Respiratory Influenza A Virus Infection Triggers Local and Systemic Natural Killer Cell Activation via Toll-Like Receptor 7

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The innate immune system senses influenza A virus (IAV) through different pathogen-recognition receptors including Toll-like receptor 7 (TLR7). Downstream of viral recognition natural killer (NK) cells are activated as part of the anti-IAV immune response. Despite the known decisive role of TLR7 for NK cell activation by therapeutic immunostimulatory RNAs, the contribution of TLR7 to the NK cell response following IAV infection has not been addressed. We have analyzed lung cytokine responses as well as the activation, interferon (IFN)-γ production, and cytotoxicity of lung and splenic NK cells following sublethal respiratory IAV infection in wild-type and TLR7ko mice. Early airway IFN-γ levels as well as the induction of lung NK cell CD69 expression and IFN-γ production in response to IAV infection were significantly attenuated in TLR7-deficient hosts. Strikingly, respiratory IAV infection also primed splenic NK cells for IFN-γ production, degranulation, and target cell lysis, all of which were fully dependent on TLR7. At the same time, lung type I IFN levels were significantly reduced in TLR7ko mice early following IAV infection, displaying a potential upstream mechanism of the attenuated NK cell activation observed. Taken together, our data clearly demonstrate a specific role for TLR7 signaling in local and systemic NK cell activation following respiratory IAV infection despite the presence of redundant innate IAV-recognition pathways.

Keywords: influenza A virus, natural killer cells, pathogen-recognition receptors, Toll-like receptor 7, innate immunity, respiratory infection

INTRODUCTION

Influenza A virus (IAV) is an Orthomyxovirus carrying a segmented, single-stranded RNA genome, and IAV infections remain a serious burden for human health during seasonal outbreaks. At the same time, there is a constant threat of newly emerging highly virulent pandemic strains. A full understanding of IAV pathogenesis and host responses will be crucial for optimizing the available prophylactic and therapeutic measures in the future.
Respiratory epithelial cells as well as alveolar macrophages, dendritic cells (DCs), and neutrophils are target cells of IAV (1, 2). Infected cells are able to recognize the virus through Toll-like receptor (TLR) 3, TLR7, RIG-I, MDA5, and the NLRP3 inflammasome, most of which sense the viral genome or replication intermediates (2) and contribute to the anti-IAV host defense in a cell-type-specific manner (3, 4). Several studies have addressed the role of TLR7 for host defense against IAV. Due to the presence of alternative innate IAV sensors and partially redundant signaling, TLR7ko mice respond to the virus and are able to survive the infection (5). Nevertheless, TLR7 has been found to affect several aspects of innate and especially adaptive B cell responses toward IAV (5–11).

Natural killer (NK) cells are innate lymphocytes that act as immune regulators through cytokine production and as cytotoxic effector cells. They are activated in vivo following IAV infection (12–14), and their main functions are the production of interferon (IFN)-γ and killing of infected host cells (15). However, the importance of NK cells in host defense against IAV is controversially discussed. Enhanced morbidity and mortality have been reported for mice depleted of NK cells and mice deficient of Nkp46, an NK cell receptor that interacts with the IAV hemagglutinin (16, 17). By contrast, another study observed increased survival and ameliorated lung pathology in mice lacking NK cells (18). Ultimately, as recently shown, in mouse models, the contribution of NK cells to anti-IAV defense is strongly dependent on the viral strain and dose as well as the host-genetic background (14). Also for humans, the role of NK cells in IAV infection is not fully clarified, whereas recent studies from the 2009 H1N1 pandemic suggest a correlation between NK cell lymphopenia and disease severity (19–21). Interleukin-12 (IL-12), IL-15, IL-18, and type I IFN (IFN I) have been identified in the lung as well as in the periphery.

Interestingly, several studies have demonstrated potent TLR7-dependent NK cell activation by immunostimulatory RNAs in the context of antitumor immunity (28–35). However, the relevance of TLR7 signaling for the NK cell response mounted toward IAV infection has not been addressed so far. Therefore, we have studied this aspect of the anti-IAV immune response in TLR7-deficient hosts and indeed identified a distinct role for TLR7 in the IAV-mediated activation of NK cell effector function in the lung as well as in the periphery.

