Enteric helminth-induced type I interferon signaling protects against pulmonary virus infection through interaction with the microbiota

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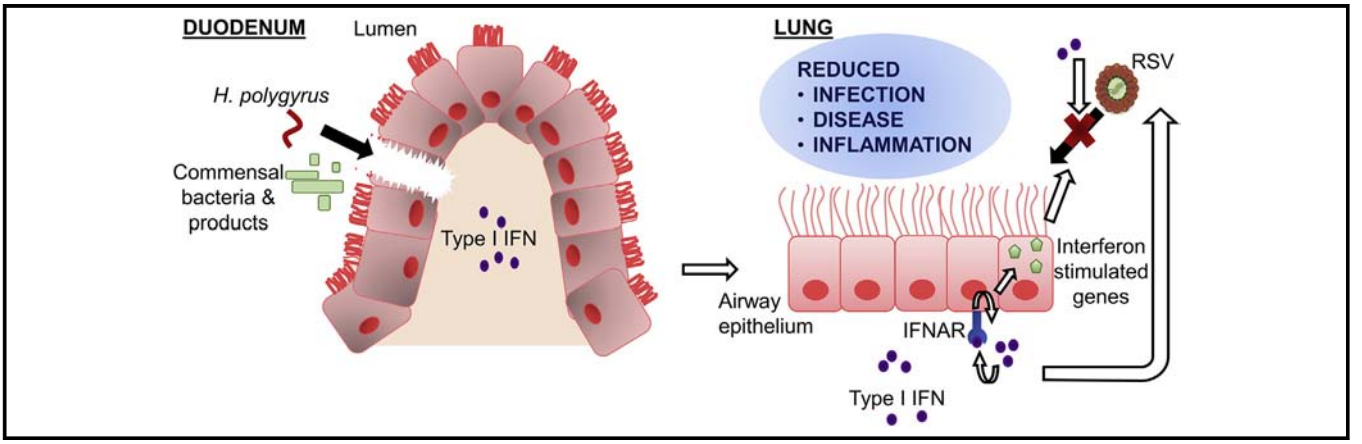
GRAPHICAL ABSTRACT



Background: Helminth parasites have been reported to have beneficial immunomodulatory effects in patients with allergic and autoimmune conditions and detrimental consequences in patients with tuberculosis and some viral infections. Their role in coinfection with respiratory viruses is not clear.

Objective: Here we investigated the effects of strictly enteric helminth infection with *Heligmosomoides polygyrus* on respiratory syncytial virus (RSV) infection in a mouse model.

Methods: A murine helminth/RSV coinfection model was developed. Mice were infected by means of oral gavage with 200 stage 3 *H polygyrus* larvae. Ten days later, mice were infected intranasally with either RSV or UV-inactivated RSV.

Results: *H polygyrus*–infected mice showed significantly less disease and pulmonary inflammation after RSV infection associated with reduced viral load. Adaptive immune responses, including T_H2 responses, were not essential because protection against RSV was maintained in *Rag1*$^{-/-}$ and *Il4rα*$^{-/-}$ mice. Importantly, *H polygyrus* infection upregulated expression of type I interferons and interferon-stimulated genes in both the duodenum and lung, and its protective effects were lost in both *Ifnar1*$^{-/-}$ and germ-free mice, revealing essential roles for type I interferon signaling and microbiota in *H polygyrus*–induced protection against RSV.

Conclusion: These data demonstrate that a strictly enteric helminth infection can have remote protective antiviral effects in the lung through induction of a microbiota-dependent type I interferon response. (J Allergy Clin Immunol 2017;140:1068-78.)

Key words: *Respiratory syncytial virus, helminths,* Heligmosomoides polygyrus*, type I interferon, microbiome*

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Abbreviations used
cDC: Conventional dendritic cell
HES: *Heligmosomoides polygyrus* excretory-secretory
IL-4R: IL-4 receptor
ISG: Interferon-stimulated gene
mCRAMP: Murine cathelicidin-related antimicrobial peptide
NK: Natural killer
OAS: 2′-5′-Oligoadenylate synthetase
RAG: Recombination-activating gene
RSV: Respiratory syncytial virus
SPF: Specific pathogen-free
UV-RSV: UV-inactivated RSV

Respiratory syncytial virus (RSV) is a major respiratory pathogen. It infects nearly all infants by the age of 2 years[1] but does not induce lasting immunity and leads to recurrent infections throughout life. Worldwide, it is estimated that 33.4 million children under 5 years of age experience RSV-induced lower respiratory tract infection (LRTI) annually, and 10% of these require hospitalization, resulting in up to 199,000 deaths.[2-4] There is also major morbidity and mortality caused by RSV in the elderly.[5] Currently, there is no effective vaccine available for RSV, and treatment is limited to supportive care. Severe RSV-induced LRTIs are associated with and thought to be caused by severe pulmonary inflammation.

In addition, severe RSV infection during infancy has also been associated with increased risk for asthma development. There is substantial evidence indicating that children hospitalized with RSV-induced bronchiolitis are more likely to experience recurrent wheezing episodes for a prolonged period of time after recovery from this illness.[6-9]

Helminths infect approximately 3 billion people worldwide. It has long been proposed that infection with helminths could suppress the development of immune-mediated disease because in countries where their prevalence is high, the prevalence of asthma, allergy, and autoimmune conditions has been found to be correspondingly low.[10] In particular, intestinal helminths have been of major interest because of their ability to modulate host immune and inflammatory responses to foreign antigens,[11-16] and several clinical trials have been carried out or are underway assessing their utility as therapeutic agents in patients with inflammatory bowel disease, multiple sclerosis, and asthma.[17]

Helminth infections rarely occur in isolation, and coinfections are very common with varying effects, such as reduced pathogen control and increased disease, as reported for HIV infection and tuberculosis.[18-21] Recent experimental models in mice report reactivation of systemic latent γ-herpesvirus and reduced control of enteric norovirus replication,[22,23] indicating that in these systems helminth infection suppresses antiviral immunity, resulting in increased viral replication. However, the effect of helminth infection on respiratory viruses is not well understood. Clinical data are lacking, but mouse models suggest reduced influenza-induced pathology in helminth coinfection.[24,25]

Here we investigated whether infection with the strictly enteric murine helminth *Heligmosomoides polygyrus* would change the course of disease and inflammation during RSV infection. This study demonstrates protective effects of helminth infection on RSV infection and reveals a novel mechanism of type I interferon induction by enteric helminth infection at a site distant from the gut.

METHODS

Animals

BALB/c, C57BL/6, IL-4 receptor α *(Il4ra)*$^{-/-}$,[26] recombination-activating gene 1 *(Rag1)*$^{-/-}$,[27] IL-33 receptor *(Il33r)*$^{-/-}$ (BALB/c background), *Ifnar1*$^{-/-}$,[28] and *Camp*$^{-/-}$[29] (bred to congenicity on a C57BL/6J Ola Hsd background) mice were bred in house at the University of Edinburgh. Germ-free BALB/c mice were obtained from the Clean Mouse Facility, University of Bern (Bern, Switzerland), and compared with specific pathogen-free (SPF) BALB/c mice from Charles River Breeding Laboratories (l'Arbresle Cedex, France). Six- to 12-week-old female mice were infected by means of oral gavage with 200 stage 3 *H polygyrus* larvae. Ten days later, mice were intranasally infected with RSV or mock infected with UV-inactivated respiratory syncytial virus (UV-RSV; standard coinfection protocol).

