 plays a key role in the immune response of lung epithelial cells to IAV. In view of these data and the fact that the functional role of TLR3 in vivo is still debated, we designed an investigation to better understand the role of TLR3 in the mechanisms of IAV pathogenesis and host immune response using an experimental murine model. The time-course of several dynamic parameters, including animal survival, respiratory suffering, viral clearance, leukocyte recruitment into the airspaces and secretion of critical inflammatory mediators, was compared in infected wild-type and TLR3−/− mice. First, we found that the pulmonary expression of TLR3 is constitutive and markedly upregulated following influenza infection in control mice. Notably, when compared to wild-type mice, infected TLR3−/− animals displayed significantly reduced inflammatory mediators, including RANTES (regulated upon activation, normal T cell expressed and secreted), interleukin-6, and interleukin-12p40/p70 as well as a lower number of CD8+ T lymphocytes in the bronchoalveolar airspace. More important, despite a higher viral production in the lungs, mice deficient in TLR3 had an unexpected survival advantage. Hence, to our knowledge, our findings show for the first time that TLR3-IAV interaction critically contributes to the debilitating effects of a detrimental host inflammatory response.

Introduction

Recent outbreaks of highly pathogenic influenza A virus (IAV) infections have had important economic repercussions and have raised concerns that a new influenza pandemic will occur in the near future. The most severe complication of influenza is acute pneumonia, which develops rapidly and may result in respiratory failure and death. The etiological agents of the disease, the single-stranded RNA influenza viruses, are classified into three types (A, B, and C), of which influenza A is clinically the most important [1]. In the United States alone, there are more than 20,000 deaths per year, and in the large pandemic of 1918, over 20 million people died worldwide [2–4]. Although vaccines and antiviral molecules to control influenza have been developed during the last years, the disease is by no means under control since these treatments are not available worldwide and their efficacy is not optimal [3–5]. Thus, a better understanding of the molecular mechanisms of IAV pathogenesis and host immune responses is required for the development of more efficient means of prevention and treatment of influenza.

The invasion of viruses is initially sensed by the host innate immune system, triggering rapid antiviral responses that involve the release of proinflammatory cytokines, and leading to the subsequent activation of adaptive immune responses. Diverse components of infecting viruses can induce the signalling pathways that regulate the cellular antiviral gene program. Among them, double-stranded RNA (dsRNA) has been viewed as the most important component. It is a common signature linked to the viral replication cycle and lysis of virus-infected cells is hypothesized to release dsRNA [6,7]. A major transducer of cell signalling generated by dsRNA is the Toll-like receptor (TLR)3, a member of a conserved family of innate immune recognition receptors that have key roles in detecting microbes, initiating innate immune responses, and linking innate and adaptive immunity [8–10].

A role for TLR3 in viral detection has been suggested by in vitro and ex vivo studies [8,11] but except for a recent study

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Abbreviations: BAL, bronchoalveolar lavage; dsRNA, double-stranded RNA; G-CSF, granulocyte colony-stimulating factor; IAV, influenza A virus; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; MyD, myeloid differentiation; pfu, plaque-forming units; qRT-PCR, quantitative RT-PCR; RANTES, regulated upon activation, normal T cell expressed and secreted; TLR, Toll-like receptor; TNF, tumor necrosis factor

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Synopsis

Influenza A virus (IAV) is responsible for highly contagious acute respiratory disease. Recent concerns have risen concerning a possible influenza pandemic in the near future. Thus, a better understanding of the molecular mechanisms of IAV pathogenesis and host immune responses is required for the development of more efficient means of prevention and treatment of influenza. The Toll-like receptor (TLR)3 is a member of a family of receptors that detects microbes and triggers host defenses. We previously demonstrated using an in vitro approach, that the TLR3 plays a key role in the response of lung epithelial cells to IAV. Here, we used a mouse model to dissect the in vivo importance of TLR3-dependent responses during influenza. The time-course of several parameters, including animal survival, respiratory distress, viral clearance, and inflammation, was compared in infected control wild-type and TLR3-deficient mice. Our findings reveal that TLR3-deficient mice have an unexpected advantage against IAV challenge as we show for the first time that a reduction of TLR3-mediated inflammatory response reduces the clinical manifestations of IAV-induced pneumonia.

