RSV infection modulates IL-15 production and MICA levels in respiratory epithelial cells

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Abstract: The cytokine interleukin-15 (IL-15), major histocompatibility complex (MHC) class I molecules and MHC class I chain-related proteins (MIC) A and B (MICA/B) are involved in cellular immune responses to virus infections but their role in respiratory syncytial virus (RSV) infection has not been studied. We aimed to determine how RSV infection modulates IL-15 production, MHC class I and MICA expression in respiratory epithelial cells, the molecular pathways implicated in virus-induced IL-15 production and how IFN-γ alters RSV-induced IL-15 production and MHC class I and MICA expression.

We infected respiratory epithelial cell lines (A549 and BEAS-2B cells) and primary bronchial epithelial cells with RSV and measured production of IL-15, expression of MHC I and MICA and the role of the transcription factor nuclear factor-kappa B (NF-κB).

We report here that RSV increases IL-15 in respiratory epithelial cells via virus replication and NF-κB dependent mechanisms. Furthermore, RSV infection of epithelial cells up-regulated cell surface expression of MICA and levels of soluble MICA. IFN-γ up-regulated RSV induction of soluble IL-15 but inhibited induction of MICA.

Up-regulation of IL-15, MHC I and MICA are likely to be important mechanisms in activating immune responses to RSV by epithelial cells.

Keywords: respiratory viruses, immune response, epithelial cell
Respiratory syncytial virus (RSV) is a common cause of severe respiratory tract diseases in infants, children and the elderly. RSV infection of respiratory epithelial cells modulates surface and soluble molecules involved in interferon (IFN)-γ production and cytotoxic activity of natural killer (NK) and T cells [1-3]. The presence of a virus-inducible region in the IL-15 promoter [4] suggests that IL-15 is an important component of host antiviral defense mechanisms, via induction of the IFN-related transcription factors nuclear factor-kappa B (NF-κB), interferon regulatory factors (IRF) and type 1 IFNs, synthesis of type 1 effector molecules (IFN-γ, granzyme and perforin) and survival and activation of NK and effector/memory CD8+ T cells [5-7]. IL-15 also up-regulated major histocompatibility complex class I chain-related proteins (MIC) A and B on intestinal epithelial cells [8]. Surface MIC molecules (encoded by genes within the human MHC and genetically linked to MHC class I HLA-B molecules), regulate innate immunity by non-specifically activating NKG2D-positive NK and CD8+ T cells [9, 10]. Therefore IL-15 is likely to play a key role in the immune response to RSV infection but no data are available regarding RSV modulation of IL-15 production and MICA/B expression in human respiratory epithelial cells.

We aimed to determine how RSV infection modulates IL-15 production in respiratory epithelial cells and the molecular pathways implicated in virus-induced IL-15 production. In addition, because RSV infection induces a type 1 immune response characterized by increased IFN-γ production [11] we determined how IFN-γ alters RSV-induced IL-15 production and the levels of MHC class I and MICA molecules in epithelial cells.

Material and methods
**Cell culture and RSV infection of respiratory epithelial cells**

RSV A2 (ATCC, Rockville, MD, USA) was grown in Hep-2 cells and virus titre determined by plaque assay. In order to assess the specificity of live RSV-mediated responses UV-inactivated, thermal-inactivated and filtered-RSV were used as controls [2, 3]. Bronchial (BEAS-2B) and alveolar (A549) epithelial cell lines (European Collection of Cell Cultures, Salisbury, UK) and human primary bronchial epithelial cells (HPBEC) (Clonetics, Cambrex) were cultured with RSV as previously described [2, 3]. Semiconfluent cell monolayers were exposed to RSV at different MOI (multiplicity of infection - infectious units/cell) or inactivated-RSV for 1h with gentle shaking, the virus was washed and fresh medium±IFN-γ (IFN-γ 50 ng/mL) (R&D Systems Ltd, Abingdon, UK) was added. Time “zero” (0 hours) was considered the moment when virus was removed. Epithelial cells were harvested for flow cytometry and supernatants, RNA or protein lysates were stored at -80°C.

