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Title: A Mouse GM-CSFR Antibody Attenuates Neutrophilia in Mice Exposed to Cigarette Smoke

Running title: Anti-GM-CSFR inhibits subchronic lung injury

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ABSTRACT

We have investigated the role of granulocyte-macrophage-colony stimulating factor (GM-CSF) in a subchronic exposure model of cigarette smoke-induced inflammation using antibodies directed against GM-CSF or the GM-CSF receptor alpha chain.

Cigarette smoke induced mononuclear and neutrophilic inflammation following four days of cigarette smoke-exposure in BALB/c mice was assessed in bronchoalveolar lavage fluid (BAL). An increase in mature dendritic cells ($CD11c^+$ and MHC II^+) and Gr-1 hi^+ neutrophils was also observed by flow cytometric analysis of whole lung tissue.

Daily intra-peritoneal injection of 400 μg of a GM-CSF or GM-CSF receptor antibody prior to daily smoke exposure attenuated the accumulation of neutrophils within the BAL by 60%. A reduction in mature dendritic cells was also observed. Anti GM-CSF receptor antibody administration did not have an effect on the percentage of lung T cells, however a significant decrease in activated $CD69^+$ $CD8^+$ T cells was observed. Anti GM-CSF receptor antibody administration decreased the mRNA and protein expression of IL-12p40 and MMP-12.

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Taken together, intervention with this receptor antibody implicates the GM-CSF pathway as an important mediator of smoke-induced inflammation.

Keywords: Cigarette smoke, granulocyte-macrophage colony stimulating factor, antibody neutralization, inflammation, neutrophils.

INTRODUCTION

Whilst lung exposure to cigarette smoke (CS) has been attributed as the leading cause of chronic obstructive pulmonary disease (COPD), the exact pathogenic mechanisms of the disease are not understood [1]. COPD is characterised by irreversible airflow obstruction and progressive lung inflammation that correlates with disease severity[2]. Whilst asthma is more associated with eosinophilic inflammation, within COPD, neutrophils and macrophages, predominate [3].

As a key regulator of myeloid cell survival and activation [4], the cytokine granulocyte macrophage colony stimulating factor (GM-CSF) has been shown to have a central role in maintaining the innate immune response in the healthy lung [5, 6]. It is released by a range of structural and inflammatory cells, such as the airway epithelium, smooth muscle cells, T cells and macrophages. It has also been shown that GM-CSF is elevated in the lung from patients with COPD [7-9] suggesting that this cytokine may also play a role in the exaggerated inflammatory response in the disease. The contribution of GM-CSF is unclear, however, it has been shown that GM-CSF not only activates macrophages and neutrophils in concert with stimuli, such as LPS, that maybe present in the COPD lung, but also promotes cytokine release and cell survival [2]. Recently it has also been demonstrated that intranasal delivery of an anti-GM-CSF antibody in a mouse model of cigarette smoke induced inflammation attenuates lung neutrophilia[10].

GM-CSF mediates its effects via a receptor that consists of two subunits, an α-subunit (GM-CSFRα), which binds the cytokine with low affinity, and a larger, β-subunit (beta common, βc), responsible for signalling, forming a ternary receptor complex [11]. Signal transduction in response to the cytokines IL-3 and IL-5 is also mediated by βc, therefore receptor specificity is due to GM-CSFRα [12]. Because CS is one of the main causative agents of COPD and a number of CS exposure models have been developed that present with phenotypes analogous to COPD such as emphysema and lung inflammation [13-15]. These models provide platforms to evaluate the efficacy and mechanisms of new therapies. Biologics are increasingly being developed for chronic diseases such as anti-TNFα antibodies and receptor antagonists in arthritis [16-18]) and anti-IgE antibodies in asthma ([19]). Moreover, antibody trials in COPD are also underway that target IL-1β (canakinumab) [20]. Currently a number of anti-GM-CSF and anti-GM-CSFRα [4] approaches are in early clinical development for rheumatoid arthritis. To date only a single study has been conducted investigating the role of GM-CSF in a CS exposure model via intranasal delivery[10]. Here, we have continued this work by evaluating both a neutralising antibody to GM-CSF (22E9) and GM-CSFRα chain (CAM-3003) antibody in the subchronic CS model dosed via the systemic compartment. Moreover we also explore the role of GM-CSF pathway blockade on downstream cytokines, its effects on dendritic cell subsets in the lung and on $CD8^+$ T cell activation.

MATERIALS AND METHODS

Animals

Female BALB/c mice (6-8wks old) were purchased from Charles River Laboratories (Montreal, PQ, Canada). IL-1 receptor knock out (KO) and wild type (WT) control C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Mice were maintained under specific pathogen-free conditions, on a 12-hr light-dark cycle, with food and water provided *ad libitum*. The Animal Research Ethics Board of McMaster University approved all experiments.

FDCP-1 cell proliferation assay

FDCP-1 cells were maintained in DMEM (Invitrogen) containing 10% FBS (JRH Biosciences, Lenexa, KS) and 5ng/ml murine GM-CSF (R&D systems). Prior to the assay, cells were cultured in the absence of GM-CSF for 30hrs at 37˚C 5% CO_2. Cells were resuspended at $1.5x10^5$ per ml in medium and 100µl added to each well of a flat bottomed 96-well plate. Cells were incubated at 37˚C 5% CO_2 with antibody in the presence of 1-2pM mGM-CSF for 16 hours. 20µl of tritiated-thymidine (5.0mCi/ml) was added to each well and incubated for 4hrs prior to harvesting on GF/C UnifilterTM plates (Perkin Elmer). Thymidine incorporation was determined using a scintillation counter (Packard Topcount). Data were analyzed using PRISM software (GraphPad, San Diego, CA).

