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Partial Attenuation of Respiratory Syncytial Virus with a Deletion of a Small Hydrophobic Gene Is Associated with Elevated Interleukin-1β Responses

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ABSTRACT

The small hydrophobic (SH) gene of respiratory syncytial virus (RSV), a major cause of infant hospitalization, encodes a viroporin of unknown function. SH gene knockout virus (RSV ΔSH) is partially attenuated *in vivo*, but not *in vitro*, suggesting that the SH protein may have an immunomodulatory role. RSV ΔSH has been tested as a live attenuated vaccine in humans and cattle, and here we demonstrate that it protected against viral rechallenge in mice. We compared the immune response to infection with RSV wild type and RSV ΔSH *in vivo* using BALB/c mice and *in vitro* using epithelial cells, neutrophils, and macrophages. Strikingly, the interleukin-1β (IL-1β) response to RSV ΔSH infection was greater than to wild-type RSV, in spite of a decreased viral load, and when IL-1β was blocked *in vivo*, the viral load returned to wild-type levels. A significantly greater IL-1β response to RSV ΔSH was also detected *in vitro*, with higher-magnitude responses in neutrophils and macrophages than in epithelial cells. Depleting macrophages (with clodronate liposome) and neutrophils (with anti-Ly6G/1A8) demonstrated the contribution of these cells to the IL-1β response *in vivo*, the first demonstration of neutrophilic IL-1β production in response to viral lung infection. In this study, we describe an increased IL-1β response to RSV ΔSH, which may explain the attenuation *in vivo* and supports targeting the SH gene in live attenuated vaccines.

IMPORTANCE

There is a pressing need for a vaccine for respiratory syncytial virus (RSV). A number of live attenuated RSV vaccine strains have been developed in which the small hydrophobic (SH) gene has been deleted, even though the function of the SH protein is unknown. The structure of the SH protein has recently been solved, showing it is a pore-forming protein (viroporin). Here, we demonstrate that the IL-1β response to RSV ΔSH is greater in spite of a lower viral load, which contributes to the attenuation *in vivo*. This potentially suggests a novel method by which viruses can evade the host response. As all *Pneumovirinae* and some *Paramyxovirinae* carry similar SH genes, this new understanding may also enable the development of live attenuated vaccines for both RSV and other members of the *Paramyxoviridae*.

Respiratory syncytial virus (RSV) is the most significant cause of bronchiolitis and pneumonia in infants for which there is no vaccine (1). Recent advances in the understanding of the infant immune response to vaccination suggest that a live attenuated vaccine given in infancy may be the most effective approach to prevent RSV infection (2), potentially in combination with maternal immunization using recombinant F protein (3). This is supported by the successful introduction of live attenuated influenza vaccine to the childhood vaccination schedule (4) in conjunction with immunization during pregnancy with the trivalent inactivated vaccine (5). One issue with live attenuated RSV vaccines has been balancing immunogenicity and safety (6). Two approaches are used to develop live attenuated vaccines: biological derivation of strains, usually by multiple passages, often at lower temperatures to mimic the upper respiratory tract, and targeted gene deletion by reverse genetics.

To most effectively attenuate a virus by reverse genetics, understanding the proteins it encodes is required. In the current generation of live attenuated RSV vaccines, genes encoding the nonstructural protein 2 (NS2) and the small hydrophobic protein (SH) have been targeted. NS2 acts as an inhibitor of the type I interferon response (7), modulating NF-κB (7), but the function of the SH protein is currently unknown. *In silico* analysis suggests that a transmembrane pentamer is the most energetically favorable conformation of SH (8). The formation of SH protein pentamers has been confirmed by cryo-electron microscopy (cryo-EM) studies (9), and SH pentamers enable the passage of ions and small molecules (9–11). When transfected into HEK293 cells, SH is located at the plasma membrane (10), and during RSV infection, SH is located at the Golgi complex (12). These studies suggest that the SH protein belongs to the family of viroporins (13); however, the role of the pore encoded by the SH gene is unknown.

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Recombinant RSV that does not express the SH protein is partially attenuated *in vivo* (14), but not *in vitro* (15). This suggests it may play a role in modulating the immune response, with earlier studies showing that RSV SH inhibits tumor necrosis factor (TNF) signaling (16). In a recent study, recombinant bovine RSV (bRSV) with an SH gene deletion was attenuated (17), inducing increased levels of the cytokines interleukin-1β (IL-1β) and TNF. Viral pore proteins have been proposed to modulate the inflammasome, a multiprotein pattern recognition complex that catalyzes the cleavage of the proforms of IL-1β and IL-18 into their active forms via caspase 1 (18).

We observed that recombinant RSV lacking the SH gene was attenuated but protective against subsequent virus challenge. We propose that the attenuation observed *in vivo* following deletion of the RSV SH gene is due to its effect on the host immune response. To test this, we compared the response to infection with wild-type (WT) RSV (strain A2) and its derivative in which the SH gene had been deleted (RSV ΔSH) *in vitro* and *in vivo*. We observed that RSV ΔSH induced significantly higher levels of IL-1β, especially from macrophages and neutrophils. These studies demonstrate increased inflammation due to the virus with SH deleted, which may contribute to its attenuation.

