MucosalImmunology

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ARTICLE

R848 or influenza virus can induce potent innate immune responses in the lungs of neonatal mice

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Innate immune responses are important to protect the neonatal lung, which becomes exposed to commensal and pathogenic microorganisms immediately after birth, at a time when both the lung and the adaptive immune system are still developing. How immune cells in the neonatal lung respond to innate immune stimuli, including toll-like receptor (TLR) agonists, or viruses, is currently unclear. To address this, adult and neonatal mice were intranasally administered with various innate immune stimuli, respiratory syncytial virus (RSV) or influenza virus and cytokine and chemokine levels were quantified. The neonatal lungs responded weakly to RSV and most stimuli but more strongly than adult mice to R848 and influenza virus, both of which activate TLR7 and the inflammasome. Notably, neonatal lungs also contained higher levels of cAMP, a secondary messenger produced following adenosine receptor signaling, than adult lungs and increased responsiveness to R848 was observed in adult mice when adenosine was coadministered. Our data suggest that the neonatal lung may respond preferentially to stimuli that coactivate TLR7 and the inflammasome and that these responses may be amplified by extracellular adenosine. Improved understanding of regulation of immune responses in the neonatal lung can inform the development of vaccine adjuvants for the young.

Mucosal Immunology (2021) 14:267–276; https://doi.org/10.1038/s41385-020-0314-6

INTRODUCTION

Our immune system must be ready to fight infections from birth, when we exit a sterile environment and suddenly become exposed to a myriad of both commensal and pathogenic microorganisms.[1] The adaptive immune responses of neonates are immature and therefore newborns rely to a greater extent on innate immunity to contain potential invaders.[2,3] This innate immune response needs to be tightly regulated as mucosal surfaces, such as the lung, should not mount over-exuberant inflammatory responses at a time when they are being colonized by novel commensal microorganisms. At the same time, neonates are also exposed to pathogens immediately after birth and are especially susceptible to lung infections. For example, respiratory syncytial virus (RSV) is the most common cause of infant hospitalization in the western world[4] and can cause severe lower respiratory tract infections especially in infants.[5] Mobilizing neonatal antiviral immune responses, especially innate immune responses, could be useful in fighting such infections and, therefore, there is a need to understand how the neonatal lung responds to innate immune stimulation and whether it is hyporesponsive to all or only some stimuli.

Innate immune responses are initiated upon detection of conserved components of pathogens known as pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by germline-encoded pattern-recognition receptors (PRRs) initiates an intracellular signaling cascade, which causes the translocation of transcription factors to the nucleus and the transcription of genes encoding proinflammatory cytokines and chemokines.[6] PRRs include cell membrane bound and cytosolic receptors. The group of membrane bound receptors is composed of toll-like receptors (TLRs) and C-type lectin receptors, while the cytosolic receptors include RIG-I-like receptors (RLRs) and STING-coupled receptors.[7] In addition to PRR triggering, inflammasome activation is important for innate immunity, especially for the secretion of mature IL-1β and IL-18.[8] These are induced as procytokines by signals from PRRs and need to be cleaved by inflammasome-dependent activated caspase-1 into their mature forms.[8,9] The expression of PRRs is similar in cells from healthy newborns, children, and in adults.[10] However, the data on PRR responsiveness to triggers are conflicting. Previous studies in humans have shown that, compared with adult monocytes, those from neonatal cord blood produce less IL-1α, IL-1β, TNF-α, IL-8, and IL-12p70 after exposure to TLR agonists.[11] However, they produce equal or higher amounts of IL-6 and IL-10 after exposure to *S. aureus* or group B streptococcal microbes.[12] In addition, R848 (resiquimod, a TLR7/8 agonist) induces the production of TNF in neonatal human blood monocytes at similar levels to adult monocytes.[11] This suggests that some but not all PRR pathways are functional in blood cells from human neonates. However, these data do not inform on responses in tissues where multiple cell types, including different leukocytes and structural cells, can respond to PAMPs. As access to human tissue is limited, we took an organ-based approach in mice to examine the differences in the ability of neonatal and adult lungs to respond to stimulation by different PRR agonists or by viruses. We report that neonatal mouse lungs are hyporesponsive to several PRR agonists and to RSV but, surprisingly, are responsive to R848 or to influenza virus infection, which both can signal via TLR7 and the inflammasome. In addition, we show that the neonatal lung contains elevated levels of cAMP and that adenosine can enhance responsiveness to R848

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Received: 5 July 2019 Revised: 16 May 2020 Accepted: 27 May 2020
Published online: 23 June 2020

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exposure in adult mice. Understanding the strengths and weaknesses of the neonatal lung immune responses may help the development of novel vaccines, adjuvants, and antiviral treatments for the very young.

RESULTS

Cytokine responses to R848, but not CpG, poly(I:C), or LPS, are more potent in the neonatal than the adult lung

To assess differences in responses to innate stimulation, adult and neonatal mice were intranasally given CpG, poly(I:C), LPS (agonists for TLR9, TLR3/RLRs, and TLR4, respectively), R848 (agonist for TLR7/8 in humans and for TLR7 in mice[13,14] and also an activator of inflammasomes,[15–18]) or PBS (as control). IL-6 levels in bronchoalveolar lavage fluid (BAL) were measured as IL-6 is a driving force for mobilizing mucosal epithelial cells and antimicrobial proteins and peptides in both neonates and adults.[2,19] When compared with neonates, adults had much greater levels of IL-6 in the BAL 12 h after exposure to CpG, poly(I:C), or LPS (Fig. 1a). In contrast, IL-6 levels were higher in BAL of neonates exposed to R848 when compared with adults (Fig. 1a). Interestingly, when exposed to imiquimod (a selective TLR7 ligand), no IL-6 was detected in the BAL from neonates or adults (data not shown). One reason for this might be the dual activity of R848 as a TLR and inflammasome stimulator.