**RESULTS**

**The Lung NK Cell IFN-γ Response Mounted following IAV Infection Is Attenuated in TLR7ko Mice**

In a previous study, we have characterized the respiratory anti-IAV response of TLR7ko mice and detected clearly reduced IFN-γ levels on day 3 and significantly reduced IFN-γ levels on day 5 post infection in comparison to that of wild-type (WT) hosts (11). As exclusively this early and not the later (day 7) IFN-γ response was affected and NK cells are typical early-acting producers of this cytokine, an underlying defect in NK cell activation was a likely cause. To further address this, we intranasally infected both WT and TLR7ko mice with a sublethal dose of IAV and confirmed reduced airway IFN-γ levels in TLR7ko mice on day 4 post infection (Figure 1A). Of note, the attenuated IFN-γ response was not a consequence of changes in the viral load between WT and TLR7ko mice (Figure 1B). Addressing the possible role of NK cells, we found that their frequency in the lung was not significantly altered between uninfected and infected or between WT and TLR7ko mice (Figure 1C). Nevertheless, a trend for a relative increase in the NK cell population in response to the infection was detectable in WT but not in TLR7ko mice on day 4 post infection (Figure 1C). Of note, the absolute number of lymphocytes isolated from the lungs on days 3 and 4 post IAV infection was not significantly altered between WT and TLR7ko mice (Figure S1A in Supplementary Material). Interestingly, however, on day 3 post infection, a significant increase in the absolute lymphocyte number, and on days 3 and 4 post infection, a significant increase in the absolute NK cell number were detectable in infected TLR7ko but not in WT mice (Figure S1A in Supplementary Material; Figure 1D). Therefore, the reduced IFN-γ response detected in IAV-infected TLR7ko mice was not a consequence of a reduced number of NK cells present in the lungs. Nevertheless, we furthermore analyzed the production of IFN-γ by lung NK cells in response to IAV infection. Importantly, on day 3 post infection, there was a clear and significant increase in the frequency of IFN-γ producing NK cells in the lungs of WT but not in TLR7-deficient mice compared to uninfected controls (Figure 1E). Taken together, these data show a clear defect in lung NK cell IFN-γ production in TLR7-deficient mice early after IAV infection that was not a consequence of attenuated NK cell recruitment. This defect in NK cell function most likely underlays the reduced IFN-γ levels detected in TLR7ko mice early after IAV infection.

**The IAV-Mediated Induction of Lung NK Cell CD69 Expression Is Significantly Delayed in TLR7ko Mice Whereas Changes in the Maturation Status Are Not Affected**

Based on these TLR7-dependent changes observed in the lung in the early phase of IAV infection, we further assessed the kinetics of NK cell activation at the site of infection by analyzing lung NK cells from IAV-infected WT and TLR7ko mice for the expression of the early activation marker CD69 (Figure 2A). Respiratory IAV infection potently induced CD69 expression by lung NK cells in WT mice on days 3 and 4 post infection. This was clearly delayed in TLR7ko mice, which displayed a significantly reduced frequency of activated CD69-expressing NK cells in the lung on day 3 post infection compared to WT mice. The induction of lung NK cell CD69 expression was, however, not fully TLR7-dependent, as significant CD69 expression was also detected in TLR7ko NK cells by day 4 post infection. In order to assess the activation status in more detail, we analyzed...
The early lung interferon-γ (IFN-γ) response following influenza A virus (IAV) infection is attenuated in toll-like receptor (TLR)7-deficient mice while the lung natural killer (NK) cell frequency is unchanged. Wild-type (WT) (black bars and symbols) and TLR7ko (open bars and symbols) mice were infected with 0.04 LD₅₀ IAV PR8 or treated with PBS and sacrificed at the indicated time points. (A) IFN-γ levels in bronchoalveolar lavage were assessed by enzyme-linked immunosorbent assay. Data show the mean ± SEM of n = 3 uninfected and n ≥ 8 infected mice per strain with samples derived from three independent experiments. (B) IAV nucleoprotein (NP) copies were quantified in whole lung tissue cDNA as a measure for the viral load. Data show individual mice and the mean/group of samples collected from two independent infection experiments. (C) Lymphocytes isolated from the lung tissue were analyzed for the frequency of NK cells (CD3⁻/NK1.1⁺). Frequency ± SEM of NK cells within the lymphocyte population from n ≥ 6 mice per group compiled from at least two independent experiments. (D) Data show the absolute number of NK cells isolated per mouse lung for individual mice and the group mean compiled from at least two independent experiments. (E) Lymphocytes isolated from the lung tissue were analyzed for NK cell (CD3⁻/NK1.1⁺) IFN-γ production by intracellular flow cytometry ex vivo. Data show the frequency ± SEM of IFN-γ-positive NK cells within the NK cell population on day 3 post IAV infection for n ≥ 5 mice per group compiled from two independent experiments. Groups were compared by two-way ANOVA with Bonferroni multiple comparisons test (*p < 0.05, **p < 0.005, ***p < 0.0005).
the co-expression of CD27 and CD11b on lung NK cells isolated from uninfected and IAV-infected WT and TLR7ko mice on days 3 and 4 post infection (36, 37) (Figure 2B). Compared to uninfected controls, respiratory IAV infection led to significant changes in the CD27/CD11b expression pattern in WT mice. While the frequency of the more mature CD27\textsuperscript{high}/CD11b\textsuperscript{low} and CD27\textsuperscript{high}/CD11b\textsuperscript{high} NK cell populations was significantly increased on day 4 post infection, the highly differentiated CD27\textsuperscript{low}/CD11b\textsuperscript{high} NK cell population, which holds a higher activation threshold, was significantly decreased at this time point. As especially the co-expression of high levels of CD27 and CD11b is associated with potent effector functions (37), these data indicated IAV infection to trigger maturation and activation of NK cells in the lung. However, the CD27/CD11b expression pattern and its IAV-induced changes were largely unchanged between WT and TLR7ko mice following IAV infection. There was only a minor but significant increase in the highly differentiated CD27\textsuperscript{low}/CD11b\textsuperscript{high} NK cell population on day 4 post infection in TLR7ko compared to that in WT mice. We also assessed the expression of Nkp46, which can act as a receptor for the IAV hemagglutinin (16), on lung NK cells following IAV infection. However, Nkp46 expression was only marginally changed following IAV infection without any significant changes between WT and TLR7ko mice. Of note, basal CD69 expression detected in splenic NK cells of uninfected mice was elevated in TLR7ko mice. However, these changes were not significant. Regarding changes of the maturation status of peripheral NK cells in response to IAV infection, the more mature CD27\textsuperscript{high}/CD11b\textsuperscript{low} NK cell population was significantly increased by day 4 post infection only in WT mice (Figure 3C). In contrast to lung NK cells, in the spleen, the frequency of both the CD27\textsuperscript{low}/CD11b\textsuperscript{high} and the CD27\textsuperscript{high}/CD11b\textsuperscript{high} NK cell populations was not changed following IAV infection in either WT or TLR7ko mice. Of note, there were significant changes of the CD27\textsuperscript{high}/CD11b\textsuperscript{high} and the CD27\textsuperscript{low}/CD11b\textsuperscript{high} NK cell populations TLR7ko mice that were independent of the infection. As for lung NK cells, Nkp46 expression on splenic NK cells was only marginally changed following IAV infection without any significant changes between WT and TLR7ko mice (Figure S2B in Supplementary Material).

