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Article

Simvastatin attenuates rhinovirus-induced interferon and CXCL10 secretion from monocytic cells in vitro

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ABSTRACT

RV infections frequently trigger exacerbations of respiratory diseases, such as asthma, yet treatment and intervention options remain limited. Statin drugs are the treatment of choice for dyslipidemia and can also modulate immune cell function. To determine whether statin drugs modify antiviral responses of human monocytic cells, we obtained blood monocytes from donors with allergies and/or asthma and treated the cells with sim prior to challenge with RV. RV-induced secretion of CXCL10 was attenuated significantly, irrespective of RV type (RV-16, -14, or -1A), which corresponded with decreases in IFN-α secretion and pSTAT1. Sim pretreatment also reduced RV-induced CXCL10 secretion from human alveolar macrophages. The addition of mev and GGPP—two intermediates of the cholesterol biosynthesis pathway—was able to rescue CXCL10 release fully, demonstrating that effects of sim were related to inhibition of cholesterol biosynthesis and not to an off-target effect. In addition, sim pretreatment attenuated IFN-α-induced pSTAT1 and CXCL10 secretion, providing evidence that sim additionally can affect type I IFNR signaling. *SOCS1* and *-3* mRNA are both induced with RV stimulation, but sim did not elevate *SOCS1* or *SOCS3* mRNA expression basally or in the presence of RV. Our findings suggest that sim inhibition of the cholesterol biosynthesis pathway leads to decreased RV-induced chemokine secretion in monocytes and macrophages. These findings suggest that statin drugs have the potential to curb the inflammatory response to RV infection. *J. Leukoc. Biol.* 95: 000–000; 2014.

Introduction

Viral infections are an established risk factor for exacerbations of chronic lower-airway diseases, such as asthma. In adults, up to 50% of asthma exacerbations are a result of viral infections, with two-thirds of those infections attributed to RV, the principal cause of the common cold [1]. Virus-induced asthma exacerbations lead to increased doctor and hospital visits, loss of control of asthma symptoms, and overall decreased quality of life [2]. Treatment options remain limited, despite the prevalence of viral-induced exacerbations.

The primary target of RV infection and site of replication are the bronchial epithelium in the upper and lower airway [3]. Recently, RV has been shown to colocalize with airway monocytes/macrophages [4]. Additionally, we have shown previously that coculture of epithelial and monocytic cells results in a synergistic release of proinflammatory mediators, such as CXCL10 [5]. Monocytic cells represent up to 90% of immune cells in the resting lung and alone contribute to the immune response to RV through the synthesis of chemokines, cytokines, and IFNs, independent of viral replication [6, 7].

RV binding and cellular internalization promote monocytic cells to produce IFN-α, which acts via the type I IFNR to activate STAT1 and promote CXCL10 secretion [7]. CXCL10 is a chemoattractant for a variety of immune cells, including T cells, NK cells, and eosinophils, all of which play a role in asthma pathophysiology. During RV illnesses and exacerbations of asthma, CXCL10 is increased in airway secretions and serum and may contribute to virus-induced inflammation [8]. As such, understanding the RV-induced sensing and signaling pathways that may modulate CXCL10 production and secretion may provide novel therapeutic targets.

HMG-CoA reductase inhibitors, also known as statin drugs, are ubiquitous and effective therapeutics for dyslipidemia, by reducing LDL levels. In addition to their cholesterol-lowering effects, statin drugs are immunomodulators [9]. The therapeutic dosage of statins for potential anti-inflammatory effects has

Abbreviations: BAL = bronchoalveolar lavage, FPP=farnesyl pyrophospate, GGPP=geranylgeranyl pyrophosphate, HMG-CoA=3-hydroxy-3-methyl-glutaryl-CoA, MCM=monocyte complete media, mev=mevalonate, MTS=3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium, pSTAT=phosphorylated STAT, qPCR=quantitative PCR, RV=rhinovirus, sim=simvastatin, SOCS=suppressor of cytokine signaling molecules, veh=vehicle

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not yet been identified and may be different from doses required for cholesterol-lowering effects. Many of the in vitro anti-inflammatory effects in immune cell lines require doses much higher than physiological statin blood levels [10]. However, there is evidence that administration of statins via inhalation increases anti-inflammatory effects by 20- to 100-fold compared with i.p. injection or gavage in a mouse model of allergic asthma [11], and local concentrations after inhalation would be quite high. The anti-inflammatory effects of statins are mainly attributed to the inhibition of cholesterol biosynthesis intermediates FPP and GGPP. Depletion of FPP and GGPP reduces intracellular concentrations of isoprenylated, small GTPase signaling molecules, important in a number of cell-signaling pathways, many of which are involved in immune responses. As such, statin drugs have been proposed for the treatment of asthma and other inflammatory diseases [12–14].

Clinical studies to test anti-inflammatory properties of statins in asthmatic patients have yielded conflicting results. In some reports, statin use reduces asthma-associated hospitalization and enhances anti-inflammatory effects of inhaled corticosteroids [15, 16]. Conversely, other findings suggest a lack of steroid-sparing effects or improvement in lung function associated with statin treatment [17, 18]. However, these studies did not consider effects of statin drugs on seasonal and environmental modulators of asthma exacerbations, such as viral infections.

With the increasing appreciation of statins as immunomodulators, there are no data in human cell models testing the role of statins in modulating the immune response to common viral pathogens known to worsen asthma symptoms in monocytic cells. To test the hypothesis that statins modulate the viral immune response to RV, we examined the effects of sim, a commonly prescribed statin drug, on monocytic cells isolated from subjects with respiratory allergies or persistent asthma. We found that sim inhibits monocyte activation in response to RV, as measured by IFN-α and CXCL10 release and pSTAT1. These data suggest that statin drugs dampen the inflammatory response to RV infections.