To extend these data, the early inflammatory response to R848 in adults and neonates was analyzed in greater detail (Fig. 1b–f). As early as 6 h post R848 administration, transcripts encoding proinflammatory cytokines (*Il6*, *Tnfa*) were higher in lung tissue from neonates compared with adults, which translated into higher levels of TNF-α and IL-6 protein in BAL (Fig. 1b, c). The expression of the neutrophil chemoattractant *Cxcl1* was also increased at 6 h post R848 exposure in the neonatal lung and a significant influx of neutrophils into the airways was observed in neonates at 12 and 24 h after R848 exposure (Fig. 1d). Furthermore, the expression of *Il1b* and *Il28b* (a member of the type III interferon (IFN) family) was increased in the neonatal lung compared with the adult lung (Fig. 1e, f). Although, these mediators could not be detected in BAL via ELISA or Luminex (data not shown). Interestingly, type I IFNs (IFN-α5 and IFN-β) were not detectable either via transcript analysis or ELISA (data not shown). However, *Ifng* and the interferon inducible gene (ISG) *Cxcl10* were induced to similar levels in adults and neonates after R848 exposure (Supplementary Fig. 1). Overall, these data suggest that the neonatal lung is hyporesponsive to many innate immune stimuli but responsive to R848, which induces high levels of proinflammatory cytokines and neutrophil recruitment.

R848 stimulation is dependent on MyD88/TRIF signaling

MyD88 and TRIF are two adaptors used for TLR signaling.[20] MyD88 also acts as an adaptor for signaling by members of the IL-1 receptor family.[21] To determine if the inflammatory response to R848 is dependent on signaling via MyD88/TRIF, wild type or *Myd88/Trif*$^{-/-}$ neonates were exposed intranasally to the stimulus. As before (Fig. 1), this induced expression of *Il6*, *Cxcl1*, *Tnfa*, *Il1b*, and *Cxcl10* in the neonatal lung of wild type neonatal mice (Fig. 2). However, in the lungs of the *Myd88/Trif*$^{-/-}$ neonates, no cytokines or chemokines transcripts were detected (Fig. 2). These data indicate that the response to R848 in neonates requires signaling via MyD88 and/or TRIF.

Neonatal alveolar macrophages and inflammatory monocytes produce IL-6 and IL-1β in response to R848

To determine which immune cells of the neonatal lung produce inflammatory mediators after R848 stimulation, mice were given R848 intranasally and, 12 h later, multiple immune cell populations (for gating strategy see Supplementary Fig. 2) were stained for IL-6, IL-1β, or TNF-α using intracellular staining and flow cytometry (Fig. 3). No TNF-α was detectable using this method (data not shown), but the two other cytokines gave a clear signal. The main source of IL-6 appeared to be neonatal AMs and inflammatory monocytes (Fig. 3a) but not neutrophils, eosinophils, CD103^{+} conventional dendritic cells (cDCs), CD11b^{+} cDCs, or monocyte-derived DCs (Supplementary Fig. 3). When compared with adults, a significantly higher percentage of neonatal AMs expressed IL-6 and there was a similar trend for inflammatory monocytes (Fig. 3b).

In addition, neonatal AMs, but not adult AMs, expressed IL-1β (Fig. 3c, d), which might correspond to the precursor and/or the mature cytokine as the antibody does not distinguish the two. Adult or neonatal inflammatory monocytes did not express (pro)-IL-1β (data not shown). Thus, AMs appear to be a significant source of IL-6 and (pro)-IL-1β in neonates after exposure to R848 while inflammatory monocytes act as an additional source of IL-6.

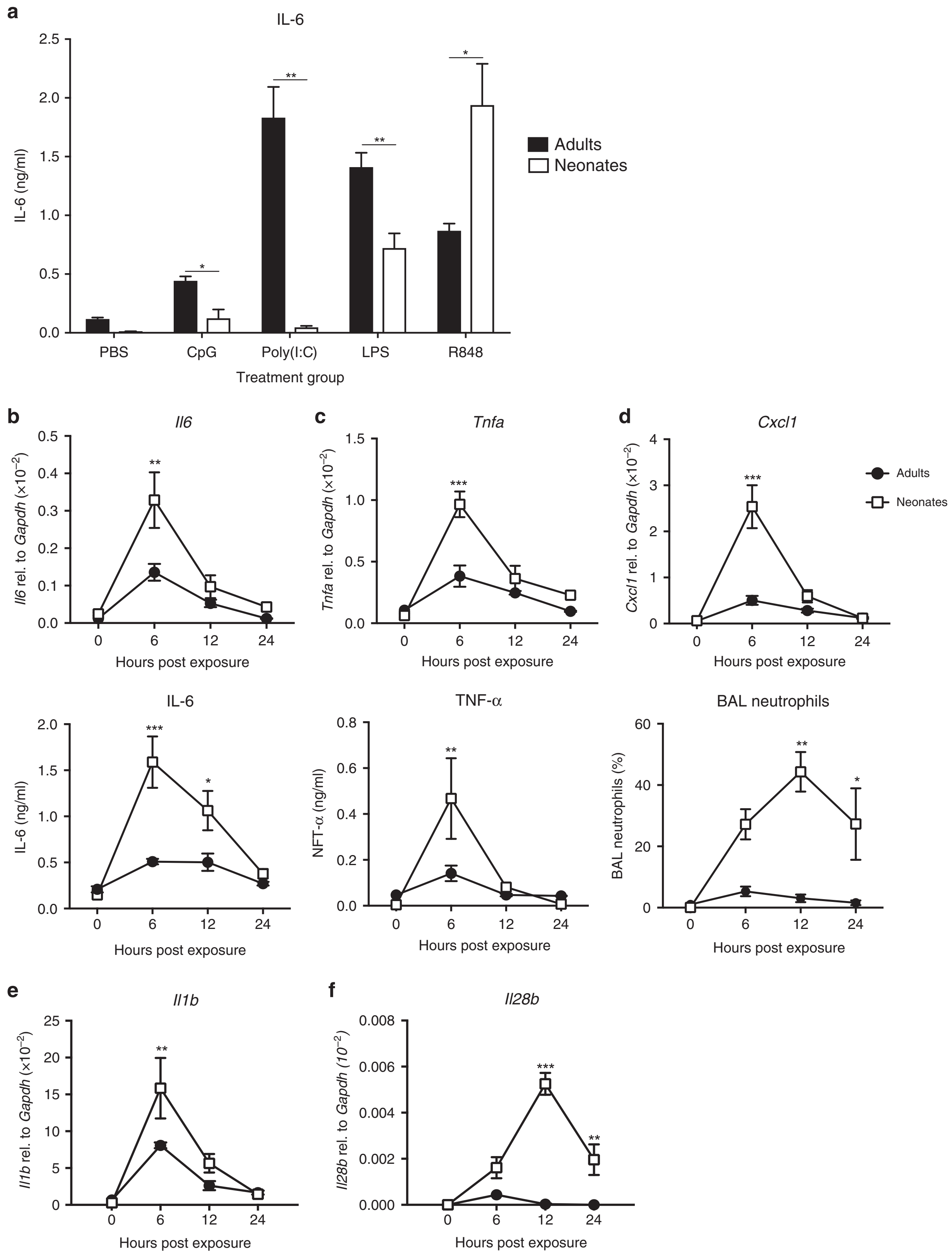
Neonate mice respond to influenza A virus but not RSV infection