that has clearly shown that TLR3 mediates West Nile virus entry into the brain, causing lethal encephalitis, the functional role of TLR3 in vivo remains unclear [12,13]. Recently, using an in vitro approach, we demonstrated that TLR3 and its signaling-associated molecule TRIF play a key role in the immune response of respiratory epithelial cells to IAV [14]. In the current study, we used TLR3-deficient (TLR3−/−) mice to study the specific role of this pattern-recognition receptor in IAV-mediated acute pneumonia. Our findings clearly indicate that TLR3 contributes to a detrimental inflammatory response.

Results

Kinetics of Pathological Features Associated with IAV Infection

The pathogenesis and immune response associated with influenza pneumonia are assumed to be very complex [15–17]. To further dissect the role of TLR3 in IAV sensing and pathogenesis, we first characterized in infected wild-type animals the time-course of major dynamic parameters, including animal mortality and weight, leukocyte recruitment into the airspaces, increase in alveolocapillary permeability, and secretion of critical mediators. Figure 1A shows that all C57Bl/6 mice inoculated intranasally with IAV at a dose of 300 plaque-forming units (pfu) per mouse died within 12 d. Signs of piloerection and anorexia were associated with a loss of weight (approximately 32%) and appeared after 4 d of infection (Figure 1B). Animals were killed at different intervals postinfection, and bronchoalveolar lavage (BAL) samples were collected to assess cellular infiltration and mediators content in the airspaces. Figure 1C (square symbol) shows a biphasic leukocyte recruitment constituted mainly of polymorphonuclear cells (circle symbol) by days 3 to 8.

Figure 1. Time-course of Dynamic Parameters in Wild-Type Mice Infected by a Lethal IAV Challenge

Male C57Bl/6 mice were infected intranasally with 300 pfu of IAV and different parameters were analyzed during the course of infection. (A) Survival of mice. (B) Body weight changes. (C) Leukocyte recruitment into the airways (●, all leukocytes; ○, polymorphonuclear cells; ▲, mononuclear cells). (D and E) RANTES and IL-6 production in BAL fluids. (F) Total protein amount in BAL fluids as an index of alveolocapillary permeability. All these results are the mean ± SD values obtained from three distinct animals and are representative of three independent experiments.

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postinfection and of mononuclear cells (triangle symbol) afterward. The kinetics of IAV-induced secretion of a major cytokine (IL-6) and a CC chemokine (RANTES) was investigated. While RANTES peaked at day 3 and decreased significantly thereafter (Figure 1D), IL-6 secretion increased steadily until day 4 and was sustained at that level until day 11 (Figure 1E). As an index of transudation from the vascular compartment into the lungs, the amount of total protein was determined in the BAL (Figure 1F). Interestingly, the protein concentration curve parallels that of total leukocyte content (Figure 1C, square symbol) confirming a connection between increased microvascular permeability and cellular infiltration during acute lung injury [18]. BAL fluids were further analyzed by an inflammatory protein array to examine at days 3 and 10 postinfection whether other major components, not measured in the initial assays, were affected during influenza pneumonia. A total of 32 mediators were measured, which included 16 cytokines, 11 chemokines, three growth factors, one metalloproteinase inhibitor, and one soluble cytokine receptor (example of such protein array blot is shown later in Figure 4A). This array analysis indicated selective major increases of granulocyte colony-stimulating factor (G-CSF), IL-6, monocyte chemoattractant protein-1 (MCP-1), MCP-5, macrophage inflammatory protein-2α, RANTES, soluble tumor necrosis factor-α (TNFα) receptor, and tissue inhibitor of metalloproteinase-1 (not illustrated).