MATERIALS AND METHODS

Reagents

Activated InSolution sim sodium salt (veh H_2O, 0.1%) was purchased from Calbiochem (Darmstadt, Germany). Mev (veh H_2O, 0.03%), FPP (veh MeOH, 0.2%), GGPP (veh MeOH, 0.2%), and atorvastatin (veh DMSO, 0.06%) were purchased from Sigma (St. Louis, MO, USA). Human rIFN-α2b (Intron A) was purchased from Schering (Kenilworth, NJ, USA). Immunoblotting antibodies anti-STAT1, anti-rabbit IgG, and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-actin from BD Transduction Laboratories (Franklin Lakes, NJ, USA); and anti-pSTAT1 pY^{701} from Cell Signaling Technologies (Danvers, MA, USA). Coating/biotinylated antibodies and the recombinant protein for CXCL10 ELISAs were purchased from R&D Systems (Minneapolis, MN, USA). Flow cytometry antibodies V450-conjugated anti-CD14 (Clone MΦPg) was purchased from BD Horizon (San Jose, CA, USA), and PE-conjugated anti-CD54 (ICAM-1, Clone HA58) was from BD PharMingen (San Jose, CA, USA).

Purification of peripheral blood monocytes and isolation of alveolar macrophages

PBMCs and BAL macrophages were received from volunteer donors under informed consent at the University of Wisconsin Hospital, in accordance with a protocol approved by the Human Subjects Committee. Donors were men and women between the ages of 18 and 50 with confirmed respiratory allergies and/or mild, persistent asthma, according to American Thoracic Society criteria [19]. Exclusion criteria included smoking, pregnant/lactating females, albuterol use within 2 weeks or corticosteroid use within the last month of the screen, unstable asthma based on self-report, and presence of other major health problems (heart disease, diabetes, other lung diseases besides asthma). Peripheral blood monocytes and BAL macrophages were purified and isolated as described previously [7]. Blood monocytes were maintained in MCM containing RPMI 1640 (Mediatech, Herndon, VA, USA), supplemented with 10% FBS (Hyclone, Logan, UT, USA), 100 U/ml penicillin/streptomycin, 2 mM sodium pyruvate, and 2 mM L-glutamine (all from Mediatech). In addition, 0.25 μg/mL amphotericin B was added to BAL macrophage media (Mediatech).

RV production and purification

RV-16, -14, and -1A were grown in HeLa cells and sucrose-purified, and infectivity (plaque-forming units) was determined by plaque assay in HeLa cell monolayers, as described previously [20]. Viruses were suspended in HBSS plus 0.1% HSA.

Cell viability

Blood monocytes were plated at 1×10^5 cells/0.1 mL media in 96-well plates (Sarstedt, Newton, NC, USA) and incubated overnight (16 h) at 37°C. The cells were treated with 0.1–10 μM sim for 24 h. The cells were then treated with MTS and the electron-coupling reagent phenazine methosulfate, according to the manufacturer's protocol (Promega, Madison, WI, USA). The cells were incubated at 37°C for 2 h, and the amount of formazan was measured by absorbance at 490 nm.

Detection of IFN-α and CXCL10 secretion

Blood monocytes were cultured at 1×10^6 cells/well and BAL macrophages at 5×10^5 cells/well in a 24-well Costar plate in 0.5 mL MCM (Corning, Corning, NY, USA). Blood monocytes were pretreated with 0.1–10 μM sim and BAL macrophages with 10 μm sim for 24 h at 37°C. For cholesterol intermediate rescue experiments, sim (10 μM)-treated cells were also cosupplemented with mev (100 μM), FPP (5 μM), or GGPP (5 μM). Cells were subsequently stimulated with control veh, RV-16, at a MOI of 10 or IFN-α (10 ng/ml) for 24 h at 34.5°C. Supernatants were collected, centrifuged at 15,800 *g* to remove nonadherent cells and debris, and stored at −40°C until analysis. IFN-α was detected using by ELISA (PBI Interferon Source, Piscataway, NJ, USA). CXCL10 concentration was determined using a two-step, sandwich-type ELISA, as described previously [21].

Flow cytometric analysis of cell-surface expression of ICAM-1

Blood monocytes were cultured at 2×10^6 cells/well in a six-well plate in 2 mL MCM. Cells were treated with veh or 10 μM sim for 24 h. Cells were lifted by pipetting and centrifuged at 200 *g*. Cells were suspended in PBS, supplemented with 1% FBS, and 5×10^5 cells were aliquoted per flow tube (BD Falcon; Becton Dickinson, Franklin Lakes, NJ, USA). With the use of fluorescence-minus one controls [22], cells were immunostained with 5 μl V450-conjugated anti-CD14 and 5 μl PE-conjugated anti-ICAM-1 for 45 min at 4°C. Cells were washed, centrifuged at 200 *g*, and suspended in 200 μl PBS + 1% FBS. Dead cells were excluded with 0.3 μg/mL PI. Ten thousand events were collected on a LSRII flow cytometer (Becton Dickinson, Bedford, MA, USA). The data were analyzed on FlowJo analysis software (TreeStar, Ashland, OR, USA). Cells positive for CD14 were gated, and the geometric mean fluorescence intensity was assessed for ICAM-1.

