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G-CSFR antagonism reduces mucosal injury and airways fibrosis in a virus-dependent model of severe asthma

Short title: Neutrophils cause airway fibrosis in severe asthma

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Abstract

Background and Purpose: Asthma is a chronic disease that displays heterogeneous clinical and molecular features. A phenotypic subset of late-onset severe asthmatics has debilitating fixed airflow obstruction, increased neutrophilic inflammation and a history of pneumonia. Influenza A virus (IAV) is an important viral cause of pneumonia and asthmatics are frequently hospitalised during IAV epidemics. This study aims to determine whether antagonising granulocyte colony stimulating factor receptor (G-CSFR) prevents pneumonia-associated severe asthma.

Experimental Approach: Mice were sensitized to house dust mite (HDM) to establish allergic airway inflammation, and subsequently infected with IAV (HKx31/H3N2 subtype). A neutralising monoclonal antibody against G-CSFR was therapeutically administered.

Key results: In IAV-infected mice with prior HDM sensitisation, a significant increase in airway fibrotic remodelling and airways hyper-reactivity (AHR) was observed. A mixed granulocytic inflammatory profile consisting of neutrophils, macrophages and eosinophils was prominent and at a molecular level, G-CSF expression was significantly increased in HDMIAV-treated mice. Blockage of G-CSFR reduced neutrophilic inflammation in the bronchoalveolar (BAL) and lungs by over 80% in HDMIAV-treated mice without altering viral clearance. Markers of netosis (dsDNA and MPO in BAL), tissue injury (LDH activity in BAL) and oedema (total BAL-fluid protein) were also significantly reduced with anti-G-CSFR treatment. In addition, anti-G-CSFR antagonism significantly reduced BAL gelatinase activity, active TFG β lung levels, collagen lung expression, airways fibrosis and AHR in HDMIAV-treated mice.

Conclusions and Implications: We have shown that antagonising G-CSFR-dependent neutrophilic inflammation reduced pathological disruption of the mucosal barrier and airways fibrosis in an IAV-induced severe asthma model.

Key words: severe asthma, influenza A virus, neutrophilic inflammation, G-CSF receptor, airway fibrosis

Abbreviations:

IAV, influenza A virus; HDM, house dust mite; AHR: airways hyper-reactivity; BAL, bronchoalveolar lavage; MPO, myeloperoxidase; G-CSF, Granulocyte colony stimulating factor; G-CSFR, Granulocyte colony stimulating factor receptor; ICS, inhaled corticosteroids; FeNO, fractional exhaled nitric oxide; OVA, ovalbumin; MCh, methacholine; SMA, smooth muscle actin; H&E, hematoxylin and eosin; AB-PAS, Alcian blue-periodic acid Schiff; MT, Masson's Trichrome; TGF β , Transforming growth factor beta; MMP, Matrix metalloproteinase; NETs:neutrophil extracellular traps.

Bullet point summary: (1-2 bullet points under each title, maximum 15 words in each bullet point)

What is already known

- A subset of severe asthmatics present with persistent neutrophilic inflammation and a history of pneumonia
- A granulopoietic cytokine G-CSF is increased in severe asthmatic airways and levels associate with neutrophilic inflammation

What this study adds

- Influenza A viral infection recruits' neutrophils, which cause mucosal injury in mice with allergic airways disease
- In addition, neutrophil activation promotes an increase in lung collagen expression and fibrotic airways deposition, which contributes to airways hyper-reactivity in this model.

What is the clinical significance

- The recruitment of neutrophils was dependent on G-CSFR signalling in this severe asthma model
- Monoclonal antibodies that neutralise G-CSFR represents a novel therapy for treating non-T2 severe asthma

Introduction

Asthma is a heterogeneous disease where biological or molecular characteristics can dictate disease severity and clinical manifestations. Severe asthma is defined as asthma which remains uncontrolled despite treatment with high doses of inhaled (ICS) or systemic corticosteroids (Chung et al., 2014). Cohort studies such as Severe Asthma Research Program (SARP) (Silkoff, Moore & Sterk, 2019) and Airway Disease Endotyping for Personalized Therapeutics (ADEPT) (Loza et al., 2016) have identified distinct phenotypes based on biological and clinical features. Approximately two-thirds of severe asthmatics are characterised by elevated levels of type-2 (T2) inflammatory biomarkers, including blood eosinophils, serum IgE and fractional exhaled nitric oxide (FeNO). Biologics against 'treatable traits' (Agusti et al., 2016) that target the well-characterised T2 inflammation pathways (e.g. IgE (Holgate et al., 2004), IL-5 (Ortega et al., 2014), IL-4/IL-13 (Rabe et al., 2018) and TSLP (Corren et al., 2017)) have been developed and used with some clinical success. However, the remaining one-third of patients with severe asthma do not present with T2-dominant inflammation. This cluster of severe asthmatics displays an altered immunological profile associated with neutrophilic or mixed-granulocytic airway inflammation that is not suppressed by high dose ICS or systemic corticosteroids (Moore et al., 2014; Silkoff, Moore & Sterk, 2019). Severe asthmatics that fall within a non-T2 dominant cluster also present with the lowest lung function and irreversible airflow obstruction, which was closely related to a history of pneumonia (Moore et al., 2014; Silkoff, Moore & Sterk, 2019). Since this important cluster of severe asthmatics cannot be effectively managed with T2 blockers alone, development of novel therapies to treat non-T2 severe asthma in the face of respiratory infection represents an important unmet medical need. To develop such new therapies, a greater understanding of the immunological and molecular mechanisms that drive infection-dependent transition to severe asthma is needed (Busse et al., 2013).

Influenza A virus (IAV) is a prototypical respiratory virus that can cause pneumonia. Asthmatics are an important high-risk group during IAV epidemics as they are over-represented during hospital admissions. In the 2009 H1N1 pandemic, asthma was the most common co-morbid condition seen in both children (29%) and adults (27%) (Jain et al., 2009). Whilst IAV is not a frequent cause of asthma exacerbations in children, the prevalence appears to be higher in adult asthmatics (20–25% exacerbation cases) admitted to an emergency department or hospital (Papadopoulos et al., 2011). In asthmatics presenting with an acute exacerbation, respiratory viruses including IAV are associated with a greater risk of treatment failure involving management with inhaled bronchodilators and systemic corticosteroids (Merckx et al., 2018). There is conflicting pre-clinical data on how underlying asthma pathology is impacted by the immunological response to IAV. For example, mice sensitised to house dust mite (HDM) allergen and subsequently infected with IAV resulted in increased mucous production and eosinophilic airway inflammation that caused airways hyper-responsiveness (AHR) in a manner that was unresponsive to inhaled corticosteroids (Ravanetti et al., 2017). dsRNA, synthesized during replication of RNA viruses has been reported to exacerbate eosinophilic inflammation and AHR in ovalbumin (OVA)-sensitized rats (Shiraishi et al., 2008). In contrast, there are reports that acute IAV infection can dampen T2 inflammation and asthma pathology. OVA induction of allergic inflammation has been shown to prime NK cells to enhance antigen-specific CD8⁺ T cells elimination of viral-infected cells, leading to reduced morbidity and mortality (Ishikawa, Sasaki, Fukui, Fujita, Kutsukake & Matsumoto, 2012). In another study, viral clearance was improved and T2 inflammation was reduced during the early phase of infection, however by day 7 post infection, OVA-sensitized mice displayed more severe weight loss and morbidity (An et al., 2018).

Since neutrophils are recruited during acute influenza infection and are a prominent inflammatory cell population in severe asthma phenotypes, we have focused our study on defining the role of this innate cell. The hematopoietic growth factor granulocyte colony-stimulating factor (G-CSF) contributes to neutrophil granulopoiesis and trafficking. The CSF3 gene resides within the major 17q12-21 asthma susceptibility locus that is linked to recurrent wheeze and asthma exacerbations (Bisgaard et al., 2009), where its product (G-CSF) binds exclusively to G-CSF receptor (G-CSFR) expressed on multiple cells including mature neutrophils. *CSF3* and *CSF3R* transcript expression positively correlate with neutrophilic inflammation in bronchial biopsies from asthmatics (Wang et al., 2019). Consistent with this finding, sputum G-CSF protein levels were significantly higher in asthmatic patients with neutrophil-dominant inflammation (Kim et al., 2020). We have recently shown that influenza A virus significantly increased blood and lung neutrophil numbers at day 3 post infection in a manner that was suppressed with anti-GCSFR treatment (Wang, Aloe, Wilson & Bozinovski, 2019). These findings have guided our treatment protocol for this study where anti-G-CSFR was delivered during the peak blood granulocytic phase (at day 2 and 5 post influenza infection) as a strategy to block the peak viral-mediated systemic and local neutrophil response. We have also shown that antagonising G-CSFR reduced neutrophilic inflammation, mucous production and AHR in HDM allergen sensitised mice that were chronically infected with pneumococcus (Wang et al., 2019). In this study, we have identified a novel role for G-CSFR dependent neutrophilic inflammation, whereby this pathway regulates the extent of airways fibrosis and mucous barrier damage in HDM-sensitised mice infected with IAV.

