**TITLE**

**Expression of the B cell differentiation factor BAFF and chemokine CXCL13 in a murine model of Respiratory Syncytial Virus infection.**

**Authors:** Wael Alturaiki1, 2, Amanda J McFarlane3, 4, Katie Rose1, Rachel Corkhill1, Paul S McNamara1, Jürgen Schwarze3, 4, Brian F Flanagan1

1Department of Women's and Children's Health
Institute of Translational Medicine, University of Liverpool,
Alder Hey Children's NHS Foundation Trust Hospital,
Eaton Road, Liverpool, L12 2AP UK.

 2Department of Medical Laboratory, College of Science, Majmaah University, King Fahad Street, PO Box1712, Al-Zulfi, Riyadh Region11932,Kingdom of Saudi Arabia.

3Centre for Inflammation Research,

Queen’s Medical Research Institute,

The University of Edinburgh,

 47 Little France Crescent, Edinburgh EH16 4TJ, UK.

4Child Life and Health,

The University of Edinburgh,

 20 Sylvan Place, Edinburgh EH9 1UW, UK.

**Corresponding Author**

Dr Brian F Flanagan

Department of Women’s and Children’s Health, Institute of Translational Medicine, University of Liverpool
Alder Hey Children's NHS Foundation Trust Hospital
Eaton Road, Liverpool, L12 2AP

E mail fla1@liverpool.ac.uk

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**Key words:** Innate Immunity; BAFF, Respiratory Syncytial Virus; CXCl13; respiratory infection; B cell.

**Highlights:**

RSV infection increases B cell growth factor BAFF and CXCL13 chemokine expression

Lung BAFF was increased at 2 and 7 days post infection, CXCL13 at 1, 2 and 7 days

These cytokines may have an important role in local airway responses to RSV

**Abbreviations:** APRIL, A proliferation-inducing ligand; BAFF, B cell activating factor; BAL, broncho-alveolar lavage; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; PE, phycoerythrin; RNA, ribonucleic Acid; RSV, Respiratory Syncytial Virus;

**Abstract**

Innate immune responses are known to influence the subsequent development of adaptive immunity. We have previously shown that RSV infection of human airway epithelial cells results in production of the B cell growth factor, BAFF. To better understand how the airway responds to RSV infection by production of this and other factors to support or enhance local B cell responses to infection, we analysed the lung expression of BAFF and B cell homeostatic chemokines CXCL12, CXCL13, CCL19 and CCL21 in a murine model of RSV infection. Following infection with A2 strain RSV, the highest RSV N gene expression was observed at day 4 after challenge with virus. In contrast, two peaks of elevated BAFF expression at days 2 and 7 were observed. CXCL13 was significantly elevated at days 1, 2 and 7. CXCL12, CCL19 and CCL21 were expressed within lung tissue from control and RSV challenged animals but no significant difference in expression was found. Immunofluorescence showed BAFF to be present throughout the tissue however CXCL13 expression was localized to cell rich areas probably constituting lymphoid aggregates. Our results define the kinetics of B cell chemoattractant and growth factor expression during RSV infection and indicate an important role for these cytokines in the airway response to RSV infection.

**1. Introduction**

Respiratory Syncytial Virus (RSV) is the commonest cause of severe respiratory disease in infants with a global RSV disease burden of around 30 million cases and up to 200,000 deaths each year (1, 2). Morbidity associated with RSV may also be long-term, with children hospitalized with RSV infection more likely to wheeze, sometimes for years, after acute infection (3, 4). Nearly half of those infected in their first year of life are re-infected in their second year (5). In older children and adults, despite previous exposure and limited antigenic diversity between viral strains, airway antibody levels decline and protective immunity is short term (6-8).

The major lymphocytes present in the lung of human infants who have died from severe RSV infection are B cells (9, 10). We have previously shown expression of the B cell growth and differentiation factor BAFF (also known as TNFSF13B or Blys) in broncho-alveolar lavage (BAL) fluid, and BAFF mRNA in airway epithelial cells from the lower airways of infants during ongoing severe RSV bronchiolitis (11). This cytokine, a major factor promoting B cell survival and differentiation (12, 13), was also present in upper airway secretions of children with other viral infection s. Recently, lung tissue B cells have been shown to increase in number following viral challenge in a murine model of RSV infection (14). To better understand how the airway innate immune response is regulated and supports or enhances the adaptive immune response to infection, we have examined B cell distribution and expression of the BAFF and the B cell homeostatic chemokines CXCL12, CXCL13, CCL19 and CCL21(15), in the same murine model of RSV infection.