Immunoblotting

Blood monocytes were pretreated with 0.1–10 μM sim for 24 h at 37°C and stimulated with control veh or RV-16 (MOI=10) for 2 h or IFN-α for 30 min at 34.5°C. Supernatants were collected and centrifuged at 15,800 *g* for 1 min to collect nonadherent cells. Cells were lysed in 2× sample buffer (20 mM Tris, pH 6.8, 2 mM EDTA, 1 mM Na_3VO_4, 2 mM DTT, 2% SDS, and 20% glycerol) and added to the cell pellet. Samples were sonicated, boiled, and centrifuged at 15,800 *g* for 5 min to pellet cellular debris. Equal volume of cell lysate was separated by electrophoresis on 10% SDS-polyacrylamide gels, transferred to PVDF membranes, and blocked in 5% BSA/TBST (for pSTAT1) or 5% nonfat dry milk in TBST (STAT1, actin). Membranes were incubated with the primary antibodies to pSTAT1 or actin, followed by secondary antibodies conjugated to HRP. For α-STAT1 blots, pSTAT1 blots were stripped with One-Minute Western Blot Stripping Buffer (GM Biosciences, Rockville, MD, USA) and incubated with α-STAT1, or the lysate from the same sample was run on a separate gel for reference but not for protein-loading control. All blots were normalized to the actin-loading control. The immunoreactive bands were imaged using Epichemi II Darkroom (UVP, Upland CA, USA), and band densitometry was quantified using ImageJ (U.S. NIH, Bethesda, MD, USA).

Real-time qPCR

Blood monocytes were cultured at 1×10^6 cells/well in a 12-well Costar plate (Corning). Cells were treated with veh or 10 μM sim for 24 h and stimulated with RV-16 for 1, 3, 6, or 12 h. The 0-h time-point represents unstimulated cells. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was obtained using the Superscript III system (Invitrogen/Life Technologies, Grand Island, NY, USA). Expression of mRNA was determined by qPCR using SYBR Green Master Mix (SABiosciences, Frederick, MD, USA). *SOCS1* (forward-GCTGGCCCCTTCTGTAGGAT, reverse-TGCTGTGGAGACTGCATTGTC)- and *SOCS3* (forward-GAGACTTCGATTCGGGACCAG, reverse-GAAACTTGCTGTGGGTGACCA)-specific primers were designed using Primer Express 3.0 (Applied Biosystems, Carlsbad, CA, USA), and sequences were compared with the human genome to determine specificity using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast; National Center for Biotechnology Information, Bethesda, MD, USA). The reference gene, β-glucuronidase (forward-CAGGACCTGCGCACAAGAG, reverse-TCGCACAGCTGGGGTAAG), was used to normalize the samples. Data are expressed as fold change compared with veh (0 h) using the ΔΔ comparative cycle threshold method.

Statistical analysis

Data were analyzed with paired Student's *t*-tests (see Figs. 1A, 2A, 3A, 5C, and 6A and B), or two-way ANOVA was used to assess differences between control and sim treatment at multiple time-points (see Fig. 6C and D). One-way ANOVA, followed by Bonferroni's corrected *t*-test for pair-wise comparisons, was used for all other statistical analyses.

RESULTS

Sim attenuates RV-induced CXCL10 release in a dose-dependent manner from human blood monocytes

Monocytic cells and bronchial epithelial cells produce CXCL10 in response to RV-16 stimulation [7, 23]. Thus, we tested the hypothesis that sim pretreatment modulates RV-induced CXCL10 secretion, which is a potential biomarker for RV-induced viral exacerbation [8]. Blood monocytes were pretreated with increasing doses of sim for 24 h and then stimulated with RV-16. Sim significantly attenuated RV-induced CXCL10 release in a dose-dependent manner (**Fig. 1A** and **B**). At 0.1 μM sim, CXCL10 levels trend 24% lower than RV treatment alone. At 1 μM and 10 μM, sim significantly inhibited RV-induced CXCL10 release by 41% and 54%, respectively. Accordingly, pretreatment with 10 μM atorvastatin, another commonly prescribed statin medication, significantly reduced RV-induced CXCL10 secretion by 57% (Supplemental Fig. 1).

Experiments using an additional major group virus, RV-14, and a minor group virus, RV-1A, yielded results similar to those obtained with RV-16. (Fig. 1C and D). Major group RVs (i.e., RV-16 and RV-14) use ICAM-1, and minor group viruses (i.e., RV-1A) use the LDLR family members as receptors to enter cells [24]. Sim has been reported to reduce ICAM-1 surface expression on circulating monocytes [25] but has no effects on surface expression of LDLR [26]. To test if sim effects on CXCL10 production were secondary to down-regulation of ICAM-1, monocytes were treated for 24 h with veh or sim, and ICAM-1 surface expression was measured on CD14-positive monocytes. Sim had no significant effects on monocyte ICAM-1 expression at 24 h (Supplemental Fig. 2). These data suggest that at the 24-h time-point, sim effects on RV-induced CXCL10 secretion are independent of RV entry receptor expression.

To confirm that the doses of sim did not affect viability and thus, chemokine secretion, metabolic activity of blood monocytes was assessed 24 h after sim treatment (Fig. 1E). There was no statistical difference in metabolic activity at any of the sim concentrations tested.

Mev and GGPP rescue RV-induced CXCL10 release from human blood monocytes

In the presence of sim, monocytes obtain all cholesterol from media, supplemented with 10% FBS. However, the cholesterol biosynthesis pathway is not reversible; thus, products dependent on mev production, such as the precursors for isoprenylated proteins FPP and GGPP, are diminished. Also, statin drugs can have off-target effects in cells, in addition to the inhibition of HMG-CoA reductase [27]. To test whether the effects of sim on chemokine responses are a result of the direct inhibition of the cholesterol biosynthesis pathway, we supplemented sim-treated monocytes with mev, the product of HMG-CoA reductase. The supplementation of sim-pretreated blood monocytes with mev rescued RV-induced CXCL10 release completely compared with cells treated with RV in the absence of sim (Fig. 1F). In addition to mev, sim-treated monocytes were supplemented with FPP and GGPP, two intermediates of the cholesterol biosynthesis pathway that are crucial for the isoprenylation of small G-protein families, such as Ras and Rho family members, respectively. GGPP supplementation rescued RV-induced CXCL10 release completely compared with veh-pretreated/RV-stimulated monocytes (Fig. 1F). FPP had similar but less striking effects. Together, these data support the idea that sim-induced inhibition of the cholesterol biosynthesis pathway is responsible for differences in RV-induced CXCL10 release.

