Neutralizing GM-CSF inhibits cigarette smoke-induced lung inflammation

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Short Title: GM-CSF and lung inflammation
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Scientific Knowledge on The Subject

Elevated levels of GM-CSF have been observed in a variety of human inflammatory lung diseases. Previously, GM-CSF has been considered a homeostatic mediator in lung macrophages, epithelial innate immunity and leukocyte survival. We have shown that antibody neutralization of GM-CSF markedly reduces smoke-induced lung inflammation and sequelae.

What This Study Adds to the Field

Antibody neutralization of GM-CSF may represent a novel means of controlling inflammatory lung disease.

Abstract

Rationale: Cigarette smoke is the major cause of COPD and there is currently no satisfactory therapy to treat people with COPD. We have previously shown that GM-CSF regulates lung innate immunity to LPS through Akt/Erk activation of NFκB and AP-1. *Objective:* The aim of this study was to determine whether neutralization of GM-CSF can inhibit cigarette smoke-induced lung inflammation *in vivo*. *Methods:* Male BALB/c mice were exposed to cigarette smoke generated from 9 cigs per day for 4 days. Mice were treated intranasally with 100 μg 22E9 (anti GM-CSF mAb) and isotype control antibody on day 2 and 4, 1 h prior to cigarette smoke or sham exposure. On the fifth day, mice were killed, the lungs lavaged with PBS and then harvested for genomic and proteomic analysis. *Measurements and Main Results:* Cigarette smoke-exposed mice treated with anti-GM-CSF mAb had significantly less BALF macrophages and neutrophils, whole lung TNF-α, MIP-2 and MMP-12 mRNA expression and lost less weight compared to smoke-exposed mice treated with isotype control. In contrast, smoke-induced increases in MMP-9 and net gelatinase activity were unaffected by treatment with anti-GM-CSF. In addition, neutralisation of GM-CSF did not affect the phagocytic function of alveolar macrophages. *Conclusion*: GM-CSF is a key mediator in smoke-induced airways inflammation and its neutralization may have therapeutic implications in diseases such as COPD.

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Introduction

Worldwide, cigarette smoke exposure is the single most important determinant of adverse lung health. Cigarette smoke is the major cause of COPD, a global health problem that is predicted to become the third largest cause of death by 2020 (1). Cigarette smoke causes lung inflammation where macrophages and neutrophils are prominent, leading to oxidative stress, emphysema, small airways fibrosis, mucus hypersecretion and progressive airflow limitation (2). Since this inflammatory response to cigarette smoke responds poorly to current anti-inflammatory treatments, there is intense research to identify more effective treatments for cigarette smoke-induced lung damage.

Granulocyte macrophage-colony stimulating factor (GM-CSF) is of interest in smoking-related lung diseases because it governs the growth, activation and survival of leukocytes directly implicated in the pathogenesis of COPD (3). Under physiological conditions, GM-CSF is produced in small amounts by lung epithelium where it directs the terminal differentiation of alveolar macrophages by activating PU-1 (4, 5). Infiltrating leukocytes may also be induced to synthesize GM-CSF by inflammatory and chemotactic stimuli (6). GM-CSF also exerts its effect by increasing cytokine gene expression and priming inflammatory cells to amplify their responses to other stimuli (7). Interestingly, GM-CSF is a direct neutrophil chemotactic factor (8) and increases neutrophil survival in the respiratory tract (9). GM-CSF is directly implicated in the development of small airway fibrosis (10, 11) and adenoviral-mediated GM-CSF gene transfer in the lung induced lung eosinophilia, macrophage expansion and airway fibrosis as well as marked macrophage accumulation (12, 13). GM-CSF also primes for sensitization to aeroallergen and is directly implicated in the inflammatory responses to the PP10

fraction (particles < 10 microns) of respirable pollutants, such as diesel exhausts, implicated in COPD exacerbations (14). The concentrations of GM-CSF in BALF are increased in stable COPD and are markedly elevated during exacerbations (15). Studies have also shown that the levels of GM-CSF in sputum are increased in asthma (16), stable COPD (16, 17) and during exacerbations of COPD (18).

We have previously shown that innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR4 (19, 20). We have now explored the effects of anti-GM-CSF antibody treatment in a mouse model of cigarette smoke-induced lung inflammation. As genetic depletion of GM-CSF causes alveolar proteinosis in mice (21) and anti-antibody formation can confound interpretation, we have restricted our study to a sub-chronic cigarette smoke challenge model (22). Intranasal administration of anti-GM-CSF antibody to mice exposed to cigarette smoke significantly reduced BALF macrophages, neutrophils, whole lung cytokine (TNF-α), chemokine (MIP-2), and protease (MMP-12) mRNA expression and weight loss. Our data provide new evidence for the role of GM-CSF in smoke-induced lung inflammation and suggest the potential therapeutic utility of blocking GM-CSF *in vivo*.

Some of the results of these studies have been previously reported in the form of an abstract (23).

Methods

Animals

Specific pathogen-free male BALB/c mice aged 7 weeks and weighing ~20 g were obtained from the Animal Resource Centre Pty. Ltd. (Perth, Australia). The animals were housed at 20°C on a 12-h day/night cycle in sterile micro-isolators and fed a

standard sterile diet of Purina mouse chow with water allowed *ad libitum*. The experiments described in this manuscript were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and conducted in compliance with the guidelines of the National Health and Medical Research Council of Australia on animal experimentation.

Cigarette smoke exposure

Mice were placed in an 18 litre perspex chamber in a class II biosafety cabinet and exposed to cigarette smoke generated from 9 cigarettes per day for 4 days as previously described (22). The mean total suspended particulate mass concentration in the chamber containing cigarette smoke was approximately 420 mg m^3. Sham-exposed mice were placed in an 18 litre perspex chamber but did not receive cigarette smoke. On the fifth day, mice were killed by an ip overdose of anaesthetic (5.6 mg ketamine/1.12 mg xylazine, Parnell Laboratories, NSW, Australia) and the lungs lavaged with PBS. Commercially available filter-tipped cigarettes (manufactured by Philip Morris, Australia) of the following composition were used: 16 mg or less of tar, 1.2 mg or less of nicotine and 15 mg or less of CO. Mice were weighed on Days 1 and 5 and group sizes of 8-10 mice per treatment were used.

Bronchoalveolar lavage

Lungs from each terminally anaesthetised mouse were lavaged *in situ* with a 400 μl aliquot, followed by three 300 μl of PBS as previously described (22). The total number of viable cells in the BALF was determined, cytospins prepared using 50-200

µl BALF and cells differentiated by standard morphological criteria. Mitotic figures, an index of cell division, were also identified as previously described (22).

Protease Expression and Activity in BALF

Zymography was used to assess protease expression in response to cigarette smoke exposure as previously described (22). Briefly, BALF from animals in each treatment group was pooled, concentrated, spun and the pellet washed and resuspended in 50 µl of 1 x non-reducing buffer. 20 µl was loaded on 10 % SDS-page mini-gels containing 2 mg/ml gelatin and run at a constant voltage of 200 V for 45 min. Gels were then washed in 2.5 % Triton X-100, incubated at 37 °C overnight in zymography buffer, stained for 45 min with Coomassie Brilliant Blue R-250 and extensively destained. Neat BALF was also tested for net gelatinase activity using fluorescence-conjugated gelatin (Molecular Probes, USA) as previously published (22).

