Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection

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Although they are continuously exposed to environmental antigens, the mucosal surfaces of the respiratory tract must restrain excessive inflammatory responses to fulfill its role of gaseous exchange and prevent bystander tissue damage. Powerful mechanical and immunosuppressive mechanisms protect the lungs against most external aggressions, yet repeat infections with variable immunopathological outcome are common. In recent years, epidemiological and animal model data have demonstrated that one respiratory tract infection alters immunity and pathology to a second unrelated pathogen, even long after the resolution of the first pathogen and in the absence of cross-reactive immunity (1, 2). The outcome of successive infections may be beneficial for the host, as shown for some respiratory virus combinations (2, 3), or it may be significantly worse, as illustrated by the increased susceptibility to life-threatening bacterial pneumonia in patients infected with seasonal and pandemic influenza (4, 5). Altogether, these data suggest that the lung undergoes some form of education or maturation that alters the way it responds to subsequent challenges.

In the absence of shared T and B cell epitopes, what does this maturation or adaptation involve? Bystander activation of unrelated memory T cells known to be retained in the lungs long after resolution of respiratory viral infection (6, 7) may modify the local cytokine balance and influence immunity and pathology to subsequent infections (2). Regulatory T cells and their associated immune suppressive cytokines, such as IL-10, may also be initiated by the first infection and reduce the amplitude of immunity to the next infection. We often attribute immune adaptation...
to acquired T and B lymphocytes and assume that innate immune compartments return to their preinfection state even after multiple waves of inflammation. However, the number of dendritic cells and their ability to prime T cells remain elevated in the murine lungs long after the resolution of an influenza or respiratory syncytial virus (RSV) infection (8, 9). Influenza infection also leads to the long-term formation of inducible bronchus-associated lymphoid tissue in the lungs where these dendritic cells are located (10). We now provide evidence that Toll-like receptor (TLR) responsiveness is “reset” after the first wave of inflammation. Specifically, we show a long-term desensitization of lung alveolar macrophages (AM) to the TLR ligands flagellin, LPS, and lipoteichoic acid (LTA). Ligation of TLRs is associated with inflammatory signaling cascades, culminating in cytokine and chemokine production that ultimately leads to pathogen clearance (11). We show that AMs, which are isolated after resolution of lung influenza infection, have impaired NF-κB nuclear translocation to TLR ligations, which is associated with reduced subsequent acute inflammation and cell recruitment. The reduced neutrophil recruitment correlates with a higher and prolonged respiratory bacterial load. Our observations are important because they reveal that a crucial pathway of innate immunity does not return to a preinfection state, which may contribute to the increased susceptibility to common secondary bacterial pneumonias associated with pandemic and seasonal influenza infection.

RESULTS AND DISCUSSION

We have previously shown that excessive inflammation and pathology associated with RSV or Cryptococcus neoformans infection is attenuated in mice previously infected with influenza virus (unpublished data) (2). This does not depend on cross-reactive adaptive immunity, and occurs even 6 mo after the initial infection. To investigate the putative role of innate immunity in this process, we introduced the bacterial TLR5 agonist flagellin, which produces transient cytokine release and rapid recruitment of neutrophils and macrophages to the lungs (12, 13), to such “post-influenza” mice, leaving an interval of at least 4 wk. At this time, virus is not detectable in the lungs, the mice have recovered their initial starting weight, and total lung cellularity and proinflammatory cytokines have returned to preinfection levels (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070891/DC1). As previously reported (8, 10), minor populations of CD8+ T cells, CD11c+ cells, and isolated lymphoid aggregates were observed in the post-influenza lungs, whereas the overall architecture of the lungs was similar to noninfected animals (Fig. S2). Administration of the TLR5 ligand flagellin into the post-influenza lungs caused a significantly reduced early neutrophil transmigration into the airways compared with control mice (88.1 ± 8.3% reduction; n = 16; P < 0.001; Fig. 1, A and C), which was confirmed by immunohistology (unpublished data) and was not caused by a delay in the kinetics of recruitment (24-h time point; Fig. 1 B). This effect was independent of the mouse genetic background (Fig. 1 A, C57BL/6 and BALB/c), and was evident even when the interval between influenza and flagellin was increased to 3 or 6 mo (Fig. 1 C).