Sim attenuates RV-induced CXCL10 secretion from BAL macrophages

There are intrinsic differences between blood monocytes and terminally differentiated tissue macrophages [28]. To deter-

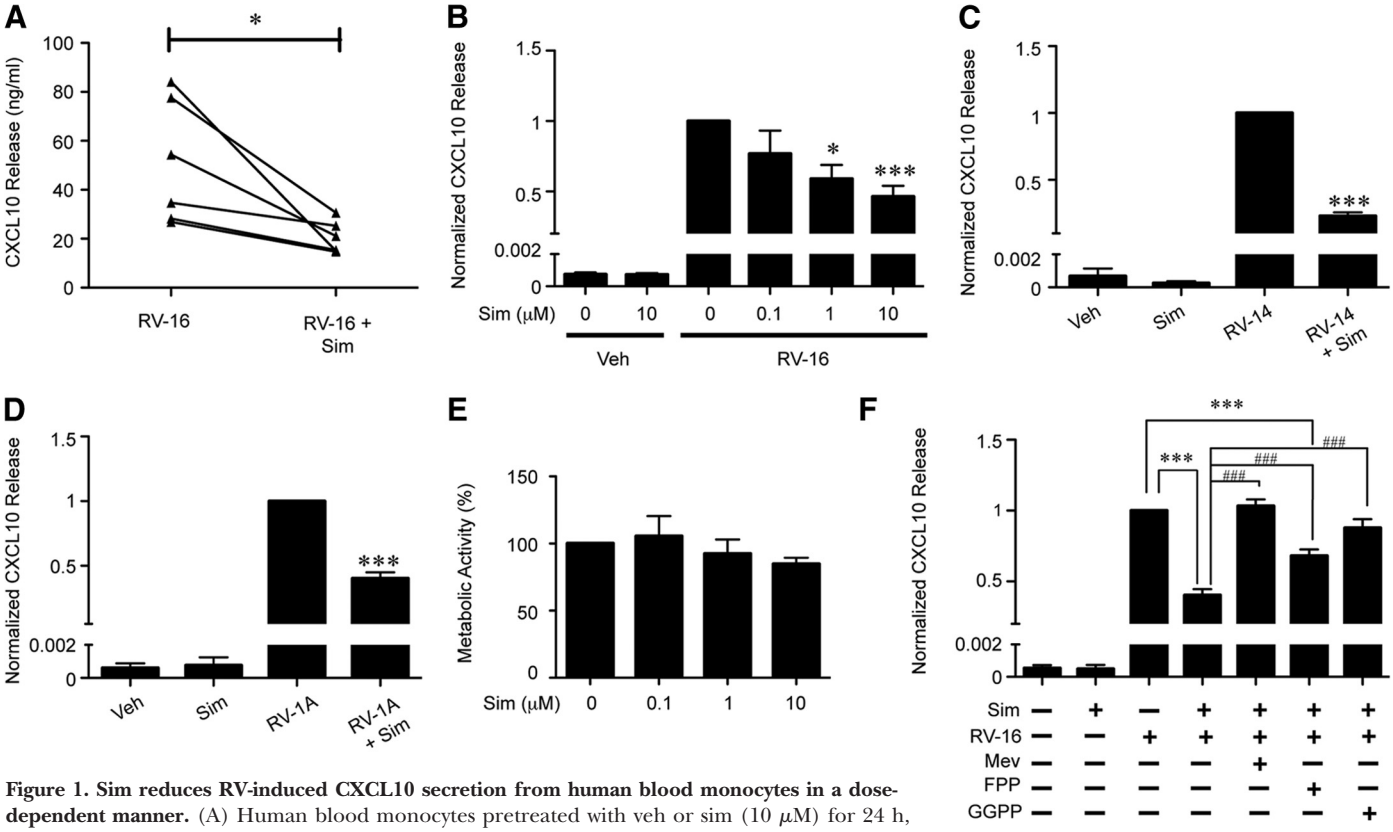

Figure 1. Sim reduces RV-induced CXCL10 secretion from human blood monocytes in a dose-dependent manner. (A) Human blood monocytes pretreated with veh or sim (10 μM) for 24 h, and RV-16 (MOI=10)-induced CXCL10 release at 24 h was measured (n=6). (B) Human blood monocytes were pretreated with increasing doses of sim (0.1, 1, or 10 μM) for 24 h and then stimulated with RV-16 (MOI=10) for 24 h. Data were pooled and normalized to veh-pretreated/RV-16-stimulated CXCL10 release (n=6). (C) Human blood moncytes were pretreated with veh or sim (10 μM) for 24 h, and RV-14 (MOI=10)- and (D) RV-1A-induced CXCL10 release at 24 h was determined. (E) Human blood monocytes were treated for 24 h with veh or increasing doses of sim (0.1, 1, or 10 μM), and metabolic activity was measured by MTS assay. Metabolic activity was normalized to veh (n=3). (F) Human blood monocytes pretreated with veh or sim (10 μM) and supplemented with mev (100 μM), FPP (5 μM), or GGPP (5μM) for 24 h. RV-16 (MOI=10)-induced CXCL10 secretion was normalized to veh-pretreated/RV-16-stimulated monocytes (n=7). Error bars indicate SEM. *$P < 0.05$, ***$P < 0.001$ compared with RV treatment alone; ###$P < 0.001$ compared with sim + RV-16.

mine whether sim exerted similar effects on airway cells, BAL macrophages were pretreated with veh or 10 μM sim for 24 h and then stimulated with RV-16 for 24 h. Similar to observations in blood monocytes, sim pretreatment reduced RV-induced CXCL10 release by 60% (**Fig. 2A** and **B**). These observations confirm that sim has similar effects on virus-induced responses of human blood monocytes and airway macrophages. Taken together, these data indicate that sim pretreatment blunts a monocytic, proinflammatory response to RV.