Next, we checked that the lethal pneumonia induced by IAV was not associated with a bacteria superinfection, as it has been reported by others [19]. We showed that bacteria counts in blood or BAL of IAV-treated mice at day 3, 6, 8, or 10 postinfection were negligible (constantly below 250 cfu/ml). In addition, intramuscular administration of the mice with a large-spectrum antibiotic (Clamoxyl; amoxicillin trihydrate-potassium clavulanate, 25 mg/kg) before and during the viral infection did not reduce or delay the survival pattern (unpublished data).

**IAV Upregulates the Lung Expression of TLR3**

We previously demonstrated that of all the mediators we tested, i.e., bacterial LPS, the cytokines TNFα and IL-1β, the protein kinase C activator PMA, IAV, and the synthetic dsRNA poly(I:C), only the two latter stimuli upregulated TLR3 expression in human pulmonary epithelial cells, suggesting that the signaling pathways controlling the induction of this gene are restricted [14]. Given the distinct complexity between in vitro and in vivo systems, there was a need to determine the regulation and the function of TLR3 in relation to the pathogenesis of influenza in an experimental animal.
The results clearly show that the disease is further prolonged survival after a challenge of 300 pfu IAV than did wild-type animals (0% survival for both groups, Figure 2B, right upper panel; n = 8). Then, to monitor the role of TLR3 in lung dysfunction induced by the viral infection, the respiratory distress index Penh and total protein amount as well as the secretion of inflammatory cytokines and chemokines were evaluated in BAL fluids of TLR3−/− and wild-type animals at day 3 postinfection by 300 pfu IAV (this time point was chosen as it corresponds to the peak of the viral load in the lungs of both animal groups, cf. Figure 5). Figure 3A shows that the Penh index was significantly diminished in TLR3−/− mice compared to control mice, i.e., 0.95 ± 0.12 versus 1.74 ± 0.2, respectively (n = 5, p = 0.005). Likewise, total protein, RANTES, and IL-6 amounts were significantly lower in TLR3−/− than in wild-type mice (Figure 3B–3D). BAL fluids were further analyzed by an inflammatory protein array to examine at day 9 whether the expression of additional mediators was directly regulated by TLR3. Figure 4A not only confirms a clearly reduced IL-6 production in the lungs of TLR3−/− mice but also reveals a similar inhibition concerning inflammatory mediators such as IL-12p40/p70, sTNFR1, and tissue inhibitor of metalloproteinase-1. On the contrary, the expression of distinct components is increased in TLR3−/− versus wild-type lungs, including not only INF-γ but also G-CSF and, to a lesser extent, IL-9 and IL-10. As additional evidence, we showed by ELISA that, although similar at day 4 to day 7 post–viral infection, the amount of interferon (IFN)-γ in TLR3−/− mice was greater (approximately 14.5 times that of wild-type animals) at day 9 (Figure 4B). Finally, Figure 4C presents a typical gross morphological view of perfused lungs isolated from noninfected wild-type and TLR3−/− mice (lower panel) and at day 9 postinfection by 300 pfu of IAV (upper panel). Lungs of wild-type animals appeared severely injured as manifested by an almost black hemorrhaged lung surface, whereas those obtained from TLR3−/− mice produced only faintly and diffuse red lungs, suggesting that the lesions induced by IAV are reduced in the absence of TLR3.

**Paradoxical Increased Lung Viral Load in TLR3−/− Mice**

The previous results indicated that a potent inflammatory reaction occurs in the lungs of wild-type mice after influenza infection and that this process is critically reduced or altered in TLR3−/− animals.

Inflammatory signaling pathways during viral infection have been interpreted in some cases as a protective response of the host, whereas in other cases the virus can utilize these pathways to enhance its replication [20]. Thus, we first used quantitative RT-PCR (qRT-PCR) to investigate whether TLR3-dependent host response might regulate the replication of IAV. Figure 5 shows that virus replication was major in both wild-type and TLR3−/− mice with very similar titers when examined on days 1 to 4 post–viral infection. Paradoxically, while control animals had significantly reduced the viral load of their lungs at day 9 postinfection, the virus persisted in TLR3−/− mice with an elevated amount (approximately nine times that of wild-type animals). Note that we also used a more traditional method to accurately determine IAV potency. Viral replication was studied in MDCK cell lines at day 9 postinfection, according to a standard protocol [21]. We confirmed that TLR3−/− mice had a significantly higher IAV amount in their lungs compared to wild-type animals (p < 0.001).