A similar impairment of neutrophil recruitment in the post-influenza airways was also observed with the TLR4 agonist LPS (Fig. 2 A), which is a major trigger of inflammation during Gram-negative bacterial infection and is often used as a model for acute lung injury. At 48 h, a reduction in macrophage recruitment was also observed (control, 5.8 ± 0.2 × 10^5; post-influenza, 3.4 ± 1.4 × 10^5; n = 4; P = 0.041), suggesting that general cell recruitment is affected in post-influenza lungs. Neutrophil recruitment to TLR2 ligation (LTA), which is associated with recognition of Gram-positive bacteria, also showed a modest reduction (Fig. 2 A). In addition, this effect was not observed with inactivated virus (unpublished data) and could be extended to the noncytopathic virus RSV (84.1 ± 10.7% reduction of neutrophils 6 h after flagellin challenge; n = 5; P = 0.028). Because desensitization to bacterial microbial-associated molecular patterns after resolution of influenza infection may explain why secondary bacterial infections occur, we next
Reduced airway cell recruitment occurs after secondary bacterial challenge. Control (PBS-treated) or post-influenza mice (6 or 2 wk after influenza, as indicated) were inoculated with 1 μg LPS or 25 μg LTA (A) or were infected with 5 × 10^6 CFU of P. aeruginosa PAK strain (B; n = 5–8 mice/group), 5 × 10^6 CFU of group B streptococcus (C; n = 6 mice/group), or 10^6 CFU of S. pneumoniae (D; n = 6–15 mice/group). The cell number in the airways was monitored at the time points indicated. The number of bacteria was also evaluated in the lungs of infected mice (B–D). *, 3 d after infection, one control mouse out of 6 did not survive the infection (17%), whereas 3 out of 5 mice died in post-influenza mice (60% death). Error bars represent the mean ± the SEM.

Figure 2.

Examined the response to the opportunistic Gram-negative bacterium *Pseudomonas aeruginosa* because early containment of this organism is known to require TLR4 and TLR5 (13, 14). As observed with flagellin and LPS, neutrophil recruitment was reduced up to fourfold at 4 h and correlated with enhanced bacterial load (Fig. 2B).

Similar results were obtained for the Gram-positive organism group B streptococcus. Airway cellularity was also reduced, and the bacterial burden was enhanced in the post-influenza lungs (Fig. 2C). *Streptococcus pneumoniae* is a common pathogen in secondary pneumonia after influenza infection. Peak neutrophil recruitment was once again affected 6 wk after influenza infection, and uncontrolled bacterial replication led to death in 60% of mice compared with 17% in control animals (Fig. 2D). In patients, secondary bacterial infections usually occur within 2 wk of influenza infection (4). Reducing the time interval to 2 wk between the 2 infections produced the same effect in our model (Fig. 2D).

Reduced neutrophilia to a second respiratory stimulus may reflect enhanced apoptosis within the lungs, an inability to traverse the epithelial layer, or a reduction of chemotactic signals to draw them there. We show it is the latter. Although some viral infections induce apoptosis of granulocytes during the acute phase of infection (15), the proportion of apoptotic neutrophils was low and comparable between post-influenza (4.7 ± 1.4%) and control mice (4.8 ± 0.9%) 6 h after flagellin challenge (n = 5). Impaired neutrophil transmigration, which is caused by possible modification of the lung microstructure after virus infection, was ruled out by showing equivalent numbers recruited to the airways of control and post-influenza mice after intranasal (i.n.) administration of the neutrophil chemoattractants, KC (CXCL1), and MIP-2α (CXCL2; Fig. 3A) (16). However, flagellin-mediated induction of KC and MIP-2α (Fig. 3B) and proinflammatory cytokines (Fig. 3C) were reduced in the post-influenza lungs and airways compared with controls. We tested and confirmed that this impaired chemotactic signal was not caused by antimicrobial factors or neutralizing antibodies secreted in the airways that could potentially neutralize flagellin (unpublished data). In addition to protein, we show that KC and MIP-2α mRNA transcripts are reduced in post-influenza mice (Fig. 3D). Altogether, these data indicate that the local induction of the TLR signaling pathway is altered by prior viral lung exposure.