Sim attenuates RV-induced IFN-α release from human blood monocytes

Secretion of type I IFNs is a hallmark of the innate immune response to RV [23, 29]. Additionally, IFN-α secretion is required for the production of CXCL10 in monocytic cells. To test whether sim additionally attenuates a monocytic cell type I IFN response, human blood monocytes were pretreated with veh or sim for 24 h and then stimulated with RV-16 for 24 h. Similarly to CXCL10, sim pretreatment reduced RV-induced IFN-α release by 65% (**Fig. 3A** and **B**).

Sim reduces RV-induced pSTAT1 in human blood monocytes

IFN-α binds to the type I IFNR and promotes pSTAT1, and this process contributes to RV-induced CXCL10 release [7]. Based on the observations that sim pretreatment decreased RV-induced IFN-α and CXCL10 secretion, we hypothesized that sim would also reduce RV-induced pSTAT1. Human blood monocytes were treated with increasing doses of sim for 24 h and then stimulated with RV-16 for 2 h. sim significantly attenuated RV-induced pSTAT1 (52% and 68% reduction at 1 μM and 10 μM sim, respectively) but did not affect total STAT1 (**Fig. 4**).

Sim reduces IFN-α-induced pSTAT1 and CXCL10 release

As RV-induced IFN-α contributes to CXCL10 production, we tested whether sim pretreatment additionally blunts IFN-α (10 ng/ml)-stimulated pSTAT1 and CXCL10 release. Blood monocytes were pretreated with veh or sim for 24 h and then stimulated with IFN-α for 30 min to detect pSTAT1 activation or 24

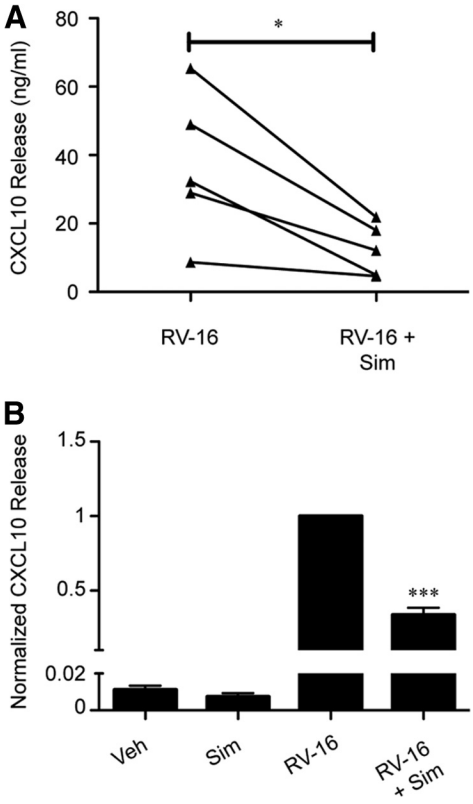

Figure 2. Sim attenuates RV-induced CXCL10 secretion from BAL macrophages. (A) BAL macrophages were pretreated with veh or sim (10 μM) for 24 h, and RV-16 (MOI=10)-induced CXCL10 release at 24 h was determined (n=5). (B) Data were pooled and normalized to veh-pretreated/RV-stimulated CXCL10 release. Error bars indicate SEM. $^*P < 0.05$, $^{***}P < 0.001$ compared with RV treatment alone.

h to assess CXCL10 release. Compared with veh-pretreated/IFN-α-stimulated monocytes, sim pretreatment attenuated IFN-α-induced pSTAT1 levels by 48% (**Fig. 5A** and **B**) and CXCL10 release by 40% (Fig. 5C and D). These data suggest that sim can affect cell-signaling pathways upstream and downstream of the type I IFNR.

Mev, GGPP, and FPP rescue sim-attenuated, IFN-α-induced CXCL10 release

To test whether attenuation in IFN-α-induced effects were also a result of inhibition of the cholesterol biosynthesis pathway, sim-pretreated blood monocytes were supplemented with mev (100 μM), FPP (5 μM), or GGPP (5μM) and then stimulated with IFN-α (10 ng/ml). All three cholesterol-biosynthesis intermediates were able to rescue sim-pretreated/IFN-α-induced CXCL10 release to levels of veh-pretreated/IFN-α-stimulated monocytes (Fig. 5E).

SOCS1 and *SOCS3* are not involved in the sim-induced blockade of type I IFN response to RV

SOCS1 and -3 regulate type I IFNR signaling by preventing pSTAT transcription factors [30]. Previous work in murine RAW264.7 macrophages and human THP-1 premonocytic cell lines found that sim induced expression of *SOCS1* and *SOCS3* mRNA [31]. Therefore, we investigated whether sim pretreatment increased *SOCS1* and *SOCS3* mRNA expression levels in blood monocytes, with or without RV stimulation. Cells were pretreated for 24 h with veh or sim and then stimulated with RV for 0–12 h, and *SOCS1* and *SOCS3* mRNA levels were determined. RV induced *SOCS1* (**Fig. 6A**) and *SOCS3* mRNA (Fig. 6B) at 12 h and 6 h, respectively, but sim did not augment either mRNA in the presence or absence of RV (Fig. 6C and D). These data suggest that sim attenuates RV-induced chemokine release independently of two known regulators of pSTAT1.

