

Prophylactic benefits of systemically delivered simvastatin treatment in a house dust mite challenged murine model of allergic asthma

Aruni Jha^{1,3,4}, Min H Ryu^{1,3,4}, Ojo OO^{1,3}, Hilary J Bews⁵, Jules C Carlson⁵, Jacquie Schwartz^{1,3}, Sujata Basu^{1,3}, Charles S Wong^{3,5} and Andrew J Halayko¹⁻⁴

Affiliation(s)

¹Departments of Physiology and Pathophysiology, and ²Internal Medicine, University of Manitoba, Winnipeg, MB, Canada; ³Biology of Breathing Group, Children's Hospital Research Institute of Manitoba, Winnipeg, MB, Canada; ⁴Canadian Respiratory Research Network, Ottawa, ON, Canada; and ⁵Richardson College for the Environment, University of Winnipeg, Winnipeg, MB, Canada.

Corresponding Author: Andrew J. Halayko, PhD, FCAHS, Department of Physiology and Pathophysiology, University of Manitoba, Rm 605 John Buhler Research Centre, 715 McDermot Avenue, Winnipeg, MB Canada R3E 3P4. Tel.: 1 204 480 1327; E-mail: andrew.halayko@umanitoba.ca

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AJ: Study design, execution, data analysis and manuscript writing

MR: Study execution and manuscript review

OOO: Study execution and manuscript review

HB: Estimation of simvastatin and simvastatin hydroxy acid in biological samples

JC: Estimation of simvastatin and simvastatin hydroxy acid in biological samples

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Abstract (Word count 241):

Background and Purpose: Systemically delivered statins can blunt airway inflammation in ovalbumin-challenged mice; however, in asthma clinical trials the beneficial effects of introducing oral statins are not compelling. We aim to reconcile this discrepancy using a clinically relevant murine model of allergic asthma, and by including a prophylactic study arm.

Experimental Approach: Adult mice were: 1) challenged with house dust mite (HDM) alone or with subcutaneous (s.c.) simvastatin for two weeks; or 2) also treated with simvastatin for one week prior to HDM challenge. We assayed lung function, inflammatory cell influx and cytokine profile, goblet cell abundance, and simvastatin concentration in serum, lung lavage and tissue.

Key Results: Ultrahigh performance liquid chromatography-tandem mass spectrometry revealed that pharmacologically active simvastatin reached peak serum concentration after 8h, but declined rapidly. Prophylactic treatment doubled peak serum simvastatin and repeated s.c. delivery established stable serum levels, but simvastatin was undetectable in the lungs. Both simvastatin treatment arms suppressed indices of HDM-induced airway inflammation and goblet cell hyperplasia, but this was significantly greater with prophylactic therapy, in particular to inhibit neutrophil and eosinophil influx, and cytokine accumulation. Conversely, neither acute nor prophylactic delivery of simvastatin prevented HDM challenge-induced airway hyperreactivity.

Conclusion and Implications: Systemically administered simvastatin accumulates in the blood, but not in lung tissues, and reduces leukocyte influx and associated lung inflammation. Prophylactic therapy has the greatest anti-inflammatory effects, but as observed in human clinical trials, systemic simvastatin therapy does not prevent allergic airway hyperreactivity.

List of Abbreviations

BALF	Bronchoalveolar Lavage Fluid
FEV1	Forced Expiratory Volume in 1 second
G	Tissue Resistance
H	Tissue Elastance
HDM	House Dust Mite
i.p.	Intraperitoneal
IFN γ	Interferon Gamma
IL	Interleukin
CXCL1	Keratinocyte Chemoattractant/ Human growth-regulated oncogene
MCh	Methacholine
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PC20	Provocative Concentration of MCh to decrease FEV1 by 20%
PEEP	Positive End Expiratory Pressure
PMSF	Phenylmethylsulfonyl Fluoride
R _N	Newtonian Resistance
s.c.	Sub-cutaneous
TGF β 1	Transforming Growth Factor β 1
TNF α	Tumor Necrosis Factor Alpha
UHPLC/MS/MS	Ultrahigh Performance Liquid Chromatography-tandem Mass Spectrometry
Z _{rs}	Respiratory Mechanical Input Impedance

List of Hyperlinks:

hydroxymethylglutaryl-CoA reductase

URL:

<http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=639>

simvastatin

URL:

<http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2955>

Lovastatin

<http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2739>

methacholine

<http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=7438>

pravastatin

<http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2953>

Background:

Statins are competitive inhibitors of hydroxylmethylglutaryl-CoA reductase, the proximal rate-limiting enzyme of the mevalonate cascade. Beyond their capacity to block *de novo* cholesterol synthesis, statins inhibit formation of isoprenoid lipid chains that are needed for membrane anchorage and activation of signaling proteins that regulate cell proliferation, migration, contraction and secretory function (Ghavami et al., 2011; Kume, 2008; Schaafsma et al., 2011a; Zeki, Franzi, Last & Kenyon, 2009). Thus, orally administered statins have pleiotropic systemic effects on endothelial function, platelet aggregation, and inflammation that enhance their therapeutic benefits on cardiovascular health (Ridker et al., 1998; Rosenson & Tangney, 1998). Retrospective epidemiological investigation shows that long-term statin use can also be associated with health benefits for asthmatics; chiefly, reduced emergency room visits and corticosteroid use (Tse et al., 2014; Tse et al., 2013). Some clinical evidence suggests that short-term statin use can enhance the anti-inflammatory effects of inhaled corticosteroids (Maneechotesuwan, Ekjitrakul, Kasetsinsombat, Wongkajornsilp & Barnes, 2010; Samson et al., 2006). However, systematic review of clinical studies, which to date have not included prospective assessment of the prophylactic effects of long-term statin use, reveals a paucity of evidence for short-term oral statin therapy having a significant impact on lung function in asthmatics (Braganza et al., 2011; Cowan, Cowan, Palmay, Williamson & Taylor, 2010; Hothersall et al., 2008; Malek Mohammad et al., 2012; Menzies, Nair, Meldrum, Fleming, Barnes & Lipworth, 2007; Yuan et al., 2012). This contrasts understanding from preclinical studies showing that acute systemic statin therapy can prevent lung inflammation, and may reduce airway hyperreactivity in ovalbumin sensitized rodents (Ahmad et al., 2011; Chiba, Arima, Sakai & Misawa, 2008; Imamura et al., 2009; McKay, Leung, McInnes, Thomson & Liew, 2004; Zeki, Franzi, Last & Kenyon, 2009).

Systemic delivery of simvastatin at the onset of ovalbumin administration in mice prevents airway inflammation by inhibiting lung leukocyte influx, promoting leukocyte apoptosis, and blunting biogenesis of pro-inflammatory cytokines and mediators (Imamura et al., 2009; Luo, Liu, Li & Wang, 2005; McKay, Leung, McInnes, Thomson & Liew, 2004; Zeki, Franzi, Last & Kenyon, 2009). Simvastatin treatment can also prevent ovalbumin exposure-induced airway goblet cell hyperplasia (Zeki, Bratt, Rabowsky, Last & Kenyon, 2010). *In vitro* studies indicate that statins can have direct effects on lung structural cells for example; simvastatin promotes apoptosis and inhibits proliferation of airway smooth muscle cells;

blocks TGF β ₁-induced extracellular matrix protein production by airway fibroblasts and airway smooth muscle cells; and, blocks cytokine release by airway epithelial cells (Ghavami et al., 2011; Schaafsma et al., 2011a; Schaafsma et al., 2011b; Takeda, Kondo, Ito, Ito, Shimokata & Kume, 2006; Zeki, Thai, Kenyon & Wu, 2012). Despite focus on the effects of statins on lung inflammation and remodeling, there is more limited investigation of airway hyperresponsiveness, a hallmark feature of asthma. Lovastatin appears to reduce contractility of bronchi isolated from ovalbumin challenged rodents (Chiba, Arima, Sakai & Misawa, 2008; Chiba, Sato & Misawa, 2008). Systemic simvastatin treatment reportedly prevents increased lung resistance in ovalbumin exposed mice (Ahmad et al., 2011; Zeki, Franzi, Last & Kenyon, 2009). Recent pre-clinical studies in mice suggest that inhaled statins may affect lung function (Wu, Yang & Wang, 2017), but delivery of statins to the lungs is not approved for humans. Real world interpretation and translation of results from pre-clinical studies of systemically delivered statins is limited: none have investigated the effects of long-term, prophylactic treatment or monitored the concentration of statins that accumulate in the lung; and, all used ovalbumin as an aeroallergen (Gregory & Lloyd, 2011; Johnson et al., 2008; Johnson et al., 2004).