**Wild-Type and TLR3−/− Mice Raise a Contrasted Leukocyte Content in Their Lungs after Infection by IAV**

Some of the foregoing findings suggested that a TLR3-mediated host immune response plays a harmful role in the model. We first showed that the expression of this receptor in the lungs of infected mice is constitutive and markedly upregulated following IAV administration, peaking at day 3 and slightly decreasing at day 9 postinfection (Figure 2A).
pathogenesis of IAV infection. In that regard, it is of note that several studies revealed a functional redundancy and synergy of different immune cells in the antiviral response to IAV [15,17,22]. Thus, to gain insight into which cell type can possibly participate in the TLR3-regulated detrimental immune response, leukocytes were harvested from the BAL of wild-type and TLR3−/− mice 9 d postinfection and the number and phenotype of the cells were characterized by flow cytometry. Infection of wild-type mice with IAV resulted in a significant increase in the number of leukocytes (cf. Figure 1C), and the accumulated cells in the BAL were composed mostly of T-lymphocytes (55 × 10^4 ± 12.5 × 10^4 BAL), neutrophils (25 × 10^4 ± 4.1 × 10^4 BAL), and macrophages (11 × 10^4 ± 2.2 × 10^4 BAL) (Figure 6, left and central panels); these numbers have to be compared to a leukocyte population in naïve mice constituted by approximately 90% macrophages (Figure 6, left panels and [23]). The absolute numbers of the whole leukocyte population in the BALs from either infected wild-type or TLR3−/− mice were similar (approximately 1 × 10^7 cells). Among the T lymphocytes, the CD8+ T cells were the predominant cell population in the lungs of infected wild-type animals (37.3 × 10^4 ± 7.8 × 10^4)
Discussion

Our understanding of how the immune system recognizes pathogens has increased exponentially in recent years due to the discovery of the TLR family. TLRs play a central role in the detection of pathogen-associated molecular patterns and in the initiation of an effective innate and adaptive immune response [9,10]. In agreement with this paradigm, there is accumulating in vivo evidence using TLR−/− mice that support a role for these receptors in antibacterial, antifungal, and antiviral defense. Moreover, several clinical reports confirm the contribution of TLRs to the pathophysiology of infectious diseases, and polymorphisms in TLR genes are associated with predisposition to severe infections [24].

In view of this major information, we anticipated at the start of our investigation that TLR3 would act as a protective component and, conversely, its absence in TLR3−/− mice would render the animals more susceptible to IAV infection. On the contrary, our study reveals that mice deficient in TLR3 have an unexpected advantage. Thus, in comparison with wild-type mice, we found in TLR3−/− animals: (i) a clearly reduced level of inflammatory mediators in the bronchoalveolar spaces, including RANTES, IL-6, and IL-12p40/p70; (ii) a lower number of the predominant leukocyte population in the airspaces, i.e., the CD8+ T cells; and, most important, (iii) a paradoxical longer survival. Based on these findings and in view of previous work describing the major role of CD8+ T lymphocytes and cytokines in the pathogenesis of influenza infection, we discuss below the fact that the enhanced resistance of IAV-infected TLR3−/− might be due to a lower TLR3-mediated release of inflammatory mediators and T cell infiltration in the lung airspaces.