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MATERIALS AND METHODS

Virus. RSV strain A2 was used as the WT and compared to an SH gene deletion recombinant on an A2 background (19). Infectious stocks of virus were grown using the human laryngeal carcinoma cell line HEp-2. The viral titer was calculated by an immunoplaque assay using biotinylated goat anti-RSV polyclonal antibody (AbD Serotec, Oxford, United Kingdom) to detect plaques. Prior to *in vitro* and *in vivo* studies, stocks were screened for lipopolysaccharide (LPS) contamination. Virus was inactivated by UV irradiation at 1.3×10^5 μJ/cm^2 for 15 min on ice in a CX-2000 cross-linker (UVP, Cambridge, United Kingdom).

Animals. Female BALB/c mice were obtained from Harlan Scientific (Brook House, United Kingdom) and used at 6 to 8 weeks of age. All procedures undertaken were approved by the local animal welfare and ethical review board and performed by personal licensees under the appropriate project license. Experiments were carried out in accordance with the Animals (Scientific Procedures) Act of 1986. The mice were infected with 2.5×10^5 PFU of virus or medium alone in a 100-μl volume intranasally while under isoflurane anesthesia. The animals were weighed prior to RSV challenge and daily thereafter. Where described, neutrophils were depleted with 0.5 mg anti-Ly6G (clone 1A8; BioXCell), and IL-1β was blocked with 0.5 mg anti-murine IL-1β (mIL-1β) (clone B122; BioXCell) *in vivo* in 500 μl delivered intraperitoneally on days −1 and +1 of primary RSV infection. For macrophage depletion, mice were treated with 100 μl of clodronate liposome (CL) suspension (Boehringer GmbH, Mannheim, Germany) or control empty liposomes (PL) intranasally. After infection, bronchoalveolar lavage (BAL) fluids, lung tissues, and serum samples were harvested as described previously (20).

RSV load. The viral load *in vivo* was assessed by extracting RNA from frozen lung tissue disrupted in a TissueLyzer (Qiagen, Manchester, United Kingdom) using TRIzol extraction and then converting it into cDNA. Quantitative real-time (RT)-PCR was carried out using bulk viral RNA for the RSV L gene and mRNA, using 900 nM forward primer (5′-GAACTCAGTGTAGGTAGAATGTTTGCA-3′), 300 nM reverse primer (5′-TTCAGCTATCATTTTCTCTGCCAAT-3′), and 100 nM probe (5′-6-carboxyfluorescein [FAM]-TTTGAACCTGTCTGAACAT-6-carboxytetramethylrhodamine [TAMRA]-3′) on a Stratagene Mx3005p (Agilent Technologies, Santa Clara, CA, USA). The L-specific RNA copy number was determined using an RSV L gene standard.

Cytokine quantification. IL-1β was measured in samples by human or mouse duoset enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Oxford, United Kingdom). IL-6 and CXCL1/KC were measured by Luminex (Bio-Rad, Hemel Hempstead, United Kingdom).

Flow cytometric analysis. Live cells were suspended in Fc block (anti-CD16/32; BD) in phosphate-buffered saline (PBS)-1% bovine serum albumin (BSA) and stained with surface antibodies (panel 1, RSV M2 82-90 pentamer R-PE [Proimmune, Oxford, United Kingdom[, CD3-fluorescein isothiocyanate [FITC] [BD, Oxford, United Kingdom], CD4-allophycocyanin [APC] [BD], CD8-APC Alexa 75 (Invitrogen, Paisley, United Kingdom), and CD19-eFluor 450 [eBioscience, Hatfield, United Kingdom]; panel 2, CD11c-FITC [BD], CD80-APC [eBioscience], major histocompatibility complex class II [MHC-II]-efluor450 [eBioscience], F4/80-phycoerythrin [PE]-Cy7 [eBioscience], and Ly6G-BV605 [BD]). For intracellular IL-1β staining, after surface staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD) and stained with IL-1β (BD). Analysis was performed on an LSR Fortessa flow cytometer (BD). Fluorescence minus one (FMO) controls were used for surface stains, and an IgG1-PE isotype control was used for IL-1β intracellular staining.

Antigen-specific ELISA. A quantitative assay (adapted from reference 21) was used to determine serum antibody levels. Plates were coated with 1 μg/ml RSV antigen and blocked with PBS-1% BSA. Bound IgG was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (AbD Serotec, Kidlington, United Kingdom). A dilution series of recombinant murine IgG was used as a standard to quantify RSV-specific antibodies. 3,3′,5,5′-Tetramethylbenzidine (TMB) with H_2SO_4 to quench the reaction was used to detect the response, and optical densities were read at 450 nm.