RNA extraction and Quantitative Real-Time PCR

Whole lungs were perfused free of blood via right ventricular perfusion with 10 ml warmed saline, rapidly excised en bloc, blotted and snap frozen in liquid nitrogen. Total RNA was extracted from 15 mg of whole lung tissue pooled from 5 mice per treatment group using RNeasy kits (Qiagen), reverse transcription with SuperScript III (Invitrogen) and triplicate real time PCR reactions with Applied Biosystems pre-developed assay reagents and 18S rRNA internal control were done as previously described (22).

Intranasal Administration of Anti-GM-CSF

Mice were anaesthetized lightly by inhalation of Penthrane vapour and 50 μl of (a)

PBS containing isotype control (rat anti-mouse IgG2a mAb of irrelevant specificity,

100 μg/mouse) and (b) PBS containing anti-GM-CSF mAb (22E9, rat anti-mouse

IgG2a ultra purified in-house, 100 μg/mouse) administered intranasally. Anti-GM-

CSF, PBS or isotype control was administered 1 h before the first cigarette smoke

exposure on days 2 and 4 of the experimental protocol.

Purification of BAL macrophages for phagocytosis assay

BAL cells were pooled from 5 animals per group, washed once with PBS, incubated

with rat anti-mouse FcγIII/II receptors (mouse BD Fc Block^{TM}, Pharmingen) for 15

min at 2-8°C and then incubated with a cocktail of biotinylated antibodies (Ter-119,

B220, CD3ϵ (ebioscience), CD8a, CD49b, Ly6G (BD Biosciences, Pharmingen)) for

20 min at 2-8°C. Cells were then washed twice using PBS with 0.5%BSA and 2 mM

EDTA and incubated with Dynabeads® biotin binder (Invitrogen) at ≥8 dynabeads

(pre-labeled with specific biotinylated antibodies) per target cell in 1ml of PBS with

0.5% BSA and 2mM EDTA, pH7.4, for 30 min with gentle tilting and rotation. Cells

were then placed in Dynal magnetic particle concentrator (Dynal MPC^{TM}-L,

Invitrogen) for 3 min and untouched cells (monocyte-macrophage enriched, >95%

purity according to differential counting of the cytospin stained cells) in the

supernatant were subsequently used in the phagocytosis assay.

For the phagocytosis assay macrophages were resuspended in RPMI + 10%

FCS, seeded at 100,000 cells/well (96 well plate) in replicates of five and left to

adhere for 2 h. The media was then aspirated and replaced with either resuspended

fluorescently-labelled *E.Coli* bio-particles in accordance with the manufacturer's

instructions (Molecular Probes, Vybrant Phagocytosis Assay Kit V-6694). Briefly,

cells were incubated with *E.Coli* bioparticles for 2 h in HBSS, aspirated and then excess fluorescence quenched with trypan blue for 1 min. Trypan Blue was then removed and fluorescence per well quantitated using a microplate reader (Flex Station, Molecular Devices). In addition, images were captured using a fluorescence microscope (Nikon Eclipse TS100). Whilst this method is an effective quantitative method for measuring internalization of bacteria, this approach does not assess the killing capacity of macrophages which is also integral to macrophage phagocytosis.

Statistical analyses

As data were normally distributed, they are presented as grouped data expressed as mean±standard error of the mean (s.e.m.); *n* represents the number of mice. Differences were determined by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test for multiple comparisons, where appropriate. All statistical analyses were performed using GraphPad Prism™ for Windows (Version 4.02). Probability levels less than 0.05 ($P<0.05$) were taken to indicate statistical significance.

RESULTS

Anti-GM-CSF blocks cigarette smoke-induced BALF neutrophilia and macrophage accumulation

In BALB/c mice exposed to cigarette smoke generated from 9 cigarettes per day for 4 days, there was a significant increase in the total number of cells, neutrophils and macrophages in BALF (Figure 1A-C) ($P<0.05$, ANOVA followed by Bonferroni post hoc test) . We have previously shown that BALF of smoke-exposed BALB/c mice

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had very few and variable numbers of lymphocytes compared to sham-exposed mice

(22). Mice treated with 22E9 (100 µg/mouse) had significantly reduced total cells,

neutrophils and macrophages compared with isotype (Figure 1A-C) ($P<0.05$, ANOVA

followed by Bonferroni post hoc test). Isotype alone antibody had no effect on

baseline total, macrophage and neutrophil cell numbers in sham- or smoke-exposed

animals. Similarly, 22E9 had no effect on baseline total, macrophage and neutrophil

cell numbers in sham-exposed animals. However, 22E9 significantly reduced

smoke-induced increases in total cell number, neutrophils and macrophages

($P<0.05$, ANOVA followed by Bonferroni post hoc test).

Animals exposed to cigarette smoke had an increased % of BAL

macrophages in mitosis, based on nuclear mitotic figures indicating active cell

division as previously described (22), compared to sham-exposed mice ($P<0.001$,

ANOVA followed by Bonferroni post hoc test) (Figure 2). 22E9 had no effect on

baseline % of BAL macrophages in mitosis in sham-exposed animals but

significantly reduced the % of BAL macrophages in mitosis in smoke-exposed

animals ($P<0.001$, ANOVA followed by Bonferroni post hoc test) (Figure 2).

Cigarette smoke not only caused an influx of macrophages but caused a

marked increase in size and activation state of these cells as indicated by large

vacuolated cytoplasm and ruffled edges (Figure 3A-D). Isotype control and 22E9

had no effect on macrophage size in either sham or smoke-exposed animals (Figure

3A-D).

Neutralization of GM-CSF reduces cigarette smoke-induced weight loss

In the present study mice exposed to cigarette smoke generated from 9 cigarettes

per day for 4 days lost a significant amount of weight (Figure 4A) ($P<0.001$, ANOVA

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followed by Bonferroni post hoc test). However, mice treated with 22E9 lost less weight than those treated with isotype control antibody by the end of the experimental protocol ($P<0.05$, ANOVA followed by Bonferroni post hoc test). In contrast, body weights for all groups were similar at the beginning of the experiment (Figure 4B).

Neutralization of GM-CSF inhibits cigarette smoke-induced increases in TNF-α, MIP-2 and MMP-12 mRNA

Cigarette smoke-exposed animals had markedly elevated mRNA levels of the cytokine TNF-α, the chemokine MIP-2 and the protease MMP-12 (Figure 5). 22E9 (100 μg/mouse) alone had no effect on baseline levels of TNF-α, MIP-2 and MMP-12 mRNA but reduced smoke-induced increases in TNF-α, MIP-2 and MMP-12 mRNA (Figure 5).