The current study compares the effects of prophylactic and acute systemic simvastatin therapy on lung inflammation, airway goblet cell remodeling, and airway function in a murine model of allergic asthma that is induced by repeated lung challenge with house dust mite (HDM). We monitor pharmacokinetics and accumulation of simvastatin in serum, lung tissue and lung lavage. We reveal that repeated systemic delivery of simvastatin results in its accumulation in serum, but not in the lungs. Prophylactic systemic delivery of simvastatin increases anti-inflammatory effects, however, consistent with clinical studies, systemic simvastatin has no effect on airway hyperreactivity.

Method

Simvastatin Formulation

Simvastatin (Toronto Research Chemicals, Toronto, ON, Canada) was prepared as described by McKay et al. (McKay, Leung, McInnes, Thomson & Liew, 2004): to 100 μ L simvastatin stock solution (40 mg/mL in ethanol), 150 μ L of 0.1N NaOH was added, incubated at 50°C (2h), then after adjusting to pH 7.0, diluted to 1 mL with phosphate buffered saline (PBS) (final concentration 4mg/mL simvastatin).

House Dust Mite Challenge and Simvastatin Treatment Protocols

Female Balb/c mice are commonly used to study asthma *in-vivo* (Kim, Ryu, Lim, Lee & Ro, 2007; McKay, Leung, McInnes, Thomson & Liew, 2004). We obtained female Balb/c mice (6-8 weeks old) from the Central Animal Care Facility at University of Manitoba and maintained in 12-hour light/dark cycles with access to food and water ad libitum in individually ventilated cages. Each mouse was assigned an identity number and randomized to different groups so that all experiments were carried out in a blinded manner. Mice were housed in a group of 3/cage. Animals were sacrificed at experimental end points by intraperitoneal (i.p.) pentobarbital sodium. All procedures were carried out according to protocols approved by the University of Manitoba animal ethics committee.

Mice were challenged with house dust mite (HDM) *Dermatophagoides farina* (Greer Labs, Lenoir, NC) by repeated exposure as we have previously described (Anaparti et al., 2016; Piyadasa, Altieri, Basu, Schwartz, Halayko & Mookherjee, 2016). The stock solution for the lot of HDM used for this study included 137.9 μ g Der P1/mL and 132.2 endotoxin units of lipopolysaccharide/mL. HDM dissolved in saline was delivered by intranasal instillation (35 μ L; 0.75 μ g HDM/ μ L) in mice sedated with isoflurane (5%). Animals were challenged daily for 5 consecutive days, followed by 2 days “rest”, and then another 5 consecutive days of HDM challenge. For an allergen naïve control, age-matched mice were challenged with intranasal saline only. Simvastatin was administered to the animals through a subcutaneous (s.c.) route, to bypass the hepatic first pass metabolism responsible for low bioavailability of simvastatin. Animals underwent one of two simvastatin treatment protocols: 1) *for acute treatment*, mice received subcutaneous (s.c.) simvastatin (40mg/kg) concomitantly with each HDM or saline-only i.n. challenge; and 2) *for prophylactic treatment*, mice received 3 s.c. administrations of simvastatin every other day over 5 days in the week prior to undergoing

the acute HDM challenge and simvastatin treatment protocol (Figure 1A). We developed the prophylactic experimental arm in an effort to mimic retrospective clinical data that suggest statin use indicated for non-respiratory disease reasons has consistently been associated with reduced asthma exacerbations and ER visits (Tse et al., 2014; Tse et al., 2013). The duration used for prophylactic treatment was determined in pilot experiments and our previous studies that revealed maximum steady-state concentrations of serum simvastatin, measured using ultrahigh performance liquid chromatography-tandem mass spectrometry, are reached within one week, thus longer periods of pre-treatment were not necessary (Bews, Carlson, Jha, Basu, Halayko & Wong, 2014).

Each time point and experimental, treatment and control group included 9 mice. The sample size, $n=6$, was estimated based on the power (0.95) calculation performed (G*Power 3.1) using preliminary data for the primary end point (airway resistance, R_N) for allergen naïve and HDM-challenged animals. An additional 3 animals in each group were also required for complementary histological indexing. Mice were studied 8 or 48h after final challenge: being subjected to lung function testing, and collection of bronchoalveolar lavage fluid (BALF), blood and lung tissue for assessing inflammation and tissue histology.

Simvastatin Measurement and Pharmacokinetics

To determine the accumulation of pharmacologically active simvastatin in serum, BALF and lung homogenates from mice in acute treatment and prophylactic treatment groups, we used our established extraction and ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC/MS/MS) protocol for the simultaneous measurement of simvastatin and pharmacologically active simvastatin-hydroxy acid (Bews, Carlson, Jha, Basu, Halayko & Wong, 2014). Notably, this protocol is unique in that it ameliorates the spontaneous interconversion between simvastatin and simvastatin-hydroxy acid that can occur during processing by minimizing the time the compound is in aqueous solution and by buffering samples to physiological pH; this preserves the endogenous abundance of pharmacologically active simvastatin in biosamples. In brief, samples were prepared via solid phase extraction, with methanol elution, then dried and reconstituted in 80:20 (v/v) acetonitrile:sodium phosphate monobasic (pH 7.4) just prior to analysis using an Agilent (Mississauga, ON) 1200 ultrahigh performance liquid chromatograph coupled to a 6410B triple quadrupole mass spectrometer with electrospray ionization in positive mode. The column used was an Agilent Zorbax C18 column (2.1 mm × 50 mm × 1.8 μm dp).

Pharmacokinetic Study: In an independent study, 8-week old, female Balb/c mice received a single s.c. injection of simvastatin (40mg/kg). Animals were anesthetized using sodium pentobarbital, 90 mg/kg (i.p.) prior to collecting blood (cardiac puncture) and lungs at 8, 16, 32 and 48 h. Three mice were investigated at each time point, including just prior to simvastatin administration (0 h). Serum was fractionated from blood samples using centrifugation (2300g, 10 min, room temperature) prior to storage. Lungs were excised, rinsed in ice cold phosphate buffer saline and stored at -80°C until being subjected to polytron homogenization in 500µL lysis buffer (composition: 10 mM Tris pH 7.5, 150 mM NaCl, 1:100 NP40, 1g/100mL Deoxycholic Acid Salt, 0.1% SDS, 1:100 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), 1:100 phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St. Louis, MO), and 1 mM PMSF, pH adjusted to 8.0). After centrifugation (2300g 15 min, 4°C) samples were briefly stored (-80°C) prior to analysis by UHPLC/MS/MS.

Inflammatory cell counting

As we have previously described (Ryu et al., 2014), immediately after lung function testing, mouse lungs *in situ* were lavaged twice with 1 mL ice cold saline. BALF was centrifuged (100g 10 mins) to pellet cells, which was re-suspended in 1 mL saline prior to determining total cell count using a hemocytometer. For differential cell counting, cytopsin preparations of suspended BALF cells were air dried and stained with modified Wright-Giemsa stain (HEMA 3 STAT PACK) (Fisher Scientific, Waltham, MA). Cell number and differential analysis was performed manually, identifying eosinophils, lymphocytes, macrophages, and neutrophils in six randomly chosen fields examined with a light microscope at 200X magnification. Average cell count was used to calculate the absolute cell numbers normalized to BALF volume. An experienced technician who was blinded to the sample group identity performed the cell counting.