We next investigated which cell types were desensitized to TLR-mediated signals. AMs and radioresistant, nonhematopoietic resident cells such as epithelium are instrumental in inducing TLR-dependent early innate responses (13, 17, 18). We identified that systemic (i.v.) administration of flagellin, which is likely to target lung endothelial cells (19), did not impair neutrophil infiltration in the lung parenchyma or at peripheral sites (Fig. 3A and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20070891/DC1), which suggests that alterations in airway apical TLR signaling is involved when flagellin is administered i.n. Therefore, we first investigated to what extent AMs were affected in post-influenza lungs. The reduction in cytokine production is not explained by reduced numbers (Fig. 1A) or an alteration in TLR levels (Fig. 4A). AMs isolated 1 h after flagellin challenge and tested directly ex vivo displayed reduced mRNA transcripts for KC, MIP-2α, and TNF-α in post-influenza compared with control mice (Fig. 4B), suggesting that transcriptional control of these genes is responsible for the reduced level of cytokines observed (Fig. 3, B and C).

Removal and analysis of cells ex vivo does not indicate whether transcriptional regulation occurs independently in this cell type or as a result of cooperation with others. Cross talk between AMs and alveolar epithelial cells (AECs) is known to occur in vivo (20). To address this issue, we next individually isolated these populations from post-influenza mice and examined flagellin-induced NF-κB activation in vitro. Nuclear translocation of the p65 subunit of NF-κB in response to flagellin was inhibited in AMs (Fig. 5A) isolated from post-influenza mice, which was associated with a defect in chemokine production (Fig. 5B). In contrast, flagellin-induced activation of NF-κB was not altered in post-influenza AECs (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20070891/DC1). We therefore propose a model whereby sustained reduction of NF-κB activation in AMs after the initial viral infection...
the influence on neutrophil recruitment to subsequent flagellin challenge was lost (Fig. 5 C). Based on the dominant response occurring after apical (i.n.) administration of TLR agonists (Fig. 3 A), this data suggests that AMs are instrumental in the long-lived altered response in the post-influenza lung. This is further supported by the slow turnover of these cells in the airways (23). However, we cannot exclude an indirect role for other CD11c-expressing populations, such as DCs and tissue macrophages. It remains to be determined whether AMs are directly affected by infection or their phenotypes are altered by interaction with recruited inflammatory cells. In addition, whether the pool of lung macrophages that give rise to AMs (23) is also affected by previous infection warrants further investigation. Importantly, we are not describing simple TLR cross-tolerance, whereby TLR-activated cells are, for a short period of time, refractory to subsequent TLR stimulation (24), as the effect persists for months after the initial infection. The molecular mechanisms responsible for long-term TLR desensitization remain to be resolved, and they may include up-regulation of intracellular antagonists such as IRAK-M, down-regulation of adaptor molecules, and/or sustained influence from other signaling pathways (25).