Cigarette smoke exposure protocol

Mice were exposed to CS from 12 2R4F reference cigarettes with filters removed (Tobacco and Health Research Institute, Lexington, KY) for approximately 50 minutes, twice daily, for four days using a whole body smoke exposure system (SIU-48, Promech Lab AB (Vintrie, Sweden) as described previously[21]. CS exposure followed an initial acclimatization period whereby, on day 1, mice were placed into the exposure box for 20 min, on day 2 for 30 min, and on day 3 for 50 min. Control animals (sham),were exposed to room air only.

Intraperitoneal (i.p.) administration of antibodies.

BALB/c mice were i.p. injected with 400 µg of anti-GM-CSF receptor (CAM-3003; mouse IgG1) or anti-GM-CSF ligand (MP122E9 clone, R&D) antibody (22E9; rat IgG2a) 12 hours prior to each of the first daily CS exposures. Mice were sacrificed 12-18 hours after the last CS exposure.

Bronchoalveolar lavage and differential cell counting

Bronchoalveolar lavage (BAL) fluid was collected as described. Briefly, lungs were dissected, cannulated, and instilled with 0.25ml of ice-cold 1x phosphate-buffered saline (1xPBS), followed by 0.2ml of 1xPBS. Total cell numbers were counted by haemocytometer. Cytospins were prepared for differential cell counts and stained with Hema 3 (Biochemical Sciences Inc., Swedesboro, NJ). 500 cells were counted per cytospin to identify mononuclear cells, neutrophils, and eosinophils.

Enzyme-Linked Immunoassays (ELISAs) and Meso Scale Discovery Analysis

ELISA kits for GM-CSF were purchased from R&D Systems and used according to manufacturers protocol. Cytokine detection of interferon-gamma (IFN-γ), interleukin (IL)-1 beta (IL-1β), IL-4, keratin-derived cytokine (KC) and IL-12 was done using the multi-array murine pro-inflammatory and Th1/Th2 cytokine panel detection system (MesoScaleDevices, Gaithersburg, MD).

mRNA Expression Fluidigm Analysis

RNA was extracted from a single lobe using the Qiagen RNeasy Fibrous Tissue kit according manufacturer's protocol (Qiagen, Germany). RNA was quantified and normalized, and integrity assessed using the Agilent RNA 6000 Nano Kit (Agilent, Santa Clara, CA). cDNA generation was carried out with the Super Script III kit (Life Technologies, Carlsbard, CA). Relative transcript expression was assessed using the Fluidigm Biomark Dynamic array loaded with probes for transcripts of interest.

Isolation of Lung Mononuclear cells

Lung mononuclear cells were isolated as described [21]. Lungs were perfused with 1x PBS and cell suspensions generated by disaggregation and incubation for 1 hour at 37^{o}C in 150U/ml collagenase III in hepes-buffered saline solution (Invitrogen, Burlington, ON, Canada). Debris was removed by passing through a nylon mesh. Cells were resuspended in 1x PBS containing 0.3% bovine serum albumin (BSA) (Invitrogen, Burlington, ON, Canada) or in RPMI supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada), 1% L-glutamine, and 1% penicillin/streptomycin (Invitrogen, Burlington, ON, Canada).

Flow cytometric analysis

1x10^{6} lung mononuclear cells were washed once with 1x PBS/0.3% BSA and stained with directly conjugated primary antibodies for 30 minutes at 4°C. 10^{5} live events were acquired on an LSR II (BD Biosciences) flow cytometer and analyzed with FlowJo software (TreeStar Inc., Ashland, Oregon). The following antibodies were used: FITC-conjugated anti-CD11c, PE-conjugated anti-CD11b, PE-Alexa Fluor 610-conjugated CD8, PE-cy5.5-conjugated anti-CD19, PE-cy7-conjugated anti-CD69, APC-conjugated anti-MHC class II, Alexa Fluor 700-conjugated anti-CD86, APC-cy7-conjugated anti-CD45, Pacific Blue-conjugated anti-CD3, Pacific Orange-conjugated anti-Gr-1. The above antibodies were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA) except for CD86 (BioLegend, San Diego, CA). Qdot605-conjugated anti-CD4 and Qdot655-conjugated anti-B220 were purchased from Invitrogen (Carlsbad, CA).

Statistical analysis

Data were analyzed using PRISM Software version 5 (Graphpad, San Diego, CA) and expressed as mean ± SEM. Statistical analysis was performed by testing for a normal distribution using Kolmogorov-Smirnov followed by a Student's t test, one-way analysis of variance (ANOVA) or a two-way ANOVA with a Bonferroni post test as stated in figure legends. Non-parametric tests were undertaken using Kruskall-Wallis with Mann Whitney post test. Differences were considered statistically significant when P values were less than 0.05.

RESULTS

Cigarette smoke-induced inflammation is associated with increased GM-CSF mRNA and protein expression.

To investigate the impact of CS exposure on GM-CSF expression in the lungs, we exposed BALB/c mice to CS for 4 days. Control animals were exposed to room air only (sham). CS exposure led to a significant increase in total cell numbers in BAL which reflected predominantly neutrophils (Figure 1A**)**. CS significantly increased the levels of GM-CSF in the lung (Figure 1B).

Mechanisms of GM-CSF expression.

While multiple pathways likely contribute to cigarette smoke-induced inflammation, we next sought to investigate whether expression of GM-CSF following smoke exposure was IL-1R1-dependent. This experiment was based on a recent report by Doz *et al.* [22] demonstrating a important role of IL-1R1 CS-induced inflammation. Consistent with Doz *et al.*, CS-induced neutrophilia was markedly attenuated in IL-1R deficient (Figure 2A) mice. Loss of IL-1R expression did not significantly affect the increase in mononuclear cells following CS exposure. We further examined GM-CSF mRNA expression in C57BL/6 (wildtype) and IL-1R deficient mice that were either sham or CS-exposed. GM-CSF mRNA expression was abrogated in the IL-1R deficient mice (Figure 2B).