We next examined how cytokine and chemokine production differed in adult and neonatal lungs during viral infection. Mice were intranasally infected with RSV or influenza PR8 virus, adjusting the virus dose to the weight of mice so that both cohorts were similarly infected. The expression of genes encoding proinflammatory mediators was measured at 18 and 48 h post exposure, time points that correspond to when these mediators peak during RSV and influenza virus infection, respectively.[22–25] Broadly, similar levels of viral RNA could be detected after either RSV or PR8 challenge, confirming a similar degree of infection in both adult and neonatal mice (Supplementary Fig. 4A). Upon infection with RSV, adult mice displayed higher expression of *Ifna5*, *Cxcl10*, *Il6*, *Il1b*, *Tnfa*, and *Cxcl1* in lung tissue at 18 h post infection compared with neonates (Fig. 4a). Surprisingly, the opposite pattern was seen with influenza virus, with neonates expressing similar or higher levels of these mediators compared with adult mice (Fig. 4b). There was no difference between adults and neonates in expression of *Tlr7* at baseline and all mice upregulated the transcript during infection although a larger upregulation was observed in adult mice during RSV infection (Supplementary Fig. 4B). Taken together, these data suggest that neonates can respond to influenza virus infection similarly to adults early after infection but this is not the case for infection with RSV.

cAMP is higher in the neonatal lung and adenosine can potentiate IL-6 responses after R848 exposure

The potential mechanism/s for the selective response to R848 and influenza A virus in the neonatal lung is currently unknown. Factors such as CD200-CD200R and surfactants are important for development, lung structure, and regulation of homeostasis and AM activation.[1,26] However, the expression of *Cd200* and *Cd200r1* was higher in the adult lung compared with neonates and adult lungs also expressed significantly higher levels of the surfactants *Sftpa1*, *Sftpc*, and *Sftpd* when compared with neonatal lung (Supplementary Fig. 5). Furthermore, the complement system, especially C1q, has been shown to alter TLR7-dependent responses.[27] Therefore, C1q levels in serum, lung lysate, and BAL from naïve adult and neonatal mice were assessed. A higher level of C1q was observed in adult compared with neonatal serum but no differences were detected in lung lysates and no C1q was detected in BAL from either age group (Supplementary Fig. 5). Thus, it is unlikely that C1q is the factor that potentiates R848 stimulation in the lungs of neonatal mice.

Adenosine has multiple roles in lung tissue development and regulation of TLR signaling,[2,28] including inhibiting TLR-mediated TNF-α production by human PBMCs and cord blood monocytes.[28,29] Extracellular adenosine is very labile but binds to A3 adenosine receptors to induce cAMP generation, which can be detected both intracellularly and extracellularly.[30,31] Therefore,



extracellular cAMP (in BAL) and intracellular cAMP (in lung homogenate) were measured as a surrogate marker for adenosine. Neonates had significantly higher levels of cAMP in lung homogenate and BAL compared with adults (Fig. 5a). However, no significant difference in the expression of the G-coupled adenosine receptors *Adora2a*, *Adora2b*, and *Adora3* was observed between adult and neonatal lung tissue (Fig. 5b). Loss-of-function experiments to investigate if adenosine blockade decreased

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Fig. 1 R848 induces a higher level of inflammatory mediators and neutrophil influx in the neonatal lung compared with the adult lung. **a** Neonatal and adult C57BL/6 mice were intranasally exposed to PBS, CpG, poly(I:C), LPS, or R848 for 12 h (LPS at 500 ng/g of body weight; CpG, poly(I:C), and R848 at 2.5 μg/g of body weight). The production of IL-6 was measured in the BAL by ELISA. Neonatal and adult C57BL/6 mice were exposed to R848 intranasally and levels of **b** IL-6 or **c** TNF-α were measured as gene expression in the lung tissue by qPCR (upper panel) or in the BAL via ELISA (lower panel). The expression of **d** *Cxcl1* in the lung tissue was measured by qPCR and the frequency of neutrophils in the BAL was quantified on H&E stained cytospin slides. **e** *Il1b* and **f** *Il28b* in lung tissue were measured by qPCR at different times post R848 exposure. The mean value for PBS exposed mice from 6 or 12 h is represented by the 0-h time point. Data are shown as mean ± SEM of $n = 3–6$ mice per group and are representative of at least two experiments. Statistical significance for the differences between the indicated groups was determined by one-way ANOVA with Tukey's post hoc test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

responses to R848 in the neonatal lung were not feasible as there are three adenosine receptors. Instead, we tried gain-of-function experiments in adult mice by coadministering adenosine with PBS, LPS, or R848. Adenosine on its own induced some IL-6 (Fig. 5c). Notably, it increased the levels of IL-6 elicited by R848 but not by LPS, the response to which was, if anything, suppressed (Fig. 5c). Our data suggest that adenosine can selectively enhance responses to R848 exposure in the adult lung and that the presence of cAMP in the neonatal lung correlates with R848 responsiveness.

