Preexposure to CpG Protects against the Delayed Effects of Neonatal Respiratory Syncytial Virus Infection

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Severe respiratory viral infection in early life is associated with recurrent wheeze and asthma in later childhood. Neonatal immune responses tend to be skewed toward T helper 2 (Th2) responses, which may contribute to the development of a pathogenic recall response to respiratory infection. Since neonatal Th2 skewing can be modified by stimulation with Toll-like receptor (TLR) ligands, we investigated the effect of exposure to CpG oligodeoxynucleotides (TLR9 ligands) prior to neonatal respiratory syncytial virus (RSV) infection in mice. CpG preexposure was protective against enhanced disease during secondary adult RSV challenge, with a reduction in viral load and an increase in Th1 responses. A similar Th1 switch and reduction in disease were observed if CpG was administered in the interval between neonatal infection and challenge. In neonates, CpG pretreatment led to a transient increase in expression of major histocompatibility complex class II (MHCII) and CD80 on CD11c-positive cells and gamma interferon (IFN- γ) production by NK cells after RSV infection, suggesting that the protective effects may be mediated by antigen-presenting cells (APC) and NK cells. We conclude that the adverse effects of early-life respiratory viral infection on later lung health might be mitigated by conditions that promote TLR activation in the infant lung.

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection during infancy and childhood, infecting about 90% of infants by 2 years of age (31). The problems associated with RSV infection not only occur during the acute phase of infection but also occur in later life, increasing the risk of wheeze for more than a decade after primary infection (27, 29). It is not known whether this is an effect of severe RSV infection on lung development or if there are preexisting factors for wheezy lungs that make these children more susceptible to infantile RSV infection. Some of the genetic polymorphisms associated with the risk of severe RSV bronchiolitis are also risk factors for allergy and asthma (31). However, the association between infantile bronchiolitis and asthma is comparable in groups with different family histories of asthma, suggesting a negative effect of severe RSV infection on the developing lung, leading to bronchiolitic wheeze.

The neonatal immune response (1), and in particular the immune response in the lung (24), is Th2 skewed. We have developed a mouse model of the delayed sequelae of early-life RSV infection (4), and using this model, we have previously shown that altering the T helper balance away from Th2 using recombinant viruses that express cytokines can reduce the disease outcome (13). One factor influencing neonatal Th2 skewing is antigen-presenting cell (APC) immaturity; there are fewer APCs in neonatal lungs (24), and they are severely deficient in major histocompatibility complex class II (MHCII) and costimulatory molecule expression (including CD40, CD80, and CD86) and exhibit reduced interleukin-12 (IL-12) production (9). The insufficient IL-12 responses and the low MHC peptide density (favoring priming of Th2-type responses [20]) may limit the Th1 response in neonates and hence lead to Th2 skewing (2).

Exposure to ligands of the Toll-like receptor (TLR) family of pattern recognition receptors can induce accelerated maturation of APCs, switching the response away from Th2 toward a protective Th1 response (24), which can be protective against the development of allergy (19). Evidence from cohorts of farm children have shown that increased exposure to lipopolysaccharide (LPS) is

a protective factor against allergy (7). Other TLRs can also have a protective effect against the development of asthma; polymorphisms in the TLR9 promoter region, which may affect expression levels, for example, by altering NF- κ B binding (22), have been associated with wheeze in later life (8, 28), and levels of CpG oligodeoxynucleotides are greater in dust from rural homes (25). In a murine model, CpG treatment protects neonatal mice from the development of allergy (6).

We wished to determine whether TLR ligands have a similar protective effect against the development of the sequelae of neonatal RSV infection by altering the phenotype of the cellular immune response. Intranasal exposure to CpG prior to neonatal RSV infection reduced the disease on subsequent adult reinfection. The reduction in disease was associated with a switch to Th1-type responses and reduced viral load, linked to APC maturation induced by exposure to TLR ligands.

MATERIALS AND METHODS

Mice and virus. Time-mated pregnant BALB/c mice (Harlan, Carthorpe, United Kingdom) were purchased at <14 days of gestation, and pups

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FIG 1 Pretreatment with unmethylated CpG reduces the delayed effects of neonatal RSV infection. Neonatal mice were treated with 10 μ g CpG (day 3 of life) at 1 day prior to RSV infection (day 4 of life), and 8 weeks later mice were reinfected with RSV. Weight loss (A), lung cell number (B), and lung viral load at day 4 (C) were measured after infection, and the effects of pretreatment with methylated and unmethylated CpG on weight loss during secondary infection were compared (D) ($n = 5$ mice per group per time point). Means $\pm$ SEM are shown. *, $P < 0.05$; **, $P < 0.01$.