Anti-GM-CSF ligand and receptor antibodies attenuate cigarette smoke-induced lung inflammation.

To test the importance of GM-CSF to CS-induced airway inflammation, we administered either anti-ligand (22E9) or receptor blocking antibodies (CAM-3003) to sham or CS-exposed mice. The activity of each antibody was confirmed in the FDCP-1 proliferation assay (Figure 3A). The IC_{50} of 22E9 and CAM-3003 was 919pM (95% CI: 694 to 1218pM) and 130pM (95% CI: 99 to 171pM) respectively. Due to the different potencies and isotypes of these antibodies fully saturating doses of each antibody were used in these studies. Pharmacokinetic analysis of CAM-3003 was undertaken to confirm antibody exposure levels between sham and smoke treated animals in both lung and blood compartments. No difference in antibody exposure was observed between sham and smoke treated animals (Figure 3B). In vivo anti-GM-CSF and anti-GM-CSFR antibody administration resulted in a comparable and significantly attenuated CS induced neutrophilic inflammation. Control mice receiving the isotype control antibody demonstrated no attentuation of CS-induced inflammation (Figure 3C and 3D). To determine changes in neutrophil numbers in the interstitium flow cytometry of lung single cell suspensions was undertaken. The percentage of GR1hi positive cells was significantly increased by CS exposure when compared with sham treated animals ($P<0.05$). No significant difference was observed between smoke exposed isotype control, anti-GM-CSF or anti-GM-CSFR treated mice (Figure 4).

Anti-GM-CSF ligand and receptor antibodies attenuate cigarette smoke-induced dendritic cell expansion and T cell activation.

It is widely accepted that GM-CSF promotes dendritic cell (DC) maturation, a critical step in antigen-driven T cell responses (reviewed in [4]). Therefore, we investigated whether intervention with anti-GM-CSF or receptor antibodies attenuated DC expansion and T cell

activation following CS exposure. Anti-GM-CSF ligand antibody intervention abrogated CS-induced increases in myeloid (m) ($CD11c^{hi}$ MHC class II^{hi}) and plasmacytoid (p) ($CD11c^{hi}$ MHC class II^{+} $B220^{+}$) DCs (Figure 5A). Administration of an anti-GM-CSF receptor antibody also reduced the frequency of mDCs (Figure 5B), while no effect was observed on pDCs.

Anti-GM-CSF ligand antibody intervention abrogated CS-induced increases in activated $CD69^{+}$ $CD4^{+}$ and $CD8^{+}$ T cells as compared to isotype control-treated animals. In contrast to ligand blocking antibody, a reduction was only observed for $CD69^{+}$ $CD8^{+}$ T cells, but not $CD4^{+}$ T cells (Figure 5C, D) in mice treated with an anti-GM-CSF receptor antibody.

Anti-GM-CSF receptor antibody treatment reduces IL-12 and MMP-12 mRNA and expression in cigarette smoke-exposed BALB/c mice.

To study the mechanisms of CS inflammation, we analyzed the expression of lung cytokines and chemokines, as well as matrix metalloproteinases using fluidigm analysis. CS exposure caused a significant increase in mRNA expression for the following cytokines (IL-12p40, IL-1β, IL-33, IL-6 , Muc5ac, TNF-α), chemokines (KC, MCP1, CCL3, CCL4, CCL9, CSF3, CXCL2) and matrix metalloproteinases (MMP9, MMP12) genes (Table 1). Anti-GM-CSF receptor antibody significantly attenuated the mRNA expression of IL-12p40 (Figure 6A) and MMP-12 (Figure 6B) relative to isotype control antibody treatment (Figure 6 and Table 1). No significant difference was observed with TNFα or CXCL2 relative to isotype control (Figure 6C, D).

To confirm the mRNA expression data, we examined expression of cytokines IL-1β KC, IFN-γ, IL-2, IL-12 and IL-4. Increased expression of IL-1β, KC and IL-12 in CS exposed animals plus isotype antibody control was observed. The anti-GM-CSF receptor antibody significantly reduced expression of IL-12 in the BAL and lung homogenates, while IL-1β or KC levels remained unchanged. A similar observation was made for IL-1β levels in animals treated with the anti-ligand antibody (Supplemental Table 1). IFN-γ, IL-2 and IL-4 levels were all below the limit of detection in sham and CS-treated groups (Table 2).

Discussion

Neutrophils and macrophages are regularly implicated in the pathogenesis of COPD. It has previously been shown that as the severity of the disease progresses the proportion of inflammatory cells in the airways increases[2]. Therefore it has been proposed that interfering with mechanisms that blunt the activity of these cells may provide clinical benefit. Consequently, GM-CSF has been implicated as a potential mediator in COPD.

Whilst GM-CSF is elevated in the lungs from patients with COPD or chronic bronchitis [8] intervention of this pathway in models of CS-induced lung inflammation have not been widely described [10] . Here we showed that, following four days of CS exposure, we observed a consistent increase in total cells in the BAL, the majority being neutrophils. Moreover, GM-CSF was elevated at both the transcript and protein levels within the lungs from these animals. Interestingly whilst Vlahos et al. [10] demonstrated attenuation of CS induced lung inflammation with antibodies to GM-CSF they were unable to measure GM-CSF protein within the BAL. Likewise they also demonstrated attenuation of neutrophils and macrophages, however in our model of cigarette smoke-induced inflammation, we only observed an increase and subsequent attenuation of neutrophils by either antibodies. It is of note that the type of cigarette smoke exposure system and route of antibody delivery were different and thus may account for the subtle differences observed between this study and that previously described.[10].