***In vitro* cells.** HEp-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) with 10% fetal calf serum (FCS), L-glutamine, and penicillin-streptomycin. THP-1 cells (a macrophage-like cell line) were grown in RPMI with 10% FCS, L-glutamine, and penicillin-streptomycin. The THP-1 cells were differentiated into phagocyte-type cells by seeding into new tissue culture plates, supplementing with 20 ng/ml phorbol myristate acetate (PMA), and incubating for 24 h before resting for a further 24 h (adapted from reference 22). For PBMC, mixed donor pools from three NC24 leukocyte clones were sourced from the NHS Blood and Transplant Unit (Colindale, United Kingdom). RosetteSep CD8 depletion cocktail (StemCell Technologies, Cambridge, United Kingdom) was used to prepare CD8-depleted PBMC populations. Cells were dispensed into tissue culture plates at the required density following the addition of 10 U/ml IL-2. For primary neutrophils, neutrophils were separated from fresh blood collected from healthy donors with written consent according to local research committee guidelines. A modified Percoll method was used (23). The neutrophils were separated on a 70% over 60% Percoll solution and centrifuged at 500 × *g* for 35 min at 22°C, with acceleration/braking at 2 m/s^2. This gave a population that was >90% neutrophils, as confirmed by differential staining.

***In vitro* viral infection.** Cells were seeded into 24-well plates at the following densities: HEp-2, 5×10^5; THP-1, 5×10^5; PBMC, 5×10^6; neutrophils, 5×10^6. The cells were incubated for a minimum of 24 h at 37°C, 5% CO_2 before infection with RSV ΔSH or RSV WT at a multiplicity of infection (MOI) of 0.5. At the time points specified in the results, supernatants were collected and subjected to cytokine analysis by ELISA.

Fluorescence microscopy and imaging. The HEp-2 and THP-1 cell lines and primary neutrophils were infected with green fluorescent protein (GFP)-tagged RSV (24) at an MOI of 0.1 24 or 48 h prior to imaging. Images were captured using a Nikon Eclipse TE2000 inverted microscope attached to a Nikon digital camera (DXM 1200F), with 20× magnification. Blue (400- to 446-nm) fluorescence filters were used to detect GFP.

