

Neutrophil TLR4 expression is reduced in the airways of infants with severe bronchiolitis.

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Abstract

In RSV bronchiolitis, neutrophils, account for >80% of cells recovered from airways in bronchoalveolar lavage (BAL) fluid. We investigated neutrophil activation and toll-like receptor (TLR) expression in the blood and lungs of infants with severe RSV bronchiolitis. **Methods:** BAL and (blood) samples were collected from 24 (16) preterm and 23 (15) term infants, ventilated with RSV bronchiolitis, and 12 (8) control infants. We measured protein and mRNA expression of CD11b, myeloperoxidase (MPO) and TLR 2,4,7,8,9 in neutrophils.

Results: Blood neutrophils had more CD11b in preterm and term bronchiolitic infants, than control infants ($P<0.025$) but similar amounts of MPO. BAL neutrophils from bronchiolitic infants had increased amounts of CD11b and MPO than blood neutrophils and BAL neutrophils from controls ($P<0.01$). Blood neutrophils from term RSV infants had less total TLR4 protein than preterm RSV infants ($P=0.005$) and both had less than controls ($P<0.04$). Total TLR4 for each group was greater in BAL than blood neutrophils. Blood neutrophils from preterm RSV infants had greater TLR4 mRNA expression than term RSV infants ($P=0.005$), which had similar expression to controls ($p=0.625$). **Conclusions:** In infants with severe RSV bronchiolitis neutrophil activation starts in the blood and progresses as they are recruited into the airways. Total neutrophil TLR4 remains low in both compartments. TLR4 mRNA expression is unimpaired. This suggests neutrophil TLR4 expression is deficient in these infants, which may explain why they develop severe RSV bronchiolitis.

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Introduction

Respiratory syncytial virus (RSV) bronchiolitis is the commonest cause of lower respiratory tract infection in children under the age of one year¹. Symptoms begin in the upper airways and it is likely that the primary site of RSV infection is the nasal epithelium² with local spread to the lower airways³. Initial infection of the airways leads to production of pro-inflammatory cytokines^{4 5} and chemokines⁶ which initiates the recruitment of inflammatory cells from the peripheral circulation. There is little evidence that RSV causes a viraemia⁷, but the immunopathogenesis of RSV bronchiolitis is poorly understood. By studying cells from the blood and airways of infants with RSV bronchiolitis and comparing them to uninfected controls, information can be elucidated about the changes that inflammatory cells undergo as they are recruited to the airways.

The clinical spectrum of RSV infection is wide, from a mild upper respiratory tract infection to severe lower respiratory tract infection¹. Risk factors for severe RSV bronchiolitis are prematurity (particularly with associated chronic lung disease)⁸ congenital heart disease⁹ and immunodeficiency¹⁰. Half of infants ventilated for RSV bronchiolitis are born at term with no risk factors^{1 11}. In previous studies, we have consistently shown that term infants have a more vigorous immune response to RSV infection in their airways than preterm infants^{4 5 12} and this may relate to the considerable maturation that occurs in the third trimester and in the first year of life^{13 14}.

Neutrophils are the predominant cell found in the inflammatory infiltrate of the bronchoalveolar lavage (BAL) fluid of infants ventilated for severe RSV bronchiolitis⁵. They are recruited to the lungs, early in the course of infection, where they are known to release cytokines¹² and show delayed apoptosis^{15 16}. As the clinical condition improves, lower neutrophil concentrations are found in the BAL^{4 5}. Neutrophil activation can be studied by looking at cell surface markers e.g. the integrins:- CD11b or internal markers e.g. myeloperoxidase (MPO) found in the neutrophil granules. Integrin expression is quickly upregulated by mobilisation of internal stores. MPO is released to the cell surface when the neutrophil is degranulating at the site of active inflammation.

Toll-like receptors (TLRs) recognise viral and bacterial pathogen associated molecular patterns and initiate a specific inflammatory response to a range of infections¹⁷. In RSV infection, TLRs may play an important role in regulating innate and adaptive immune responses¹⁸⁻²⁰. Immune responses mature with gestation and during the first few months of life, when infants encounter RSV infection²¹.

Circulating neutrophils express all human TLRs, except TLR3²² but the contribution of TLRs, to the pathogenesis of RSV disease, is not fully understood. There has been considerable interest in the role of TLR4 in regulating RSV infection since RSV F protein has been reported to be a ligand for TLR4²³. A clinical study found increased TLR4 expression in blood monocytes of infants with RSV bronchiolitis²⁴ and a genetic susceptibility study identified two common TLR4 gene mutations which are associated with an increased risk (odds ratio 5.1(95% CI: 1.4-18.1) and 4.0 (95% CI: 1.3-12.5) of severe RSV bronchiolitis compared to mild disease²⁵.

