Resveratrol inhibits the TRIF dependent pathway by up-regulating SARM, contributing to anti-inflammatory effects after respiratory syncytial virus infection.

Running title: SARM regulated IFN-γ after RSV infection

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Abstract

Respiratory syncytial virus (RSV) is the most important cause of lower respiratory tract infection in young children, and the leading cause of infant hospitalization worldwide. Uncontrolled response to RSV is mediated by a toll-like receptor (TLR)-mediated immune response. Resveratrol possesses anti-RSV activity and is an inhibitor of the TRIF/TBK1/IRF-3 complex. We hypothesize that resveratrol inhibits the TRIF-dependent pathway through up-regulation of SARM post-RSV infection. BALB/c mice were infected with RSV and were injected with resveratrol one hour post-inoculation. SARM siRNA was administrated to RSV-infected and resveratrol-treated mice. Lung function was measured by whole-body plethysmography, lung histopathology was examined, and lymphocytes in bronchoalveolar lavage fluid were quantified. SARM and TRIF protein expression were detected in lung by western blot analyses. The expression of interferon-γ in BALF was evaluated by ELISA. SARM expression was reduced and...
TRIF expression was increased after infection with RSV. Resveratrol increased SARM expression and decreased TRIF expression after RSV infection. SARM knockdown in resveratrol-treated mice enhanced interferon-γ production, RSV-induced airway inflammation, and AHR. Resveratrol decreased TRIF expression and prevented the RSV-mediated reduction of SARM expression.

Resveratrol-mediated inhibition of the TRIF-dependent pathway may be dependent on SARM expression.

**Importance**

Our study provides insights into the regulation of innate immunity in response to RSV infection. The results suggest that resveratrol-mediated alterations in SARM may have therapeutic potential against RSV immunopathology caused by deregulation of the TLR-mediated immune response. Ultimately, improved insight into the complex interplay between TLR adaptor proteins and the occurrence of severe RSV infection might lead to novel therapeutic treatment strategies such as TLR adjuvants.

**Introduction**

Respiratory syncytial virus (RSV) is the most important cause of viral bronchiolitis in infants and young children and the leading cause of infants hospitalization worldwide (1). Nearly all children have been infected with RSV before they reach 2 years of age (2). Surprisingly, infection at a young age does not provide life-long
protection. Immunocompromised children, and the elderly, are also at high risk of developing RSV-associated disease (3, 4). According to WHO, RSV causes 64 million infections annually and approximately 160,000 deaths per year (5). Altogether, RSV causes significant economic burden. Unfortunately there is no RSV vaccine or effective anti-viral drug available today.

Toll-like receptors (TLRs) recognize specific structural motifs expressed by microbes that are defined as pathogen-associated molecular patterns (PAMPs). They are type-1 transmembrane proteins composed of an extracellular leucine-rich (LR) domain and a cytoplasmic tail that contains a conserved TIR domain (6). Viral PAMPs bind to TLRs causing the activation of transcription factors (7). This leads to the induction of cytokines, chemokines, and interferons that create an antiviral state and mature the adaptive immune response (8).

To date, five intracellular TLR adaptor proteins containing a TIR domain have been identified: myeloid differential primary response protein (MyD88), MyD88-adaptor-like (Mal or TIRAP), TIR domain-containing adaptor inducing interferon-β (TRIF or TICAM-1), TRIF-related adaptor molecule (TRAM or TICAM-2), and sterile-alpha and Armadillo motif protein (SARM). SARM was the last of the adaptor proteins to be discovered. It contains sterile-α (SAM) and HEAT/armadillo (ARM) motifs (9, 10) and is highly conserved from arthropod to human. SARM is the most conserved TIR domain-containing protein (11). In Caenorhabditis elegans, TIR-1, a SARM homologue, plays a fundamental role in host
defense against bacterial and fungal infections. Human SARM functions as a negative regulator of the TRIF-dependent pathways in innate immunity (12, 13). It has been shown to inhibit both TRIF- and MyD88-mediated activation of the transcription factor AP-1 (14). SARM contributes to the initiation, elongation, and maintenance of dendritic arbors, and influences axonal death and neuronal polarization (15-17). Recent studies have shown that SARM directly binds to mitochondria (18) and induces apoptosis in T cells (19). However, whether human SARM has an anti-viral role has not yet been elucidated.