were weaned at 3 weeks of age. BALB/c mice were infected intranasally (i.n.) with 4×10^4 PFU/gram body weight RSV A2 at 4 days (neonatal; $\sim 10^5$ PFU in 20 μ l) or 4 to 6 weeks of age (adult; $\sim 5 \times 10^5$ PFU in 100 μ l) under isoflurane anesthesia. Mice were reinfected i.n. at 8 weeks with 10^6 PFU in 100 μ l. The RSV A2 strain was grown in HEP-2 cells and viral titer determined by plaque assay. Where stated, mice were treated with 10 μ g unmethylated or methylated CpG ODN 1826 (5'-TCCATGACGTTCCCTGACGTT, where underlined Cs are methylated in control CpG) in 20 μ l phosphate-buffered saline (PBS). RSV viral load was assessed by reverse transcription-PCR (RT-PCR) for the L gene using 900 nM forward primer (5'-GAAGTCACTGTAGGTAGAATGTTTGCA-3'), 300 nM reverse primer (5'-TTCAGCTATCATTTTCTCTGCCAAT-3'), and 100 nM probe (5'-FAM-TTTGAACCTGCTGAACATCCCGGTT-TAMRA-3') and normalized against 18S rRNA levels.

RSV-specific antibody quantification. Serum antibody was assessed by enzyme-linked immunosorbent assay (ELISA) as described previously (12). Antigen was prepared by infecting HEP-2 cells with RSV at 1 PFU/cell. Microtiter plates were coated overnight with either RSV or HEP-2 antigen. After blocking with 1% bovine serum albumin (BSA) for 1 h, dilutions of test samples were added and left for a further 1 h. Bound antibody was detected using peroxidase-conjugated rabbit anti-mouse Ig (Dako) and *o*-phenylenediamine as a substrate. RSV-specific antibody was determined by subtracting the RSV absorbance from the HEP-2 absorbance for the same sample. Specific isotypes were measured following the same protocol, changing the primary antibody.

Cell preparation and flow cytometry. After infection, animals were culled using intraperitoneal (i.p.) pentobarbitone. Cells were harvested as described previously (32). Prior to staining, cells were blocked with CD16/32. For surface staining, antibodies against the surface markers CD3, CD4, CD8, CD11c, MHCII, CD80, and CD86 (BD) were added at a 1:100 dilution and live/dead discrimination was performed using 7-amino-actinomycin D (7AAD); percentages were based on live cells. For intracellular staining, cells were stimulated for 4 h at 37°C in the presence of 10 μ g/ml brefeldin A, 100 μ g/ml phorbol myristate acetate (PMA), and 10 μ g/ml ionomycin. Cells were permeabilized with 0.5% saponin and stained with directly conjugated anti-gamma interferon (anti-IFN- γ) or anti-IL-4. Samples were run on an LSR instrument (BD) and analyzed using Winlist (Verity).

IFN- γ cytokine ELISA. Cytokine levels were assessed in bronchoalveolar lavage (BAL) fluid or lung mash supernatants by ELISA using a pair of capture and biotinylated detection antibodies (cytokine [BD] or chemokine [R&D Systems]) following the manufacturer's instructions. Mediator concentrations were quantified by comparison to recombinant cytokine standards.

Statistical analysis. Results are expressed as means $\pm$ standard errors of the means (SEM); statistical significance was calculated by ANOVA, followed by Tukey tests when there were greater than 3 groups and *t* tests for the comparison of 2 groups, using GraphPad Prism software. All calculations were performed using GraphPad Prism software.

RESULTS

CpG pretreatment reduces the delayed sequelae of neonatal RSV infection. Neonatal RSV infection primes for a long-lasting propensity for enhanced disease during adult reinfection with RSV (32). To test the effect of TLR ligands on this response, neonatal mice were pretreated with intranasal CpG (using 10 μ g CpG ODN 1826) or PBS on day 3 of life prior to RSV infection on day 4 of life. This was followed by adult RSV reinfection at 8 weeks of age. Daily weight changes were monitored during reinfection and are presented as percentages of the weight on day 0 (Fig. 1A). During adult reinfection, mice infected with RSV as neonates (nnRSV) started losing weight on day 1, peaking at day 6. In contrast, the onset of weight loss was delayed in the CpG-pretreated group (nnCpG-RSV), and these mice lost weight on days 3 and 4 only. The weight loss in CpG-pretreated mice peaked at about 10% of their original weight, and they fully recovered by day 7. There were significant differences between the nnCpG-RSV and nnRSV groups from day 3 to 7 ($P < 0.01$ from day 3 to 5; $P < 0.001$ on days 6 and 7). CpG-pretreated mice had fewer cells recruited to the lungs than nnRSV mice (Fig. 1B). The CpG-pretreated group had a significantly lower viral titer than the nnRSV group (Fig. 1C) ($P < 0.05$). These results demonstrate that neonatal CpG pretreatment provides a level of protection from the delayed sequelae of neonatal RSV infection.

