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Targeting the human β_c receptor inhibits inflammatory myeloid cells and lung injury caused by acute cigarette smoke exposure

Nok Him Fung[1] | Hao Wang[1] | Ross Vlahos[1] | Nick Wilson[2] | Angel F. Lopez[3] | Catherine M. Owczarek[2] | Steven Bozinovski[1]

[1]School of Health & Biomedical Sciences, RMIT University, Bundoora, Victoria

[2]CSL Limited, Parkville, Victoria, Australia

[3]Centre for Cancer Biology, SA Pathology and UniSA, Adelaide, South Australia, Australia

Correspondence
Steven Bozinovski
Email: steven.bozinovski@rmit.edu.au

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CSL Limited

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Abstract

Background and objective: Chronic obstructive pulmonary disease (COPD) is a devastating disease commonly caused by cigarette smoke (CS) exposure that drives tissue injury by persistently recruiting myeloid cells into the lungs. A significant portion of COPD patients also present with overlapping asthma pathology including eosinophilic inflammation. The β_c cytokine family includes granulocyte monocyte-colony-stimulating factor, IL-5 and IL-3 that signal through their common receptor subunit β_c to promote the expansion and survival of multiple myeloid cells including monocytes/macrophages, neutrophils and eosinophils.

Methods: We have used our unique human β_c receptor transgenic (hβ_cTg) mouse strain that expresses human β_c instead of mouse β_c and β_{IL3} in an acute CS exposure model. Lung tissue injury was assessed by histology and measurement of albumin and lactate dehydrogenase levels in the bronchoalveolar lavage (BAL) fluid. Transgenic mice were treated with an antibody (CSL311) that inhibits human β_c signalling.

Results: hβ_cTg mice responded to acute CS exposure by expanding blood myeloid cell numbers and recruiting monocyte-derived macrophages (cluster of differentiation 11b$^+$ [CD11b$^+$] interstitial and exudative macrophages [IM and ExM]), neutrophils and eosinophils into the lungs. This inflammatory response was associated with lung tissue injury and oedema. Importantly, CSL311 treatment in CS-exposed mice markedly reduced myeloid cell numbers in the blood and BAL compartment. Furthermore, CSL311 significantly reduced lung CD11b$^+$ IM and ExM, neutrophils and eosinophils, and this decline was associated with a significant reduction in matrix metalloproteinase-12 (MMP-12) and IL-17A expression, tissue injury and oedema.

Conclusion: This study identifies CSL311 as a therapeutic antibody that potently inhibits immunopathology and lung injury caused by acute CS exposure.

KEYWORDS

β_c cytokine, inflammation, chronic obstructive pulmonary disease, eosinophils, acute cigarette smoke exposure, pre-clinical model

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is an incurable disease caused by inhalation of noxious particles including long-term cigarette smoke (CS) exposure. An important driver of COPD pathophysiology is chronic lung inflammation, orchestrated by key leukocyte populations including neutrophils and macrophages that accumulate around the airways as the severity of COPD progresses.[1] There are also significant changes that occur to resident alveolar macrophages (AMs) because of chronic CS exposure and COPD. Morphologically, AMs become enlarged in appearance due

Nok Him Fun and Hao Wang contributed equally to this study.

to engulfment of oxidized phospholipids generated during CS exposure.[2,3] At a transcriptional level, gene expression changes cluster into multiple functional categories including anti-oxidant/detoxifying genes, the immune and inflammatory response and proteinase/anti-proteinase balance.[4,5] Matrix metalloproteinase-12 (MMP-12) is a protease classified as an M2 marker that is highly induced by CS, and MMP-12$^+$ AMs increase in number in smokers and COPD patients.[6,7]

The advancement of immunophenotyping techniques is identifying an increasing number of alternative macrophage populations in the lung. AMs primarily located within the lung lumen are defined by a distinct immunophenotype including surface expression of F4/80, Siglec F and cluster of differentiation 11c (CD11c). In addition to AMs, interstitial macrophages (IMs) reside within the lung tissue and differentially express multiple markers including F4/80$^+$/Siglec F$^-$/CD11b$^+$/CD11c$^-$. IMs originate from the bone marrow postnatally to maintain a tissue resident macrophage population and mature into at least three distinct IM subsets.[8] IM populations have been characterized as being pro-inflammatory, and less phagocytic compared to AMs,[8] a finding that has been replicated in lung macrophages isolated from COPD patients.[9] CD11b$^+$ IM populations expand in response to CS exposure in a manner that is dependent on monocyte recruitment and IL-1α signalling.[10] Furthermore, an elastase-induced emphysema model promoted the expansion of monocyte-derived CD11b$^+$ macrophage lung population described as IMs, which were identified as a major source of the MMP-12 elastase.[11]

A separate CD11b$^+$ macrophage population has also been described as exudative macrophages (ExMs), which originate from Ly6C$^+$ (lymphocyte antigen 6 complex, locus C) monocytes and markedly expand in numbers during acute inflammation.[12] ExMs express both CD11b and CD11c and are a major source of inflammatory cytokines during the early phases of an inflammatory event and are capable of stimulating T-cell activation.[13] This inflammatory response is typically self-limiting as ExMs phenotypically switch to producing anti-inflammatory mediators such as IL-1RA[14] and, once the insult is eliminated, gradually contract in number. There is ongoing debate as to whether ExMs may overlap with IM subsets that share similarities, such as IM3 that expresses CD11b and CD11c, and are dependent on circulating CCR2$^+$ monocyte precursors.[8] For the purposes of our study, we have defined the following three lung macrophage populations including AMs (F4/80$^+$/Siglec F$^+$/CD11c$^+$/CD11b$^-$), IMs (F4/80$^+$/Siglec F$^-$/CD11b$^+$/CD11c$^-$) and ExMs (F4/80$^+$/Siglec F$^-$/CD11b$^+$/CD11c$^+$).

Airway neutrophilia is also a hallmark immunological feature of COPD where increased sputum numbers correlate with lung function decline in COPD patients.[15] Recruited neutrophils exhibit dysregulated degranulation, leading to increased release of proteolytic enzymes such as MMP-9 and neutrophil elastase as COPD severity progresses,[16,17] which can degrade the extracellular matrix (ECM) and surrounding connective tissues. There is also a significant subset of COPD patients (10%–40%) where eosinophilic inflammation is a prominent immunological feature.[18] The IL-5 cytokine is a central mediator of eosinophilic inflammation as it can prime quiescent eosinophils to become more responsive to chemotaxis, degranulation and cytokine production.[18,19] Increased levels of IL-5 and eosinophils were detected in the lungs of CS-exposed mice, where aldehydes in CS were required for this inflammatory response.[20] There is a growing list of monoclonal antibodies that block single cytokines (IL-5, granulocyte monocyte-colony-stimulating factor [GM-CSF] and IL-4) as a therapeutic strategy to treat chronic inflammatory conditions including asthma and rheumatoid arthritis. In the context of CS exposure, anti-GM-CSF antibodies effectively inhibited bronchoalveolar lavage (BAL) neutrophil and AM numbers without compromising phagocytic function in the pre-clinical setting.[21]