Methods

Animals

All animal experiments were approved by the Animal Ethics Committee at RMIT University (AEC #1805) and conducted in compliance with the guidelines of National Health and Medical Research Council (NHMRC) of Australia and ARRIVE. Six week old female BALB/cArc mice (RRID:IMSR_ARC:BC, referred to as BALB/c from here onwards) were purchased from the Animal Resources Centre (Perth, Australia) and were acclimatised prior to experiments. To induce allergic airway responses, mice were intranasally inoculated with house dust mite extract under light isoflurane anaesthesia (HDM, *Dermatophagoides pteronyssinus*, 3343 endotoxin units/mg protein, Stallergenes Greer, US; 25 µg in 35 µL saline) 3 times (Monday, Wednesday & Friday) a week for 3 weeks. Twenty-four hours following the last HDM treatment, mice were intranasally inoculated with 100 PFU influenza A virus (HKx31/H3N2 subtype) under light isoflurane anaesthesia. Saline was used as vehicle control for HDM and IAV. Seven days post infection, mice were culled for endpoint analysis. In a separate set of experiments, mice were treated with HDM and IAV as described above and on day 2 and day 5 post IAV infection, mice were injected intraperitoneally with an anti-G-CSFR antibody or isotype control (50 µg, 5E2-VR81 clone, CSL Limited, Australia). On day 7 post infection, mice were culled for endpoint analysis where lung function tests, flowcytometry and BAL were performed, and lung tissue was collected.

Reverse Transcriptase Quantitative PCR (RT-qPCR) for gene expression analysis

Total RNA was extracted from frozen lung tissue with a RNeasy kit (Qiagen, Germany). cDNA was prepared with a High Capacity cDNA Kit (Life Technologies, USA). qPCR was performed using bioinformatically validated TaqMan probes (Life Technologies, USA). The threshold cycle values (Ct) were normalized to a reference gene (glyceraldehyde phosphate

dehydrogenase; GAPDH) and the relative fold change was determined by the $\Delta\Delta C_t$ value. To determine lung viral load, qPCR on polymerase A subunit (PA) gene was performed on total RNA extractions to measure influenza A virus in the lung tissue using TaqMan® Fast Virus 1-Step Master Mix (Life Technologies, USA) as previously described (Wang et al., 2017). Lung viral load is expressed as fold of change vs saline controls.

Lung function, BAL and tissue collection

Mice were anaesthetised by ketamine (125 mg/kg) and xylazine (25 mg/kg), after which tracheotomy was performed by inserting an 18G canular into the trachea. Airway reactivity in response to increased doses of methacholine (MCh) was measured *in vivo* with the Flexivent FX1 (SCIREQ® Montreal, QC, Canada) as previously described (Wang et al., 2019). This was followed by bronchoalveolar lavage (BAL), where BAL-fluid was collected and BAL cells were enumerated. Cytospins were prepared and stained with Shandon™ Kwik-Diff™ Stains (Life Technologies, US) for differential cell counts. Lungs were perfused with ice-cold PBS to remove blood. The superior lobe of the lungs was digested for flowcytometry analysis and the left lobe was fixed in 10% neutral-buffered formalin for histology. The rest of the lungs were snap-frozen in liquid nitrogen prior to -80°C storage.

Flowcytometry

Following BAL, the inferior lobe of the lungs was finely minced and digested in Liberase™ (Sigma, US) at 37 °C with constant shaking. Single cell suspension was prepared by passing the digested tissue through a 25G needle and then a 40 µm cell strainer. Cells were then pelleted and red blood cells were lysed with ACK lysis buffer. Cells were further stained with an antibody cocktail that contained FITC-CD45, PE-Siglec F, APC-F4/80, eFluor 450-CD11b, PE/Cy7-CD11c, PerCp/eFluor710-Ly6G (all from Life Technologies, US) and analysed on a

BD FACSAria. A strict gating strategy was used to exclude cell debris and cell aggregates and subset macrophage populations as previously described (Abdulnour et al., 2016; Anthony et al., 2020). Briefly, CD11b and CD11c were used to differentiate three distinct lung macrophage populations including resident alveolar macrophages (AM; CD45⁺ F4/80⁺ SSC^{High} CD11c^{High} CD11b⁻), exudative macrophages (ExMacs; CD45⁺ SSC^{High} F4/80⁺ CD11c^{High} CD11b⁺) and infiltrating monocytes (monocytes; CD45⁺ SSC^{High} F4/80⁺ CD11c^{Low} CD11b⁺).

Histology and Immunohistochemistry

The left lobe of the lungs was fixed in 10% neutral-buffered formalin for 24 hours before storing in 70% ethanol. Tissues were processed, paraffin-embedded and sectioned vertically at a thickness of 4 µm. Coronal sections that uniformly contain bronchi and > 8 airways were then stained with hematoxylin and eosin (H&E), Alcian blue-periodic acid Schiff (AB-PAS) and Masson's Trichrome (MT) for the assessment of airway inflammation, airway mucus and airway collagen (Wang et al., 2019). To measure airway smooth muscle mass, sections were immune-stained with a monoclonal mouse anti-human smooth muscle actin antibody (1:300 dilution, SMA, IgG2a, Clone 1A4, Cat: M085129-2, RRID:AB_2811108, Dako, Glostrup, Denmark) after antigen retrieval and blocking. Immunohistochemistry has been conducted the experimental detail provided conforms with British Journal of Pharmacology Guidelines (Alexander et al., 2018). Stained whole sections were scanned with an Olympus VS-120 slide scanner and morphometric analysis were performed on Olympus CellSens Software (RRID:SCR_014551, Olympus, Japan). A minimum of four small to medium size (100 to 350 µm luminal diameter) airways per lung section were analysed (Wang et al., 2019). Airway inflammation was scored blindly by two individual researchers where a score between 0 to 5 was given (Cyphert-Daly, Yang, Ingram, Tighe & Que, 2019). To quantify airway AB-PAS, MT and SMA, a band of region of interest (20 µm band width for SMA, 30µm for AB-PAS

and 40 μm for MT) was drawn around airways and stain-positive areas were calculated as area fractions of region of interest.

Lung TGF β , peroxidase activity and BALF assays

The superior lobe, middle lobe and post-caval lobe of the lungs were snap-frozen upon excision. Active transforming growth factor beta (TGF β) in fresh-frozen lungs was measured by an ELISA assay kit according to the manufacturer's instructions (Life Technologies, US). Peroxidase activity as a surrogate marker for tissue neutrophils and eosinophils in the lungs was measured as previously described (Wang et al., 2019). Gelatinase activity in BALF were measured by an EnzChek™ Gelatinase Assay Kit according to company instructions (Life Technologies, US). dsDNA levels in BALF were measured by a Quant-iT™ PicoGreen™ dsDNA Assay Kit as a NETosis marker, whereas LDH and proteins contents in BALF were measured by LDH Cytotoxicity Assay and Pierce and BCA Protein Assay Kit as lung injury markers (all from Life Technologies, USA).

Data and analysis

The experimental design and analysis comply with the recommendations and requirements of the British Journal of Pharmacology (Curtis et al., 2018). Animals were randomised prior to treatment to generate groups of equal size and blinded data analysis was performed where applicable (e.g. lung function tests & histological analysis). For group size calculation, we assumed type I error rate of 0.05 and a statistical power of at least 90% as previously reported with similar experimental designs (Jha et al., 2018; Wang et al., 2019). Data are presented as the mean $\pm$ SEM except for viral load which is presented as median $\pm$ interquartile range. Whist raw measurements showing standard units were presented in most figures, data normalisation was performed where standard units were not applicable: gene expression was presented as

fold mean of the controls and enzymatic activities as percentages of the controls. Viral loads measured by copies of viral gene were log-transformed as we previously reported. All data presented were statistically analysed using GraphPad Prism 7.0 (RRID:SCR_002798, Graphpad, San Diego, CA) with group size $n \geq 5$. 'n' represents the number of animals or the number of individual samples derived from these animals. These independent values, on which statistical analyses were performed, are not technical replicates. Where detailed and appropriate, two-tailed Students t-tests or one-way analyses of variance (ANOVA) with Tukey or Bonferroni's post-hoc tests were used. $p < 0.05$ was considered to be statistically significant. Post-hoc tests were conducted only if the F-value in ANOVA achieved statistical significance and there was no significant variance inhomogeneity. Outliers, predefined as individual data points exceeding $2 \times SD$, were excluded in data presentation and analysis, which were detailed in figure legends.

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., 2018) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

Results

IAV infection exacerbated AHR and airways fibrosis in HDM-sensitized mice.