DISCUSSION

Shortly after birth the lungs are colonized by microbial species. Tight regulation of PRR signaling is therefore crucial, as exuberant response to microbes that occupy the commensal niche or to harmless environmental antigens could be devastating.[1] A conserved evolutionary regulation of the PRR sensitivity and perhaps the signaling pathways that restrain cells of the lung from mounting an excessive immune response to microbes has been suggested.[10,29] However, immune responses to pathogenic microorganisms have to be elicited to protect the neonates from life-threatening infections.[32] Therefore, it is possible that neonates display some selectivity in responding to PAMPs rather than being globally desensitized. Our work shows that the neonatal mouse lung is less responsive than the adult lung to CpG, poly(I:C), LPS, or RSV. However, it displays a stronger response to R848 and to influenza A virus infection than the adult lung. This was associated with elevated baseline cAMP levels in the lungs of neonates, perhaps indicative of activation of receptors for extracellular adenosine, which may selectively potentiate proinflammatory responses to R848 exposure.

RSV infections in the first 6 months of life are associated with increased morbidity[33,34] but, interestingly, this is not the case for infections with influenza virus.[35] TLR7 and RLRs are key PRRs for detecting viral RNA and their dual importance has been shown during influenza virus infection.[36–39] In contrast, during RSV infection TLR7 signaling contributes to IL-17 and mucus induction while production of IL-6, IFNs, and TNF-α relies predominantly on RLR signaling.[22,40–42] Given our finding that neonates selectively respond to R848, an agonist of TLR7 in mice, and to influenza A virus infection, this may therefore mean that TLR7 signaling is especially active in young mice even if levels of receptor in total lung tissue do not differ appreciably from those in adult mice. Interestingly, R848 and influenza A virus shares the additional similarity of acting as inflammasome activators, which is in contrast to RSV. Indeed, release of mature IL-1β is frequently observed during influenza infection[9,43,44] but very little IL-1β is detected after RSV infection.[24,45,46] Here, we found that pro-IL-1β is not present in lungs of unchallenged neonatal mice or in R848-challenged neonates lacking TLR signaling adaptors MyD88/TRIF, indicating that it needs to first be induced by PRR signaling. But pro-IL-1β induced by TLR7 signaling in response to either R848 or influenza A virus would be expected to be processed into mature cytokine, allowing for amplification of inflammatory responses in a paracrine and autocrine manner. Whether this is the underlying cause of increased resistance of neonates to influenza virus compared with RSV remains unknown. The contribution of the inflammasome to the responses observed here was not investigated but it is interesting to speculate that the TLR7 and the inflammasome pathways are functional in neonates in order to detect and respond to specific threats. These might be mimicked by compounds such as R848 that, like influenza virus, dually activate TLR7, and inflammasomes.

In which cell type/s does inflammasome and/or TLR7 activation take place? Previous studies have found that human cord blood monocytes produce TNF when stimulated with R848.[11,12] In this study, we found AMs and monocytes to be the main leukocytes producing IL-1β and/or IL-6 after lung exposure to R848. Interestingly, R848 has also been shown to induce a cytokine response in the noses of mice and humans and in the gills of fish.[47] Whether, in addition to the leukocytes studied here, structural cells such as lung mesenchymal stromal cells or epithelial cells also respond to R848 and contribute to cytokine and chemokine production remains to be addressed. Reporter mice for cytokine synthesis and inflammasome activation[22,48–50] will prove helpful tools in investigating these issues.

In an effort to understand the basis for the relative hyper-responsiveness of neonates to R848, we found that the levels of cAMP were elevated in neonatal lung tissue and airways. cAMP can be induced via the activation of many G-coupled receptors including adenosine receptors.[31] It has previously been shown that neonatal plasma contain more adenosine than adult blood plasma and that human neonatal cord blood monocytes display increased sensitivity of the adenosine A3 receptor.[28] There are multiple receptors for extracellular adenosine and experiments to block them in neonates proved unfeasible. Instead, we performed a gain-of-function experiment and showed that adult mice given adenosine displayed exacerbate responses to R848 but not LPS. These findings indicate a possible role for the adenosine system in modulating innate immune responses in the lung. Future work using genetic loss-of-function may prove useful in dissecting the exact contribution of the adenosine system to lung immunity in neonates and adults.

Understanding neonatal immunity is important for the development of new vaccines for use in early life[51] as newborns fail to respond optimally to most vaccines.[52] TLR agonists have great potential as prophylactic and/or therapeutic agents against bacterial or viral infections.[16,53] Creams containing TLR7/8-agonists have been successfully used in adults for the therapy of external genital warts, superficial basal cell carcinoma and actinic keratosis.[54] Furthermore, administration of RSV F trimers with a TLR7/8-targetting adjuvant protect mice from intranasal challenge with RSV[55] and R848 administration decreases the severity of asthma in an ovalbumin sensitization mouse model.[56,57] In addition, alum-adjuvanted pneumococcal conjugate vaccine 13 administered together with 3M-052 (TLR7/8 agonist) resulted in enhanced protective responses in infant macaques.[52] Together with the findings reported here, these data suggest that TLR7/8 targeting, perhaps combined with inflammasome activation, may be a useful strategy for the development of adjuvants and vaccines for infants against