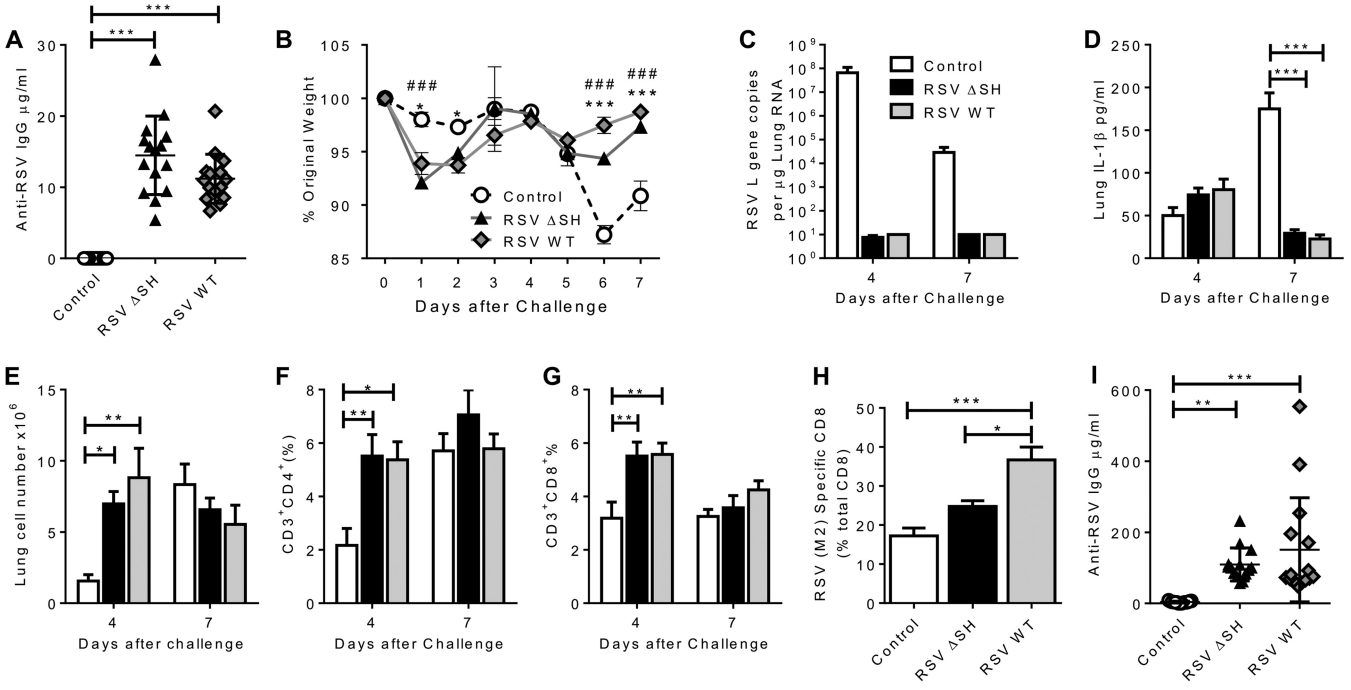

FIG 1 RSV ΔSH is protective against RSV infection. Mice were infected intranasally with 2.5×10^5 PFU of either RSV WT (gray symbols) or RSV ΔSH (black symbols) or control treated (white symbols), and 4 weeks later, all the mice were challenged with RSV WT. (A) Anti-RSV IgG was measured by ELISA 1 day before RSV challenge. (B to E) Weight change (B), lung viral load (C), lung IL-1B (D), and lung cell numbers (E) were measured after secondary infection. (F to H) Lung $CD4^+$ (F) and $CD8^+$ (G) T cells on day 4 and day 7 and lung RSV-specific CD8 T cells on day 7 (H) were measured by flow cytometry. (I) Anti-RSV IgG was measured by ELISA on day 7 after infection. (B to H) The points represent individual animals. The data represent means ± standard errors of the mean (SEM) ($n = 5$ animals). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ comparing WT RSV and the control; #, $P < 0.05$; and ###, $P < 0.001$ comparing RSV ΔSH and the control, measured by multiple *t* tests with Holm-Sidak correction (B) or analysis of variance (ANOVA) (A and C to I).

Statistical analysis. The calculations described in the figure legends were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

RSV ΔSH infection is protective against subsequent viral challenge. SH gene deletion has been proposed as a possible attenuating mutation for the generation of vaccine strains of RSV. To confirm that RSV ΔSH protected against RSV challenge, mice were infected intranasally with either RSV WT or RSV ΔSH at 2.5×10^5 PFU in a 100-µl volume or with medium alone as a control and then 28 days later challenged intranasally with 2.5×10^5 PFU RSV WT in 100 µl. Sera were collected prior to challenge infection, and RSV WT and ΔSH infection generated an RSV-specific IgG response, with the response to ΔSH slightly, but not significantly, greater than that to RSV WT (Fig. 1A). Mice that had primary infection with either ΔSH or WT were protected against disease following subsequent RSV challenge; on days 6 and 7 after RSV challenge infection, control-treated mice lost significantly more weight ($P < 0.001$) (Fig. 1B). Interestingly, as we have previously observed (25), there was acute weight loss on days 1 and 2 in the primed groups. There was no detectable viral load in either primed group, but RSV L gene-specific RNA was detectable in the control group on both days 4 and 7 after RSV infection (Fig. 1C). There was no difference in the IL-1β level in the lungs (Fig. 1D) on day 4 after infection, but the control group had significantly more IL-1β in the lungs on day 7. The primed groups recruited more cells to the lungs (Fig. 1E) on day 4, with a greater proportion of $CD4^+$ and $CD8^+$ T cells on day 4. On day 7 after infection, the mice previously infected with RSV WT had significantly more RSV-specific CD8 cells (Fig. 1H). Both previously infected groups had significantly higher anti-RSV IgG responses than the control-treated mice on day 7 and an increase from the level prior to challenge, indicating a memory response to RSV (Fig. 1I). We thus confirmed that RSV ΔSH can induce a protective immune response.

RSV lacking the SH gene is attenuated *in vivo* but induces a greater IL-1β response. To assess the role of the SH protein during RSV infection, BALB/c mice were infected with 2.5×10^5 PFU RSV WT or RSV ΔSH (19). The ΔSH virus was attenuated; mice infected with RSV WT lost significantly more weight than mice infected with RSV ΔSH on days 5 to 7 after infection ($P < 0.05$) (Fig. 2A), and markedly less RSV RNA was detected in the RSV ΔSH groups than in RSV WT-infected animals (Fig. 2B). There was no difference in the total cell counts recovered from the lungs (Fig. 2C). However, there was significantly greater recruitment of $CD4^+$ ($P < 0.05$) (Fig. 2D), $CD8^+$ ($P < 0.05$) (Fig. 2E), and $DX5^+$ NK ($P < 0.05$) (Fig. 2F) cells in the lungs of mice infected with RSV ΔSH on day 2 after infection. Lung CXCL1 ($P < 0.001$) (Fig. 2G) and IL-6 ($P < 0.001$) (Fig. 2H) levels were significantly greater in RSV WT-infected mice on day 1 after infection. IL-1β levels were slightly, but not significantly, higher in mice infected with RSV ΔSH on days 2 and 4 after infection (Fig. 2I). This was striking, because the viral load was so much lower in the RSV ΔSH-infected mice, suggesting that the IL-1β response was modulated by the SH gene. IL-1β was blocked during RSV ΔSH infection to determine its contribution to the attenuated phenotype of RSV