It is established that IAV replicates in epithelial cells and leukocytes, resulting in the production of chemokines and cytokines that favors the recruitment of mononuclear cell population to the site of infection. There is growing evidence that the mediators that have a central role in the resolution of influenza are the same that can be the cause of many clinical signs related to this pathology [25–27]. We confirm in the present study that influenza infection leads to the synthesis of major inflammatory cytokines and chemokines, including IL-6, G-CSF, IL-12p40/p70, MCPs, macrophage inflammatory proteins, and RANTES. Remarkably, IL-6 exhibits multifunctional activities that are largely proinflammatory and its release has been correlated with the symptom pathogenesis during acute influenza [26]. IL-12 administration was also found to have an adverse effect on the course of influenza infection [27]. Other studies reported the involvement of cytokines in influenza pathogenesis, but the blockade of one individual cytokine appears somewhat partial as there is a substantial redundancy between cytokines [15,17]. Notably, our study clearly establishes that TLR3 plays a major role in the inflammatory cytokine response to IAV, the lack of TLR3 resulting in a significant decrease of cytokine synthesis, including that of IL-6, IL-12p40/p70, and RANTES. Ongoing loss-of-function in vitro studies with human pulmonary epithelial cells also demonstrate an essential function for TLR3 in the production of inflammatory cytokines, including IL-6, after IAV challenge (unpublished data). On the contrary, the expression of some other mediators is increased in TLR3−/− versus wild-type lungs. The consequences and the mechanisms of this TLR3-regulated differential expression of cytokines and chemokines are rather complex to hypothesize at this stage due to the pleiotropic and multiple effects of these mediators on diverse cell types. Moreover, these molecules induce or inhibit the production of other cytokines or mediators from their targets in a complex array of positive and negative feedback loops [15,17].

Regardless, consistent with the role of TLR3 in virus-induced inflammation, two of us recently established that infection of TLR3−/− mice with the West Nile virus induces a lower secretion of cytokines, including IL-6 and TNF-α, compared to wild-type mice, that leads to reduced neuronal injury and increased survival in TLR3−/− mice [13]. Similarly, in a study examining the role of another TLR in the host response to herpes simplex virus, Kurt-Jones et al. [28] demonstrated an attenuated cytokine response parallel to a reduction in symptoms of encephalitis in TLR2−/− compared to control animals. It is of note, however, that our results still demonstrate high mortality when the TLR3−/− strain was infected by a high viral amount of IAV, although the difference between this group and wild-type animals is highly significant (p < 0.0001). This finding suggests that TLR3 contributes to the shaping of a harmful innate and adaptive immune response at an extent that varies under the IAV load, i.e., that supplementary signaling pathways may be involved at high viral load. In that regard, it is of note that while TLR3 has emerged as a key sensor of viral dsRNA, very recent studies show that cells also express RNA helicases that...
function as alternative pattern-recognition receptors that
detect actively replicating viruses in cytoplasm (reviewed in
[29]). Hence, depending on the IAV dose, it is tempting to
speculate that the activity of one dsRNA receptor or the other
may be transiently switched on or off. The in vivo evidence
that RNA helicases–IAV interaction contributes to the
debilitating effects of a detrimental inflammation awaits
further studies.

Figure 6. Wild-Type and TLR3−/− Mice Raise a Contrasted Leukocyte Content in Their Lungs after IAV Challenge

BAL cells were collected from IAV-infected mice at day 9 postinfection. To characterize the recovered leukocyte cell types, polymorphonuclear
neutrophils (PMN, Gr1⁺, CD11b⁺), macrophages (Mφ, F4/80⁺), CD4⁺ T lymphocytes (CD4), and CD8⁺ T lymphocytes (CD8) were stained with
fluorescently labeled specific antibodies. Far right and far left dot-plots: Representative BAL cell composition of naive wild-type and TLR3−/− mice. Right
and left dot-plots: Representative BAL cell composition of wild-type mice and TLR3−/− mice at day 9 postinfection by 300 pfu of IAV. Central histograms:
Results are the mean ± SD obtained from 12 wild-type mice and seven TLR3−/− mice (**p < 0.01, ***p < 0.001, ****p < 0.0001).