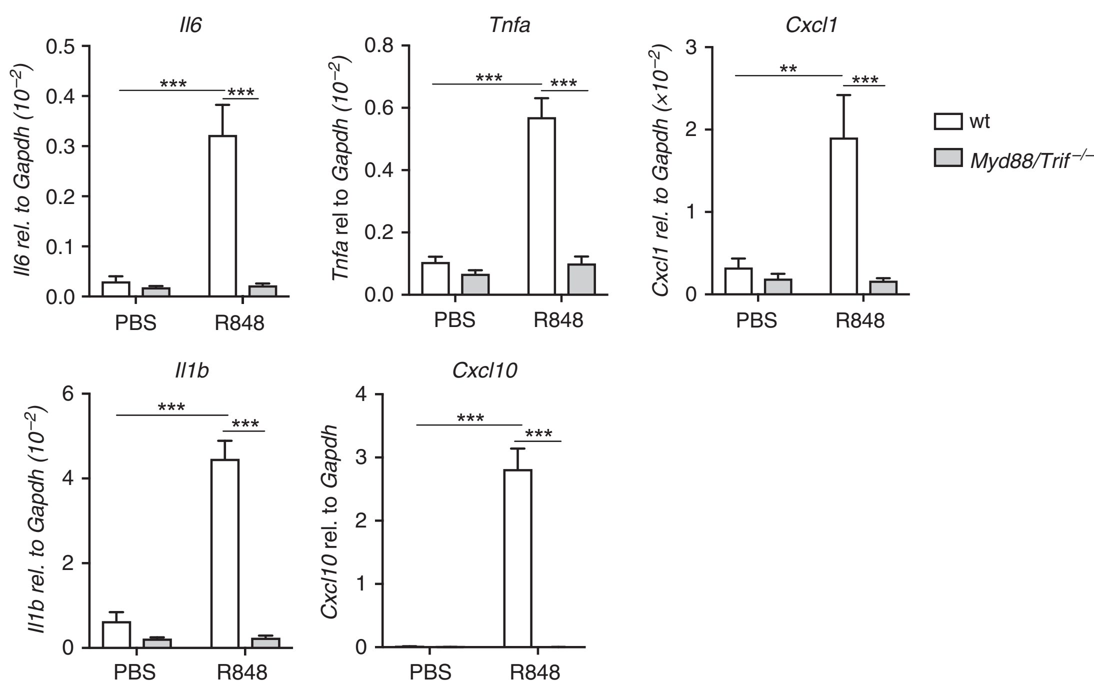

Fig. 2 R848 responses are dependent on the MyD88/TRIF signaling pathways. Neonatal wild type (wt) and *Myd88/Trif*$^{-/-}$ mice were intranasally exposed to R848 (2.5 µg/g of body weight) or PBS for 6 h. Gene expression of *Il6*, *Tnfa*, *Cxcl1*, *Il1b*, and *Cxcl10* were measured in the lung tissue by qPCR. Data are shown as mean ± SEM of $n = 8$–13 mice per group pooled from at least two experiments. Statistical significance for the differences between the indicated groups was determined by one-way ANOVA with Tukey's post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

respiratory viral pathogens and in the treatment of respiratory infections.

MATERIALS AND METHODS

Mice

C57BL/6 adult mice were purchased from Charles River or Harlan, UK. *Ifna6gfp*$^{+/-}$ and *Ifna6gfp*$^{+/-}$ *Myd88Trif*$^{-/-}$ mice were bred in-house (obtained from S. Akira, Japan.[50]) The GFP signal has not been quantified in this work so the mice will be denoted as wt and *MyD88/Trif*$^{-/-}$ mice, respectively. All mice were bred and maintained in pathogen-free conditions, and gender and age-matched mice (8–12-week-old adults; 4–6-day-old neonates) were used for each experiment. All animal experiments were reviewed and approved by the Animal Welfare and Ethical Review Board within Imperial College London and approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 and the ARRIVE guidelines.

Virus infections

Plaque-purified human RSV (originally the A2 strain from ATCC, USA) was grown in HEp2 cells. Influenza A virus, A/Puerto Rico/8/1934(H1N1); PR8 was obtained from C. Reis e Sousa (The Francis Crick Institute, UK). For infections, mice were lightly anaesthetized and virus was intranasally administered. For RSV, adults received 1×10^6 focus forming units (FFU) of RSV in 100 µl and neonates received 2×10^5 FFU in 20 µl. For PR8 infections, adults received 500 plaque forming units (PFU) in 100 µl and neonates received 100 PFU in 20 µl.

Innate stimulations

Adult mice received TLR ligands intranasally in a volume of 100 µl; whereas administration to neonates was 20 µl. R848 and poly(I:C) were administered at 2.5 µg/g body weight, CpG at 1.25 µg/g of body weight and LPS at 500 ng/g of body weight (all ligands were obtained from InvivoGen). Adenosine (Sigma-Aldrich A4036-25G) was coadministered with TLR ligands or PBS at 2.5 µg/g body weight.

Lung cell isolation

Mice were sacrificed at different time points and the lungs were perfused with PBS. Lung lobes were placed into cryovials and snap-frozen in liquid nitrogen or placed in C-tubes (Miltenyi Biotec) containing complete DMEM (cDMEM; supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin), 5 ng/ml Brefeldin A (Sigma-Aldrich), 30 µg/ml DNase I (Invitrogen), 1 mg/ml Collagenase D (Roche), and dissociated using the gentleMACS dissociator according to the manufacturer's instructions (Miltenyi Biotech). Shredded tissue was incubated for 1 h (37 °C, 5% CO_2) and the red blood cells were lysed using ACK. Remaining tissue was filtered through a 100 µm filter (BD). For lung tissue homogenate for cAMP quantification, lung tissue was homogenized according to the manufacturer's instruction (R&D Systems).

