Alveolar macrophages can control Respiratory Syncytial Virus infection in the absence of type I interferons

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**ABSTRACT**

Respiratory syncytial virus (RSV) is a common cause of lower respiratory tract infections. Immunity to RSV is initiated upon detection of the virus by pattern recognition receptors, such as RIG-I-like receptors (RLRs). RLRs signal via MAVS to induce the synthesis of pro-inflammatory mediators, including type I interferons (IFNs), which trigger and shape antiviral responses and protect cells from infection. Alveolar macrophages (AMs) are amongst the first cells to encounter invading viruses and the ones producing type I IFNs. However, it is unclear whether IFNs act to prevent AMs from serving as vehicles for viral replication. In this study, primary AMs from MAVS (Mavs\(^{-/-}\)) or type I IFN receptor (Ifnar1\(^{-/-}\)) deficient mice were exposed to RSV ex vivo. Wildtype (wt) AMs but not Mavs\(^{-/-}\) and Ifnar1\(^{-/-}\) AMs produced inflammatory mediators in response to RSV. Furthermore, Mavs\(^{-/-}\) and Ifnar1\(^{-/-}\) AMs accumulated more RSV proteins than wt AMs but the infection was abortive. Thus, RLR-MAVS and IFNAR signalling are important for the induction of pro-inflammatory mediators from AMs upon RSV infection but this signalling is not central for controlling viral replication. The ability to restrict viral replication makes AMs ideal sensors of RSV infection and important initiators of immune responses in the lung.
INTRODUCTION

Lung infections must be carefully managed by the host in order to prevent detrimental effects to gas exchange and lung tissue integrity. A pivotal balance must be maintained where the pathogen is eradicated rapidly while inflammation remains tightly controlled. Alveolar macrophages (AMs) are cells residing in the alveoli at the airway epithelium and air interface and account for over 95% of the leukocytes in the airways. These macrophages are a unique subset of mononuclear phagocytes in both phenotype and function [1,2]. They originate from fetal monocytes [3] and are believed to play a central role in maintaining tissue homeostasis and removing cellular debris [1,2]. AMs are also crucial for the initiation of immune responses against invading respiratory viruses [4-9]. Respiratory syncytial virus (RSV) can cause severe lower respiratory tract infections especially in infants, the immunocompromised and the elderly [10]. Approximately 2-3% of RSV infected children develop severe bronchiolitis/viral pneumonia and require hospitalisation [10] and many of these children are prone to recurrent wheeze and asthma-like symptoms in their adolescence [11]. Several studies suggest that the innate immune response is indispensable for the lung to maintain its function while clearing RSV [10]. RSV is recognized by pattern recognition receptors (PRRs), which induce the production of type I IFNs and other cytokines that enhance the antiviral responses [12]. Type I IFNs exert both cell-intrinsic effects, which limit viral replication and cell-extrinsic anti-viral effects, such as recruitment and activation of immune cells [12,13]. Work from our group showed that during RSV infection type I IFNs have a central role in both interfering with viral replication and driving lung inflammation during RSV infection [4,14]. AMs are the main producers of type I IFNs in response to RSV and this production is
initiated via recognition of RSV by cytosolic MAVS-coupled PRRs [4,15]. Although studies suggest that AMs are early producers of inflammatory mediators, there is very little evidence as to how this production is initiated and especially how type I IFNs contribute to viral control in AMs. We therefore studied primary murine AMs deficient in MAVS and IFNAR1 exposed to RSV ex vivo. Our results show that AMs can produce several cytokines and chemokines after exposure to RSV and that this production is dependent on type I IFNs. However, AMs are not a source of all mediators known to be important early after RSV infection. Furthermore, we found that although the viral replication was abortive, transcription of RSV genes in both MAVS and IFNAR1 deficient AMs was increased in comparison to wt AMs. Thus, AMs efficiently control RSV infection and are an important source of cytokines and chemokines during RSV infection highlighting their role as important guards of the lower airways.
MATERIALS AND METHODS