To determine whether the GM-CSF pathway played a significant role in the recruitment of inflammatory cells into the BAL, we evaluated both an anti-GM-CSF neutralising antibody (22E9) and an anti-GM-CSFRα chain antibody (CAM3003) in this model. To rule out the potential of antibody dependent cellular cytotoxicity (ADCC) the anti GMCSFR antibody was specifically expressed as a mouse IgG1 antibody. Consequently, no evidence of neutrophil depletion in peripheral blood has been observed with this antibody (data not shown). Using either approach, we inhibited the recruitment of neutrophils in the BAL by dosing via the systemic compartment, however we did not reduce neutrophil numbers in the tissue as defined by flow cytometry. This observation in the BAL is consistent with the previous observation that intranasal delivery of the same GM-CSF antibody (22E9) was reported to blunt CS induced neutrophil and macrophage recruitment in the BAL [10, 23]. Whilst we did not observe a significant increase in macrophages and lymphocytes upon smoke exposure, we did observe a modest but significant change in the percentage of mature lung DCs that was attenuated with either antibody. In vitro, it has been shown that diesel exhaust particle conditioned medium from epithelial cells can induce a GM-CSF dependent DC maturation [24], suggesting that smoke exposure may also contribute to DC maturation in a similar manner. This is supported by the observation that in turn both CD4+ and CD8+ T cells increase CD69 expression following smoke exposure as previously described[21] and that this was partially inhibited by blockade of the GM-CSF pathway. Interestingly only CD8+ T activation was suppressed by both mechanisms but the CD4+CD69+ activated T cells weren't affected by treatment with anti GM-CSFR. The reason for this is unclear and merits further investigation. Nevertheless, T cells do not express the GM-CSFRα chain; therefore these data suggest that a reduction in the smoke induced T cell activation maybe an indirect effect of GM-CSF stimulation of myeloid cells rather than a direct effect of GM-CSF on T cells.

Recent studies have shown [22] that MyD88, TLR4 and IL-1R deficient mice all had reduced lung neutrophilia and cytokine levels in the lung following tobacco smoke. Therefore we investigated GM-CSF expression in CS-exposed IL-1R deficient mice. In this system we confirmed that CS-induced neutrophilia was suppressed and that KC mRNA levels were significantly inhibited (data not shown). Based on our observations it may be concluded that GM-CSF plays a central role in CS lung inflammation potentially downstream of the IL-1R

pathway. A similar observation has also been observed in a mouse model of IL-1β driven monoarthitis[25].

To determine the mechanism of GM-CSF blockade we investigated the change in expression levels of various proteins and mRNA in the lung. Whilst smoke exposure enhanced the expression of a range of cytokines, blocking the GM-CSF pathway did not appear to suppress cytokines typically associated with neutrophilic inflammation such as IL1β and KC. GM-CSF is known to activate neutrophils, promoting adhesion in pulmonary vascular endothelium [26], recruitment [27] and sensitization to chemokines [28]. The lack of a direct effect on KC, IL1β and TNF, suggests that GM-CSF may operate downstream of these cytokines, potentially rendering neutrophils less responsive to the direct effect of these aforementioned molecules and may also account for there being no apparent difference between the percentage of neutrophils in lung homogenates from CS exposed mice treated with either an anti-ligand antibody or anti-receptor antibody. It has also been demonstrated that GM-CSF promotes neutrophil survival by preventing apoptosis[29], and thus GM-CSF blockade may also have enhanced neutrophil apoptosis and clearance by macrophages. Further studies are warranted to better understand how neutrophil numbers in the BAL are attenuated and the activation status of resident neutrophils within the lung.

However, inhibition of the GM-CSF pathway during CS exposure did inhibit IL-12p40 and IL-12 production at the transcript and protein level respectively. In vitro, GM-CSF has previously been shown to induce IL-12 from both DCs and macrophages [30]. In addition, mice deficient in GM-CSF exposed either to a viral [31] or bacterial pathogen [32] demonstrated a reduction in IL-12 production. Furthermore, over-expression of GM-CSF in the lungs of normal mice stimulates an increase in IL-12 production [33]. Similarly we showed that IL-12p40 was up-regulated in the lungs of CS exposed mice and that these levels were attenuated with either antibody indicating that IL12p40 expression is downstream of smoke-induced GM-CSF production. Chronic smoke exposure in mice plus the addition of virus or viral mimetics (polyI:C) has been shown to promote an increase in IL-12p40 production [34], however this is the first time that this elevation has been shown in such an subchronic model. Nevertheless, relatively little is known about the expression of this molecule and its role in COPD [24] and therefore further studies are warranted.

In addition to an up-regulation of IL-12p40, subchronic smoke exposure also stimulated an increase in MMP-12 mRNA consistent with previous reports [35]. Tissue remodelling and proteolytic damage is a hallmark of COPD and proteases, such as MMP9 and MMP12, have been shown to be elevated within the COPD lung [36, 37]. Furthermore, mice deficient in MMP-12 [38] do not present with an emphysematous phenotype following chronic smoke exposure. Both neutrophils and macrophages are known to be a rich source of MMP-12 [39] and their numbers correlate with disease severity in COPD [40]. In another model of CS induced lung inflammation [35], macrophages were described as being the main source of this protease. Here, we have demonstrated that blocking GM-CSF signalling attenuated MMP-12 expression in lung tissue. These data are consistent with the observation that GM-CSF can induce MMP-12 production from human peripheral blood monocyte-derived macrophages [41, 42]. Interestingly, whilst we did observe a change in MMP-12 mRNA levels in the lung, as recently described [10], we did not observe a statistically significant change in either TNF-α mRNA or MIP2α (Figure 6C, D) in this system suggesting that the route of delivery may have subtle differences on mRNA profiles within the lung. This difference was not due to antibody exposure as pharmacokinetic analysis of CAM-3003 confirmed significant levels of antibody in both serum and lung homogenates (Figure 3B).