Cytokine Assay

We quantified a panel of 10 cytokines and chemokines in duplicate in BALF supernatant, using the V-PLEX™ Proinflammatory Panel 1 Mouse Kit as per manufacturer's instructions (Meso Scale Diagnostics, Rockville, MD, USA). The mediators assayed included: IFN- γ , IL-1 β , IL-10, IL-12 p70, IL-2, IL-4, IL-5, IL-6, CXCL1 (KC/Gro), and TNF- α . Data was normalized to BALF volume.

Lung Histology

Lungs were collected from mice that were not subjected to lung function testing or BALF collection. In situ, a polyethylene tracheal cannula was inserted by tracheostomy then lungs were carefully removed from the chest cavity and both inflated (1mL 10% formalin) and submerged in 10% formalin for 24 h. The left lobe of the lung was excised then subjected to serial alcohol dehydration prior to embedding in paraffin wax. Sections (6µm thick) were cut using a microtome along the longitudinal axis to the midline of the lobe then subjected to periodic acid Schiff (PAS) staining to identify airway goblet cells. Images of stained sections were obtained by an upright Olympus light microscope equipped with SPOT CCD camera and Image Pro Plus Software. To quantify goblet cell, the basement membrane was traced using manual tracing tool and the perimeter of the basement membrane was converted from pixel to µm (30pixel/µm) and then using color segmentation the number of goblet cell was calculated. Only airways of similar perimeter were included in the analysis.

Lung Function Testing

As we have previously described (Ma et al., 2007; Piyadasa, Altieri, Basu, Schwartz, Halayko & Mookherjee, 2016; Ryu et al., 2014), lung function of anesthetized mice (i.p. sodium pentobarbital, 90 mg/kg) tracheostomised with a 20-gauge polyethylene catheter was measured using a flexiVent ventilator (Scireq Inc. Montreal, PQ). Mice were ventilated at 2.5Hz using a tidal volume of 10 ml/kg body weight, and 3 cmH₂O positive end expiratory pressure (PEEP) Mice were subjected to increasing concentrations (0-50mg/mL) of nebulized methacholine (MCh) via an in-line ultrasonic nebulizer to assess respiratory mechanics. Changes in respiratory mechanics were assessed by low frequency forced oscillation, normalized to body weight. Respiratory mechanical input impedance (Zrs) was derived from the displacement of the ventilator's piston and the pressure in its cylinder. Correction for gas compressibility, and resistive and accelerative losses in ventilator, tubing and catheter were performed according manufacturer instructions. Zrs was fitted to a constant phase model to extrapolate Newtonian resistance (R_N) (an index of conducting airway resistance), peripheral tissue and airway resistance (G), and lung stiffness (H). Values were calculated as the mean of 20 perturbation cycles performed after each MCh challenge. Fall in FEV1 by 20% in response to MCh challenge (PC₂₀) is equivalent to 43.5% rise in respiratory resistance (Short, Anderson, Manoharan & Lipworth, 2015). Thus in our model the MCh PC₂₀ was calculated assessing the MCh concentration required to increase R_N by 43.5% from the base.

Data and Statistical Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Data are presented as mean values $\pm$ standard error mean (SEM). Means were compared by univariate or one-way ANOVA and Tukey multiple comparisons test, using SPSS_20 software (IBM Canada Ltd., Markham, ON). A p-value <0.05 was used to define statistically significant differences between mean values. The univariate analysis, we performed for analysing the lung function data using SPSS, is a Two Way ANOVA that incorporates a nested variable, methacholine dose, because it is not a completely independent variable.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2017), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017a; Alexander et al., 2017b).

Results

Simvastatin Pharmacokinetics

The peak serum concentration of pharmacologically active simvastatin hydroxy acid (151 ± 87.5 $\mu\text{g/L}$) was reached 8 h after a single s.c. administration of simvastatin (40mg/kg); this declined sharply 16 and 32 h post-administration (9.1 ± 3.4 and 4.8 ± 4.8 $\mu\text{g/L}$, respectively), and 48 h after a single s.c. injection was non detectable (Figure 1B). Levels of simvastatin hydroxy acid were below the level of detection in lung tissue and BALF at all time points. Having established the kinetics of single dose simvastatin, we next profiled pharmacologically active simvastatin that developed at experimental endpoints for both the acute and prophylactic treatment regimens. Eight hours after the final administration of simvastatin, serum concentration was 46.7 ± 9.7 $\mu\text{g/L}$ in animals receiving acute treatment, and this was more than doubled in animals that received prophylactic treatment (116 ± 24.5 $\mu\text{g/L}$, $n=3$) (Figure 1C). In this study for $n=3$, we calculated power to be > 0.95 , using $\alpha = 0.05$ to predict significant differences among groups. On this basis, and to implement the 3Rs (reduce, refine, replace) for animal research we restricted the number of biological replicates to three. Both treatment regimens established a similar steady state concentration of simvastatin in the systemic circulation as measured 48h after final administration (80.3 ± 29.2 $\mu\text{g/L}$ and 57.7 ± 0.7 $\mu\text{g/L}$ for acute and prophylactic protocols, respectively; $n=3$). This suggests that serum simvastatin hydroxy acid concentration follows a dynamic pattern after each s.c. administration, and with repeated treatment establishes stable levels in systemic circulation. In striking contrast, neither the acute nor the prophylactic treatment protocol was sufficient to establish levels of simvastatin hydroxy acid within the LOQ for UHPLC/MS/MS of lung tissue or BALF.

Inflammatory cell count

8-Hours Post-HDM Challenge

Lung influx of inflammatory cells was induced markedly by HDM challenge, reaching $100.6 \pm 12.7 \times 10^4$ cells/mL BALF, as compared to the allergen naïve mice ($2.0 \pm 0.2 \times 10^4$ cells/mL). Acute treatment with simvastatin significantly blunted HDM challenge-induced inflammatory cell number in BALF by approximately 25% ($75.3 \pm 10.8 \times 10^4$ cells/mL). Prophylactic simvastatin treatment significantly prevented HDM challenge-induced total inflammatory cell influx by 70% ($30.4 \pm 5.7 \times 10^4$ cells/mL), an inhibitory impact that was significantly better and nearly twice as high as that achieved with the acute treatment protocol (Figure 2A). HDM challenge induced pronounced lung neutrophilia ($45.2 \pm 5.9 \times 10^4$ vs. naïve),

acute treatment with simvastatin was not sufficient to significantly inhibit neutrophil accumulation. However, prophylactic treatment did significantly blunt neutrophil accumulation in BALF by 77%. HDM challenge-induced eosinophilia ($25.9 \pm 3.0 \times 10^4$ cells/mL BALF) was not reduced by acute treatment, however eosinophil accumulation was significantly inhibited by 78% with prophylactic treatment. Neither acute nor prophylactic treatment was sufficient to change the number of macrophages/monocytes after HDM challenge, whereas prophylactic (but not acute) simvastatin treatment significantly inhibited HDM-induced lung lymphocyte influx by over 75%.

48-Hours Post-HDM Challenge

HDM challenge-induced lung inflammatory cell accumulation in BALF was sustained for 48 h beyond final allergen exposure ($104.6 \pm 42.4 \times 10^4$ cells/mL; $p < 0.05$ vs. naive; $n = 6$). Both acute simvastatin treatment and prophylactic treatment significantly prevented HDM-induced total lung leukocyte infiltration ($50.6 \pm 0.9 \times 10^4$; $p < 0.05$, and $23.4 \pm 5.5 \times 10^4$, respectively) (Figure 2B). At this time point, in HDM-challenged mice eosinophils predominated in BALF ($43.5 \pm 10.2 \times 10^4$ cells/mL). Of note, eosinophil accumulation was significantly reduced by 55% with acute simvastatin treatment ($19.7 \pm 4.3 \times 10^4$ cells/mL vs. HDM) and even more so by prophylactic treatment that reduced eosinophil number by nearly 85% ($6.9 \pm 1.7 \times 10^4$ cells/mL; $p < 0.05$ vs. HDM). Lung neutrophilia persisted 48h after HDM challenge ($22.4 \pm 7.6 \times 10^4$ cells/mL BALF); this was blunted significantly by prophylactic treatment ($3.1 \pm 1.12 \times 10^4$ cells/mL vs. HDM) but not by the acute treatment regime. As we observed 8 h post-allergen challenge, neither acute nor prophylactic treatment was sufficient to limit the number of macrophages/monocytes 48 h after HDM challenge, whereas prophylactic (but not acute) simvastatin treatment significantly inhibited HDM-induced lung lymphocyte influx by 61%.

