

Oxidative stress-induced antibodies to carbonyl-modified protein correlate with severity of COPD

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PK: Conceived & designed the study, participated in both clinical and animal studies & drafted the manuscript. GC: Helped design & participated in the clinical studies & drafting the manuscript. PC: Participated in the immunohistochemical studies. ME: Helped in design & participated in the animal studies. BS, KT, FH & MP: Participated in the animal studies. YK, LH, LS and MY: Helped in experimental work and clinical samples. MY, AP, PB FC & IA: Participated in the study design & coordination and drafting of the manuscript.

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**At a Glance Commentary:
Scientific Knowledge on the Subject**

There is increasing evidence for the presence of autoantibodies in chronic obstructive pulmonary disease (COPD), but it is yet unclear as to how this is linked to one of the major pathogenic drivers present in COPD, chronic oxidative stress.

What This Study Adds to the Field

The detection of antibodies in COPD against novel carbonyl-modified self-antigens provides a plausible mechanistic link between chronic oxidative stress and the development of a potentially destructive autoimmune component in COPD.

Abstract

Rationale: There is increasing evidence for the presence of autoantibodies in COPD. Chronic oxidative stress is an essential component in COPD pathogenesis and can lead to increased levels of highly reactive carbonyls in the lung which could result in the formation of highly immunogenic carbonyl adducts on 'self' proteins.

Objective: To determine the presence of autoantibodies to carbonyl-modified protein in COPD patients and in a murine model of chronic ozone exposure. To assess the extent of activated immune responses towards carbonyl-modified proteins.

Methods: Blood and peripheral lung was taken from COPD patients, age matched smokers and non-smokers with normal lung function, as well as patients with severe persistent asthma. Mice were exposed to ambient air or ozone for 6 weeks. Antibody titres were measured by ELISA, activated complement deposition by immunohistochemistry and cellular activation by ELISA and FACs.

Results: Antibody titre against carbonyl-modified 'self' protein was significantly increased in GOLD stage III COPD patients compared to controls. Antibody levels inversely correlated with disease severity and showed a prevalence towards an IgG1 isotype. Deposition of activated complement in the vessels of COPD lung as well as autoantibodies against endothelial cells was also observed. Ozone-exposed mice similarly exhibited increased antibody titres to carbonyl-modified protein, as well as activated antigen presenting cells in lung tissue and splenocytes sensitized to activation by carbonyl-modified protein.

Conclusions: Carbonyl-modified proteins, arising as a result of oxidative stress, promote antibody production, providing a link by which oxidative stress could drive an auto-immune response in COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is currently a leading cause of morbidity and mortality worldwide (1) with the main cause being long term cigarette smoking in the Western World (1, 3) . Inflammation and remodelling of the small airways are major determinants for the progression and severity of COPD, as defined by the decline in forced expiratory volume in one second (FEV₁) (5). Accumulation of inflammatory mucous exudates in the lumen and infiltration of the wall by innate and adaptive inflammatory immune cells, such as CD4+ cells, CD8+ cells, B cells, macrophages and neutrophils, and the formation of lymphoid follicles are all features of the observed inflammation, that correlate with the severity of COPD (5, 6).

Previous studies have suggested that autoimmune mechanisms may contribute to the pathogenesis of COPD. Serum autoantibodies against elastin (9) and bronchial epithelial cells along with corresponding immunoglobulin (IgG) and complement (C3) deposition (10) have been observed in COPD lung. It has therefore been proposed that cigarette smoke-derived antigens may be responsible for driving this disease process in COPD (7, 10 - 12), but until now this has not been investigated. In addition, complement activation in the lung which is usually direct evidence of autoimmune activation has not been examined in COPD, particularly in progressive disease (1, 13).

Oxidants which are a major constituent of cigarette smoke can cause the formation of carbonyl adducts on proteins (14). They are formed *in vivo* as a result of lipid peroxidation which then in turn modify proteins, but can also be directly incorporated into proteins through direct oxidation of amino acid side-chains as well as oxidative cleavage of proteins (15). These have been implicated in the pathogenesis of many chronic inflammatory and/or autoimmune diseases (14, 16). In COPD patients, carbonyl adducts have been found both within the lung (16) and in the circulation (17), and their levels correlated with disease severity, measured by the decline in FEV₁.