In conclusion, the present study reports that systemic delivery of anti-GM-CSF or antiGM-CSFR antibodies attenuate smoke-induced neutrophilia. Furthermore, GM-CSF

appears to play a role in activating resident lung DCs and lymphocytes, providing a link between the initial innate response to subchronic cigarette exposure and supporting the adaptive response. Further studies are warranted to characterise further this relationship and shed light on the potential role of this cytokine in COPD.

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	Room Air	Smoke	Room Air + Control IgG	Room Air + anti-GM-CSF R	Smoke + Control IgG	Smoke + anti-GM-CSF R
IL-12p40	1.03±0.13	2.92±0.66	0.99 ± 0.07	0.43 ± 0.1	1.32 ± 0.36	0.4 ± 0.14*
IL-1β	1.03±0.12	2.16±0.42	1.09±0.24	1.28±0.28	1.28±0.24	0.88±0.25
IL-33	1.05±0.2	1.92±0.37	1.16±0.23	1.4±0.17	1.2±0.31	0.57±0.18
IL-6	1.24±0.38	4.17±0.8	1.28±0.33	1.29±0.15	2.1±0.45	3.33±1.53
TNFα						
KC	1.17±0.28	10.2±1.82	0.87±0.18	0.81±0.19	5.35±0.88	5.39±1.7
MCP-1	1.028±0.12	14.01±2.5	0.8±0.14	0.56±0.06	8.56±1.2	5.53±1.4
CCL3	1.09±0.2	19.13±4.6	0.97±0.2	1.172±0.27	10.9±2.27	5.682±1.5
CCL4	1.08±0.17	6.87±1.4	0.84±0.2	1.14±0.21	5.04±0.78	4.04±1.0
CCL9	1.028±0.1	7.42±1.6	1.14±0.28	1.04±0.15	5.25±0.7	3.05±0.78
CSF3	1.18±0.37	6.16±1.31	0.4±0.15	0.34±0.28	1.8±0.5	1.4±0.4
CXCL2	1.19±0.33	13.47±2.7	1.28±0.26	0.92±0.18	7.9±1.6	5.64±1.7
Muc5ac	1.07±0.18	6.8±1.15	0.84±0.2	0.7±0.26	3.15±0.78	1.45±0.3
MMP-9	1.02±0.1	1.11±0.2	1.03 ± 0.15	1.33 ± 0.24	0.88 ± 0.14	0.48 ± 0.1*
MMP-12	1.03±0.12	10.31±2.1	1.08 ± 0.20	0.46 ± 0.08	6.16 ± 1.28	0.8 ± 0.26**

Table 1. Fluidigm mRNA expression Analysis of four day smoke-exposed BALB/c mice injected with anti-GM-CSF receptor antibody. Four day room air (RA) or CS-exposed mice received mouse IgG2a (CAT004) or anti-GM-CSF receptor antibody (CAM3003) as described in methods. Fluidigm mRNA gene expression analysis was performed on lung tissue samples, all values were normalised to 3 housekeeping genes and expressed as a relative fold change to room air (sham) exposed mice. Results shown are a mean ± sem of five mice per group. Statistical Analysis was performed comparing smoke + control IgG vs smoke + anti-GM-CSF receptor antibody groups, *$P < 0.05$, **$P<0.003$, Mann Whitney U test.

	BAL Fluid			Lung Homogenate		
	Room Air	**Smoke + Control IgG**	**Smoke + Anti-GM-CSF R**	**Room Air**	**Smoke + Control IgG**	**Smoke + Anti- GM-CSF R**
IL-1β	**3 ± 1**	**46 ± 4***	**35 ± 5**	**132 ± 32**	**508 ± 65***	**419 ± 40**
KC	**112 ± 28**	**434 ± 56***	**420 ± 55**	**257 ± 26**	**1616 ± 90***	**2143 ± 123**
IL-12	**133 ± 33**	**1805 ± 208***	**653 ± 112***	**753 ± 63**	**2656 ± 18***	**1665 ± 117***

Table 2. Protein expression (pg/ml) following room air or subchronic 4 day smoke exposure ± anti-GM-CSF receptor antibody. Protein levels were determined by MSD multi-array platform using the murine pro-inflammatory and Th1/Th2 cytokine panels. Data are expressed as the mean ± SEM. n=8-10 mice/group from 2 independent experiments, *P<0.0001 comparing room air vs smoke exposed control IgG treated mice and *p<0.0005 comparing smoke exposed control IgG vs anti-GM-CSFR treated mice, Mann-Whitney U test.

	BAL Fluid			Lung Homogenate		
	Room Air	**Smoke + Control IgG**	**Smoke + Anti-GM-CSF**	**Room Air**	**Smoke + Control IgG**	**Smoke + Anti- GM-CSF**
IL-1β	**0.22 ± 0.05**	**4.18 ± 0.78***	**3.18 ± 0.76**	**3.57± 0.78**	**33.15 ± 7***	**15.86± 4**

Supplemental Table 1. Protein expression (pg/ml) following room air or subchronic 4 day smoke exposure ± anti-GM-CSF antibody (22E9). Protein levels were determined by a custom designed MSD multi-array ELISA. Data are expressed as the mean ± SEM. n=5 mice/group, *P<0.01 comparing room air vs smoke exposed control IgG treated mice, Mann-Whitney U test. No significant difference was observed between control IgG and anti-GMCSF groups in this study.