Effect of anti-GM-CSF on protease expression and activity

There was a marked increase in protease expression in BALF from BALB/c mice exposed to cigarette smoke as assessed by zymography (Figure 6A). A major band of protease expression was identified at approximately 90 kDa, corresponding to the molecular size of the active form of MMP9 (Figure 6A). Smoke-exposed mice treated with 22E9 (100 μg/mouse) had similar levels of MMP-9 to smoke-exposed mice treated with isotype control (Figure 6A). Consistent with the zymography, there was an increase in net gelatinase activity in the BALF from mice exposed to cigarette smoke but smoke-exposed mice treated with 22E9 (100 μg/mouse) had similar levels of net gelatinase activity to smoke-exposed mice treated with isotype control (Figure 6B).

Effect of anti-GM-CSF on BAL macrophage phagocytic function

In experiments designed to explore the effects of 22E9 on BAL macrophage phagocytic function, we found that macrophages harvested from mice treated with isotype control antibody and exposed to cigarette smoke had increased phagocytic function compared with sham + isotype-treated mice (Figure 7). Neutralisation of GM-CSF did not affect the phagocytic function of macrophages (Figure 7). Similarly, the phagocytic function of macrophage obtained from sham mice treated with isotype control antibody or 22E9 were similar (Figure 7).

Discussion

The principle objective of the present study was to characterize in detail the effects of neutralizing GM-CSF in a sub-chronic mouse model of cigarette smoke-induced lung inflammation. It was not possible to use GM-CSF deficient mice as they develop fatal alveolar proteinosis, or to give anti-GM-CSF for extended periods due to mouse-anti-rat immune responses, both of which confound interpretation. GM-CSF has pro-inflammatory properties because of its action on neutrophils and cells of the monocyte/macrophage lineage (3, 7) and we have shown that GM-CSF contributes to NFκB-dependent lung inflammation in part via activation of the upstream kinase Akt/PKB (19). Recombinant GM-CSF augments leukocyte leukotriene and superoxide anion production (24), enhances allergic sensitization (25), and when over-expressed in rat lung epithelium, GM-CSF promotes inflammation and fibrosis (11).

GM-CSF is often difficult to detect at the protein level because is exerts its effects at very low concentrations, it is very rapidly consumed by receptor internalization and the absolute density of internalizing receptors increase markedly in inflammation with the influx of leukocytes (26). In this study we could not detect elevated levels of GM-CSF protein in BALF of mice treated with cigarette smoke but found that whole lung GM-CSF mRNA is increased by cigarette smoke exposure and, given the marked inhibitory effects of 22E9, revealed a GM-CSF-dependent disease process under conditions where GM-CSF itself remains below the detection sensitivity of current methods.

Since it is known that GM-CSF is a survival factor for neutrophils and macrophages and that GM-CSF inhibits neutrophil apoptosis (7), the ability of anti-GM-CSF antibody to reduce cigarette smoke-induced BALF neutrophils and macrophages was probably due in part to apoptosis. We were unable to detect apoptotic cells *ex vivo*, presumably because of rapid clearance by macrophages. In addition, since infiltration of neutrophils we observed was in accordance with the induction of MIP-2, a potent murine neutrophil chemotactic factor, reduced MIP-2 (and potential other mediators such as KC, not measured in this study) caused by anti-GM-CSF may also account for reduced neutrophils. We observed GM-CSF-sensitive macrophage replication that is consistent with the ability of GM-CSF to induce lung macrophage replication *in vitro* (27) and the increase in macrophage numbers observed after targeted GM-CSF overexpression in the lung (11).

Macrophages are markedly increased in the lung parenchyma of smokers and patients with COPD (28) where their number correlates with disease severity (29). Macrophages can be directly activated by cigarette smoke to release inflammatory mediators including TNF-α, MCP-1, reactive oxygen species and neutrophil

chemotactic factors. As we have previously observed mononuclear lineage chemotactic factor induction (22) it is likely that the increase in macrophages we observed represents both the progeny of resident alveolar macrophages as well as influx of blood monocytes adopting an alveolar macrophage like morphology ("alveolar monocytes"), as has been demonstrated after LPS challenge (30). If this is the case it is likely that recruited monocytes contribute to further neutrophil accumulation (31). The induction of CSF-1 and its receptor as well as GM-CSF by cigarette smoke (22) is consistent with this interpretation as well as the observation that a substantial fraction of macrophages in smoke-exposed lungs were in mitosis. It was interesting to note that although neutralization of GM-CSF reduced macrophage numbers, it did not affect their size, shape and phagocytic function. Although lymphocytes infiltrate the lungs of long-term smokers we have previously published that they were not prominent in this model and FACS profiling indicates they constitute a very small fraction of total cells in this model comprising both CD3+CD4+ and CD3+CD8+ lymphocytes (22). CD3+ CD8+ lymphocytes have recently been directly implicated in long-term smoke-induced inflammation and lung damage suggesting that more sustained inflammation is necessary to drive their recruitment or proliferation (32).

TNF-α is considered to be a key inflammatory mediator in lung pathology, and its levels are elevated in lung diseases where neutrophilic and macrophage inflammation is prominent (33). In concert with chemokines, TNF-α also promotes release of reactive oxygen species, elastase, and other proteases including MMPs that are implicated in lung tissue damage (33). Churg and colleagues showed that mice lacking TNF-α receptors exposed to cigarette smoke for 6 months had significantly less neutrophils, macrophages, MMP-12 and emphysema (34). In the

present study we have shown that neutralization of GM-CSF significantly inhibited smoke-induced TNF-α expression. We previously investigated the molecular pathways that mediate this reinforcement of TNF-α levels *in vivo* and in particular demonstrated the GM-CSF-dependent activation of the transcription factors AP-1 and NFκB in response to LPS (19). Of possible relevance to the neutrophilia observed in our cigarette smoke studies is the fact that TNF-α itself has variable effects on neutrophil survival, but it augments GM-CSF-induced survival of neutrophils (35). It is presently unclear whether GM-CSF regulation of TNF-α also contributed to the lack of effect on cigarette smoke-induced MMP-9 (assessed by zymography) expression observed in the present study, as cigarette smoke, LPS and TNF-α induce MMP-9 in diverse cell systems (3).

Proteases which break down connective tissue components are found in increased amounts in people with COPD. In patients with emphysema there is an increase in BALF concentrations and macrophage expression of MMP-1 (collagenase) and MMP-9 (gelatinase) (36). Alveolar macrophages from normal smokers express more MMP-9 than those from normal subjects (37) and there is an even greater increase in cells from patients with COPD (38), which have greatly enhanced elastolytic activity (39). In the present study, anti-GM-CSF did not alter smoke-induced increases in MMP-9 as assessed by zymography. Similarly, anti-GM-CSF did not affect smoke-induced increases in net gelatinase activity.

The accumulation of macrophages may also explain, in part, the very marked increase in MMP-12 message we observed. MMP-12 is markedly increased in induced sputum from stable COPD patients (40), is discretely localized into macrophages in human lung disease (41) and cigarette smoke increases the expression of MMP-12 in the lungs of mice and that macrophages and DCs produce

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MMP-12 (42). Moreover, emphysema induced by chronic cigarette smoke exposure is prevented in MMP-12$^{-/-}$ mice (43). Levels of MMP-12 protein were below detection using zymography in our study. Lung proteolysis is actively counteracted by anti-proteases. We did not measure antiproteases in this study however; the zymographic and fluorogenic substrate approaches we used have the advantage of defining actual net matrix degrading activity over more sensitive but less informative methods such as immunocytochemistry and ELISA.