Prompted by the significant activation of splenic NK cells that was detectable through increased \textit{ex vivo} CD69 expression on day 4 following respiratory IAV infection, we additionally analyzed the effector functions of splenic NK cells isolated from IAV-infected hosts \textit{in vitro} at that time point. Interestingly, following \textit{in vitro} stimulation, more splenic NK cells isolated from IAV-infected WT mice expressed IFN-γ than those from uninfected mice. Importantly, significantly more IFN-γ producing splenic WT NK cells than TLR7ko splenic NK cells were detected (Figure 4A). Of note, there was no difference in the frequency of IFN-γ expressing lung NK cells isolated from IAV-infected WT and TLR7ko mice following \textit{in vitro} stimulation, whereas the IFN-γ production on a per cell basis was significantly attenuated in TLR7ko NK cells (data not shown).
The induction of natural killer (NK) cell CD69 expression in the lung following respiratory influenza A virus (IAV) infection is delayed in Toll-like receptor (TLR)7-deficient hosts. Wild-type (WT) (black bars) and TLR7ko (white bars) mice were infected with 0.04 LD50 IAV PR8 or treated with PBS and sacrificed at the indicated time points. Lung NK cells (CD3\(^{-}\)/NK1.1\(^{+}\)) were analyzed for the expression of CD69 (A) and the co-expression of CD11b and CD27 (B) by flow cytometry. Data show the mean ± SEM of ≥6 mice/group from at least two independent experiments and representative flow-cytometric results for an uninfected and an IAV-infected WT mouse on day 4 post infection. Data were compared by two-way ANOVA with Bonferroni multiple comparisons test (*p < 0.05, ****p < 0.0001).

**FIGURE 2.** The induction of natural killer (NK) cell CD69 expression in the lung following respiratory influenza A virus (IAV) infection is delayed in Toll-like receptor (TLR)7-deficient hosts. Wild-type (WT) (black bars) and TLR7ko (white bars) mice were infected with 0.04 LD50 IAV PR8 or treated with PBS and sacrificed at the indicated time points. Lung NK cells (CD3\(^{-}\)/NK1.1\(^{+}\)) were analyzed for the expression of CD69 (A) and the co-expression of CD11b and CD27 (B) by flow cytometry. Data show the mean ± SEM of ≥6 mice/group from at least two independent experiments and representative flow-cytometric results for an uninfected and an IAV-infected WT mouse on day 4 post infection. Data were compared by two-way ANOVA with Bonferroni multiple comparisons test (*p < 0.05, ****p < 0.0001).
The expression of CD69 and CD27/CD11b by splenic natural killer (NK) cells in response to respiratory influenza A virus (IAV) infection is significantly altered in toll-like receptor (TLR)7-deficient mice. Wild-type (black bars) and TLR7ko (white bars) mice were infected with 0.04 LD50 IAV PR8 or treated with PBS and sacrificed at the indicated time points. Splenic NK cells were analyzed for the expression of intracellular interferon-γ (IFN-γ) (A) and surface CD69 (B) as well as CD11b/CD27 (C) by flow cytometry ex vivo. (A) Data show the mean ± SEM of 6 mice/group from two independent experiments. (B) Data show the mean ± SEM of ≥6 mice/group from at least two independent experiments. (C) Data show the mean ± SEM of ≥6 mice/group from at least two independent experiments. Data were compared by two-way ANOVA with Bonferroni multiple comparisons test (*p < 0.05, **p < 0.005, ***p < 0.0005).

Stegemann-Koniszewski et al. TLR7-Dependent Influenza-Mediated NK Cell Activation

The il-12p40 and IFN I Response Is Attenuated in TLR7-Deficient Mice

Regarding the mechanisms of NK cell activation following IAV infection, IL-12 has been shown to contribute to early NK cell-dependent IFN-γ production (25), and especially IFN I has been identified as a key mediator of NK cell activation (12, 26, 27). Furthermore, also IL-15 and IL-18 have been implicated to play a role in IAV-mediated NK cell activation (12, 18, 38). In order to identify possible upstream defects that underlie the impaired NK cell activation in TLR7-deficient hosts following IAV infection, we assessed the levels of IL-12p70, IL-12p40, IL-18, and IFN I in the respiratory tract of IAV-infected WT and TLR7ko mice (Figure 6). Furthermore, we have previously characterized the respiratory cytokine response in WT and TLR7ko mice over the course of IAV infection in our model (11) and not detected IL-15 at...
Any time point (data not shown). On days 3 and 4 post infection, there was no induction of IL-12p70 in comparison to uninfected mice (Figure 6A). By contrast, a significant production of IL-12p40 was detectable in WT mice by day 4 post infection, whereas only a marginal and not significant increase in IL-12p40 was detected in TLR7-deficient mice (Figure 6B). At the same time, no increase in IL-18 levels was detectable in the respiratory tract of WT or TLR7ko mice on days 3 and 4 post IAV infection (Figure 6C). As IFN I is produced very rapidly following viral infections and as we have previously described a significantly impaired very early respiratory IFN I response in TLR7ko mice following lethal IAV infection (39), we now assessed the very early kinetics of respiratory IFN I production following sublethal infection on days 2, 3, and 4 post infection (Figure 6D). Indeed, a significant delay in the early IFN I response of TLR7ko mice became apparent. An efficient and significant induction of IFN I was detectable from day 3 post infection in WT mice but only by day 4 in TLR7ko mice, and the mean IFN I levels were reduced in IAV-infected TLR7ko compared to those in WT mice at all tested time points.