FIGURE LEGENDS

Figure 1. *BAL cellular profile and GM-CSF expression in cigarette smoke-exposed mice*. BALB/c mice were sham- (white bars) or CS-exposed (black bars) for four days. Panel (A) shows BAL total cell number (TCN), mononuclear cells (MNC), and neutrophils (NEU). Data represent mean ± SEM, n=5 per group. Panel (B) shows GM-CSF mRNA (mean ± SEM, n=3 sham, n=8 CS) and protein expression (n=5 per group) in the lungs of sham and CS-exposed mice. Statistical analysis was performed using Non-parametric Kruskal-Wallis with Mann Whitney post test. * represents $P < 0.05$, ***P<0.001.

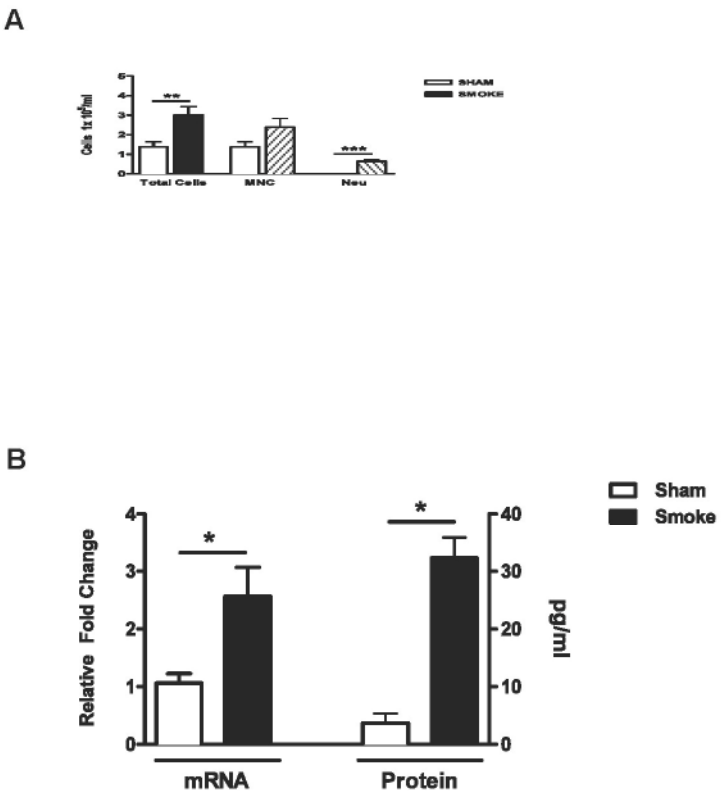

Figure 1

Figure 2. *BAL cellular profile and GM-CSF expression in cigarette smoke-exposed IL-1 receptor knockout (KO) mice*. (A) wild type (white bars) and IL-1R KO (cross hatched) mice were exposed to room air (sham) or CS for four days. Data show BAL total cell number (TCN), mononuclear cells (MNC), and neutrophils (NEU). Panel (B) show GM-CSF mRNA expression in sham- and CS-exposed wild type IL-1R KO mice,. Data represent mean ± SEM, n=5 per group. Statistical analysis was performed using two-way ANOVA with a Bonferroni post-test for wildtype versus knockout. *** represents $P < 0.001$.

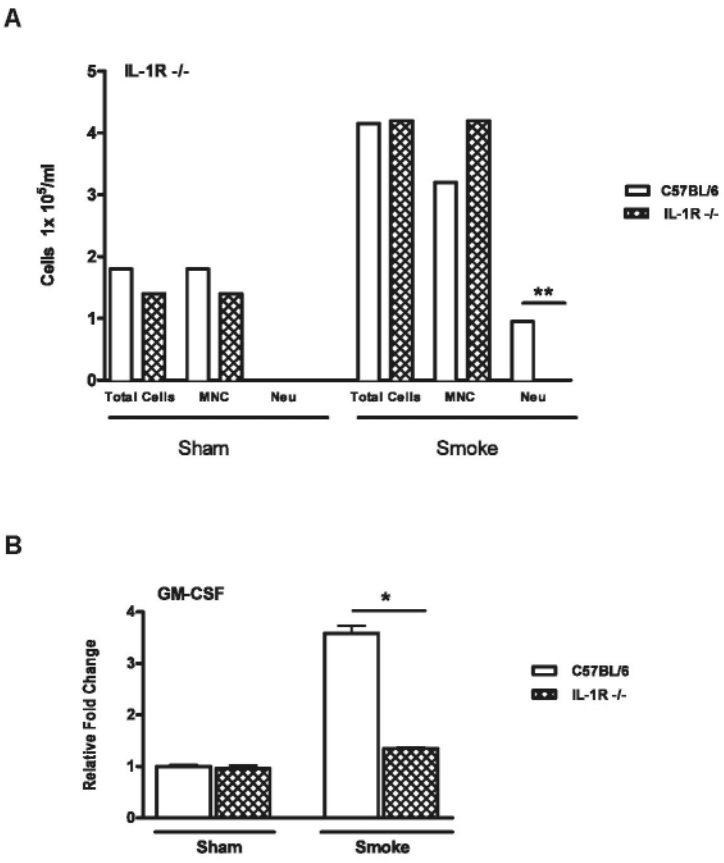

Figure 2

Figure 3. *GM-CSF neutralizing activity and the BAL cellular profile in smoke-exposed mice injected with anti-GM-CSF ligand or anti-GM-CSF receptor (anti-GM-CSFR) antibodies.* (A) Potency comparison of the GM-CSF antibody (open circles) and GM-CSF receptor (closed circles) antibody in a mouse GM-CSF FDCP-1 proliferation assay. Cells were incubated with mouse GM-CSF and a dilution series of antibody for 16 hours. Cell proliferation was quantified following a further 4 hr incubation with tritiated thymidine. (B) Terminal exposure of CAM-3003 levels in serum and lung homogenates from sham and smoked exposed mice (C) and (D) BALB/c mice were exposed to CS for four days. Mice were injected intraperitoneally (i.p.) with either anti-GM-CSF ligand (B, hashed bars) or an anti-GM-CSFR (C, black bars) antibodies or isotype control antibodies (white bar) 18 hours prior to daily CS exposures. Data show total cell numbers (TCN), mononuclear cells (MNC), and neutrophils (NEU) (mean ± SEM, n=5 per group). Statistical Analysis was performed using a two-way ANOVA with a Bonferroni post-test for sham versus smoke groups. * $P < 0.05$, ** $P < 0.01$..