Mice
C57BL/6 mice were purchased from Charles River or Harlan, UK and Ifnar1<sup>−/−</sup> mice on a C56BL/6 background were obtained from C Reis e Sousa, The Francis Crick Institute, UK. Ifna6<sup>gfp</sup> Mavs<sup>−/−</sup> mice (obtained from S. Akira, Japan) were screened to ensure the genotype was maintained and will be designated as Mavs<sup>−/−</sup> to denote the fact that the mice may or may not have a copy of the gfp insert at the Ifna6 locus. All mice were bred and maintained in pathogen-free conditions and gender and age-matched mice aged 8-14 weeks were used for each experiment. All animal experiments were reviewed and approved by the Animal Welfare and Ethical Review Board (AWERB) within Imperial College London and approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986 and the ARRIVE guidelines.

Isolation of alveolar macrophages
Mice were sacrificed and primary AMs were collected by bronchoalveolar lavage through flushing the lungs 3 times with 1 ml of PBS supplemented with 5 mM EDTA (Life Technologies). The lavage was repeated twice and AMs from several mice were pooled. The purity of the AMs was >98% as determined by flow cytometry and on cytopsins counts [4]. Collected cells were incubated in a flat-bottom 96-well plate (1.25x10<sup>5</sup> cells/well) in complete DMEM (Invitrogen) containing 10% FCS (heat-inactivated from Gibco), 2mM L-glutamine (Invitrogen) and 1000 U/ml Penicillin-Streptomycin (Sigma-Aldrich) for 3h. After washing, the adherent cells were exposed to various stimuli (see below).
**Virus infections, cell lines and innate stimulation**

Plaque-purified human RSV (originally A2 strain from ATCC, US) was grown in HEp2 cells [16]. Inactivation was performed by exposing virus to UV light for 2 min (UV-RSV) in a CX-2000 UV cross-linker (UVP). The mouse epithelial cell line LA4 was grown in Ham’s F12K medium containing 10% FCS (heat-inactivated from Gibco), 2mM L-glutamine (Invitrogen) and 1000 U/ml Penicillin-Streptomycin (Sigma-Aldrich).

AMs or LA4 cells were stimulated with different MOIs of RSV, UV-RSV or to TLR ligands (R848 or LPS from Invivogen; 1 ng/ml) and incubated for 20h. The supernatant was collected and the cells were resuspended in 200μl of Trizol for subsequent RNA extraction.

**RNA isolation and qPCR**

RNA extraction from AMs or LA4 was performed using Trizol reagent (Invitrogen) according to manufacturer’s instructions. The RNA then underwent DNAse treatment according to manufacturer’s instructions (Life Technologies). 0.5-1μg of RNA was reverse-transcribed using the High Capacity RNA-to-cDNA kit according to manufacturer’s instructions (Applied Biosystems). To quantify mRNA levels in AMs or LA4 cells qPCR was performed. qPCR reactions for Ifnb, Ifng, Ifnl, Tnfa, Oas1a, Rsad2, Eif2ak2 and RSV L and N gene were performed using primers and probes previously described [4]. For the RSV strand-specific qPCR was performed as previously described [17]. Briefly, RNA extracted from AMs or LA4 cells was converted using (Qiagen) with gene specific primers for the positive and negative sense RSV nucleocaspid region RNA.
Since the primers contain a tag sequence, a tag-specific primer was used in the qPCR was performed [17]. Analysis was performed using the Quantitect Probe PCR Master Mix (Qiagen) and the 7500 Fast Real-time PCR System (Applied Biosystems). Results were normalized to levels of Gapdh, a housekeeping gene (Applied Biosystems). For absolute quantification, the exact number of copies of the gene of interest was calculated using a plasmid DNA standard curve for each gene. For relative quantification, the expression of RSV positive and negative-sense strand [17], Ifna6, Cxcl10, Il6, Ccl3, Il1a, Il1b, Il2, Il4, Il5, Il10, Il12p40/p70, Il13, Il17, Cxcl1, Ccl2, Cxcl9, Csf2 and Vegfa (all from Applied Biosystems) was expressed relatively to the expression of Gapdh. First, the $\Delta C_t$ (Ct=cycle threshold) between the target gene and Gapdh for each sample was calculated. Then the expression was calculated as $2^{-\Delta C_t}$. Analysis was performed using 7500 Fast System SDS Software (Applied Biosystems).