Allergic airways inflammation was established by sensitizing BALB/c mice with HDM allergen over 3 weeks, followed by subsequent IAV infection and cull on day 7 post infection as detailed in Figure 1A. At day 7 post infection, low copies of the viral polymerase *PA* gene were detected in the lungs of IAV infected mice, and the level of detection was similar in HDMIAV-treated mice (Figure 1B). Baseline lung function responses were not different across the treatment groups, however Newtonian or central airway resistance (R_n) increased in a dose dependent manner to methacholine (MCh), peaking at 30 mg/mL in VEH/IAV/HDM-treated mice and 100 mg/mL in HDMIAV-treated mice (Figure 1C). Maximal MCh responses were compared across all groups and data demonstrates >40% increase in R_n in HDMIAV-treated mice (Figure 1D; $p < 0.05$ vs VEH). HDM-sensitisation increased tissue resistance (G), however this was not further exacerbated by IAV infection (Figure 1E; $p < 0.05$ HDM vs VEH). A trend of increase in G was observed in IAV alone group ($p = 0.09$ IAV vs VEH).

Airway inflammation, mucus overproduction, smooth muscle hypertrophy and collagen deposition are common pathological features underlying airway hyperresponsiveness (AHR). We next performed H&E, Alcian Blue-Periodic acid Schiff (AB-PAS), smooth muscle actin (SMA) and Masson's Trichrome (MT) staining on lung sections to evaluate these pathological processes (Figure 2A). HDM allergen or IAV alone induced airway inflammation, which was significantly higher in HDMIAV-treated mice (Figure 2A&B; $p < 0.05$ vs VEH, $p < 0.05$ HDM vs HDMIAV). Goblet cell hyperplasia was observed in HDM-treated mice but not in IAV-infected mice as demonstrated by AB-PAS staining (Figure 2A&C). IAV infection reduced the expansion of mucus-producing cells by about 50% in HDMIAV-treated mice compared to HDM alone. Consistently, a similar trend in mucin gene *MUC5AC* expression was observed

(Figure S2A). Airway smooth muscle was not altered in any of the groups, evidenced by the unchanged SMA-positive area (Figure 2A & D) and SMA gene *ACTA2* expression (Figure S2B). Airway fibrosis was also observed in HDM-treated mice, demonstrated by increased collagen deposition around the airways (Figure 2A&E). IAV infection in the background of HDM sensitisation further increased airway collagen area ($p < 0.05$ vs HDMIAV). This fibrotic airway staining pattern was consistent with the significant increase in *COL1A1*, *COL3A1* and *COL5A1* gene expression in HDMIAV-treated mice (Figure 2F-H). TGF β , a master regulator of collagen gene expression, was upregulated by HDM (Figure S2C; $p < 0.05$ vs VEH). However, there was no further increase in TGF β expression after IAV infection, suggesting a post-translational mechanism, such as protease-mediated activation of latent TGF β .

IAV infection induced mixed-granulocytic inflammation in the background of HDM sensitisation.

To investigate the cellular mechanisms responsible for AHR and lung remodelling, we analysed the subtypes of infiltrated inflammatory cells in the BALF compartment. Macrophages numbers tripled in response to repeated HDM challenge, which was further increased by 35% in HDMIAV treated mice (Figure 3A; $p < 0.05$ vs HDMIAV). Neutrophil trafficking to airways was promoted by IAV infection, which was not altered by prior HDM allergen exposure (Figure 3B). In contrast, BAL eosinophils recruited into the airways in response to HDM sensitisation were significantly reduced by IAV infection (Figure 3C; $p < 0.05$ HDM vs HDMIAV). Despite this reduction, IAV-infected mice with prior HDM sensitisation clearly presented with a unique mixed-granulocytic profile, with both neutrophils and eosinophils being abundant in the airways. This inflammatory profile was also observed in lung tissue, where FACS analysis adapted from the published gating strategies (Abdulnour et al., 2016; Wozniak, Kolls & Wormley, 2012) revealed accumulation of lung neutrophils

(CD45⁺Ly6G⁺) and eosinophils (CD11b⁻CD11c^{low}Siglec F⁺) exclusively in HDMI-*A*V-infected mice (Figure 3D-F).

Additionally, exudative macrophages (EM, CD11b⁻CD11c⁺Siglec F⁻) and infiltrating monocytes (CD11b⁺CD11c⁻Siglec F⁻) were significantly increased by HDMI-*A*V treatment (Figure 3H&I; $p < 0.05$ vs VEH) while alveolar macrophages (AM, CD11b⁻CD11c^{high}Siglec F⁺) were unaltered (Figure 3G). The disparate results observed between AM numbers in the BAL and lungs may reflect that mice were lavaged prior to extracting the lung lobe for FACS analysis. Accordingly, the majority of the AMs were removed during the lavage process, with only the residual AMs or interstitial macrophages detected in the lung tissue. In contrast, exudative macrophages and monocytes were increased in IAV and HDMI-*A*V infected mice, which is consistent with reports showing that monocyte derived exudative macrophages are increased and pathogenic during severe influenza infection in mice (Lin, Suzuki, Nakano, Ramsburg & Gunn, 2008). In addition, we assessed inflammation-associated lung injury and mucosal barrier disruption underlying AHR (Heijink et al., 2020). Consistent with the heightened neutrophilic response, NETosis marker, dsDNA (Figure S3A) was significantly increased in the BALF of HDMI-*A*V-treated mice ($p < 0.05$ vs VEH). This was accompanied with IAV-exacerbated mucosal injury and lung oedema that were induced by HDM, measured by the high levels of LDH (Figure S3B) and leaked serum proteins (Figure S3C) in BALF ($p < 0.05$ vs VEH & HDM).

IAV and HDM alters expression of Th2 markers and leukocyte chemokines.

We next measured gene expression of multiple molecular markers that regulate Th1/Th2 immunity and leukocyte trafficking. IAV infection stimulated a Th1 response evidenced by *IFNG* upregulation, which was not altered by prior HDM exposure (Figure 4A). HDM sensitisation elicited a robust Th2 response with significant increases in *IL-5*, *IL-4* and

transcription factor *GATA-3* that regulates Th2 cytokines (Figure 4B-D; $p < 0.05$ vs VEH). Interestingly, *IL-5*, *IL-4* and *GATA-3* expression levels were significantly reduced in HDMIAV-infected mice (Figure 4B-D; $p < 0.05$ vs HDM), which is consistent with reduced BAL eosinophil numbers. Monocyte chemokine *CCL-2/MCP1* is strongly induced by IAV and its levels were not altered by prior HDM exposure (Figure 4E). *CCL3/MIP1 α* levels were significantly increased in both HDM and HDMIAV treated mice (Figure 4F; $p < 0.05$ vs VEH), whereas *CCL5/RANTES* was only significantly increased in HDMIAV treated mice (Figure 4G; $p < 0.05$ vs. VEH). Neutrophil chemokine *CXCL1* was not significantly increased in HDMIAV treated mice, suggesting its limited role in sustaining neutrophilic inflammation in this setting (Figure 4H). In contrast, *G-CSF* expression was significantly increased in HDMIAV treated mice (Figure 4I; $p < 0.05$ vs VEH). *GM-CSF*, a leukocyte survival factor implicated in Th1 and Th2 immunity, was increased in HDM and HDMIAV treated mice (Figure 4J; $p < 0.05$ vs VEH).

G-CSFR mAb potently reduced neutrophilic inflammation and airway fibrosis.

To determine whether neutralising G-CSFR can block neutrophilic inflammation and reduce lung pathology, we treated mice with a murine anti-G-CSFR (α GR) or isotype control (ISO) mAb on day 2 and day 5 post IAV infection (Figure 5A). No difference in viral clearance was observed in mice treated with α GR or ISO (Figure 5B). BAL macrophage numbers were not altered, whereas BAL neutrophil numbers were significantly reduced by α GR treatment by over 80% (Figure 5C&D; $p < 0.05$ ISO vs α GR). Elevated eosinophil numbers in HDM/IAV-infected mice were not reduced in the BAL compartment or lung tissue with α GR treatment (Figure 5E & G), whereas lung neutrophil numbers were reduced to control levels with α GR treatment (Figure 5F). Elevated lung peroxidase activity was not reduced by α GR treatment

(Figure 5H), consistent with the high abundance of eosinophils in the lung. The accumulation of AMs, EMs and monocytes was not altered by α GR treatment (Figure 5I-K).

We next quantified neutrophil-associated lung injury and mucosal barrier disruption markers following α GR treatment. Notably, markers of NETosis and neutrophil degranulation, namely dsDNA (Figure 6A) and MPO (Figure 6B) were potently suppressed by α GR treatment (both $p < 0.05$ ISO vs α GR). Mucosal tissue injury, measured by the release of LDH to BALF, was significantly reduced in α GR-treated mice (Figure 6C; $p < 0.05$ ISO vs α GR). Lung oedema, measured by the leakage of serum proteins into the BAL compartment, was also partially prevented (Figure 6D; $p < 0.05$ ISO vs α GR). We then assessed airway remodelling by histology. α GR treatment did not reduce airway inflammation (Figure 7A&B) or mucous production in HDMI-1V-infected mice (Figure 7A&C). However, there was a significant reduction in airway collagen in α GR-treated mice (Figure 7A&D; $p < 0.05$ ISO vs α GR).

