Alveolar Macrophages Can Control Respiratory Syncytial Virus Infection in the Absence of Type I Interferons

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Key Words
Macrophages · Type I interferons · Pattern recognition receptors

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. RIG-I-like receptors signal via MAVS to induce the synthesis of proinflammatory 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. Wild-type (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, RIG-I-like receptor-MAVS and IFNAR signalling are important for the induction of proinflammatory 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) reside 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, which induce the production of type I interferons (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 antiviral 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 pattern recognition receptors [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 wild-type (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.

Fig. 1. AMs produce cytokines and chemokines after RSV exposure. Primary wt AMs were exposed for 20 h to medium or the indicated 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 means ± 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.
Materials and Methods

Mice
C57BL/6 mice were purchased from Charles River or Harlan, UK, and Ifnar1−/− mice on a C57BL/6 background were obtained from C. Reis e Sousa, The Francis Crick Institute, UK. Ifna6+/− Mavs−/− mice (obtained from S. Akira, Japan) were screened to ensure the genotype was maintained and are designated as Mavs−/− 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 AMs
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 cytospin counts. Collected cells were incubated in a flat-bottom 96-well plate (1.25 × 10^5 cells/well) in complete DMEM (Scientific Procedures) Act 1986 and the ARRIVE guidelines.

Virus Infections, Cell Lines, and Innate Stimulation
Plaque-purified human RSV (originally the A2 strain from ATCC, US) was grown in HEp2 cells [16]. Inactivation was performed by exposing the 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), 2 mM L-glutamine (Invitrogen), and 1,000 U/ml penicillin-streptomycin (Sigma-Aldrich) for 3 h. After washing, the adherent cells were exposed to various stimuli (see below).

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). 0.5–1 µg of RNA was reverse-transcribed using the High Capacity RNA to-cDNA kit according to the manufacturer’s instructions (Applied Biosystems). qPCR was performed to quantify mRNA levels in AMs or LA4 cells. qPCR reactions for Il1a, Il1b, Il2, Il4, Il5, Il10, Il12p40/p70, Il13, Il17, Il18, Ifn-λ, Ifn-γ, CXCL1, CCL2, CXCL9, GM-CSF, and VEGF were performed using the QuantiTect Probe PCR Master Mix (Qiagen) with gene-specific primers for the positive- and negative-sense RSV nucleocapsid region RNA. Since the primers contain a tag sequence, a tag-specific primer was used in the qPCR reaction at the denatured from Gibco), 2 mM L-glutamine (Invitrogen), and 1,000 U/ml penicillin-streptomycin (Sigma-Aldrich) for 3 h. After washing, the adherent cells were exposed to various stimuli (see below).

Table 1. Inflammatory mediators not or partially detected using Luminex, ELISA, or RT-PCR after 20 h of exposure of AMs to RSV MOI of 2

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Assay</th>
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<tr>
<td>IL-1α</td>
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<td>IL-1β</td>
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<td>IL12p40/p70</td>
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Data are representative of at least two experiments.

Chemokine and Cytokine Detection
Chemokines and cytokines were quantified by a Cytokine Mouse Magnetic 20-Plex Panel for Luminex (Life Technologies), and a ProcartaPlex mouse IFN-α/IFN-β 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 derived from the Bio-Plex 6 software (Bio-Rad Laboratories). The concentration of CXCL10, CCL2, and TNF-α was additionally measured using mouse DuoSet ELISA (R&D) according to the 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 analysed using SoftMax software (version 5.2).

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Fig. 2. Cytokines and chemokine production by AMs is dependent on MAVS and IFNAR1 signalling. Primary AMs from wt, Mavs$^{-/-}$, and Ifnar1$^{-/-}$ mice were exposed to medium or MOI of 2 of UV-RSV (UV) or RSV for 20 h. IFN-α and IFN-β (a) were detected in culture supernatant by Luminex and CXCL10, TNF-α, and IL-6 (b) 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 20 h 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 UV-RSV (UV) or RSV for 20 h. The data are shown as means ± SEM of 4–6 cultures per stimulation pooled from 3 independent experiments (a), 4–15 cultures per stimulation pooled from 2–6 independent experiments (b), 4–8 cultures per stimulation pooled from 2–4 independent experiments (c), and as 3–4 individual RNA samples pooled from 3–4 independent experiments (d). Statistical significance of differences between indicated groups was determined by unpaired Student’s t test. *p < 0.05; **p < 0.01; ***p < 0.001.
**Western Blot Analysis**
Lysates from $8 \times 10^5$ primary AMs extracted using RIPA Buffer (Sigma) containing cOmplete Protease Inhibitors (Mint; Roche). Briefly, the cells were resuspended in RIPA buffer and placed on a rotator at 4°C for 30 min before being centrifuged at 12,000 g for 10 min at 4°C. The supernatant was quantified using the Pierce® BCA protein assay kit (Thermo Scientific) following the manufacturer’s instruction. For Western blots, 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 (NuPAGE MES SDS) before being transferred to a polyvinylidene difluoride filter paper sandwich with 0.45-μm pore membrane (Novex) using Tris-glycine transferase (Novex). The membranes were blocked for 1 h and then exposed to a goat-anti-RSV antibody (1:2,000; Abcam) or Direct-Blot™ HRP anti-β-actin (1:1,000; 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 (1:5,000; Jackson ImmunoResearch) for 1 h 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 an unpaired Student’s t test or one-way ANOVA with Tukey’s post hoc test. For all tests, $p < 0.05$ was considered significant.