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Interestingly, cytokines play critical roles in shaping subsequent adaptive T cell responses. This is the case for IFN-γ, which is generally considered as essential for the control of many microbial infections, and its important role in the antiviral immune defense is highlighted by the fact that several viruses encode proteins designed to interfere with IFN-γ signaling [30]. However, most current evidence studies suggest that it may also exert a strong negative effect on the CD8⁺ T cell, a critical cellular component of the adaptive immune response. Thus, T cells treated with IFN-γ show reduced proliferation and/or increased apoptosis [31]. Moreover, IFN-γ can act as a negative feedback regulator to control Th1-mediated immune responses [32]. In view of those data, it may be conceivable that the excessive IFN-γ recovered from the airspaces of TLR3⁺⁺ mice might contribute to a decrease in CD8⁺ T cell number and to a limitation of T cell–mediated inflammation in this group of animals, in comparison with wild-type mice. Indeed, although the recruitment of CD8⁺ T cells is essential for protective responses, it is becoming increasingly evident that they can also be associated with the development of influenza-related immunopathological sequelae [33,34]. We confirmed that CD8⁺ T cells are prominent in the airways of IAV-challenged immunocompetent wild-type mice. The drastic decrease in CD8⁺ T lymphocyte infiltration concomitant with the prolonged survival of the TLR3⁻⁻ mice suggests that a dysregulated TLR3-dependent CD8⁺ T cell response may lead to sustained lung injury. Accordingly, this idea sheds new light on previous studies that have reported that mice given antilymphocyte serum [35] or the immunosuppressive drug cyclophosphamide had a lesser pulmonary pathology when treated earlier [8,46]. Moreover, antisera against TLR3 were observed at least eight times with C57Bl/6 to ensure similar genetic backgrounds. Mice strains were bred in an animal facility in accordance with Pasteur Institute guidelines in compliance with the European animal welfare regulation.

**Materials and Methods**

**Virus preparation and quantification.** Influenza A/Scotland/2074 (H3N2) virus was generously provided by N. Escrivo and S. Van Der Werf (Unité de Génétique Moléculaire des Virus Respiratoires, Institut Pasteur, Paris, France). The virus was prepared as previously described [14]. Viral RNA was isolated from the viral stock with an RNAeasy mini kit (Qiagen, Hilden, Germany) and quantified with a Nanodrop ND-1000 spectrophotometer (Rockland, Delaware, United States). CDNA derived from the viral RNA sample was used as a standard for a qRT-PCR. qRT-PCR was then performed using specific primers (sense: 5’ AAG ACC AAT CCT GTC ACC TCT GA 3’ and antisense: 5’ CAA AGC GTC TAC GCC GCA GTC C 3’). Prolog, Biogen, France) that complement 20 temporally and spatially divergent influenza A matrix protein gene sequences, as previously described [45].

**Mouse strains.** Mice were bred in an animal facility with C57Bl/10 mice purchased from the Centre d’Elevage R. Janvier (Le Genest Saint-Ise, France) and were used at about 8 wk of age. MyD88⁻⁻ and TLR3⁻⁻ mice were obtained from Dr. V. V. N. Akira (Osaka University, Osaka, Japan). Each type of mice was backcrossed at least eight times with C57Bl/6 to ensure similar genetic backgrounds. Mice strains were bred in an animal facility in pathogen-free conditions. Mice were fed normal mouse chow and water ad libitum and were housed under standard conditions with air filtration. For experiments of infection by IAV, mice were housed in cages inside stainless steel isolation cabinets that were ventilated under negative pressure with HEPA-filtered air. Mice were treated in accordance with Pasteur Institute guidelines in compliance with the European animal welfare regulation.

**Animal fluid collection.** Mice were anesthetized by a mixture of ketamine-xylazine (1 and 0.2 mg per mouse, respectively) and infected intranasally with 50 μl of PBS containing 300, 100, or 30 pfu IAV. Mice were observed daily for signs of morbidity. All mice, were killed at different time points by intra-peritoneal injection of 300 mg/kg sodium pentobarbital, and 1 ml of heparinized blood was collected by the vena cava. After centrifugation at 3000 g, the resulting plasma was stored. Airways were washed twice with 1 ml of saline, and the BAL was collected to further determine cell differential counts and percentages using a Coulter counter (Coulter-Electronics, Margency, France) as well as a Diff-Quik staining (Baxter-Dale, Dudingen, Germany) of cytosin slides. Aliquots of BAL fluids were stored for total protein and cytokine measurement. For flow cytometry analysis, cells were eventually counted and stained with Cytofix-Cytoperm (Sero, Omaha, Nebraska, United States). Under these conditions, cells could be stored for 1 wk at 4 °C before labeling for flow cytometry analysis.

