Pre-exposure to CpG protects against the delayed effects of neonatal RSV infection

Running title: Protective effect of CpG on neonatal lungs

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Abstract

Severe respiratory viral infection in early life is associated with recurrent wheeze and asthma in later childhood. Neonatal immune responses tend to be skewed towards 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 TLR ligands, we investigated the effect of exposure to CpG oligodeoxynucleotides (TLR9 ligand) prior to neonatal RSV infection in mice. CpG pre-exposure 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 was observed if CpG was administered in the interval between neonatal infection and challenge. In neonates, CpG pre-treatment led to a transient increase in expression of MHCII and CD80 on CD11c positive cells and IFNγ production by NK cells after RSV infection, suggesting that the protective effects may be mediated by 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.

Key Words: Innate, Neonatal, TLR, respiratory tract infection, RSV, mouse
Introduction

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection during infancy and childhood, infecting about 90% of infants by two years of age (32). 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 (28,30). It is not known whether this is an effect of severe RSV infection on lung development, or if there are pre-existing factors for wheezy lungs that makes 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 (32). 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 post bronchiolitic wheeze.

The neonatal immune response (1) and in particular the immune response in the lung (25) 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 (14). One factor influencing neonatal Th2 skewing is APC immaturity, there are fewer APC in neonatal lungs (25), they are severely deficient in MHC class II and co-stimulatory molecule expression (including CD40, CD80 and CD86) and exhibit reduced IL-12 production (10). The insufficient IL-12 responses and the low MHC-peptide density (favouring priming of Th2 type responses (21)) may limit the Th1 response in neonates and hence lead to Th2-skewing (2).

Exposure to ligands of the Toll like receptor family (TLR) of pattern recognition receptors can induce accelerated maturation of APC switching the response away from Th2 towards a protective Th1 response (25), which can be protective against the development of allergy (20). Evidence from cohorts of farm children have shown that increased exposure to LPS is a protective factor against allergy (8). Other TLR 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-κB binding (23), have been associated with wheeze in later life (9,29) and levels of CpG oligodeoxynucleotides are greater in dust from rural homes (26). 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 re-infection. 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.
Methods

Mice and Virus. Time mated pregnant BALB/c (Harlan, Carthorpe, UK) were purchased at <14d gestation and pups were weaned at 3wk old. BALB/c mice were infected i.n. with 4x10⁴ PFU/gram body weight RSV A2 at 4 days (neonatal ~ 10⁵ PFU in 20μl) or 4-6 weeks of age (adults ~ 5 x 10⁵ PFU in 100μl) under isoflurane anaesthesia. Mice were re-infected i.n. at 8wk, with 10⁶ PFU in 100μl. RSV A2 strain was grown in HEp-2 cells and viral titre determined by plaque assay. Where stated mice were treated with 10 μg unmethylated or methylated CpG ODN 1826 in 20 μl PBS (5'-TCCATGA.CGCTTCCTGAGT where Cs are methylated in control CpG). RSV viral load was assessed by RT-PCR for the L gene using 900 nM forward primer (5'-GAACCTAGGTAGGATGTTGGCA-3'), 300 nM reverse primer (5'-TTCAGCTATCATTTTTCTGCAAT-3'), and 100 nM probe (5'-FAM-TTTGAACCTGTCTGAACATTCCCGGTT-3') and normalised against 18s rRNA levels.

RSV-specific antibody quantification. Serum antibody was assessed by ELISA as described previously (13). 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% BSA for 1h, dilutions of test samples were added for a further 1h. 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 i.p. pentobarbitone. Cells were harvested as described previously (33). Prior to staining cells were blocked with CD16/32. For surface staining antibodies against the surface markers CD3, CD4, CD8, CD11c, MHCII, CD80, CD86 (BD) were added in 1:100 dilution and live/ dead discrimination was performed using 7AAD, percentages were based on live cells. For intracellular staining, cells were stimulated for 4h at 37°C in the presence of 10 μg/ml Brefeldin A, 100μg/ml PMA and 10μg/ml ionomycin. Cells were
permeabilised with 0.5% saponin and stained with directly conjugated anti-IFNγ or anti-IL4. Samples were run on an LSR (BD) and analysed using Winlist (Verity).