**Results**

**AMs Produce an Array of Cytokines and Chemokines after RSV Exposure**
AMs 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 (6–96 h) was performed and 18–20 h was determined as the optimum time point to study both cytokine mRNA and protein induction (data not shown). At 20 h after 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 (see online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000446824). This cytokine and chemokine response was not triggered by UV-RSV and was positively correlated with RSV dose (MOI; fig. 1; online suppl. 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 during viral infection.

**The Cytokine and Chemokine Production from AMs Depends on MAVS and IFNAR Signalling**
We used primary AMs from wt, Ifnar1−/−, and Mavs−/− mice, and exposed them 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, b). As a
Type I IFN-Deficient AMs Can Control RSV Replication

wt Mavs–/– Ifnar1–/– Medium

RSV DAPI

wt Mavs–/– Ifnar1–/– UV RSV

UV RSV

wt Mavs–/– Ifnar1–/– RSV

RSV

wt Mavs–/– Ifnar1–/– RSV

RSV DAPI

Color version available online.
Ifnar1−/−

Medium
UV RSV

RSV

RSV-N

Lamp-1

DAPI

(For figure 4d and legend see next page.)
control for responsiveness, AMs were stimulated with LPS and R848, which are known to trigger TLR4 and TLR7, respectively, independently of MAVS and IFNAR. LPS and R848 stimulation resulted in the production of IL-6 and TNF-α from all genotypes of AMs (fig. 2c). However, Ifnar1<sup>−/−</sup> AMs showed decreased production of TNF-α after LPS stimulation and Ifnar1<sup>−/−</sup> and Mavs<sup>−/−</sup> 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 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, d).

**IFNAR and MAVS Deficiency Leads to Increased Intracellular Viral Protein Accumulation and to the Presence of Inclusion Bodies**

In order to assess if the lack of a type I IFN response and ISG induction would alter the presence and localization of RSV proteins within AMs, fluorescence microscopy was performed. Staining for RSV proteins 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-stain with LAMP-1, suggesting that they were indeed IBs (fig. 4c). Furthermore, intensity analysis of the IB 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-exposed (MOI of 2) AMs from wt, Mavs<sup>−/−</sup>, and Ifnar1<sup>−/−</sup> mice was performed (fig. 5). This analysis revealed proteins suggestive of RSV G, N, P, and M2-1 [23–25] in all samples (UV-RSV and RSV). However, in RSV-exposed Mavs<sup>−/−</sup> and Ifnar1<sup>−/−</sup> 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 1 to 48 h after 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...
We compared this to the mouse lung epithelial cell line LA4, which showed a 100- to 1,000-fold increase in L and N gene transcripts between 1 and 48 h after 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; online suppl. 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 strands with a slightly higher level of positive-sense strand RSV in Ifnar1–/– AMs at 24 h 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 bronchoalveolar lavage. AMs are the main producers of type I IFNs 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 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 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 antiviral state in infected and neighbouring cells, and for...
Fig. 6. Control of replication of RSV in AMs is independent of MAVS and IFNAR signalling. a AMs from wt, Mavs−/−, and Ifnar1−/− mice and LA4 cells were exposed to medium or MOI of 2 of UV-RSV (AM UV or LA4 UV) or RSV for 1, 24, and 48 h. RSV L and N gene copies were determined by RT-PCR and the data are shown as fold increase over the copies present at 1 h after 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 means ± SEM of 3–4 individual RNA samples pooled from 3 independent experiments (a) and 4–6 individual RNA samples pooled from 4 independent experiments (b). 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−/− AMs and ‘#’ between wt and Mavs−/− AMs. * p < 0.05; ** p < 0.01; *** p < 0.001.
IBs are less obvious because the virus forms filaments on the plasma membrane and abortive replication, 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 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, the viral cycle was less restricted, and more viral genes and proteins were detected within the cytosol compared to wt AMs. Yet, despite the increased accumulation of viral proteins in the type I IFN-deficient AMs, RSV only underwent abortive replication, 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, such as 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, collagen 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 immunofluorescence 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 to control RSV infection would seem to render them as ideal sensors of infection. Human epithelial cells have been suggested to be the ideal host for RSV [17]. Although RSV also 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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Disclosure Statement

The authors declare no competing financial interests.

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