DISCUSSION

Herein, we demonstrate that sim can modulate the immune response to RV in primary human monocytic cells. Sim pretreatment attenuates RV-induced CXCL10 secretion in BAL macrophages and blood monocytes. This corresponds with decreases in IFN-α release and pSTAT1 in blood monocytes. Sim did not alter expression of ICAM-1, indicating that this effect

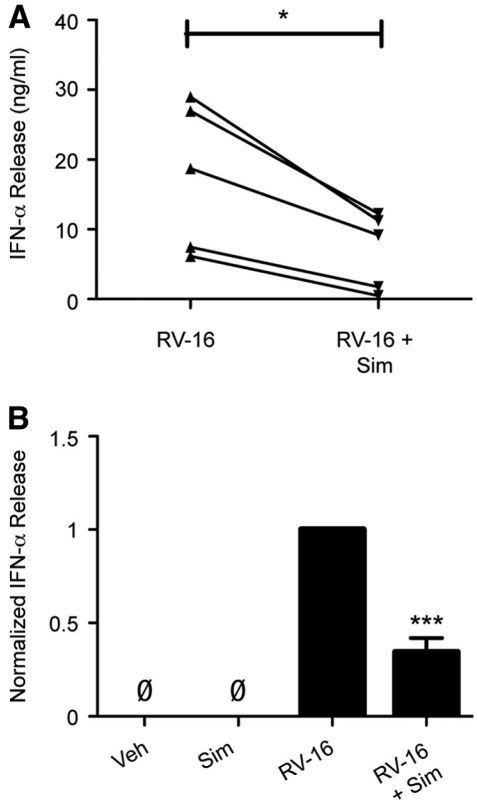

Figure 3. Sim attenuates RV-induced IFN-α release from human blood monocytes. (A) Human blood monocytes, pretreated with veh or sim (10 μM) for 24 h, and RV-16 (MOI=10)-induced IFN-α (pg/mL) release at 24 h were measured (n=5). (B) Data were pooled and normalized to veh-pretreated, RV-16-stimulated IFN-α release (n=5). Error bars indicate SEM. $^*P < 0.05$, $^{***}P < 0.001$ compared with RV-16 treatment alone; ØBasal level of IFN-α below detection limit of 12.5 pg/ml.

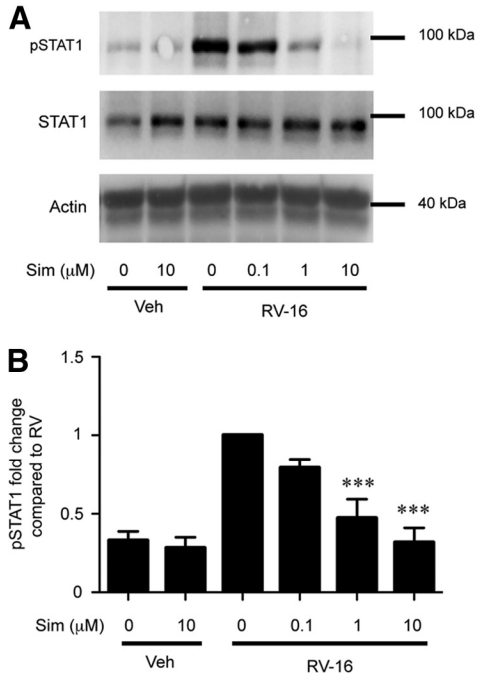

Figure 4. Sim pretreatment decreases RV-induced pSTAT1 in human blood monocytes in a dose-dependent manner. (A) Representative immunoblots for RV-16 (MOI=10) induced pSTAT1, total STAT1, and actin (loading control) at 2 h from human blood monocytes pretreated with veh or sim (0.1, 1, or 10 μM) for 24 h. (B) Band densitometry averages for pSTAT1 corrected with actin and normalized to RV treatment (n=5). Error bars indicate SEM. ***$P < 0.001$ compared with RV treatment alone.

was not a result of reduced binding capacity of the virus. Additionally, sim blocks IFN-α-stimulated pSTAT1 and CXCL10 release, suggesting that sim affects upstream and downstream of the type I IFNR. However, *SOCS1* and *SOCS3* do not appear to be involved in sim inhibition of RV- or IFN-α-induced STAT signaling and CXCL10 release. Overall, these data provide evidence that statin drugs may attenuate RV-induced inflammatory responses.

Significant immunomodulatory effects of sim were seen at levels higher than those achieved with oral administration [32, 33]. However, inhaled sim in a murine model of allergic asthma was well-tolerated and reduced inflammatory responses, such as Th2 cytokines and chemokines, and physiologic outcomes, such as airway remodeling and overall lung function [11]. Additionally, in the dsRNA-induced mouse pneumonia model, intranasal administration of sim decreased STAT3 activation, RANTES release, and neutrophilia [34]. Inhaled sim would be associated with high drug concentrations in the lung; thus, the investigation of the effects of higher levels of sim is of interest.