**IFNγ Cytokine ELISA.** Cytokine levels were assessed in 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 mean ± S.E.M.; 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 pre-treatment reduces the delayed sequelae of neonatal RSV infection.

Neonatal RSV infection primes for a long-lasting propensity to enhanced disease during adult re-infection with RSV (33). To test the effect of TLR ligands on this response, neonatal mice were pre-treated with intranasal CpG (using 10μg 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 re-infection at 8 weeks of age. Daily weight changes were monitored during re-infection and presented as percentages of the weight on day 0 (Figure 1A). During adult re-infection, 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 pre-treated group (nnCpG-RSV) and they lost weight on days 3 and 4 only. The weight loss in CpG pre-treated 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 group from day 3 to 7 (p<0.01 from day 3 to 5, p<0.001 on day 6 and 7). CpG pre-treated mice had fewer cells recruited to the lungs than nnRSV mice (Figure 1B). The CpG pre-treated group had a significantly lower viral titre compared to the nnRSV group (Figure 1C, p<0.05). These results demonstrate that neonatal CpG pre-treatment provides a level of protection from the delayed sequelae of neonatal RSV infection.

The protective effect of CpG pre-treatment 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 pre-treated group were protected from further weight loss while the other groups continued losing weight until the day 4 peak (Figure 1D, p<0.05). From these studies we observe that CpG pre-treatment is protective against disease on adult RSV re-challenge.

CpG pre-treatment increases Th1 responses on rechallenge.
We reasoned that CpG pre-treated mice might show improved humoral immunity, providing better protection from rechallenge infection and lower viral titres in than untreated nnRSV mice. However, there was no difference in anti-RSV serum antibody and the ability of the sera to neutralise virus four days after RSV re-challenge between groups with or without CpG pre-treatment (data not shown). Since levels of total anti-RSV antibodies are similar in the neonatally primed groups, a reduction of the viral titre in CpG pre-treated mice could instead be caused by strong antiviral cellular responses. There was significantly more IFNγ in the airways on day 4 post infection in the CpG pre-treatment group (Figure 2A, p<0.05) and a decrease in IL-5 levels on d7 (Figure 2B). There was also a significant NK cell recruitment to the lungs on day 4 post infection (Figure 2C, p<0.01). There was an increase in CD4 cell number (Figure 2D) and the CpG pre-treated group had significantly more IFNγ producing CD4+ T cells (p<0.05, Figure 2E) and significantly fewer IL-4 producing CD4+ T cells than the RSV group (p<0.05, Figure 2F). There were also significantly more CD8+ T cells in the CpG pre-treated mice on day 7 (p<0.01, Figure 2G). Supporting a shift to a Th1 phenotype was a reduction in eosinophil recruitment (p<0.05, Figure 2H) and a switch of IgG isotype to IgG2a (p<0.001, Figure 2I, J and K). These results suggest that CpG pre-treatment alters the responses increasing Th1 responses, improving viral clearance during re-infection and reducing disease.

CpG has been shown to have a protective effect as immunotherapy against allergic sensitisation (15). We wished to determine whether the protective effect of CpG only occurred 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 re-infected mice as adults. CpG treated mice lost significantly less weight than untreated mice (p<0.05, Figure 3A). The reduced illness may be explained by boosting 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 depicted), and there was no difference in IgG1 (Figure 3B) or IgG2a (Figure 3C) levels, suggesting that...
these are set at the time of the initial infection. There was a significant increase in IFNγ (p<0.01, Figure 3D) and a decrease in both IL-5 (p<0.05, Figure 3E) and eosinophil number (Figure 3F) in the airways on d4 post infection. But CpG did not significantly alter the numbers of NK (Figure 3G), CD4 (Figure 3H) or CD8 (Figure 3I) cells recruited to the lungs post infection. 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 lung**

