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Azithromycin induces anti-viral responses in bronchial epithelial cells

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Running title: Anti-viral activity of macrolide antibiotics

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Abstract

The majority of asthma exacerbations are caused by rhinovirus. Currently the treatment of asthma exacerbations is inadequate. Previous evidence suggests that macrolide antibiotics have anti-inflammatory and anti-viral effects however the mechanism is unknown. We investigated the anti-rhinoviral potential of macrolides through the induction of anti-viral gene mRNA and protein. Primary human bronchial epithelial cells were pre-treated with the macrolides Azithromycin, Erythromycin and Telithromycin, and infected with minor group rhinovirus 1B and major group rhinovirus 16. The mRNA expression of the anti-viral genes, type I interferon-β and type III interferon-λ1, interferon-λ2/3, and interferon stimulated genes (retinoic acid inducible gene I, melanoma differentiation associated gene 5, oligoadenylate synthase, MxA and Viperin) and pro-inflammatory cytokines (IL-6 and IL-8), and rhinovirus replication and release were measured. Azithromycin, but not Erythromycin or Telithromycin significantly increased rhinovirus 1B and 16 induced interferons and interferon stimulated gene mRNA expression and protein production. Furthermore, Azithromycin significantly reduced rhinovirus replication and release. Rhinovirus induced IL-6 and IL-8 protein and mRNA expression were not significantly reduced by Azithromycin pre-treatment. In conclusion, the results demonstrate that Azithromycin has anti-rhinoviral activity in bronchial epithelial cells, and during rhinovirus infection increases the production of interferon stimulated genes.

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Introduction

Respiratory viral infections are associated with the majority of asthma exacerbations (approximately 80%) in both adults [1,2] and children [3,4]. Asthma exacerbations are the major cause of morbidity, mortality and healthcare costs in asthma [5]. The mechanisms of virus induced asthma exacerbations are poorly understood [6]. Inhaled corticosteroids reduce asthma exacerbation frequency [7-9]; however, this effect is only partial even in combination with long-acting β_2-agonists [10,11], more effective therapies are therefore necessary. Rhinoviruses (RV) are responsible for approximately 60% of virus induced exacerbations [1,4]. Asthmatics have an increased susceptibility to RV infection compared to normal subjects [1]. A novel mechanism for this increased susceptibility has been identified recently: asthmatics exhibit lower levels of interferon (IFN)-β and IFN-λ gene and protein expression upon RV infection [12,13], and IFN-λ levels negatively correlate with exacerbation severity [13].

Type I IFNs consist of the single gene IFN-β and numerous IFN-αs. The recently discovered type III IFNs consist of IFN-λ1,2,3 (respectively IL-29, IL-28A and IL-28B). The major IFNs produced by bronchial epithelial cells are IFN-β and- λ [14]. Despite using different receptor complexes, both types of IFNs have functional similarities, including antiviral effects through stimulation of the innate and adaptive immune responses. After secretion, IFNs bind to the surface of infected and neighbouring cells to initiate the Janus activated kinases (JAKs) and the signal transducers and activators of transcription (STATs). Activation of the JAK-STAT pathway leads to the induction of many interferon stimulated genes (ISGs), which

prevent viral replication such as MxA [15], Viperin [16] or the 2'-5' oligoadenylate synthesase (OAS) family [17], and also recognise viral RNA, such as retinoic acid inducible gene I (RIG-I) [18], and melanoma differentiation associated gene 5 (MDA5) [19]. As asthmatics have defective IFN-β and IFN-λ expression, one potential approach to therapy of asthma exacerbations is to augment host IFN responses and induction of anti-viral ISG expression.

Macrolides include Erythromycin (EM), Azithromycin (AM) and the ketomacrolide Telithromycin (Tel). They have well established anti-bacterial [20] and anti-inflammatory effects [21-23], and preliminary evidence showed that they may also have anti-viral effects [24-27]. The anti-bacterial action of macrolides is through inhibition of protein synthesis via binding to the 50S subunit of bacterial ribosomes. Macrolides also inhibit IL-6 and IL-8 protein production and reduce neutrophil attraction to the site of infection, likely through inhibiting NF-κB [21,28]. Tel was reported to have a beneficial effect in treatment of asthma exacerbations, with Tel treated patients experiencing greater improvements in lower airway symptoms and lung function compared to placebo treated patients though the mechanisms of this therapeutic effect are unknown [29]. *In vitro* studies report an anti-rhinoviral and anti-inflammatory effect of some macrolide antibiotics [24,26,27], possibly via a reduction of ICAM-1 mRNA expression after major group RV infection, which use ICAM-1 as their receptor. In contrast, anti-viral effects were also observed for minor group RVs, which utilise a different receptor [26,27]. The mechanisms for this effect are therefore unknown.

We hypothesised that the beneficial effects of macrolide antibiotics in asthma exacerbations may be due, at least in part, to their ability to exhibit anti-viral activity. In the current study, we used *in vitro* models of normal primary human bronchial epithelial cell (HBEC) culture and investigated the effects of the macrolides AM, EM and Tel on IFN and ISG gene expression, ISG protein induction and also the anti-viral activity during RV infection.

Materials and Methods

Cell and virus culture

Human bronchial epithelial cells (HBEC, Lonza, Cleveland, USA) were cultured in BEGM according to manufacturers recommendations. RV1B and RV16 (ATCC, Rockville, USA) were prepared as previously described [30], and were titrated on HeLa cells to ascertain their 50% tissue culture infective dose (TCID50)/mL [31]. The identity of each virus was confirmed by inhibition with serotype specific antibody using titration assays. Both viruses were negative for *Mycoplasma* infection.