Many mechanisms have been proposed to explain enhanced bacteria at the time of pandemic and seasonal influenza infection (4) or RSV (26), including a disruption of epithelial integrity, up-regulation of bacterial adhesion molecules, and/or an alteration in antibacterial peptides (27). We now propose an additional mechanism, TLR desensitization, which is associated with reduced neutrophils and heightened secondary bacterial load. Furthermore, we are the first to examine long-term alterations of an innate immune pathway after resolution of respiratory viral infection. It is important to point out, however, that in addition to the reduced chemokine leads to reduced inflammatory response and neutrophil recruitment. To support this hypothesis, we then conditionally depleted CD11c+ cells, including AMs, from post-influenza mice using CD11c–diphtheria toxin (DT) receptor (DTR) chimeric mice (21, 22). These mice were infected with influenza and depleted of CD11c+ cells using i.n. DT after resolution of infection (2 wk later). This resulted in an ~90% reduction in AMs (Fig. 5 C), as well as DCs and lung macrophages (Fig. S5), which is similar to that described by Landsman and Jung (23). The pool of AMs was regained 3 wk later. In this scenario, the influence on neutrophil recruitment to subsequent flagellin challenge was lost (Fig. 5 C). Based on the dominant response occurring after apical (i.n.) administration of TLR agonists (Fig. 3 A), this data suggests that AMs are instrumental in the long-lived altered response in the post-influenza lung. This is further supported by the slow turnover of these cells in the airways (23). However, we cannot exclude an indirect role for other CD11c-expressing populations, such as DCs and tissue macrophages. It remains to be determined whether AMs are directly affected by infection or their phenotypes are altered by interaction with recruited inflammatory cells. In addition, whether the pool of lung macrophages that give rise to AMs (23) is also affected by previous infection warrants further investigation. Importantly, we are not describing simple TLR cross-tolerance, whereby TLR-activated cells are, for a short period of time, refractory to subsequent TLR stimulation (24), as the effect persists for months after the initial infection. The molecular mechanisms responsible for long-term TLR desensitization remain to be resolved, and they may include up-regulation of intracellular antagonists such as IRAK-M, down-regulation of adaptor molecules, and/or sustained influence from other signaling pathways (25).

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**Figure 3.** The recruitment defect in post-influenza mice is associated with an impaired TLR-induced cytokine production. (A) Airway and lung neutrophil recruitment in post-influenza mice (2 mo after influenza) or control mice (2 mo after PBS treatment) were compared after i.n. or i.v. administration of flagellin, 1 μg FliC, or after co-instillation of 1 μg of recombinant KC and MIP-2α. n = 4. (B) MIP-2α and KC levels were measured in the BAL fluid and lung homogenates of post-influenza or control mice 6 and 24 h after FliC administration. (C) Levels of other pro-inflammatory cytokines induced by TLR5 signaling in the airways of post-influenza compared with control mice. For B and C, n = 3–5 mice/time point; this is representative of 4 experiments. (D) Relative fold increase of KC and MIP-2α mRNA transcripts purified from the lungs of control and post-influenza mice at different time points after flagellin (1 μg) i.n. challenge. Data were normalized to levels of β-actin mRNA and are represented relative to the mRNA levels in PBS-treated mice. n = 3. Error bars represent the mean ± the SEM.
production, other TLR-dependent antimicrobial mechanisms, such as the production of microbicidal products, may also play a role.

The pertinent question is why would the lung leave itself vulnerable to bacterial infection in this way? In certain compartments, such as the lung, inflammatory cascades need to be regulated to prevent bystander tissue damage, which itself can be life threatening to the host. The lung, although it contains a heavy microbial load in the upper respiratory tract, is essentially sterile below the larynx, and it is devoid of any significant resident organized lymphoid tissue. Pathogens bypassing passive antimicrobial strategies can cause significant indirect pathology in the lungs because of the recruitment of excessive numbers of immune cells that occlude the airspaces. An alteration in responsiveness to TLRs is therefore beneficial for those infections associated with excessive immunity, but at the expense of other pathogens, such as bacteria. This phenomenon, although detrimental in a subpopulation of co-infected individuals, is likely to represent an evolutionary advantage for the respiratory tract and shares interesting similarities with immunosuppressive mechanisms operating at other mucosal sites.