**Monoclonal antibodies for flow cytometry analysis.** MAbs reactive to CD11b (Mac-1, M170, rat IgG2a), Ly-6G-Gr1 (clone RB6-8C5, rat IgG2a), Ly-6C (clone 5G12, rat IgG2b), and CD4 (clone RM4–5, rat IgG1) were purchased from BD Pharmingen (San Diego, Calif, and purchased from Caltag Laboratories, Burlingame, Calif, United States) as conjugated to fluorescein isothiocyanate, phycoerythrin, or cd-Chrome. Phycoerythrin-conjugated F4/80 (clone C1:7A3–4, rat IgG2b) was purchased from Caltag Laboratories, Burlingame, Calif, United States. Before flow cytometry analysis, cells were washed in PBS containing 5% FCS and stained for 30 min at 4 °C with the conjugated Abs. Cells were further washed twice and analyzed on a FACScan flow cytometer (BD Pharmingen).

**RANTES, IL-6, and IFN-γ ELISA.** Murine RANTES, IL-6, and IFN-γ concentrations in BAL were determined using DuoSet ELISA kits obtained from R&D Systems (Minneapolis, Minn, United States).

**Inflammatory protein array.** A commercial antibody-based protein array designed to detect 32 inflammatory mediators was used according to the manufacturer’s instructions (RayBio Mouse Cyto-kine Array II; RayBiotech, Atlanta, Georgia, United States). Membrane arrays were hybridized with BAL fluids to compare different types of mice and different time points and were always processed simultaneously. Array images were recorded after amplification with an Ultra-Lum system (Ultra-Lum, Claremont, Calif, United States), and all scanned images accurately reproduced spots seen on film (Q).
RNA. PCR was performed using specific primers (Proligo, Evry, France) for mouse TLR3 (sense: 5′ GCT CAT TGC CCC TGT CTC AC 3′; antisense: 5′ CCC GAA AAC AAT CTC CTA AA 3′). As an internal control, we used primers for mouse β-actin (sense: 5′ GGA CTC CTA TGT GGG TGA CGA 3′; antisense: 5′ GGG AGG ACA TAG CCC TCG TAG AT 3′). Amplifications were performed in a Peltier thermal cycler (MJ Research, Watertown, Massachusetts, United States) using the Qiio2q polymerase (Qbiogene, Illkirch, France). To detect mTLR3, the thermocycling protocol was 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min. To detect β-actin, only 30 cycles were used and the annealing temperature was 62 °C. Amplification products were resolved on 1.5% agarose gel containing ethidium bromide. Band intensities on gels were recorded after amplification with an Ultra-Lum system. Samples for each point were serially diluted to verify that PCR was performed in the linear phase of the amplification reaction (unpublished data).

Assessment of the basal respiratory function. Unrestrained conscious mice, infected or not by IAV, were placed in a whole body plethysmographic chamber (Buxco Electronics, Sharon, Connecticut, United States) to analyze the respiratory waveform. The system measured the magnitude and the slope of the chamber pressure. After a 10-min stabilization period, the basal respiratory capacity of each individual mouse was estimated by recording the enhanced pause pressure expressed as “Penh,” calculated as Penh = [Te (expiration time) - Tc (relaxation time)] × 1 ÷ Pp (peak inspiratory flow)/Pf (peak inspiratory flow). The values of Penh expressed per minute were averaged from three determinations recorded every 20 s. A Penh increase is an indicator of deterioration in airway mechanics.

Statistical analysis. Statistical significance between the individual groups was analyzed using the unpaired Student's t test with a threshold of p < 0.05. Survival of mice was compared using Kaplan-Meier analysis and log-rank test.

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