Treatment with macrolides and infection of HBECs with RV1B

HBEC cells were cultured in 12-well tissue culture plates (Nunc, Rochester, NY) until 80-90% confluent before being treated with AM, EM (Sigma-Aldrich, St Louis MA), and Tel (a kind gift from Sanofi Aventis) at a concentration of 10μM or 50μM in bronchial epithelial basal medium (BEBM) for 24h. The diluent used for all three macrolides was 96% ethanol, at a final concentration of 0.05% for 50μM macrolides and was confirmed in preliminary experiments not to have any effect on measured outcomes (data not shown). HBECs were then infected with RV1B, at multiplicity of infection (MOI) 1 or 0.25, or RV16 (MOI of 1) for 1h with shaking at room temperature and replaced with 1mL of BEBM. After 8, 24 and 48h, supernatants and cells were harvested in RLT-buffer (Qiagen, Crawley, UK) for RNA isolation, or in 2% Tris-glycine SDS sample buffer (Invitrogen, Paisley, UK) for protein. Supernatants and lysates were stored at -80°C until analysis.

RNA isolation and cDNA synthesis

Total RNA isolation was performed using RNeasy® mini kit (Qiagen) according to the manufacturers' recommendations. cDNA was prepared using random primers (Promega, Maddison, WI) and Omniscript RT kit (Qiagen) according to manufacturers recommendations and stored at -80˚C.

TaqMan real time PCR

In a 25μL PCR reaction, 2μL cDNA was amplified in a 7500 Sequence Detector, using the 2x QuantiTect Probe PCR Master Mix (Qiagen), 50nM forward primer and 300nM reverse primer and 100nM probe were used for RV, 300nM primers and 100nM probe were used for the 18S rRNA internal control, 300nM forward primer, 900nM reverse primer and 100nM probe for OAS, IL-29 and IFN-β and 900nM primers and 100nM probes for MxA, Viperin, IL-28, IL-6, IL-8, RIG-I and MDA5 (all FAM-TAMRA labelled). Sequences of primers and probes used are listed in Table 1. The copy numbers were determined via standard curves of each gene constructed as dsDNA plasmids, and normalised with the housekeeping gene 18S rRNA. Data is presented as % of copy number of control wells (RV1B), from approximately $2.5x10^5$ cells/well.

ELISA

IL-6 and IL-8 were quantified by ELISA using commercially available paired antibodies and standards, following the manufacturer's instructions (R&D Systems, Abingdon, UK). The sensitivity of each assay was 10pg/mL.

SDS PAGE and Western Blotting

Total protein lysates were run on 4-12% Bis-Tris polyacrylamide gels, and transferred onto nitrocellulose membranes (Invitrogen), blocked in 5% skimmed milk, and probed with antibodies specific for human RIG-I 0.083µg/mL (Cell Signalling, Danvers, MA, USA), RSAD2/Viperin 0.3µg/mL (ProteinTech Group, Chicago, USA), OAS1 0.5µg/mL (Abgent, San Diego, USA), MxA 0.2µg/mL (Santa Cruz Biotechnology Inc) or α-tubulin 0.2µg/mL (Santa Cruz Biotechnology Inc). Secondary antibodies used were goat anti-mouse HRP, 0.08µg/mL and sheep anti-rabbit HRP, 2µg/mL (AbD Serotec, Oxford, UK). Blots were developed using ECL (GE Healthcare, Chalfont St Giles, UK).

Virus titration assay

Supernatants were serially diluted in DMEM containing 4% FCS (Invitrogen) and titrated on HeLa cells to determine the TCID50/mL of the RV in the supernatants (42). Each dilution was assayed in eight replicates, and TCID50/mL was calculated according to the Spearman-Karber formula [31].

Statistical analysis

All data were expressed as means±SEM. Comparisons of different groups were analyzed using one-way analysis of variance (ANOVA) with Bonferroni post testing (Prism, GraphPad Software, San Diego, CA). Values were considered significant if $p<0.05$. All experimental work was performed as 3-5 independent experiments.

Results

RV1B increases type I and III IFN and ISG mRNA expression

At 8h after infection, RV1B (MOI 0.25) significantly induced IL-28 mRNA expression ($p<0.01$, data not shown) and at MOI 1 there was a significant induction of IL-28, MxA, RIG-I, MDA-5 ($p<0.05$) and OAS ($p<0.01$) compared to medium treated cells (data not shown). At 24h after infection, RV1B significantly induced type I and III IFNs and the ISGs measured at both RV1B concentrations compared to medium treated cells. RV1B increased IFN-β mRNA levels from $8.9x10^3$ ($\pm4.4x10^3$) copy number per $2.5x10^5$ cells to $1.6x10^5$ ($\pm6.2x10^4$, $p<0.05$) at MOI 0.25 and to $5.6x10^5$ ($\pm2.1x10^5$, $p<0.05$) at MOI 1, IL-28 from $1.3x10^1$ ($\pm1.1x10^1$) to $1.1x10^5$ ($\pm4.3x10^4$, $p<0.05$) at MOI 0.25 and to $5.6x10^5$ ($\pm3.0x10^5$, $p<0.05$) at MOI 1 and IL-29 mRNA from $1.6x10^1$ ($\pm0.6x10^0$) to $3.2x10^3$ ($\pm6.9x10^2$, $p<0.05$) at MOI 0.25 and to $5.2x10^6$ ($\pm3.3x10^4$, $p<0.05$) at MOI 1 24h post infection. ISG mRNA levels were also significantly increased by RV1B 24h post infection, Viperin was increased from $2.5x10^1$ ($\pm2.3x10^1$) copy number per $2.5x10^5$ cells to $9.2x10^5$ ($\pm4.4x10^5$, $p<0.05$) at MOI 0.25 and to $1.2x10^6$ ($\pm3.9x10^5$, $p<0.05$) at MOI 1, MxA from $1.1x10^4$ ($\pm1.4x10^3$) to $3.5x10^6$ ($\pm1.4x10^6$, $p<0.05$) at MOI 0.25 and to $3.7x10^6$ ($\pm1.3x10^6$, $p<0.05$) at MOI 1, OAS from $1.8x10^5$ ($\pm4.4x10^4$) to $4.3x10^6$ ($\pm1.3x10^6$, $p<0.01$) at MOI 0.25 and $4.0x10^6$ ($\pm1.2x10^6$, $p<0.01$) at MOI 1, RIG-I from $2.0x10^5$ ($\pm4.7x10^4$) to $1.5x10^6$ ($\pm4.7x10^5$, $p<0.05$) at MOI 0.25 and $3.2x10^6$ ($\pm1.1x10^6$, $p<0.05$) at MOI 1 and MDA5 from $3.2\ x10^4$ ($\pm6.7\ x10^3$) to $2.5x10^5$ ($\pm8.0x10^4$, $p<0.05$) at MOI 0.25 and $2.8x10^5$ ($\pm6.8x10^4$, $p<0.01$) at MOI 1.