Intracellular TLRs (3,7,8 and 9) recognise viral and bacterial nucleic acid in the endosomes of infected cells. RSV has been demonstrated to interfere with TLR7 and TLR9 signalling pathways in plasmacytoid dendritic cells, resulting in significant reductions in Type I interferon production²⁰. RSV may also induce cytokine secretion and mucus production in airway epithelial cells via TLR3 signalling²⁶.

We have undertaken a large study to investigate neutrophil activation and TLR expression in the blood and BAL of infants with severe RSV bronchiolitis (term and preterm) compared to control infants to delineate what changes occur in neutrophils as they migrate to the lungs from the systemic circulation.

Patients and Methods

Study Population and Sample Collection

We recruited 47 consecutive infants, aged less than one year who were admitted with severe RSV bronchiolitis to the intensive care unit at Royal Liverpool Children's Hospital, Alder Hey over three winter seasons. RSV bronchiolitis, was confirmed by direct immunofluorescence of nasopharyngeal aspirates. After informed consent samples of peripheral blood and BAL were collected, as previously described.^{4 5 12} We recruited 24 participants who were born prematurely (<37 weeks gestation) and 23 participants who were born at term (>= 37 weeks gestation). Data from infants born at term were analysed separately from those infants born preterm. Infants with underlying cardiorespiratory disease were excluded. The control group were 12 uninfected healthy infants of the same age as the bronchiolitis infants, ventilated prior to elective non-cardiac surgery, from which blood and BAL samples were collected immediately after induction of anaesthesia and intubation of the airway. The local paediatric research ethics committee approved the study and parents/guardians of participants gave consent for all samples to be taken. No recruited infant was subsequently withdrawn from the study. All samples were processed and data analysed from each recruited infant. During the first year of the study, we collected BAL, but not blood samples.

Preparation of BAL samples

Standard techniques were used to assess total and differential cell counts^{8 9 16 27}. Briefly BAL fluid was filtered (60 µm pore size gauze (Sefar Nitex 03-48/31, Sefar Inc, Switzerland) to remove bulk mucus and then centrifuged. The supernatant was removed and stored at -70°C. The cellular component was resuspended in RPMI 1640 and the neutrophils purified using sedimentation gradient techniques on PolymorphPrep© (Axis-Shield PoC AS, Norway) We took 59 BAL samples, 47 from infants with RSV bronchiolitis (24 preterm and 23 term infants) and 12 from healthy control infants. The number of cells was highest in those infants with RSV bronchiolitis born at term and as in previous studies^{4 5 12}, neutrophils were the predominant cell.(Table 1). After enrichment >93% of the cells were neutrophils, with >94% viability, the median percentage (SD) of other cells were: macrophages 4 (3), lymphocytes 2 (1.0).

Preparation of blood neutrophils. Neutrophils were purified from whole blood by sedimentation gradient as described above. We took 39 blood samples, 31 from infants with RSV bronchiolitis (16 preterm and 15 term) and 8 from healthy control infants. There was no difference in the total cell count between cases and control infants (Table 1). After enrichment >95% of the cells were neutrophils with >96% viability, the median percentage (SD) of other cells were: monocytes 2(1), lymphocytes 2 (1.0).

Table 1. Characteristics of patients and BAL and blood samples before enrichment .
Data mean (SEM) unless stated.