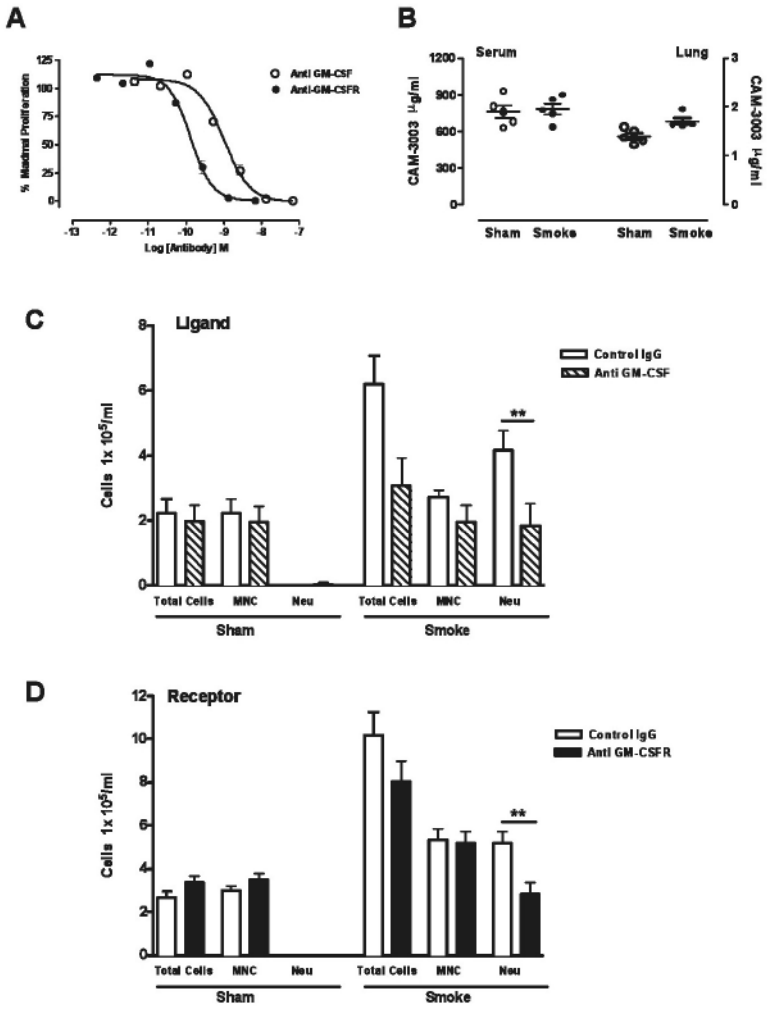

Figure 3

Figure 4. *Flow cytometric analysis of neutrophils (Gr^{Hi+ve}) in sham and smoke-exposed mice injected with anti-GM-CSF ligand or anti-GM-CSF receptor antibodies.* BALB/c mice were exposed to CS for four days. Mice were injected intraperitoneally (i.p.) with either anti-GM-CSF ligand (A) hashed bars or an anti-GM-CSFR (B) antibodies (black bars) or isotype control antibodies (white bar). Neutrophils ($Gr1^{hi}$ MHC II^{hi}) were examined in whole lung single cell suspensions by flow cytometry. Statistical Analysis was performed using a two-way ANOVA with a Bonferroni post-test for sham versus smoke.

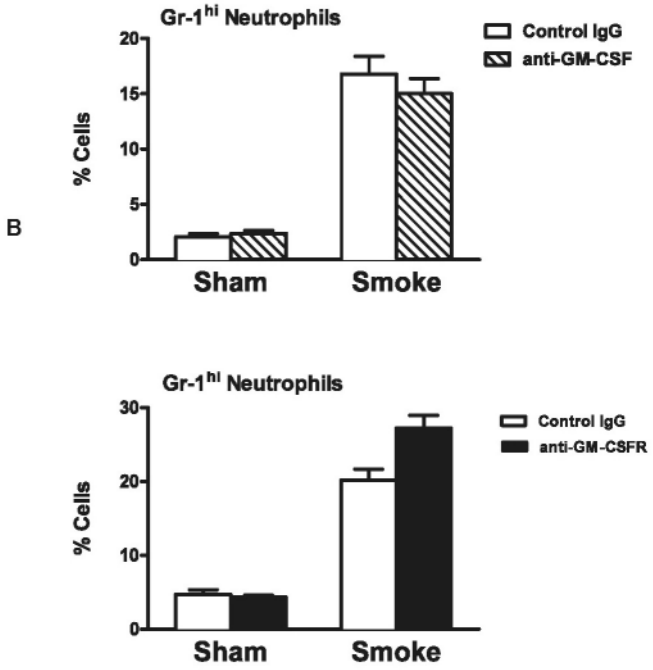

Figure 4

Figure 5. *Flow cytometric analysis of dendritic cell subsets (DCs) and activated T cells in smoke-exposed mice injected with anti-GM-CSF ligand or anti-GM-CSF receptor antibodies.* BALB/c mice were exposed to CS for four days. Mice were injected intraperitoneally (i.p.) with either anti-GM-CSF ligand (A,C) antibodies (hashed bars) or an anti-GM-CSFR (B, C) antibodies (black bars) or isotype control antibodies (white bar). Myeloid DCs ($CD11c^{hi}$ MHC II^{hi} $B220^{-ve}$) and plasmacytoid DCs ($CD11c^{hi}$ MHC II^{hi} $B220^{+ve}$) or (C) activated $CD69^{+}$ $CD4^{+}$ and $CD8^{+}$ T cell subsets were examined in whole lung single cell suspensions by flow cytometry. Statistical Analysis was performed using a two-way ANOVA with a Bonferroni post-test for sham versus smoke. * $P < .05$.