An interesting observation in this study was that neutralization of GM-CSF reduces cigarette smoke-induced weight loss. We have previously shown that the weight loss observed in our model could be attributed to reduced food intake and loss of appetite (44) but food intake was not measured in the present study. Alternatively, the significantly lower BALF inflammation and whole lung TNFα in anti-GM-CSF treated smoke-exposed mice may partly explain the reduced weight loss given that TNFα has been associated with muscle wasting in COPD (45).

In summary, the marked suppression of cigarette smoke-induced inflammation by anti-GM-CSF when administered prophylactically suggests that GM-CSF is a key mediator of cigarette smoke-induced lung inflammation. The striking effects of anti-GM-CSF in our model suggest the possible therapeutic utility of blocking GM-CSF in human lung disease where high neutrophil and macrophage numbers, protease induction, and cytokine and chemokine overproduction are believed to be central agents in disease pathogenesis.

References

1.   Lopez AD, Murray CC. The global burden of disease, 1990-2020. *Nat Med* 1998;4(11):1241-1243.

2.   Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of Chronic Obstructive Pulmonary Disease. NHLB/WHO global initiative for Chronic Obstructive Lung Disease (gold) workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-1276.

3.   Vlahos R, Bozinovski S, Hamilton JA, Anderson GP. Therapeutic potential of treating Chronic Obstructive Pulmonary Disease (COPD) by neutralising granulocyte macrophage-colony stimulating factor (GM-CSF). *Pharmacol Ther* 2006; 112(1):106-115.

4.   Shibata Y, Berclaz PY, Chroneos ZC, Yoshida M, Whitsett JA, Trapnell BC. GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through pu.1. *Immunity* 2001;15(4):557-567.

5.   Bonfield TL, Raychaudhuri B, Malur A, Abraham S, Trapnell BC, Kavuru MS, Thomassen MJ. Pu.1 regulation of human alveolar macrophage differentiation requires granulocyte-macrophage colony-stimulating factor. *Am J Physiol Lung Cell Mol Physiol* 2003;285(5):L1132-1136.

6.   Shinagawa K, Trifilieff A, Anderson GP. Involvement of ccr3-reactive chemokines in eosinophil survival. *Int Arch Allergy Immunol* 2003;130(2):150-157.

7.   Hamilton JA. GM-CSF in inflammation and autoimmunity. *Trends Immunol* 2002;23(8):403-408.

8.   Gomez-Cambronero J, Horn J, Paul CC, Baumann MA. Granulocyte-macrophage colony-stimulating factor is a chemoattractant cytokine for human

neutrophils: Involvement of the ribosomal p70 s6 kinase signaling pathway. *J Immunol* 2003;171(12):6846-6855.

9. Barnes PJ, Shapiro SD, Pauwels RA. Chronic Obstructive Pulmonary Disease: Molecular and cellular mechanisms. *Eur Respir J* 2003;22:672-688.

10. Piguet PF, Grau GE, de Kossodo S. Role of granulocyte-macrophage colony-stimulating factor in pulmonary fibrosis induced in mice by bleomycin. *Exp Lung Res* 1993;19(5):579-587.

11. Xing Z, Ohkawara Y, Jordana M, Graham F, Gauldie J. Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J Clin Invest* 1996;97(4):1102-1110.

12. Worgall S, Singh R, Leopold PL, Kaner RJ, Hackett NR, Topf N, Moore MA, Crystal RG. Selective expansion of alveolar macrophages in vivo by adenovirus-mediated transfer of the murine granulocyte-macrophage colony-stimulating factor cDNA. *Blood* 1999;93(2):655-666.

13. Xing Z, Braciak T, Ohkawara Y, Sallenave JM, Foley R, Sime PJ, Jordana M, Graham FL, Gauldie J. Gene transfer for cytokine functional studies in the lung: The multifunctional role of gm-csf in pulmonary inflammation. *J Leukoc Biol* 1996;59(4):481-488.

14. Stampfli MR, Wiley RE, Neigh GS, Gajewska BU, Lei XF, Snider DP, Xing Z, Jordana M. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J Clin Invest* 1998;102(9):1704-1714.

15. Balbi B, Bason C, Balleari E, Fiasella F, Pesci A, Ghio R, Fabiano F. Increased bronchoalveolar granulocytes and granulocyte/macrophage colony-

stimulating factor during exacerbations of chronic bronchitis. *Eur Respir J* 1997;10(4):846-850.

16.   Saha S, Doe C, Mistry V, Siddiqui S, Parker D, Sleeman M, Cohen ES, Brightling CE. Granulocyte-macrophage colony-stimulating factor expression in induced sputum and bronchial mucosa in asthma and COPD. *Thorax* 2009;64(8):671-676.

17.   McManus TE, Marley AM, Baxter N, Coyle PV, Kidney JC. GM-CSF is elevated in COPD patients in comparison to non-obstructed smokers [abstract]. *Proceedings of The American Thoracic Society* 2005;2:A394.

18.   Tsoumakidou M, Tzanakis N, Chrysofakis G, Siafakas N. Nitrosative stress, heme oxygenase-1 expression and airway inflammation during severe exacerbations of COPD. *Chest* 2005;127(6):1911-1918.

19.   Bozinovski S, Jones JE, Vlahos R, Hamilton JA, Anderson GP. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NFκB and AP-1 in vivo. *J Biol Chem* 2002;277(45):42808-42814.

20.   Bozinovski S, Jones J, Beavitt SJ, Cook AD, Hamilton JA, Anderson GP. Innate immune responses to lps in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of Tlr-4. *Am J Physiol Lung Cell Mol Physiol* 2004;286(4):L877-885.

21.   Stanley E, Lieschke GJ, Grail D, Metcalf D, Hodgson G, Gall JA, Maher DW, Cebon J, Sinickas V, Dunn AR. Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *PNAS USA* 1994;91(12):5592-5596.

22. Vlahos R, Bozinovski S, Jones JE, Powell J, Gras J, Lilja A, Hansen MJ, Gualano RC, Irving L, Anderson GP. Differential protease, innate immunity, and NFκB induction profiles during lung inflammation induced by sub-chronic cigarette smoke exposure in mice. *Am J Physiol Lung Cell Mol Physiol* 2006;290(5):L931-945.

23. Vlahos R, Bozinovski S, Irving L, Smallwood DM, Hamilton JA, Anderson GP. GM-CSF is a pathogenic mediator in experimental COPD [abstract]. *Proceedings of The American Thoracic Society* 2005;2:A143.

24. Silberstein DS, Owen WF, Gasson JC, DiPersio JF, Golde DW, Bina JC, Soberman R, Austen KF, David JR. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by biosynthetic (recombinant) granulocyte-macrophage colony-stimulating factor. *J Immunol* 1986;137(10):3290-3294.