Resveratrol (trans-3,5,4-trihydroxystilbene), one of the nonflavonoid polyphenolic phytoalexins found in grapes and red wines (20), is a potent inhibitor of TRIF dependent signaling (21, 22). Resveratrol has been shown to prevent cancer (23), cardiovascular disease (24) and ischemic injuries (25), and to possess anti-RSV activity (26, 27). It also protects against airway remodeling and airway hyper-reactivity in asthma (28). Resveratrol can reduce RSV titers in the lung, the number of infiltrating lymphocytes present in bronchoalveolar lavage fluid (BALF), and inflammation (21, 22). It reduces airway inflammation following RSV infection, significantly decreases interferon-γ (27) and down-regulates interferon-γ-inducible inflammatory genes in macrophages (29).

Previously, we reported that RSV infection induced TLR3 and activated TRIF dependent signaling, which was associated with the induction of interferon-γ (27); Interferon-γ from Th1 cells is required for the induction of severe airway
Furthermore, we demonstrated that resveratrol inhibits TLR3 signaling, M2R expression, and interferon-γ production.

Taken together, these data indicate that RSV functions as a trigger to activate innate immune responses, and that the anti-inflammatory function of resveratrol involves TLR associated signaling. However, several interesting issues remain to be determined, including whether resveratrol inhibits the TRIF dependent pathway through the up-regulation of SARM after RSV infection.
Materials and Methods

Cell line and cell culture condition

The 9HTEo cell line was provided gratis from Hans D. Ochs (University of Washington School of Medicine, Seattle, Washington, USA). 9HTEo cells and HEp2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM: GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 U/mL penicillin (Invitrogen, Carlsbad, CA), and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA) at 37°C under 5% CO₂.

Virus preparation and titration

The RSV viral stock (A2-strain) was obtained from the viral laboratory at Beijing Children’s Hospital (Capital University of Medical Sciences, Beijing, China), and was grown in HEp2 cells as elsewhere described (31). RSV A2 was treated with UV light irradiation at 9×10⁴ μJ/cm² for 30 minutes using a UV cross-linker (Thermo, USA). Titers of viable virus were determined by plaque assay (32). A master stock and working stock of RSV were prepared as described previously (33).

Infection of 9HTEo cell

An overnight culture 9HTEo cells in a 6-well plate was infected with RSV at a multiplicity of infection (MOI) of 10 for 2 hours (h). To remove extracellular RSV, the cells were washed twice with 1 ml of PBS. The infection was allowed to continue for 12h, 24h, 36h, 48h, or 72h at 37°C under 5% CO₂. Cells were collected for analysis at each time point.
Animals

6-8 week old female BALB/c mice, free of specific pathogens, were purchased from Chongqing Medical University Animal Laboratory and housed in individual filtered cages. Cages, bedding, food, and water were sterilized before use. Room temperature was maintained at 23°C, and provided a 12-h on / 12-h off light cycle. All animal handling procedures were performed under clean-bench policy conditions. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Chongqing Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Chongqing Medical University (Permit Number: SYXK-(YU) 2012-0001). All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering. Experiments were performed three times using three mice per group unless otherwise noted.

Experimental design and sample collection

Mice were infected intranasally with $4.5 \times 10^7$ PFU of RSV in a 100μL volume. Mock-infected mice were inoculated intranasally with the same amount of HEp-2 cell culture supernatant in parallel. One hour post inoculation, mice were injected intraperitoneally with either resveratrol (Sigma-Aldrich Corp., St. Louis, MO) or placebo (PBS, phosphate buffered saline) as previously described (27). The lung function of the mice was measured at serial time points after infection (days 3, 5, 7) prior to sacrificing the animal. The lungs were removed at each time point for
detection of virus, protein extraction, and histopathological analysis. BALF was collected at 5 days after infection to determine the total number of cells, cell phenotypes and cytokine expression.