The decline in mucosal injury and airways fibrosis translated to a decrease in AHR, where total resistance (R_{rs}) was only significantly increased in HDMI-1V-ISO treated mice (Figure 7E). When lung function was compartmentalised, α GR treatment completely normalised central airways resistance (R_n) to control levels, whereas tissue resistance (G) was not significantly reduced (Figure 7F&G). The activity of leukocyte proteases in the BALF was assessed by measuring the net enzymatic degradation of FITC-labelled gelatin, where increased gelatinase activity in HDMI-1V-infected mice was significantly reduced with α GR treatment (Figure 8A). A similar profile was seen when active TGF β proteins in the lung tissue were measured, where increased TGF β levels in HDMI-1V-infected mice was significantly reduced with α GR treatment (Figure 8B). Consistent with this finding, increased expression of the collagen genes

COL1A1, *COL3A1* and *COL5A1* in the lungs of HDMIAV-ISO mice were reduced to levels detected in vehicle treated control mice with α GR treatment (Figure 8A-E).

Discussion

In this study, we have identified a novel pathogenic role for G-CSFR-dependent neutrophilic inflammation in an allergic airways disease model that was worsened by IAV respiratory infection. This model displays characteristic features of severe asthma including increased AHR that was associated with fibrotic remodelling of the airways. Elevated expression of types I and III collagens in patients with moderate-to severe asthma is insensitive to corticosteroid treatment (Chakir et al., 2003). The steroid refractory nature of fibrosis in severe asthma is consistent with our findings, as corticosteroids are known to promote neutrophil survival (Cox, 1995). Clinically, airways fibrosis does not appear to be a uniform feature of severe asthma, where excessive collagen deposition and fibrosis is found in some but not all severe asthma cohort studies (Benayoun, Druilhe, Dombret, Aubier & Pretolani, 2003; Chakir et al., 2003; Chu, Halliday, Martin, Leung, Szefer & Wenzel, 1998; Kaminska et al., 2009). Our data suggests that airways fibrosis may be a more prominent feature of severe asthmatics with mixed granulocytic inflammation and a history of pneumonia. Quantifying airways fibrosis according to distinct asthma phenotypes will determine whether non-T2 severe asthmatics with fixed airflow obstruction and prior pneumonia, display more fibrotic airway remodelling of the distal airways. Accordingly, our findings identify severe asthmatics with mixed granulocytic inflammation, lower lung function and a history of pneumonia as a phenotype that could benefit from α GR treatment, particularly during the peak and resolution phases of infection.

Fibrosis is a central feature of asthma pathology whereby excessive deposition of ECM components including collagen I, collagen III and collagen V can result in airway wall fibrosis and increased airflow limitation (Postma & Timens, 2006). TGF β is a potent profibrotic cytokine produced by multiple cell types including bronchial epithelial cells. In this study, lung TGF β transcript levels were increased in HDM-sensitized ‘asthmatic’ mice and levels were not

further elevated in IAV-infected 'asthmatic' mice at day 7 post infection. Once gene expression is initiated, TGF β is synthesised and secreted as a latent complex that requires conversion to its active form, which primarily controls the levels of bioactive TGF β . This conversion process can occur via multiple mechanisms including the proteolysis of latent TGF β by serine proteases and metalloproteases such as MMP-9 into its active form (Yu & Stamenkovic, 2000). Once activated, TGF β initiates expression of collagen genes via a SMAD-dependent signalling cascade. We observed that *COL1A1* and *COL3A1* lung expression levels were only increased in HDM-sensitised mice infected with IAV, which was consistent with increased airway collagen deposition in this group. In contrast, *COL5A1* gene expression was increased by HDM alone, which suggests that *COL5A1* promoter is controlled by a molecular transcriptional network that is distinct from *COL1A1* and *COL3A1*.

During IAV infection, the secretion of neutrophil-derived gelatinases such as MMP-9 degrade the basement membrane to facilitate greater mobilisation of circulating neutrophils into the site of infection, which was required for controlling the virus (Bradley, Douglass, Chatterjee, Akira & Baaten, 2012). We propose that the virus-specific proteolytic response involving release of neutrophil-derived proteases may have a secondary effect of converting latent TGF β into active TGF β and exacerbating airways fibrosis. This is supported by our finding that increased net gelatinase activity is blocked with anti-G-CSFR treatment in HDMIAV infected mice, which demonstrates that neutrophils are an important source of gelatinases in this model. Furthermore, the levels of active TGF β were similarly increased in the lungs of HDMIAV treated mice in a manner that was reduced with anti-G-CSFR treatment. Consistent with the reduction in gelatinase and active TGF β levels, anti-G-CSFR treatment reduced *COL1A1* and *COL5A1* levels in HDMIAV infected mice, to suggest that neutrophil derived proteases are regulating collagen deposition in this model. In contrast, *COL3A1* levels were not significantly

reduced by anti-G-CSFR treatment despite being increased in HDMIAV infected mice. It is plausible that IAV instigates a transcriptional network that increases *COL3A1* gene expression independent of TGF β , however this requires further investigation.

Excessive neutrophil activation during severe IAV infections has been associated with oxygenation impairment and is predictive of death (Tang et al., 2019). At a molecular level, the excessive release of neutrophil extracellular traps (NETs) that consists of dsDNA, MPO and proteases during IAV infection can cause significant damage to the microvascular bed resulting in oedema (Narasaraju et al., 2011). Hence, therapies that selectively reduce excessive neutrophil trafficking and/or activation should be considered as an important strategy to combat the pathological consequences of pneumonia causing respiratory viral infections. However, targeting neutrophilic inflammation during acute infections does raise safety questions, as total depletion of neutrophils with a monoclonal antibody that causes neutropenia, results in more severe pneumonia and higher rates of mortality in mice infected with IAV (Tate, Deng, Jones, Anderson, Brooks & Reading, 2009). The protective function of neutrophils during acute IAV infections involves regulating the function of cytotoxic CD8⁺ T cells, whereby neutrophil depletion reduced cytokine production and cytotoxic effector function of IAV-specific CD8⁺ T cells (Tate, Brooks, Reading & Mintern, 2012). In addition, neutrophils can release the T cell chemokine CXCL12 whilst trafficking into the lungs during infection, and this trail of CXCL12 provides a chemotactic gradient for subsequent cytotoxic CD8⁺ T cell migration (Lim et al., 2015). Our recent study showed that α GR treatment reduced airway neutrophils by over 70% and normalised blood neutrophil counts to uninfected/control levels whilst preserving anti-viral immunity and viral clearance in an acute IAV infection model (Wang, Aloe, Wilson & Bozinovski, 2019). Another potential complication of IAV infections is the increased susceptibility to secondary bacterial infections such as pneumococcus. In the same study, we

have also shown that α GR treatment reduced neutrophilic inflammation during acute pneumococcal lung infection without compromising bacterial clearance (Wang, Aloe, Wilson & Bozinovski, 2019). (Cox, 1995) and increase risk of developing pneumonia (Kew & Seniukovich, 2014). Th17 cells that can coordinate neutrophilic inflammation increase with asthma severity, however, brodalumab, a human anti-IL-17RA monoclonal antibody did not produce a treatment effect in subjects with asthma (Busse et al., 2013), which may reflect its multifactorial role in immunity. In addition, targeting the neutrophil chemokine CXCR2 receptor with antagonists such as AZD5069 did not significantly improve symptom scores in severe asthmatics (O'Byrne et al., 2016) and CXCR2 deficiency increased susceptibility to pneumococcal infection in mice (Herbold et al., 2010). Our recent studies support antagonising G-CSFR signalling as an effective strategy to combating neutrophil dependent pathology in severe asthma. We found that in bronchial biopsies obtained from severe asthmatics with mixed granulocytic inflammation and chronic airway bacterial infection, G-CSF and G-CSFR transcript levels were significantly higher (Wang et al., 2019). In addition, antagonising G-CSFR in HDM-sensitized mice chronically infected with pneumococcus significantly improved AHR by reducing neutrophilic inflammation and mucous production (Wang et al., 2019). Furthermore, systemic depletion of neutrophils resulted in a compensatory increase in lung G-CSF levels, which primed type 2 innate lymphoid cells (ILC2s) to increase T2 inflammation, mucus production and airways resistance in HDM sensitized mice (Patel et al., 2019). In our HDMIAV model, increased production of G-CSF was not associated with an increase in T2 inflammation as Th2 markers and eosinophil numbers were decreased relative to HDM-sensitized mice. Our data suggests that the Th1/IFN γ and innate response to IAV infection in HDM sensitised mice dampens established type-2 inflammation. This data is consistent with the virus-induced IFN γ response inducing the T-bet transcription factor, which can redirect polarised Th2 cells into Th1 cells (Szabo, Kim, Costa, Zhang, Fathman &

Glimcher, 2000). However, this reduction in T2 inflammation was not sufficient to reduce AHR, rather an increase in central airways resistance was observed.