**Chemokine and cytokine detection**

Chemokines and cytokines were quantified by a Cytokines Mouse Magnetic 20-plex panel for Luminex (Life Technologies) and a ProcartaPlex mouse IFN alpha/IFN beta panel (eBioscience) according to the manufacturer’s instructions and data were acquired using a Bio-plex 200 system (Bio-Rad laboratories, UK). The concentration of cytokines in each sample was determined according to the standard curve using the Bio-plex 6 software (Bio-Rad laboratories). The concentration of CXCL10, CCL2 and TNF-\(\alpha\) was additionally measured using mouse DuoSet ELISA (R&D) according to manufacturer’s instructions. IL-6 was detected by ELISA using MP5-20F3 capture antibody and biotinylated MP5-32C11 detection antibody (both from BD Pharmingen). Data were
acquired on a SpectraMax Plus plate reader (Molecular Devices) and analyzed using SoftMax software (version 5.2).

**Staining for fluorescence microscopy**

3.75x10^5 primary AMs were seeded in a 24-well flat bottom plate containing glass coverslips. The cells were allowed to adhere for 3h before exposure to medium, RSV or UV-RSV. After 21h the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at RT followed by blocking with 3% BSA in 0.2%Triton X/PBS for a further 10 min. The cells were stained with polyclonal anti-RSV (1:200; Abcam) or monoclonal anti-RSV N (1:300; Abcam) and anti-LAMP-1 (1:300; Abcam) antibodies for 1.5h at RT. This was followed by staining with species-specific secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 586 (1:1000; Invitrogen) for 45 min in the dark at RT. The coverslips were mounted onto glass slides with ProLong® Gold Antifade Mountant with DAPI (ThermoFisher Scientific). The Zeiss confocal laser-scanning microscope (LSM)-510 on a 100x/1.4 Plan-Apochromat oil lens was used for obtaining images of the cells. Analysis of the images was performed on the Fiji, an open-source imaging processing software (ImageJ).

**Western blot analysis**

Lysates from 8x10^5 primary AMs extracted using RIPA Buffer (Sigma) containing cOmplete-protease inhibitors (Mini Roche). Briefly, the cells were resuspended in RIPA buffer and placed on a rotator at 4°C for 30 minutes before being centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was quantified using the Peirce™ BCA protein
assay kit (Thermo scientific) following the manufacturer’s instruction. For westerns, 5 µg of protein was added in each lane in a 1:1 dilution with laemmli buffer containing bromophenol blue (Sigma). Proteins were loaded onto a 10-lane 4-12% Bis-Tris gel (Novex) containing the MagicMark™XP Western Protein Standard (Invitrogen) markers. Gels were run in Running buffer (NugePage MES SDS) before being transferred to a Polyvinylidene difluoride (PVDF) filter paper sandwich with 0.45 µm pore membrane (Novex) using Tris-Glycine Transferase (Novex). The membranes were blocked for 1h and then exposed to a goat-anti-RSV antibody (1:2000; Abcam) or Direct-Blot™ HRP anti-β-actin (1:1000; Biolegend) at 4°C overnight in blocking buffer (TBS containing 5% BSA and 0.01% TWEEN20). The membranes were then incubated with a secondary anti-goat HRP antibody (1:5000; Jackson Immunoresearch) for 1h before being developed using EZ-ECL (Biological Industries). The membrane was read using a Fusion FX reader (Vilber Lourmat) and analysis performed using the Vilber Lourmat software program.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc.). Comparisons were performed using either unpaired Student’s t test or One-way ANOVA with Tukey’s post hoc test. For all tests a value of P<0.05 was considered as significant. *p<0.05; **p<0.01; ***p<0.001; ns, not significant; ND, not detectable.
RESULTS