AM increases RV1B induced type I and type III IFN mRNA expression

RV1B (MOI 1 and 0.25, Fig. 1) induced type I and III IFN and the effects of AM, EM and Tel pre-treatment on this were determined using real-time PCR. RV1B infection (MOI 1 and 0.25, respectively Fig. 1A,B,C and Fig. 1D,E,F) increased type I and III IFN mRNA expression in HBECs 24h post infection. RV1B (MOI 1) induced IFN-β (Fig.1A), IL-28 (Fig.1B) and IL-29 (Fig.1C) mRNA expression was non-significantly increased by 10μM and 50μM AM compared to RV1B infected cells. RV1B (MOI 0.25) induced IFN-β (Fig.1D), IL-28 (Fig.1E) and IL-29 (Fig.1F) mRNA expression and this was non-significantly increased at 10μM AM and significantly increased at 50μM AM (p<0.05) compared to control (RV1B infected cells). The effects of EM and Tel on RV induced type I and III IFN mRNA expression were also investigated, but neither macrolide had any significant effect on RV1B induced IFN or ISGs (data not shown).

AM increases RV1B induced ISG mRNA and protein expression

RV1B (MOI 1 and 0.25, respectively Fig. 2 and Fig. 3) induced ISG mRNA and the effects of AM, EM and Tel pre-treatment on this were determined using real-time PCR. RV1B (MOI 1) induced ISG mRNA expression was increased by AM at 10μM and 50μM at 24h post infection, RIG-I (Fig.2A), OAS (Fig.2C), Viperin (Fig.2D) and MxA (Fig.2E) mRNA expression were non-significantly increased by 10μM and significantly increased at 50μM AM (respectively p<0.01, p<0.001, p<0.01 and p<0.05) compared to control (RV1B MOI 1). MDA5 mRNA expression was non-significantly increased at 10μM and 50μM AM compared to control (RV1B MOI 1, Fig.2B). To confirm the effects of AM on ISG mRNA, using western blotting, RV1B (MOI 1, Fig. 4) induced ISG protein and the effects of AM on this were determined. RV1B induced Viperin, RIG-I, OAS and MxA protein were further increased by AM

in a dose dependent manner. AM did not induce any of these ISGs when administered alone (Fig. 2F).The cytoskeletal protein α-tubulin was used as load control. RV1B (MOI 0.25), induced ISG mRNA expression was increased by AM at 10μM and 50μM at 24h post infection, RIG-I (Fig.3A), OAS (Fig.3C), Viperin (Fig.3D) and MxA (Fig.3E) mRNA expression were non-significantly increased by 10μM and significantly increased at 50μM AM ($p<0.01$, $p<0.001$ and $p<0.01$ respectively) compared to control (RV1B MOI 0.25). MDA5 mRNA expression was non-significantly increased at 10μM AM and 50μM AM compared to control (RV1B, Fig.3B). The effects of EM and Tel on RV induced ISG mRNA expression were also investigated, but neither macrolide had any significant effect (data not shown).

AM increases RV16 induced ISG mRNA and protein expression

HBECs were also infected with major group RV16 following pre-treatment with AM. Fig 4 shows that RV16 induced IFN-β (Fig 4A), MxA (Fig 4B) and Viperin (Fig 4C) was enhanced by 10μM AM and significantly augmented with 50μM AM ($p<0.05$, $p<0.05$, $p<0.01$ versus RV16 control, respectively). To confirm the effects of AM on ISG induction, RV16 induced ISG protein by western blot and the effects of AM on this were determined. RV16 induced Viperin, and MxA protein were further increased by AM in a dose dependent manner. AM did not induce any of these ISGs when administered alone. The cytoskeletal protein α-tubulin was used as load control. (Fig. 4D).

AM has no significant effects on RV1B induced pro-inflammatory cytokines

The effects of AM, EM and Tel on RV1B induced IL-6 and IL-8 mRNA levels were determined by real-time PCR and RV1B induced IL-8 and IL-6 protein levels

determined by ELISA (Fig.5) AM reduced IL-6 and IL-8 mRNA expression and protein levels modestly, but not statistically significant at either concentration at 24h post infection (Fig.5). The effects of EM and Tel on RV1B induced IL-8 and IL-6 mRNA were also investigated, but both macrolides did not have any significant effect (data not shown).

AM suppresses RV replication and release

The effects of AM, EM and Tel on RV1B and RV16 release and RNA was measured using a virus titration assay and quantitative PCR for viral RNA. RV1B RNA (Fig. 6A) at 24h and 48h post infection was non-significantly reduced by 10μM AM and significantly reduced by 50μM AM ($p<0.001$). At 48h post infection 10μM AM also significant reduced RV1B RNA abundance ($p<0.001$). Furthermore, the reduction by 50μM AM was significantly more than by 10μM AM ($p<0.001$).RV1B release (Fig. 6B) was significantly reduced by 50μM AM at 24h ($p<0.05$) and 48h ($p<0.001$). AM at 10μM had no significant effect on RV1B release. RV16 RNA abundance was significantly decreased by 50 μM AM at 24h ($p<0.01$, Fig 6C). RV16 RNA at 48h was also decreased by AM, significantly so 50μM AM ($p<0.001$, Fig 6C). RV16 release was not affected at 24h post infection however was significantly affected using AM at 50μM ($p<0.05$, Fig 6D). The effects of EM and Tel on RV1B release and replication were also investigated, but both macrolides did not have any significant effect on RV replication (data not shown).