Cytokine Analysis

As the profile of leukocytes in BALF was different 8 and 48 h after HDM challenge, being predominantly neutrophilic and eosinophilic, respectively, we profiled a panel of 10 pro-inflammatory cytokines and chemokines in BALF at both experimental end points.

8-Hours Post-HDM Challenge

Compared to allergen naïve mice, HDM challenge induced significant accumulation of a number of mediators, including: a 100-fold rise in TNF α ; 45-fold increase in CXCL1; 300-fold higher IL-6 levels; 190-fold increase in IL-4; 240-fold increase in IL-5; 800-fold rise in

IFN- γ ; and, 80-fold accumulation of IL-1 β (Figure 3A). Neither IL-2 nor IL-12 were detected in naïve mice, however post HDM challenge levels in BALF of both cytokines rose to 5.2 ± 1.1 pg/mL and 19.9 ± 4.9 pg/mL, respectively. Both acute and prophylactic simvastatin treatment protocols inhibited HDM challenge-induced accumulation of a number of pro-inflammatory cytokines by 60-95%, including: TNF α , CXCL1, IL-6, IL-4, IL-1 β , and IFN- γ . The level of IL-12 in BALF was also reduced to below detection limits by both treatment protocols. In contrast, neither acute nor prophylactic treatment affected HDM challenge-induced IL-2 (not shown). Notably, though acute treatment had no significant impact on IL-5 in BALF from HDM challenged mice, prophylactic treatment significantly inhibited IL-5 level by 95%. Interestingly, for the anti-inflammatory cytokine, IL-10, acute and prophylactic simvastatin treatment suppressed HDM challenge-induced accumulation in BALF by $84.6 \pm 6.9\%$ and $94.5 \pm 1.4\%$, respectively.

48-Hours Post-HDM Challenge

As shown in Figure 3B, as we observed 8h after HDM challenge, and compared to naïve mice, 48 h after allergen exposure we measured significant elevation in four pro-inflammatory cytokines in BALF, including: 23-fold rise in IL-1 β ; 7-fold accumulation of IL-4; 38-fold increase in IL-5; and, 6-fold rise in CXCL1. HDM challenge also supported a 7-fold increase in the anti-inflammatory cytokine, IL-10 (Figure 3B). Conversely, though a trend for elevated levels was apparent, we did not observe a statistically significant change in the abundance of IFN- γ , IL-12, IL-2, IL-6 or TNF α compared to allergen naïve animals (data not shown). Acute and prophylactic treatment with simvastatin prevented HDM challenge-induced accumulation of IL-1 β by 70 % and 85%, respectively. Notably, prophylactic simvastatin treatment, but not the acute therapy regimen, markedly blunted HDM-induced accumulation of IL-4, IL-5 and by over 75%, and prophylactic treatment was significantly more effective than acute treatment in damping allergen challenge-associated accumulation of the Th2-cytokine, IL-4, and the pro-neutrophilic chemokine, CXCL1. In contrast to the effects we measured 8h after allergen challenge neither acute nor prophylactic simvastatin treatment abrogated the accumulation of anti-inflammatory IL-10 in BALF 48h after allergen exposure.

Lung Histology

Lung histology was performed to assess airway goblet cell hyperplasia using PAS staining and quantitative microscopy. Goblet cells were absent in peripheral airways of allergen naïve

animals (mean airway perimeter ($161.1 \pm 28.5 \mu\text{m}$) μm , $n = 3$ animals/group and at least 9 airways/animal was analyzed). In contrast, in similar sized airways, both 8 and 48 h after completing allergen challenge robust goblet cell staining per unit of basement membrane was evident (Figure 4). Both acute and prophylactic simvastatin treatment were sufficient to virtually abrogate the development of goblet cell hyperplasia after allergen challenge. Since lung histology analysis was complementary to the other parameters studied, biological replicates were kept at $n = 3$, which still ensured power > 0.95 , using $\alpha = 0.05$ to predict significant differences among groups, and to implemented 3R policy to reduce animal use in research.

Lung function

We used a small animal ventilator to measure airway reactivity to inhaled MCh, 8 and 48 h after completing the HDM exposure protocol. Consistent with our prior studies (Piyadasa, Altieri, Basu, Schwartz, Halayko & Mookherjee, 2016; Ryu et al., 2014), airway resistance (R_N) was not statistically different from that in allergen naïve animals at the 8h experimental end point, but 48 h after final HDM challenge we observed a significant increase in R_N in response to 25 and 50 mg/mL MCh (Figure 5A). Therefore, we only assessed the impact of acute and prophylactic simvastatin treatment on lung function 48 h after completing HDM challenge. In contrast the inhibitory effects of simvastatin therapy on allergen-induced lung inflammation and goblet cell hyperplasia, neither acute nor prophylactic simvastatin treatment prevented airway hyperreactivity – increased R_N - 48h after HDM exposure (Figure 5A). Similarly, simvastatin therapy was without effect on increased lung elastance (H) caused by allergen challenge (Figure 5B). Tissue damping (G) - an indicator of the combined contribution of small airways resistance and lung elastic recoil - was increased after HDM challenge, but this effect was blunted significantly by both acute and prophylactic simvastatin treatment when mice were exposed to high concentrations of MCh (Figure 5C). In line with the hyperreactivity, HDM challenge significantly induced hypersensitivity to MCh (PC_{20} for HDM challenged mice was $3.2 \pm 2.2 \text{ mg/mL}$ compared $25.2 \pm 4.5 \text{ mg/mL}$ for naïve mice) that remained untouched by both acute and prophylactic treatment with simvastatin.

Discussion

This study addresses a lack of full concordance of results for the effects of systemically delivered statins on asthma pathophysiology from pre-clinical small animal model studies and human clinical trials. With a murine model of allergic asthma that is induced by repeated delivery to the lungs of a clinically relevant aero-allergen, HDM, for asthma (Desai & Brightling, 2012; Gregory & Lloyd, 2011), we show that acute systemic delivery of simvastatin in parallel with allergen challenge blunts more than 50% of allergic inflammation in the lung - including eosinophil and neutrophil influx, and pro-inflammatory cytokine release - as well as abrogating airway goblet cell hyperplasia. Moreover, prophylactic simvastatin treatment, which establishes sustained levels of pharmacologically active simvastatin in the blood, blunts all indices of allergic lung inflammation even more effectively than acute treatment, and abrogates airway goblet cell hyperplasia. Despite the marked inhibitory effects of acute or prophylactic systemic simvastatin therapy on the accrual of lung inflammation, they were without effect on airway hyperreactivity, a hallmark of asthma. These findings indicate that with systemic delivery pharmacologically active simvastatin does not accumulate in the lungs, indicating that beneficial effects to prevent lung inflammation are likely associated with a mechanism of action in the systemic circulation and the behavior of leukocytes therein.