Western blot analysis

Total protein extracts from lung tissues or cells were obtained using a total protein extraction kit (KeyGEN, Nanjing, China). The protein concentration was determined using BCA assay reagent (Biotek) according to the manufacturer’s protocol. Equal amounts of the isolated proteins from lung or cell extracts were separated on an 8% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were probed with primary antibodies against SARM (1:500; SANTA, USA), TRIF (1:500; Abcam, Cambridge, MA) or β-actin (1:5,000; cwbiotech, Beijing, China). Alkalinephosphatase-conjugated goat anti-rabbit secondary antibody (1:10,000; MultiSciences, China) and goat anti-mouse antibody (1:10,000; MultiSciences, China) were used to detect the presence of the respective protein bands. Signals were quantified by use of Quantity One software (Bio-Rad, Hercules, CA) and normalized relative to β-actin.

Total cells differential cell count in BALF

To obtain the BALF, the trachea of each mouse was cannulated to access the lungs. The lungs were washed three times with 0.5 ml ice-cold PBS. For total cell counts, BALF cells were pelleted by centrifugation at 1,000 × g for 5 minutes at 4 °C.
The cells were resuspended in 1 ml of PBS and counted with a hemocytometer. The BALF was centrifuged and supernatants were collected and stored at −80 °C to test for cytokine production. The cells were pelleted to cytospin slides, air dried and used to quantify lymphocytes in the BALF. Cytospin slides were fixed and stained with DiffQuik (Baxter Healthcare Corp, Deerfield, Miami, FL) for leucocyte differential analysis. The number of monocytes, lymphocytes, neutrophils and eosinophils in at least 200 cells per slide were counted. These experiments were performed three times with three mice per group.

Lung histopathology

The lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h and then embedded in paraffin. The blocks were cut into 5μM thick sections and stained with H & E solution (hematoxylin, Sigma MHS-16; and eosin, Sigma HT110-1-32). Tissue was subsequently mounted and coverslipped using Dako-mounting medium (Dakocytomation, Denmark, CA). The degree of airway inflammatory cell infiltration was scored in double-blind screening by two independent investigators as described previously (34).

Pulmonary function tests

Five days post RSV infection AHR was assessed in conscious and unrestrained mice by means of whole-body plethysmography (Emca instrument; France). Each mouse was placed in a plastic chamber and exposed to aerosolized PBS, which was followed by increasing concentrations of aerosolized methacholine solutions (3.125,
Bronchoconstriction was recorded for 5 minutes after each dose of methacholine. The highest Penh value (airway resistance) obtained during each methacholine challenge was expressed as a proportion of the basal Penh value seen in response to PBS challenge.

Preparation and administration of in vivo siRNA

SARM siRNA and negative control siRNA tagged internally with GFP were purchased from Invitrogen (Shanghai, CHINA). The siRNA sequences were:

SARM:237868(3-1):

sense: TGCTTGAAAGAAGCGCAGACTGCTGACAC
antisense: CCTGTCAAGCAGGCTTCTTCA;

237868 (Negative control):

sense: tgctgAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT; and
antisense: cctgAAATGTACTGCGGAGACGAGTCAGTCAGTGCCAAAACGTCTCCACGCAGTACATTTc.

The siRNA was dissolved in a solution of 5% glucose and in vivo jetPEI (Polyplus Transfection, New York, NY, USA) to an N/P ratio of 7 (number of nitrogen residues of jetPEI per RNA phosphate), according to the manufacturer’s instructions.
A total of 80µl of siRNA-jetPEI complex was administrated intranasally to RSV infected and resveratrol treated mice as previously described (35). The siRNA knockdown experiments were performed at least three times with five mice.

**Confocal analysis**

Lungs from wild type control or transgenic mice expressing GFP fluorescence were freshly excised and frozen sectioned. Airway sections were stained with DAPI (Beyotime, China) for nuclear stain. Confocal images were acquired using a confocal microscope (Nikon, A1R, Japan). These experiments were performed three times with five mice.

**Cytokines level in BALF were measured by ELISA**

The level of interferon-γ contained in BALF was measured using a specific mouse interferon-γ ELISA kit (Sizhengbai, Beijing, China). ELISAs were performed per the manufacturer’s specifications.