Discussion

The current treatment of asthma consists of corticosteroids and/or β_2-agonists, and only partially prevents asthma exacerbations. A vital need exists for new treatment regimes for this form of asthma. Macrolide antibiotics have anti-inflammatory activity and possibly anti-viral activity in airway epithelial cell lines [24,26,27], although the exact a mechanism of this is unclear. In our study, AM, but not EM and Tel, increased RV induced IFN and ISG responses and reduced RV replication and release in HBECs. The macrolides had no anti-inflammatory effect.

Several macrolide antibiotics have anti-inflammatory effects, demonstrated by inhibition of the production of pro-inflammatory cytokines *in vitro* [22,24-28,32]. This effects occurs via suppression of NF-κB activation in human bronchial epithelial cells [22,24,32]. Reduction of cytokines such as IL-6 and IL-8 can be an advantageous mechanism that leads to attenuation of airway inflammation. Other studies have reported anti-inflammatory activity of Clarithromycin, Bafilomycin A1 and EM on major group RV infection, and minor group RV infection for EM, in airway epithelial cells showing reduction of RV induced pro-inflammatory cytokines [24,26,27]. In the present study we showed no significant reduction of RV1B induced IL-6 and IL-8 mRNA and protein by AM, EM or Tel. This data questions the anti-inflammatory potential of macrolides and the mechanism of this in bronchial epithelial cells.

The conflicting results observed in studies with various macrolides may be explained by differences in experimental model. In the present study, using primary bronchial epithelial cells, we used shorter incubation times with the macrolides and sampled at

24 to 48h post RV infection, as primary bronchial epithelial cells are more susceptible to RV infection than cell lines, requiring a shorter duration of experimentation. The reduction of RV induced anti-inflammatory cytokines previously observed by macrolides is also modest, at best. The reduction of pro-inflammatory molecules by macrolides in micromolar concentrations range from 80-25% [24,26,27]. In contrast, corticosteroids reduce RV induced pro-inflammatory cytokine production by 70-85% in bronchial epithelial cells using nanomolar concentrations [33]. Therefore the anti-inflammatory effects observed with macrolides are modest if compared to the anti-inflammatory effects of corticosteroids, and require much higher doses, questioning their real use as anti-inflammatory agents.

A possible mechanism of anti-RV activity of macrolides has been demonstrated as reduction of ICAM-1, the receptor of major group RVs. However the anti-rhinoviral effects of macrolides can not be completely explained by this. A range of studies have suggested that macrolides inhibit replication of both major and minor groups of RV [24,26,27]. Furthermore, a study by Suzuki et al observed anti-viral effects of EM on minor group RV2, but no reduction in the LDL receptor, the receptor for minor group RV [27]. Our study further supports that the anti-viral effects of macrolides are not completely dependent on reduction of ICAM-1, as our results show suppression of viral replication with minor group RV1B and major group RV16, which therefore can not be explained by reduction of the major group RV receptor, ICAM-1.

In our studies, AM reduced RV1B and RV16 replication and release into supernatants, and increased ISG gene and protein expression. The ISGs studied have been shown to have anti-viral activity. Recently Viperin, one of the RV induced ISGs

increased by AM, is required for the host defence against RV infection in human bronchial epithelial cells [34]. The anti-viral role for the other ISGs investigated in the present study have not been yet established to have rhinoviral activity in bronchial epithelial cells, yet are well established in other viral infections [35,36].

AM also induced the mRNA and protein of the important intracellular dsRNA and ssRNA pattern recognition molecules RIG-I and MDA5, required for IFN-β induction [18]. Hence not only are macrolides responsible for direct induction of anti-viral genes, but they also upregulate the sensors of viral infection. This may be desirable for use as a therapeutic treatment given shortly after viral infection, whereby macrolides if given topically to the airway, during viral infection, could potentially increase the number of sensory molecules ready to detect and respond to replicating viral nucleic acid.

We found that the macrolides did not induce anti-viral genes by themselves. Currently we do not understand the mechanism of how AM induces anti-viral activity in the presence of RV infection. Anti-viral ISGs can be induced via IRF transcription factors following virus infection [37]. One possible mechanism of AM could be activation of signalling pathways including IκB-kinase (IKK)-β [38], IKK-ι/ε [37] and TBK-1 [39], producing IRF activation. Alternatively, macrolides may act via the type I IFN receptor complex (IFNAR1), inducing IRF9, IRF7 and STAT1/2, producing the transcription factor ISGF3 [40,41]. AM could in theory act on the IFNAR1 complex, or the receptor complex for IL-29 and IL-28 [42].

In vivo studies of macrolides have shown contradictory results. Clarythromycin had no effect on the development of cold symptoms and nasal inflammation In contrast, the TELICAST study demonstrated the potential benefits of the macrolide antibiotic Tel in the treatment of asthma exacerbations [29]. Whether the success of the TELICAST study was in part due to anti-viral activity is unknown. Interestingly, we found no anti-viral activity of Tel *in vitro*, suggesting that the positive effect of Tel in asthma exacerbations is either unrelated to virus infection or through anti-viral properties in other cell types and different immune responses during infection. This data demonstrates that although studies in cell lines are important and useful in defining mechanisms, results need to be confirmed *in vivo*. A similar study examining the effects of AM as a treatment for asthma exacerbations would therefore be appealing.

In summary, we present a possible new mechanism of anti-rhinoviral activity of macrolides in human bronchial epithelial cells. This is the first study showing anti-viral effects of macrolides in primary human bronchial epithelial cells, which is the site of infection of RV *in vivo* and therefore important in rhinovirus induced asthma exacerbations [43,44]. While only modest, the upregulation of IFNs and ISGs and suppression of RV replication may have consequences on asthma exacerbations and may further explain the anti-viral effects seen in previous studies. The importance and protective role of IFN-β and IFN-λ in asthma exacerbations have recently been shown [12,13], and epidemiological studies have provided a model of the disease burden associated with RV induced asthma exacerbations. Whether the anti-viral effects of AM can be used as a therapeutic treatment for asthma exacerbations requires further study in appropriate animal and human models.