Though the pharmacodynamics of oral statin therapy has been described in humans (US FDA, 2015), for lung diseases it has not been a primary point of assessment for systemically delivered statins in pre-clinical studies. We show that serum simvastatin concentration increases sharply after s.c. delivery, but this declines rapidly for a single administration and is not detectable 48 hours later. This is consistent with human pharmacokinetic trials that show peak plasma concentrations within 4 hours of oral simvastatin therapy, and that this is reduced to only 10% of the peak concentration 8 hours later (US FDA, 2015). A caveat of our study is that we did not measure peak simvastatin hydroxyl acid until 8 hours after treatment, thus we may not have determined the maximum peak that could have been attained. Nonetheless, we did use our previously established UHPLC/MS/MS protocol that is tuned to specifically monitor pharmacologically active simvastatin hydroxy acid (Bews, Carlson, Jha, Basu, Halayko & Wong, 2014) to show that prophylactic treatment established a steady-state serum concentration of simvastatin hydroxy acid $\sim 50\mu\text{g/L}$. Similar circulating levels of pharmacologically active simvastatin were likely also achieved with acute treatment in

parallel with HDM challenge. Notably we show that peak levels of active simvastatin 8 hours after drug delivery were more than doubled, $>120 \mu\text{g/L}$, with the prophylactic treatment protocol. The disparate peak level of circulating, pharmacologically active simvastatin that is achieved by prophylactic therapy, combined with the kinetics of circulating simvastatin hydroxy acid that we describe, is important to consider in deciphering the possible mechanisms for the effects of simvastatin on the evolution of lung inflammation in HDM-challenged mice.

Our findings are consistent with a number of studies that show the systemic delivery of statins is associated with blunted influx of inflammatory cells and goblet cell hyperplasia upon allergen challenge (Imamura et al., 2009; Kim, Ryu, Lim, Lee & Ro, 2007; McKay, Leung, McInnes, Thomson & Liew, 2004; Zeki, Franzi, Last & Kenyon, 2009). These prior studies used mice subjected to ovalbumin sensitization and challenge to induce eosinophilic lung inflammation, thus the primary benefit of statin therapy is reported to be the prevention of lung eosinophilia and eosinophil-associated cytokines such as IL-5 and eotaxin-1 (McKay, Leung, McInnes, Thomson & Liew, 2004; Zeki, Franzi, Last & Kenyon, 2009; Zeki, Thai, Kenyon & Wu, 2012). The HDM-challenge model uses aero-exposure to a complex allergen that includes multiple- antigenic peptides, some with proteolytic activity to induce inflammation (Johnson et al., 2004). We observed overlapping neutrophil, eosinophil, macrophage/monocyte and lymphocyte accumulation in the lung. This includes an early peak in neutrophil accumulation (8 hours post allergen) and a later peak in eosinophil accumulation (48 hours post allergen). Therefore, our experiments can discriminate the effects of simvastatin therapy on different immune cell types. Prophylactic simvastatin treatment is significantly more effective than acute treatment in suppressing neutrophil, eosinophil and lymphocyte recruitment to the lungs at all experimental end points. Indeed, acute simvastatin therapy was ineffective in controlling the neutrophilia observed with HDM challenge. The differential effects of prophylactic treatment could be attributed to the higher serum concentration of simvastatin hydroxy acid. This is consistent with previous observations by McKay, Leung, McInnes, Thomson and Liew (2004) who reported only high doses of intra peritoneal simvastatin support a histological reduction in inflammatory infiltrates in the lungs, an effect that may be linked to processes that involve vascular cell adhesion and interstitial cell adhesion molecules (Kim, Ryu, Lim, Lee & Ro, 2007; McKay, Leung, McInnes, Thomson & Liew, 2004). In line with these studies, Zeki et al. (2015)

demonstrated that intra-tracheal instillation of pravastatin, which is retained in the lung and does not accumulate significantly in plasma, is not sufficient to reduce BALF total leukocyte or eosinophil cell count in ovalbumin exposed mice. Collectively, our observations support a general concentration-dependent role for simvastatin hydroxy acid in the circulation to disrupt the influx and accumulation of inflammatory cells in the lung in response to allergen challenge.

In line with previous reports using ovalbumin challenged mice, we show that acute and prophylactic simvastatin treatment is sufficient to prevent goblet cell hyperplasia (McKay, Leung, McInnes, Thomson & Liew, 2004; Zeki, Franzi, Last & Kenyon, 2009; Zhu et al., 2012). This effect is likely attributable to our findings that simvastatin therapy inhibits eosinophil infiltration and associated release of mediators that promote mucous production (McBrien & Menzies-Gow, 2017).

To complement our studies comparing the magnitude of the effect of acute and prophylactic simvastatin therapy on lung inflammatory cell influx and goblet cell hyperplasia, we also used 10-plex technology for quantitative profiling of pro- and anti-inflammatory cytokines and chemokines in BALF. Consistent with HDM-associated human asthma (Desai & Brightling, 2012; Gregory & Lloyd, 2011) HDM challenge in animals induced a complex inflammatory response, increasing pro-inflammatory cytokines that support eosinophilic and neutrophil inflammation, Th1- and Th2-like responses, as well as the anti-inflammatory cytokine, IL-10. As reported in prior studies using ovalbumin-exposed mice (Imamura et al., 2009; McKay, Leung, McInnes, Thomson & Liew, 2004; Zeki, Franzi, Last & Kenyon, 2009; Zeki, Thai, Kenyon & Wu, 2012), we also observed a broad inhibitory effect of systemic simvastatin therapy on cytokine accumulation in the lungs. This was most evident at 8 hours after HDM challenge when a broader spectrum of cytokines and chemokines existed and were inhibited by both acute and prophylactic therapy: IFN γ , IL-12, IL-1 β , IL-6, and TNF α . We observed a statistically unique effect of prophylactic simvastatin therapy to prevent lung neutrophilia; this effect is mirrored by the inhibition of CXCL1 accumulation in BALF 8 and 48 hours after HDM challenge. Similarly, pro-eosinophilic IL-5 and IL-4 in BALF are inhibited by prophylactic simvastatin therapy 8 and 48 hours after allergen challenge, a pattern that appeared to be mirrored by acute treatment, though the trend for the latter was not validated by statistical analyses. Interestingly, prophylactic treatment prevented the accumulation of anti-inflammatory IL-10 and the stereotypic Th2 cytokine, IL-4, 8 and 48

hours after HDM challenge but acute simvastatin therapy only slowed their accumulation as peak allergen-induced levels were still reached 48 hours after HDM challenge. This is inconsistent with some reports that simvastatin can upregulate IL-10 in human subjects with chronic obstructive lung disease (Maneechotesuwan, Wongkajornsilp, Adcock & Barnes, 2015). A limitation of our experiments is that though we observed a suppression of lymphocyte influx with simvastatin therapy, our cytokine array did not include IL-17, which is downregulated by statin treatment in ovalbumin primed mice (Imamura et al., 2009; Wu, Yang & Wang, 2017). Change in IL-17 abundance could have shed light on the impact of therapy on Th17 / Treg cell balance as a mechanism for Treg-associated suppression of Th2 cell-mediated inflammation. To overcome these limitations we are incorporating broader cytokine arrays in our ongoing and future work. Overall our data indicate that in parallel with inhibitory effects on inflammatory cell influx, compared to acute simvastatin treatment, prophylactic therapy more effectively suppresses the breadth and magnitude of the cytokine and chemokine milieu that develops in the lung with repeated HDM challenge.

A principal goal of any asthma therapy is to improve lung function by mitigating debilitating tendency for paroxysmal bronchial spasm associated with airway hyperresponsiveness. *In vivo*, preclinical studies by Zeki, Franzi, Last and Kenyon (2009) show that systemically delivered simvastatin tempers ovalbumin exposure-induced increased respiratory resistance at baseline and in response to low concentration inhaled MCh. Using a chronic ovalbumin challenge model of airway remodeling, but with low levels of lung inflammatory cell infiltrate, Ahmad et al. (2011) show that simvastatin treatment improves indices of lung and airway resistance. There is a general lack of concordance of such studies with clinical trials in asthmatics, which have not revealed any substantive improvement in airway function resulting from oral statin therapy. Indeed one study suggests statin treatment may lead to a decline in FEV1 (forced expiratory volume in 1 second) (Ostroukhova, Kouides & Friedman, 2009), whilst others show no effects of statin treatment on lung function (Braganza et al., 2011; Hothersall et al., 2008; Moini, Azimi & Farivar, 2012; Yuan et al., 2012). One randomized controlled trial does report that in a subgroup of asthmatics that can tolerate withdrawal of inhaled corticosteroid therapy, oral simvastatin (40mg/day) showed a 150mL improvement in pre-bronchodilator FEV1 (Cowan, Cowan, Palmay, Williamson & Taylor, 2010). For the current study we used a repeat HDM challenge protocol to mimic the complex inflammatory and tissue remodeling microenvironment associated with human allergic asthma. Though we show that acute and prophylactic systematic simvastatin treatment

diminish lung inflammation, neither is sufficient to prevent increased respiratory resistance, central airway resistance (RN) – which accounts for 80% of total respiratory resistance - or lung elastance (H) in HDM challenged mice. Interestingly, similar to published data (Ahmad et al., 2011), at the highest concentration of MCh challenge, both acute and prophylactic simvastatin did significantly inhibit the HDM-induced increase in alveolar tissue constriction (G). This index of lung function reflects small airway function, as well as lung parenchymal tissue changes associated with edema and inflammatory cell influx. Thus, our demonstration of a protective effect of systemic simvastatin therapy on G is consistent with the fact that lung inflammation is also significantly reduced.