**Flow cytometry**

Epithelial cells were harvested at different time points and processed as described elsewhere [2, 3]. Surface MHC class I and MICA were detected on respiratory epithelial cells by direct staining with mouse antihuman mAb: PE-labelled human MIC-A (R&D Systems) and APC-labelled human HLA class I (BD Bioscience).

We acquired at least 10,000 events using a BD LSR flow cytometer (BD Bioscience). Results were expressed as mean fluorescent intensity (MFI) after subtracting the MFI of the control cells stained with the appropriate isotype control antibodies. Stimulation
experiments were expressed as fold increase of MFI of experimental condition over medium-treated cells of at least three separate experiments.

**ELISA for IL-15 and soluble MICA**

Levels of IL-15 were measured in culture supernatants by ELISA using commercially available matched–pair antibodies (R&D Systems) and recombinant IL-15 (Biosource). The lower limit of sensitivity of the assay was 0.25 pg/mL (Laza-Stanca et al, PLoS Pathogens, in press). Levels of soluble MICA were determined with Human MICA ELISA development kit, DuoSet (R&D Systems) (sensitivity 31.25 pg/mL).

**RNA extraction, reverse transcription, and TaqMan real-time PCR**

Total RNA was extracted with RNeasy Mini Kit (Qiagen) and 2 µg used for cDNA synthesis (Omniscript RT Kit, Qiagen). Quantitative RT-PCR was performed by using specific primers and probe for IL-15 designed by us (Laza-Stanca et al, PLoS Pathogens, in press).

Data were analysed using version 1.4, ABI Prism 7000 SDS software (Applied Biosystems). IL-15 gene expression were normalized to 18S rRNA (used as endogenous loading control) and presented as fold-increase relative to medium.

**Nuclear factor-kappa B activation**

The effect of NF-κB activation on RSV-induced IL-15 production was evaluated using an IKK2 inhibitor (AS602868) as previously described [12]. AS602868 inhibits the activation of IKK kinase responsible for the degradation of NF-κB inhibitors, and
consequently decreases intracellular levels of NF-κB [12]. We used the IKK2 inhibitor AS602868 at a concentration previously shown to be optimal for rhinovirus induced TNF-α inhibition and without cell toxicity [12].

**Functional analysis of IL-15 promoter activity**

Plasmids containing the full-length IL-15 promoter-reporter construct (hIL-15sAP), plasmids containing deletions of the IL-15sAP and the NF-κB mutant plasmids (mtNF-κB-897-seAP) were gifts from Dr N Azimi [13]. To determine the regions of the IL-15 promoter at which RSV up-regulation occurs, the plasmids coupled to secreted alkaline phosphatase (sAP) reporter were transfected into uninfected or RSV-infected A549 cells using electroporation by the calcium phosphate method (CalPhos mammalian transfection kit, BD Clontech). At different time points the supernatants were collected, protein content measured (Biorad Laboratories), and equivalent amounts assayed for reporter activity by standard secretory alkaline phosphatase assay (SEAP reporter gene assay, Roche Diagnostics). Results were normalized for transfection efficiency by using cotransfection of β-galactoside reporter construct. The promoter activity was expressed as the fold induction compared with that of the negative control.

**Statistical analysis**

The results were analyzed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA). Results of at least three separate experiments were expressed as mean±standard error of the mean (SEM). Analysis of variance
ANOVA) followed where appropriate by paired Student’s t-test was used for the comparisons between different experimental conditions. P values < 0.05 were considered statistically significant.

RESULTS

RSV up-regulates IL-15 production in epithelial cells

Alveolar A549 and bronchial BEAS-2B cells constitutively express low levels of IL-15 and infection by RSV significantly increased levels of IL-15 in A549 (Figure 1a left panel) and BEAS-2B cell (Figure 1a right panel) supernatants in a concentration- and time-dependent manner up to 72 hours post-infection (Figure 1b). RSV infection also increased IL-15 production in HPBEC (Figure 1c).