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Table 1: Primer and probe sequences 5'-3'

Gene	Forward primer	Reverse primer	Probe (5'-FAM, 3'-TAMRA)
18s rRNA	CGCCGCTAGAGGTGA AATTCT	CATTCTTGGCAAATG CTTTCG	ACCGGCGCAAGACG GACCAGA
IFN-β	CACGGATACAGAACC TATGG	ACGAACAGTGTCGCC TACTA	TCAGACAAGATTCAT CTAGCACTGGCTGGA
IL-28	CTGCCACATAGCCCA GTTCA	AGAAGCGACTCTTCT AAGGCATCTT	TCTCCACAGGAGCTG CAGGCCTTTA
IL-29	GGACGCCTTGGAAGA GTCACT	AGAAGCCTCAGGTCC CAATTC	AGTTGCAGCTCTCCT GTCTTCCCCG
RIG-I	CCAAGCCAAAGCAGT TTTCAA	CACARGGATTCCCCA GTCATG	TTGAAAAAAGAGCA AAGATATTCTGTGCC CGAC
MDA-5	GATTCAGGCACCATG GGAAGT	AGGCCTGAGCTGGAG TTCTG	GGGATGCTCTTGCTG CCA CAT TCT CTT
OAS	CTGACFCTGACCTGG TTGTCT	CCCCGGCGATTTAAC TGAT	CCTCAGTCCTCTCAC CACTTTTCA
MXA	CAGCACCTGATGGCC TATCAC	CATGAACTGGATGAT CAAAGG	AGGCCAGCAAGCGC ATCTCCAG
Viperin	CACAAAGAAGTGTCC TGCTTGGT	AAGCGCATATATTTC ATCCAGAATAAG	CCTGAATCTAACCAG AAGATGAAAGACTCC
IL-6	CCAGGAGCCCAGCTA TGA AC	CCCAGGGAGAAGGC A ACTG	CCTTCTCCACAAGCG CCTTCGGT
IL-8	CTGGCCGTGGCTCTC TTG	CCTTGGCAAAACTGC ACCTT	CAGCCTTCCTGATTT CTGCAGCTCTGTGT
RV	GTGAAGAGCCSCRTG TGCT	GCTSCAGGGTTAAGG TTAGCC	TGAGTCCTCCGGCCC CTGAATG

Figure Legends

Figure 1: AM increased minor group RV1B induced IFN gene expression. HBECs were pre-treated with AM or medium for 24h and infected with RV1B MOI 1 (A,B.C) or MOI 0.25 (D,E,F), or treated with medium. AM and RV1B induced mRNA levels of IFN-β (A,D), IL-28 (B,E) and IL-29 (C,F), measured by real time PCR at 24h post infection compared to control (RV1B infected cells, *n*=5).

Fig 1

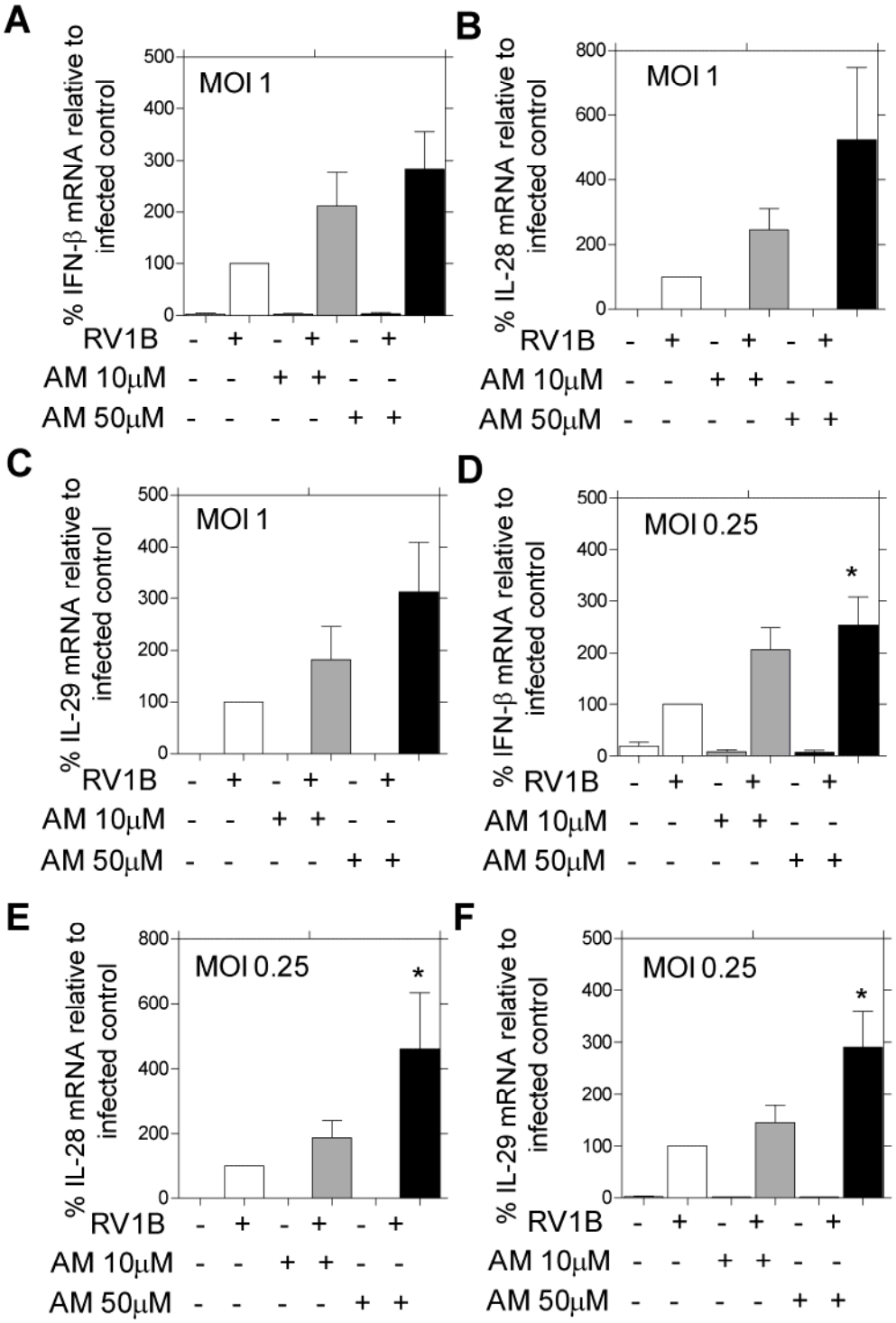
A
% IFN-β mRNA relative to infected control
MOI 1
RV1B
AM 10μM
AM 50μM
B
% IL-28 mRNA relative to infected control
MOI 1
C
% IL-29 mRNA relative to infected control
MOI 1
D
% IFN-β mRNA relative to infected control
MOI 0.25
E
% IL-28 mRNA relative to infected control
MOI 0.25
F
% IL-29 mRNA relative to infected control
MOI 0.25