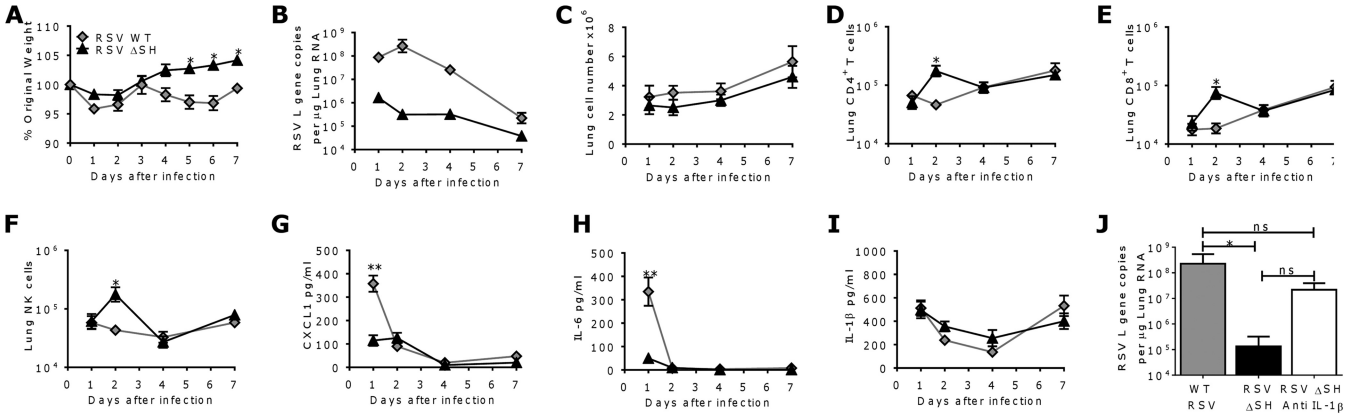

FIG 2 RSV ΔSH is attenuated *in vivo* but induces a greater IL-1β response than the wild type. Mice were infected intranasally with RSV WT or RSV ΔSH. (A to C) Weight loss (A), lung viral load (B), and lung cell numbers (C) were measured after infection. (D to F) Lung $CD4^+$ T (D), $CD8^+$ T (E), and $DX5^+$ NK (F) cells were measured by flow cytometry. (G to I) CXCL1 (G), IL-6 (H), and IL-1β (I) were measured in lung supernatants by ELISA. (J) Mice were treated with anti-IL-1β prior to infection with RSV ΔSH, and the viral load was measured on day 4 after infection. The points represent means ± SEM ($n > 5$ mice). *, $P < 0.05$; **, $P < 0.01$; calculated by multiple *t* tests with Holm-Sidak correction.

ΔSH. Mice were treated with anti-IL-1β on days −1 and +1 of infection, and the viral load was measured on day 4 after infection. RSV ΔSH was significantly attenuated compared to the wild-type virus, but anti-IL-1β treatment restored the ΔSH viral load to wild-type levels (Fig. 2J).

RSV lacking the SH gene has growth kinetics similar to those of the wild-type virus but induces a greater IL-1β response *in vitro*. To dissect the effect of SH gene deletion on the IL-1β response, a range of cell types were infected *in vitro*. HEp-2 cells were infected at an MOI of 0.5 with RSV WT or RSV ΔSH, and supernatants were collected at various time points after infection. RSV ΔSH has been observed to replicate to titers similar to those of wild-type virus *in vitro*, and we observed a similar effect in HEp-2 cells (Fig. 3A). The levels of IL-1β induced by viral infection of HEp-2 cells were low, but RSV ΔSH induced more IL-1β than RSV WT (Fig. 3B). We have previously observed that macrophages contribute to the inflammatory response to RSV infection (26) and wished to compare the IL-1β responses in these cells.