In summary, we demonstrate that G-CSFR-dependent neutrophilic inflammation worsens AHR in an IAV-induced severe asthma model. Mechanistically, we identify a role for G-CSFR signalling in exacerbating mucosal lung injury, oedema and markers of netosis in the airways. Consistent with our model, netosis markers have been shown to be elevated in a subset of severe asthmatics with neutrophilic inflammation, where NETs caused cytotoxicity in airway epithelial cells (Lachowicz-Scroggins et al., 2019) and disrupt the microvascular bed to promote oedema (Narasaraju et al., 2011). Hence, by antagonising G-CSFR dependent neutrophilic inflammation and netosis, the integrity of the mucosal barrier was improved, thereby reducing bronchoconstrictor access to airway smooth muscle cells. In addition, we show that G-CSFR dependent neutrophilic inflammation contributes to excessive airways fibrosis in HDMIAV-infected mice. TGF β is a well-recognised master regulator of fibrosis, where proteases including MMP-9 can proteolytically cleave latent TGF β into active TGF β (Yu & Stamenkovic, 2000). Once activated, TGF β utilises the smad transcription factor family to initiate expression of ECM proteins including a suite of collagen genes (Verrecchia, Chu & Mauviel, 2001). MMP9 is a potent gelatin degrading enzyme that is stored by neutrophils in tertiary granules, which are released during neutrophil degranulation and netosis. We show for the first time that antagonising G-CSFR markedly reduced gelatinase levels in the airways, normalised active TGF β and collagen expression lung levels, and reduced collagen deposition in the airways of HDMIAV-infected mice. Hence, antagonising G-CSFR signalling offers a new therapeutic opportunity to reduce deleterious fibrotic airway remodelling, which may prevent the transition from mild to severe asthma.

Conflict of interest statement: N.W. is employed by CSL Ltd. The other authors declare no competing interests.

Declaration of transparency and scientific rigour: This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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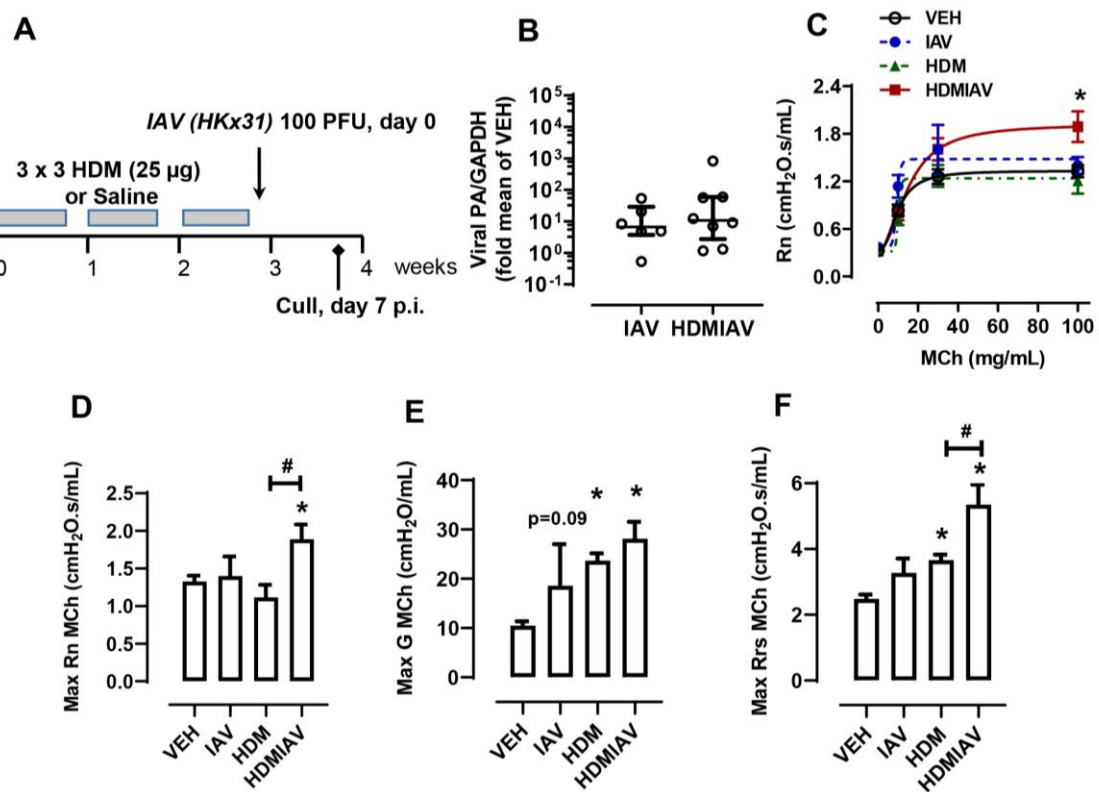


Figure 1. IAV infection exacerbated HDM induced BAL inflammation.

BALB/c mice were treated with HDM or saline (VEH) for 3 weeks. Twenty-four hours after the last HDM treatment, mice were inoculated with 100 PFU IAV (HKx31) or saline (VEH) and culled on day 7 post infection as detailed in (A). Lung viral titres were measured by quantifying viral polymerase *PA* gene expression (B). Lung function was performed on all groups in response to increasing doses of methacholine (MCh, 0 mg/mL, 10 mg/mL, 30 mg/mL, 100 mg/mL) using the Flexivent FX1 system. Dose response curve of central airways resistance (Rn, newtonian resistance) was presented and peak resistance was identified (C). Comparison of maximal Rn across all four groups demonstrated a significant increase only in HDMIAV-infected mice (D), whereas maximal tissue resistance (G) was increased in both HDM-sensitized mice and HDMIAV-infected mice (E). $n = 8$ for all groups, except for the IAV group ($n=6$), as two mice did not complete the protocol. Also, in panel (F), one outlier was excluded from the HDMIAV group ($n = 7$). * $p < 0.05$, one-way ANOVA vs VEH; # $p < 0.05$, one-way ANOVA vs HDMIAV.

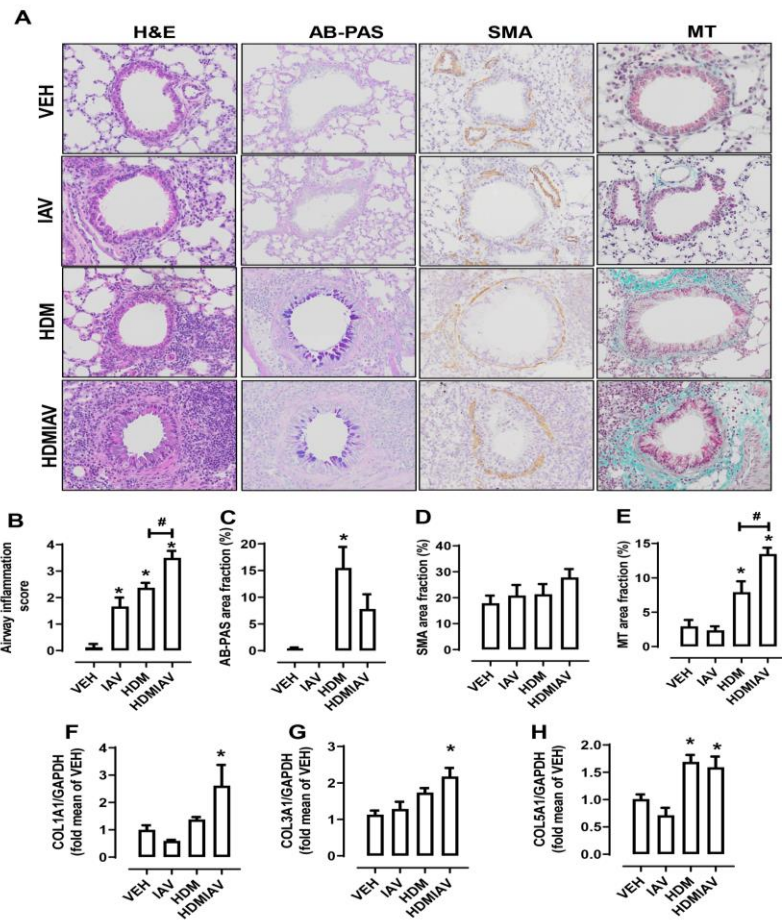


Figure 2. Collagen deposition contributed to airway hyperreactivity in HDMIAV-infected mice.

Lung sections were stained with H&E, Alcian Blue-Periodic acid Schiff (AB-PAS), smooth muscle actin (SMA) and Masson's Trichrome (MT) (A). Airway inflammation was scored (B) on H&E stained lung sections. Airway mucus production was quantified by area fraction on AB-PAS stained lung sections (C). Airway smooth muscle mass was quantified by area fraction on SMA stained lung sections (D). Airway collagen was quantified by area fraction on MT stained lung sections (E). The increase in collagen deposition around the airways was associated with an increase in collagen gene expression in the lung (F-H). $n = 6$ for the IAV group and $n = 8$ for all other groups. * $p < 0.05$, one-way ANOVA vs VEH; # $p < 0.05$, one-way ANOVA vs HDMIAV.