In this study, we have focused on targeting the β_c cytokine family as a whole rather than individual cytokines involved in myeloid cell proliferation, survival and function. The β_c cytokine family includes GM-CSF, IL-5 and IL-3, which share the β_c receptor subunit that forms a high affinity complex with specific α subunits of the IL-3, IL-5 or GM-CSF receptor (GM-CSFR).[22] As the β_c subunit is required for downstream signalling upon engagement by these three ligands, it represents an important single therapeutic candidate to reduce lung immunopathology driven by multiple β_c cytokines. For example, antisense therapy against β_c and CCR3 significantly reduced sputum eosinophils numbers by about 50% in mild asthmatics who were challenged with allergen.[23] Inhibition of β_c signalling in a model of allergic contact dermatitis also suppressed ear pinna thickening by reducing accumulation of neutrophils, mast cells and eosinophils in the skin.[24] To date, no study has tested the efficacy of a pan antagonist that targets the common cytokine-binding site within the human β_c receptor in an acute CS exposure model. Here, we used our unique transgenic mouse model (human β_c receptor transgenic [hβ_cTg] mice) expressing human β_c[24] to show that when acutely exposed to CS, the anti-β_c CSL311 antibody effectively blocks β_c cytokine signalling with high potency.[25–29] We demonstrate that β_c receptor signalling promotes infiltration of CD11b$^+$ macrophages including IMs and ExMs, neutrophils

SUMMARY AT A GLANCE

Myeloid cells, including macrophages, neutrophils and eosinophils, are important cellular drivers of inflammation and injury. In this study, we blocked granulocyte monocyte-colony stimulating factor, IL-5 and IL-3 signalling with an anti-β_c receptor antibody (CSL311), which greatly reduced lung inflammation and injury in a pre-clinical model of acute cigarette smoke exposure.

and eosinophils in a manner that is potently amenable to inhibition by CSL311 treatment.

METHODS

Animal experimental protocol

Humanized transgenic mice where the endogenous mouse β_c and $\beta_{IL\text{-}3}$ receptors have been knocked out and replaced with the human (h)β_c receptor were used.[24] Under in vitro conditions, haematopoietic cells from hβ_cTg respond to mouse GM-CSF and IL-5 but not to IL-3, which indicates that the mouse IL-3Rα subunit does not efficiently interact with the human β_c receptor subunit.[24] Mice were exposed to nine cigarettes per day period in an 18-L Perspex container that allows for free movement for four or 11 consecutive days, as previously described.[21,30] CSL311, a human monoclonal antibody that blocks β_c cytokine signalling or matching isotype control antibody,[24,25] was administered via intravenous injection (50 mg/kg) on day 1, day 4, day 8 and day 11 prior to the CS exposure session.

BAL, histology, RTqPCR and injury analysis

On day 12, hTgβc mice were euthanized, blood collected and BAL performed as previously described.[31,32] Cytospin slides stained with a Hemacolor® Rapid Staining Kit (Sigma-Aldrich, USA) were used for differential cell counting. The left lung was fixed in 10% neutral-buffered formalin for histology, the right superior lobe was excised for flow cytometry and the remaining lobes was snap-frozen in liquid nitrogen and stored at −80°C as previously described.[31,32] Lung injury was blindly scored as previously described,[33] where the degree of inflammatory cell infiltration, epithelial/endothelial destruction and alveolar septal thickening was used to generate an aggregate score. Lactate dehydrogenase (LDH) levels in the BAL fluid (BALF) were measured using the Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, USA). Lung oedema was determined by measuring serum albumin levels in the BALF using the Bromocresol Green Albumin Assay kit (Sigma-Aldrich, USA). Gelatinase activity in the BALF was measured using the EnzChek™ Gelatinase Kit (Thermo Fisher Scientific, USA). Cell-free double-stranded DNA (dsDNA) in the BALF was determined using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, USA). Total RNA and real-time quantitative PCR (RTqPCR) were then performed as previously described.[33,34]

Flow cytometry

The right superior lobe was digested in Liberase™ (Sigma-Aldrich, USA) and single-cell suspensions were stained with a panel of antibodies consisting of FITC-CD45, PE-Siglec F, APC-F4/80, eFluor 450-CD11b, PE/Cy7-CD11c and PerCp/eFluor710-Ly6G. Data were analysed on a BD LSRFortessa™ X-20 (BD Biosciences, USA), as previously been described[33,34] and presented in Figures S1 and S2 in the Supporting Information. For the purposes of our study, we have defined the following three lung macrophage populations including AMs (F4/80$^+$/SiglecF$^+$/CD11c$^+$), IMs (F4/80$^+$/SiglecF$^-$/CD11b$^+$/CD11c$^-$) and ExMs (F4/80$^+$/Siglec F$^-$/CD11b$^+$/CD11c$^+$).

Statistical analysis

All statistical analyses were performed with GraphPad Prism 9.0 (GraphPad, USA) and graphical data are presented as mean ± SEM. One-way/two-way analysis of variance or Student's *t*-tests were performed where appropriate. Statistical significance is declared where $p < 0.05$.

RESULTS

CS induces systemic inflammation and lung injury in hβcTg mice

We first compared the CS response in hβ_cTg mice with wild-type (WT) mice in an acute 4-day CS exposure model as previously described.[35] hβ_cTg mice lost a significant degree of body weight (Figure S1A in the Supporting Information) and displayed a blunted BAL inflammatory profile compared to WT mice, the latter of which was mainly attributed to lower BAL macrophage numbers (Figure S1B,C in the Supporting Information). hβcTg mice were further exposed to CS (9 cigarettes/day) over 11 days as summarized in Figure 1A. CS exposure caused a significant 2% loss in body weight, whereas room air-exposed mice gained 4% body weight by the end of the protocol (Figure 1B). Whilst the total white blood cell (WBC) count was not significantly altered by CS exposure (Figure 1C), there was an approximately two-fold increase in circulating neutrophils and monocytes, whereas lymphocyte numbers were not altered (Figure 1D–F). CS exposure also caused significant BAL inflammation, determined by the increase in total BAL cells (Figure 1G). Differential cell analysis demonstrated a three-fold increase in BAL macrophage numbers, a large influx in BAL neutrophils and smaller increase of BAL lymphocytes in CS-exposed mice (Figure 1H–J). Flow cytometry was employed to analyse myeloid cell populations in lung tissue. Similar to the BAL compartment, significant recruitment of inflammatory cells into the lungs was observed. There were approximately 3.5 times more neutrophils in the lungs of CS-exposed mice compared to room air-exposed mice (Figure 1K). Macrophages were further immunophenotyped, which revealed a significant increase in IMs (CD11b$^+$ CD11c$^-$) and ExMs (CD11b$^+$CD11c$^+$) (Figure 1L,M). Lung eosinophils showed an approximate 30% increase in the number in CS-exposed mice (Figure 1N).