Of note, we also analyzed systemic IL-12, IL-18, and IFN I levels with regard to the systemic NK cell activation observed following IAV infection, but did not detect any increase in the serum levels in response to IAV infection at any of the time points analyzed. Of note, there was, however, a significant decrease in serum IL-12p40 in TLR7ko mice following infection (Figure S3 in Supplementary Material). Ultimately, the attenuated IL-12p40 and the impaired and delayed IFN I response observed in the airways of TLR7ko mice following sublethal IAV infection correlated well with their attenuated NK cell response.

**DISCUSSION**

Prompted by the attenuated early respiratory IFN-γ response of TLR7ko mice following sublethal IAV infection, our study addressed the role of TLR7 signaling in IAV-mediated NK cell activation. Indeed, we discovered a clear contribution for TLR7 in the induction of a timely local and systemic NK cell response following IAV infection.
In line with previous studies (12–14, 21, 26), we detected significant activation of NK cells following respiratory IAV infection through CD69 expression by lung and splenic NK cells and IFN-γ production by lung NK cells ex vivo. In vitro, we detected clear cytotoxicity and degranulation of splenic NK cells from IAV-infected mice in response to target cells as well as IFN-γ production in response to unspecific stimulation. Interestingly, no significant but only very marginal degranulation of NK cells was detectable ex vivo following isolation from the site of infection. While cytotoxicity and degranulation of lung NK cells from IAV-infected mice toward target cells has been shown in vitro (12, 26, 38), our findings are in line with a report by others showing no changes in ex vivo surface CD107a expression over the early course of IAV infection in C57Bl/6 mice (14). Ultimately, this lack of detectable degranulation at the site of infection suggests that cytokine production rather than cytotoxicity is the predominant effector function of lung NK cells following IAV infection. Regarding CD27/CD11b expression, we found the majority of lung and also splenic NK cells in uninfected mice to display a more functionally mature phenotype, as has also been reported by others (13, 40). NK cell differentiation has been described to subsequently progress from CD27low/CD11bhigh over CD27^high/CD11b^low and CD27^high/CD11b^high stages to CD27^low/CD11b^high. Here, the CD11b^high populations are reported to possess more potent effector functions, and of these the CD27^high/CD11b^high NK cell population displays the most potent effector cells (36). In line with a previous report (40), we observed a clear shift toward the less differentiated but more functionally mature phenotypes for both lung and splenic NK cells in the course of IAV infection, supporting the concept of site-specific maturation in the periphery (41). Importantly, we detected a strong increase in the CD27^high/CD11b^high effector NK cell population in the lung by day 4 post IAV infection, reflecting IAV-induced activation of NK cells at the site of infection. Surprisingly and in contrast to other reports, however (14), we did not observe a significant increase in the overall population size or the absolute NK cell numbers in the lungs of WT mice during the first 4 days post IAV infection despite the rapid and strong activation of NK cells. Surprisingly and in contrast to WT mice, we found NK cell counts to be significantly increased in infected versus uninfected TLR7ko mice, possibly displaying a compensatory mechanism for attenuated NK cell activation. Ultimately, the details of recruitment of NK cells of different maturation states and/or the proliferation and local maturation at the site of IAV infection as well as the discrimination between tissue-resident and peripheral NK cells will have to be addressed specifically by future investigations.