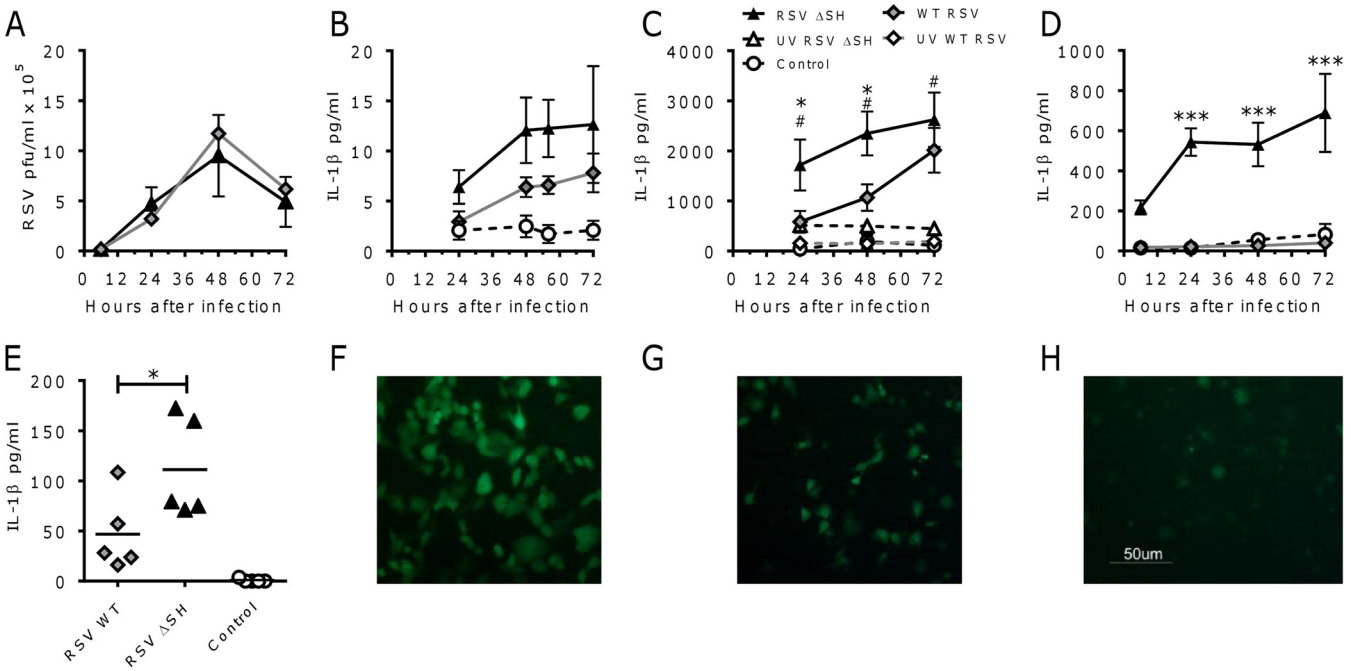

FIG 3 Recombinant RSV lacking the SH gene induces a greater IL-1β response than the wild type *in vitro*. (A) HEp-2 cells were infected with RSV WT or RSV ΔSH (MOI = 0.5), and the viral load was assessed by plaque assay. (B to E) Supernatants were collected and analyzed for IL-1β levels by ELISA following infection of HEp-2 cells (B), THP-1 cells (C), PBMC (D), and neutrophils (E). (F to H) HEp-2 (F) and THP-1 (G) cells were infected with RSV GFP (MOI = 0.1), and primary neutrophils (H) were infected with RSV (MOI = 0.25) and imaged at 24 h after infection. The points represent means ± SEM ($n = 3$ repeats) of HEp-2, PBMC, and THP-1 cells and 3 individual PBMC and neutrophil donors. *, $P < 0.05$, and ***, $P < 0.001$ between RSV ΔSH and RSV WT; #, $P < 0.05$ between ΔSH and UV-inactivated ΔSH; calculated by two-way ANOVA (A to D) or ANOVA (E). The images are representative of 2 studies.

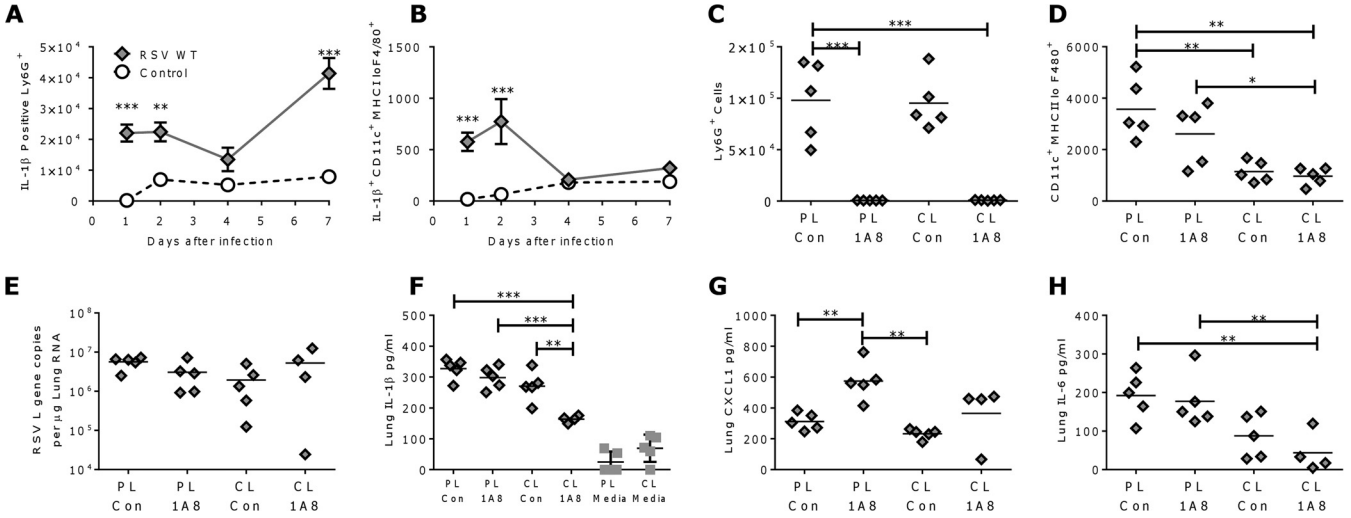

FIG 4 IL-1β is produced by neutrophils and macrophages *in vivo*. (A and B) Mice were infected with RSV WT or control treated intranasally. Expression of IL-1β by $Ly6G^+$ neutrophils (A) and $CD11c^+$ $F480^+$ MHC-II low alveolar macrophages (B) was measured by flow cytometry at various time points after infection. (C to H) Mice were treated with anti-Ly6G-depleting antibody (1A8) or control antibody (Con) intraperitoneally and CL or PL intranasally prior to RSV WT infection. Neutrophil (C) and alveolar macrophage (D) numbers were analyzed by flow cytometry and the RSV L gene (E) by RT-PCR, and lung IL-1β (F), CXCL1 (G), and IL-6 (H) were measured on day 1 after infection. The points represent means ± SEM ($n = 5$ mice) (A and B) and individual animals; the bars represent means ($n = 5$) (C to H). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; calculated by multiple *t* tests with Holm-Sidak correction (A and B) or ANOVA (C to H). Gray squares, media-alone groups.