**AMs produce an array of cytokines and chemokines after RSV exposure**

Alveolar macrophages have the ability to rapidly respond to invading pathogens including RSV [4]. To investigate which inflammatory mediators are produced by AMs after encountering RSV, primary AMs were exposed to RSV *ex vivo* and quantification of secreted mediators and mRNA was performed by Luminex and RT-PCR, respectively. Initially a time course (6–96h) was performed and 18-20h was determined as the optimum time point to study of both cytokine mRNA and protein induction (data not shown). At 20h post RSV exposure, primary AMs produced IFN-α, IFN-β, CXCL10, TNF-α, IL-6 and CCL3 as detected both by Luminex (Fig. 1) and mRNA (Fig. S1). This cytokine and chemokine response was not triggered by UV-inactivated RSV (UV-RSV) and was positively correlated with RSV dose (multiplicity of infection (MOI); Fig. 1 and Fig. S1). An MOI of 2 was then chosen for subsequent experiments. Interestingly, there were several inflammatory mediators that primary AMs exposed *ex vivo* to RSV did not produce such as IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p40/p70, IL-13, IL-17, IL-18, IFN-λ, IFN-γ, CXCL1, CCL2, CXCL9, GM-CSF and VEGF, as assessed by Luminex and ELISA (Table 1). A weak mRNA signal but no protein was detected for *Il1a, Il1b, Cxcl1, Ccl2* and *Cxcl9* (Table 1). Thus, AMs can produce many but not all of the early mediators detected *in vivo* in response to RSV infection [14], suggesting that they, together with other cell types, contribute to inflammatory lung responses to viral infection.
The cytokine and chemokine production from AMs depends on MAVS and IFNAR signalling

We used primary AMs from wt, Ifnar1\textsuperscript{−/−} and Mavs\textsuperscript{−/−} mice and exposed them \textit{ex vivo} to RSV MOI of 2. We found that both IFNAR and MAVS deficient AMs lacked the production of IFN-α, IFN-β, CXCL10, IL-6 and TNF-α by AMs after RSV exposure (Fig. 2A and B). As a control for responsiveness, AMs were stimulated with LPS and R848, known to trigger TLR4 and TLR7, respectively independently of MAVS and IFNAR. LPS and R848 stimulation resulted in production of IL-6 and TNF-α from all genotypes of AMs (Fig. 2C). However, Ifnar1\textsuperscript{−/−} AMs showed decreased production of TNF-α after LPS stimulation and Ifnar1\textsuperscript{−/−} and Mavs\textsuperscript{−/−} AMs of IL-6 after LPS and R848 production compared to wt AMs (Fig. 2C). This is possibly due to the importance of type I IFNs in potentiating proinflammatory responses [14].

Type I IFNs are known to induce several IFN-stimulated genes (ISGs) that interfere with viral replication. Since AMs are one of the first cells to encounter virus particles reaching the lower airways we investigated the induction of some ISGs, i.e. Viperin, OAS-1 and PKR in AMs after \textit{ex vivo} exposure to RSV (MOI of 2; Fig. 2D). These ISGs have all been implicated in restricting RSV replication [18-21] and were upregulated in wt AMs but not in IFNAR1 or MAVS deficient AMs, in line with their lack of response to or induction of type I IFNs (Fig. 2A and D).

IFNAR and MAVS-deficiency leads to increased intracellular viral protein accumulation and to presence of inclusion bodies
In order to assess if the lack of type I IFN response and ISG induction would alter the presence and localisation of RSV proteins within AMs fluorescence microscopy was performed. Staining for anti-RSV protein showed that wt AMs displayed similar punctate staining whether the cells were exposed to RSV or UV-RSV (Fig. 3). In contrast, IFNAR1 and MAVS deficient AMs showed more of a cytoplasmic staining with larger aggregated staining of viral proteins that resembled inclusion bodies (IBs; Fig. 3B-C). Consistent with that notion, these large cytoplasmic bodies could be detected with an antibody against the RSV N protein, which is abundant in IBs [22], in IFNAR1 and MAVS deficient AMs but not in wt AMs (Fig. 4). To distinguish phagolysosomes from IBs, staining for LAMP-1 was performed and demonstrated that the larger aggregates of viral proteins found in IFNAR1 and MAVS deficient AMs did not co-stained with LAMP-1 suggesting that they were indeed IBs (Fig. 4C). Furthermore, intensity analysis of the IBs areas (Fig. 4D) and preliminary z-stack analysis (data not shown) confirmed that IBs detected in IFNAR1 and MAVS deficient AMs are not within phagolysosomes.