Figure 5. Inhibition of TLR-induced NF-κB in AMs is responsible for reduced cell recruitment in post-influenza mice. (A) AMs isolated from control and post-influenza mice were stimulated for the indicated time with 1 μg/ml FliC and further stained for p65 (green) or a nuclear marker (propidium iodide, red). Colocalization of p65 and propidium iodide is visible in control AM after 60 min of flagellin activation (yellow). The percentage of cells with p65 translocation is shown. (B) AMs were stimulated overnight with FliC and LPS at indicated concentrations and KC and MIP-2α were measured in the supernatant. Results are expressed as fold increase compared with medium level to normalize the data obtained from different cultures obtained from individual (n = 3) or pooled mice (n = 10). (C) Irradiated wild type C57BL/6 mice were reconstituted with bone marrow from mice expressing the DT receptor under the control of the CD11c promoter. These mice were then infected with influenza or received PBS as control. 2 wk later, half of the mice in each group received an intratracheal injection of DT leading to the depletion of AM and further left 3 wk for naive AM to reconstitute the airways. Nondedpleted mice received PBS as control (no depletion). Neutrophil airway recruitment in control and after influenza mice was then evaluated 6 h after i.n. flagellin challenge in DT-treated and nontreated mice (right). The number of neutrophils before flagellin treatment was comparable between all groups and similar to naive mice (not depicted). Error bars represent the mean ± the SEM.
In the gut, the microbial flora and associated TLR ligands play an essential role in mucosal homeostasis by actively inhibiting intestinal innate responses (28). Furthermore, responsiveness of intestinal epithelial cells to TLR activation is impaired immediately after birth by exposure to exogenous endotoxin (29). Although the distal lung cannot afford a bacterial flora, repetitive exposure to microbial products may induce a similar beneficial hyperresponsiveness. Associated with attenuated acute inflammation, lung dendritic cells in post-influenza mice are more efficient at presenting antigens and at promoting T cell responses (8). Therefore, by reducing excessive inflammation and improving its ability to induce a specific adaptive response, the “experienced” lungs are better equipped to fight a secondary infection. The unfortunate consequence of attenuated TLR responsiveness may be susceptibility to bacterial infection. Although our studies investigate interactions between successive respiratory pathogens, similar effects may also occur in the lung to more soluble antigens, such as allergens.

### MATERIALS AND METHODS

#### Mouse infection and treatment.
Female BALB/c, C57BL/6 mice (Harlan) and CD11c-DTR mice (obtained from S. Jung, The Weizmann Institute of Science, Rehovot, Israel) were kept in specific pathogen–free conditions according to Institutional and Home Office guidelines. Animal protocols used in this study were approved by the United Kingdom Home Office. Mice were between 6–8 wk of age, kept in individually ventilated cages, and provided with autoclaved food, water, and bedding. Groups of 4–5 mice were infected i.n. with 50 HA of X31 (a gift from A. Douglas, National Institute for Medical Research, London, England), 50 HA of UV-irradiated X31, or 10⁶ PFU RSV A2 (American Type Culture Collection) in 50 μl of sterile PBS or PBS as control, and then left to recover for at least 4 wk. Mice were monitored daily, and weight loss was measured throughout infection. Mice were then challenged with 1 μg flagellin (Apotech), 10 μg LPS (Sigma–Aldrich), or 1 μg each of the recombinant chemokines KC and MIP-2α (R&D Systems) by the i.n. or intravenous route. In some experiments, mice were challenged with 5 × 10⁵ mid-log phase P. aeruginosa PAK strain (a gift from S. Lory, Harvard Medical School, Boston, MA) grown in Luria–Bertani broth (LB), S. pneumoniae strain D39 (American Type Culture Collection), and human clinical isolate NCTC10/84 serotype V Group B Streptococcus grown in Todd–Hewitt broth (THB) medium. The number of CFU was measured by plating dilutions on LB or THB plates.

For conditional depleion of AM, WT mice were irradiated and reconstituted with bone marrow cells from CD11c-DTR transgenic or WT mice (control) to ensure that the DT receptor was only expressed on hematopoietic cells. Mice were infected 5 wk later with X31 or PBS as control. 2 wk later, half of the mice received an intratracheal injection of 50 ng DT (Calbiochem) to deplete CD11c⁺ cells or PBS for nondepleted controls. Mice were challenged with flagellin 3 wk later.