	Bronchiolitis Infants		Control Infants
	Preterm	Term	
Clinical information			
Number of patients	24	23	12
Median (Range) age on admission, weeks	3.5(-5,11.7)	16(4,56)	12(6,20)
Weight on admission, kg	3.3 (0.35)	5.1 (0.36)	4.3 (2.0)
Median (Range) gestation at birth, weeks	34 (27,36)	40 (39,42)	40 (37,40)
BAL cell counts			
Total cell concentration, x 10 ⁶ cells/ml	1.8(0.8)	1.9(0.5)	1.5(0.8)
Total cell count, x 10 ⁶ cells	4.5(1)	4.8(1.2)	3(0.5)
Percentage of viable cells	95(2.5)	94(2)	95(1)
Median (Range) percentage neutrophils	84(2.5)	83(2)	37(3)
Median (Range) percentage alveolar macrophages	12.5(1.5)	11.6(1.0)	47(3)
Median (Range) percentage lymphocytes	5(0.5)	3.5(1.0)	7(3)
Blood cell counts			
Number of patients	16	15	8
Total cell concentration, x 10 ⁹ cells/l	9.95(1.67)	9.95(1.64)	9.44(1.5)
Percentage of viable cells	97(2)	96(1)	96(1)
Mean concentration x10 ⁹ /l neutrophils	4.93(1.46)	5.11(1.48)	4.5(1.56)
Mean concentration x10 ⁹ /l monocytes	0.99(0.15)	1.11(0.17)	1.0(0.18)
Mean concentration x 10 ⁹ /l lymphocytes	3.67(0.3)	3.57(0.44)	3.63(0.35)
Mean concentration X 10 ⁹ /l eosinophils	0.088(0.04)	0.078(0.029)	0.082(0.045)

FACS Analysis: protein expression of CD11b, MPO and TLR 2,4,7,8,9

All Cells for FACS analysis were triple stained with Ethidium Monoazide Bromide (EMA) (Biotium, UK) to allow exclusion of non-viable cells, a neutrophil marker (MPO or CD16) and the test antibody (TLR 2,4,7,8,9, CD11b, MPO) or isotype control

For cell surface expression of CD11b, TLR2,4 neutrophils were labelled and fixed (BD cytofix: 1% paraformaldehyde).

For total expression of CD11b, MPO, TLR2,4,7,8,9 neutrophils were permeabilised, fixed (BD Cytofix and Cytoperm (BD Biosciences) and labelled. Samples were analysed on a FACSsort flow cytometer (Becton Dickinson) collecting a minimum of 10,000 events. Data were processed using WinMDI Version 2.8. Neutrophils were gated by size, granularity, viability and CD16 or MPO expression. TLR expression was standardised from the Mean Fluorescent Intensity (MFI) into Antigen Binding Capacities (ABC)- relative units²⁸ to allow quantification of the antigen binding sites between different commercial antibodies, each with different binding affinities, as per manufacturer instructions. (Simple Cellular Antigen-Bangs Laboratories, Inc) (Table 2).

Table 2. Antibodies used for flow cytometry

Antibody raised against:	Antibody Type:	Manufacturer:
TLR2	FITC mouse monoclonal IgG2a	Imgenex
TLR4	FITC mouse monoclonal IgG2a	
TLR7	FITC mouse monoclonal IgG1	
TLR8	FITC mouse monoclonal IgG1	
TLR9	FITC mouse monoclonal IgG1	
CD16	PE mouse monoclonal IgG1	BD BioSciences
Myeloperoxidase (MPO)	PE mouse monoclonal IgG1	DakoCytomation
CD11b	FITC Mouse monoclonal IgG1	BD Biosciences
Isotype antibodies:		
	FITC/PE mouse IgG1 control	Imgenex
	FITC/PE Mouse IgG2a control	

Real time polymerase chain reaction (RT-PCR).

RNA was prepared from 0.5×10^6 neutrophils using Trizol (Gibco, Basingstoke, UK), 5µg of DNase treated total RNA was used as a template for first strand cDNA synthesis in a 20µl reaction using Multiscribe reverse transcriptase (Applied Biosystems).

The mRNA expression of TLR 2,4,7,8,9 was assessed using Taqman primer/probe sets. (Applied Biosystems). All PCR reactions were in triplicate using an Applied Biosystems 7300 and standardised to the housekeeping gene L32 using the Δ Ct method.

Statistical analysis

Protein expression of CD11b, MPO and protein and mRNA expression of TLR2,4,7,8,9 were compared from enriched neutrophil blood and BAL samples in preterm RSV, term RSV and control groups. Differences in means between patient groups were examined using one-way analysis of variance and independent sample t-tests. For comparison of the same neutrophils in blood and BAL, as paired data were not available on all infants, pairing was ignored, leading to more conservative analyses, but an analysis using only paired data is available online (Table 3) . Data were analysed using SPSS 15 (SPSS Inc, Chicago, IL). A probability level of 95% ($p < 0.05$) was considered as the threshold for statistical significance.

Results

Protein expression of neutrophil activation markers is greater in RSV disease.