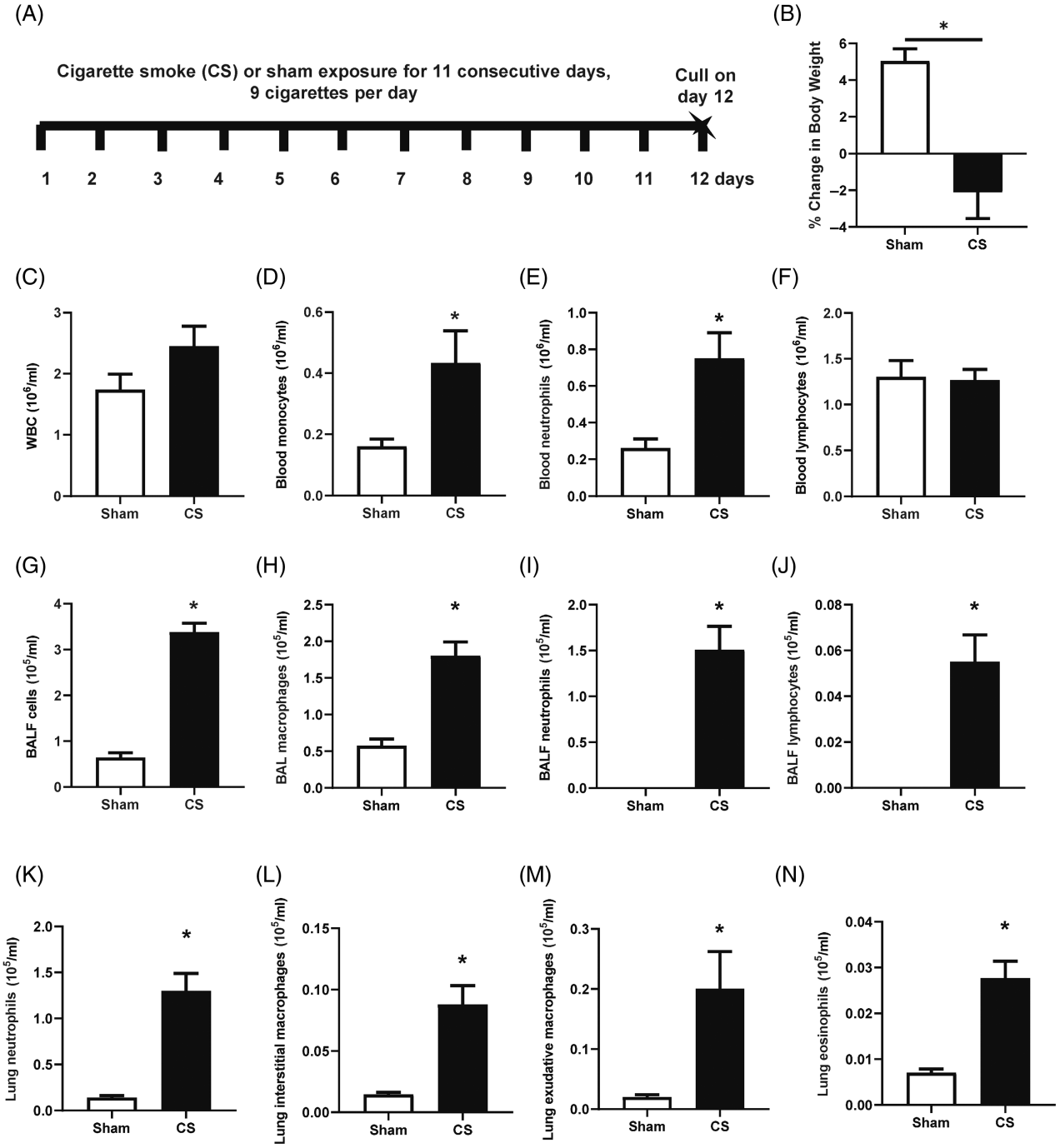

FIGURE 1 Cigarette smoke (CS) induced weight loss and inflammation in human β_c receptor transgenic (hβ_cTg) mice. (A) Mice were exposed to 11 consecutive days of room air (sham) or CS and culled the following day. (B) Body weight was monitored over the experimental protocol demonstrating a reduction in CS-exposed mice at the end of the protocol. (C) Total white blood cell (WBC), (D) blood monocytes, (E) blood neutrophils and (F) blood lymphocytes were quantified by haematological analysis. (G) Total bronchoalveolar lavage (BAL) cell counts were determined, and differential cell counts performed on cytospots to quantify (H) BAL macrophages, (I) BAL neutrophils and (J) BAL lymphocytes. Flow cytometry was also performed to quantify lung (K) neutrophils, (L) interstitial macrophages, (M) exudative macrophages and (N) eosinophils. Sham, $n = 5$; CS, $n = 6$. *$p < 0.05$, Student's t-test versus sham.

CS exposure resulted in alveolar, perivascular and peribronchiolar inflammation throughout the lung lobe sections (Figure 2A,B). The degree of inflammation and injury was scored across the entire lung lobe sections, where CS-exposed lungs averaged a significantly higher injury score compared to sham mice (Figure 2C). Serum albumin levels in BALF were increased in CS-exposed mice, consistent with compromised alveolar–capillary barrier integrity leading to lung oedema (Figure 2D). In addition, LDH levels were also increased by over seven-fold in the BALF of CS-exposed