The protective effect of CpG pretreatment could have been due to any synthetic DNA fragments with or without unmethylated CpG motifs; thus, neonatal mice were treated with methylated (mCpG) or unmethylated CpG oligodeoxynucleotides of exactly the same sequence. Only the unmethylated CpG-pretreated group was protected from further weight loss, while the other groups continued losing weight until the day 4 peak (Fig. 1D) ($P < 0.05$). From these studies, we observe that CpG pretreatment is protective against disease on adult RSV rechallenge.

CpG pretreatment increases Th1 responses on rechallenge. We reasoned that CpG-pretreated mice might show improved humoral immunity, providing better protection from rechallenge infection and lower viral titers in than untreated nnRSV mice. However, there was no difference in anti-RSV serum antibody and the ability of the sera to neutralize virus at 4 days after RSV rechallenge between groups with or without CpG pretreatment (data not shown).

Since levels of total anti-RSV antibodies are similar in the neonatally primed groups, a reduction of the viral titer in CpG-pretreated mice could instead be caused by strong antiviral cellular

FIG 2 Treatment with CpG prior to neonatal RSV increases antiviral cellular immunity. On day 3 of life, neonatal mice were treated with 10 μ g CpG (●, black bars) or PBS (○, white bars) at 1 day prior to RSV infection (day 4 of life), and 8 weeks later mice were reinfected with RSV. (A and B) IFN- γ (A) and IL-5 (B) measured in bronchoalveolar lavage (BAL) fluid by ELISA. (C to H) Lung DX5⁺ NK cells (C), CD4 T cells (D), IFN- γ (E)- and IL-4 (F)-producing CD4 T cells, CD8 T cells (G), and airway eosinophilia (H) were measured by flow cytometry on day 4 and/or day 7. (I to K) RSV-specific IgG1 (I) and IgG2a (J) and the ratio (K) in serum were measured on day 4 postchallenge by ELISA. Data are representative of 2 experiments, and bars represent means for 4 mice per group $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$.

responses. There was significantly more IFN- γ in the airways on day 4 postinfection in the CpG pretreatment group (Fig. 2A) ($P < 0.05$) and a decrease in IL-5 levels on day 7 (Fig. 2B). There was also a significant NK cell recruitment to the lungs on day 4 postinfection (Fig. 2C) ($P < 0.01$). There was an increase in CD4 cell number (Fig. 2D), and the CpG-pretreated group had significantly more IFN- γ -producing CD4⁺ T cells ($P < 0.05$) (Fig. 2E) and significantly fewer IL-4-producing CD4⁺ T cells ($P < 0.05$) (Fig. 2F) than the RSV group. There were also significantly more CD8⁺ T cells in the CpG-pretreated mice on day 7 ($P < 0.01$) (Fig. 2G). Supporting a shift to a Th1 phenotype was a reduction in eosinophil recruitment ($P < 0.05$) (Fig. 2H) and a switch of IgG isotype to IgG2a ($P < 0.001$) (Fig. 2I, J, and K). These results suggest that CpG pretreatment alters the responses, increasing Th1 responses, improving viral clearance during reinfection, and reducing disease.

CpG has been shown to have a protective effect as immunotherapy against allergic sensitization (14). We wished to determine whether the protective effect of CpG occurred only prior to

neonatal infection or if it could modulate the recall immune response after it had developed. To test this, we administered CpG intranasally 4 weeks after neonatal RSV infection and then reinfected mice as adults. CpG-treated mice lost significantly less weight than untreated mice ($P < 0.05$) (Fig. 3A). The reduced illness may be explained by boosting of the amount of RSV-specific serum antibody at 4 weeks old, but the amount of total antibody was not altered by interval CpG treatment (data not shown), and there was no difference in IgG1 (Fig. 3B) or IgG2a (Fig. 3C) levels, suggesting that these are set at the time of the initial infection. There was a significant increase in IFN- γ ($P < 0.01$) (Fig. 3D) and a decrease in both IL-5 ($P < 0.05$) (Fig. 3E) and eosinophil number (Fig. 3F) in the airways on day 4 postinfection. However, CpG did not significantly alter the numbers of NK (Fig. 3G), CD4 (Fig. 3H), or CD8 (Fig. 3I) cells recruited to the lungs postinfection. Therefore, the pathogenic cellular recall response following neonatal RSV response is not permanently imprinted and can be switched by later exposure to CpG.