Most importantly, our study shows that in a number of aspects, the activation of both local and systemic NK cells in response to respiratory IAV infection significantly depends on TLR7. The production of IFN-γ by lung NK cells and the induction of CD69 expression by lung and splenic NK cells triggered by respiratory IAV infection were clearly affected in TLR7-deficient hosts. Importantly, the priming for in vitro IFN-γ production and target-cell-directed cytotoxicity of splenic NK cells through respiratory IAV infection was fully dependent on TLR7. This finding is well in line with the TLR7-dependent induction of NK cell antitumor activity through immunostimulatory RNA molecules (28, 35, 42). The role of TLR7 in IAV-mediated NK cell activation has, however, not been addressed so far, and our study now shows that also IAV infection activates and primes for NK cell effector function in a TLR7-dependent manner. This specific role for TLR7 is remarkable in the light of the fact that viral infections trigger broader and more versatile immune responses compared to the treatment with immunostimulatory RNAs or even specific TLR ligands. Mechanistically, NK cell activation through immunostimulatory molecules has been described to mainly depend on the presence of DCs that signal to NK cells via IL-12 and IFN I (28, 33, 42, 43). Regarding the mechanisms of NK cell activation following IAV infection, contributing roles have been described for IL-15 (12, 18), IL-12 (25, 27), IL-18 (38), and IFN I (12, 26, 27, 44), with IFN I displaying the key player (12, 44). As for the induction of NK cell antitumor activity by immunostimulatory molecules, activation following IAV infection has been demonstrated to occur in an accessory cell-dependent manner (27, 44), with a major function for plasmacytoid DC (pDC) (44). Interestingly, the production of large amounts of IFN I by pDC is a major function specific for TLR7, also following IAV infection (10, 45, 46). Accordingly, we have previously described a delayed...
and attenuated IFN I response in the lungs of TLR7ko mice following lethal IAV infection (39) even though TLR7-deficient hosts are generally able to mount IFN I responses following viral infection via alternative receptors (5). Importantly, here we show a significantly delayed IFN I response in TLR7-deficient mice following sublethal IAV infection that correlates with the observed defects in NK cell activation. Most likely, there is a mechanistic link via TLR7-dependent IAV recognition and IFN I production by pDC. Nevertheless, TLR7ko mice did mount an IFN I response comparable to the WT by day 4 post infection, well in line with the delayed but nevertheless significant activation of lung NK cells also in these mice. Furthermore, we show an attenuated Il-12p40 response in the airways of TLR7ko mice post infection that possibly also contributes to the attenuated NK cell activation observed in the lung. Generally, NK cell activation, i.e., the significant induction of CD69 expression and also the shift toward a higher frequency of CD27<sup>high</sup>CD11b<sup>low</sup> NK cells, was stronger at the site of infection than in the periphery. This observation possibly explains why in splenic NK cells, CD69 expression and the frequency of CD27<sup>high</sup>CD11b<sup>low</sup> cells were significantly increased only in infected WT and not in TLR7ko mice and only by day 4 post infection. Importantly, we did, however, not detect any increase in II-12, II-18, or IFN I in serum samples of infected WT or TLR7ko mice, leaving open the questions of whether minimal but not detectable early systemic IFN I or II-12 originating from the lung and present only in WT mice due to a threshold effect was sufficient to activate splenic NK cells, whether there are alternative fully TLR7-dependent mechanisms of systemic NK cell activation, or whether this issue is a matter of recirculation of NK cells locally activated in the lung to the periphery (47). Ultimately, also the question regarding the contribution of direct viral recognition by NK cells, which do express TLR7 (33, 48), to their activation following IAV infection arises. For a first insight into this question, we adoptively transferred TLR7-deficient NK cells into WT recipients and analyzed their CD69 and IFN-γ expression in the lung following IAV infection (Figure S4 in Supplementary Material). There was little activation of TLR7ko NK cells in the lungs of WT recipients following IAV infection, which was, however, not as clear and strong as in WT NK cells adoptively transferred to WT recipients as control. These data suggest that both accessory cell-dependent and direct activation via TLR7 play a role in TLR7-dependent IAV-mediated NK cell activation, and the detailed contributions will need to be clarified in future investigations. Though mostly not significant, to our knowledge, we are the first to report altered baseline CD69 as well as CD27/CD11b expression of lung and splenic NK cells in uninfected TLR7ko compared to that in uninfected WT mice,
and therefore also a contribution of intrinsic effects of TLR7-deficiency on NK cell maturation and activation to the observations made cannot fully be excluded. Nevertheless, our data show a clear role of TLR7 in the timely and efficient activation of NK cells in response to IAV infection. A number of studies of the past, including our own, have demonstrated TLR7 to play only a minor role in viral clearance and survival to IAV infection but to rather fine-tune innate responses (5, 6, 9, 53–55). Therefore, delayed and incomplete NK cell activation possibly contributes to the defects in adaptive immune responses observed in TLR7-deficient mice following IAV infection. Severe secondary bacterial infections are a major complication of IAV infections, and we have previously shown a benefit for TLR7ko mice in antibacterial clearance following IAV/Streptococcus pneumoniae coinfection (11). As IFN-γ is known to have detrimental effects in coinfections (56, 57), the attenuated NK cell IFN-γ response observed in TLR7ko mice correlates well to this finding, and the modulation of antibacterial defense in secondary bacterial infection displays a possible downstream effect of NK cell activation following IAV infection.

Taken together, our study adds a novel piece of knowledge to our understanding of the induction of NK cell responses following IAV infection. Importantly, we have identified a previously unrecognized specific function of TLR7. Such knowledge will ultimately be essential for a full understanding of IAV pathogenesis and in turn for the development of efficient prophylactic and therapeutic measures.

MATERIALS AND METHODS

Mice
All experiments were performed in female mice 8–12 weeks of age with an average weight of 23 g. TLR7-deficient mice (58) (provided by S. Bauer) were bred at the animal facility of the Helmholtz Centre for Infection Research (HZI) and were backcrossed to the C57BL/6J background for a total of 10 generations. C57BL/6J mice were obtained from Harlan (now Envigo) or bred at the HZI.

IAV Infection
Madin–Darby canine kidney cell-derived IAV PR8/A/34(H1N1) was obtained as described previously (59). Following anesthesia through inhalation of isoflurane or intraperitoneal injection of ketamine–xylazine, mice were intranasally infected with 0.04 MLD50 diluted in PBS. Control animals were treated with PBS.