Bronchoalveolar lavage

At each endpoint the lungs were flushed with PBS supplemented with 0.5 mM EDTA (Life Technologies) three times using 1 ml for adults and 200 µl for neonates. For cAMP quantification BAL was performed using PBS. BAL cells were enumerated on counting slides (Immunesystems) using trypan blue and transferred onto microscope slides using Cytospin 4 centrifuge (Thermo Fisher). Slides were stained with hematoxylin and eosin (Quick-Diff staining, Reagena). Cells were categorized as macrophages, lymphocytes/monocytes, neutrophils, and eosinophils based on morphology, coloring, and size under a light microscope.[22,58]

Flow cytometry

Lung and BAL cells were incubated for 20 min at 4 °C with a purified rat IgG2b anti-mouse CD16/CD32 receptor antibody (BD

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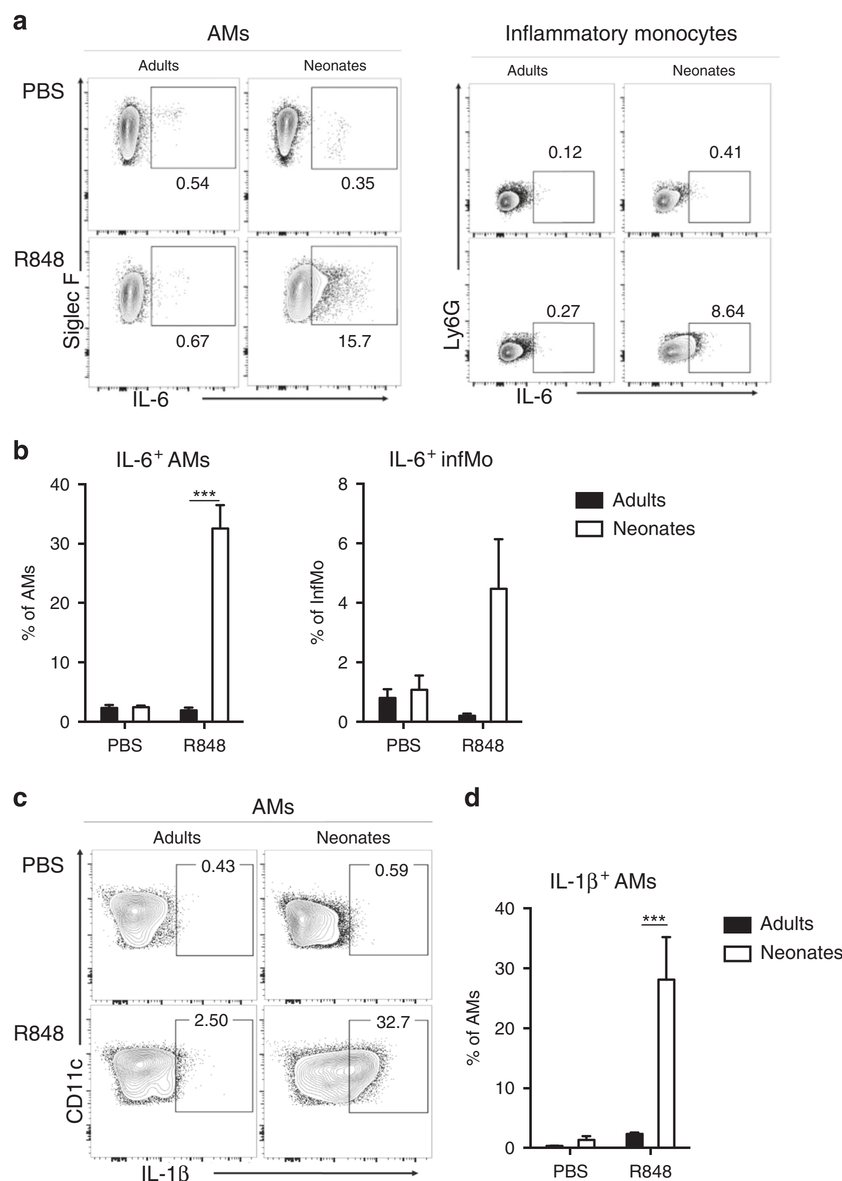

Fig. 3 Alveolar macrophages are a source of IL-6 and IL-1β and inflammatory monocytes are a source of IL-6 in the neonatal lung exposed to R848. Neonatal and adult C57BL/6 mice were intranasally exposed to R848 (2.5 μg/g of body weight) or PBS for 12 h. **a** Representative flow cytometry plots for IL-6^{+} alveolar macrophages (AMs) and IL-6^{+} inflammatory monocytes. **b** The percentage of IL-6 expressing AMs and inflammatory monocytes. **c** Representative flow cytometry plots for IL-1β^{+} AMs and **d** percentage of IL-1β expressing AMs. Data are shown as mean ± SEM of 3–5 mice, representative of two experiments. Statistical significance for the differences between the indicated groups was determined by two-tailed, unpaired Student's *t* test. *$p < 0.05$; ***$p < 0.001$.

Biosciences). Cells were stained with fluorochrome-conjugated antibodies against CD11b (M1/70, AF700), CD11c (HL3, PE-CF594), CD45 (30F11, eFluor780), CD64 (X54-5/7.1, FITC), CD103 (2E7, PerCP/Cy5.5), Ly6C (HK1.4, eFluor450), Ly6G (1A8, BV570), Siglec-F (E50-2440, PE), Siglec-H (E50-2440, eBio440c), for 25 min at 4 °C in PBS containing 1% BSA, 5 mM EDTA, 0.05% NaN3, and 20 μg/ml of GolgiStop (BD). Cells were incubated with fixable live-dead Aqua dye (Invivogen) for 30 min at 4 °C. Cells were fixed in BD Cytofix/Cytoperm (BD) containing purified rat IgG2b anti-mouse CD16/CD32 receptor antibody for 30 min at 4 °C, followed by staining for IL-1β (NJTEN3, PerCp-eFluor710), TNF-α (MP-XT22 (rat IgG1,kappa), BV650), or IL-6 (MP-520F3, APC) 60 min at 4 °C. Samples were analyzed on BD LSR Fortessa-SORP equipped with 50-mW 405-nm, 50-mW 488-nm, 50-mW 561-nm, and 20-mW 633-nm lasers and an ND1.0 filter in front of the FSC photodiode. The acquisition was set to 250,000 live/CD45^{+} cells. All antibodies were purchased from BD, Biolegend or eBioscience and data were analyzed using FlowJo Software (Tree Star).

Protein measurement

Cytokines in the BAL was measure using ELISA. The concentration of TNF-α was measured using mouse DuoSet ELISA (R&D Systems) according to the manufacturer's instruction. IL-6 was detected by ELISA using the MP-20F3 capture antibody and biotinylated MP5-32C11 detection antibody (both from BD).[22] Data were acquired on a FLUOstar Omega plate reader (BMG LABTECH) and analyzed using MARS data analysis (BMG LABTECH). The measurement of C1q in the lung, BAL, and serum was performed as previously published[27] by Marina Botto's lab (Imperial College London). cAMP was measured in lung homogenate and BAL using a Mouse cAMP Parameter Assay kit (R&D Systems).