Our findings also suggest sim as a specific tool to better understand the effects of cholesterol pathways on antiviral responses. We provide evidence that sim modulates the sensing and response pathways of monocytic cells to RV, both upstream of IFN-α production and downstream of the type I IFNR, leading to attenuation of CXCL10 release. Notably, CXCL10 is a potential therapeutic target for viral-induced asthma exacerbation, and the corticosteroid budesonide can decrease RV-induced CXCL10 secretion [8, 35]. The effects of sim on RV-induced CXCL10 are reversed by the cholesterol biosynthesis intermediates mev and GGPP but not FPP. This suggests that Rho small GTPase family members, which are isoprenylated by GGPP, may play a role in RV-induced CXCL10 production. Rho family members are involved in inflammatory cell activation via MAPK and NF-κB signaling [36]. In plasmacytoid DCs, sim decreased TLR9 ligand stimulation of IFN regulatory factory 7 nuclear translocation and IFN-α production in a GGPP-dependent mechanism [37]. Our model suggests that sim may inhibit RV-induced IFN-α and CXCL10 release by affecting transcription factor activation in response to RV, leading to downstream decreases in chemokine secretion. Further understanding the mechanism by which sim attenuates RV-induced CXCL10 will give insight into the regulatory components of this pathway in monocytic cells.

RV infection stimulates IFN production in epithelial and monocytic cells, and IFN secretion secondarily enhances chemokine production (such as CXCL10) and immune cell recruitment to clear the virus. In asthmatics, an excessive proinflammatory response to stimulants, such as RV, can trigger an asthmatic episode [38]. However, whether IFN production is increased or decreased during virus-induced asthma exacerbation remains controversial [39]. In our study, sim pretreatment reduced RV-induced IFN-α release, on average, by 60% (Fig. 4). Previous findings indicate that IFN-α and IFN-λ-1 levels in respiratory secretions tended to be higher in children with wheezing RV infection compared with nonwheezing RV infection [40]. In contrast to these results, infants predisposed to lower antiviral responses, such as lower IFN responses, are more likely to be affected by more severe respiratory infections early in life, which can lead to wheezing, atopy, and higher risk for future asthma development [1]. Furthermore, BAL macrophages isolated from adult asthmatics stimulated with RV-16 have significantly lower IFN-α and IFN-β responses compared with normal patients [41]. Additional studies are warranted to determine whether reduction of IFN-α and CXCL10 impacts the antiviral responses of other cell types to delineate the balance between excessive inflammatory responses to RV seen in asthmatic patients versus a dampened host-defense response to a viral infection.

Our group and others have shown that statins can inhibit the Jak/STAT signaling cascade, which is downstream of IFN release and the type I IFNR [42, 43]. Dysregulation of this pathway has been implicated in the pathogenesis of inflammatory diseases, such as asthma [44]. Even with high-dose stimulation with IFN-α, pSTAT1 expression was still reduced (Fig. 6), suggesting that this pathway can be targeted independently of RV-induced IFN-α secretion. To our knowledge, this is the first study to show that RV induces *SOCS1* and *SOCS3* mRNA, two negative regulators of the Jak/STAT pathway, in primary human monocytic cells. However, we found no evidence that sim induced *SOCS1* or *SOCS3* mRNA, contrary to data from transformed cell lines. There are reports that SOCS family