**Q-PCR**

Lungs were harvested five days post RSV infection. The RSV A N gene-specific primers and probe were:

- **RSV-F:** 5’-AGATCAACTTCTGTCATCCAGCAA-3’; RSV-R:
  5’-TTCTGCACATCATAATTAGGAGTATCAAT-3’;
- **RSV-P:** FAM-5’-CACCATCCAACGGAGCACAGGAGAT-3’ -BHQ1(36). The plasmid amplified target fragment was cloned into the pMD19-T vector (TaKaRa Biotechnology, Dalian, China) and verified by sequencing. The Real-Time PCR
instrument (Applied Biosystems) used the following conditions: one cycle at 50°C for
2 min, one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and one cycle at
60°C for 1 min. RSV load values were expressed as log_{10} copy number of
RSV-RNA/ml. RSV subtype A plasmid was the positive control. Negative controls
and serial dilutions of positive controls were included in every PCR assay.

Statistical analysis

Statistical analyses were performed with a two-way ANOVA or a Student t test
between all groups using Prism GraphPad Software (La Jolla, CA). p<0.05 was
considered to be significant in the present experiments.
Results

Live RSV but not UV-inactivated RSV reduces SARM expression

To investigate SARM expression in 9HTEo cells at the protein level, we infected 9HTEo cells with RSV at MOI of 10. After 12 h, 24 h, 36 h, 48 h or 72 h of infection, the cells were harvested and the expression of SARM protein was determined by immunoblotting. RSV was able to suppress SARM expression within 36 h of infection. SARM expression levels continued to gradually decrease with time (Figure 1 A1, A2). In contrast, TRIF expression was increased in a time dependent manner (Figure 1 A1, A3).

To determine whether RSV inhibited SARM expression in vivo BALB/c mice were infected intranasally with live RSV or UV-inactivated RSV. Plaque assay confirmed that the mice were infected by the RSV-challenge. Similar to results in the 9HTEo cell line, RSV reduced SARM expression 3 days after infection (Figure 1 B1, B2). The levels of SARM remained low, but the levels of TRIF increased at day 5 and day 7 post infection, (Figure 1 B1, B2). Unlike live virus, UV-inactivated RSV was not able to reduce SARM expression in a time-dependent manner in vivo (Figure 1 C1, C2).

SARM is a functional inhibitor of RSV-mediated activation of TRIF-dependent pathway induced by resveratrol

Resveratrol, and SARM, are inhibitors of TRIF and MyD88-independent pathways (21). To investigate the interplay between resveratrol and SARM expression...
after RSV infection, BALB/c mice were treated with resveratrol for 1h post RSV infection. RSV increased TRIF expression by western blot while at the same time infection reduced SARM expression. In contrast, in mice treated with resveratrol after RSV infection, TRIF expression was inhibited and SARM expression increased. These results indicated that resveratrol mediated inhibition of the TRIF-dependent pathway might rely on SARM expression (Figure 1D1-3).

Resveratrol reduces airway inflammation and AHR by up-regulating SARM expression

We have previously established that IFN-γ causes severe airway inflammation in a model of RSV infection in immunocompromised mice (27). To understand whether the airway inflammation we observed was SARM mediated mice were treated with cyclophosphamide (CYP) as described previously (37). Briefly, CYP was administered in a single dose of 100 mg/kg of body weight, and 5 days later, mice were intranasally infected with RSV. SARM expression was elevated in the lungs of BALB/c mice treated with resveratrol after RSV infection compared to infection with RSV alone. Small interfering (siRNA) was used to knock down SARM mRNA level and reduce the protein expression level in resveratrol treated mice. To test whether transgenic SARM siRNA-GFP was able to function in lungs of treated mice, confocal microscopy was used to confirm the proper insertion and function of siRNA-GFP (Figure 2A). The SARM siRNA construct effectively suppressed the level of SARM protein expression in the lungs of RSV infected BALB/c mice treated with resveratrol.
and the negative siRNA vector had no effect. However, following SARM knock down
TRIF expression was increased and the TRIF dependent pathway was induced (Figure
2 B1-3).