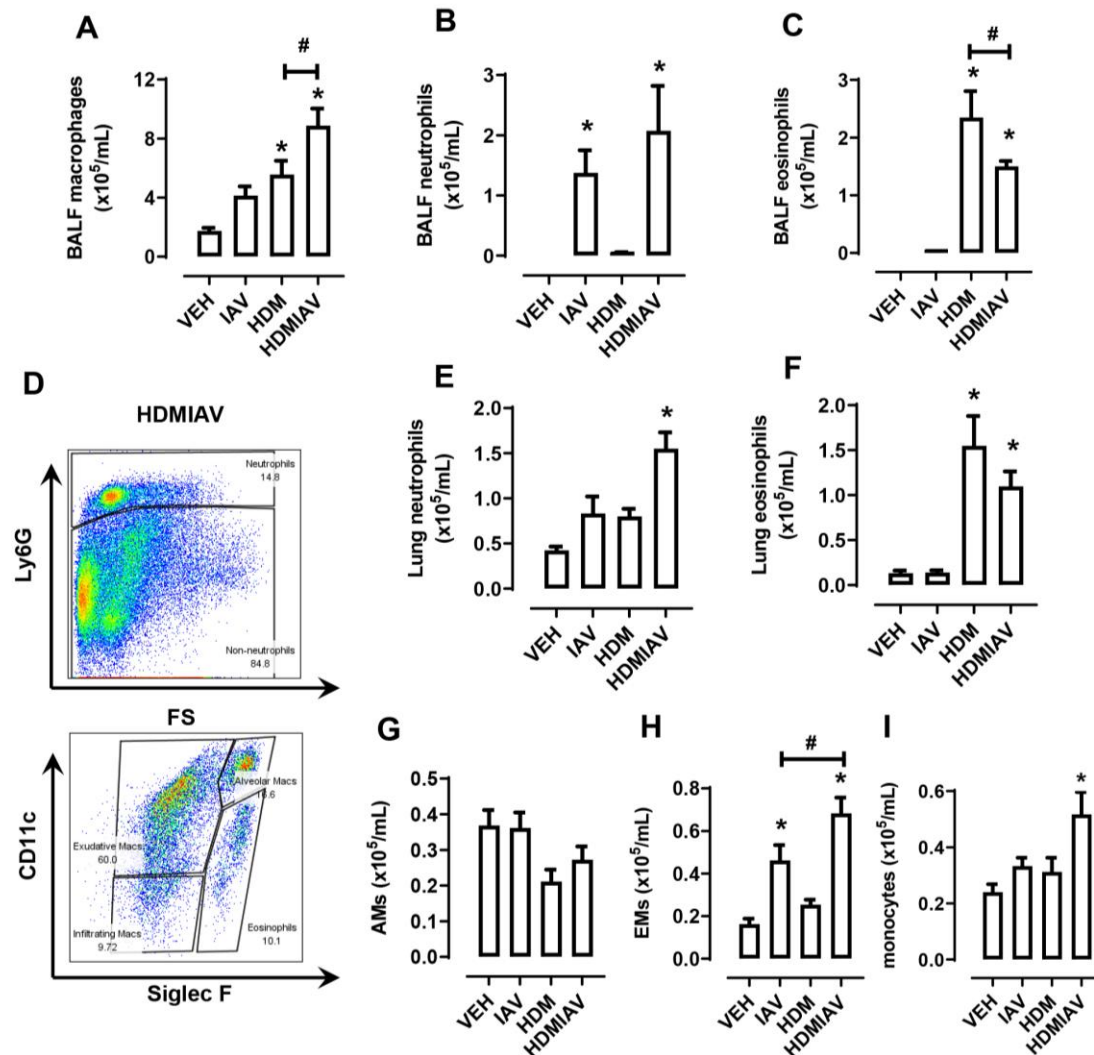


Figure 3. IAV infection in HDM-sensitized mice initiates mixed granulocytic inflammation.

BAL cytoposits were prepared and differential cell counts of macrophages (A), neutrophils (B) and eosinophils (C) were performed, demonstrating that HDMIAV-infected mice were the only group to display a mixed granulocytic inflammatory phenotype. Single cells were prepared from digested lungs and a gating strategy was employed for flowcytometric analysis of neutrophils ($\text{CD45}^+\text{Ly6G}^+$) and non-neutrophils ($\text{CD45}^+\text{Ly6G}^-$) (D & E). F4/80^+ non-neutrophils (D, lower panel) were further gated into eosinophils ($\text{CD11b}^-\text{CD11c}^{\text{low}}\text{Siglec F}^+$, F), alveolar macrophages (AM, $\text{CD11b}^-\text{CD11c}^{\text{high}}\text{Siglec F}^+$, G), exudative macrophages (EM, $\text{CD11b}^-\text{CD11c}^+\text{Siglec F}^-$, H), and monocytes ($\text{CD11b}^+\text{CD11c}^-\text{Siglec F}^-$, I). $n=6$ for IAV group and $n=8$ for all other groups. * $p < 0.05$, one-way ANOVA vs VEH; # $p < 0.05$, one-way ANOVA vs HDMIAV.

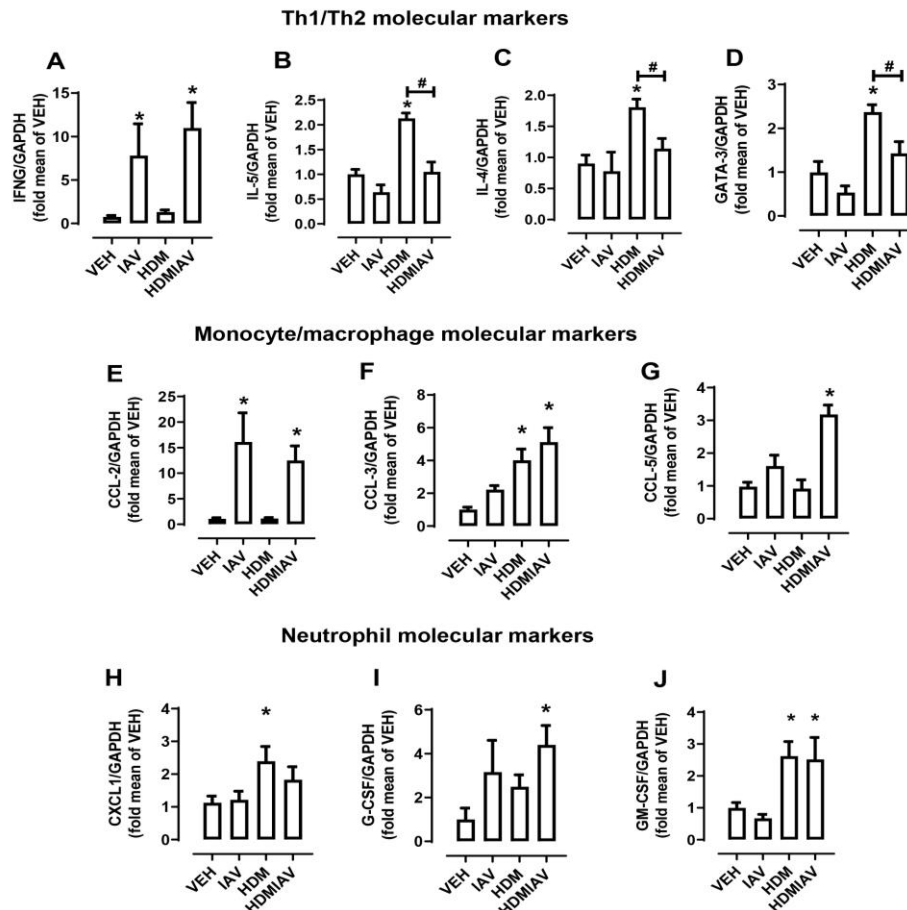


Figure 4. Profiling molecular drivers of Th2 and innate inflammation.

RT-qPCR was performed on lung tissue to measure gene expression of the classic Th1 marker *IFNG* (A) and the Th2 markers *IL-5* (B), *IL-4* (C) and *GATA3* (D), demonstrating that elevated expression of Th2 markers in HDM-sensitized mice was reduced by IAV infection. The macrophage chemokines *CCL-2* (E), *CCL-3* (F) and *CCL-5* (G) were significantly increased in HDMIAV-infected mice. The neutrophil chemokine *CXCL1* (H) was not increased in this model, whereas *G-CSF* expression was significantly increased in HDMIAV-infected mice (I). *GM-CSF* levels were increased in HDM-sensitized mice and this response was not altered by IAV infection (J). $n = 6$ for IAV group and $n = 8$ for all other groups. * $p < 0.05$, one-way ANOVA vs VEH; # $p < 0.05$, one-way ANOVA vs HDMIAV.