25. Lei XF, Ohkawara Y, Stampfli MR, Gauldie J, Croitoru K, Jordana M, Xing Z. Compartmentalized transgene expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in mouse lung enhances allergic airways inflammation. *Clin Exp Immunol* 1998;113(2):157-165.

26. Metcalf D, Nicola NA, Mifsud S, Di Rago L. Receptor clearance obscures the magnitude of granulocyte-macrophage colony-stimulating factor responses in mice to endotoxin or local infections. *Blood* 1999;93(5):1579-1585.

27. Nakata K, Akagawa KS, Fukayama M, Hayashi Y, Kadokura M, Tokunaga T. Granulocyte-macrophage colony-stimulating factor promotes the proliferation of human alveolar macrophages in vitro. *J Immunol* 1991;147(4):1266-1272.

28. Retamales I, Elliott WM, Meshi B, Coxson HO, Pare PD, Sciurba FC, Rogers RM, Hayashi S, Hogg JC. Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am J Respir Crit Care Med* 2001;164:469-473.

29.   Di Stefano A, Capelli A, Lusuardi M, Balbo P, Vecchio C, Maestrelli P, Mapp CE, Fabbri LM, Donner CF, Saetta M. Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med* 1998;158(4):1277-1285.

30.   Maus U, Herold S, Muth H, Maus R, Ermert L, Ermert M, Weissmann N, Rosseau S, Seeger W, Grimminger F, et al. Monocytes recruited into the alveolar air space of mice show a monocytic phenotype but upregulate CD14. *Am J Physiol Lung Cell Mol Physiol* 2001;280(1):L58-68.

31.   Maus UA, Waelsch K, Kuziel WA, Delbeck T, Mack M, Blackwell TS, Christman JW, Schlondorff D, Seeger W, Lohmeyer J. Monocytes are potent facilitators of alveolar neutrophil emigration during lung inflammation: Role of the ccl2-ccr2 axis. *J Immunol* 2003;170(6):3273-3278.

32.   Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, Maestrelli P, Ciaccia A, Fabbri LM. CD8+ T-lymphocytes in peripheral airways of smokers with Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 1998;157(3 Pt 1):822-826.

33.   Barnes PJ. Mediators of Chronic Obstructive Pulmonary Disease. *Pharmacol Rev* 2004;56(4):515-548.

34.   Churg A, Wang RD, Tai H, Wang X, Xie C, Wright JL. TNFα drives 70% of cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med* 2004;170(5):492-498.

35.   van den Berg JM, Weyer S, Weening JJ, Roos D, Kuijpers TW. Divergent effects of tumor necrosis factor alpha on apoptosis of human neutrophils. *J Leukoc Biol* 2001;69(3):467-473.

36.   Finlay GA, O'Driscoll LR, Russell KJ, D'Arcy EM, Masterson JB, FitzGerald MX, O'Connor CM. Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am J Respir Crit Care Med* 1997;156(1):240-247.

37.   Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ, Fan Chung K. Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. *Am J Respir Crit Care Med* 2000;162(4 Pt 1):1355-1360.

38.   Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J, Barnes PJ. Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with Chronic Obstructive Pulmonary Disease. *Am J Respir Cell Mol Biol* 2002;26(5):602-609.

39.   Russell RE, Thorley A, Culpitt SV, Dodd S, Donnelly LE, Demattos C, Fitzgerald M, Barnes PJ. Alveolar macrophage-mediated elastolysis: Roles of matrix metalloproteinases, cysteine, and serine proteases. *Am J Physiol Lung Cell Mol Physiol* 2002;283(4):L867-873.

40.   Demedts IK, Morel-Montero A, Lebecque S, Pacheco Y, Cataldo D, Joos GF, Pauwels RA, Brusselle GG. Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax* 2006;61(3):196-201.

41.   Molet S, Belleguic C, Lena H, Germain N, Bertrand CP, Shapiro SD, Planquois JM, Delaval P, Lagente V. Increase in macrophage elastase (MMP-12) in lungs from patients with Chronic Obstructive Pulmonary Disease. *Inflamm Res* 2005;54(1):31-36.

42.   Bracke K, Cataldo D, Maes T, Gueders M, Noel A, Foidart JM, Brusselle G, Pauwels RA. Matrix metalloproteinase-12 and cathepsin d expression in pulmonary

macrophages and dendritic cells of cigarette smoke-exposed mice. *Int Arch Allergy Immunol* 2005;138(2):169-179.

43.   Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;277(5334):2002-2004.

44.   Chen H, Vlahos R, Bozinovski S, Jones J, Anderson GP, Morris MJ. Effect of short-term cigarette smoke exposure on body weight, appetite and brain neuropeptide y in mice. *Neuropsychopharmacology* 2005;30(4):713-719.

45.   Remels AH, Gosker HR, van der Velden J, Langen RC, Schols AM. Systemic inflammation and skeletal muscle dysfunction in chronic obstructive pulmonary disease: State of the art and novel insights in regulation of muscle plasticity. *Clinics in Chest Medicine* 2007;28(3):537-552, vi.

Figure 1 Anti-GM-CSF inhibits cigarette smoke-induced increases in BALF total cell number (A), neutrophils (B) and macrophages (C) in BALB/c mice. Data are shown as mean ± SEM for n=8 mice per treatment group. Clear bars represent no treatment, black bars represent isotype control-treated mice and hatched bars represent 22E9 (100 μg/mouse)-treated mice. *P<0.001 vs respective control (ANOVA and Bonferroni's *post hoc* test), #P<0.05 vs respective control (ANOVA and Bonferroni's *post hoc* test), † P<0.001 vs isotype-treated smoke-exposed mice (ANOVA followed by Bonferroni's *post hoc* test).

Figure 2 Anti-GM-CSF inhibits cigarette smoke-induced increases in % BALF macrophages in division. Data are shown as mean ± SEM for n=8 mice per treatment group. Clear bars represent isotype control-treated mice and black bars represent 22E9 (100 μg/mouse)-treated mice. *P<0.001 vs respective control (ANOVA and Bonferroni's *post hoc* test), † P<0.001 vs isotype-treated smoke-exposed mice (ANOVA followed by Bonferroni's *post hoc* test).

Figure 3 Effect of 22E9 (100 μg/mouse) on macrophage cell size. Panels (A, B) show representative DiffQuik-stained cytospin preparations of BALF from sham-exposed mice pretreated with isotype control antibody (A) or mice exposed to smoke and treated with isotype control antibody (B). Note in panel (A) that sham-exposed mouse lavage comprises >98 % resting alveolar macrophages whereas panel (B) shows smoke-exposed mouse lavage comprising activated macrophages with vacuolated cytoplasm and recruitment of neutrophils. Panels (C) and (D) show representative DiffQuik-stained cytospin preparations of BALF from sham-exposed

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mice pre-treated with 22E9 (C) or mice exposed to smoke and treated with 22E9 (D). Magnification as indicated by scale bar.