Mice infected with RSV had severe airway inflammation compared with
uninfected control mice. However, treatment with resveratrol reduced inflammation in
mice infected with RSV. The effect of resveratrol was reduced when SARM
expression was knocked down using siRNA. Resveratrol treated mice with SARM
knocked down had similar histologic findings as mice inoculated with RSV in the
absence of resveratrol (Figure 3A1-3).

RSV infected mice had significantly more cells in the BALF than uninfected mice.
However, treatment with resveratrol reduced the cellularity of the BALF (Figure 3B).
SARM knockdown was sufficient to significantly increase the total number of cells
present in the BALF of resveratrol treated mice compared to the negative control
siRNA treated group. There were qualitative differences in cell types observed
between treatment groups. The group treated with resveratrol and siRNA 3-1-treated
RSV-infected mice had significantly increased lymphocyte numbers (P<0.05)
compared to resveratrol and siRNA negative-treated RSV-infected mice (Figure 3B).

Aerosolized methacholine elicited significantly increased AHR in mice infected
with live RSV compared with all other groups. 5 days after inoculation, mice infected
with live RSV had significantly greater AHR than uninfected controls at methacholine
concentrations of between 12.5 and 50.0 mg/ml. Treatment with resveratrol
significantly reduced AHR caused by RSV. However, in mice with SARM knocked down after transfection, AHR was significantly higher than in mice transfected with the negative control siRNA vector and mice treated with resveratrol after RSV infection (Figure 3C).

Depletion of SARM may cause more severe airway inflammation. To investigate a possible mechanism interferon-γ expression level in the BALF was assessed using ELISA-based assays. RSV infection significantly increased the interferon-γ level in BALF compared to the uninfected control mice (Figure 3D). Resveratrol treatment showed some protection and significantly reduced the level of interferon-γ compared to the RSV group. However, SARM knockdown abrogated the protective effect of resveratrol treatment (Figure 3D). The level of interferon-γ was significantly increased in the SARM knockdown group compared to the negative control siRNA vector group (Figure 3D). RSV-infected mice and RES-treated SARM siRNA 3-1 transfected RSV-infected mice had a similar RSV viral titer (Figure 3E).
In the present study, we discovered that SARM expression was reduced after infection with live, but not inactivated, RSV in vivo and in vitro. Furthermore, we found that resveratrol was able to prevent the RSV-mediated reduction of SARM expression level. Resveratrol was also able to decrease the airway inflammation and AHR caused by RSV. The anti-inflammatory function of resveratrol involved TLR-TRIF associated signaling.

TLRs are expected to play an essential role in innate immune activity against RSV infection. The activation of cytokines, chemokines and IFNs in the host after binding viral PAMPs leads to an antiviral state and activates the adaptive immune response. Upon RSV infection, the signal initiated from TLR3 and TLR4 activates the adaptor protein TRIF (7, 27, 30). Specifically, the RSV fusion protein can bind to TLR4 which induces the production of IL-1β, IL-6, IL-8 and TNF-α (38). RSV is a negative-strand, nonsegmented RNA pneumovirus. Double stranded RNA replication intermediates formed during the RSV replication cycle activate TLR3 (39) and the RIG-like helicases RIG-I (40) and MDA-5 (41). Our previous studies showed that RSV replication activates TLR3 and activates a TRIF dependent signaling pathway (27, 42). Some reports have demonstrated that mice deficient in TLR4 showed an impaired ability to clear the virus (43). In contrast, Douville et al showed that responses to RSV infection are not dependent upon TLR4-mediated stimulation and associated with substantial innate immunity and robust Th1 activation (44).
Moreover, SARM is an inhibitor of TRIF-dependent signaling (13). Peng et al showed that SARM inhibits both TRIF- and MyD88-mediated AP-1 activation (14) thus indicating that SARM is a negative regulator in TLRs mediated innate immunity. The results presented here suggest that RSV suppressed SARM expression in vivo and in vitro. UV-inactivated RSV which cannot replicate in the host was not able to reduce the expression of SARM in vivo. This suggests that active viral replication is necessary to reduce SARM expression.