Figure 2: AM increased high dose RV1B induced ISGs. HBECs were pre-treated with AM or medium for 24h and infected with RV1B MOI 1 or treated with medium. AM and RV1B induced mRNA levels of RIG-I (A), MDA5 (B), OAS (C), Viperin (D) and MxA (E), measured by real time PCR at 24h post infection compared to control (RV1B infected cells, *n*=5). Both 10 and 50μM AM increased RV1B induced ISG protein in HBECs compared with RV1B infected cells (the above figure is representative of 3 independent experiments, F).

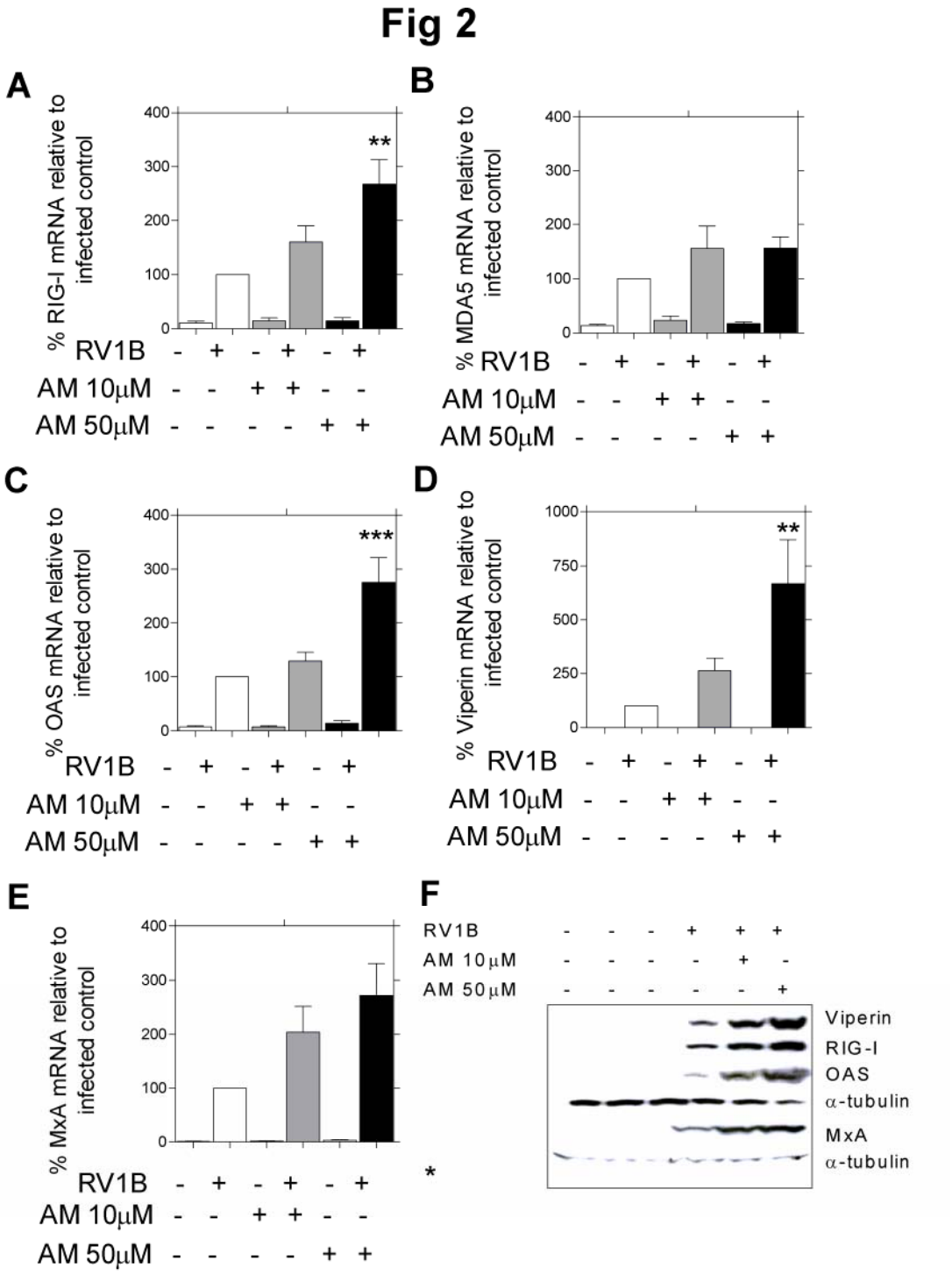

Figure 3: AM increased low dose RV1B induced ISGs. HBECs were pre-treated with AM or medium for 24h and infected with RV1B MOI 0.25 or treated with

medium. AM and RV1B induced mRNA levels of RIG-I (A), MDA5 (B), OAS (C), Viperin (D) and MxA (E), measured by real time PCR at 24h post infection (*n*=5).