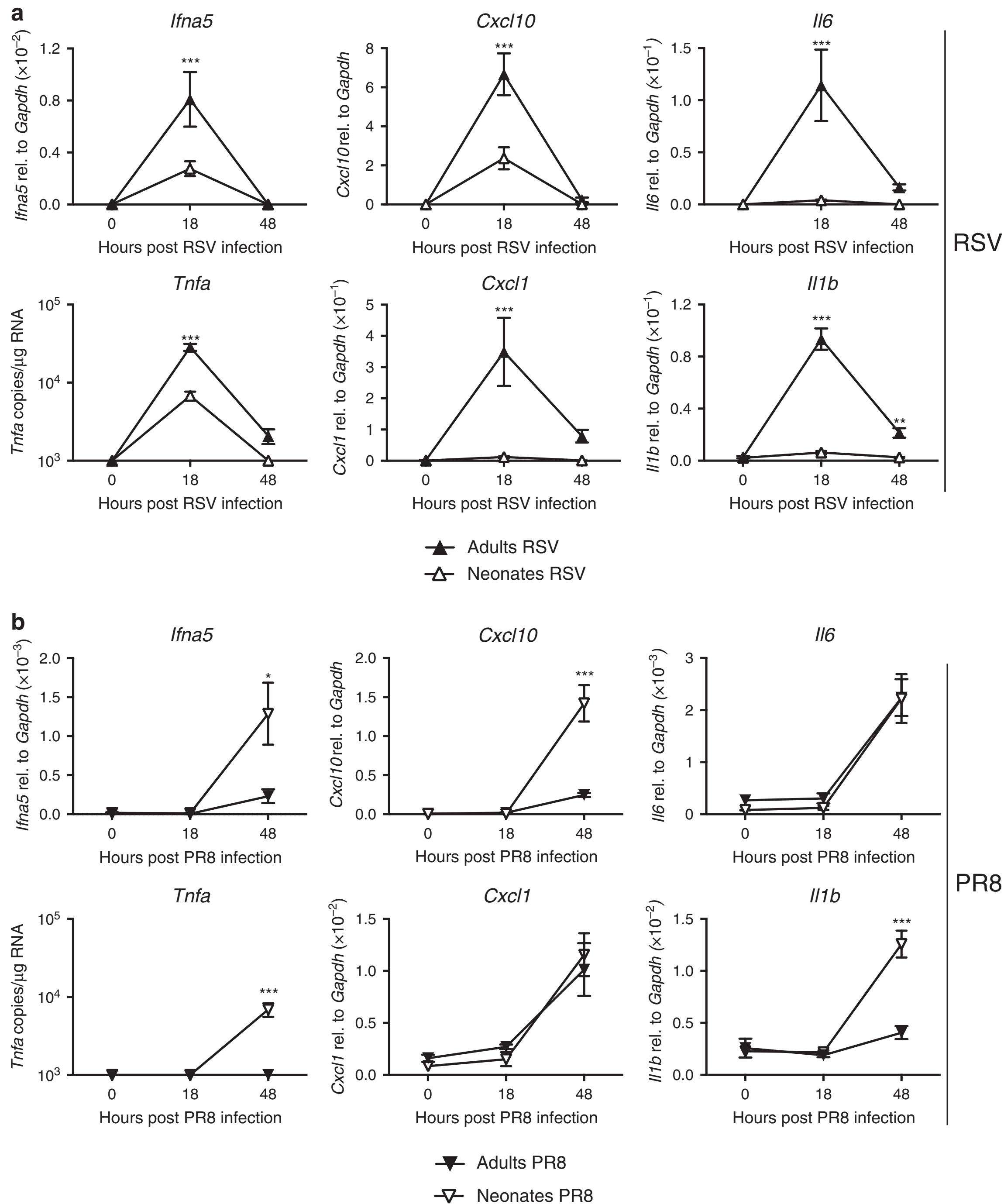

Fig. 4 Neonatal mice express cytokines/chemokine genes in response to influenza virus. Neonatal and adult C57BL/6 mice were intranasally exposed to **a** RSV or **b** influenza A virus (A/Puerto Rico/8/1934(H1N1); (PR8)) and the levels of cytokines and chemokines in the lung tissue were measured by qPCR. The expression of *Ifna5, Cxcl10, Il6, Tnfa, Cxcl1*, and *Il1b* was measured in adult and neonatal lungs at 18- and 48-h post infection by qPCR. *Tnfa* copy numbers were determined using a plasmid standard and the results were normalized to *Gapdh* levels. The mean value for naïve or PBS exposed mice from 6 or 12 h is represented by the 0-h time point. The data are shown as mean ± SEM of 3–8 mice, representative of two experiments. Statistical significance for the differences between the indicated groups was determined by one-way ANOVA with Tukey's post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

RNA isolation and RT-qPCR
Lung tissue was homogenized using a TissueLyser LT (Qiagen), and total RNA was extracted using the RNeasy Mini kit including DNA removal (Qiagen) according to the manufacturer's instructions. Overall, 1–2 μg of RNA was reverse-transcribed using the High Capacity RNA-to-cDNA kit according to the manufacturer's instructions (Applied Biosystems). qPCR was performed to quantify lung RNA levels. To quantify *Ifng*, *Tnfa*, and L gene primers and FAM-TAMRA probes previously described were used.[22] The absolute number of gene copies was calculated