Figure 4 Anti-GM-CSF reduces cigarette smoke-induced weight loss. Mouse body weights are shown for the last (A) and first (B) days of the experimental protocol. Data are shown as mean ± SEM for n=8 mice per treatment group. Clear bars represent isotype control-treated mice and black bars represent 22E9 (100 μg/mouse)-treated mice. *P<0.001 vs sham + isotype (ANOVA and Bonferroni's *post hoc* test), **P<0.05 vs sham + 22E9 (ANOVA followed by Bonferroni's *post hoc* test), † P<0.05 vs smoke+isotype (ANOVA and Bonferroni's *post hoc* test).

Figure 5 Anti-GM-CSF inhibits cigarette smoke-induced increases in TNF-α, MIP-2 and MMP-12 mRNA. mRNA expression for all genes was measured simultaneously under identical conditions using quantitative real-time PCR. Responses are shown as fold expression relative to 18S. Data are shown as mean ± SEM of 3 replicates as previously published (22). Clear bars represent isotype control-treated mice and black bars represent 22E9 (100 μg/mouse)-treated mice.

Figure 6 Effect of anti-GM-CSF on MMP-9 expression and net gelatinase activity. Panel (A) shows MMP-9 assayed by gelatin zymography under reducing conditions. The BALF from mice in each treatment group (n=8) was pooled. Panel (B) shows net free gelatinase activity by degradation of flurogenic gelatin substrate, using neat BALF assayed from individual mice, providing a measure of the balance of protease/anti-protease. Data in (B) are shown as mean ± SEM for n=8 mice per treatment group. Clear bars represent isotype control-treated mice and black bars

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represent 22E9 (100 μg/mouse)-treated mice.   *$P<0.05$ vs respective control (ANOVA and Bonferroni's *post hoc* test).

Figure 7 Effect of anti-GM-CSF (22E9, 100 μg/mouse) on macrophage phagocytic function. Images in panel (A) were captured by fluorescent microscopy. Data in panel (B) are shown as mean ± SEM of 5 replicates. Clear bars represent isotype control-treated mice and black bars represent 22E9 (100 μg/mouse)-treated mice.

A

B

C
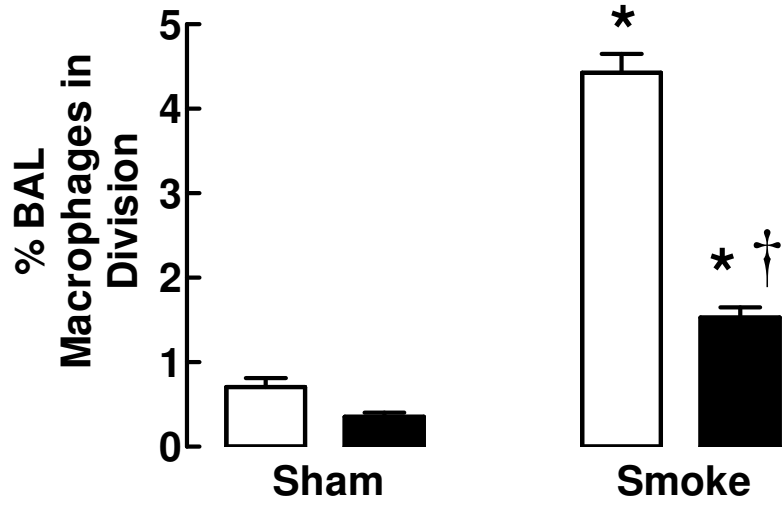
Figure 1

Figure 2

A   **Sham + isotype**
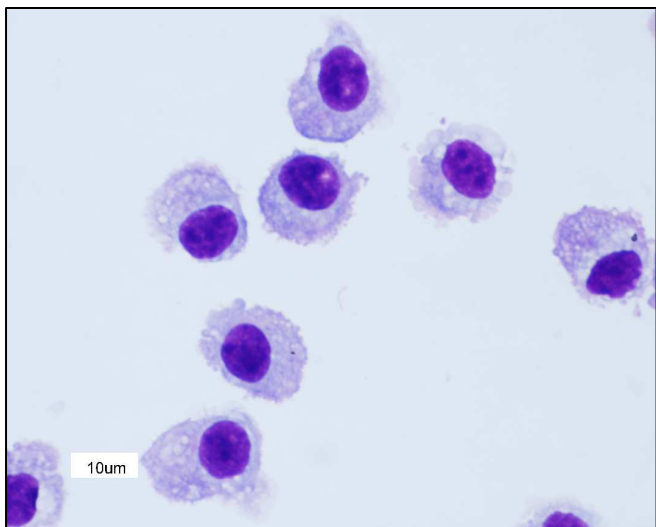
B   **Smoke + isotype**
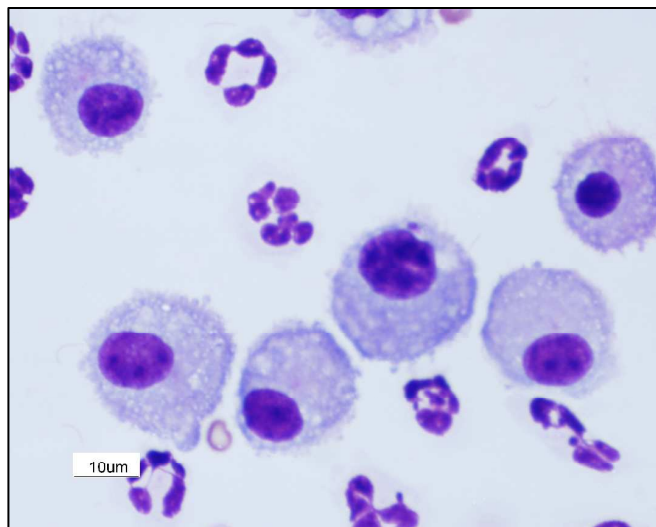
C   **Sham + 22E9**