**2. Materials and methods**

*2.1 Infection of mice and collection of tissues*

BALB/c mice were bred in house at the University of Edinburgh. All mice were kept in specific pathogen-free conditions. Eight to 12 week old animals were infected intranasally with 4 × 105 Plaque forming units (in 100μl) of RSV (A2 strain grown in HEp-2 cells) or were mock infected with UV light inactivated RSV HEp2 lysate under anaesthesia, as described previously (16). Mice were weighed and monitored daily thereafter. At each time point post-challenge lung tissue was collected and stored at -80oC for subsequent preparation of, RNA, whole tissue extracts for western blotting, tissue homogenate supernatants for elisa ,or frozen in OCT media for later preparation of tissue sections. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the UK animals (scientific procedures) act 1986 and associated guidelines.

*2.2 Isolation of RNA and measurement of Cytokine mRNA or RSV RNA by real time PCR*

Total RNA was extracted from lung tissue (left lower lobe) using RNeasy Mini kits according to the manufacturer’s instructions (QIAGEN). Total RNA (2μg) was first reverse transcribed using Superscript II (Life Technologies) and random primers. RSV N gene expression was measured by Taqman real time PCR as previously described (11), and cytokine mRNA expression measured using Taqman real time PCR probes (Life Technologies: BAFF Mm00446347, CXCL13 Mm 004445333, and CCL19 Mm00839967 CCL21). Cytokine expression was normalised to the housekeeping gene L32 as previously described (11).

*2.3 Cytokine measurement by ELISA*

Lung homogenate supernatents were prepared in cold PBS containing protease inhibitor cocktail (Sigma) before being clarified by centrifugation at 18,0000g for five minutes at 4˚C. CXCL12, CXCL13, CCL19 and CCL21 were measured using DuoSet ELISA assays as described by the manufacturer (R&D systems). Murine BAFF was measured using Quantikine ELISA assays (R&D systems). Total protein concentrations were determined by BCA protein assay (Biorad) according to the manufacturer’s instructions and expression per milligram of lung homogenate calculated.

*2.4 Western Blot analysis.*

BEAS-2B cell pellets or lung tissue samples were homogenized in 100 µl of denaturing lysis buffer (10% glycerol, 125mM Tris, 3% SDS, 0.2% bromophenol blue, 10%DTT and 1% protease inhibitor pH6.8). Samples were denatured by boiling before separation on 12% polyacrylamide gels (Biorad) using a Biorad mini protean tetra system and transferred to PVDF membrane using a Biorad transblot turbo apparatus as described by the manufacturer. Membranes were then blocked with 5% skimmed milk in Tris-buffered saline with 1% Tween 20 (TBST) for 30 minutes and then washed with TBST, incubated with the primary antibody rat anti BAFF (1:1000) (Abcam ab16081) or anti-actin control antibody (SIGMA,cat#A2668) overnight at 40C. Membranes were then washed for 30 minutes with 1% TBST and incubated with HRP-conjugated secondary antibodies (Abcam) for 1 hour at room temperature before being visualized with ECL reagent (GE healthcare).

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*2.5 Preparation and staining of Tissue sections*

Microscope slides were coated with poly-L-lysine (Sigma, UK) diluted in distilled water (1:10) for 5 minutes. Slides were then air-dried at room temperature overnight. Mouse lung tissue was snap frozen immediately after extraction and stored at -80˚C. Prior to staining, 10µm cold acetone fixed frozen tissue sections were first rehydrated in PBS for 3 min and then blocked with PBS containing 5% milk powder for 1 hour, before staining with antibodies against BAFF (Buffy2, ab16082, Abcam), CD20 (ab171203, Abcam), CXCL13 (AF470, R&D systems) and matched isotype controls as described by the manufacturers. After staining, sections were washed twice for 5 minutes with PBS plus 1% Tween 20, counterstained with DAPI (Sigma) and then analysed using a LEICA DM 2500 confocal microscope.

*2.6 Statistical Analysis*

Data were expressed as mean ± standard deviation. Two-way ANOVA followed with Bonferroni post-hoc test was used to calculate statistical significance using GraphPad Prism 5.0 software. P-values indicate significance. (\* p<0.05, \*\* p<0.01 and \*\*\* p<0.001).

**3. Results**

*3.1 Pulmonary BAFF mRNA and protein expression increases following RSV infection.*

To determine if BAFF expression is increased following experimental RSV infection, mice were challenged with RSV or control UV inactivated RSV preparations and BAFF expression measured at days 1, 2, 4, and 7 post-infection. In this model, RSV N gene expression, a measure of viral infection, was elevated at days 1, 2 and 4 post-infection with only low levels detectable at day 7 (Figure 1A).