Despite effects of systemic simvastatin therapy on peripheral lung damping induced by high dose methacholine, our findings reveal this is not sufficient to manifest as an improvement in respiratory resistance. The failure for systemic simvastatin to significantly improve airway resistance in HDM challenged mice is consistent with results of human clinical trials for oral statin therapy in asthma, as clinical indices for airway conductance, such as FEV₁, are not improved (Silva, Couto, Delgado & Moreira, 2012). Our pharmacokinetic data suggest that the refractory nature of airway hyperreactivity to systemically administered simvastatin may be associated with the failure of its pharmacologically active form to accumulate in the lungs, thereby preventing direct or sustained effects on resident epithelium, smooth muscle and other structural cells. The absence of simvastatin or its hydroxy acid form in the lungs may be due, in part, to its lipophilicity that likely favors deposition in lipid rich organs, thus compromising already-low bioavailability <5% (US FDA, 2015). Despite this we did expect that some circulating simvastatin would reach to the lungs through the bronchial circulation. However, since bronchial circulation is only a fraction of cardiac output it is likely that abundance of simvastatin reaching the lungs by this route is very small. Another mechanism for limiting accumulation of simvastatin is the presence of the simvastatin metabolizing enzyme, CYP3A11 in mouse lung (CYP3A4 in humans) (Biggs et al., 2007; Haag, Fautrel, Guillozou, Frossard & Pons, 2003; Li, Ross-Viola, Shay, Moore & Ricketts, 2009; Shah, Chuttani, Mishra & Pathak, 2011). Recent evidence indicates that direct delivery of statins to the lung may be needed to reverse airway hyperreactivity and hypersensitivity (Wu, Yang & Wang, 2017; Zeki et al., 2015). Thus our evidence indicates that systemic administration of simvastatin is ineffective to mitigate airway dysfunction, despite its capacity to prevent immune cell influx and release of pro-inflammatory cytokines.

In summary, using a murine model of allergic asthma that employs repeated challenge with the clinically relevant aero-allergen, HDM, we demonstrate that systemic delivery of simvastatin is sufficient to inhibit lung infiltration by inflammatory cells, and release of Th1 and Th2 cytokines. Moreover, prophylactic treatment that establishes a steady state circulating concentration of simvastatin hydroxy acid prior to allergen challenge is more effective than commencing treatment at the onset of allergic insult. In a manner that parallels human clinical trials, we confirm that systemic delivery of simvastatin, acutely or prophylactically, is without impact on allergen challenge-induced airway hyperreactivity. Our findings support need for developing approaches for the delivery of statins directly to the lung, perhaps in concert with systemic delivery, to assess the real world potential of this therapeutic approach for asthma control.

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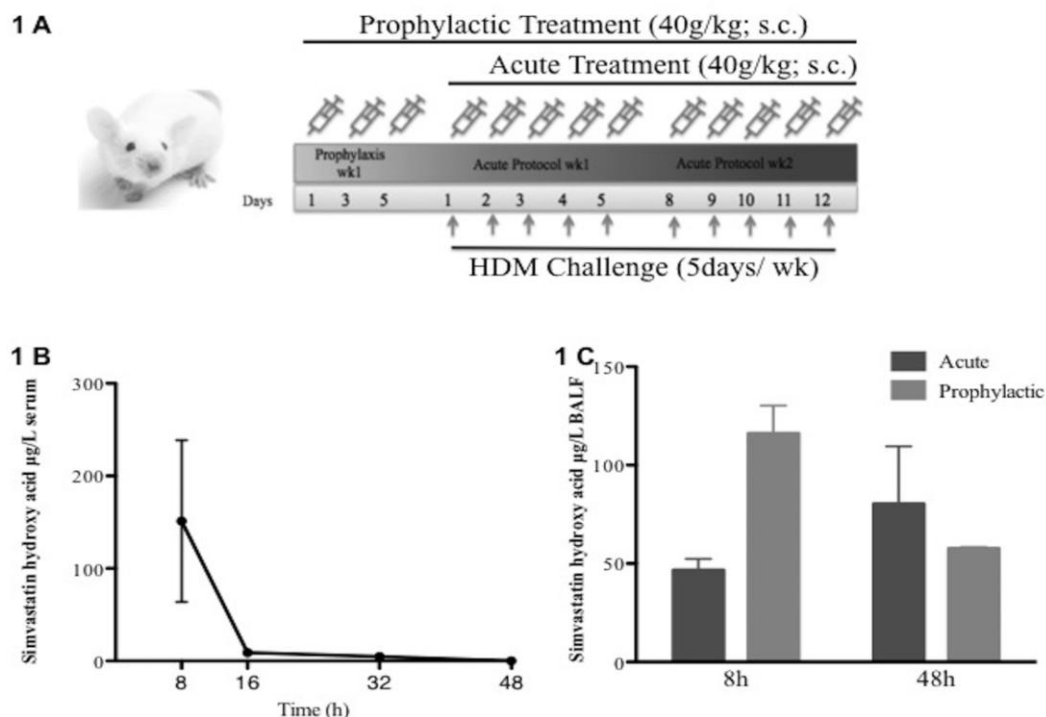


FIGURE 1

Figure 1: Serum kinetics of simvastatin injection. A) Schematic of 2-wks of HDM challenge with or without acute and prophylactic simvastatin treatment B) Simvastatin hydroxy acid concentration in serum 8 h after a single administration of simvastatin (40mg/kg; s.c) as measured by UHPLC-MS/MS. C) Serum concentration of simvastatin hydroxy acid 8 h and 48 h after acute and prophylactic treatment protocols. All data represent the mean from 3 animals at each time point and treatment. Data are shown as mean $\pm$ SEM.