Isolation of Lymphocytes from the Respiratory Tract and Spleen
Lungs were once flushed through the trachea with 1 ml cold sterile PBS to obtain bronchoalveolar lavage (BAL). Lavaged lungs were perfused with PBS, excised and minced on ice, followed by enzymatic digestion for 45 min at 37°C in Iscove’s modified Dulbecco’s medium containing 0.2 mg/ml Collagenase D (Roche), 0.01 mg/ml DNase (Sigma-Aldrich), and 5% fetal calf serum. After the addition of EDTA (5 mM final concentration), suspensions were filtered (70 µm) and pelleted by centrifugation. Enrichment for lymphocytes was performed using Ficoll–Paque PLUS (GE Healthcare Life Sciences) or EasySep (1.124 g/ml; Merck Millipore) following erythrocyte lysis by osmotic shock. Splenocytes were isolated by homogenization of spleens through a 70-µm cell strainer in PBS using a syringe plunger, centrifugation of the cell suspension, and erythrocyte lysis by osmotic shock.

Stimulation for Intracellular IFN-γ Staining
Lymphocytes isolated from the lung tissue were incubated with 10 ng/ml Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich) and 1 µg/ml Ionomycin (Sigma-Aldrich) for 4 h; 5 µg/ml Brefeldin A (Sigma-Aldrich) was added after the first 2 h of incubation. Splenocytes were incubated in a medium containing 10 ng/ml PMA for 5 h, and 5 µg/ml Brefeldin A was added after 1 h.

YAC-1 Target Cell Co-Incubation
Isolated splenocytes were counted and co-incubated with YAC-1 target cells at a 10:1 ratio at 37°C and 5% CO2. After 1 h, 0.006 µg/ml Monensin and 5 µg/ml Brefeldin A (Sigma-Aldrich) were added. After a total of 5 h, cells were stained for flow cytometry.

Flow Cytometry
Cell suspensions were incubated with a CD16/CD36 (2.4G2) antibody for the blocking of Fc receptors and stained with fixable live/dead stain (Molecular Probes) as indicated. Antibody stainings on the cell surface were performed for CD3 (145-2C11), NK1.1 (PK136), CD107a (1D4B). Intracellular staining for IFN-γ (XMG1.2) was performed following fixation with 2% paraformaldehyde and permeabilization with 0.1% Igepal CA-630 (Sigma-Aldrich). Data were acquired using a BD Fortress and analyzed using FlowJo (Tree Star). Following exclusion of dead cells and gating on singlets and lymphocytes, NK cells were defined as the CD3-/NK1.1+ population. For the calculation of absolute cell numbers, 20,000 polystyrene beads (Comp Beads Plus negative control beads, BD Biosciences) were added to each sample, and absolute cell numbers were calculated in relation to the acquired bead population.

51Cr Release Assay for the Detection of Specific Target Cell Lysis
Splenocytes isolated at day 4 post infection were used as effector cells at a concentration of 1 × 106/ml and co-cultured with 51chromium-labeled YAC-1 cells, a well-known target for NK cells. YAC-1 target cells were incubated in RPMI (Invitrogen Life Technologies) without FCS and labeled with 100 µCi of 51Cr (Amersham) for 1.5 h at 37°C and 5% CO2. Effector and target cells
were incubated at an effector/target ratio of 10:1 for 4 h at 37°C and 5% CO2. Subsequently, cells were centrifuged, and the radioactivity present in the supernatant was measured by scintillation counting. Spontaneous lysis was detected in untreated target cells, whereas the maximal lysis was determined after adding 100 µl of 5% Triton X-100 (Carl Roth). Results are expressed as the percentage of lysed cells according to the calculation \[
\{\text{sample} - \text{spontaneous lysis}\} / \{\text{maximal lysis} - \text{spontaneous lysis}\} \times 100\].

**Quantification of Cytokines**

Bronchoalveolar lavage samples were centrifuged for 10 min at 4°C and 10,000 × g. IL-18, IFN-γ, IL-12/IL-23(p40), and IL-12(p70) were detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (MBL for IL-18, BioLegend ELISA Max for all others). For the quantification of IFN I, IFN-sensitive epithelial cells from Mx2-Luc reporter were treated with BAL or serum and analyzed for luciferase activity as previously described (38, 60).

**Quantification of the Viral Load**

The viral load in lung tissue homogenates was determined as IAV nucleoprotein (NP) RNA copies by absolute qRT-PCR. Perfused lung tissue was stored in RNAlater solution (Ambion), and RNA was extracted using the RNA easy kit (Qiagen) following homogenization using a manual disperser (Kinematica). For cDNA synthesis 1 µg of RNA was transcribed using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Absolute qRT-PCR was performed on a LightCycler 480 II (Roche) using FastStart Essential DNA Green Master (Roche). Per reaction 25 ng reversely transcribed RNA was used and compared to a plasmid standard containing defined copy numbers of the IAV NP gene. NP primers were GAGGGGTGAGAATGGACGAAAAAC (5’-NP) and CAGGCAGGCAGGACGAAGCTT (3’-NP) and were used in a final concentration of 500 nmol/l.

**Statistical Analyses**

Groups were compared by the indicated statistical test using the GraphPad Prism software, and \( p \leq 0.05 \) was considered indicative of statistical significance.