Parasites, parasite products, and viral stocks

Parasites were maintained, as previously described.[30] For some experiments stage 3 *H polygyrus* larvae were irradiated at 100, 200, or 300 Gy by using a GSR-C1 irradiator at a rate of 6.2 Gy/min before administration by oral gavage. Axenic *H polygyrus* larvae were produced, as previously described.[31] Plaque-purified human RSV (Strain A2; ATCC, Manassas, Va) was grown in Hep-2 cells, as previously described.[32]

Whole-body plethysmography

Baseline respiratory effort was assessed in individual mice by using whole-body plethysmography (Buxco, Wilmington, NC). Mice were placed into individual chambers, and baseline measurements were recorded for 5 minutes. Enhanced pause values were recorded, averaged, and expressed as absolute values, as previously described.[33]

RSV immunoplaque assay

RSV titers were assessed, as previously described,[34] in lung homogenates by means of titration on HEp-2 cell monolayers in 96-well, flat-bottom plates. Twenty-four hours after infection, monolayers were washed, fixed with methanol, and incubated with biotin-conjugated goat anti-RSV antibody (Bio-rad, Watford, United Kingdom). Infected cells were detected with 3-amino-9-ethylcarbazole, and infectious units were enumerated by means of light microscopy.

Lung cell isolation and flow cytometry

Right lung lobes were excised, cut into small pieces, incubated on a shaker with collagenase A (0.23 mg/mL PBS; Sigma, St Louis, Mo) at 37°C for 45 minutes, and sheared through a 19-gauge needle. After red blood cell lysis (Sigma), the single-cell suspension was passed through a 40-μm cell strainer and stained with the viability dye eFluor 780 (eBioscience, Hatfield, United Kingdom). The following anti-mouse antibodies were used to phenotype lung immune cells: PDCA-1 (EBIO-927), Ly6G (RB6-C5), NKp46 (29A1.4), and B220 (RA3-6B2), all eFluor 450 conjugated (eBioscience, San Diego, Calif); Ly6C (AL-21) and CD8 (Ly-2), both fluorescein isothiocyanate conjugated (BD Biosciences, San Jose, Calif); CD11b (M1/70) and CD4 (RM4-5), both phycoerythrin conjugated (eBioscience); CD45 (30-F11), eFluor 605 Nanocrystal (NC605) conjugated (eBioscience); CD49B (DX5), CD19 (6D5; BioLegend, San Diego, Calif), and F480 (Cl:A3-1), all Alexa Fluor 647 conjugated (AbD Serotec, Bio-Rad Laboratories, Hercules, Calif); MHC class II (M5/114.15.2) and CD3 (145-2C11), both peridinin-chlorophyll-protein complex Cy5.5 conjugated (BioLegend); CD19 (EBIO1D3), CD3 (17A2; eBioscience), and Ly6G (1AB), all Alexa Fluor 700 conjugated (BD Biosciences); and CD11c (N418), phycoerythrin-Cy7 conjugated (eBioscience). Isotype control antibodies were used on pooled samples. Cells

TABLE I. Primers used for real-time PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Probe (FAM-TAMRA 5′-3′)
Oas1a	TCCTGGGTCATGTTAATACTTCCA	GAGAGGGCTGTGGTGGAGAA	CAAGCCTGATCCCAGAATCTATGCC
Viperin	CGAAGACATGAATGAACACATCAA	AATTAGGAGGCACTGGAAAACCT	CCAGCGCACAGGGCTCAGGG
RSV L	GAACTCAGTGTAGGTAGAATGTTTGCA	TTTCAGCTATCATTTTCTCTGCCAAT	TTTGAACCTGTCTGAACATTCCCGGTT

were gated as viable and $CD45^+$ and subsequently phenotyped based on their markers as follows: $Ly6G^-CD19^-CD3^-CD49B^+NKp46^+$ natural killer (NK) cells, $Ly6G^-CD19^-CD3^-$MHC class $II^+CD11B^+CD11C^+$ conventional dendritic cells (cDCs) and $Ly6G^-CD19^-CD3^+CD4^+$ or $CD8^+$ T cells, and $Ly6G^-CD3^-CD19^+CD19^+B220^+$ B cells. Samples were collected with the LSR Fortessa II (BD Biosciences). Postacquisition analysis was performed with FlowJo software (version 7.6.5; TreeStar, Ashland, Ore).

Real-time PCR

The lung and duodenum were harvested and homogenized in 1 mL of TRIzol (Invitrogen, Carlsbad, Calif) by using a TissueLyser. cDNA was made from the extracted RNA by using the Qiagen QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. One microgram of RNA was used for reverse transcription. Primers were diluted in TE buffer to a final concentration of 0.025 nM/μL, and probes were diluted to 0.005 nM/μL. Custom primers and probes were purchased from Jena Bioscience (Jena, Germany) or Applied Biosystems (Foster City, Calif). PCR amplification was carried out in a 25-μL volume made up of custom 7 μL of primer probe mix (300 nM primers and 200 nM probe), 12.5 μL of TaqMan Mastermix (Applied Biosystems), 1.75 μL of H_2O, 1.25 μL of 18S (Applied Biosystems), and 2.5 μL of DNA template. Premade primer probe mix (1.25 μL) was used in the following mixture: 12.5 μL of Mastermix, 5 μL of H_2O, 1.25 μL of 18S, and 2.5 μL of DNA template. *Ifnb* (Mm00439552_s1) and *Camp* (Mm00438285) primers and probes were bought premade from Life Technologies (Grand Island, NY). Custom primers used are shown in Table I.

ELISA

IFN-α and IFN-β levels were measured with an ELISA kit (PBL, Interferon Source, Piscataway, NJ), according to the manufacturer's instructions.

Statistical analysis

All data were analyzed with Prism 6 software (GraphPad Software, La Jolla, Calif). Analysis of 2 groups used an unpaired *t* test. Analysis of 3 or more groups used either 1-way ANOVA with the Tukey or Bonferroni posttest or 2-way ANOVA with the Bonferroni posttest. Unless otherwise stated, the differences are nonsignificant. We tested for outliers using the Grubb test, and outliers were removed.

Study approval

All procedures were carried out with institutional ethical approval under Home Office licenses. Germ-free animal experiments were performed according to institutional guidelines and Swiss Federal and Cantonal laws on animal protection.

RESULTS

H polygyrus protects against RSV-related disease and inflammation and reduces viral load

Mice were infected with *H polygyrus*, and 10 days later, when adult worms emerge into the lumen of the gut, mice were infected with RSV. *H polygyrus* coinfection protected against RSV-induced weight loss (Fig 1, *A*) and reduced RSV-induced increases in enhanced pause, which are indicative of increasing baseline respiratory effort (Fig 1, *B*).

RSV infection in the mouse model induces pulmonary inflammation with cellular infiltration, specifically of NK cells, $CD8^+$ T cells, and cDCs.[34,35] In mice coinfected with *H polygyrus*, RSV-induced increases in NK cell, B-cell (see Fig E1, *A* and *B*, in this article's Online Repository at www.jacionline.org), and $CD8^+$ T-cell numbers were absent (Fig 1, *C*), and the increase in cDC numbers was significantly reduced (Fig 1, *D*). Early proinflammatory cytokine production of IL-6 and TNF-α on day 2 was induced to a significantly lower level in *H polygyrus*–infected mice compared with those infected with RSV alone (see Fig E1, *C* and *D*). IFN-γ levels increased with RSV infection but were not significantly suppressed in coinfected mice, indicating selective inhibition of a pathway independent of IFN-γ (see Fig E1, *E*).

Given these changes in RSV-induced signs of disease, we investigated whether *H polygyrus* suppresses the immune response or directly alters the magnitude of RSV infection. Lung RSV titers, as assessed by using the plaque assay, were reduced after *H polygyrus* infection, without changes in the kinetics of replication (Fig 1, *E*). In C57BL/6 mice *ex vivo* plaque assays for RSV are unreliable because of the low viral load,[36] and therefore we tested the effects of coinfection in C57BL/6 mice by measuring expression of the RSV L gene in the lung by RT-PCR, as an indicator of viral load. L gene expression was significantly reduced in *H polygyrus*–infected mice in this strain (see Fig E1, *F*).

These findings demonstrate a potent inhibition of RSV-induced disease, early proinflammatory cytokine production, and recruitment of a broad range of immune cells to the lung in the setting of *H polygyrus* coinfection, presumably because of an early reduction in viral infection.