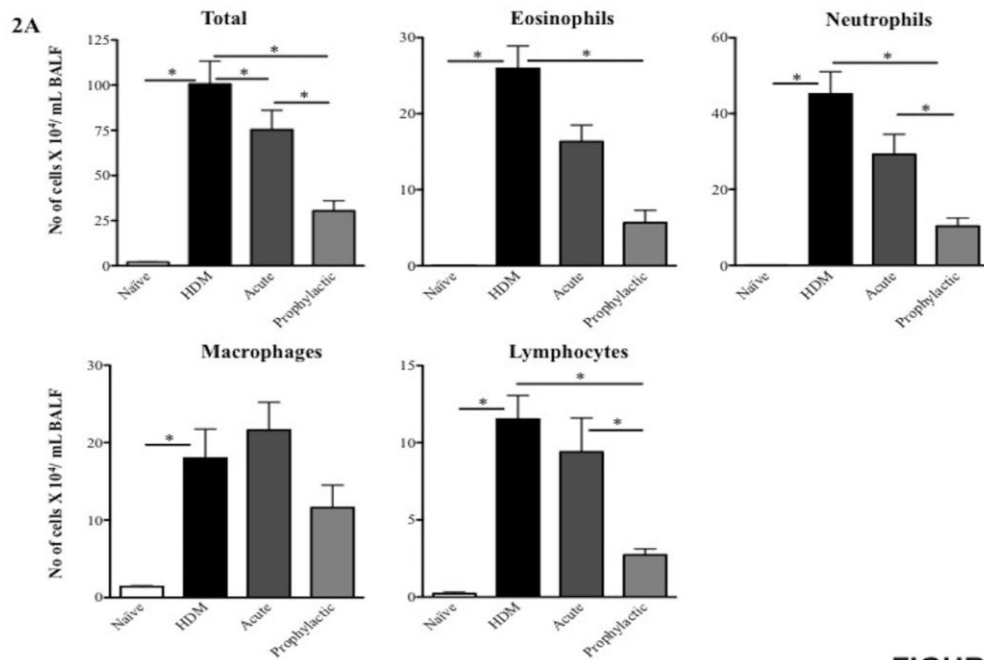


FIGURE 2

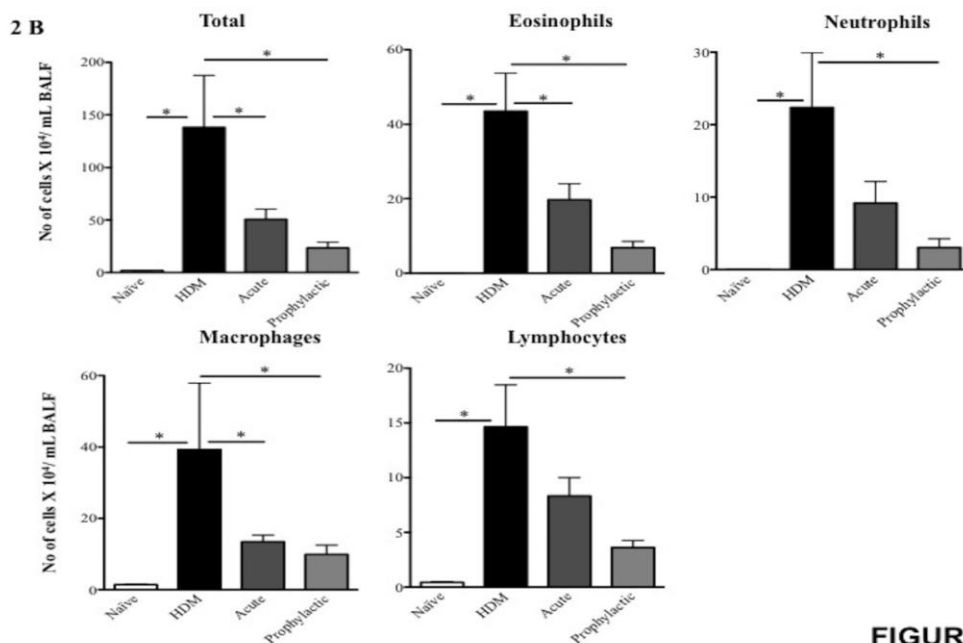


FIGURE 2

Figure 2: Effects of HDM challenge and acute and prophylactic simvastatin treatment on leukocyte number and differential in BALF. Cell counts determined A) 8h and B) 48h after completing HDM challenge, with and without simvastatin treatment. For all data, cell number is normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using One Way ANOVA with Tukey multiple comparison test: * $p < 0.05$.

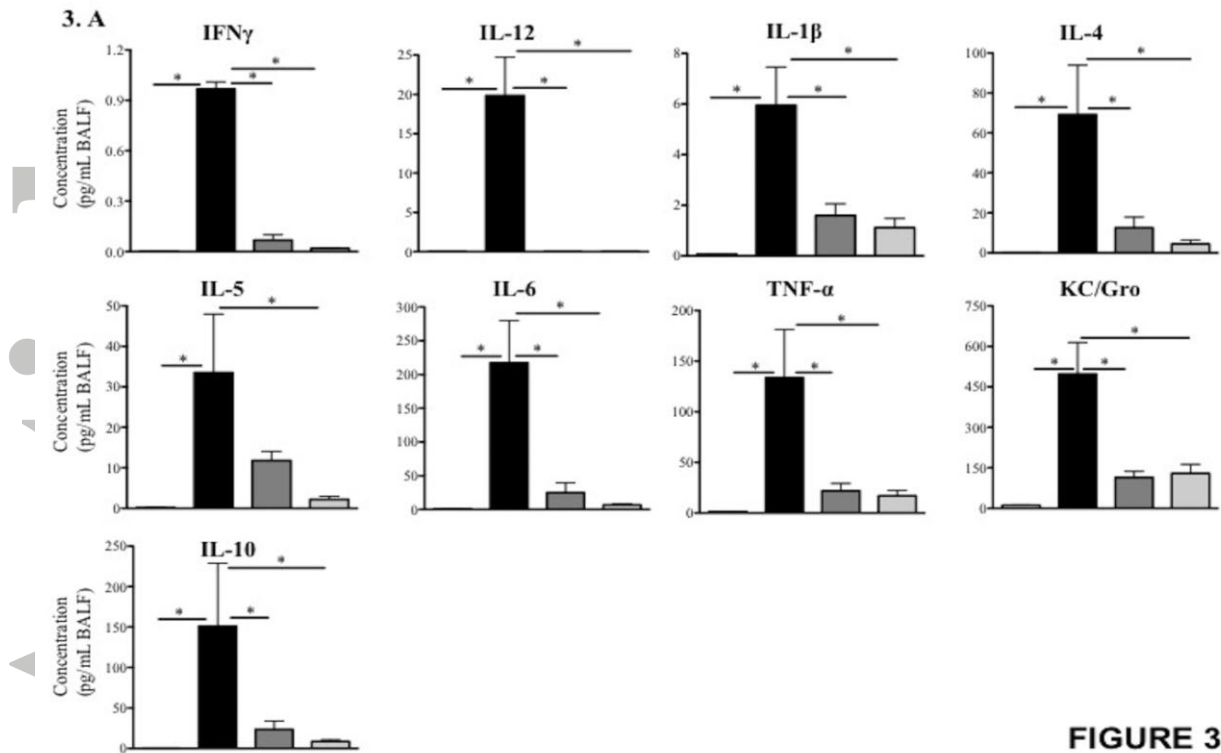


FIGURE 3

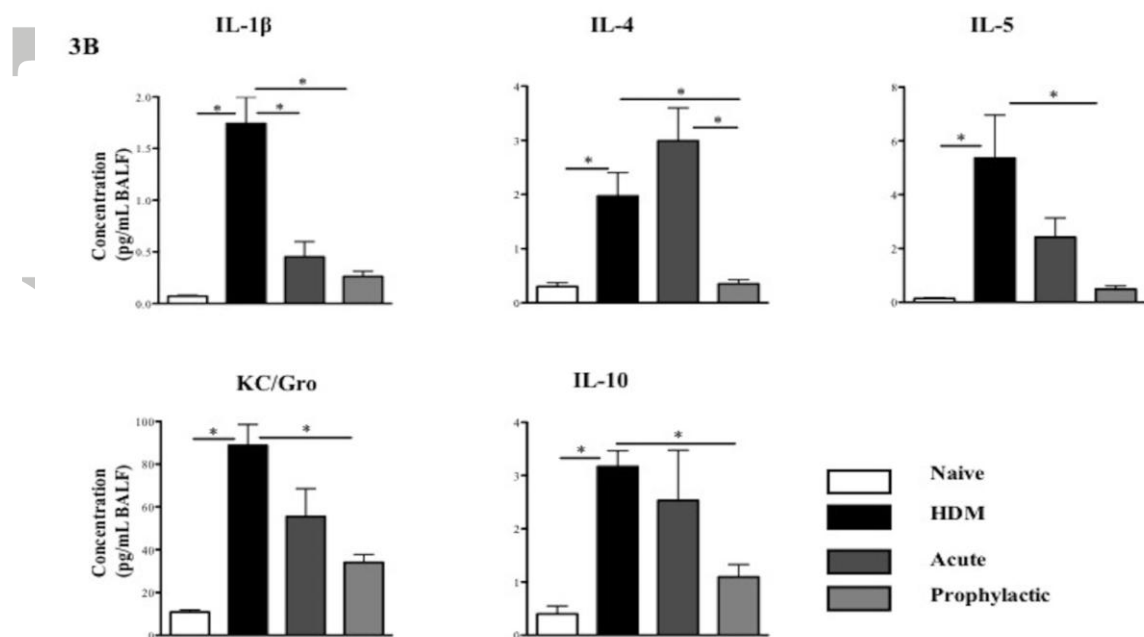


FIGURE 3

Figure 3: Effects of HDM challenge and acute and prophylactic simvastatin treatment on cytokine accumulation in BALF. Cytokine abundance determined by 10-plex MSD array determined A) 8h and B) 48h after completing HDM challenge with and without simvastatin treatment. For all data, cell number is normalized to initial BALF volume and presented as mean $\pm$ SEM from 3 animals per group. Analysis was done using One Way ANOVA with Tukey multiple comparison test: * $p < 0.05$.