All neutrophils in the blood and BAL were positive for expression of CD11b and MPO. All data presented are expressed as relative ABC units (mean ABC x 10⁶ (SEM).) The amounts of CD11b in blood neutrophils from both preterm RSV [0.05(0.005)] and term RSV [0.055(0.005)] infants were greater than control infants [0.02(0.005)](P<0.025). The amounts of CD11b in BAL neutrophils in preterm RSV [0.08(0.001)] and term RSV [0.07(0.002)] were greater than controls 0.025(0.005)[P<0.012]. (Figure 1a and 1b)

There was no difference between any of the groups in the amount of blood neutrophil MPO; preterm RSV [0.15(0.03)], term RSV [0.12(0.02)]; controls 0.13(0.01)[P>0.05]. MPO was greater in BAL neutrophils from both preterm RSV [0.28(0.02)] and term RSV [0.30(0.02)] infants than control [0.14(0.01)](P<0.05) infants. (Figure 1c) There were no significant differences in expression of CD11b or MPO between preterm and term infants in blood or BAL neutrophils. For both groups of infants with severe RSV bronchiolitis there were greater amounts of CD11b and MPO in neutrophils from the BAL compared to the blood. There was no difference between these compartments for either marker in control infants.

TLR4 expression was decreased in infants with RSV bronchiolitis but TLR2,7,8,9 expression was the same in neutrophils from RSV bronchiolitis and control infants.

All neutrophils in the blood and BAL were positive for expression of TLRs 2, 4, 7, 8 and 9. There was no difference between cell surface TLR2 expression on neutrophils from blood or BAL or between preterm, term or control infants (figure 2a,2b) nor was there any difference between groups or compartments for total TLR2 (figure 2a,c).

There was no difference in neutrophil cell surface TLR4 expression between the three groups in either the blood or the BAL (figure 3a,3b)

However in blood neutrophils; total TLR4 for term RSV infants [0.51(0.023)] was significantly less than preterm RSV infants [0.7(0.038)](P=0.036) and both were less than control infants [1.0 (0.0353)](p=0.005) (Figure 3b)

In the BAL, total neutrophil TLR4 in neutrophils was significantly less in term RSV infants [0.7(0.02)] than preterm RSV infants[0.92(0.02)](P=0.03) and both were less than control infants (1.7 (0.028) p=0.001) (figure 3a,c) For each patient group there was significantly more total neutrophil TLR4 expressed in the BAL than in the blood (P<0.05).

For the TLRs expressed intracellularly, (TLRs 7,8,9) there was considerably less overall expression in neutrophils from blood and BAL than those expressed on the cell surface (TLRs 2,4). The total ABC for TLRs 7,8 and 9 ranged from 982 to 9,070 compared to the much higher range of total TLR2 and TLR4 which was of 0.138 to 1.7 x 10⁶. There were no significant differences in neutrophil expression of the intracellular TLRs 7, 8 and 9 between term and preterm infants with bronchiolitis and controls in either blood or BAL neutrophils or between BAL and blood within each group. (data not shown)

Neutrophil expression of TLR mRNA in severe RSV bronchiolitis compared to healthy infants. Neutrophil TLR4 mRNA is similar in term RSV and control infants. TLR2,4,7,8,9 mRNA expression predominantly occurs in the blood.

In the blood, neutrophil mRNA expression for TLR4 was similar for the term RSV [34.84(2.168)] and control infants [48.16 (2.758)](P=0.625), (data expressed as mean logfold ratio of TLR mRNA to L32 mRNA (SEM)). Blood neutrophil TLR4

mRNA expression of preterm RSV infants [566.27 (19.825)] was much greater than term RSV infants ($p=0.005$) or controls ($p=0.021$) (figure 4a).

In the BAL neutrophil TLR4 mRNA expression was significantly less than in the blood for each patient group. Similarly BAL neutrophil mRNA expression for TLR4 was greater for preterm RSV [4.38(0.122)] compared to both term RSV [2.46(0.129)($P=0.037$)] and control [1.12 (0.577)($P=0.034$)]. As in the blood, BAL neutrophil TLR4 mRNA expression for term RSV infants was similar to control ($P=0.6$)(Figure 4b)

To determine if the increased expression of TLR4 mRNA in the blood compared to the BAL was also observed for the other TLRs, the mean mRNA expression of TLR 2,7,8,9 in blood neutrophils for the two RSV and control groups was measured (Figure 4c). For TLR 7 and 9 there was a similar pattern of greatly increased neutrophil mRNA expression in preterm RSV infants compared to the other two groups. Neutrophil TLR7 mRNA was undetectable in controls. There were only low and similar levels of expression of neutrophil TLR2 in the three groups and for TLR8 mRNA in term RSV infants and controls ($P>0.05$). There was no detectable expression of neutrophil TLR8 mRNA in preterm RSV infants. In the BAL, there was very little neutrophil mRNA expression of TLRs 2,7,8,9 compared to blood (data not shown).