THP-1 cells and CD8-depleted, adherent PBMC were cultured with RSV WT or RSV ΔSH. RSV WT induced an IL-1β response that was greater than that of control-treated cells, at a level similar to that observed in previous studies (Fig. 3C and D) (27). RSV ΔSH induced a significantly greater IL-1β response than RSV WT in both THP-1 cells ($P < 0.05$) (Fig. 3C) and PBMC ($P < 0.05$) (Fig. 3D). To determine whether viral replication or virus-associated pathogen-associated molecular patterns (PAMPs) were driving the IL-1β response, the IL-1β responses in THP-1 cells exposed to live or UV-inactivated virus were compared. Inactivation of the virus was confirmed by *in vitro* plaque assay (data not shown). Live RSV induced a significantly greater IL-1β response than UV-inactivated virus with both the ΔSH and WT viruses, indicating that the virus needs to replicate to induce cytokine release in macrophages. Since neutrophils are also a significant source of inflammatory cytokines in the lungs after infection, IL-1β levels were measured at 24 h after RSV infection of primary human neutrophils. Infection of neutrophils with RSV ΔSH led to a significantly greater IL-1β response than RSV WT ($P < 0.05$) (Fig. 3E). From these studies, we speculate that the SH protein of RSV antagonizes the IL-1β response.

IL-1β release following exposure to RSV may be in response to intracellular infection of the cell (in *cis*) or in response to extracellular danger signals (in *trans*). To understand the responses in different cell types, we determined whether RSV infects these cell types. Cells were cultured with GFP-expressing RSV and imaged by fluorescence microscopy. Green fluorescence was detectable in HEp-2 (Fig. 3F) and THP-1 (Fig. 3G) cells after infection with RSV, indicating that there is transcription of virus-encoded RNA. More green HEp-2 cells were detectable than THP-1 cells for an equivalent MOI of RSV. Very limited green fluorescence was observed in primary neutrophils at 24 h after infection, even with a higher MOI of RSV, suggesting they do not support viral replication and are therefore responding to extracellular virus, though further studies are required to confirm this (Fig. 3H).

IL-1β is produced in response to RSV by macrophages and neutrophils *in vivo*. Having observed differences in the amounts of IL-1β produced by different cell types *in vitro*, we wished to determine which cell types produce IL-1β *in vivo*. Cells were isolated from the lungs after RSV WT infection or mock infection with medium alone, and IL-1β-positive cells were identified by intracellular cytokine staining in the absence of stimulation. The number of $IL\text{-}1\beta^+$ $Ly6G^+$ cells (neutrophils) (Fig. 4A) was significantly greater in the infected group than in the uninfected group. Interestingly, the percentage of Ly6G cells positive for IL-1β increased over the course of infection. Detectable expression of IL-1β by $CD11c^+$ $F480^+$ MHC-II low cells (alveolar macrophages [28]) (Fig. 4B) was highest on days 1 and 2 after infection but returned to control levels by day 4 after infection. No lymphocytes were observed to be IL-1β positive. A similar profile of IL-1β-positive cells was observed following infection with RSV ΔSH.

Cell-specific depletion was used to determine whether a specific cell type was the major contributor to lung IL-1β after infection. The effects on acute RSV infection (day 1 after infection) of alveolar macrophage depletion (by CL treatment) or neutrophil depletion (using anti Ly6G [clone 1A8] antibody) were compared. 1A8 treatment led to a significant reduction in the number of neutrophils detectable in the lungs (Fig. 4C) and BAL fluid (data not shown). CL treatment led to a significant decrease in the percentage of $CD11c^+$ $F480^+$ MHC-II low cells (Fig. 4D). Cell depletion had no effect on the viral load at day 1 after infection (Fig. 4E). In spite of the high percentage of IL-1β-positive neutrophils detected, neutrophil depletion by 1A8 treatment alone had no effect on IL-1β (Fig. 4F). Interestingly, more CXCL1 was detected in the lungs of these mice (Fig. 4G), and there was no effect on IL-6 levels (Fig. 4H). CL treatment alone significantly reduced the level of IL-6 but had no effect on lung IL-1β or CXCL1 levels. Mice that received both clodronate and 1A8 treatments had significantly reduced IL-1β, suggesting that either there is some redundancy in the cells that produce IL-1β or these cells act cooperatively. CL

treatment alone without RSV infection did not significantly increase IL-1β production in the lungs compared to control PL treatment alone (Fig. 4F). We therefore conclude that IL-1β is produced by a number of different cellular sources, including neutrophils and CL-sensitive cells, presumably alveolar macrophages.