To further evaluate the presence of viral proteins in AMs, Western blot analysis using anti-RSV antibodies on lysate from UV-RSV or RSV (MOI of 2) exposed AMs from wt, Mavs\textsuperscript{−−} and Ifnar1\textsuperscript{−−} mice was performed (Fig. 5). This analysis revealed proteins suggestive of RSV G, N, P and M2-1 (on size and [23-25]) in all samples (UV-RSV and RSV). However, in RSV exposed Mavs\textsuperscript{−−} and Ifnar1\textsuperscript{−−} AMs more N was detected (Fig. 5) suggesting RSV protein production. Thus, when compared to wt AMs, lack of signalling through IFNAR1 or MAVS in AMs allows viral replication to advance further and enables the virus to form IBs.
RSV replication in AMs is restricted even in the absence of MAVS and IFNAR signalling

To assess the viral load in AMs exposed to RSV ex vivo, RSV L and N genes were quantified. RSV L and N genes were detected in AMs from all genotypes from 1h after exposure up to 48h post exposure. In the absence of type I IFNs, IFNAR1 and MAVS deficient AMs had marginally more L gene copies but significantly more N gene copies compared to wt AMs after RSV exposure (Fig. 6A and Fig. S2). We compared this to a mouse lung epithelial cell line LA4, which showed a 100-1000 fold increase in L and N gene transcripts between 1h to 48h post infection (Fig. 6A). In addition, AMs or LA4 cells exposed to UV-inactivated RSV (AM UV and LA4 UV) showed no increase in L and N gene copies (Fig. 6A and Fig. S2). Viral load was also assessed by immunoplaque assay to quantify infectious particles produced by AMs but no plaques could be detected (data not shown). Furthermore, using a RSV strand-specific qPCR assay [17], AMs showed similar levels of both RSV positive- and negative-sense strand with a slightly higher level of positive-sense strand RSV in Ifnar1−/− AMs at 24h after RSV exposure. However, LA4 cells showed a significant increase in both RSV strands (Fig. 6B). Altogether, these data indicate that viral replication is slightly elevated in MAVS and IFNAR deficient AMs compared to wt AMs but that RSV replicates more efficiently in the lung epithelial cells (LA4) compared to AMs.
DISCUSSION

AMs are key cells of the lower airways in both health and disease. They have an important role in surfactant catabolism and clearing up debris as well as in sensing invading pathogens and initiating immune responses [1]. Here, we show that AMs rely on MAVS and IFNAR1 signalling to produce cytokines and chemokines, including type I IFNs, in response to RSV exposure. However, even in the absence of type I IFN production or signalling, AMs can control viral replication indicating that they possess mechanisms independent of the canonical IFN pathway to restrict viral infection.

AMs constitute a unique subset of macrophages that develops from fetal liver monocytes, is exposed to air and is phenotypically and functionally different from other tissue macrophages [2]. Therefore, in order to study these cells, primary AMs must be obtained from the lung most commonly via brochoalveolar lavage. AMs are the main producers of type I IFNs \textit{in vivo} during RSV and Newcastle disease virus infection [4,6]. Human, ovine and murine AMs have also been suggested to produce several cytokines and chemokines after \textit{ex vivo} exposure to RSV [4,15,23,26-31]. We confirmed the ability of AMs to produce IFN-α, IFN-β, TNF-α, IL-6, CCL3 and CXCL10 after \textit{ex vivo} exposure to RSV. Interestingly, several cytokines and chemokines such as CXCL1, CCL2 and IL-1β are known to be present in the lungs early after RSV infection [14] yet were not produced by AMs. This highlights the delicate interplay between cells within the lung in that multiple cell types are needed in order to induce a proper inflammatory response to viral infections.