D   **Smoke + 22E9**

Figure 3

A

B

Figure 4

TNFα

A

MIP-2

B

MMP-12

C

Figure 5

A

Sham   Smoke
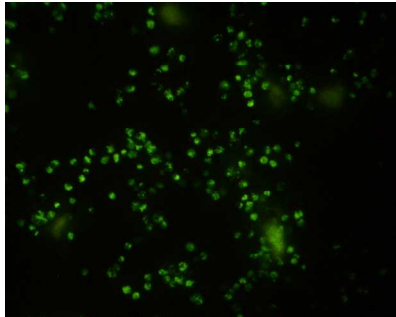
MMP-9

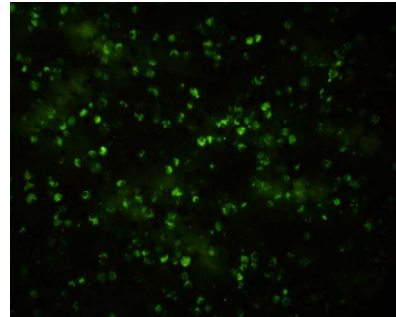isotype   +   -   +   -

22E9   -   +   -   +

B
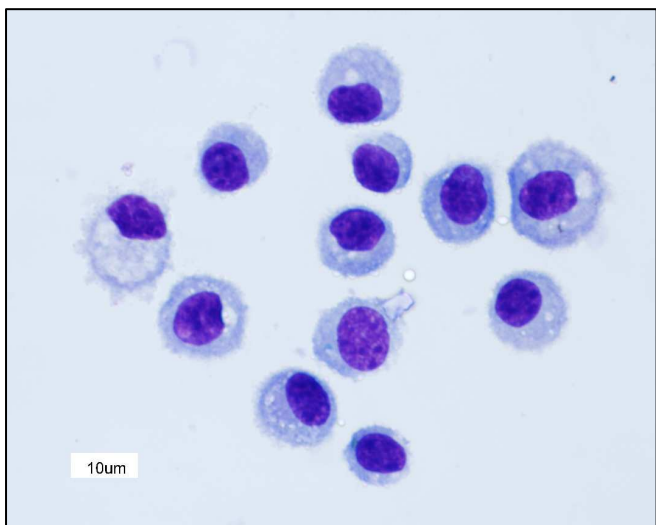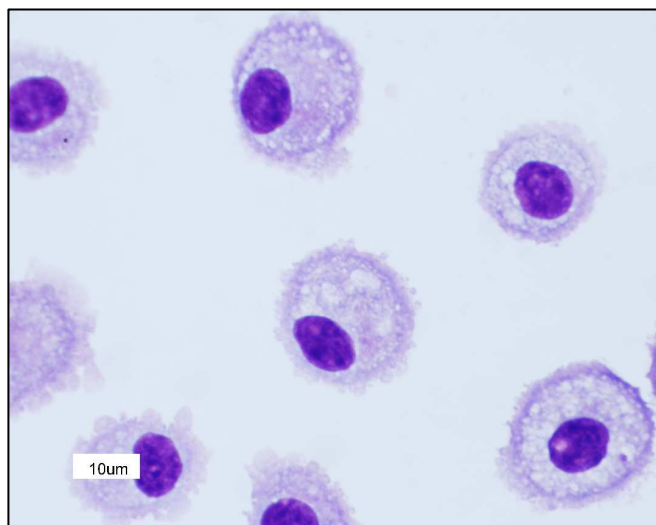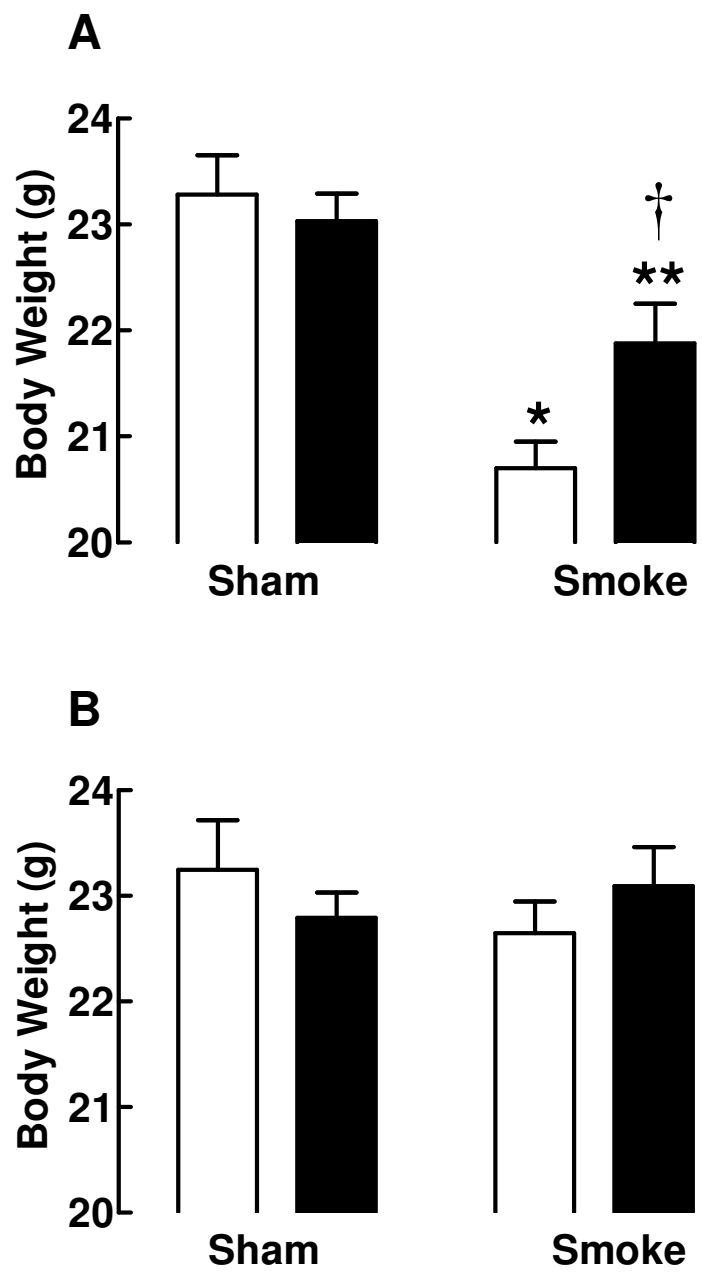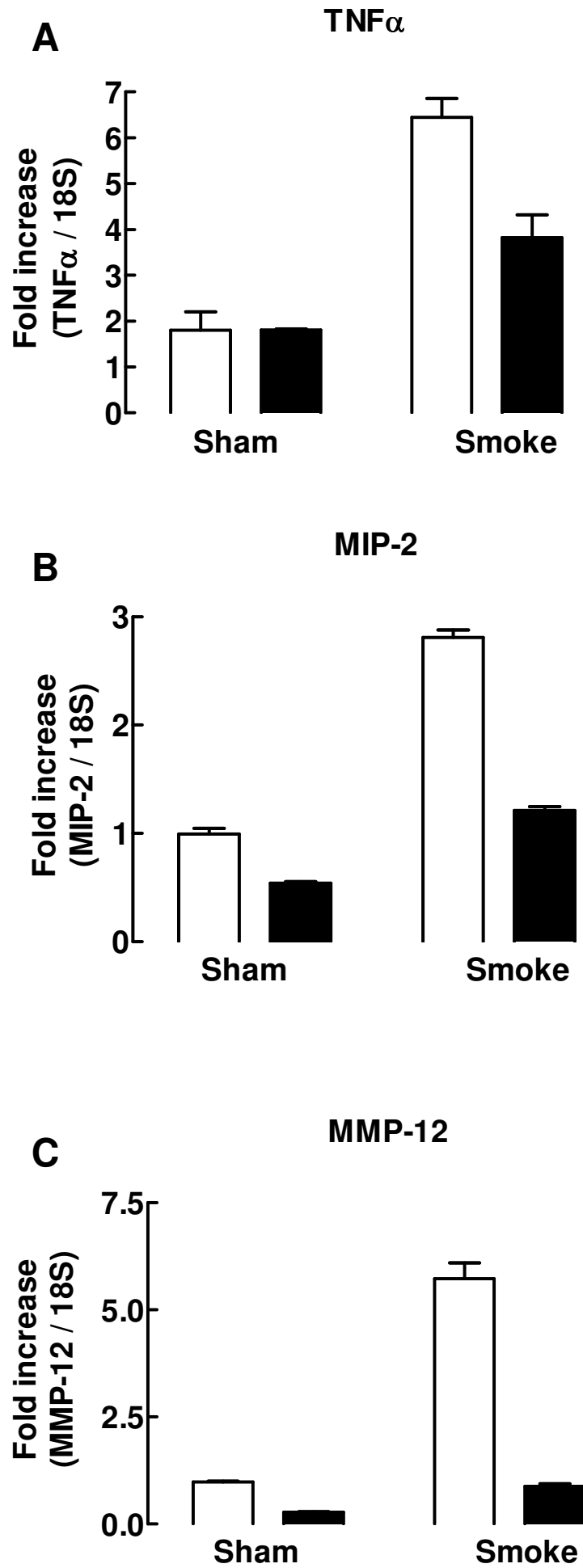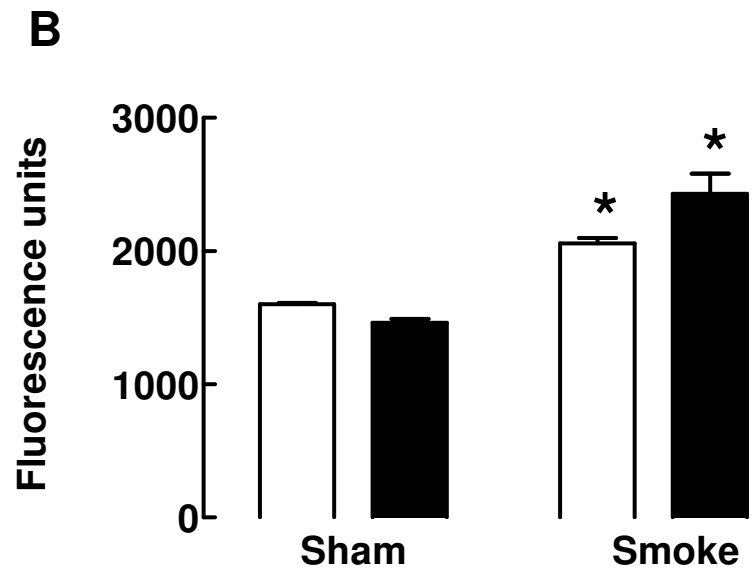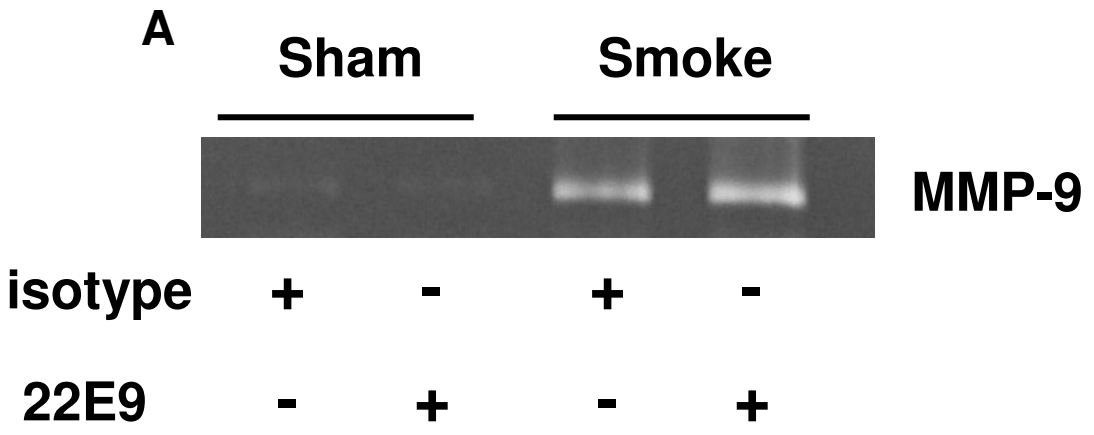
Figure 6