To establish whether virus replication was required for induction of IL-15, we next determined IL-15 protein release in supernatants of RSV-infected respiratory epithelial cells and in cells treated with non-replicative virus (UV- or heat-inactivated) or inoculums from which virus had been removed by molecular weight filtration. RSV significantly induced increased levels of IL-15 48 hours post-infection as compared with A549 epithelial cells treated with UV-, heat-inactivated, filtered-virus or medium only (Figure 2a), indicating that IL-15 induction in bronchial epithelial cells was due to live virus active replication and not to soluble factors in the virus inoculum.

RSV increases IL-15 gene expression and promoter activation in epithelial cells, and IL-15 induction is NF-κB dependent
In order to investigate whether RSV increases IL-15 protein by a pre-transcriptional mechanism via up-regulation of mRNA levels or by increasing the release of constitutive intracellular protein, we measured IL-15 mRNA expression. In RSV-infected A549 cells, IL-15 mRNA levels were increased at 12, 48, and 72 hours post-infection as compared with the control (Figure 2b). IL-15 mRNA also increased in human primary bronchial cells following RSV infection at 24 hours (Figure 2b).

As IL-15 induction in other systems has been reported to be NF-κB dependent [14], we next investigated the role of NF-κB in RSV induced IL-15 production in epithelial cells. RSV-induced IL-15 expression in A549 cells was significantly decreased in a concentration-dependent manner upon addition of the pharmacological IKK2 inhibitor AS602868 (Figure 3a left panel), and induction in HPBEC was also significantly inhibited by IKK2 inhibition (Figure 3a right panel). These data suggest that RSV induction of IL-15 is dependent on IKK2 and likely involves the canonical NF-κB pathway.

In order to investigate whether RSV induction of IL-15 involved IL-15 promoter activation, and to confirm the role of NF-κB in activation of the IL-15 promoter by RSV we used plasmids containing full-length IL-15 promoter (-897-seAP), plasmids with IL-15 promoter almost absent (-15-seAP) and full-length promoter plasmids containing mutations at the -75/-65 NF-κB biding site (mtNF-κB-897-seAP). RSV infection induced activation of full-length IL-15 promoter construct (fold increase 4.45±0.92, P<0.01) when compared with uninfected transfected cells. Cells transfected with the promoters -15-seAP and mtNF-κB-897-seAP did not significantly increase expression of IL-15
promoter when infected with RSV (Figure 3b). These data provide evidence that RSV induction of IL-15 involves IL-15 promoter activation and further evidence that the NF-κB ligation site is necessary for the activation of IL-15 promoter by RSV.

**RSV and surface MHC class I and MICA/MICB molecules**

Because MHC class I molecules are related to MIC molecules we investigated how RSV modulates these molecules. MHC class I molecules have an important role in the innate (by controlling NK cell activity) and, via antigen-presentation, in acquired antiviral immunity.

RSV infection increased surface levels of MHC class I molecules in A549 ($P<0.05$, Figure 4a), BEAS-2B ($P<0.05$, Figure 4b) and HPBEC ($P<0.01$, Figure 4c) at 24 hours in a replication-, dose-response and time-dependent manner (data not shown).

We were interested to determine further, how RSV modulates MHC class I chain-related A and B (MICA and MICB) molecules. It was reported that surface MICA molecules enhance non-specific activation of NKG2D$^+$ NK and CD8$^+$ T cells and that in some circumstances released soluble forms of MICA inhibit the immune response [15].

Using at the beginning of our study an anti-MIC antibody (recognizing both MICA and MICB molecules) (6D4.6 antibody, gift from Veronica Groh) [16], we found that HPBEC, A549 and BEAS-2B cells constitutively expressed MICA/MICB molecules (representative histogram in HPBEC shown in Figure 5a) as previously reported [17, 18]. We continued our studies by using a specific MICA antibody which became commercially available. We found RSV infection increased surface levels of MICA in
BEAS-2B cells in a time- and dose-dependent manner (Figures 5b and 5c respectively). RSV infection also increased levels of soluble MICA levels produced by BEAS-2B cells in a dose- and time-dependent manner (Figure 5d).