**REFERENCES**

1. Hufford MM, Richardson G, Zhou H, Manicassamy B, Garcia-Sastre A, Enelow RI, et al. Influenza-infected neutrophils within the infected lungs act as antigen presenting cells for anti-viral CD8(+) T cells. PLoS One (2012) 7:e46581. doi:10.1371/journal.pone.0046581
2. Iwasaki A, Pillai PS. Innate immunity to influenza virus infection. Nat Rev Immunol (2014) 14:315–28. doi:10.1038/nri3665
3. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity (2005) 23:19–28. doi:10.1016/j.immuni.2005.04.010
4. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature (2006) 441:101–5. doi:10.1038/nature04734
5. Koyama S, Ishii KJ, Kumar H, Tanimoto T, Coban C, Uematsu S, et al. Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol (2007) 179:4711–20. doi:10.4049/jimmunol.179.7.4711
6. Budimir N, de HA, Meijerhof T, Wajer S, Boon L, Gostick E, et al. Critical role of TLR7 signaling in the priming of cross-protective cytotoxic T lymphocyte responses by a whole inactivated influenza virus vaccine. PLoS One (2013) 8:e63163. doi:10.1371/journal.pone.0063163
7. Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, Kopf M, et al. TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. J Immunol (2007) 178:2182–91. doi:10.4049/jimmunol.178.4.2182
8. Jeisy-Scott V, Davis WG, Patel JR, Bowzard JB, Shihe WJ, Zaki SR, et al. Increased MDSC accumulation and Th2 biased response to influenza A virus infection in the absence of TLR7 in mice. PLoS One (2011) 6:e25242. doi:10.1371/journal.pone.0025242
9. Jeisy-Scott V, Kim JH, Davis WG, Cao W, Katz JM, Sambhara S. TLR7 recognition is dispensable for influenza virus A infection but important for

**ETHICS STATEMENT**

This study was carried out in accordance with the recommendations of national and international guidelines. The protocol was approved by the “Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit.”

**AUTHOR CONTRIBUTIONS**

SS-K designed and performed experiments and wrote the manuscript; SB, JB, and IH performed experiments; PR designed and performed experiments; AK designed experiments and provided research materials; CG and JS provided research materials; MG designed experiments and provided research materials; DB designed experiments, provided research materials, and wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00245/full#supplementary-material.
the induction of hemagglutinin-specific antibodies in response to the 2009 pandemic split vaccine in mice. *J Virol* (2012) 86:10988–98. doi:10.1128/JVI.01064-12.

10. Kaminski MM, Ohnemus A, Cornitescu M, Staechiel P. Plasmacytoid dendritic cells and Toll-like receptor 7–dependent signaling promote effective protection of mice against highly virulent influenza A virus. *J Gen Virol* (2012) 93:555–9. doi:10.1099/vir.0.039065-0

11. Stegemann-Konisiewski S, Gereke M, Orrskog S, Lienenklaus S, Pasche B, et al. TLR7-dependent influenza-mediated NK cell activation. *J Innate Immun* (2013) 5:84–96. doi:10.1159/000345112

12. Hwang I, Scott JM, Kakarla T, Duriancik DM, Choi S, Cho C, et al. Activation mechanisms of natural killer cells during influenza virus infection. *PloS One* (2012) 7:e51858. doi:10.1371/journal.pone.0051858

13. Wang J, Li F, Zheng M, Sun R, Wei H, Tian Z. Lung natural killer cells in mice: phenotype and response to respiratory infection. *Immunology* (2012) 137:37–41. doi:10.1111/j.1365-2657.2012.03607.x

14. Zhou K, Wang J, Li A, Zhao W, Wang D, Zhang W, et al. Swift and strong NK cell responses protect mice against high-dose influenza virus infection. *J Immunol* (2016) 196:1842–54. doi:10.4049/jimmunol.1501486

15. Schultz-Cherry S. Role of NK cells in influenza infection. *Curr Top Microbiol Immunol* (2015) 386:109–20. doi:10.1007/82_2014_403

16. Gazit R, Gruda R, Elboim M, Arnon TI, Katz G, Achdout H, et al. Lethal influenza infection in the absence of the negative regulatory receptor gene Ncr1. *Nat Immunol* (2006) 7:517–23. doi:10.1038/ni3522

17. Stein-Streilein J, Guffe J. In vivo treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. *J Immunol* (1986) 136:1435–41.

18. Abdul-Careem MF, Mian MF, Yue G, Gillgrass A, Chenoweth MJ, Barra NG, et al. Critical role of natural killer cells in lung immunopathology during influenza infection in mice. *J Infect Dis* (2012) 206:167–77. doi:10.1093/infdis/jia340

19. Fox A, Le NM, Horby P, van Doorn HR, Nguyen VT, Nguyen HH, et al. Changes in cytokine levels and NK cell activation associated with influenza in the lung by integrating pathogen- and microenvironment-derived signals. *MBio* (2016) 7:e00276–16. doi:10.1128/mBio.00276-16

20. Beli E, Clinkenhorje FJ, Duriancik DM, Hwang I, Kim S, Gardner CM. Natural killer cell function is altered during the primary response of aged mice to influenza infection. *Mech Ageing Dev* (2011) 132:503–10. doi:10.1016/j.mad.2011.08.005

21. Pinehas N, Sternberg-Simon M, Chiossone L, Shahaf G, Walzer T, Vivier E, et al. Murine peripheral NK-cell populations originate from site-specific immature NK cells more than from BM-derived NK cells. *J Immunol* (2009) 183:6078–86. doi:10.4049/jimmunol.2010054847