Adaptive immune responses, including T_H2 responses, are not required for *H polygyrus*–induced protection against RSV infection

Type 2 immune responses are crucial during most helminth infections, aiding in wound healing and immunity to helminths.[37-40] *Il4ra*–deficient mice cannot respond to IL-4 or IL-13 signals and present strongly diminished type 2 immune responses.[41] Consistent reductions in RSV titers were observed in *H polygyrus*–coinfected $Il4r\alpha^{-/-}$ mice, similar to those seen in wild-type BALB/c mice (Fig 2, *A*). We further assessed innate type 2 immune responses after *H polygyrus* infection and found a nonsignificant trend for increased IL-13–producing type 2 innate lymphoid cells and IL-33 levels in the lung tissue compared to RSV- and UV-RSV–infected control mice (see Fig E2, *A* and *B*, in

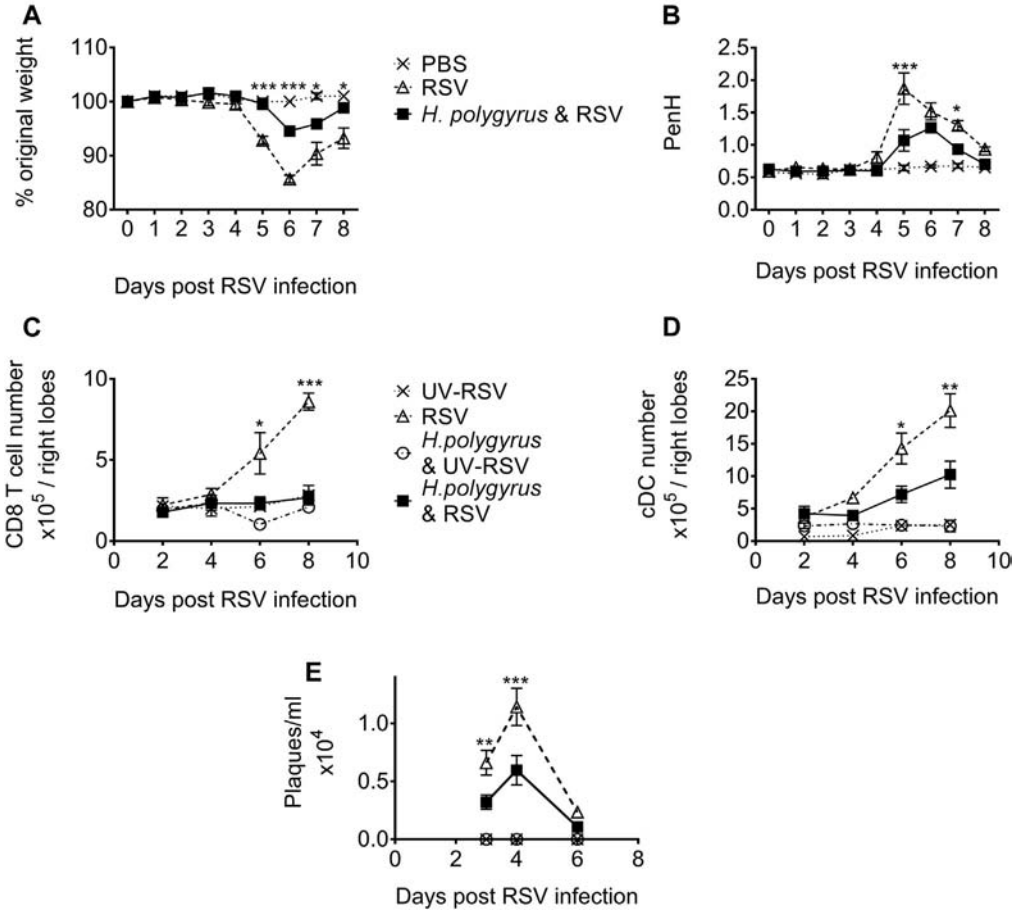

FIG 1. *H polygyrus* infection attenuates RSV disease and inflammation and reduces RSV viral load. The standard coinfection protocol was used as follows: female BALB/c mice were given 200 *H polygyrus* L3 larvae by oral gavage at day −10 or left naive. At day 0, 6×10^5 plaque-forming units (PFU; **A** and **B**) or 4×10^5 PFU **(C-E)** RSV or UV-inactivated RSV was administered intranasally. Fig 1, *A*, Mice were weighed daily, and percentage of original weight is shown. Fig 1, *B*, Enhanced pause *(PenH)* was assessed by whole-body plethysmography. Fig 1, *C* and *D*, Samples were taken at indicated time points after RSV infection for flow cytometric analysis. Numbers of $CD3^+CD8^+$ T cells (Fig 1, *C*) and MHC class $II^+CD11b^+CD11c^+$ cDCs (Fig 1, *D*) per right lung lobe are shown. Fig 1, *E*, Lungs were harvested on days 3, 4, and 6 after RSV infection, and plaque assays were performed. All data are depicted as means ± SEMs. Data in Fig 1, *A* and *B*, were pooled from 2 independent experiments (total n = 8 per group), and those in Fig 1, *C*, *D*, and *E*, were from 2 independent experiments (total n = 6 per group per time point). Statistical significance of differences between RSV-infected groups was determined by using 2-way ANOVA with the Bonferroni *post hoc* test. $*P < .05$, $**P < .01$, and $***P < .001$.

this article's Online Repository at www.jacionline.org). To investigate any protective role of IL-33 in response to RSV infection, we used *Il33r*$^{-/-}$ mice. The RSV load was similar between *Il33r*$^{-/-}$ and wild-type control mice (see Fig E2, *C*) and was reduced to similar levels in both groups by *H polygyrus* coinfection, indicating that there is no essential role for IL-33 in protection against RSV infection.

To determine whether any adaptive immune responses are required for *H polygyrus*–mediated protection against RSV infection, we used *Rag1*-deficient mice, which lack all T and B cells. Once again, RSV titers were significantly suppressed in both *Rag1*$^{-/-}$ mice and wild-type control mice after coinfection with *H polygyrus* (Fig 2, *B*). Together, these observations show that adaptive immune responses and IL-4Rα–dependent or IL-33 receptor–dependent type 2 cytokine responses are not required for the protective effect of *H polygyrus* on RSV infection.

H polygyrus infection induces expression of type I interferons and interferon-stimulated genes in both the duodenum and lung

Type I interferons are major players in the initial response to viral entry into the mucosa.[42] Because adaptive and innate type 2 immune responses were not essential for protection against RSV infection, we hypothesized that *H polygyrus* enhances the mucosal innate interferon response, conferring an antiviral state. 2′-5′-Oligoadenylate synthetase (*Oas*) and viperin are 2 of many interferon-stimulated genes (ISGs) that have been found to play a protective role in RSV infection and can be driven by type I interferon signaling.[43-46] Gene expression of *Ifnb*, viperin, and *Oas1a* tended to increase in the duodenum from day 3 after *H polygyrus* infection (see Fig E3 in this article's Online Repository at www.jacionline.org). Importantly, expression of these genes was also subsequently increased in the lung (Fig 3, *A-C*), despite the strictly enteric nature of *H polygyrus*,

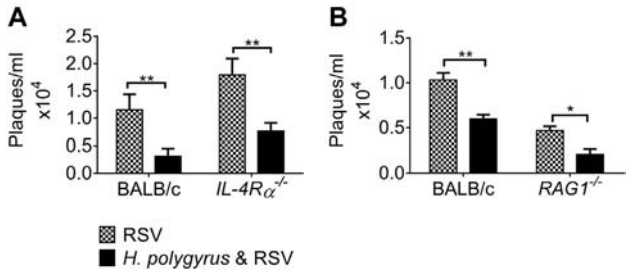

FIG 2. Adaptive immune responses, including T_H2 responses, are not required for the *H polygyrus*–mediated attenuation of RSV viral titers. The standard coinfection protocol was followed in BALB/c *Il4ra*–deficient mice **(A)** and BALB/c *Rag1*-deficient mice **(B)**. Lungs were harvested on day 4 of RSV infection, and plaque assays were performed to determine RSV titers. All data are depicted as means ± SEMs. Data in Fig 2, *A*, are pooled from 2 individual experiments (total n = 4-8 per group). Data in Fig 2, *B*, are representative of 2 independent experiments (n = 3-4 per group). Statistical significance between groups was determined by using 1-way ANOVA with the Tukey *post hoc* test. $*P < .05$ and $**P < .01$.

and remained increased 1 hour after RSV infection (Fig 3, *D-F*) if this was preceded by *H polygyrus*. By 6 to 12 hours after RSV infection, *Ifnb* transcripts reached the same levels in RSV monoinfected and coinfected mice (Fig 3, *G* and *H*). IFN-β protein levels measured by ELISA were below the detection limit at 1 hour after RSV infection and were found at similar levels between groups by 6 hours after infection, reflecting the RT-PCR data. However, IFN-α protein levels were significantly increased in *H polyrgyrus* coinfected mice at 6 hours after RSV infection (Fig 3, *I*). These data suggest that pre-existing upregulation of pulmonary type I interferons, viperin, and *Oas1a* before RSV infection could underpin *H polygyrus*–induced protection against RSV infection in the lung.