**IFN-γ increases RSV-induced IL-15 levels but down-regulates RSV induction of MICA**

It has been reported that IFN-γ increases IL-15 levels in bronchial epithelial cells from lung cancer patients and in the BEAS-2B cell line [19, 20], we therefore finally investigated whether IFN-γ influenced IL-15 levels, MHC Class I expression or surface or soluble levels of MICA, either alone, or in combination with RSV infection. The presence of IFN-γ in the culture milieu significantly increased IL-15 secretion, and enhanced RSV-induced soluble IL-15 levels in BEAS-2B cells at 24 hours (P<0.01, Figure 6a). Treatment of epithelial cells with IFN-γ alone also significantly increased surface MHC class I expression on epithelial cells at 24 hours (P<0.01 for BEAS-2B Figure 6b right panel, and P<0.001 for HPBEC, Figure 6c). The presence of IFN-γ in culture of RSV-infected cells had no effect on MHC class I in both epithelial cell lines and HPBEC. In contrast, treatment with IFN-γ significantly decreased both surface MICA and soluble MICA in BEAS-2B cells at 24 hours (Figure 7a) and 48 hours (Figure 7b). IFN-γ also inhibited the RSV-induced increase surface MICA in BEAS-2B cells at 24 hours and blocked the release of soluble MICA. The same inhibitory effect was observed at 48 hours (Figure 7b).
DISCUSSION

We show here evidence that *in vitro* RSV infection up-regulates airway epithelial cell IL-15 production via virus replication, activation of transcription factor NF-κB and up-regulation of the IL-15 gene. RSV infection also up-regulated surface MHC class I molecules and surface and soluble MICA molecules. RSV infection of respiratory epithelial cells in a type 1 cytokine milieu rich in IFN-γ increased IL-15 production and MHC class I molecules, but down-regulated surface and soluble MICA levels.

IL-15 has been shown to play an essential role in the survival of memory/effector CD8\(^+\) T cells and to activate cytolytic function independent of TCR specificity (NK-like killing) in effector CD8\(^+\) T cells through NKG2D [8], helping to eliminate target cells. IL-15 gene and protein have been previously reported in respiratory epithelial cells [21, 22] and IL-15 gene and protein expression have been detected in bronchial biopsies from healthy controls as well as from patients with inflammatory lung diseases [21]. RSV up-regulates IL-15 mRNA in a monocyte cell line but the effect of RSV on IL-15 production in respiratory epithelial cells has not been reported. We found that RSV infection increased production of both IL-15 mRNA and protein in respiratory epithelial cell lines and primary bronchial epithelial cells. By using a small molecule IKK inhibitor and promoter constructs we also determined that the canonical NF-κB pathway plays an essential role in RSV-induced IL-15 expression. Up-regulation of IL-15 by respiratory epithelial cells in response to RSV infection is likely to play an essential role in activating the cellular components of the innate immune system. We have reported deficient IL-15 production in asthmatics (Laza-Stanca et al, PLoS Pathogens, in press) and this may be one mechanism of increased susceptibility to respiratory virus infections in asthmatics.
We also found that RSV infection of respiratory epithelial cells up-regulates both MHC class I and MICA molecules. MHC class I molecules on respiratory epithelial cells have an important role in activating antigen-specific CD8+ T cells but may inhibit NK cells with a negative impact on controlling viral replication. Expression of MICA on epithelial cells leads to increased cytotoxicity of NKG2D-positive NK and CD8+ T cells [16] and lysis of infected cells. It has been reported that the expression of surface MIC on human tumor cells is sufficient to overcome the inhibitory effects of MHC class I expression on NK cell killing, and NKG2D-positive cytotoxicity will occur despite MHC class I expression [23]. Therefore up-regulation of MICA on epithelial cells in response to RSV infection will assure the activation of local cytotoxic NKG2D+ NK and T cells and the eradication of virus-infected cells. However RSV infection also up-regulated levels of soluble MICA produced by respiratory epithelial cells. Soluble NKG2D ligands such as MICA impair NKG2D-mediated cytotoxicity by NK and T cells by receptor internalization, receptor masking or degradation of surface NKG2D [10, 15]. Therefore higher levels of soluble MICA would be expected to impair viral clearance. It is possible that up-regulation of soluble MICA has a role in preventing excessive NKG2D-mediated cytotoxicity so averting cytolysis of bystander non-infected cells, but this hypothesis requires further investigation.