Effect of CpG on antigen-presenting cells in the neonatal

FIG 3 Interval CpG application abrogates neonatal disease. Four-day-old BALB/c mice were infected i.n. with 10^5 PFU RSV or sham infected with PBS. Four weeks later, mice were given i.n. CpG (open symbols and bars) or PBS (filled symbols and bars), and 8 weeks later mice were challenged with 10^6 PFU of RSV. (A) Weight was monitored following infection. (B and C) RSV-specific IgG1 (B) and IgG2a (C) subtypes were measured by ELISA. (D to F) Airway IFN- γ (D), IL-5 (E), and eosinophilia (F) were measured on day 4 after infection. (G to I) Lung NK (G), CD4 cells (H), and CD8 cells (I) were measured on day 4 and 7 postinfection. Data are representative of 2 independent repeats with 5 mice per group per time point; means $\pm$ SEM are shown. *, $P < 0.05$; **, $P < 0.01$.

lung. To further examine the mechanism by which CpG exposure was protective against secondary challenge with RSV, we focused on the role of antigen-presenting cells (which are known to be highly TLR responsive). It has been observed that dendritic cells (DCs) in the neonatal lung have a Th2 bias but that this can be modified by exposure to LPS or *Mycobacterium bovis* BCG (24). To determine if a similar effect occurs with CpG, we compared phenotypes of CD11c⁺ APCs in adult and neonatal mice to give a general view of what is happening to lung DCs in response to RSV versus RSV plus CpG. Seven-day-old neonatal mice and 8-week-old adult mice were infected with RSV (4×10^4 PFU per gram body weight) and compared to age-matched PBS-treated controls. Four mice were sacrificed from each group on days 4, 7, and 11 after infection, and lung samples were collected for further analysis.

Significantly fewer cells were recruited to the lungs of neonatal mice than to those of adult mice following primary infection ($P <$

0.001) (data not shown). To measure numbers of APCs, cells from the lung were stained for the surface marker CD11c, MHC class II, and the marker of activation CD86, and cells were scored as a percentage of total live (7-AAD-negative) cells. There were no significant differences in the percentage or number of activated CD11c⁺ APCs in the RSV-infected neonates compared to their age-matched PBS-treated controls at any time point (Fig. 4A). However, in adult mice both the percentage and the number of activated CD11c⁺ APCs increased after RSV infection compared to those in the PBS group and the neonatal RSV group, peaking on day 11 (Fig. 4A) ($P < 0.001$).

It is known that CpG treatment causes the recruitment and maturation of splenic APCs in neonates following subcutaneous immunization (10). To test whether CpG altered APC activation in the lungs of neonatal mice after viral infection, 6-day-old mice were infected with RSV, with or without intranasal CpG pretreatment. CpG pretreatment had no effect on lung viral load during

FIG 4 CpG pretreatment increases APC activation. Neonatal or adult mice were infected with RSV intranasally or left uninfected. (A) At days 0, 4, 7, and 11 postinfection, lungs were removed and the number of CD11c⁺ cells expressing MHCII and CD86 characterized by flow cytometry. ***, $P < 0.001$, comparing Ad RSV with NN RSV; ###, $P < 0.001$, comparing Ad RSV with Ad PBS. (B to G) Neonatal mice were treated with 10 μ g CpG 1 day prior to RSV infection. Lungs were removed and viral load was measured on day 4 postinfection (B), and CD11c⁺ cell number (C) and mean fluorescence intensity (MFI) of MHCII (D) and CD80 (E) were measured by fluorescence-activated cell sorting (FACS) on days 1 and 4 postinfection. Numbers of DX5⁺ NK cells (F) and IFN- γ producing NK cells (G) were also evaluated in the lungs by FACS. Bars and points represent means $\pm$ SEM for 4 mice; *, $P < 0.05$; **, $P < 0.01$.