Fig 3

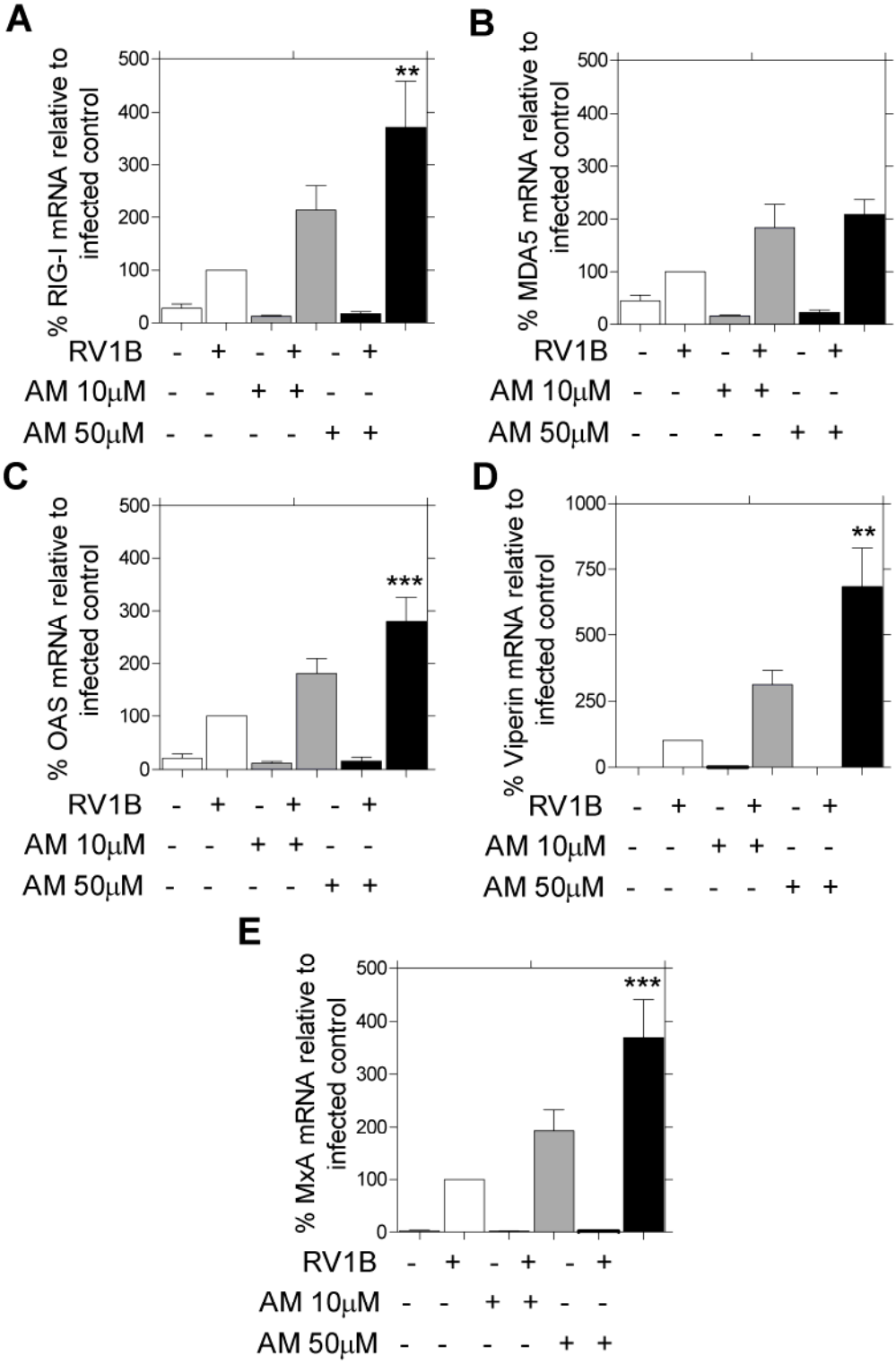

Figure 4: **AM increased major group RV16 induced ISGs** AM HBECs were pre-treated with AM or medium for 24h and infected with RV16 MOI 1 or treated with medium. AM and RV1B induced mRNA levels of IFN-β (A), MxA (B) and Viperin (C) measured by real time PCR at 24h post infection (*n*=5). Both 10 and 50μM AM increased RV16 induced ISG protein in HBECs compared with RV16 infected cells (the above figure is representative of 3 experiments, F).