Discussion

We have shown, for the first time, that neutrophils from infants with severe RSV bronchiolitis are activated both in the peripheral circulation and, to a greater degree, in the lower airways. This was associated with reduced neutrophil TLR4, in both compartments, with the lowest amounts for term RSV infants. Expression of neutrophil TLRs 2,7,8,9 in both blood and BAL were similar in all three groups. This finding of a reduced TLR4 protein expression raises the possibility of an impaired innate immune response and contrasts with findings of many clinical studies which have shown highly over exuberant inflammatory responses during RSV bronchiolitis. This is the first study to investigate the role of TLRs in both airway and circulation of infants with RSV bronchiolitis. We studied the expression of the human TLRs currently believed to be involved in viral recognition in a group of infants with the most severe manifestations of RSV disease and no known risk factors. We analysed infant neutrophils, the biology and immune function of which is very different from neutrophils of adults²⁹.

This work has some limitations. As only infants with severe disease were intubated we were unable to collect BAL samples from infants with mild or moderate disease to determine whether these changes were more marked with increasing disease severity as suggested by genetic susceptibility studies²⁵. Secondly, although our findings may suggest a constitutive reduction in neutrophil TLR4 expression in some infants, we do not know whether expression was altered before onset of RSV disease or after recovery. RSV F protein has been demonstrated to bind to TLR4 on monocytes³⁰ and we have some preliminary data which suggests that RSV virus may bind to, or associate with, neutrophils in the BAL of our RSV infected infants. Whether this results from an interaction with between RSV and a specific receptor, such as TLR4, has not been determined. We have undertaken some preliminary *in vitro* work, with neutrophils from healthy adults, which shows no evidence of interference by RSV with TLR4 antibody binding or TLR4 detection, in our assay. Additional evidence to suggest that RSV F protein binding is not interfering with TLR4 detection is provided by the observation that TLR4 expression on the surface

of neutrophils is similar between our three groups and between the blood and the BAL within the groups, whereas there are differences, for these comparisons, in total TLR4 expression.

As the neutrophils from the peripheral circulation of infants with severe RSV bronchiolitis expressed higher levels of CD11b than uninfected controls, although recirculation from the lung cannot be excluded, it appears that they become partially activated or primed, in the peripheral circulation before recruitment into the lung. In the lung, neutrophils express higher levels of total and cell surface CD11b and MPO which suggests either progressive activation³¹⁻³³ and release of intracellular MPO by degranulation at this site or, alternatively, the selective recruitment of highly activated neutrophils from the peripheral circulation.

The observed decrease in neutrophil intracellular TLR4 protein expression could be due to reduced synthesis, mobilisation to the cell surface, or increased degradation. The first two of these suggestions seem unlikely as, compared with controls, neutrophil TLR4 mRNA expression was greatly increased in preterm and similar in term infants and TLR4 expression on the surface of neutrophils was similar between the three groups. A precedent for TLR4 degradation comes from epithelial cells where TLR4/MD2 complexes are degraded once engaged by an agonist³⁴. The third explanation alone seems unlikely, as neutrophil TLR4 was not only reduced in BAL neutrophils, where RSV is present in large titres, but also on blood neutrophils. Currently there is no evidence to suggest that RSV causes a viraemia⁷.

As only TLR4 and not expression of other TLRs is markedly reduced in the main infiltrating inflammatory cell during active infection, this suggests both a specific effect on TLR4 expression and that neutrophils and TLR4 play an important role in host defence against RSV infection. TLR4 signalling has primarily been studied in airway epithelial cells during RSV infection. There is very little work on RSV infection and TLR pathways in blood-derived cells and none, to our knowledge, in clinical studies that investigated neutrophils. Deficiencies in TLR pathways have been shown to be important in other viral infections and modulate host defence for example *Vaccinia* virus protein, A46R, blocks TLR4 signalling³⁵ in human epithelial tumour cell lines (HeLA cells) and *Vaccinia* virus lacking A46R has attenuated virulence.