Type I IFNs are important mediators to induce an anti-viral state in infected and neighbouring cells and for the recruitment and activation of immune cells. Human AMs
have been shown to be less susceptible to RSV infection than nasal and bronchial epithelial cells [32]. We therefore hypothesised that the lack of production of \((Mavs^{−/−})\) or signalling by \((Ifnar1^{−/−})\) type I IFNs in AMs would render these cells more susceptible to RSV replication. As expected, the \(Mavs^{−/−}\) and \(Ifnar1^{−/−}\) AMs were unable to induce the ISGs Viperin, OAS-1 and PKR and the viral cycle was less restricted and more viral genes and proteins were detected within the cytosol in these AMs compared to wt AMs. Yet, despite the increased accumulation of viral proteins in the type I IFN deficient AMs, RSV only underwent abortive replication in these cells, which resulted in detection of IBs. This is in contrast to cells susceptible to RSV infection, such as in the human epithelial cell line HEp2, where the virus forms filaments on the plasma membrane and IBs are less obvious [33,34]. How type I IFN deficient AMs still managed to control the virus and how far into the viral life cycle is control achieved was not elucidated in this study. Nevertheless, increased presence of RSV N was detected in \(Mavs^{−/−}\) and \(Ifnar1^{−/−}\) AMs, which would suggest that initiation of viral replication occurs in these cells. It could therefore be speculated that there is an arrest either in assembly or budding. As we have not systematically compared the ability of RSV to infect multiple cell types, it remains possible that the restriction in viral replication observed in AMs is not unique. A possible reason for their ability to control viral replication might be due to particular characteristics of AMs for instance their high degree of phagocytosis. Furthermore, a growing number of cellular factors have been reported to be required for RSV replication, such as Rab11 pathway proteins, RhoA, Cofilin 1, Caveolin proteins, HSP90 and mitochondrial proteins. It is thus possible that lack of certain host factors [35-38] do not allow productive viral replication in AMs. Alternatively, AMs might have an altered lipid content of their cell
membrane or lipid rafts compared to susceptible cells such as epithelial cells which could prevent release of infectious virions \[39,40\]. The immunohistochemistry analysis of the RSV proteins in the wt AMs showed a very similar punctate staining of RSV or UV-RSV exposed AMs. We were not able to confirm if the RSV proteins were inside or bound to the surface of the cell. However, in the type I IFN deficient AMs there were more RSV proteins detected within the cytosol and the Western blot analysis and RT-PCR also showed an increase in RSV N proteins and genes. Altogether, this raises the possibility that AMs could contribute to viral clearance by mopping up virus from the airways preventing productive infection of epithelial cells.

The ability of AMs would seem to render them as ideal sensors of infection. Human epithelial cells have been suggested to be the ideal host for RSV \[17\]. However, RSV replicates in the mouse lung \[17,41\] and in mouse epithelial cells (LA4). AMs did not appear to support viral replication to the same degree as LA4 cells. Thus, AMs are important for initiation of the immune response in the lower airways after RSV infection and they are able to restrict viral replication even in the absence of type I IFNs. This positions them as excellent sensors of infection on the mucosal barrier.
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FIGURE LEGENDS