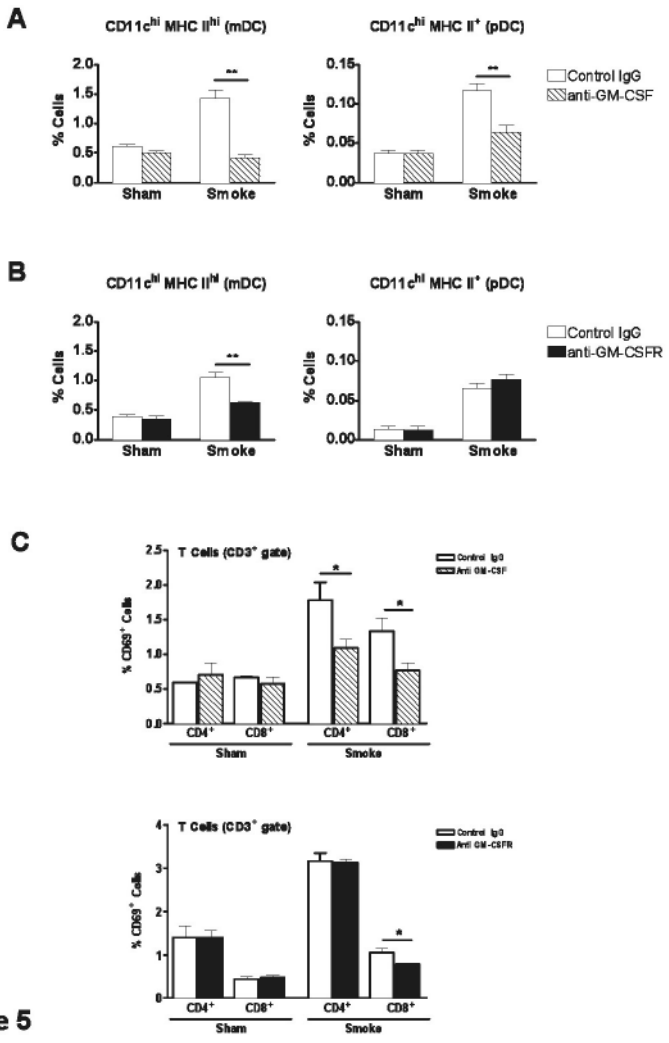

Figure 5

Figure 6. IL-12p40 (A), MMP-12 (B), TNFα (C) and CXCL2 (D) mRNA expression. BALB/c mice were exposed to room air (sham, white bars) or CS (black bars) for four days. Mice were injected intraperitoneally (i.p.) with anti GM-CSF receptor or isotype control antibodies. All values were normalized to 3 housekeeping genes and expressed as the relative fold change to sham mice. Data are expressed as the mean ± SEM, n=5 per group. Statistical analysis was performed comparing smoke exposed control IgG vs anti GM-CSFR antibody treated mice, **P<0.001, Mann-Whitney U test.

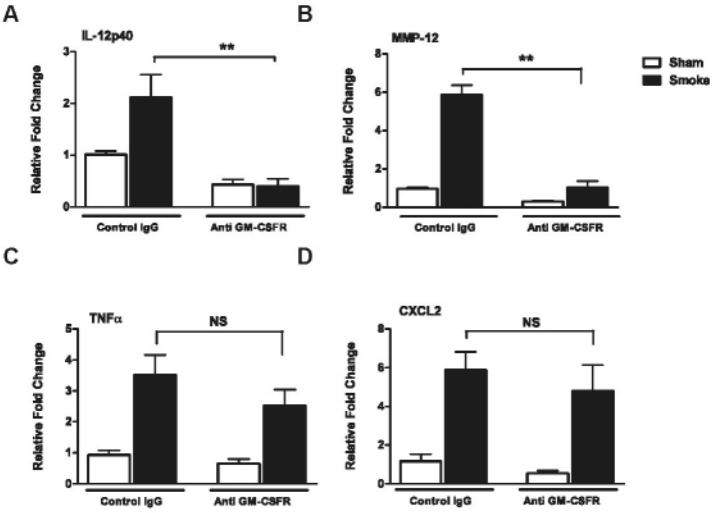
A
IL-12p40
**
Relative Fold Change
Control IgG
Anti GM-CSFR
B
MMP-12
**
Relative Fold Change
Control IgG
Anti GM-CSFR
Sham
Smoke
C
TNFα
NS
Relative Fold Change
Control IgG
Anti GM-CSFR
D
CXCL2
NS
Relative Fold Change
Control IgG
Anti GM-CSFR

Figure 6

Supplemental Figure 1. *Flow Cytometric Analysis of Gr-1^{hi} Neutrophils and CD11c^{hi} MHC class II^{hi} Dendritic cells (DCs) in smoke-exposed Mice injected with anti-GM-CSF ligand antibody.* (A) Representative FACS plots of Gr-1^{hi}-expressing neutrophils (FACS plots show Gr-1 staining versus MHC class II staining) from whole lung tissue of sham (room air) or smoke-exposed mice treated with isotype control or anti-GMCSF antibody. (B) Representative FACS plots of a flow cytometric analysis of CD11c^{hi} and MHC class II (MHC II)^{hi} myeloid dendritic cells (gates for low and high expression of MHC class II are shown) from whole lung tissue of sham (room air) or smoke-exposed mice treated with isotype control or anti-GMCSF antibody.

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