#### Cell isolation and flow cytometry.
After terminal anesthesia, lungs were inflated 6 times with 1.5 ml of EMEM/EDTA to obtain bronchoalveolar lavage (BAL) fluid, and then perfused with PBS + 1 mm EDTA via the right ventricle. The left lobe was snap-frozen for RNA and cytokine analysis, and the remaining lobes were cut in small pieces and digested for 30 min at 37°C with agitation with 0.14 Wunsh unit of Liberase III enzyme (Roche) in the presence of 50 μg/ml of DNase (Roche) in Dulbecco’s minimum essential medium containing 5% fetal calf serum (Invitrogen). Cell number was quantified using a hemocytometer and trypan blue exclusion, and was further checked by differential counting on cytopsins. Single-cell suspensions were stained for surface markers in PBS containing 0.1% sodium azide and 1% BSA for 30 min at 4°C, and then fixed with 2% PFA. The different cell types were characterized according to their forward and side scatter profile and by their phenotype, as follows: neutrophils (Gr1high, CD11bhigh, and CD11c⁺), DCs (Gr1⁺, CD11b⁺, CD11c⁺, MHCIIPhigh, and CD86⁺), macrophages (CD11blow, CD11c⁺, MHCIIPlow, CD86low, and highly autofluorescent), plasmacytoid DCs (CD11c⁺, B220⁺, PDCA-1⁻, and 24G8⁺), and T lymphocytes (CD3⁺, CD4/8⁺, and TCroB⁺). All antibodies were purchased from BD Biosciences. Aseptic cells were detected using the In Situ Cell Death Detection kit (Roche). Samples were analyzed on an LSR flow cytometer (BD Biosciences) and analyzed using CellQuest software.

#### Quantitative RT-PCR.
RNA was extracted from frozen lung lobe using the NucleoSpin RNA kit (Macherey-Nagel) and cDNA generated for each sample using the SuperScript II RT kit using random hexamers (Invitrogen). Real-time PCR was performed using platinum SybrGreen super mix (Invitrogen) and the Rotor-Gene 6000 (Corbett). Primers used in this study are available as in the Supplemental materials and methods (available at http://www.jem.org/cgi/content/full/jem.20070891/DC1). The copy number of the gene of interest was calculated as arbitrary units using the two standard curves method and normalized to 18S. Similar results were obtained using GAPDH or β-actin as housekeeping genes.

#### Cytokine quantification.
The levels of cytokines in BAL fluid and lung homogenates were determined using ELISA kits (R&D Systems) or using the 20-plex Luminex kit (Luminex). The protein concentration in lung homogenates was determined using the BSA protein assay kit (Thermo-Scientific) and the Rotor-Gene 6000 (Corbett). Primers used in this study are available as in the Supplemental materials and methods (available at http://www.jem.org/cgi/content/full/jem.20070891/DC1).

#### Immunohistochemistry.
For neutrophil staining, the lungs were gently inflated with OCT and frozen. 7-μm sections were dehydrated and fixed in acetone and stained with goat primary antibodies specific for Gr-1 (BD Biosciences). Alexa Fluor 564 anti–goat (Invitrogen) was used as a secondary antibody, and the slides were visualized using a confocal microscope (Nikon). For NF-κB activation, AMs were fixed in 4% PFA, permeabilized in 0.2% Triton X–100, and stained with a rabbit anti-p65 antibody (Santa Cruz Biotechnology) and Alexa Fluor 488 anti–rabbit antibody (Invitrogen). Propidium iodide was used for nuclear staining.

#### Statistical analysis.
Statistical significance was calculated using an unpaired Mann–Whitney test and Prism software. All P values of ≤0.05 were considered significant and are referred to as such in the text. All data are represented as the mean ± the SEM.

#### Online supplemental material.
Fig. S1 shows that influenza-induced inflammation is resolved by the time of flagellin administration. Fig. S2 shows by immunohistochemistry and flow cytometry that certain cell populations remain in the lungs after influenza infection has resolved. Fig. S3 shows that the neutrophil recruitment is not affected in peripheral organs if flagellin is administered intravenously. Fig. S4 shows that TLR5–induced NF-κB activation is not affected in post-influenza AEs. Fig. S5 shows the depleion of different cell types in CD11c⁺-DTR BM chimera after instillation of DT. A Supplemental materials and methods is also available. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070891/DC1.

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