One of our striking findings was that, in neutrophils of preterm RSV infants compared to controls, despite reduced protein expression of neutrophil TLR4 in the blood and BAL, there were vastly increased levels of TLR4 mRNA in the blood. In preterm infants this may represent immaturity of the TLR4 transcription pathway. However, in previous, unpublished studies, we measured mRNA for TLRs 2,4,7 and 9 in two groups of controls, those born at term and those born preterm, and found no differences in expression between the groups. In the preterm infants this may represent an appropriate response to RSV infection with increased degradation and utilisation of TLR4 dependent pathways to help clear the infection, or these findings may reflect the immunological immaturity of these infants. As the expression of TLRs has been shown to increase with gestational age²¹ the finding that total neutrophil TLR4 is much less in term infants compared to preterm infants with severe RSV disease may suggest that term infants with severe disease have a constitutive abnormality in TLR4 expression. This may be related to the genetic polymorphisms in TLR4, that are associated with an increased risk of severe disease^{24 25} and associated with impaired TLR4 signalling in response to RSV F protein²⁷.

This study has highlighted important aspects about the role of neutrophils in RSV disease and the differences in the immunopathogenesis in preterm and term infants.

In vitro studies are needed to determine whether the abnormalities in TLR4 expression contribute to or are a result of RSV disease. TLR4 agonists are currently undergoing phase II clinical trials, so elucidating these mechanisms further will have important therapeutic implications for infants with severe RSV bronchiolitis.

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Table 3: Protein expression between blood and BAL neutrophils using paired analysis.

All data expressed as Mean units of ABC x 10⁶

Mean Diff= Mean difference in protein expression between blood and BAL neutrophils.

Table 3(a)

Group	Total MPO		Total CD11b	
	Mean diff	SEM	Mean diff	SEM
Preterm (n=16)	-0.12	0.04	-0.035	0.001

	P=0.011	P=0.032
Term (n=15)	-0.24 0.05 P=0.025	-0.021 0.001 P=0.03
Control (n=8)	-0.002 0.001 P=0.89	-0.001 0.001 P=0.73

Table 3(b)

Group	Cell Surface TLR2		Cell Surface TLR4		Total TLR2		Total TLR4	
	Mean diff	SEM	Mean diff	SEM	Mean diff	SEM	Mean diff	SEM
Preterm (n=16)	-0.0006	0.001	-0.001	0.001	-0.003	0.001	-0.22	0.03
	P=0.56		P=0.32		P=0.80		P=0.024	
Term (n=15)	-0.0005	0.001	-0.0005	0.001	-0.001	0.001	-0.15	0.02
	P=0.43		P=0.46		P=0.63		P=0.036	
Control (n=8)	-0.003	0.001	-0.001	0.001	-0.003		-0.55	0.01
	P=0.69		P=0.2		P=0.89		P=0.026	