H polygyrus–induced protection against RSV infection requires type I interferon receptor signaling

Because ISGs, including viperin and *Oas1a*, are expressed upon type I interferon receptor signaling, we used *Ifnar1*-deficient mice, which do not signal in response to IFN-α and IFN-β. In $Ifnar1^{-/-}$ mice the reduction of RSV load in the setting of *H polygyrus* coinfection was lost, implying an essential role for this pathway in *H polygyrus*–induced protection against RSV infection (Fig 4, *A*). Furthermore, the ISG induction seen in wild-type mice is also lost in $Ifnar^{-/-}$ mice after *H polygyrus* infection (Fig 4, *B* and *C*).

The cathelicidin-related AMP is upregulated during *H polygyrus* infection but is not required for expression of type I interferon and ISGs

Cathelicidins are a family of small cationic peptides with microbicidal and immunomodulatory properties.[47] Human subjects and mice have only one cathelicidin, LL-37 and murine cathelicidin-related antimicrobial peptide (mCRAMP), respectively, both of which have direct antiviral activity against RSV.[32,48] Cathelicidins have also been shown to promote type I interferon production by dendritic cells (DCs)[49,50] and enhance responses to viral RNA.[51] Interestingly, expression of *Camp* (encoding mCRAMP) was also found to be upregulated in both the duodenum and lung (see Fig E4, *A* and *B*, in this article's Online Repository at www.jacionline.org) during *H polygyrus* infection, with expression peaking before peak type I interferon and ISG expression and remaining increased 1 hour after RSV infection (see Fig E4, *C*). These data suggested that *Camp* expression might be upstream of these responses. Thus *H polygyrus*–induced type I interferon and ISG expression was investigated in cathelicidin-deficient $(Camp^{-/-})$ mice and found to be intact (see Fig E4, *D-F*). This indicates that, although potentially contributing to the innate defense against RSV infection, mCRAMP is not the initiator of and is not required for the protective antiviral immune response induced by *H polygyrus* infection.

H polygyrus adult excretory-secretory products are not responsible for effects on RSV infection, whereas larval stages alone confer protection

Much interest has been building around the prospect of helminth excretory-secretory products as potential therapeutics.[52] *Heligmosomoides polygyrus* excretory-secretory (HES) products, as secreted by adult worms collected from the intestinal lumen, have been shown to have systemic effects in models of disease and to mimic the effects of live infection.[53] HES products were administered in various regimens through the intranasal and intraperitoneal routes the day before RSV infection, for a week before infection, and both before and after infection and also by means of continuous HES product treatment through an intraperitoneal osmotic minipump. None of these protocols resulted in significant reduction in viral titers when compared with those in RSV-infected control mice without HES product treatment (see Fig E5 in this article's Online Repository at www.jacionline.org).

The lack of protection afforded by adult worm products, together with the lack of requirement for an adaptive immune response, caused us to question whether adult worms play any role in the interaction with RSV or whether larval stages of *H polygyrus* and the damage associated with their initial invasion of submucosal tissue is key. Therefore we irradiated stage 3 *H polygyrus* larvae as a nonlethal means of preventing their maturation to adulthood.[54] Consequently, larvae are able to penetrate the duodenal wall and enter into the submucosa, causing initial trauma associated with infection, but do not re-emerge into the lumen as adults. Irradiated larvae also reduced RSV titers and induced *Ifnb*, *Oas1a*, and viperin expression (Fig 5). No adults were found in the lumen in the 300 Gy–treated group, and numbers were severely reduced after 100 Gy irradiation of larvae, but granulomas were observed in all groups on the duodenal serosa (data not shown), confirming that the irradiated larvae were still able to invade the intestinal mucosal epithelium.[55]

The presence of gut microbiota is essential for *H polygyrus*–induced protection against RSV infection

Larval stages of *H polygyrus* protected against RSV infection, and this effect could be attributed to either the direct damage caused on larval penetration of the submucosa, the consequent translocation of intestinal bacteria into the mucosal tissues, or both. To ascertain whether the microbiota play an important