22. Berger M, Ablasser A, Kim S, Bekeredjian-Ding I, Giese T, Endres S, et al. TLR8-driven IL-12-dependent reciprocal and synergistic activation of NK cells and monocytes by immunostimulatory RNA. *J Immunother* (2009) 32:262–71. doi:10.1007/jcli.010318.0111

23. Bourquin C, Schmidt I, Lanz AI, Storch B, Wurzenberger C, Anz D, et al. Immunostimulatory RNA oligonucleotides induce an effective antitumoral response in the lung after intratumoral delivery. *J Virol* (2011) 85:3893–908. doi:10.1128/JVI.01908-10

24. Gregoire C, Chasson L, Luci C, Tomasello E, Geissmann F, Vivier E, et al. The trafficking of natural killer cells. *Immunol Rev* (2007) 220:169–82. doi:10.1111/j.1600-065X.2007.00563.x

25. Sivori S, Falco M, Della CM, Carlomagno S, Vitale M, Moretta L, et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine and cytotoxic T cell responses. *Oncoimmunology* (2016) 5:1232219. doi:10.2147/PP.2016.123219

26. Pries R, Wulf S, Kesselring R, Borngen K, Xie L, Wollenberg B. Up-regulation of NK cell function against head and neck cancer in response to ss-RNA requires TLR7. *J Immunol* (2008) 183:993–1000. doi:10.4049/jimmunol.200800687

27. Hayakawa Y, Smyth MI. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J Immunol* (2006) 176:1517–24. doi:10.4049/jimmunol.176.4.1517

28. Liu B, Mori I, Hassian MJ, Dong L, Takeda K, Kimura Y. Interleukin-18 improves the early defense system against influenza virus infection by augmenting natural killer cell-mediated cytotoxicity. *J Virol* (2004) 85:423–8. doi:10.1128/JVI.10936-04

29. Stegemann-Konisiewski S, Jeron A, Gereke M, Geffers R, Kroger A, Gunzer M, et al. Defective NK cell response through the TLR7.

30. Doorduin EM, Smijter M, Salvatori DC, Silvestri S, Maas S, Arens R, et al. CD4+ T cell and NK cell interplay key to regression of MHC class I low tumors upon TLR7/8 agonist therapy. *Cancer Immunol Res* (2015) 3:564–73. doi:10.1158/2326-6066.CIR-14-0334
of cytokine release and cytotoxicity against tumors and dendritic cells. Proc Natl Acad Sci U S A (2004) 101:10116–21. doi:10.1073/pnas.0403744101
49. Altfeld M, Fadda L, Frleta D, Bhardwaj N. DCs and NK cells: critical effectors in the immune response to HIV-1. Nat Rev Immunol (2011) 11:176–86. doi:10.1038/nri2935
50. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol (2008) 9:503–10. doi:10.1038/ni1582
51. Ge MQ, Ho AW, Tang Y, Wong KH, Chua BY, Gasser S, et al. NK cells regulate CD8+ T cell priming and dendritic cell migration during influenza A infection by IFN-gamma and perforin-dependent mechanisms. J Immunol (2012) 189:2099–109. doi:10.4049/jimmunol.1103474
52. Liu Y, Zheng J, Liu Y, Wen L, Huang L, Xiang Z, et al. Uncompromised NK cell activation is essential for virus-specific CTL activity during acute influenza virus infection. Cell Mol Immunol (2017). doi:10.1038/cmi.2017.10
53. Cbling JM, Matloubian M. B cell-intrinsic TLR7 signaling is required for optimal B cell responses during chronic viral infection. J Immunol (2013) 191:810–8. doi:10.4049/jimmunol.1300244
54. Mandl JN, Akondy R, Lawson B, Kozyr N, Staprans SI, Ahmed R, et al. Distinctive TLR7 signaling, type I IFN production, and attenuated innate and adaptive immune responses to yellow fever virus in a primate reservoir host. J Immunol (2011) 186:6406–16. doi:10.4049/jimmunol.1001191
55. Walsh KB, Teijaro JR, Zuniga EL, Welch MJ, Fremgen DM, Blackburn SD, et al. Toll-like receptor 7 is required for effective adaptive immune responses that prevent persistent virus infection. Cell Host Microbe (2012) 11:643–53. doi:10.1016/j.chom.2012.04.016
56. Duvigneau S, Sharma-Chawla N, Boianelli A, Stegemann-Koniszewski S, Nguyen VK, Bruder D, et al. Hierarchical effects of pro-inflammatory cytokines on the post-influenza susceptibility to pneumococcal coinfection. Sci Rep (2016) 6:37045. doi:10.1038/srep37045
57. Sun K, Metzger DW. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. Nat Med (2008) 14:558–64. doi:10.1038/nm1765
58. Henmi K, Kato H, Takeuchi O, Sato S, Sanjo H, Hoshino K, et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol (2002) 3:196–200. doi:10.1038/ni758
59. Stegemann S, Dahlberg S, Kroger A, Gereke M, Bruder D, Henriques-Normark B, et al. Increased susceptibility for superinfection with Streptococcus pneumoniae during influenza virus infection is not caused by TLR7-mediated lymphopenia. PLoS One (2009) 4:e4840. doi:10.1371/journal.pone.0004840
60. Nandakumar R, Finsterbusch K, Lipps C, Neumann B, Grashoff M, Nair S, et al. Hepatitis C virus replication in mouse cells is restricted by IFN-dependent and -independent mechanisms. Gastroenterology (2013) 145:1414–23. doi:10.1053/j.gastro.2013.08.037

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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