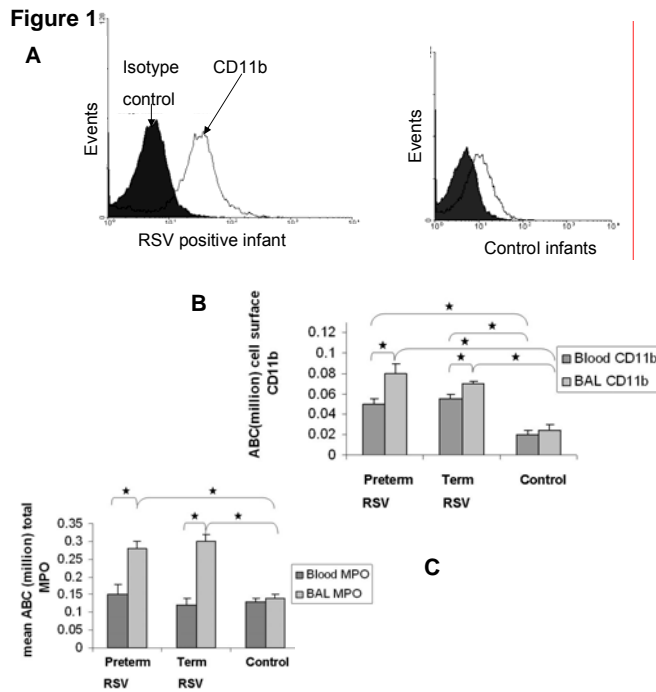


Figure 1 Protein expression of neutrophil activation markers is up-regulated in RSV disease. Data are presented as mean arbitrary units of Antigen Binding Capacity (ABC x 10⁶) (SEM). **A.** Histograms of flow cytometry data from two representative patients—an infant with RSV bronchiolitis (left) and a control infant (right) showing the mean protein expression of CD11b in the BAL compared to isotype control (shaded). **B.** To determine mean cell surface CD11b expression, blood and airway neutrophils were stained, fixed and analysed by flow cytometry. CD11b expression were similar for term RSV (n=15) and preterm RSV infants (n=16) and both were significantly greater than controls (n=8). There was significantly greater cell surface CD11b protein expression in the BAL neutrophils than in the blood neutrophils. **C.** To determine mean total MPO expression neutrophils from the Blood and BAL were fixed and permeabilised before staining and analysed by flow cytometry. For blood neutrophils there was no difference in protein expression of MPO between infants with bronchiolitis (n=31) and controls (n=8). For the BAL neutrophils both term RSV (n=15) and preterm RSV infants (n=16) had significantly more MPO expression than control infants (n=8). There was significantly more MPO in BAL than in the blood. *P<0.05

Figure 2

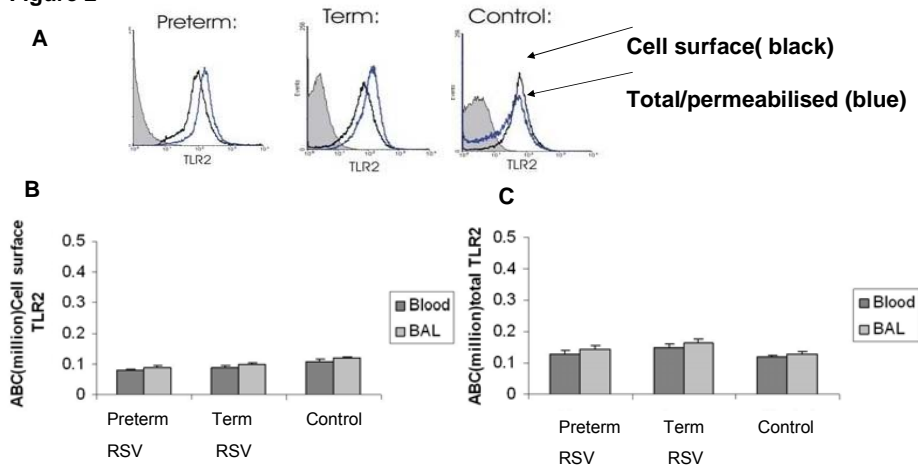


Figure 2 TLR2 protein expression is unaltered in infants with severe RSV bronchiolitis. All data presented as mean arbitrary units of ABC x 10⁶ (SEM) A: Representative histograms from flow cytometric staining of TLR2 on BAL neutrophils from three infants, a preterm infant with RSV bronchiolitis, a term infant with RSV bronchiolitis and a control infant. **B:** The mean expression of cell surface TLR2 was determined by staining and fixing neutrophils prior to analysis by flow cytometry. The mean expression of cell surface TLR 2 was similar between all three patient groups for airway and blood neutrophils [preterm infants (n=16), term infants (n=15) and controls (n=8)] and for each group between blood and BAL neutrophils. **C** To determine total TLR2 the neutrophils were fixed and permeabilised prior to analysis by flow cytometry. Total neutrophil TLR2 was similar between all three patient groups in both blood and BAL (preterm RSV n=16, Term RSV n=15, Control n=8) and between blood and BAL for each group.