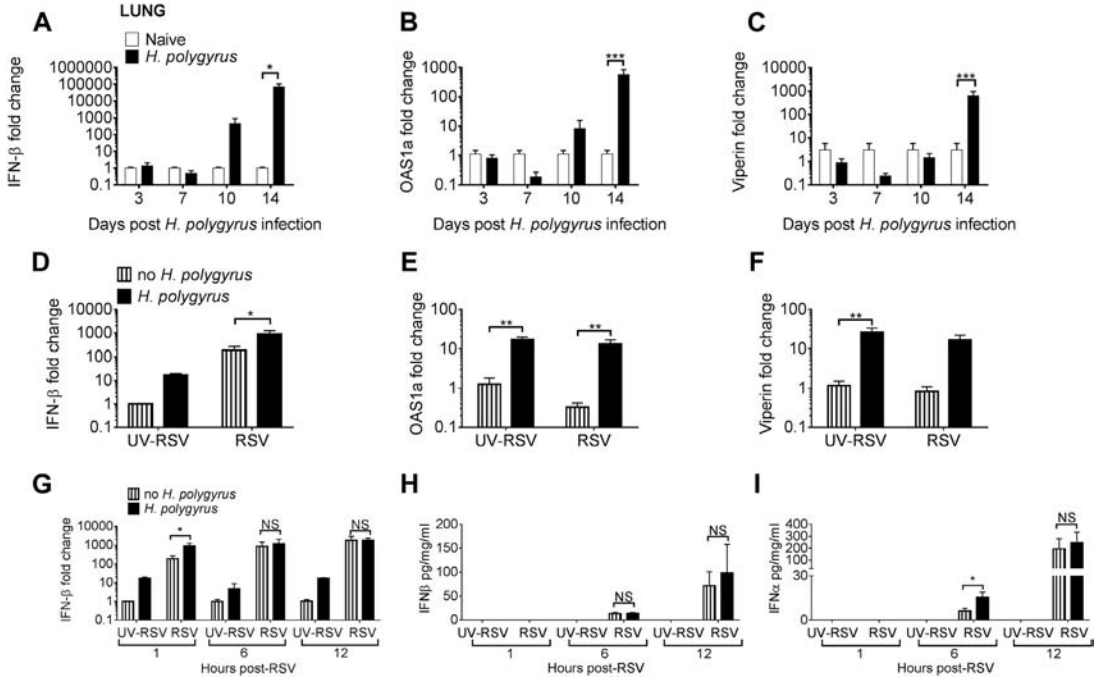

FIG 3. *H polygyrus* induces type I interferons and ISG expression in the lung. **A-C,** BALB/c mice were given 200 L3 *H polygyrus* larvae or left naive. At indicated time points after *H polygyrus* infection, half of the large left lung lobe was placed in TRIzol, and RT-PCR was performed for expression levels of *Ifnb* (Fig 3, *A*), *Oas1a* (Fig 3, *B*), or viperin (Fig 3, *C*) in lung tissue comparing *H polygyrus*–infected with naive mice. **D-I,** The standard coinfection protocol was followed in BALB/c mice. One hour after RSV infection, half of the large left lung lobe was placed in TRIzol, and RT-PCR was performed to measure expression levels of *Ifnb* (Fig 3, *D*), *Oas1a* (Fig 3, *E*), and viperin (Fig 3, *F*). Fig 3, *G*, One (data from Fig 3, *A*), 6, and 12 hours after RSV infection, half of the large left lung lobe was placed in TRIzol and RT-PCR was performed for expression levels of *Ifnb*. Fig 3, *H* and *I*, The large left lung lobe was homogenized, and IFN-β (Fig 3, *H*) and IFN-α (Fig 3, *I*) protein levels were analyzed by ELISA. In Fig 3, *A-G*, results were normalized to *18S* expression and represented as fold change in expression over that in naive control mice (Fig 3, *A-C*) and UV-RSV–infected control mice (Fig 3, *D-G*). Data are depicted as means ± SEMs. Data are pooled in Fig 3, *A-H*, from 2 independent experiments (total n = 6-8 per group) and in Fig 3, *I*, from 2 individual experiments (total n = 10 per group). Statistical significance of differences between groups was determined in Fig 3, *A-C*, by using the 1-way ANOVA with the Bonferroni *post hoc* test and in Fig 3, *D-I*, by using 2-way ANOVA with the Bonferroni *post hoc* test. **P* < .05, ***P* < .01, and ****P* < .001. *NS*, Nonsignificant.

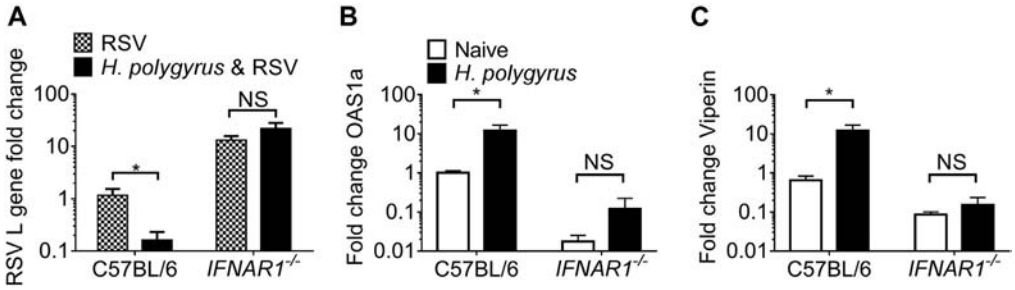

FIG 4. Type I interferon signaling is essential for *H polygyrus*–induced protection against RSV. **A,** The standard coinfection protocol was followed in C57BL/6 wild-type or *Ifnar1*-deficient mice or were given 200 L3 *H polygyrus* larvae or left naive. Three days after RSV infection, half of the large left lung lobe was placed in TRIzol, and RT-PCR was performed for expression of RSV L gene. **B** and **C,** Ten days after *H polygyrus* infection, half of the large left lung lobe was placed in TRIzol, and RT-PCR was performed to measure expression levels of *Oas1a* (Fig 4, *B*) or viperin (Fig 4, *C*) in lung comparing *H polygyrus*–infected mice with naive mice. All results were normalized to *18S* expression and represented as fold change in expression over naive/RSV control values. Data are depicted as means ± SEMs. Data are pooled from 2 independent experiments (total n = 6-10 per group). Statistical significance of differences between groups was determined by using 2-way ANOVA with the Bonferroni *post hoc* test. **P* < .05. *NS*, Nonsignificant.

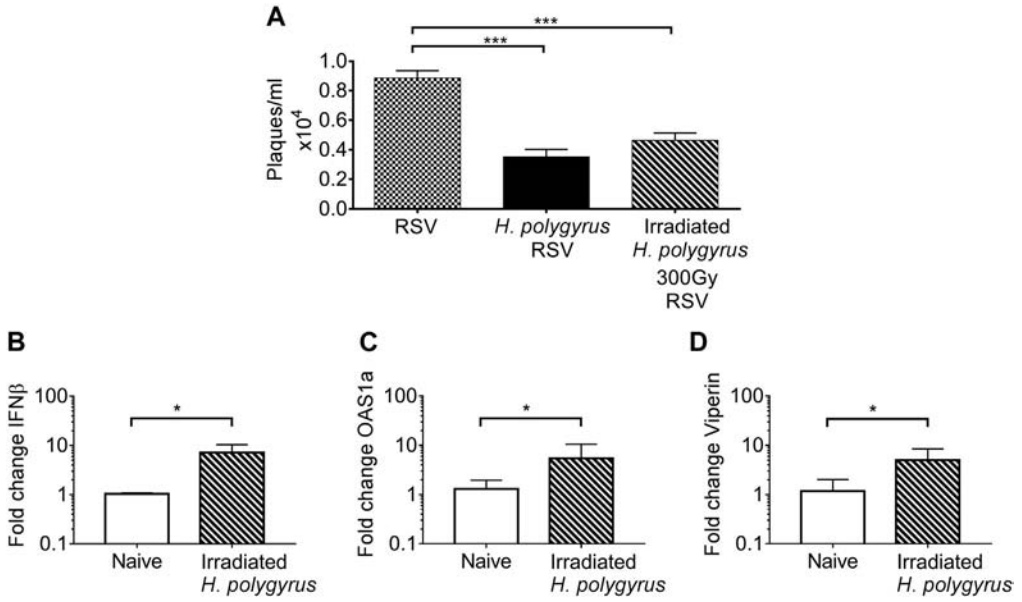

FIG 5. *H polygyrus* larval stages are sufficient to protect against RSV infection. Two hundred L3 *H polygyrus* larvae were irradiated at 300 Gy and compared with nonirradiated larvae in a standard coinfection protocol **(A)** or naive control mice after *H polygyrus* infection alone **(B-D)**. Fig 5, *A*, Lungs were harvested on day 4 of RSV infection, and plaque assays were performed. Fig 5, *B-D*, On day 10 of *H polygyrus* infection, the right lung lobes were removed and placed in TRIzol for RT-PCR for *Ifnb*, *Oas1a*, and viperin. All results were normalized to *18S* expression and represented as fold change in expression over control values. All data are depicted as means ± SEMs. The entire figure is representative of 2 individual experiments (total n = 3-4 per group). Statistical significance of differences between groups was determined in Fig 5, *A*, by using 1-way ANOVA with the Tukey *post hoc* test and in Fig 5, *B-D*, by using the unpaired *t* test. $*P < .05$ and $***P < .001$.

role in protection, we studied RSV infection in germ-free mice in the presence or absence of *H polygyrus* infection.

In contrast to fully colonized SPF mice, in germ-free mice RSV titers and RSV L gene expression were not suppressed by *H polygyrus* coinfection (Fig 6, *A* and *B*). Furthermore, the upregulation of type I interferon expression seen in the lungs and duodenum of *H polygyrus*–infected SPF mice was absent in *H polygyrus*–infected germ-free mice (Fig 6, *C* and *D*). These data support a model in which the microbiota plays a critical role in the induction of type I interferons and ISGs during *H polygyrus* infection, which in turn leads to functional antiviral protection in the lung.

DISCUSSION

Here we demonstrate that a strictly enteric helminth can have protective effects against RSV infection in the lung through a mechanism mediated by microbiota-dependent type I interferon production. First, we established that coinfection with *H polygyrus* ameliorated RSV-induced disease (manifesting as weight loss and increased respiratory effort), as well as reducing the production of proinflammatory cytokines and infiltration of immune cells (NK cells, cDCs, $CD8^+$ T cells, and B cells) into the lungs. Unexpectedly, this was associated with and presumably a consequence of a reduction in RSV load after *H polygyrus* coinfection. These protective effects were found to be independent of adaptive immune responses, including T_H2 responses, as demonstrated in $Rag^{-/-}$ and $Il4r\alpha^{-/-}$ mice, respectively. In addition, these protective effects could not be replicated with HES product treatment instead of live infection. Finally, enteric helminth infection upregulated antiviral type I interferon, ISG, and *Camp* gene expression in both the duodenum and lung, and the protective effects of *H polygyrus* on RSV infection were dependent on type I interferon receptor signaling and the presence of microbiota, as demonstrated in $Ifnar1^{-/-}$ and germ-free mice, which were not protected against RSV infection by *H polygyrus*.