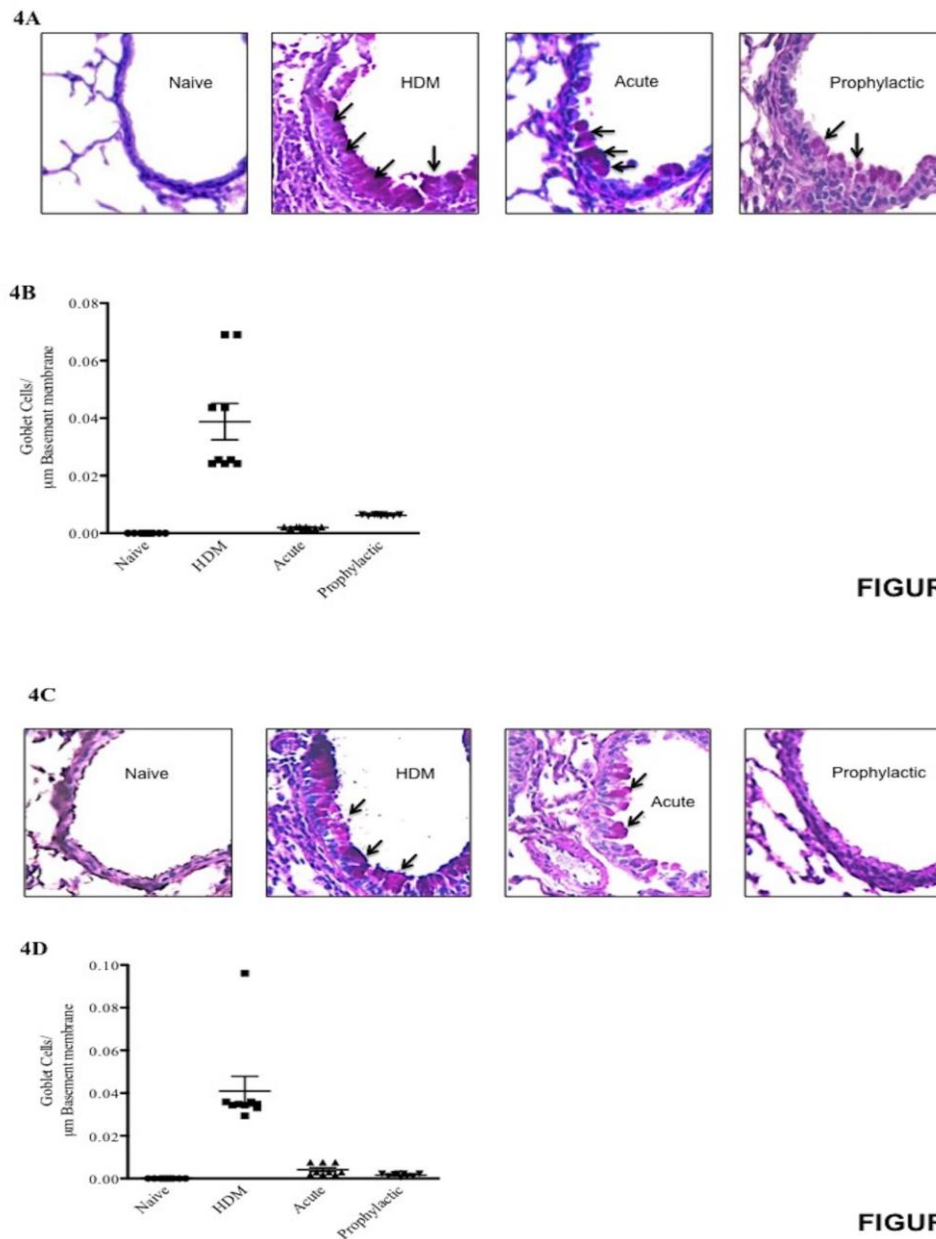


FIGURE 4

FIGURE 4

Figure 4: Effects of HDM challenge and acute and prophylactic simvastatin treatment on airway goblet cell number. Images of typical PAS staining of goblet cells in bronchioles A) 8h and C) 48h after completing HDM challenge, with and without simvastatin treatment. Images were acquired using 200x magnification. Arrows indicate presence of purple mucous staining. Quantification of goblet cells using Image Pro software B) 8h and D) 48h after completing HDM challenge, with and without simvastatin treatment was used to determine goblet cell number in similar sized airways in each mouse (perimeter $161.1 \pm 28.5 \mu\text{m}$). Data in panels B and D are presented as dot plots, with mean values indicated by horizontal lines. Error bars indicate $\pm$ SEM from a total of 9 different airways from 3 animals per group.

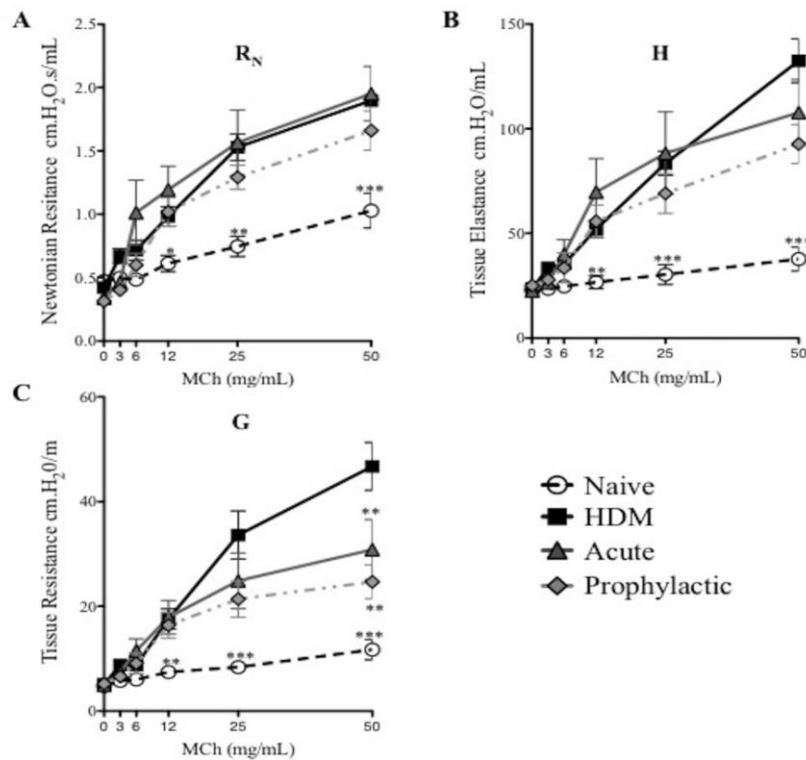


FIGURE 5

Figure 5: Effects of HDM challenge and acute and prophylactic simvastatin treatment on respiratory mechanics. Lung function was assessed using a flexiVent small animal ventilator 48h after completing HDM challenge with and without simvastatin treatment. A) Newtonian Resistance (R_N), B) Tissue Elastance (H) and C) Tissue Resistance (G) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50mg/mL). Data are presented as mean $\pm$ SEM from 6 animals per group. Comparisons of naïve and each treatment were performed against HDM challenge using Univariate analysis followed by Tukey multiple comparison test: * $p < 0.05$.

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