We hypothesised that carbonyl stress arising as a result of chronic exposure to oxidants in cigarette smoke drives the production of potentially damaging neo- or autoantibodies to carbonyl-modified ‘self’ protein in COPD. In order to test this hypothesis, we modified self-proteins with a number of different carbonyl adducts known to be present in COPD, which were then used to screen sera from COPD patients and controls for antibodies against ‘self’ and carbonyl-modified ‘self’ protein. In addition, the ability of carbonyl-modified self’ proteins to trigger lymphocyte activation *in vitro* was also examined. The presence of IgG deposition and of complement activation were examined in lung tissue of COPD patients and controls. Finally, in a chronic animal model of oxidative stress-induced lung inflammation, we examined whether an immune response against carbonyl-modified ‘self’ protein could also be triggered.

Materials and Methods

Reagents

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma Aldrich Inc., (St. Louis, MO, USA). Research grade cigarettes (Reference code 2R1/1R3F) were obtained from the University of Kentucky, USA. 4-hydroxynonenol (4-HNE) was obtained from Calbiochem. Bis-malonyldialdehyde (MDA) was acquired from Alpha Diagnostics Intl. (Texas, USA). Antibodies; peroxidase labeled monoclonal anti human IgG1, 2, 3 and 4, anti-human IgM, peroxidase labeled polyclonal goat anti-human IgG (#A6029) were all acquired from Sigma Aldrich Inc., (St. Louis, MO, USA). Murine IL-2 ELISA kit was purchased from R&D systems (UK). Ultraculture medium was obtained from Biowhittaker (UK).

Clinical samples

Subjects were recruited from the Section of Respiratory Medicine of the University Hospital of Ferrara, Italy., with approval by the local Ethics Committee. Serum and tissue samples were acquired after written informed consent was obtained and pulmonary function tests were performed as previously described (18) Predicted values for the different measures were calculated from the regression equations published by Quanjer (19). COPD was defined, according to international guidelines (post-bronchodilator FEV₁/FVC ratio <70%) and the severity of COPD was classed according to current GOLD criteria (1). Serum and lung tissue was processed as detailed in the online supplemental data and subject details are summarised in Tables 1 and 2 respectively. Serum samples from patients with severe asthma as defined by the ATS Guidelines (ref) were obtained from the Royal Brompton Hospital after written informed consent was obtained as disease controls.

Animals and treatments

Pathogen-free, 6-8 week old male BALB/c mice (Harlan, UK) were exposed to 2.5 ppm ozone for 3 hours in a sealed Perspex container either once (acute) or every 3 days for 6 weeks (chronic) (20). Control animals were exposed to air over the equivalent period. Twenty-four hours after the last exposure, the mice were culled and the lung, spleen, lymph nodes and blood removed. Dendritic cells and lung macrophages were purified by positive selection using MACs. Further details are described in the online supplement.

Antigen preparation, ELISA, Immunohistochemistry, Western blot analysis and Flow cytometry

Human or mouse serum albumin (Sigma; cat# A3782) was modified with acrolein, 4-hydroxynonenol, malonyldialdehyde (MDA) or cigarette smoke condensate for 24 hours at 37°C. Patient or mouse serum was screened for antibodies against carbonyl-modified proteins by ELISA using 96-well Nunc Maxisorb immunoplates coated with the carbonyl-modified protein prepared above. Bound antibodies were assessed for either total IgG or specific class and isotype using appropriate secondary antibodies. Murine IL-2 was assessed using a commercial ELISA from R&D systems (UK). Immunohistochemical staining for activated C4d complement was performed using rabbit anti-human C4d (Biomedica, UK). Lung tissue carbonylated proteins were detected by Western blot using the oxyblot assay (Millipore, UK). Activated dendritic cells and lung macrophages were assessed by flow cytometry after staining for CD11c, CD80, CD86, CD54 (all from BD Biosciences) and F4/80 (Caltag-MedSystems Ltd). Further details are described in the online supplement.

Statistical analysis

Differences between patient groups were calculated with GraphPad Prism software using a non-parametric Kruskal-Wallis test with Dunn's multiple comparison *post-test* analysis or Mann-Whitney test as indicated. All data are expressed as mean \pm SEM and differences were considered significant if $p < 0.05$.