RSV induces a type 1 immune response with increased levels of IFN-γ, which, together with type I interferons, IL-15 and costimulatory molecules, has an important role in virus eradication and terminating the acute infection. IFN-γ present during in vitro RSV infection of respiratory epithelial cells inhibited the viral up-regulation of surface MICA.
Soluble MICA levels were also down-regulated by RSV infection in the presence of IFN-\(\gamma\) suggesting that the decrease in MICA surface levels by IFN-\(\gamma\) was not due to proteolytic shedding of the molecules from cell surface and that IFN-\(\gamma\) probably inhibits the cell surface transport. The biological significance of IFN-\(\gamma\) down-regulating MICA in respiratory epithelial cells is not known. We hypothesize that airway epithelial cells infected with RSV initially up-regulate surface MICA leading to activation of cytotoxic NKG2D-positive NK and CD8\(^+\) T cells. The IFN-\(\gamma\) released by these activated cells then acts as a negative feedback to down-regulate MICA expression so terminating non-specific activation of NKG2D-positive cells, preventing excessive non-specific immune activation and allowing the switch to antigen-specific anti-RSV adaptive immunity. This hypothesis will require further investigation in animal models of RSV infection. The effect of chronic elevated IFN-\(\gamma\) levels is unknown, but if associated with impaired up-regulation of MICA in response to RSV infection could lead to impaired viral clearance. It was recently reported that in human tumour cells, concurrently with the up-regulation of surface MHC class I, IFN-\(\gamma\) down-regulates the levels of surface MICA [24, 25]. Interestingly chronic obstructive pulmonary disease (COPD) is associated with an exaggerated type I immune cell inflammatory infiltrate [7, 26]. Chronic RSV infection has been reported in COPD [27] and acute COPD exacerbations are associated with RSV infection [28, 29]. Borchers et al demonstrated that NKG2D ligand expression is induced on pulmonary epithelial cells in response to oxidative stress and protein levels of cell-associated MICA were significantly increased in COPD patients who smoke [17, 30]. Further investigation of the relationship between IFN-\(\gamma\), surface and soluble MICA and RSV infection in COPD is warranted.
In conclusion we report that RSV infection of respiratory epithelial cells is associated with increased production of IL-15 and up-regulation of MHC I and MICA. These are likely to be essential for an adequate immune response to RSV infection and dysregulation of these mechanisms may contribute to impaired viral clearance.
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FIGURE LEGENDS

Figure 1. RSV increases IL-15 production in a dose- and time-dependent manner in respiratory epithelial cells. Panel 1a. A549 cells and BEAS-2B cells were infected with RSV, cultured for 48 and 72 hours respectively, supernatants collected and IL-15 levels were measured by ELISA. Panel 1b. A549 cells and BEAS-2B cells were infected with RSV, supernatants collected at subsequent time points (6 to 72 hours) and IL-15 levels measured by ELISA. Panel 1c. HPBEC were infected with RSV, supernatants collected at 24 hours and IL-15 levels measured by ELISA. All results are shown as mean±SEM, N=3-6. *p<0.05, **p<0.01, ***p<0.005.
Figure 2. RSV induces IL-15 via virus replication and by increasing gene expression. Panel 2a. Inactivation of RSV abolished IL-15 induction in A549 cells at 48
18 hours. N=3, *p<0.05, **p<0.01 vs. live RSV. Panel 2b. A549 cells (N=3) and HPBEC (N=6) (2b) were infected with RSV, total RNA was extracted from cell lysates (6 to 72 hours for A549 and at 24 hours for HPBEC) and IL-15 mRNA measured by qRT-PCR (TaqMan®). Results are fold-increase of the mRNA copy numbers in infected cells over non-infected control. All data are mean±SEM. *p<0.05, **p<0.01.