BAFF mRNA was expressed in all lung RNA samples, even in the absence of infection, but was elevated significantly following RSV infection at days 1 and 7 (p<0.05) in comparison to animals challenged with a UV inactivated RSV preparation, indicative of increased BAFF expression (Figure 1B) most variability in expression being observed at day 4.

BAFF protein can be found at both the cell membrane as an integral membrane protein, and in a soluble form, released on cell activation through proteolytic cleavage (12). Measurement of soluble BAFF in lung homogenate supernatant by ELISA showed peaks of increased expression at day 2 (p<0.05) and day 7 (p<0.001) (Figure 1C). Highest expression, mean 1700pg/ml (p<0.001), was observed at day 7 after infection, following peak RSV RNA expression at day 4. Supporting the ELISA data, Western blot analysis of whole tissue revealed a 20Kd, molecular weight BAFF form comparable to soluble BAFF and lower in molecular weight than that observed with the epithelial cell line BEAS-2B which express a 32Kd membrane bound form (Figure 1D).

Alternate spliced transcripts of BAFF, without exon 4 or 5 sequences present, which may have different biological activity, have been described (17). PCR analysis using primers spanning exons 3 to 6 or 3 to 7 showed only the standard form of BAFF to be expressed, and no alternately spliced transcripts were detected (data not shown).

*3.2 Increased pulmonary expression of chemokine CXCL13 but not CXCL12, CCL19 or CCL21 during experimental RSV infection*

Homeostatic chemokines, CXCl12, CXCL13, CCL19 and CCL21, are known to influence lymphoid cell migration and subsequent formation of inducible bronchial associated lymphoid tissue (15). CXCL13 mRNA expression (Figure 2A) was significantly increased at days 1 (p<0.05), 2 (p<0.05) and 7 (p<0.05). Similarly, CXCL13 protein expression was also significantly elevated at days 1 (p<0.01), 2 (p<0.05) and 7 (p<0.01). Although found in all RSV-infected and control tissue samples, no significant differences in CXCL12, CCL19 or CCL21, protein (Figure 2C, D and E) or mRNA (CCL19 or CCL21, data not shown) expression was observed at any time point.

*3.3 Tissue distribution of CD20 positive cells, BAFF and CXCL13 proteins*

Immunofluorescence was used to examine the tissue localisation of BAFF, CD20 positive B cells, and CXCL13 (Figure 3). BAFF staining indicated widespread expression throughout the tissue including the epithelia. This methodology cannot distinguish between membrane bound and soluble BAFF and there was no visible difference in the pattern of protein localization at different time points. CD20 staining revealed that B cells can be seen in the tissue primarily in cell rich foci, possibly lymphoid cell aggregates, particularly at later time points (Figure 3). Immunofluorescence staining showed CXCL13 expression by cells within structurally similar cell rich foci

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**4. Discussion**

This study characterizes for the first time in a murine model of RSV infection how BAFF and B cell attractant chemokine responses, particularly CXCL13, change over time. Our data indicate that BAFF is constitutively expressed and increases following RSV infection, with immunofluorescence demonstrating expression throughout the lung, including airway epithelial cells. CXCL12, CXCL13, CCL19 and CCL21 were all expressed in normal lung tissue but only CXCl13 increased following RSV infection.

We have previously shown that BAFF expression is increased in human RSV infection, including release of the soluble form of BAFF both *in vitro* and *in vivo* (11). Here, in this murine model of human RSV infection, BAFF expression increased, particularly at days 2 and 7 post infection. ELISA and western blot results indicate release of the soluble form of BAFF. Early BAFF expression at days 1 and 2 was to be expected as interferon beta, known to be produced rapidly after RSV infection of epithelial cells (18), induces BAFF expression (11). However, the highest levels of protein expression were observed at day 7, after peak virus levels had been reached and at a time point when viral RNA expression was low. It remains to be determined which types of epithelial cells or if also cells recruited during the inflammatory response, such as neutrophils or NK cells, are the source of this cytokine at this later time point. Importantly, these data demonstrate that BAFF is expressed at later time points when, as shown here, CD20 B cells are present in the tissue and Class II MHC positive B cells in the lung have been reported to be increased (14).