To further examine the mechanism by which CpG exposure was protective against secondary challenge with RSV, we focussed on the role of antigen presenting cells (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 BCG (25). To determine if a similar effect occurs with CpG, we compared phenotypes of CD11c+ APC in adult and neonatal mice, to give a general view of what is occurring to lung DCs in response to RSV vs RSV+CpG. 7-day-old neonatal mice and 8-week-old adult mice were infected with RSV (4 x 10⁴ pfu per gram weight) and compared to age matched PBS treated controls. Four mice were sacrificed from each group on day 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 adult mice following primary infection (p<0.001, data not depicted). To measure numbers of APC, cells from the lung were stained for the surface marker CD11c, MHC class II and the marker of activation CD86, 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+ APC in the RSV infected neonates compare to their age-matched PBS controls at any time point (Figure 4A). However, in adult mice both the percentage and the number of activated CD11c+ APC increased after RSV infection compared to the PBS group and the neonatal RSV group, peaking on day 11 (Figure 4A, p<0.001).
It is known that CpG treatment causes the recruitment and maturation of splenic APC in neonates following subcutaneous immunisation (11). 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 pre-treatment. CpG pre-treatment had no effect on lung viral load during primary infection (Figure 4B). The number of CD11c+ cells in neonatal lungs on day 1 and 4 was not altered by CpG pre-treatment or RSV infection (Figure 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 (Figure 4D, p<0.01). The expression of CD80 on CD11c+ cells was also significantly increased by pre-treatment compared to the naïve group (Figure 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 ± 2.7% compared to 12.3 ± 2.3% of the lung cells, respectively, 10.9 ± 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 pre-treatment had no effect on CD4 or CD8 cell number in the lungs (data not depicted), however there was an increase in the number of DX5+ NK cells (Figure 4F) and a significant increase in the number of IFNγ secreting NK cells on day 1 post infection (p<0.05, Figure 4G). The data show that CpG pre-treatment changed the APC phenotype in the lungs at a very early time point which may switch the response away from Th2.
We show that CpG exposure prior to neonatal RSV infection led to increased protection against adult re-infection, and to reduced disease on adult re-exposure 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 re-infection. 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 (24). The ideal protective response reduces viral load without excess local inflammation, whereas the adverse pathogenic responses causes inflammation without effectively controlling viral load. For example, in adult RSV infection, CCL3 depletion increased pro-inflammatory RSV-specific cells but reduced the total number of cells (35). The data from our previous studies suggest that neonatal RSV infection induces hyper-inflammatory cellular responses during re-infection and reducing this cellular infiltrate can reduce disease (33), but the interaction with APC was critical in determining outcome (34). 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 (7). But these responses are relatively plastic, for example the incidence of post bronchiolitic wheeze decreases with age. This may reflect subsequent exposures to TLR ligands modulating the immune response and our interval CpG treatment experiment support 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 re-infection following the use of a recombinant virus expressing IFNγ (14) and the addition of recombinant IFNγ has been shown to have a protective effect (18). However there were differences
between CpG and IFNγ treatment, whilst both treatments suppressed Th2-type responses during rechallenge infection only CpG treatment enhanced Th1 responses. Furthermore only CpG treatment reduced the viral titre on re-infection. CpG has previously been shown to alter the Th2 skewing of neonatal APC (16). It is of note that strongly skewed Th1 responses can also be pathogenic, when IFNγ expressing RSV was used in adult mice, it led to increased disease on rechallenge (12), therefore a balanced cellular response is desirable.

Our data suggest that deficient activation of neonatal APC is involved in the delayed sequelae of RSV infection, neonatal CD11c⁺ cells failed to up-regulate MHC class II and co-stimulatory molecules on RSV infection. Delayed maturation of neonatal APC 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 APC are deficient in a number of key signalling molecules including IRF3 (3) and IRF7 (5). However some TLR ligands can activate neonatal APC including R848 acting on TLR7/8 (19), LPS acting on TLR4 (25) and CpG acting on TLR9 (31). One question is why RSV infection does not induce a similar maturation of neonatal APC, given that RSV has been shown to interact with TLR3 (27) and TLR4 (17). One possibility is that the virally encoded genes suppress the innate response and it has been recently demonstrated that the RSV NS1 protein suppresses Th1 type responses (22), in the context of the pre-skewed 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 post bronchiolitic 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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Figure Legends

Figure 1. Pre-treatment with unmethylated CpG reduces the delayed effects of neonatal RSV infection. Neonatal mice were treated with 10μg CpG (day 3 of life) one day prior to RSV infection (day 4 of life), 8 weeks later mice were re-infected with RSV. Weight loss (A), lung cell number (B) and lung viral load at day 4 (C) were measured after infection. The effect of pre-treatment with methylated and unmethylated CpG on weight loss during secondary infection was compared (D). n = 5 mice per group per timepoint. Mean ± SEM. * p<0.05, ** p<0.01.