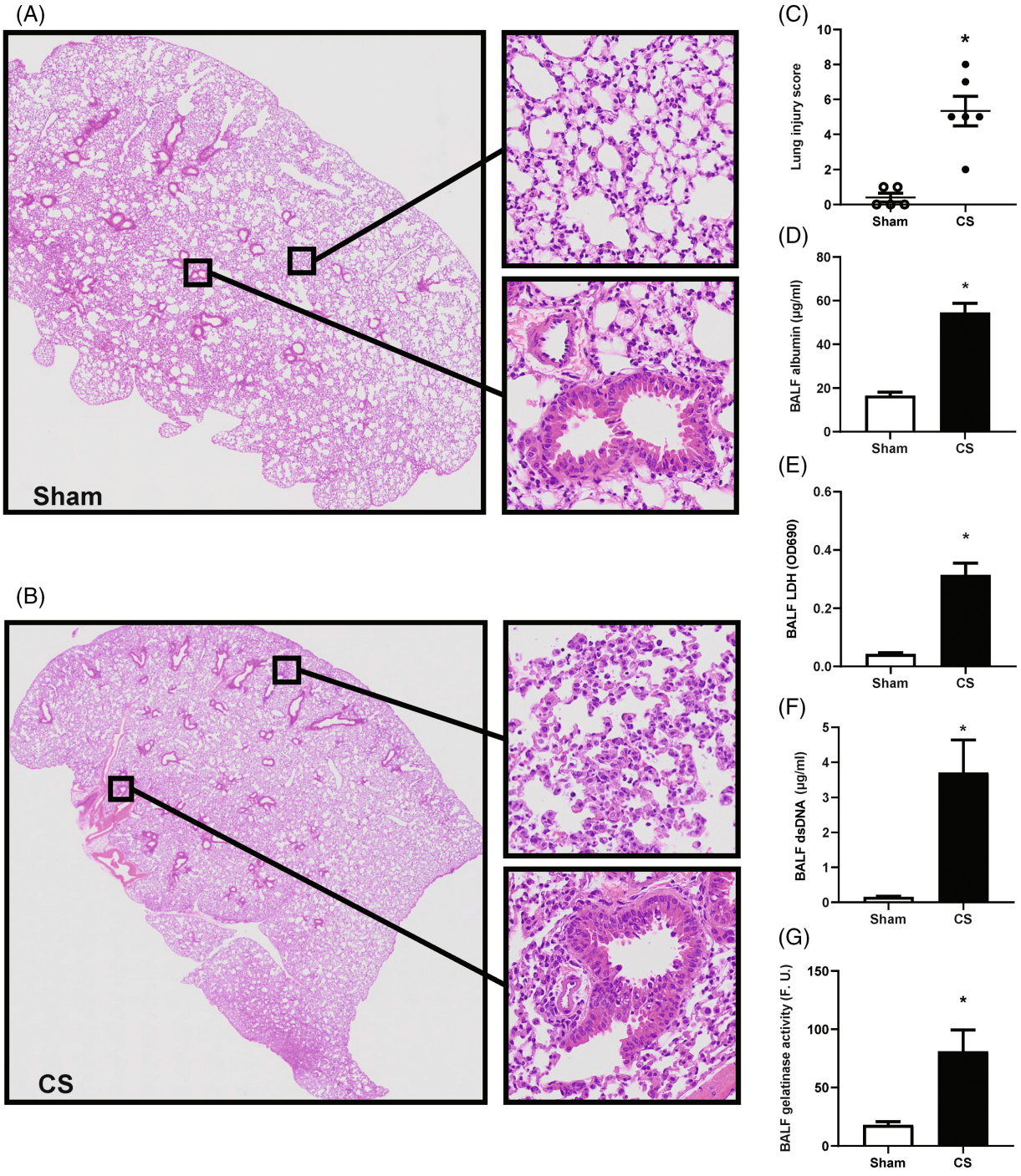

FIGURE 2 Acute cigarette smoke (CS) caused lung injury and oedema. Haematoxylin and eosin (H&E)-stained lung sections of (A) sham mice and (B) CS-exposed mice demonstrated that CS promotes perivascular, peribronchiolar and parenchymal immune cell infiltration. (C) Aggregated lung injury score quantified from whole H&E-stained lung sections. Lung oedema and injury markers were quantified in the bronchoalveolar lavage fluid (BALF) including (D) albumin and (E) lactate dehydrogenase (LDH). Markers of leukocyte degranulation were quantified in BALF including (F) double-stranded DNA (dsDNA) and (G) gelatinase activity. Sham, $n = 5$; CS, $n = 6$. $^*p < 0.05$, Student's t-test versus sham.

mice, further indicating that CS exposure induced lung epithelial/endothelial cell death and tissue damage (Figure 2E). These changes were accompanied by a 4.5-fold increase in gelatinase activity and a 20-fold increase in cell-free dsDNA content in the BALF supernatant (Figure 2F,G), which are markers of neutrophil degranulation, NETosis and cell death.

CS increases the expression of inflammatory cytokines in lung tissue

To further elucidate the inflammatory profile induced by CS in hβcTg mice, a panel of inflammatory markers were analysed using RTqPCR. The expression of the monocyte

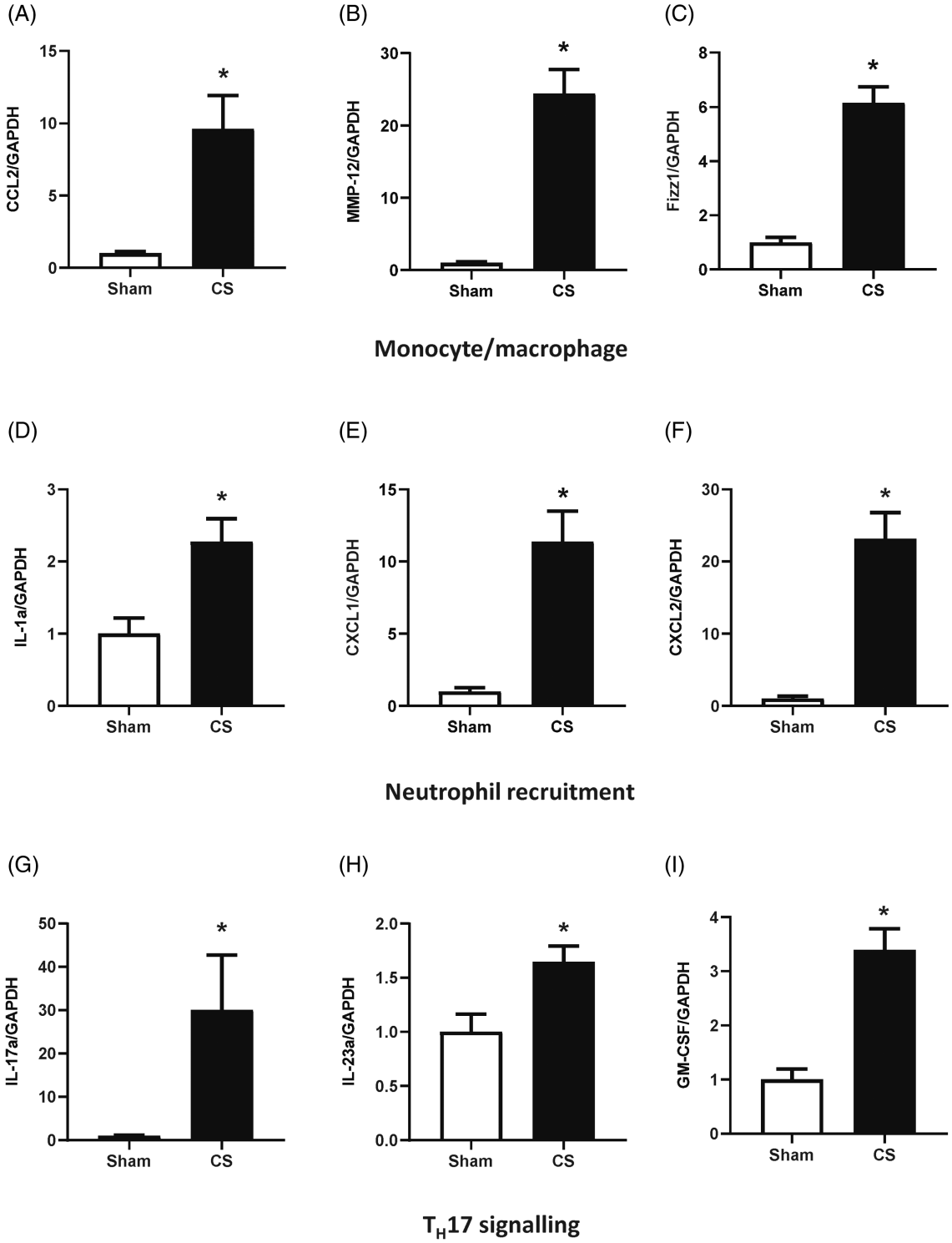

FIGURE 3 Acute cigarette smoke (CS) promotes the expression of multiple immune regulators. Real-time quantitative PCR was performed on lung tissue including (A) the monocyte chemokine C-C motif chemokine ligand 2 (CCL2), (B) matrix metalloproteinase-12 (MMP-12) and (C) Fizz1 (found in inflammatory zone). The neutrophil recruitment mediators (D) IL-1α, (E) C-X-C motif ligand 1 (CXCL1) and (F) C-X-C motif ligand 2 (CXCL2) were also quantified. The IL-23/IL-17A axis was also investigated by quantifying (G) IL-17A, (H) IL-23 and (I) granulocyte monocyte-colony-stimulating factor (GM-CSF) expression. Sham, $n = 5$; CS, $n = 6$. *$p < 0.05$, Student's t-test versus sham.