Resveratrol significantly reduces influenza virus replication by inhibiting protein kinase C (PKC) phosphorylation and its dependent pathways, JNK and p38 MAPK (45). Resveratrol also reduces the inflammation and AHR caused by enhanced interferon-γ after RSV infection (27). However, the mechanism underlying the link between resveratrol and interferon-γ remains unclear. Resveratrol exerts its broad-spectrum anti-inflammatory effects though inhibition of the TRIF/TBK1/IRF-3 complex (22). Furthermore, SARM acts as an specific inhibitor of TRIF dependent signaling (13). In this study, resveratrol acted as a promoter of SARM, and thus inhibited TRIF expression and reduced airway inflammation and AHR after RSV infection. Furthermore, SARM knockdown reversed the protective effects of resveratrol when SARM siRNA was transfected in the lungs of BALB/c mice.

The Caenorhabditis elegans SARM homologue, TIR-1, plays a crucial role for efficient immune responses against bacterial infections (12). SARM has been shown to restrict West Nile Virus infection and influence TNF-α production, microglia
activation and neuronal cell death (46). RSV infection activates TLRs and induces the production of inflammatory cytokines that direct the differentiation of naïve Th0 cells to Th1 or Th2-type CD4+ T-helper cells (47). This gives rise to Th1-type response (48). Importantly, TRIF-dependent signaling is associated with interferon-γ production (49). Recent reports suggest that interferon-γ produced by Th1 cells is required in the induction of severe AHR (30). During RSV infection, TRIF-dependent signaling was induced and the level of interferon-γ was elevated (27). Our results show that SARM interacts with TRIF and that once SARM expression is induced by resveratrol, the level of interferon-γ is reduced as well as airway inflammation and AHR. On the contrary, SARM knock down established an uncontrolled immune response against RSV in which TRIF expression was enhanced. It is important that a robust Th1-response was induced and the interferon-γ level was elevated which subsequently caused an exacerbation of airway inflammation and AHR.

In summary, these results suggest that resveratrol-mediated alterations in SARM may have therapeutic potential against RSV immunopathology caused by deregulation of the TLR-mediated immune response.

Acknowledgments

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References


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Figure Legends:

Figure 1. RSV reduced SARM expression in vivo and in vitro. Panel A: Cells were infected with RSV at a MOI of 10. A1: SARM and TRIF protein levels were detected by immunoblotting at the time points indicated. A2: RSV reduced the SARM/β-actin ratio compared to the control group; *p<0.05 48 h and 72 h after RSV infection vs. control. A3: RSV increased the TRIF/β-actin ratio compared to the control group; **p<0.01 48 h after RSV infection vs. control, *p<0.05 72 h after RSV infection vs. control. Panel B: BALB/c mice were infected by live RSV at 4.5×10⁷ pfu. The lungs of the infected mice were harvested at days 3, 5, and 7 after infection. B1: SARM and TRIF protein levels were detected by immunoblotting at the time points indicated B2: RSV reduced the SARM/β-actin ratio compared to the control group; ***p<0.0001 5 and 7 days after RSV infection vs. control. B3: RSV increased the TRIF/β-actin ratio compared to the control group; **p<0.01 5 and 7 days after RSV infection vs. control. Panel C: BALB/c mice were infected by UV-inactivated RSV. C1-C2: Days 3, 5, 7 after infection the lungs of the infected mice were harvested and the protein levels were detected by immunoblotting. Panel D: BALB/c mice were infected with live RSV (4.5×10⁷ pfu) and then treated
intraperitoneally with either resveratrol (RES) or placebo. D1: The lungs were harvested and the protein expression of TRIF and SARM was determined by immunoblotting. D2: RSV reduced SARM/β-actin ratio compared to the control group, ***p<0.0001 5 days after RSV infection vs. control; SARM/β-actin ratio was elevated in RES-treated RSV-infected mice compared to untreated RSV-infected mice. **p<0.001 RES-treated RSV-infected mice vs. RSV-infected mice. D3: RSV increased the TRIF/β-actin ratio compared to control, **p<0.01 5 days after RSV infection vs. control; TRIF/β-actin ratio was reduced in RES-treated RSV-infected mice compared to untreated RSV-infected mice. **p<0.01 RES-treated RSV-infected mice vs. RSV-infected mice. All experiments were performed three times with three mice per group.