Figure 1. AMs produce cytokine and chemokine after RSV exposure. Primary wt AMs were exposed for 20h to medium or the indicated multiplicities of infection (MOI) of RSV or UV-RSV (MOI of 2; UV2). Secretion of IFN-α, IFN-β, CXCL10, TNF-α, IL-6 and CCL3 were detected in culture supernatant by Luminex. The data are shown as mean ± SEM of 4-9 individual cultures pooled from 2-3 experiments. Statistical significance of differences between indicated groups was determined by one-way ANOVA. *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Cytokines and chemokine production by AMs is dependent on MAVS and IFNAR1 signalling. Primary AMs from wt, Mavs<sup>−/−</sup> and Ifnar1<sup>−/−</sup> mice were exposed to medium or multiplicities of infection (MOI) of 2 of RSV or UV-inactivated RSV (UV-RSV) for 20h. (A) IFN-α and IFN-β were detected in culture supernatant by Luminex and (B) CXCL10, TNF-α and IL-6 were detected in culture supernatant by ELISA. (C) Secretion of TNF-α and IL-6 from primary AMs of the indicated genotypes after ex vivo exposure for 20h to medium or 1 ng/ml of LPS or R848. (D) OAS1a, Viperin and PKR transcripts were detected by RT-PCR after exposure to medium or MOI of 2 of RSV or UV-inactivated RSV (UV-RSV) for 20h. The data are shown as mean ± SEM of (A) 4-6 cultures per stimulation pooled from 3 independent experiments, (B) 4-15 cultures per stimulation pooled from 2-6 independent experiments, (C) 4-8 cultures per stimulation pooled from 2-4 independent experiments and (D) as 3-4 individual RNA samples pooled from 4 independent experiments. Statistical significance of differences between indicated groups was determined by unpaired Student’s t test. *p<0.05; **p<0.01; ***p<0.001.
Figure 3. Accumulation of RSV proteins in Mavs\textsuperscript{−/−} and Ifnar1\textsuperscript{−/−} primary AMs.

Primary AMs from wt, Mavs\textsuperscript{−/−} and Ifnar1\textsuperscript{−/−} mice were pooled and exposed to medium or MOI of 2 of RSV or UV-inactivated RSV (UV-RSV) for 21h before being fixed, stained for polyclonal-anti-RSV and DAPI and analysed by fluorescence microscopy. (A) Representative brightfield images of medium-exposed AMs. (B) Representative images of UV-RSV exposed primary AMs or (C) RSV exposed primary AMs where location of ‘inclusion-like bodies’ are presented by white arrows. Scale bar represents 10μm. Images characterize the appearance of the majority of AMs from at least four independent experiments.

Figure 4. Inclusion bodies are only present in AMs deficient in MAVS and IFNAR1 signalling. Primary AMs from wt, Mavs\textsuperscript{−/−} and Ifnar1\textsuperscript{−/−} mice were pooled and exposed to MOI of 2 of RSV for 21h. Cells were fixed and stained with RSV-anti-N [green], anti-LAMP-1 [red] and DAPI [blue] and examined by fluorescence microscopy. (A) Representative image of medium-exposed AMs and (B) UV-RSV exposed primary AMs. (C) RSV exposed primary AMs where location of inclusion bodies is presented by white arrows. Images are representing the appearance of the majority of AMs from three independent experiments. The scale bars represent 10μm. Monochrome images were converted to composite images using the Fiji software program (ImageJ). (D) Graphs indicate the pixel intensity of RSV-N, Lamp-1 and DAPI along the white line indicated on the micrographs.
Figure 5. The presence of RSV nucleocapsid protein is increased in MAVS and IFNAR1 deficient AMs. Primary wt, Mavs<sup>−/−</sup> and Ifnar1<sup>−/−</sup> AMs were exposed to UV-inactivated RSV (UV-RSV) or RSV (MOI of 2) for 21h. Cell lysate was generated using RIPA buffer and then subjected to Western blot analysis. The blots were exposed to a polyclonal anti-RSV antibody or to an anti-β-actin antibody. The protein standard is indicated to the right of the blot and the detected RSV proteins (G, N, L and M proteins) are indicated at the right of the blots. The blots are representative of at least 3 experiments.