chemokine C-C motif chemokine ligand 2 (CCL2) was significantly increased in CS-exposed mice (Figure 3A). Furthermore, the M2 macrophage activation markers, Fizz1 (found in inflammatory zone) and MMP-12, were also significantly increased (Figure 3B,C), consistent with an increase in alternatively activated macrophages in the

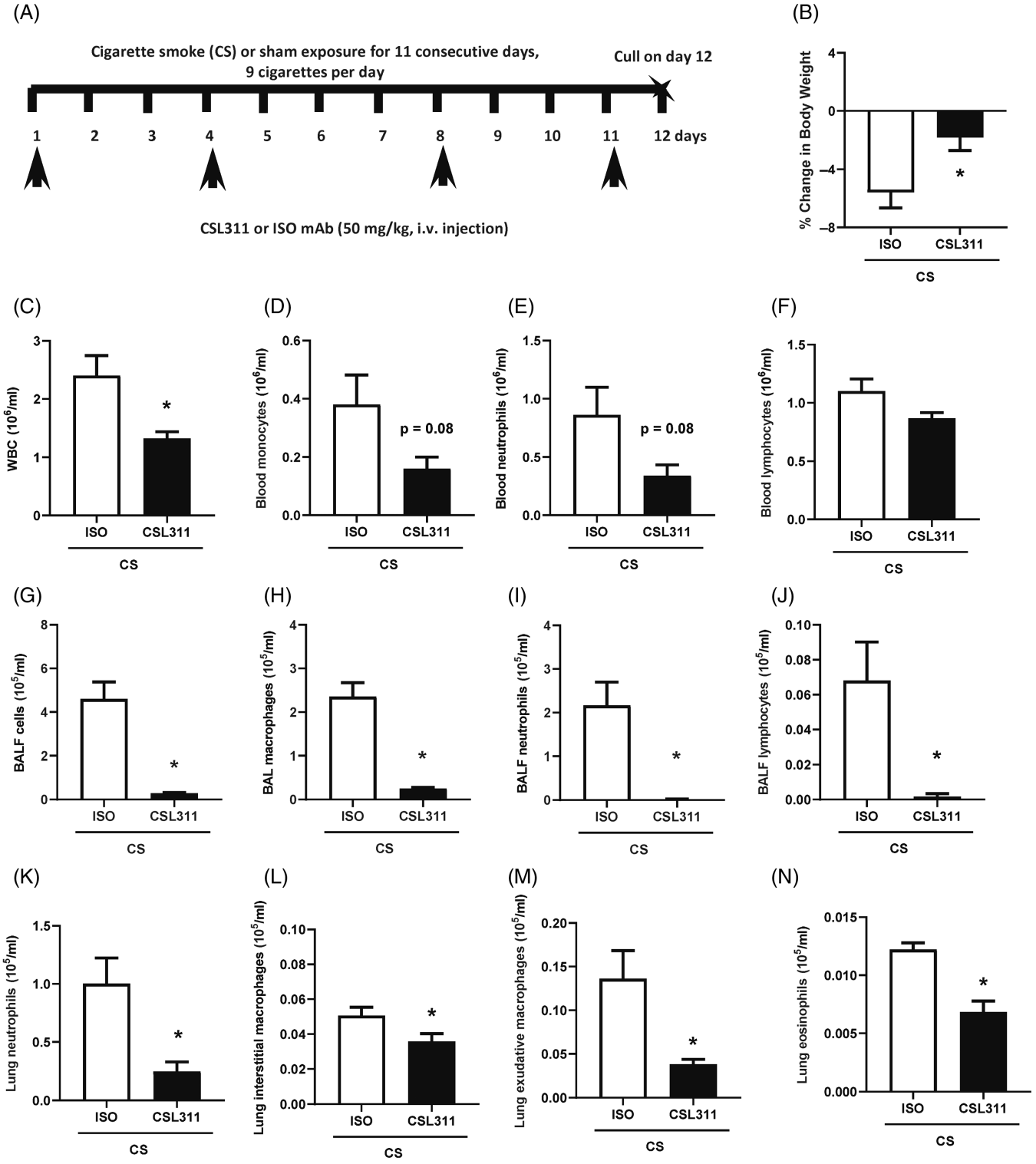

FIGURE 4 CSL311 reduced weight loss and inflammation caused by cigarette smoke (CS) exposure. (A) Human β_c receptor transgenic (hβ_cTg) mice were exposed to 11 days of CS and treated with four doses of CSL311 (iv) as detailed in the schematic. (B) Body weight was monitored, where CSL311 significantly reduced body weight loss caused by CS exposure compared to isotype control. Haematological analysis of blood included (C) total white blood cell count, (D) blood monocytes, (E) blood neutrophils and (F) blood lymphocytes. (G) Total bronchoalveolar lavage (BAL) cell counts were determined, and differential cell counts performed on cytospots to quantify BAL (H) macrophages, (I) neutrophils and (J) lymphocytes. Flow cytometry was also performed to quantify lung (K) neutrophils, (L) interstitial macrophages, (M) exudative macrophages and (N) eosinophils. ISO, $n = 5$; CSL311, $n = 6$. *$p < 0.05$, Student's *t*-test versus ISO.