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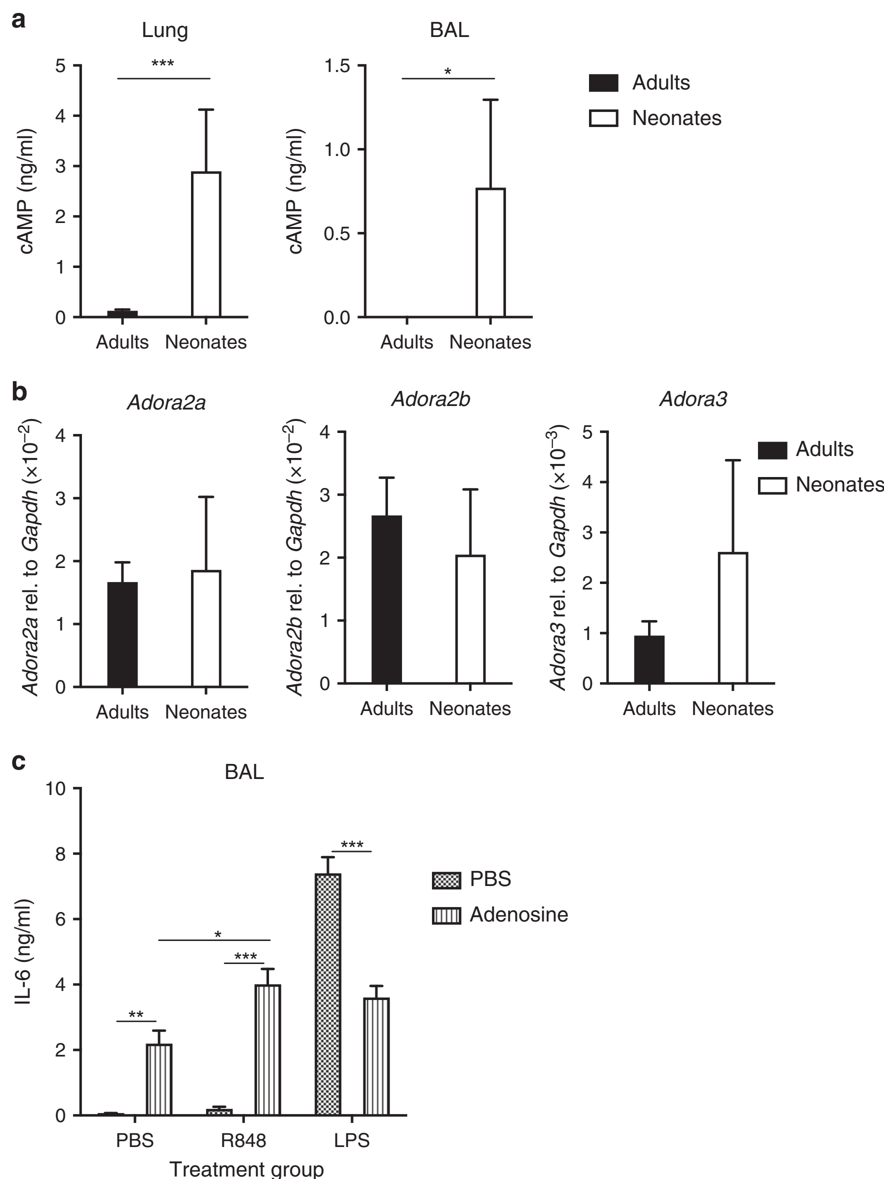

Fig. 5 Elevated cAMP levels in the airways and lungs of neonatal mice and the adenosine system increases IL-6 levels after R848 stimulation. a The levels of cAMP were assessed in lung homogenate and BAL of naïve C57BL/6 adult and neonatal mice. **b** The expression of adenosine receptors *Adora2a*, *Adora2b*, and *Adora3* was quantified using qPCR. **c** C57BL/6 adult or neonatal mice were intranasally exposed to R848 (2.5 μg/g of weight), adenosine (2.5 μg/g of weight), LPS (500 ng/g), R848 + Adenosine (each 2.5 μg/g of weight) or LPS + Adenosine (2.5 mg/g and 500 ng/g of weight for adenosine and LPS, respectively) for 6 h. The levels of IL-6 in the BAL fluid were assessed by ELISA. Data are shown as mean ± SEM from **a** 8–9 mice, **b** 5 mice, or **c** 3–4 mice and are representative of two experiments. Statistical significance for the differences between the indicated groups was determined by **a** and **b** two-tailed, unpaired Student's *t* test and **c** one-way ANOVA with Tukey's post hoc test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

using a plasmid DNA standard curve and the results were normalized to levels of *Gapdh (Applied Biosystems)*. For relative quantification, the expression of *Adora2a, Adora2b, Adora3 Cd200*, *Cd200r1*, *Cxcl1*, *Cxcl10*, *Ifna5*, *Il1b*, *Il6*, *Il28b Sftpa1*, *Sftpc*, *Sftpd*, *Tlr7* (all from Applied Biosystems run with the mastermix QuantiTech Probe PCR kit (Qiagen)), and influenza NP gene (primers from ref. [59] run with SYBR green master mix (Thermo Fisher)) were expressed relatively to the expression of *Gapdh*. First, the ΔCT (Ct = cycle threshold) between the target gene and the *Gapdh* for each sample was calculated. Then the expression was calculated as $2^{-\Delta Ct}$. Analysis was performed using 7500 Fast System SDS software (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc.). Comparisons were performed using either an unpaired Student's *t* test or one-way ANOVA with Tukey's post hoc test. For all tests, $p < 0.05$ was considered significant.

ACKNOWLEDGEMENTS

We thank Marina Botto (Imperial College London) for quantifying C1q. We thank Yanping Guo of the St Mary's flow cytometry facility for her help and the staff of the St Mary's animal facility for their assistance. We thank Caetano Reis e Sousa and Freja Kirsebom for critically reading the manuscript. S.M. was supported by a PhD

Fellowship from the National Heart and Lung Institute Foundation (registered charity number 1048073). C.J. and S.M. were supported by a grant from the Rosetrees Trust and Stoneygate Trust (M370). C.J. was also supported by a Career Development Award from the Medical Research Council (Grant G0800311).

AUTHOR CONTRIBUTIONS

S.M. designed, developed techniques, performed and analyzed the experiments, and wrote the paper. C.J. supervised the project, designed, performed and analyzed the experiments, and wrote the paper.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41385-020-0314-6) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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