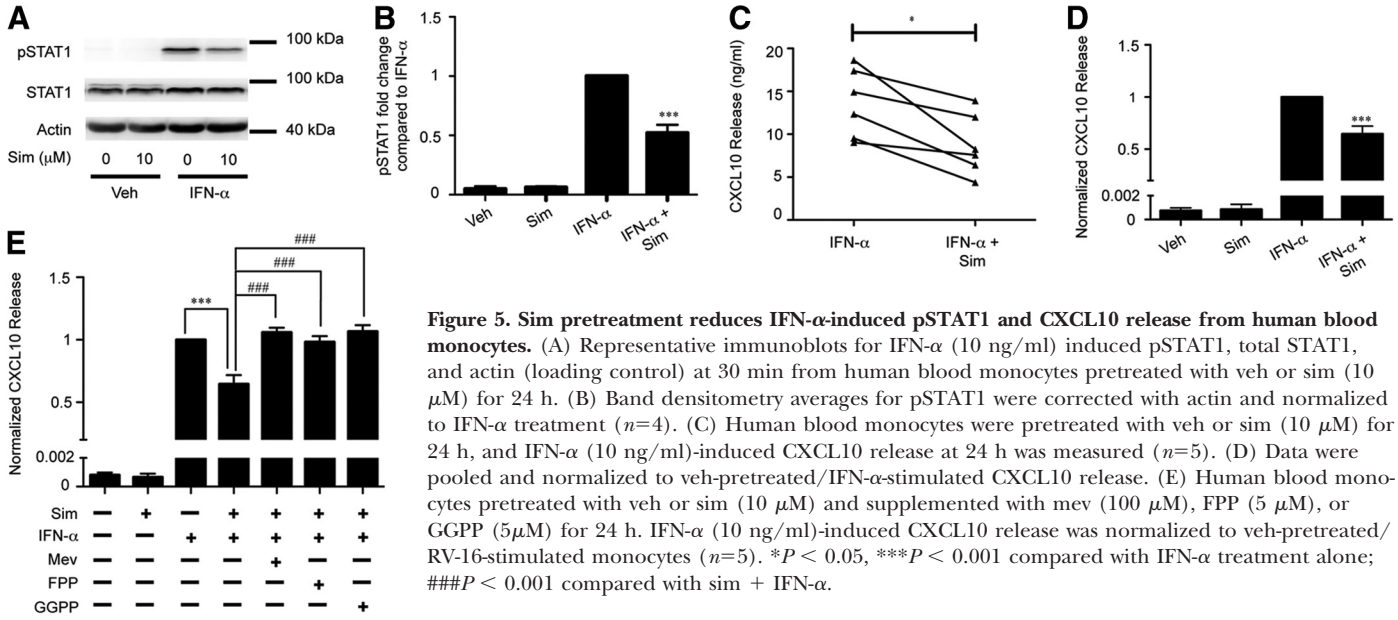

Figure 5. Sim pretreatment reduces IFN-α-induced pSTAT1 and CXCL10 release from human blood monocytes. (A) Representative immunoblots for IFN-α (10 ng/ml) induced pSTAT1, total STAT1, and actin (loading control) at 30 min from human blood monocytes pretreated with veh or sim (10 μM) for 24 h. (B) Band densitometry averages for pSTAT1 were corrected with actin and normalized to IFN-α treatment (n=4). (C) Human blood monocytes were pretreated with veh or sim (10 μM) for 24 h, and IFN-α (10 ng/ml)-induced CXCL10 release at 24 h was measured (n=5). (D) Data were pooled and normalized to veh-pretreated/IFN-α-stimulated CXCL10 release. (E) Human blood monocytes pretreated with veh or sim (10 μM) and supplemented with mev (100 μM), FPP (5 μM), or GGPP (5μM) for 24 h. IFN-α (10 ng/ml)-induced CXCL10 release was normalized to veh-pretreated/RV-16-stimulated monocytes (n=5). *$P < 0.05$, ***$P < 0.001$ compared with IFN-α treatment alone; ###$P < 0.001$ compared with sim + IFN-α.

members are subject to post-transcriptional regulation [45], and it is possible that sim affects the activity of the SOCS protein through this process. Alternatively, there are a number of tyrosine phosphatases involved in the negative regulation of the Jak/STAT pathway that could be affected directly or indirectly by sim treatment [44].

To our knowledge, this is the first study investigating the immunomodulatory properties of statins to the common respiratory virus, RV, in monocytic cells. There are limited data testing effects of sim on outcomes of other viral infections. For example, cotreatment with sim and antiviral treatments, including supplemental IFN-α, synergistically promoted clearance of hepatitis C from human-derived cell lines [46]. Conversely, in mouse models of influenza, researchers found no difference in viral replication and clearance or overall survival with statin treatment [47]. However, there is clinical evidence that statins reduce hospitalization and mortality rates secondary to influenza [47, 48].

This study has a number of strengths and some limitations that should be considered in interpreting the data. Two commonly prescribed statin drugs—sim and atorvastatin—both reduced RV-induced CXCL10 release significantly. Furthermore, the monocytic immune response to three strains of RV representing major (RV-16, RV-14) and minor (RV-1A) groups, as well as type A (RV-16, RV-1A) and type B (RV-14) viruses, was similarly affected by sim. All experiments used primary human cells (blood monocytes and BAL macrophages), and effects were consistent in blood and airway cells. However, all cells were obtained from patients with allergies or mild asthma, and responses in normal individuals could be distinct. Additionally, the duration of sim pretreatment in all of the presented experiments was 24 h, and the long-term effects of statin use could result in differences in the monocytic immune response to RV stimulation. Finally, additional experiments may be warranted to evaluate effects of statins on metabolic activity of alveolar macrophages, as this was not tested in our study.

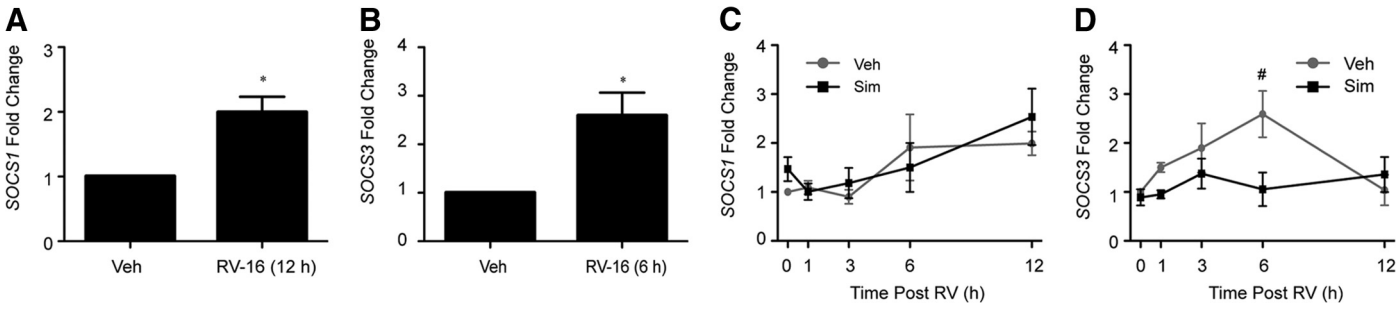

Figure 6. Sim does not induce *SOCS1* or *SOCS3* mRNA. Human blood monocytes stimulated with veh or RV-16, and (A) *SOCS1* mRNA at 12 h or (B) *SOCS3* mRNA at 6 h were determined (n=4). (C and D) Human blood monocytes pretreated with veh or sim (10 μM) for 24 h and stimulated with RV-16 (0–12 h). *SOCS1* mRNA (C) or *SOCS3* mRNA (D) is represented as fold change relative to cells pretreated with veh alone for 24 h (n=4). Error bars indicate SEM. *$P < 0.05$ compared with veh; #$P < 0.05$ compared with sim + RV at 6 h.

In sum, we provide evidence that sim modulates monocytic cell responses to RV infection and IFN-α stimulation. If these effects also occur in vivo, then it is possible that sim may modify the clinical course of RV respiratory infections. The understanding of the clinical effects of a commonly prescribed drug, sim, on viral infections may be especially important for patients with chronic respiratory diseases, such as asthma.

AUTHORSHIP

L.E.W. designed, performed, and analyzed experiments and wrote the manuscript. M.R.K. assisted with experiments and helped edit the manuscript. A.A. and J.E.G. assisted with the design of experiments and editing the manuscript. P.J.B. assisted with the conception of the project and design of experiments.

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DISCLOSURES

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