Figure 3. RSV increases IL-15 promoter activation via NF-κB activation. Panel 3a. A549 cells cultured for 8 hours in the presence of increasing concentrations of IKK inhibitor AS602868, were infected with RSV MOI 1 for 1 hour, washed and the IKK
inhibitor added again (left panel). The culture supernatants were collected at 14 hours post-infection and IL-15 levels measured by ELISA. The results are mean±SEM of 3 independent experiments. HPBEC treated with 10 μM AS602868 were infected with RSV MOI of 1, culture supernatants were collected at 24 hours post-infection and IL-15 levels measured by ELISA (right panel). Panel 3b. A549 cells transfected with (i) plasmids containing reporter construct of intact IL-15 promoter (-897-seAP), (ii) plasmids with deletion of almost entire IL-15 promoter (-15-seAP), and (iii) plasmids containing reporter construct of IL-15 promoter with NF-κB ligation site mutated (mtNF-κB-897-seAP) were infected with RSV (MOI 1) and supernatants collected at 24 hours. seAP activity was measured and results expressed as a ratio between RSV-infected and cells in medium. Data are mean±SEM, N=3. *p<0.05, **p<0.01, ***p<0.005.
Figure 4. RSV replication increases MHC class I levels. A549 (Panel 4a), BEAS-2B cells (Panel 4b) and HPBEC (Panel 4c) were cultured with RSV MOI 1, UV-inactivated RSV or filtered-RSV for 24 hours, cells harvested and HLA class I surface molecules determined by flow cytometry. Data are mean±SEM, N=3. *p<0.05, **p<0.01.
Figure 5. RSV induces surface and soluble expression of MICA in respiratory epithelial cells. Panel 5a. HPBEC constitutively express surface MICA/B molecules. Red histogram represents unstained cells. Black histogram represents isotype control. Green histogram represents MICA/B staining. One representative experiment out of 3. Panels 5b and 5c. BEAS-2B cells were cultured with medium, filtered-RSV and RSV MOI 0.03, 0.1 and 1 for up to 72 hours. Cells were harvested at 24 and 48 hours to determine surface MICA by flow cytometry. Supernatants were harvested at 24, 48 and
72 hours to determine soluble MICA by ELISA (Panel 5b). Data are mean±SEM, N=3. *p<0.05, **p<0.01, ***p<0.005.

**Figure 6. IFN-γ increases RSV-induced IL-15 and surface MHC class I levels.** Panel 6a. BEAS-2B cells were pre-treated with IFN-γ (50 ng/mL), infected or not with RSV MOI 1, cultured up to 72 hours and IL-15 measured. Data shown are mean±SEM from 4 individual experiments at 24 hours. Panels 6b and 6c. A549 cells (N=3, left panel), BEAS-2B cells (N=5, right panel) and HPBEC (N=5, panel 6c) were treated with
medium, IFN-γ, RSV and RSV+IFN-γ and MHC class I surface molecules measured by flow cytometry at 24h hours. *p<0.05, **p<0.01, ***p<0.005.
Figure 7. IFN-γ down-regulates levels of and suppresses RSV induction of surface and soluble MICA. BEAS-2B cells were treated with IFN-γ, RSV and RSV+IFN-γ, surface MICA measured by flow cytometry and soluble MICA by ELISA at 24 hours (Panel 7a) and 48 hours (Panel 7b). Data are shown as mean±SEM from 3 to 5 individual experiments. *p<0.05, **p<0.01, ***p<0.005.
References