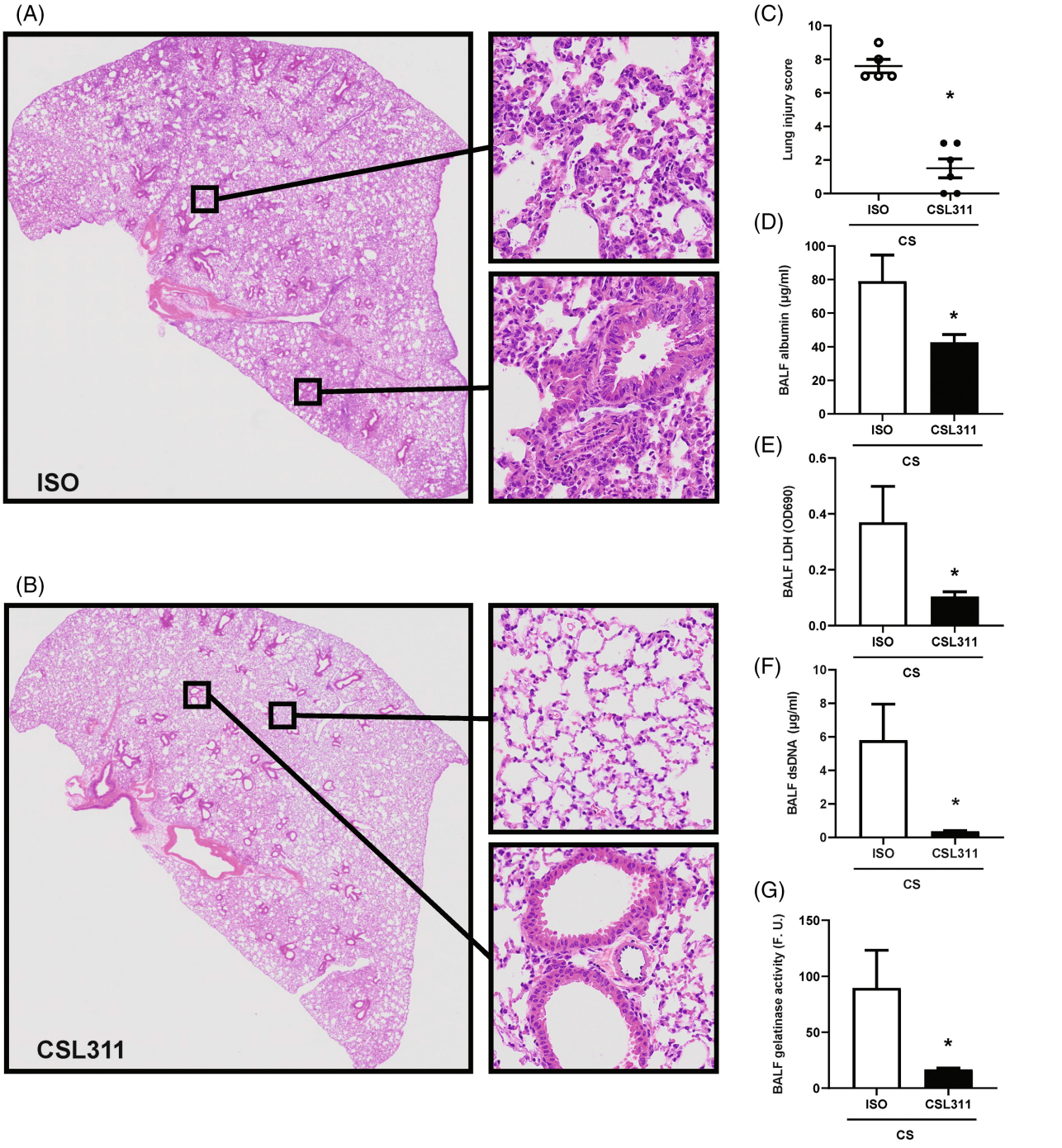

FIGURE 5 CSL311 reduced lung oedema and injury caused by acute cigarette smoke (CS) exposure. Haematoxylin and eosin (H&E)-stained lung sections were used to investigate perivascular, peribronchiolar and parenchymal immune cell infiltration in CS-exposed (A) isotype control treated (ISO) mice and (B) CSL311-treated mice. (C) Aggregated lung injury score was quantified from whole H&E-stained lung sections. Lung oedema and injury markers were quantified in the bronchoalveolar lavage fluid (BALF) including (D) albumin and (E) lactate dehydrogenase (LDH). Markers of leukocyte degranulation were quantified in BALF including (F) double-stranded DNA (dsDNA) and (G) gelatinase activity. ISO, $n = 5$; CSL311, $n = 6$. *$p < 0.05$, Student's t-test versus ISO.

lungs of CS-exposed mice. CS exposure also induced the expression of the inflammatory cytokine IL-1-alpha (IL-1α), which was two-fold higher compared to sham mice. In addition, the neutrophil chemokines C-X-C motif ligands 1 and 2 (CXCL1 and CXCL2) were increased more than 10-fold and 20-fold, respectively (Figure 3E,F), which is also consistent with prominent neutrophil trafficking into the lungs and BAL compartment. As T_H17 signalling can regulate inflammation in CS-exposed mice,[36] we measured IL-17A and IL-23A, which were

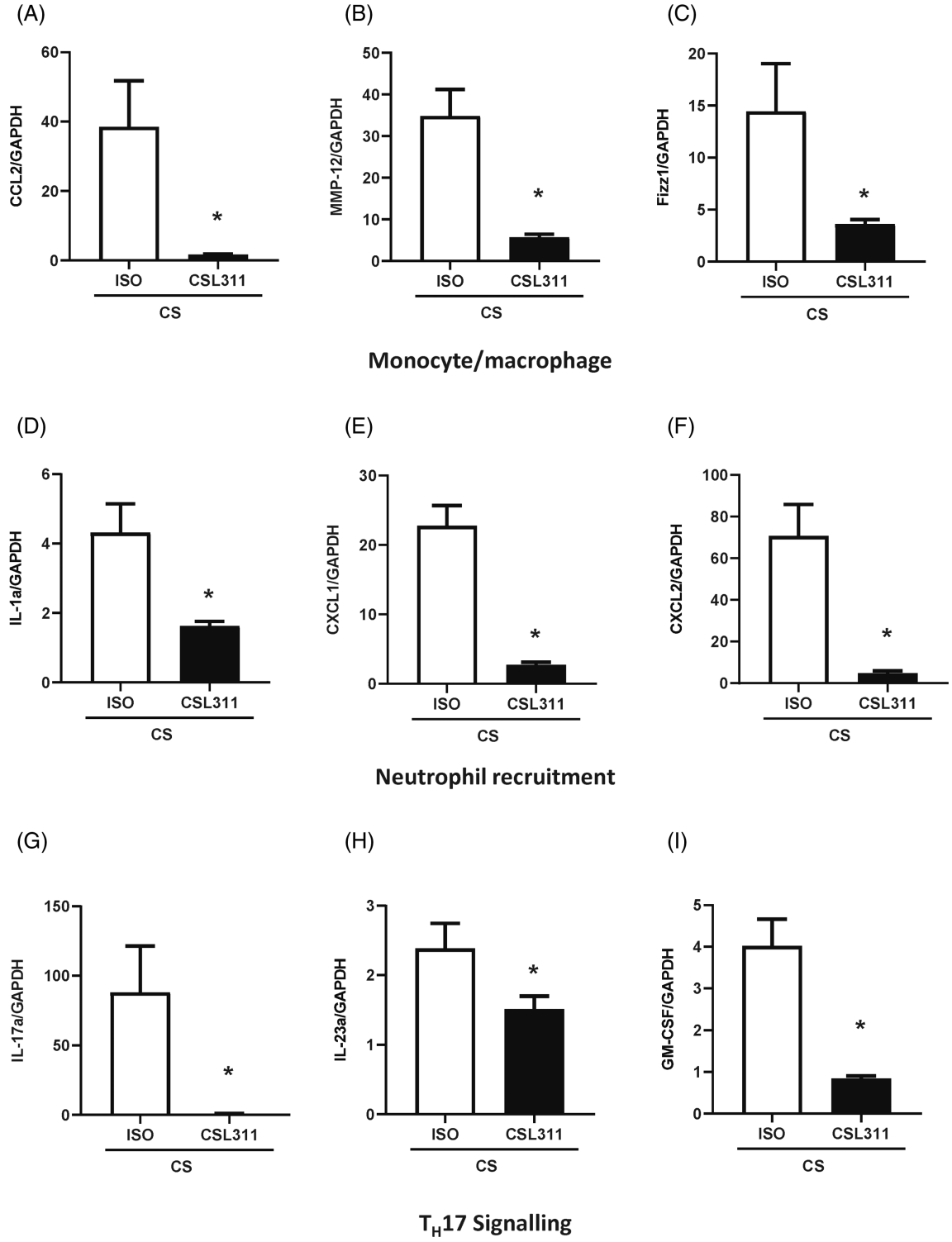

FIGURE 6 CSL311 inhibited the expression of multiple inflammatory mediators. Real-time quantitative PCR was performed on lung tissue including (A) the monocyte chemokine C-C motif chemokine ligand 2 (CCL2), (B) matrix metalloproteinase-12 (MMP-12) and (C) Fizz1 (found in inflammatory zone). The neutrophil recruitment mediators (D) IL-1α, (E) C-X-C motif ligand 1 (CXCL1) and (F) C-X-C motif ligand 2 (CXCL2) were also quantified. The IL-23/IL-17A axis was also investigated by quantifying (G) IL-17, (H) IL-23 and (I) granulocyte monocyte-colony-stimulating factor (GM-CSF) expression. ISO, $n = 5$; CSL311, $n = 6$. *$p < 0.05$, Student's t-test versus ISO.