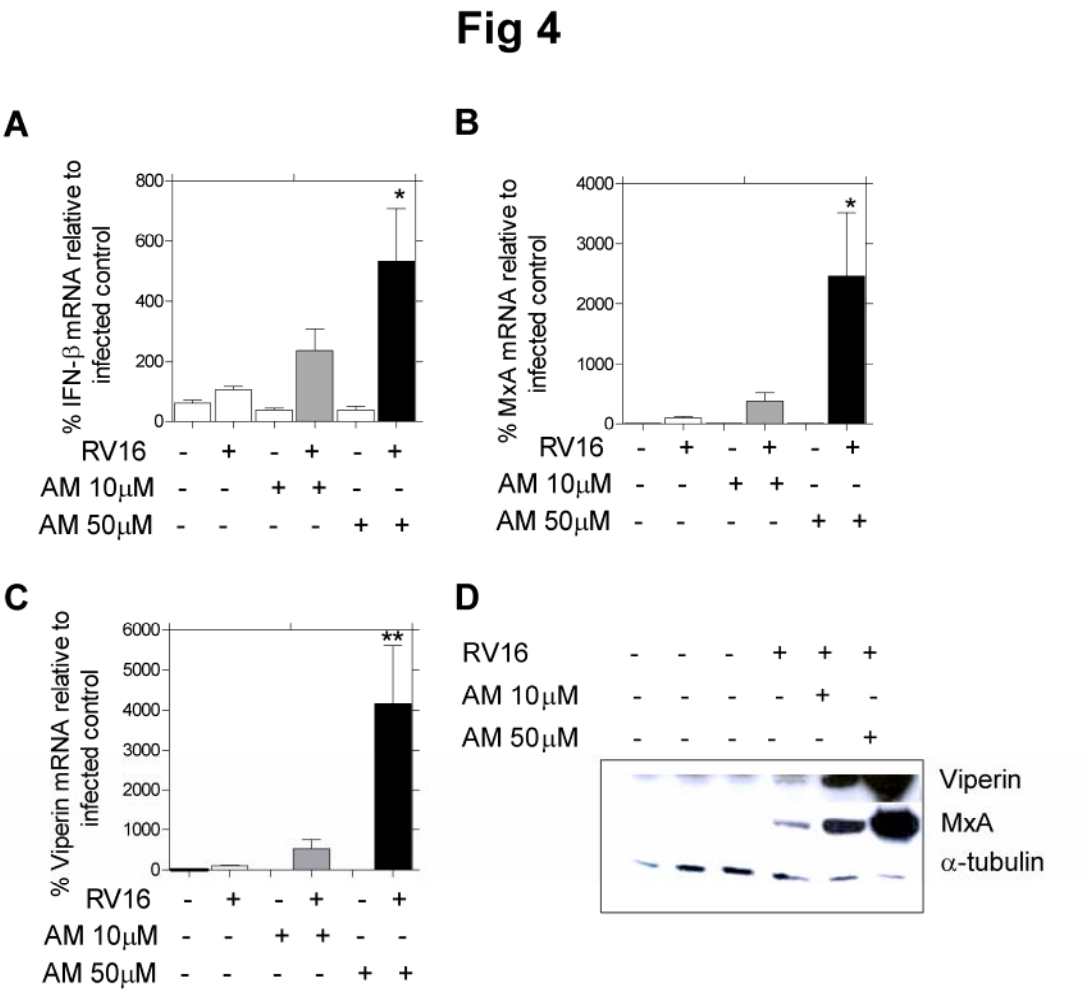

Figure 5: AM had no effect on RV induced pro-inflammatory cytokines. HBECs were pre-treated with AM or medium for 24h and infected with RV1B MOI 1 or treated with medium. AM and RV1B at either 10 or 50μM did not significantly

increase RV1B induced IL-8 mRNA (A), IL-6 mRNA (B), as measured by real time PCR at 24h post infection or IL-8 protein (C) or IL-6 protein (D) as measured by ELISA at 24h post infection compared to control (RV1B infected cells, *n*=5).

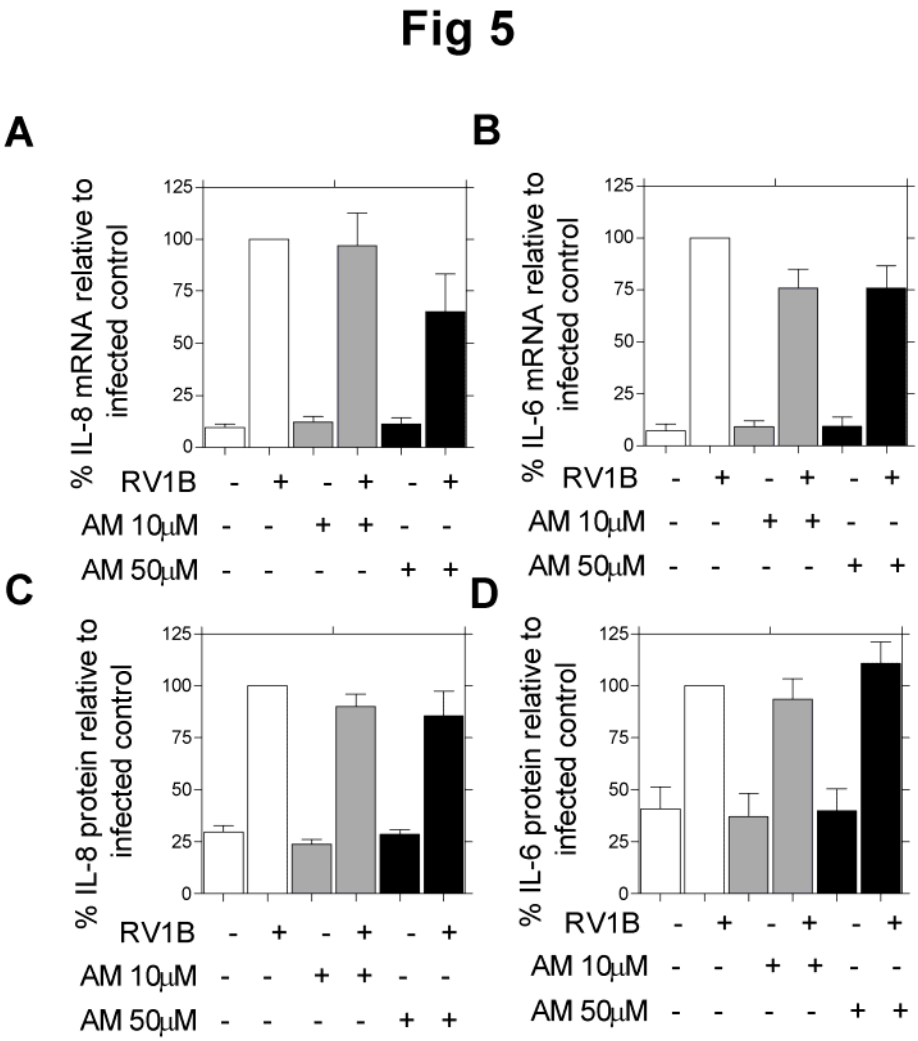

Figure 6: AM reduced RV1B and RV16 replication. HBECs were pre-treated with AM or medium for 24h and infected with RV1B or RV16 MOI 1. At 50μM, AM decreased RV1B RNA at 24h, and also at 10 and 50μM at 48h post infection, as assessed by real time PCR (A). AM at 50μM reduced RV1B release at 24h and 48h (B) (*n*=8). At 50μM, AM decreased RV16 RNA at 24h, and also at 50μM at 48h post

infection, as assessed by real time PCR (C). AM at 50μM reduced RV16 release at 48h (D, *n*=5).

Fig 6