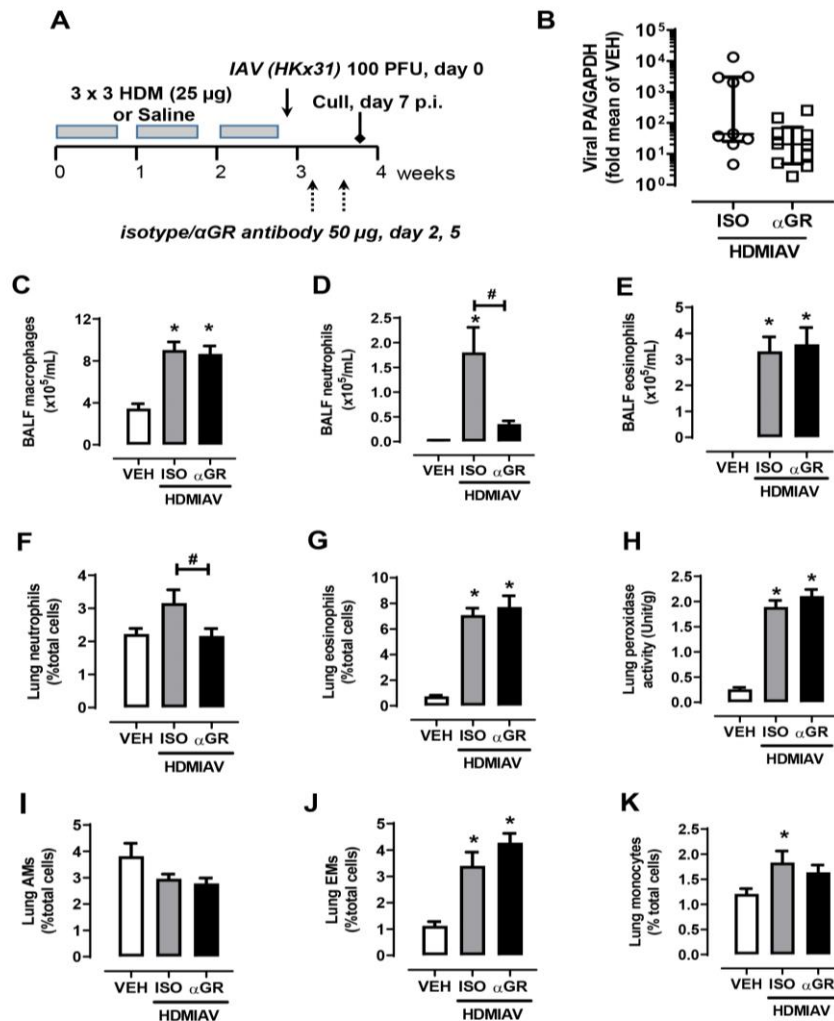


Figure 5. α GR mAb reduced neutrophilic inflammation in BALF.

BALB/c mice were treated with HDM or saline (VEH) and 24 hours post the last HDM treatment, infected with 100 PFU IAV (HKx31). On day 2 and day 5 post IAV infection, mice were injected intraperitoneally with α GR mAb or isotype control (ISO) and on day 7 post IAV infection, mice were culled as detailed in (A). Lung viral titres were measured by quantifying viral polymerase PA gene expression (B). BAL cytoplots were prepared and differential cell counts of macrophages (C), neutrophils (D) and eosinophils (E) were performed, demonstrating that α GR mAb treatment reduced neutrophilic inflammation in HDMIAV-infected mice by about 80%, without altering BAL macrophage and eosinophil counts. Flowcytometric analysis of lung tissue demonstrated that neutrophils (CD45⁺Ly6G⁺) were significantly reduced in α GR mAb treated mice (F), whereas eosinophils (CD11b⁻CD11c^{low}Siglec F⁺ (G) and total peroxidase activity (H) were unaltered. Analysis of alveolar macrophages (AM, CD11b⁻CD11c^{high}Siglec F⁺, I), exudative macrophages (EM, CD11b⁻CD11c⁺Siglec F⁻, J), and monocytes (CD11b⁺CD11c⁻Siglec F⁻, K) revealed no difference in α GR mAb treated mice. $n = 10$ for all groups except in panels (B) & (H), where an outlier was excluded from the HDMIAV_ISO group ($n = 9$). * $p < 0.05$, one-way ANOVA vs VEH; # $p < 0.05$, one-way ANOVA vs HDMIAV_ISO.

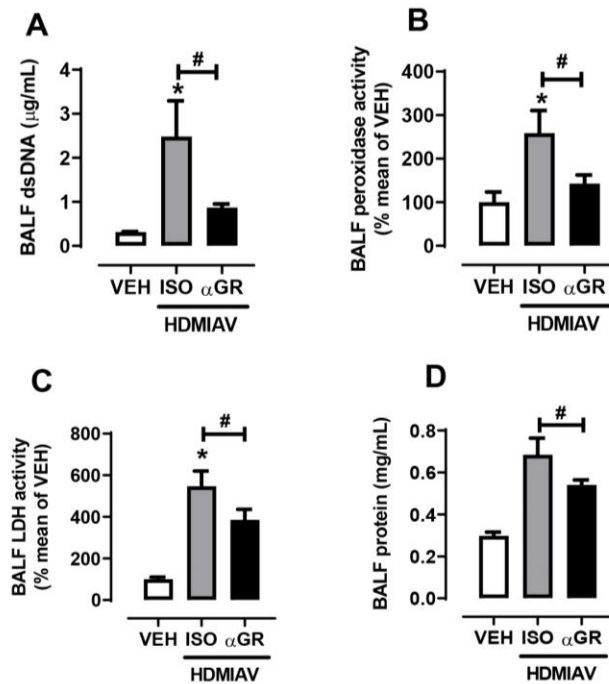


Figure 6. α GR mAb reduced markers of NETosis and lung injury.

Markers of NETosis including dsDNA levels (**A**) and peroxidase activity (**B**) were measured in the BALF, demonstrating that elevated levels in HDMIAV-infected mice were significantly reduced by α GR mAb treatment. Markers of lung injury and epithelial/endothelial barrier integrity/oedema including LDH levels (**D**) and total protein content (**F**) were also measured in BALF, demonstrating that elevated levels in HDMIAV-infected mice were significantly reduced by α GR mAb treatment. $n = 10$ for all groups except in panel (**C**) where an outlier was excluded from the HDMIAV_ α GR group ($n = 9$). * $p < 0.05$, one-way ANOVA vs VEH; # $p < 0.05$, one-way ANOVA vs HDMIAV_ISO.

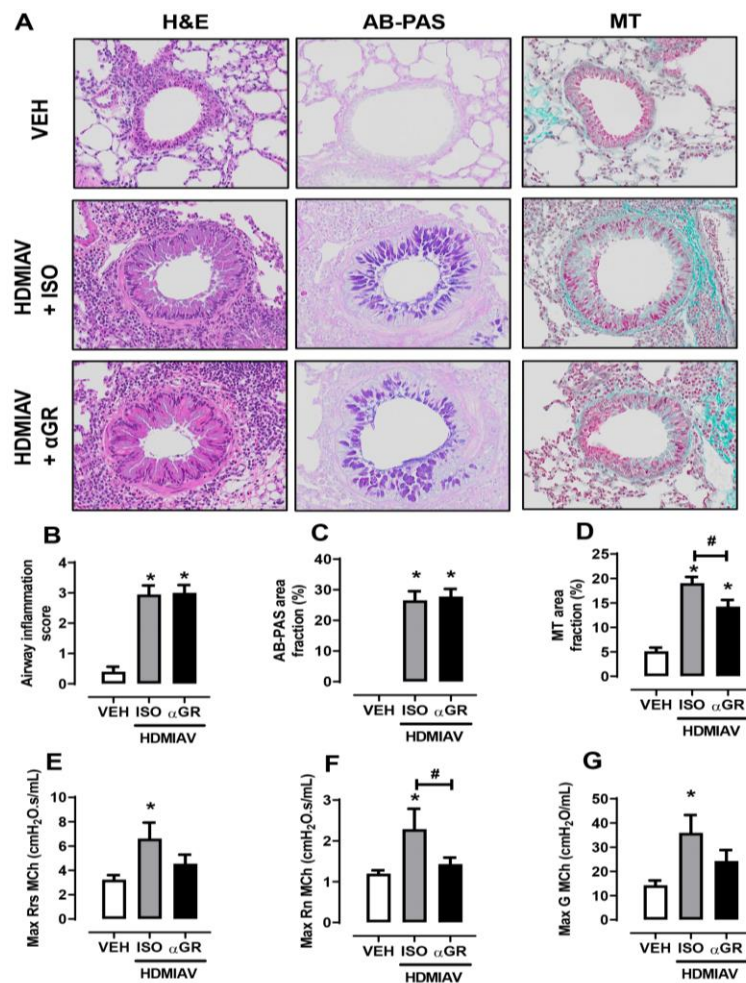


Figure 7. α GR mAb reduced airway collagen deposition and AHR.