significantly increased in CS-exposed mice (Figure 3G,H). T_H17 cells have been identified as an important cellular source of CSF2 (GM-CSF), which was also increased in the lungs of CS-exposed mice (Figure 3I). There was no difference in IL-5 lung expression and IL-3 was below our detection limit (not shown).

CS-induced inflammation and lung injury are blocked by CSL311 antibody treatment

hβ$_c$Tg mice exposed to CS were treated with CSL311 or ISO antibody on days 1, 4, 8 and 11 as summarized in Figure 4A. CSL311 treatment reduced the percentage weight loss compared to isotype-treated mice (Figure 4B; 1.8% vs. 5.6%). The total WBC was significantly decreased from 2.4×10^6/ml (isotype control) to 1.3×10^6/ml with CSL311 treatment (Figure 4C). There was a trend towards reduced blood monocyte numbers (Figure 4D; ISO; $p = 0.08$) in CS-exposed mice. Likewise, there was a trend towards decreased blood neutrophil numbers (Figure 4E; $p = 0.08$) in CS-exposed mice. No difference in blood lymphocytes was observed (Figure 4F). We next evaluated BAL inflammation where CSL311 treatment markedly reduced total BAL cells, BAL macrophage, BAL neutrophil and BAL lymphocyte numbers (Figure 4G–J), essentially blocking immune cell infiltrate to sham/control numbers. Flow cytometry comparing CS-exposed mice treated with CSL311 versus ISO antibody demonstrated that CSL311 effectively reduced lung neutrophil numbers, lung IMs ($CD11b^+CD11c^-$), lung ExMs ($CD11b^+CD11c^+$) and lung eosinophil numbers (Figure 4K–N).

Examination of histological lung sections showed that CS-exposed mice treated with isotype-matched antibody exhibited high levels of alveolar, peribronchiolar and perivascular infiltration of immune cells (Figure 5A), which were significantly lower in CS-exposed mice treated with CSL311 (Figure 5A–C). The reduction in lung injury was confirmed by significant decreases in markers of lung oedema, where there was a 50% reduction in albumin BALF levels in CSL311-treated mice compared to isotype-treated mice (Figure 5D). LDH levels were also significantly reduced by over three-fold, consistent with reduced lung mucosal cell damage and death (Figure 5E). Additionally, dsDNA and gelatinase levels were found to be at significantly lower levels in the BALF of CSL311-treated mice compared to isotype control (Figure 5F,G). Antagonism of the hβ$_c$ receptor with CSL311 also significantly decreased the expression of the monocyte chemokine CCL2, as well as M2 macrophage markers MMP-12 and Fizz1 (Figure 6A–C), which was consistent with the reduction in IM and ExM numbers in the lungs. Furthermore, CSL311 also reduced the expression of IL-1α and the neutrophil chemokines CXCL1 and CXCL2 (Figure 6D–F), consistent with the reduction of neutrophils in the BAL compartment. The T_H17 marker IL-17A was almost completely reduced (Figure 6G) and there was a significant reduction in the expression of IL-23 and GM-CSF (Figure 6H,I). These data suggest that signalling by β$_c$ is upstream of IL-17A and IL-23 and supports the notion that CSL311 also acts by inhibiting T_H17-mediated inflammation. Masson's trichrome-stained lung sections detected no difference in collagen deposition in CS-exposed mice (Figure S3A in the Supporting Information). However, there was a trend towards increased COL1A1 expression in CS-exposed mice, which was reduced with CSL311 treatment (Figure S3B in the Supporting Information). Likewise, no change was observed in alcian blue-periodic schiff stain (AB-PAS)-stained lung sections in this acute model of CS exposure (Figure S4A in the Supporting Information). However, MUC5AC expression trended to increase in CS-exposed mice in a manner that was reduced with CSL311 treatment (Figure S4B in the Supporting Information).

DISCUSSION

As the β$_c$ receptor regulates GM-CSF, IL-5 and IL-3 signalling, its antagonism represents an important strategy to simultaneously block all three β$_c$ cytokines with a single antibody in chronic diseases where they are prominent. We demonstrate that CSL311 effectively reduced multiple myeloid populations that expanded in the lungs in response to acute CS exposure. More specifically, there was a marked expansion of $CD11b^+$ IMs and ExMs in the lungs of hβ$_c$Tg mice exposed to CS, and CSL311 potently inhibited these populations. ExMs are normally self-limiting and capable of promoting the resolution of inflammation[14] by facilitating the clearance of apoptotic granulocytes in the acute inflammatory setting.[37,38] However, they can be reprogrammed to become immunopathogenic when exposed to external stimuli such as respiratory viruses and allergen, where they express M2 markers, produce more IL-13 and promote increased airways hyper-responsiveness.[39] In the context of CS exposure and COPD, the local lung expansion of a $CD11b^+$ lung macrophage population was identified as a major source of increased MMP-12 expression in a preclinical model of emphysema, although their immunophenotyping strategy did not differentiate IMs and ExMs.[11] Consistent with this, CSL311 significantly reduced the expression of MMP-12 and the alternatively activated macrophage marker, Fizz1. Hence, CSL311 effectively reduced lung macrophage populations, such as IMs and ExMs, that produce high levels of MMP12, which is a major protease involved in ECM breakdown and airspace enlargement.

Importantly, CSL311 strongly reduced the expression of IL-23 and IL-17A in CS-exposed mice, demonstrating that this inflammatory axis is dependent on β$_c$ receptor signalling. Macrophages are a major producer of IL-23, a heterodimeric cytokine involved in inflammation through its capacity to stimulate IL-17A expression from multiple cell types including T_H17 cells, natural killer/natural killer T (NK/NKT) and gamma delta T cells (γδ T cells).[40] The IL-23/IL-17A axis is a central driver of chronic inflammation in many autoimmune and chronic inflammatory diseases, including smoking-related lung diseases. $IL\text{-}17A^+$ cells are increased in frequency in the bronchial submucosa of chronic smokers and COPD patients.[41] In addition, the genetic ablation of the IL-17R prevented CCL2 expression, lung macrophage expansion, MMP-12 expression and airspace enlargement/emphysema in mice exposed to CS.[42] We have previously shown that the recruitment of neutrophils and monocytes in response to CS exposure was dependent

on IL-17A signalling, where NK/NKT and γδ T cells were identified as major cellular sources of IL-17A.[36] Hence, we propose that by reducing the number of IL-23-expressing ExMs in the lungs of CS-exposed mice, CSL311 subsequently inhibits the expression of the key inflammatory cytokine, IL-17A, that is responsible for the persistent recruitment and activation of inflammatory cells. CSL311 also reduced the expression of GM-CSF in CS-exposed hβ$_c$Tg mice, and T_H17 cells have been identified as an important cellular source of GM-CSF in several autoimmune and inflammatory diseases.[43] Therefore, blocking β$_c$ signalling may reduce the persistent production of GM-CSF in the inflamed tissue by blocking the differentiation and expansion of IL-17A-expressing lymphocytic populations, although further work is needed to confirm this.