Figure 3

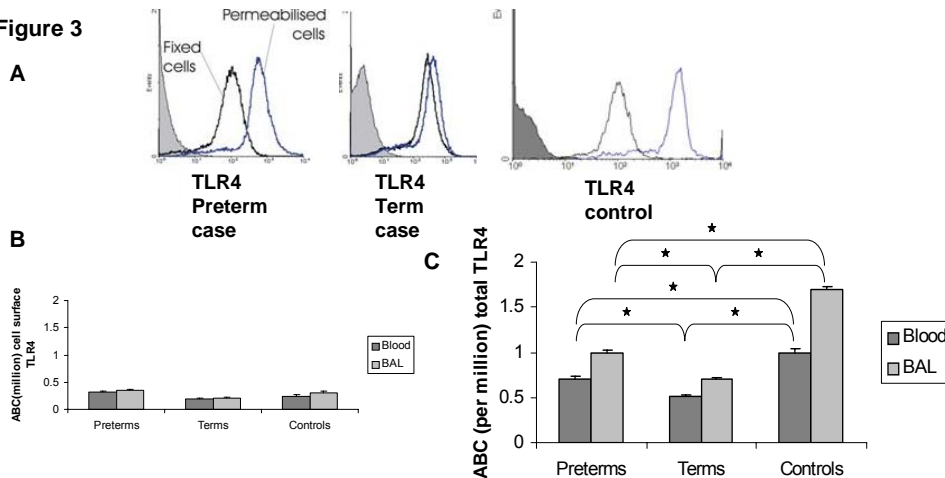


Figure 3 TLR4 protein expression is down regulated in infants with severe RSV bronchiolitis. Data presented as arbitrary mean units of ABC x 10⁶ (SEM) A: Representative histograms from flow cytometric staining of TLR4 on BAL neutrophils from three infants, a preterm and a term infant with RSV bronchiolitis and a control infant. TLR4 expression was greater than TLR2. For TLR4, term infants with bronchiolitis had less total TLR4 than preterm infants with bronchiolitis and control infants. Shaded histograms are isotype controls. **B:** Expression of cell surface TLR 4 was analysed by flow cytometry after staining and fixing neutrophils. There was no significant difference between patient groups for cell surface expression of neutrophil TLR4 for either BAL neutrophils [preterm RSV(n=24), term RSV(n=23) and control infants (n=12)] or blood neutrophils [preterm RSV infants (n=16), term RSV infants (n=15) and controls (n=8)] or between blood and BAL neutrophils for each patient group. **C** To determine total neutrophil TLR4 expression, cells were fixed and permeabilised before analysis by flow cytometry. In both blood and BAL neutrophils, term RSV and preterm RSV infants had significantly less TLR4 expression than control infants. Term RSV infants had significantly less total TLR4 than preterm infants with bronchiolitis (p=0.03). *p<0.05. Total neutrophil TLR4 in BAL was significantly greater (P<0.05) than in blood for all three groups (not shown).

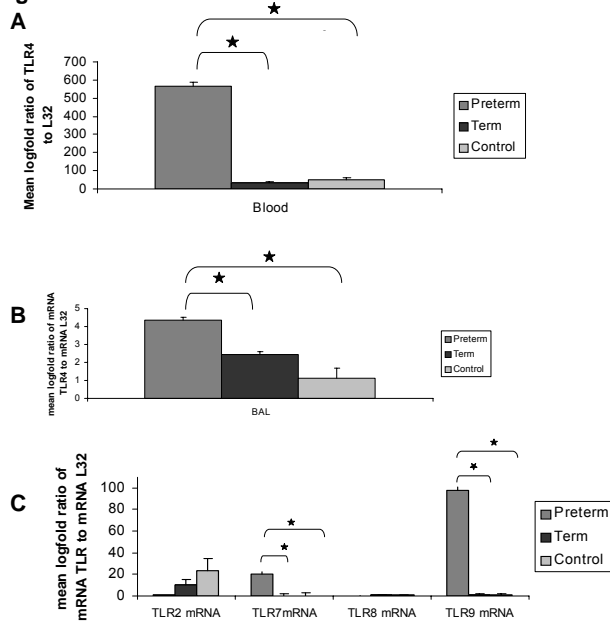
Figure 4

Figure 4. Neutrophil TLR4 mRNA expression predominantly occurs in the blood compared to the BAL and is similar in term and control infants. Data presented as mean logfold ratio (SEM) after Expression of mRNA assess by rt-PCR for all TLRs was standardised as a ratio to mRNA for the housekeeping gene L32. **A:** Neutrophil TLR4 mRNA was significantly increased in blood neutrophils, mRNA expression was greater in preterm infants (n=16) compared to term infants with bronchiolitis (n=15) but there was no significant difference between term infants with bronchiolitis and controls (N=8). **B:** Neutrophil TLR4 mRNA was significantly increased in BAL neutrophils from preterm RSV infants (n=16) compared to term infants (n=15) which was similar to control infants (n=8). There was considerable less mRNA TLR4 for each patient group in the BAL than the blood (not shown). **C:** As the blood seemed to be the main site of TLR4 mRNA production the Neutrophil mRNA expression of TLR 2,7,8,9 was determined for blood neutrophils for preterm RSV (n=16), term RSV (n=15) and controls (n=8) infants. There was no significant difference in the expression of blood neutrophil mRNA TLR2 between the three patient groups. Preterm infants had significantly greater expression of TLR7 and 9 in blood neutrophils than term or control infants. (P<0.05) There low levels of expression of neutrophil TLR8 in term RSV infants which were similar to control infants, there were undetectable levels in preterm RSV infants. *p<0.05.



Neutrophil TLR4 expression is reduced in the airways of infants with severe bronchiolitis

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