The role of helminths in coinfections is not well understood.[21] In particular, viral respiratory tract infection in the context of coinfection with helminths has not been investigated in epidemiologic studies or in any great detail in animal models. *H polygyrus* coinfection has previously been shown to reduce influenza virus titers and antibodies against the virus regardless of the lifecycle stage of helminth used.[24] In addition, *Trichinella spiralis* was found to have protective effects against influenza infection that were dependent on the intestinal phase of infection, enhancing weight gain after influenza-induced weight loss and reducing cellular infiltration into the lung.[25] These observations are similar to the reduced weight loss observed in the *H polygyrus* and RSV coinfection model reported here and the reduced cellular infiltrate into the lung. However, the mechanisms involved in this protection were not elucidated in previous studies. More recently, chronic infection with *Schistosoma mansoni* provided significant protection against lethal influenza infection and infection with pneumovirus of mice.[56] This was found to be dependent on the presence of eggs, which are known to cause significant damage to the gut wall. *S mansoni* induced TNF-α–dependent induction of Muc5ac and led to goblet cell hyperplasia in the lung, indicating increased epithelial barrier function. However, this was independent of type I interferon production and without any increase in type I interferon levels in the lungs of *S mansoni*–infected mice over control values.

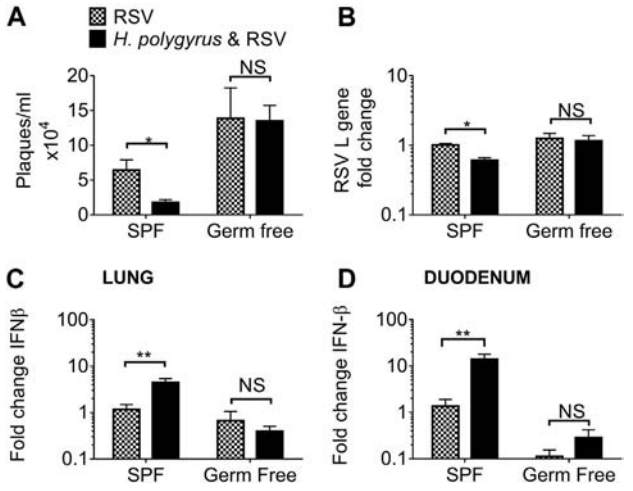

FIG 6. Microbiota are required to protect against RSV infection. The standard coinfection protocol was followed in BALB/c germ-free and SPF mice by using 400 L3 germ-free *H polygyrus* larvae and 3×10^7 sterile RSV in 100 μL. **A,** On day 4 after RSV infection, the left lung lobe was removed, and plaque assays were performed. **B** and **C,** Right lung lobes were removed and placed in TRIzol for RT-PCR for RSV L gene (Fig 6, *B*) or *Ifnb* expression (Fig 6, *C*). **D,** The first centimeter of the duodenum was removed and placed in TRIzol, and RT-PCR was performed to determine expression of *Ifnb*. Results in Fig 6, *B-D*, are normalized to *18S* and represented as fold change in expression over SPF RSV-infected control values. All data are depicted as means ± SEMs. All data are representative of 2 individual experiments (total n = 3-4 per group). Statistical significance of differences between groups was determined by using the unpaired *t* test. $*P < .05$ and $**P < .01$. *NS,* Nonsignificant.

Helminths induce a strong T_H2 immune response, which is characterized by high levels of IL-4, IL-5, and IL-13; infiltration of eosinophils, basophils, and alternatively activated macrophages; and high IgE production.[38-40] In recently reported murine models, helminths induced type 2 immune responses, and associated alternative macrophage activation aggravated γ-herpesvirus and norovirus infection.[22,23] However, our data show clearly that the T_H2 response is not involved in protection against RSV, which was maintained in *Il4rα*$^{-/-}$ mice. In fact, the helminth-induced adaptive immune response was altogether dispensable for protection, indicating an important role for the innate antiviral immune response.

Type I interferons are an important part of the innate antiviral immune response that can be triggered through activation of pathogen recognition receptors by viral components. They not only have direct antiviral activity but also have the ability to upregulate the expression of ISGs, which have further antiviral potential, thus limiting viral infection and spread. Type I interferons and ISGs are rapidly upregulated after RSV infection and decrease by 24 hours after infection.[57,58] The ISGs viperin and OAS, have previously been found to play a role in inhibiting RSV infection and have potent antiviral activity.[44,46] In murine models of RSV infection, prior administration of type I interferons results in a decrease in replication and pathology on RSV infection.[59,60] In addition, IFN-β treatment has also been shown to have antiviral effects against RSV through protease induction.[61] Administration of recombinant type I interferons in human subjects has been limited thus far to IFN-α in the context of RSV infection,[62-64] and nasal, but not intramuscular, administration before RSV challenge reduced the signs and symptoms of upper respiratory tract infection.[63] Administration of recombinant ISGs has not been widely explored; however, RSV infection in chinchillas was reduced after transduction of the airways with vectors encoding viperin.[44] However, there is very little evidence linking helminths and type I interferons in the literature. Aksoy et al[65] found that double-stranded structures found in *S mansoni* egg RNA triggered Toll-like receptor 3 activation, which in turn lead to activation of the type I interferon response.[65] In the setting of *H polygyrus* infection, the type I interferon response has been reported previously to inhibit granuloma formation around larval parasites, but expression of the cytokines in direct response to infection was not measured.[66]

H polygyrus infection induced upregulation of *Ifnb* transcript and IFN-α protein levels in the lung at very early (<6 hours after infection) time points. This result, combined with the observation that the protective effect of *H polygyrus* coinfection was lost in *Ifnar1*-deficient mice, indicates that upregulation of type I interferon expression by *H polygyrus* is critical to its antiviral effects. Although we were unable to detect IFN-β protein after *H polygyrus* monoinfection or very early after RSV infection, the extensive gene expression data and in particular the induction of ISGs suggests that *H polygyrus* induces type I interferon production at levels too low to be detected by means of ELISA. We hypothesize that helminth infection, associated bacterial exposure, or both act as weak signals for cells to produce low levels of type I interferon, which, after feedback through the IFNAR, induce ISG transcription. This can prime cells to elicit rapid and strong type I interferon and ISG responses upon encounter of a strong stimulus, such as RSV.[67] Such priming would benefit the host by enabling the fine balance between necessary and rapidly efficient antiviral responses triggered by type I interferons and detrimental inflammation and autoimmunity associated with chronic type I interferon responses.[68] We observed a significant increase in IFN-α protein levels in the lungs of coinfected mice 6 hours after RSV infection. Previous reports indicate that IFN-β is effective in inducing IFN-α production (but not *vice versa*),[69] and therefore we speculate that the early increase in IFN-β production could lead to the observed increase in IFN-α levels. Based on the recently described central role of alveolar macrophages in the production of type I interferons during RSV infection,[70] we speculate that these cell are also the likely source of helminth-induced type I interferons in the lung.