Figure 6. Control of replication of RSV in AMs is independent of MAVS, IFNAR and ISG signalling. (A) AMs from wt, Mavs<sup>−/−</sup> and Ifnar1<sup>−/−</sup> mice and LA4 cells were exposed to medium or MOI of 2 of UV-inactivated RSV (AM UV or LA4 UV) or RSV for 1h, 24h and 48h. RSV L and N gene copies were determined by RT-PCR and the data are shown as fold increase over the copies present at 1h post inoculation. The UV AM show pooled measurements of L and N genes from all genotypes of AMs. (B) Positive and negative sense strands of RSV were detected using RT-PCR. The data are shown as mean ± SEM of (A) 3-4 individual RNA samples pooled from 3 independent experiments and (B) 4-6 individual RNA samples pooled from 4 independent experiments. Each RNA sample was extracted from 2-3 pooled individual cultures of AMs or LA4 cells. Statistical significance of differences between indicated groups was determined by unpaired Student’s t test. In (A) “**” represents differences between LA4 and wt AM, “$” between wt and Ifnar1<sup>−/−</sup> AMs and “#” between wt and Mavs<sup>−/−</sup> AMs. *p<0.05; **p<0.01; ***p<0.001.
**Table 1.** Inflammatory mediators not or partially detected using Luminex, ELISA or RT-PCR after 20h exposure of AMs with RSV MOI 2. Data are representative of at least two experiments.

| Mediator       | Assay          |       | Gene expression |
|----------------|----------------|-------|-----------------|
|                | Luminex | ELISA |                 |
| IL-1α          | -       |       | +               |
| IL-1β          | -       | -     | +               |
| IL-2           | -       |       |                 |
| IL-4           | -       |       |                 |
| IL-5           | -       |       |                 |
| IL-10          | -       |       |                 |
| IL12p40/p70    | -       |       |                 |
| IL-13          | -       |       |                 |
| IL-17          | -       |       |                 |
| IL-18          | -       |       |                 |
| IFN-λ          |         |       |                 |
| IFN-γ          | -       |       |                 |
| CXCL1          | -       | -     | +               |
| CCL2           | -       | -     | +               |
| CXCL9          | -       |       | +               |
| GM-CSF         | -       |       |                 |
| VEGF           | -       |       |                 |
Fig 1 - Makris et al.
Figure 2 - Makris et al.
Figure 3 - Makris et al.
Figure 4 - Makris et al.
|          | UV-RSV | RSV |
|----------|--------|-----|
| wt       |        |     |
| Mavs⁻    |        |     |
| Ifnar¹⁻⁻ |        |     |

| 98 kDa   |        |     |
| 62 kDa   |        |     |
| 49 kDa   |        |     |
| 38 kDa   |        |     |
| 28 kDa   |        |     |
| 17 kDa   |        |     |

β-Actin

Figure 5 - Makris et al.
Figure 6 - Makris et al.

A

L gene

N gene

B

Positive-sense strand RSV

Negative-sense strand RSV

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Supplemental information for:

Alveolar macrophages can control Respiratory Syncytial Virus infection in the absence of type I interferons

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Figure S1. Gene expression of cytokine and chemokine after RSV exposure of AMs. Primary wt AMs were exposed for 20h to medium or the indicated multiplicities of infection (MOI) of RSV or UV-RSV (MOI of 2; UV2). Levels of Ifna6, Ifnb, Cxcl10, Tnfa, Il6 and Ccl3 transcripts in AMs were measured by quantitative RT-PCR. Each point depicts data from an individual experiment with RNA pooled from 2-3 individual cultures per stimulation.

Figure S2. RSV L and N gene copies in AMs and LA4 cells. AMs from wt, Mavs⁻/⁻ and Ifnar1⁻/⁻ mice and LA4 cells were exposed to medium or MOI of 2 of UV-inactivated RSV (AM UV and LA4 UV) or RSV for 1h, 24h and 48h. RSV L and N gene copies were determined by RT-PCR. The UV AM show pooled measurements of L and N genes from all genotypes of AMs. The data are shown as mean ± SEM of 3-4 individual RNA samples pooled from 3 independent experiments. Statistical significance of differences between indicated groups was determined by unpaired Student’s t test. “*” represents differences between LA4 and wt AM, “$” between wt and Ifnar1⁻/⁻ AMs and “#” between wt and Mavs⁻/⁻ AMs. *p<0.05; **p<0.01; ***p<0.001.
Fig. S1 - Makris et al.
Fig. S2 - Makris et al.