Lung sections were stained with H&E, Masson's Trichrome (MT), Alcian Blue-Periodic acid Schiff (AB-PAS) and smooth muscle actin (SMA) (A). Airway inflammation was scored (B) on H&E stained lung sections. Airway mucous levels were quantified (C) on AB-PAS stained lung sections. Airway collagen deposition was quantified (D) on MT stained lung sections demonstrating that elevated levels of collagen deposition in HDMIIV-infected mice was significantly reduced by α GR mAb treatment. Lung function was performed using the Flexivent FX1 system, where maximal total resistance (Rrs) and tissue resistance (G) was significantly increased in HDMIIV_ISO treated mice, but not in HDMIIV_ α GR treated mice (E & G). Elevated central airways resistance (Rn) in HDMIIV-infected mice was significantly reduced with α GR mAb treatment (F). $n = 10$ for all groups except in panels (E-G), where one mouse from the HDMIIV_ISO group failed lung function testing ($n = 9$). * $p < 0.05$, one-way ANOVA vs VEH; # $p < 0.05$, one-way ANOVA vs HDMIIV_ISO.

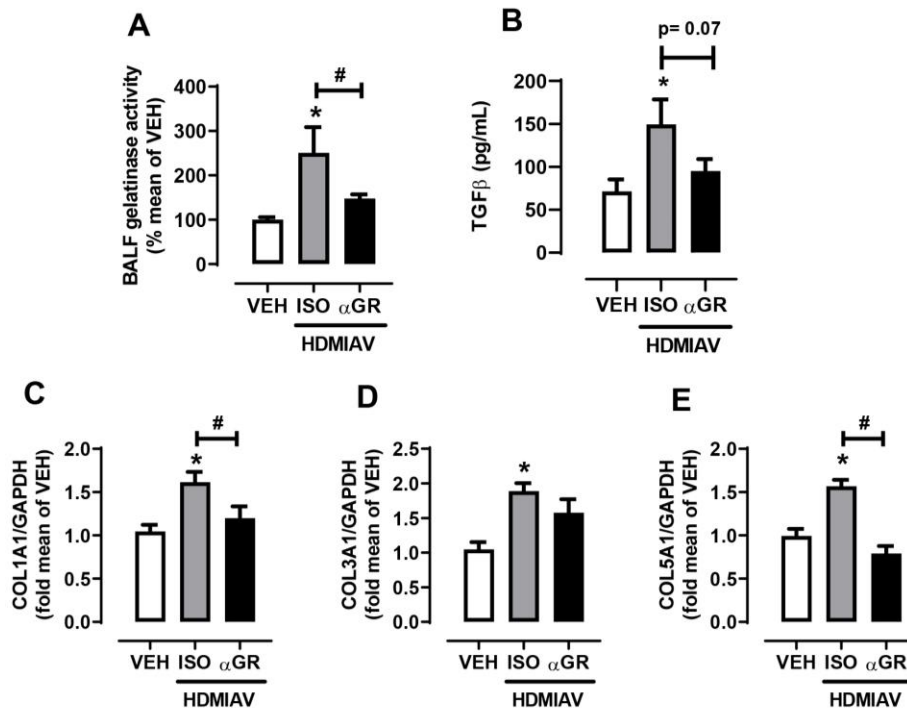


Figure 8. α GR mAb reduced gelatinase levels, active TGF β levels and collagen gene expression.

Net gelatinase activity in the BAL compartment was quantified using FITC-labelled gelatin as a substrate and showed that increased activity in HDMI AV-infected mice was significantly decreased by with α GR treatment (**A**). Active TGF β levels in the lung tissue were quantified and showed that levels were only significantly increased in HDMI AV-ISO treated mice but not in HDMI AV- α GR treated mice (**B**). There was also a decrease in the gene expression of *COL1A1* (**C**), *COL3A1* (**D**) and *COL5A1* (**E**) in lung tissue with α GR treatment. For the gelatinase assay in panel (**A**), $n = 10$ for all groups except for the HDMI AV-ISO group, where one outlier was excluded ($n = 9$). For the TGF β assay in panel (**B**), $n = 8$ for all groups. * $p < 0.05$, one-way ANOVA vs VEH; # $p < 0.05$, one-way ANOVA vs HDMI AV-ISO.

Supplement Figures

Figure S1

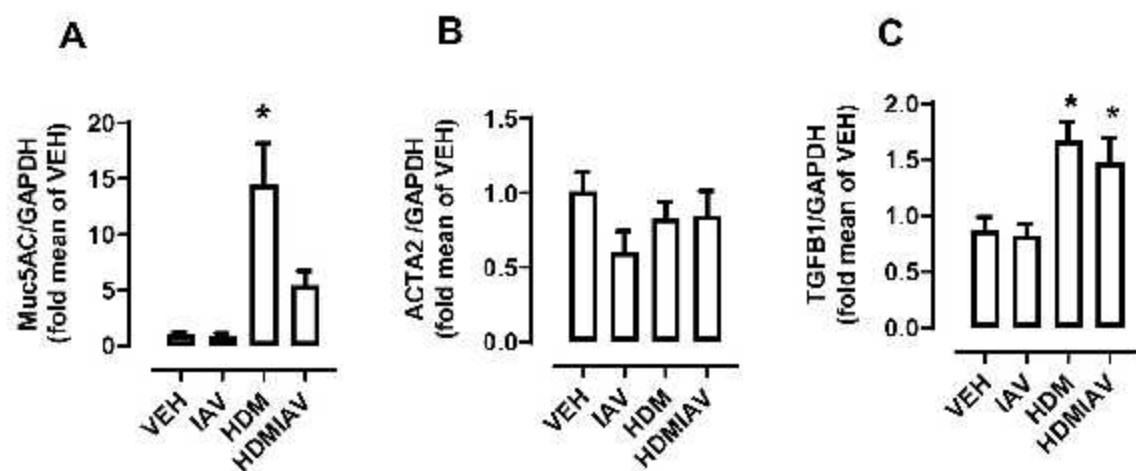
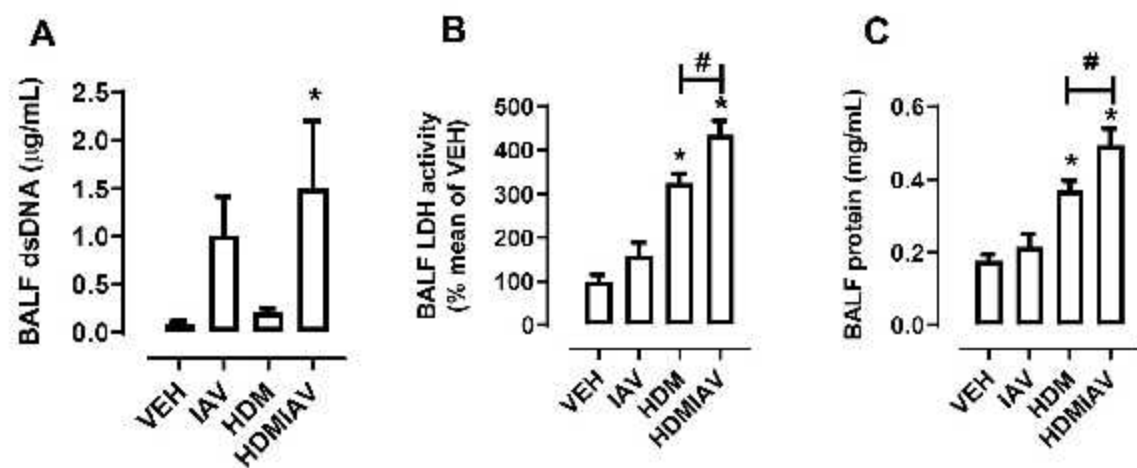


Figure S2



Supplement figure legends**Figure 1. HDMIAV induced lung expression of *Muc5AC*, *ACTA2* and *TGFB-1*.**

BALB/c mice were treated with HDM or saline (VEH) and 24 hours after the last HDM treatment, inoculated with 100 PFU IAV (HKx31) or saline (VEH) and culled 7 days post. RT-qPCR was performed on lung tissue to measure the expression of mucin gene *MUC5AC*, alpha smooth muscle actin gene *ACTA2* and *TGFB-1*. n =6 for IAV group and n = 8 for all other groups. * p < 0.05, one-way ANOVA vs VEH; # p < 0.05, one-way ANOVA vs HDMIAV.

Figure 2. HDMIAV induced NETosis and lung injury.

Levels of dsDNA (**A**) were measured in the BALF, demonstrating an elevation in HDMIAV-infected mice. Markers of lung injury and epithelial/endothelial barrier integrity/oedema, namely LDH activity (**B**) and total protein content (**C**) were also measured in BALF, demonstrating an elevation by HDM exposure and a further increase by IAV-infection. n =6 for IAV group and n = 7 for all other groups. * p < 0.05, one-way ANOVA vs VEH; # p < 0.05, one-way ANOVA vs HDMIAV.