Irradiation of stage 3 *H polygyrus* larvae has been shown previously to inhibit their maturation but allows larval migration into the intestinal submucosa, after which the larvae do not develop further into adults.[54] By taking this approach, we demonstrated that larval stages are sufficient to induce IFN-β and ISG gene expression and to confer protection against RSV infection. Further investigation in germ-free mice revealed a requirement for microbiota in helminth-induced IFN-β upregulation and resistance to viral replication. Therefore it is plausible to speculate that the damage caused by initial penetration of larvae into the submucosa can result in bacterial translocation from the gut and activation or priming of the innate immune response. Indeed, upregulation of type I interferons at epithelial barrier surfaces can reduce bacterial translocation by upregulating tight junctions.[71] Thus bacterial translocation in the intestine during *H polygyrus* infection might induce upregulation of type I interferons systemically to limit such translocation. In addition, commensal, but not pathogenic, bacteria have been shown to induce type I interferon production

and can also provide protection against influenza infection.[72-74] Furthermore, helminth infection has been shown to alter the microbiome in the intestines of both human subjects and mice. A study conducted in Malaysia indicated that helminth-infected subjects had a greater bacterial number and richer diversity, with increases in specific bacterial taxa, than uninfected control subjects.[75] Likewise in mice, parasites, including *Trichuris muris* and *H polygyrus*, have been found to alter the balance of commensals in the intestine.[76,77] A specific increase in *Lactobacillus* species has been noted during *H polygyrus* infection.[77] Interestingly, the administration of *Lactobacillus* species before RSV infection, either through the intranasal or oral routes, can increase antiviral immunity, including an increase in IFN-β levels in the bronchoalveolar lavage fluid and therefore resistance to RSV infection.[78,79]

Viral LRTI with RSV and rhinoviruses in the first years of life has been linked to the development of asthma,[80-82] which helminth infections have been shown to protect against in mouse model systems.[83,84] In parallel, intestinal helminth infections in human subjects have been reported to increase bacterial translocation.[85] Thus we speculate that helminth infection can protect against severe viral respiratory tract infections in early life and that this effect in turn can contribute to a reduced potential for asthma development.

In conclusion, we show that intestinal helminth infection can be beneficial in viral respiratory tract infections. On the basis of our findings, we hypothesize that helminth infection in the gut triggers type I interferon production through bacterial interactions, which leads to systemic type I interferon induction, thus raising preparedness of remote sites, such as the lung, to mount an effective innate response against incoming unrelated viral pathogens. Further work will be required to elucidate the exact mechanisms of *H polygyrus*–induced antiviral effects and thus inform potential translation toward new helminth-based approaches to the prevention and treatment of viral respiratory tract disease.

We thank Richard Gallo (University of California San Diego) for the *Camp*$^{-/-}$ mice, Judith Allen for the *Il4rα*$^{-/-}$ and *Rag*$^{-/-}$ mice and also Silke Currie, Lauren Melrose, and Emily Gwyer-Findlay for technical assistance.

Key messages

- **Strictly enteric helminth infection induces type I interferon production and ISG expression in both the duodenum and lung.**
- **Helminth-induced type I interferon signaling and the presence of the microbiota are critical for protection against RSV infection.**

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METHODS

Cytometric Bead Array

Half of the left lung lobe was homogenized with a TissueLyser (Qiagen) in 0.5 mL of 1× cell lysis buffer (Cell Signaling, Danvers, Mass) containing 1 μg of phenylmethylsulfonyl fluoride (Sigma). Cytokines present in the lung homogenate were detected through use of a Cytokine Bead Array Flex Set (BD Biosciences), according to the manufacturer's protocol. Samples were collected on the FACS Array (BD Biosciences) and analyzed with FlowJo software (version 7.6.5).

ELISA

IL-33 levels were measured with the R&D Systems ELISA kit, according to the manufacturer's instructions.

Osmotic minipump surgery

Minipumps (Alzet, Cupertino, Calif) were filled with the appropriate volume and concentration of HES products before implantation and primed in saline at 37°C overnight. Mice were started under general anesthesia by using inhalable isoflurane and were given 0.1 mg/kg subcutaneous buprenorphine. The peritoneal cavity area was shaved, and the area was swabbed with alcohol to provide a sterile environment. A midline incision was made just below the ribcage, about 1 cm in length. The musculoperitoneal layer was lifted with forceps to avoid internal damage, and an incision was made in the peritoneal wall beneath. The primed minipump was then inserted into the cavity, with the delivery port entering first, and the wound was then closed with interrupted sutures. Mice were monitored on recovery from anesthetic and were given a further 0.1 mg/kg subcutaneous buprenorphine postoperatively.

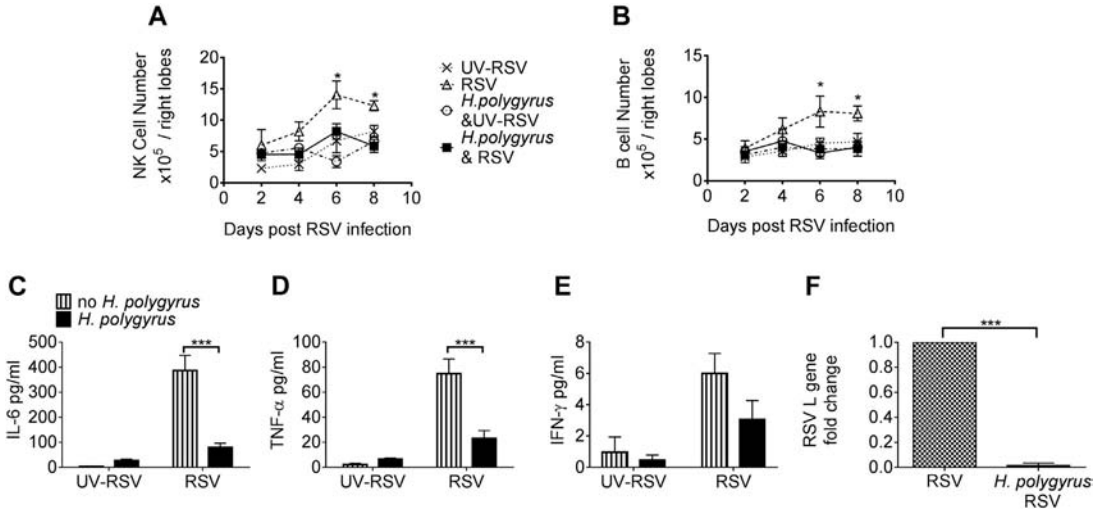

FIG E1. *H polygyrus* infection attenuates RSV inflammation and reduces viral load. The standard coinfection protocol was followed. Samples were taken at the indicated time points after RSV infection for flow cytometric analysis. **A,** Total number of $CD49B^+NKP46^+$ NK cells per right lung lobe. **B,** Total number of MHC class $II^+CD19^+B220^+$ B cells per right lung lobe. **C-E,** Half of the left lung lobe was homogenized, and cytokine levels were analyzed by using the Cytometric Bead Array for levels of IL-6 (Fig E1, *C*), TNF-α (Fig E1, *D*), and IFN-γ (Fig E1, *E*). **F,** The standard coinfection protocol was followed in female C57BL/6 mice. Three days after RSV infection, half of the large left lung lobe was placed in TRIzol, and RT-PCR was performed for expression of the RSV L gene. All data are depicted as means ± SEMs and are pooled from 2 independent experiments (total n = 6 per group per time point). Statistical significance of differences between RSV-infected groups were determined in Fig E1, *A-E*, by using 2-way ANOVA with the Bonferroni *post hoc* test and in Fig E1, *F*, by using the unpaired *t* test. $*P < .05$ and $***P < .001$.