A
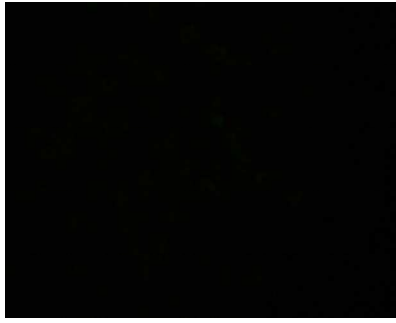
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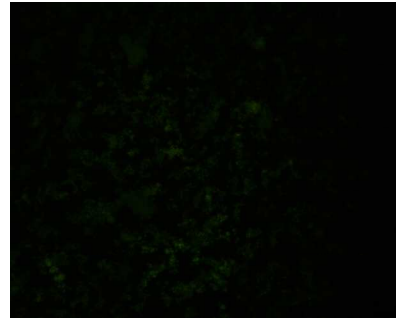
No cells + *E.coli*

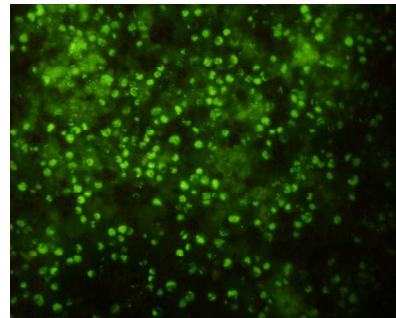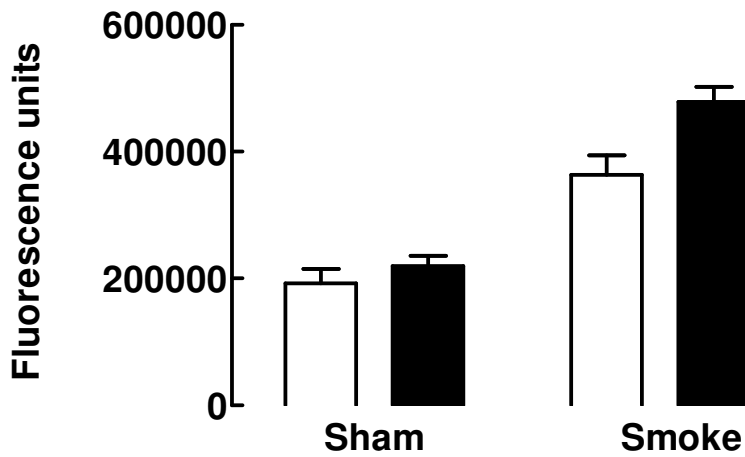Sham + isotype

Sham + 22E9
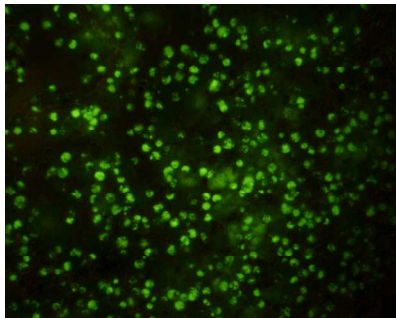
Smoke + isotype

Smoke + 22E9

B

Figure 7