Figure 2. Treatment with CpG prior to neonatal RSV increases anti-viral cellular immunity. On day 3 of life Neonatal mice were treated with 10μg CpG (●, black bars) or PBS (○, white bars) one day prior to RSV infection (day 4 of life), 8 weeks later mice were re-infected with RSV. IFNγ (A) and IL-5 (B) measured in BAL by ELISA. Lung DX5+ NK cells (C), CD4 T cells (D), IFNγ (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. RSV specific IgG1 (I), IgG2a (J) and the ratio (K) were measured in serum on day 4 post challenge by ELISA. Data representative of 2 experiments, bars represent n=4 mice per group +/- SEM. *p<0.05, **p<0.01.

Figure 3. Interval CpG application abrogates neonatal disease. 4 day old BALB/c mice were infected i.n. 10^5 PFU RSV or sham infected with PBS. 4 weeks later mice were given i.n. CpG (open symbols and bars) or PBS (filled symbols and bars), 8 weeks later mice were challenged with 10^6 PFU of RSV. Weight was monitored following infection (A). RSV specific IgG1 (B) and IgG2a (C) subtypes were measured by ELISA. Airway IFNγ (D), IL-5 (E) and eosinophilia (F) on day 4 after infection. Lung NK (G), CD4 cells (H) and CD8 cells (I) were measured on day 4 and 7 post infection. Data representative of 2 independent repeats, n = 5 mice per group per timepoint. Mean ± SEM. * p<0.05, ** p<0.01.
Figure 4. CpG pre-treatment increases APC activation. Neonatal or adult mice were infected with RSV intranasally or left uninfected. At d 0, 4, 7 and 11 post infection, lungs were removed and the number of CD11c+ cells expressing MHCII and CD86 characterised by flow cytometry (A), *** p<0.001 comparing Ad RSV with NN RSV, ### p<0.001 comparing Ad RSV with Ad PBS. Neonatal mice were treated with 10ug CpG one day prior to RSV infection. Lungs were removed and viral load (B) measured on d4 post infection and CD11c+ cell number (C) and MFI of MHCII (D) and CD80 (E) measured by FACS on d1 and d4 post infection. Numbers of DX5+ NK cells (F) and IFNγ producing NK cells (F) were also evaluated in the lungs by FACS. Bars/ points represent mean +/- SEM of n=4 mice, * p<0.05, ** p<0.01.
Figure 1

A

**A**

**B**

**C**

**D**

Figure 2

A

B

C

D

E

F

G

H

I

J

K
Figure 3

A. % original weight

B. Anti-RSV IgG1 (A490)

C. Anti-RSV IgG2a (A490)

D. Airway IFN-γ (ng/ml)

E. Airway IL-5 (pg/ml)

F. BAL eosinophils (x10^4)

G. NK cells (%)

H. CD4+ T cells (%)

I. CD8+ T cells (%)

Legend:
- nRSV-PBS-RSV
- nRSV-CpG-RSV

Day post re-infection:
- 0
- 1
- 2
- 3
- 4
- 5
- 6
- 7

Sera Dilution:
- 10
- 1
- 10
- 2
- 10
- 3
- 10
- 4

A490:
- 0.0
- 0.5
- 1.0
- 1.5
- 2.0

Airway IFN-γ:
- 0.0
- 0.5
- 1.0
- 1.5
- 2.0

Airway IL-5:
- 0.0
- 0.5
- 1.0
- 1.5
- 2.0

BAL eosinophils:
- 0
- 2
- 4
- 6
- 8
- 10

CD4+ T cells (%):
- 0
- 5
- 10
- 15

CD8+ T cells (%):
- 0
- 5
- 10
- 15
- 20
Figure 4

A. CD11c+ cells expressing MHC class II and CD86 (x10^5)

B. RSV L gene copy number

C. CD11c+ cells (x10^5)

D. Lung tissue weight (mg)

E. CD80 (MFI)

F. DX5+ cells (x10^5)

G. IFNγ+ DX5+ cells (x10^3)

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