DISCUSSION

One rationale for understanding the function of the SH gene is to target it for deletion in live attenuated RSV vaccines. Here, we demonstrate enhanced inflammation after infection with RSV lacking the SH gene, particularly in the IL-1β pathway. In the current study, we demonstrated that RSV ΔSH protected against viral challenge in the mouse model. A recent study has demonstrated that a bRSV ΔSH vaccine is highly protective in infant calves, even in the presence of maternal antibody (29), and a live attenuated RSV ΔSH vaccine with additional point mutations (Medi-559) has been shown to be safe and immunogenic in clinical trials in children (30). Data from chimpanzee studies (31), which showed moderate attenuation, suggest SH alone would be insufficient for a safe live vaccine, though it has been shown to be highly effective in cattle (29). While addition of ΔSH to other temperature-sensitive vaccines had a marginal effect (32, 33) on attenuation, we speculate that SH has an immunomodulatory function and that its deletion as part of a live attenuated RSV vaccine design would be beneficial.

The observation that deletion of RSV SH leads to an increase in IL-1β is supported by studies in bRSV, where a ΔSH vaccine strain induced higher levels of IL-1β in the lungs of infected cattle (17). In the current study, blockade of IL-1β prior to infection increased the viral load, supporting the idea that SH might enable immunomodulation. Interestingly, bRSV ΔSH also induced greater levels of apoptosis, which can be associated with inflammasome activation. In contrast, a study with different live attenuated RSV vaccines showed that the most heavily attenuated vaccine (rA2cp248/404/1030/ΔSH) induced lower responses in nasal wash samples to a number of cytokines than other live attenuated vaccines (6). However, the virus tested was highly attenuated, and the reduced cytokine response was likely a consequence of strong attenuation of replication related to the point mutations in the backbone of the virus rather than the SH deletion. A recent study by Triantafilou et al. proposed that the RSV SH protein activates the inflammasome (34). Unlike the current study, the virus used by Triantafilou et al. had the SH gene replaced with GFP. We believe that there are flaws in the published study by Triantafillou et al. that warrant further investigation: figures showing the relative growth of the two viruses are not included in the study, the study was performed at only a single time point in a single cell line using a high MOI of virus, and there was no statistical analysis comparing the responses between the viruses. In contrast to the published study, we have carefully dissected the inflammasome response to RSV infection, with and without the SH protein, both *in vivo* and *in vitro*, performing multiple time course studies using primary cells, cell lines, and a mouse model.

We observed that both neutrophils and clodronate-sensitive alveolar macrophages contribute to the lung IL-1β response (*in vivo* and *in vitro*). We propose a model where the alveolar macrophages act as sentinels for infection, initiating the immune response, and the neutrophils then amplify the signal. Of note, in our hands, epithelial cells and macrophages supported transcription of the viral genome, but neutrophils did not. While we (26, 35) and other groups (36, 37) have explored the role of alveolar macrophages in initiating the immune response to RSV infection by cytokine production, the role of neutrophils in RSV infection is not known. Studies have shown IL-9 (38), TNF (39), and chemokine (40) production by neutrophils in response to RSV. Neutrophils are a source of IL-1β in lung injury models (41), but this is the first study that has demonstrated IL-1β production by neutrophils in response to respiratory viral infection. Airway neutrophilia is observed in patients with bronchiolitis (42), and an increase in neutrophils is detected in blood prior to the influx of CD8 T cells (43). We have previously observed persistent airway neutrophilia following regulatory T cell depletion (44), suggesting they are associated with disease, but other studies have demonstrated that neutrophils can have a protective (45) or antiviral (46) role. While RSV RNA and protein have been observed in airway neutrophils from infected infants (47), the data generated using GFP-expressing virus suggest that macrophages, but not neutrophils, can support viral replication.

We speculate that SH is an immune evasion protein and that this mechanism of immune evasion may be conserved across other viruses. SH genes are found in all members of the subfamily *Pneumovirinae* and some of the *Paramyxovirinae*, including mumps virus (48) and human parainfluenza virus 5 (hPIV5) (49). The SH proteins from human metapneumovirus (hMPV) (50), bRSV (51), hPIV5 (49), and mumps virus (52) are nonessential for *in vitro* growth, and an immunomodulatory function has been described for the SH proteins from PIV5 (49), bRSV (17), and hMPV (53), which also encodes a viroporin (54). We hypothesize that the pore structure of the SH protein (10) facilitates this immunomodulatory function, counteracting the activation of NLRP3 by ionic changes induced by RSV infection (27). However, not all viroporins inhibit inflammasome function; for example, the M2 protein of influenza A virus is a viroporin that has been demonstrated to activate the inflammasome (55). The differences between RSV SH and influenza virus M2 may be due to the differences in structure (13). Of note, another viroporin with a structure similar to that of RSV SH, influenza B virus NB, has a similar, limited effect on the replication of influenza virus *in vitro* (56). Alternatively, SH has been shown to form a complex with the G protein (57), which has also been shown to have an immunomodulatory function (58), and the complex is potentially important; more research is required to determine the exact mechanism of action. In addition to depletion in live vaccines, targeting the SH protein may open up novel antiviral therapies, and a recent study has demonstrated that its function as an ion pore can be blocked with a small-molecule inhibitor, pyronin B (11). In the current study, we show that deletion of the SH gene changes the immune response on infection, increasing IL-1β responses by an as yet undetermined mechanism, and we believe that further research on this aspect of SH gene function is important in the development of an RSV vaccine.

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