CXCL12, CXCL13, CCL19 and CCL21 are known to influence B cell migration and to be expressed by cells including epithelial, endothelial and dendritic cells (19-21). The kinetics of increased CXCL13 expression observed from day 1 differs from that reported following influenza infection where increased CXCL13 expression was first observed at day 4 (19). Nevertheless, it raises the possibility that B cells found in the lungs in both human and murine RSV infection (10,14) are being actively recruited to the airway by a CXCL13 dependent mechanism, at least at early time points. The results described here for CCL19 and CCL21, contrast with those seen in a bacterial challenge model of airway infection, in which CCL19 and CCL21 levels increased progressively over a 7 day period (20). However, our results are comparable to those reported in influenza infection, where CCL21 increased only at later time points (19, 21). These results may simply reflect differences between models or methods of sampling

Our results showing induction of increased BAFF expression at both early and late time points, and increased early CXCL13 expression, raise several important questions. Previous studies of BAFF and CXCL13 demonstrate roles in B cell survival, chemotaxis and lymphoid follicle formation (21-24). Here this indicates a role in promoting local B cell survival and maturation or inducible bronchial associated lymphoid tissue formation following RSV infection, potentially enhancing airway antibody responses (21). A similar role for epithelial derived BAFF in promoting airway B cell activity occurs in chronic obstructive pulmonary disease (22, 23) and a BAFF-mediated role in enhancing B cell precursor activity in a mouse model of allergic airway inflammation has recently been reported (24). It remains to be determined how BAFF or CXCL13, particularly at early time points, may influence the activity of other cells such as innate lymphoid cells.

Collectively these results provide further insight into how local airway responses to RSV are regulated and suggest that the significance of factors such as BAFF and CXCl13 deserve further study.

**Financial Support**

This work was supported by Majmaah University, Saudi Arabia to Wael Alturaiki and Alder Hey Charitable Funds support to Rachel Corkhill.

**Contributions**

PSM, BFF, JS, WA conceived and designed experiments. WA, AM, BFF, RC, KR, performed experiments. WA, JS, PSM, KR, BFF, AM analyzed the data. PSM, AM KR WA, BFF, collected samples, contributed materials or analysis tools. BFF, WA, KR, JS and PSM wrote the paper.

**Conflicts of Interest:** None

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β actin

**Figure 1**



**A**

**B**



**D**

**C**



 Days after RSV infection

rBAFF BEAS-2B 0 1 2 4 7



32Kd

20Kd

****

Β Actin

β actin

Fig.1. RSV, BAFF mRNA and Protein expression during RSV infection. A) Level of RSV infection measured by real time PCR for RSV N gene in comparison to animals treated with UV treated RSV control. B) Kinetics of BAFF mRNA expression in RNA isolated from whole lung tissue after RSV or UV-RSV challenge. Values are shown as fold expressions relative to L32 control gene expression. C) BAFF protein expression in lung homogenate supernatant following RSV or control UV-RSV challenge measured by ELISA. Significant increased expression was observed following RSV treatment at days 1, 2, and 7. D) Western blot analysis showing the BAFF protein isoform expression in lung tissue following RSV infection. Lane 1 recombinant soluble BAFF (rBAFF) Mr 17 kD used as positive control. Lane 2 BEAS2B cell line expressing membrane bound BAFF. Following RSV infection the predominant form found had an apparent molecular weight of 20KD consistent with soluble BAFF expression. All data shown from 1 representative experiment of two. . Mean and SD are shown P-values are indicated as \* p<0.05, \*\* p<0.01 and \*\*\*p<0.001 (two-way ANOVA with Bonferroni post-hoc test).

 Figure 2



**B**

**A**

**C**

**D**



**E**



**Fig. 2.** **Airway expression of chemokines CXCl13, CCl19, CCL21 and CXCL12 following RSV infection**. A) CXCL13 mRNA expression in lung tissue measured by real time PCR. B-E) Chemokine protein expression in lung homogenate measured by ELISA, B) CXCl13, C) CCL19, D) vCCL21 and E) CXCL12. Result shown is from one representative experiment of two independent experiments conducted. Mean and SD are shown (two-way ANOVA with Bonferroni post-hoc test, \*P <0.05, \*\* p<0.01).

Figure 3



**Fig. 3.** **Immunohistochemical localisation of BAFF, CD20 and CXCL13 in murine lung at 7 days after challenge with UV RSV or RSV**. Frozen tissue sections were prepared from lung tissue collected at day 7 after challenge with RSV infection (left and centre columns) and UV treated RSV (right column). Sections were stained with either matched isotype control antibody (left column) or specific antibody (centre and right column). Row 1, anti-BAFF Buffy-2 primary FITC labelled (green). Row 2, rat anti mouse CD20 PE (red). Row 3, Anti –mouse CXCL13 with PE (red) secondary. All slides were counter stained with DAPI (Blue). BAFF was found to have a wide spread distribution including the epithelia. CD20 positive cells were found within localised areas or cell foci in tissue from animals infected for 7 days. Control animals showed only isolated CD20 positive cells. CXCL13 expression at both 1 and 7 days was associated with individual cells within structurally similar cell rich areas.