GM-CSF is a central mediator of inflammation in the context of CS exposure and COPD/emphysema. GM-CSF levels are increased in CS exposure models[21,44] and transgenic mice that produce high levels of pulmonary GM-CSF spontaneously develop emphysema and secondary polycythaemia, accumulate lung macrophages, produce more MMP-9 and MMP-12 in the lungs and display increased mortality. GM-CSF is also a central orchestrator of neutrophilic inflammation in pre-clinical models of lung inflammation, where antibodies that block this ligand potently reduce neutrophil numbers.[21,45] We have previously shown that the expansion of BAL macrophages and neutrophils is dependent on GM-CSF using the 22E9 blocking antibody in CS-exposed mice.[21] We demonstrate that CSL311 is equally effective at blocking macrophage and neutrophilic lung inflammation and importantly reveal that CSL311 also reduced lung injury and oedema associated with neutrophil dysfunction. Mechanistically, lung macrophages produce higher levels of the IL-1α cytokine in CS-exposed mice, and IL-1α signalling is necessary for the recruitment of neutrophils into the lungs.[46] We also demonstrated that IL-1α mRNA was increased in CS-exposed mice and CSL311 significantly reduced IL1α expression.

Whilst inflammatory IMs and ExMs expand in the lungs during CS exposure, AMs have been shown to decline in number due to increased apoptosis.[10] In our study, there was an increase in BAL macrophages, which likely reflects the recruitment and expansion of ExMs in this compartment. Using flow cytometry, we quantified IMs and ExMs in the lung tissue to demonstrate that IMs and ExMs markedly increase with CS exposure in a manner that is effectively reduced with CSL311. A limitation of our study is that we do not have accurate flow cytometry data on AMs, as flow cytometry was performed on lung tissue, whereas AMs are primary located in the BAL compartment. We did observe that CSL311 treatment reduced BAL macrophages numbers in CS-exposed mice to levels seen in naive/untreated mice. Future studies are warranted to investigate whether homeostatic AM functions, such as pathogen eradication and surfactant catabolism, are maintained in CS-exposed mice treated with CSL311. We have previously investigated CSL311 in an acute model of influenza infection, where this treatment did not compromise expansion of NK/NKT cells and the clearance of influenza.[47] Furthermore, chronic antagonism of the GM-CSFR does not alter surfactant catabolism in mice, which is consistent with safety data from clinical trials where AMs maintain homeostatic competency.[48]

A potential advantage of CSL311 over biologics that target the single GM-CSF/GM-CSFR axis (mavrilimumab or lenzilumab) is that it will also inhibit GM-CSF, IL-3 and IL-5 signalling. Monoclonal antibodies that target IL-5 or its receptor (mepolizumab, reslizumab, benralizumab) are approved for the treatment of steroid-resistant asthma because they reduce eosinophilic inflammation and reduce asthma exacerbation rates. Sputum eosinophils are elevated in about 30% of COPD patients and are associated with important clinical parameters including COPD severity and susceptibility to acute exacerbations.[49–51] Based on these findings, clinical trials have tested mepolizumab for potential benefit in COPD patients, where they observed that this antibody reduced exacerbation rates in patients with an elevated blood eosinophil count.[52] Here, we observed that CSL311 significantly reduced lung eosinophil numbers by about 40% in CS-exposed mice compared to isotype-treated mice, consistent with its effectiveness at reducing granulocytic infiltration and histopathology in a model of allergic contact dermatitis.[24] In addition to GM-CSF and IL-5, CSL311 inhibits signalling initiated by IL-3. Immune cells from hβ$_c$Tg mice respond to mouse GM-CSF and IL-5 but not IL-3 under in vitro conditions, which may be due to instability of the complex formed between mIL-3, mIL3Rα and hβ$_c$.[24] As we observed reduced BAL macrophages in CS-exposed hβ$_c$Tg mice compared to WT mice, these data suggest that IL-3 is contributing to the expansion of pathogenic macrophages in CS-exposed mice. Hence, we predict that the simultaneous blocking of GM-CSF, IL-5 and IL-3 signalling with CSL311 will be more effective than single target therapies in blocking pathogenic myeloid cells in COPD.

In summary, transgenic mice engineered to express human β$_c$ in a mouse $\beta_c^{-/-}$ and $\beta_{IL\text{-}3}^{-/-}$ background mount a robust response to acute CS exposure, and the ensuing immune response involves the persistent accumulation of multiple myeloid cell populations that contribute to lung tissue injury. This innate response is known to be dependent on lymphocyte subsets that expand in response to CS exposure and produce IL-17A. The β$_c$ receptor subunit is central to this immunopathological response as its inhibition with CSL311 markedly reduced multiple myeloid populations including monocytes, IMs, ExMs, neutrophils and eosinophils. We propose that inflammatory ExMs that express mediators such as IL-23 and IL-1α are reduced with CSL311 treatment, and consequently, the IL-17A-dependent inflammatory response is blocked. CSL311 represents an attractive therapeutic strategy in CS-related diseases such as COPD, particularly in patients with mixed granulocytic inflammation and an abundance of activated macrophages in the lungs.

AUTHOR CONTRIBUTION

Nok Him Fung: Formal analysis (equal); investigation (equal); writing – original draft (equal). **Hao Wang:**

Conceptualization (equal); formal analysis (equal); investigation (equal); project administration (equal); supervision (equal); writing – original draft (equal). **Ross Vlahos:** Supervision (equal); writing – review and editing (equal). **Nick Wilson:** Conceptualization (equal); funding acquisition (equal); writing – review and editing (equal). **Angel F. Lopez:** Formal analysis (equal); writing – review and editing (equal). **Catherine M. Owczarek:** Formal analysis (equal); funding acquisition (equal); methodology (equal); writing – review and editing (equal). **Steven Bozinovski:** Conceptualization (equal); formal analysis (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing – original draft (equal).

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CONFLICTS OF INTEREST

Nick Wilson and Catherine M. Owczarek are CSL Ltd employees, and CSL Ltd has funded this research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ANIMAL ETHICS APPROVAL DECLARATION

All animal experiments were approved at RMIT University (AEC#1928) in accordance with the National Health and Medical Research Council of Australia (NHMRC) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

ORCID

Ross Vlahos https://orcid.org/0000-0001-8443-7071
Steven Bozinovski https://orcid.org/0000-0001-6533-8641

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SUPPORTING INFORMATION

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