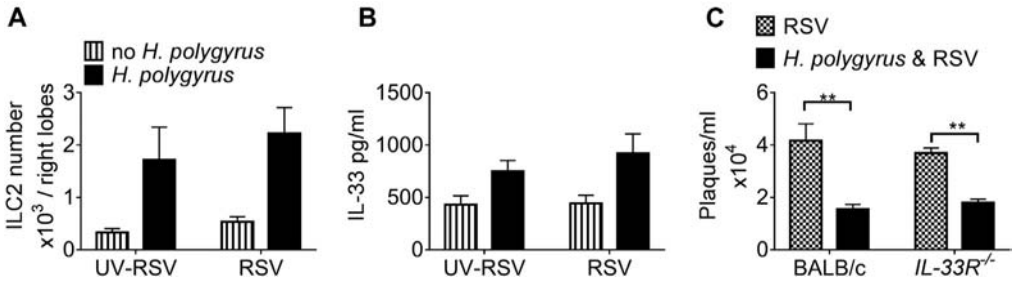

FIG E2. IL-33 is not essential for protection against RSV. The standard coinfection protocol was followed in BALB/c (**A** and **B**) or BALB/c *Il33r*$^{-/-}$ **(C)** mice. Fig E2, *A*, Samples were taken 1 hour after RSV infection for flow cytometric analysis of numbers of ICOS^{+}IL-13^{+} innate lymphoid cells per right lung lobe. Fig E2, *B*, Half of the left lung lobe was homogenized, and cytokine levels were analyzed by means of ELISA. Fig E2, *C*, The standard coinfection protocol was followed in BALB/c *Il33r*$^{-/-}$ mice. Lungs were harvested on day 4 of RSV infection, and plaque assays were performed to determine titers. All data are depicted as means ± SEMs. Data are pooled from 2 independent experiments (total n = 6-8 per group). Statistical significance of differences between groups was determined by using 2-way ANOVA with the Bonferroni *post hoc* test. $^{**}P < .01$.

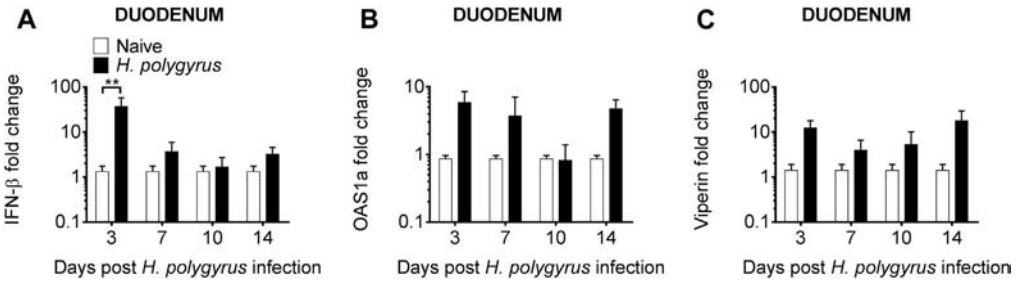

FIG E3. *H polygyrus* induces type I interferon and ISG expression in the intestine. BALB/c mice were given 200 L3 *H polygyrus* larvae or left naive. The first centimeter of the duodenum was placed in TRIzol, and RT-PCR was performed for expression levels of *Ifnb* **(A)**, *Oas1a* **(B)**, and viperin **(C)** comparing *H polygyrus*–infected with naive mice. Results were normalized to *18S* expression and represented as fold change in expression over naive control values. Data are depicted as means ± SEMs. Data are pooled from 2 independent experiments (total n = 6-8 per group). Statistical significance of differences between groups was determined by using 1-way ANOVA with the Bonferroni *post hoc* test. $^{**}P < .01$.

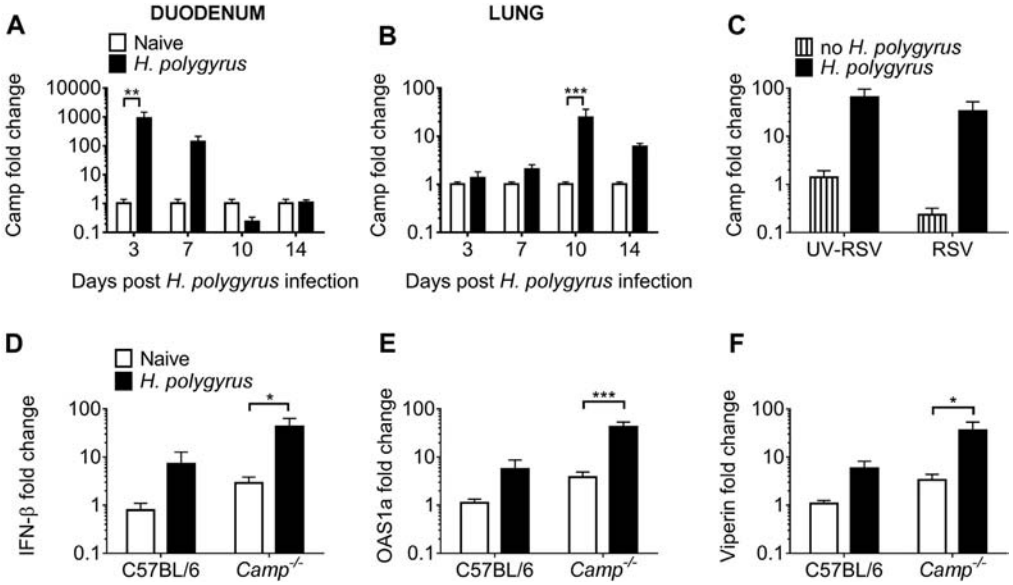

FIG E4. *Camp* does not drive type I interferon ad ISG expression in the lung. BALB/c mice were given 200 L3 *H polygyrus* larvae or left naive. **A** and **B,** The first centimeter of the duodenum (Fig E4, *A*) and half of the large left lung lobe (Fig E4, *B*) were placed in TRIzol, and RT-PCR was performed to measure expression levels of *Camp* comparing *H polygyrus*–infected with naive mice. **C,** The standard coinfection protocol was followed in BALB/c mice. One hour after RSV infection, half of the large left lung lobe was placed in TRIzol, and RT-PCR was performed for expression levels of *Camp*. **D-F,** C57BL/6 or *Camp*$^{-/-}$ mice were given 200 L3 *H polygyrus* larvae administered by means of oral gavage or left naive. Half of the large left lung lobe was placed in TRIzol, and RT-PCR was performed for measurement of expression levels of *Ifnb* (Fig E4, *D*), *Oas1a* (Fig E4, *E*), and viperin (Fig E4, *F*). All results were normalized to *18S* expression and represented as fold change in expression over naive control values (Fig E4, *A* and *B*), UV-RSV–infected control values (Fig E4, *C*), or C57BL/6 naive control values (Fig E4, *D-F*). Data are depicted as means ± SEMs. Data are pooled from 2 independent experiments (total n = 6-8 per group). Statistical significance of differences between groups was determined in Fig E4, *A* and *B*, by using 1-way ANOVA with the Bonferroni *post hoc* test and in Fig E4, *C-F*, by using 2-way ANOVA with the Bonferroni *post hoc* test. **P* < .05, ***P* < .01, and ****P* < .001.

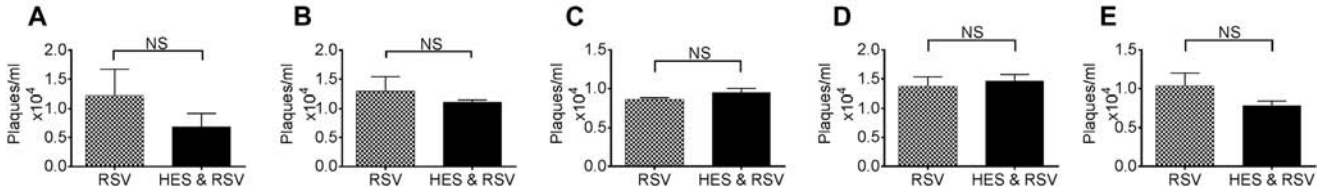

FIG E5. RSV titers are not inhibited by HES product administration. **A-C,** Five micrograms of HES product was administered to mice intranasally on days −1 and 0 (Fig E5, *A*); intranasally on days −7, −4, −1, and 0 (Fig E5, *B*); and intraperitoneally on days −7, −4, and −1 (Fig E5, *C*). **D** and **E,** Osmotic minipumps containing HES products were surgically implanted on days −7 (Fig E5, *D*) and −10 (Fig E5, *E*), releasing 0.25 μL of HES products per hour for 10 or 14 days, respectively. On day 0, 4×10^5 PFU of RSV was administered intranasally. Lungs were harvested on day 4 of RSV infection, and plaque assays were performed. All data are depicted as means ± SEMs (total n = 4 per group). Statistical significance of differences between groups was determined by using the unpaired *t* test. *NS*, Nonsignificant.