Figure 2. SARM siRNA3-1-GFP or SARM siRNA negative-GFP organization and protein expression. Panel A: The SARM siRNA-GFP signal in freshly harvested lungs demonstrates polymer formation by SARM siRNA transgene, i.e., GFP in lung of transgenic mice compared to wild type control. Images show cross-sections of mouse lung proximal and distal airway sections from transgenic mice or wild type control stained with DAPI, for nuclear stain. Corresponding DIC/DAPI images are shown. WT images are shown as a control. Panel B: B1: Western blot was used to evaluate the SARM and TRIF expression in wild type control mice, RSV-infected mice and RSV-infected mice treated with resveratrol.
RES- (lanes 1-3). RSV-infected mice treated with RES were also either SARM siRNA-negative transfected mice (lane 4) or SARM siRNA-3-1 transfected mice (lane 5). B2: SARM siRNA reduced the SARM/β-actin ratio in the lungs of RES-treated RSV-infected mice, **p<0.001 RES-treated SARM siRNA 3-1 transfected RSV-infected mice vs. RES-treated siRNA negative transfected RSV-infected mice. B3: SARM siRNA increased the TRIF/β-actin ratio in the lungs of RES-treated RSV-infected mice, **p<0.01 RES-treated SARM siRNA 3-1 transfected RSV-infected mice vs. RES-treated siRNA negative transfected RSV-infected mice. These experiments were performed three times with three mice per group.

**Figure 3. SARM knockdown increased inflammation in RSV infected lungs.**

Panel A: A1: Histological examination of lung tissues was performed 5 days post-resveratrol treatment. The lung tissues were fixed and stained with H&E (magnifications, ×100). A2: Lung tissue inflammatory cell infiltration scores, ***p<0.001 resveratrol (RES)-treated SARM siRNA 3-1 transfected RSV-infected mice vs. RES-treated siRNA negative transfected RSV-infected mice. Panel B: Cells were isolated by cytospin and stained with DiffQuik. Cells were counted using a hemocytometer. Values are expressed as the means; error bars are SD (n=3/group). Total cells present in the BALF of the respective treatment groups ***= P<0.001 RSV-infected vs. RES-treated RSV-infected mice and RES-treated SARM siRNA
3-1 transfected RSV-infected mice vs. RES-treated siRNA negative transfected RSV-infected mice. Differences in cell types between treatment groups:

Lymphocytes: **= P<0.001 RSV-infected vs. RES-treated RSV-infected mice and RES-treated SARM siRNA 3-1 transfected RSV-infected mice vs. RES-treated siRNA negative transfected RSV-infected mice. Panel C: AHR was measured 5 days post-resveratrol treatment and 1 day post-siRNA transfection in mice treated with increasing methacholine concentrations (3.125 to 50.0 mg/ml) by pleth-lysmography. Values are expressed as means SD (n =5/group). *** = P<0.0001 RES and SARM siRNA 3-1-treated RSV-infected mice vs. RES and siRNA negative transfected RSV-infected mice; **= P<0.01 RSV-infected mice vs. RES-treated RSV-infected mice. Panel D: IFN-γ was measured by ELISA from BALF harvested 5 days post RSV infection (n=8/group). IFN-γ levels in BALF are shown. Values are expressed as means-SD (n=8/group). *= P<0.05 RSV-infected mice vs. RES-treated RSV-infected mice, ***= P<0.0001 RES-treated SARM siRNA 3-1 transfected RSV-infected mice vs. RES-treated siRNA negative transfected RSV-infected mice. Panel E: Viral titer was measured by Q-PCR, RSV infected lungs were harvested 5 days post-RES treatment and 1 day post-SARM siRNA transfection. Values are expressed as mean±SD (n=5/group). There was no significant difference between RSV-infected mice and RES-treated SARM siRNA 3-1 transfected RSV-infected mice.
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