primary infection (Fig. 4B). The number of CD11c⁺ cells in neonatal lungs on days 1 and 4 was not altered by CpG pretreatment or RSV infection (Fig. 4C). However, the CpG-RSV-treated mice showed a significant increase in the expression levels of MHC class II molecules on CD11c⁺ cells, compared to the naïve as well as RSV infection-only groups (Fig. 4D) ($P < 0.01$). The expression of CD80 on CD11c⁺ cells was also significantly increased by pretreatment compared to that in the naïve group (Fig. 4E) ($P < 0.05$). There was a 1.75-fold increase in the percentages of lung CD11c⁺ cells expressing both MHC class II and CD80 between the CpG-RSV and RSV-only groups ($21.4\% \pm 2.7\%$ compared to $12.3\% \pm 2.3\%$ of the lung cells, respectively [$10.9 \pm 1.5\%$ for the naïve group]). These differences were observed only at the day 1 time point and were not sustained until day 4. CpG pretreatment had no effect on CD4 or CD8 cell number in the lungs (data not shown); however, there was an increase in the number of DX5⁺ NK cells (Fig. 4F) and a significant increase in the number of IFN- γ -secreting NK cells on day 1 postinfection ($P < 0.05$) (Fig. 4G). The data show that CpG pretreatment changed the APC phenotype in the lungs at a very early time point, which may switch the response away from Th2.

DISCUSSION

We show that CpG exposure prior to neonatal RSV infection led to increased protection against adult reinfection and to reduced disease on adult reexposure to RSV. Similar effects were also seen if CpG was delivered intranasally after the complete resolution of primary infection, in the period between neonatal challenge and adult reinfection. These effects were associated with a notable increase in Th1 responses in the lung, probably due to an increase in APC activation caused by the CpG exposure.

We and others have shown that cellular immune responses to RSV infection can be both protective and pathogenic (23). The ideal protective response reduces the viral load without excess local inflammation, whereas the adverse pathogenic response causes inflammation without effectively controlling the viral load. For example, in adult RSV infection, CCL3 depletion increased proinflammatory RSV-specific cells but reduced the total number of cells (34). The data from our previous studies suggest that neonatal RSV infection induces hyperinflammatory cellular responses during reinfection and that reducing this cellular infiltrate can reduce disease (32), but the interaction with APCs was critical in determining outcome (33). The current study shows that the response following neonatal infection can be switched from pathogenic to protective using TLR ligands. These and other studies suggest that the context of the initial exposure to virus or viral antigen determines the outcome of subsequent exposures, and parallels can be drawn with the development of asthma and allergy in early life (35). However, these responses are relatively plastic; for example, the incidence of postbronchiolitic wheeze decreases with age. This may reflect subsequent exposures to TLR ligands modulating the immune response, and our interval CpG treatment experiment supports this idea.

The T helper balance of the response to RSV is important in determining whether it will be pathogenic or protective. Previously we have observed a reduction in weight loss on adult reinfection following the use of a recombinant virus expressing IFN- γ (13), and the addition of recombinant IFN- γ has been shown to have a protective effect (17). However, there were differences between CpG and IFN- γ treatment; while both treatments suppressed Th2-type responses during rechallenge infection, only CpG treatment enhanced Th1 responses. Furthermore, only CpG

treatment reduced the viral titer on reinfection. CpG has previously been shown to alter the Th2 skewing of neonatal APCs (15). It is of note that strongly skewed Th1 responses can also be pathogenic; when IFN- γ -expressing RSV was used in adult mice, it led to increased disease on rechallenge (11), and therefore a balanced cellular response is desirable.

Our data suggest that deficient activation of neonatal APCs is involved in the delayed sequelae of RSV infection; neonatal CD11c⁺ cells failed to upregulate MHC class II and costimulatory molecules on RSV infection. Delayed maturation of neonatal APCs may be a mechanism to reduce harmful inflammatory responses to self or benign environmental antigens in early life (2). It has been shown that neonatal APCs are deficient in a number of key signaling molecules, including IRF3 (3) and IRF7 (5). However, some TLR ligands can activate neonatal APCs, including R848 acting on TLR7/8 (18), LPS acting on TLR4 (24), and CpG acting on TLR9 (30). One question is why RSV infection does not induce a similar maturation of neonatal APCs, given that RSV has been shown to interact with TLR3 (26) and TLR4 (16). One possibility is that the virally carried genes suppress the innate response, and it has been recently demonstrated that the RSV NS1 protein suppresses Th1-type responses (21); in the context of the preskewed lung environment in early life, this suppression may be particularly potent.

In conclusion, we show that prior exposure to the TLR9 ligand CpG is protective against the delayed effects of neonatal RSV infection. These delayed effects may model some of the features of postbronchiolitic wheeze and viral exacerbations of asthma, and therefore TLR ligand administration may have therapeutic potential in prevention of respiratory morbidity in childhood. These results may also go some way to explain how exposure to bacterial products in early life can protect against the development of asthma.

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