Results

Autoantibodies to carbonyl modified self protein are present in COPD

Serum from stable COPD patients with different stages of severity, as well as from control subjects were screened against various forms of carbonylated-modified ‘self’ protein, in order to establish the presence of auto-antibodies against carbonyl-modified epitopes (**Fig 1**). Human serum albumin was chosen as a generic ‘self’ protein as it was easily available, not expected to have an autoimmune response directed against it and could therefore be used to screen against carbonyl epitopes. There was a significant increase in autoantibody titre against carbonyl-modified protein by GOLD stage 3 (**Fig 1**) which was inversely correlated with FEV₁ (% predicted) particularly for MDA- and acrolein-modified protein (MDA: $r^2=0.31$ $p<0.01$, Acrolein: $r^2=0.27$ $p<0.01$, 4-hydroxynonenal: $r^2=0.17$ $p<0.05$, CSE: $r^2=0.17$ $p<0.05$). There was also a small, but significant, increase in autoantibody titre against unmodified HSA in GOLD 3 COPD subjects only. There was no significant increase in antibody titre against MDA- and acrolein-modified HSA in smokers. In contrast, apart from a significant response to acrolein-modified HSA ($p<0.001$) there was no significant increase in antibody titre to unmodified, MDA-, 4HNE- or CSE-modified HSA in sera from patients with severe persistent asthma. Overall, GOLD 3 COPD subjects exhibited an increase in total antibody titre levels compared to asymptomatic non-smokers ($p<0.001$; **Fig 1f**). Severe asthmatics had a similar total anti-carbonyl titre levels as asymptomatic smokers.

Numerous carbonyl-modified proteins were present in the lung parenchyma from COPD patients as determined by Western blotting (**Fig 2a** lanes 1, 3 and 5). The most abundant carbonyl modified protein had a molecular weight similar to that for HSA. In contrast, no non-specific bands were observed in the control lanes (**Fig 2a** - lanes 2, 4 and 6) although protein loading was similar across all 6 lanes (**Fig 2b**).

A significant increase in IgG1 isotype responses in smokers and COPD patients compared to non-smokers (**Supplementary data - Fig E1**) was observed. Moreover, there was a trend towards increasing immunoreactivity for this isotype response with increased disease severity. Except for a significant increase in IgG2 immunoreactivity against carbonyl-modified protein in GOLD stage 3 COPD (**Supplementary data - Fig E1b**), there were no significant responses against the other immunoglobulin isotypes tested (IgG3, IgG4 and IgM). In contrast to carbonyl-modified HSA, significant immunoreactivity towards unmodified HSA was only observed in GOLD stage 3 patients, and was of an IgG2 isotype (**Supplementary data - Fig E1g**). No significant response was observed against HSA for IgG1, IgG3, IgG4 and IgM.

Chronic ozone exposure leads to activated immune response to carbonyl-modified self

The effect of 6 weeks exposure to ozone, an oxidant stress, on antibodies against carbonyl modified proteins in mice was also determined. Ozone exposure led to a significant increase in antibody titre against MDA-modified murine serum albumin (MSA) compared to a control group of mice exposed to air only (**Fig 3**). In contrast, acute exposure to ozone (one exposure) showed no increase in titre above control.

Splenocytes isolated from chronic ozone exposed mice when treated with MDA-modified albumin exhibited significantly greater proliferation and increased release in IL-2, when compared to splenocytes isolated from control air-exposed mice (**Supplementary data - Fig E2**). In contrast, unmodified murine serum albumin had no significant impact on splenocytes from either air-exposed or chronic ozone-exposed mice. Finally, chronic-ozone exposure lead to activation of lung APCs, from both within the lung (**Supplementary data- Fig E3**) and draining lung lymph nodes (**Supplementary data- Fig E4**) as demonstrated by increased expression of the surface expression markers CD80, CD86 and CD54.

Complement deposition and immunoreactivity against endothelial cells in the peripheral lung of stable COPD patients

Immunohistochemical staining for C4d was mainly confined to lung vessels and the number of C4d+ve lung vessels was significantly increased in smokers, with or without COPD, compared to non-smokers (**Fig 4**). However, there were no significant differences between GOLD 2 COPD subjects and control smokers. In addition, auto-antibody levels against endothelial cells were significantly elevated in subjects with COPD compared to healthy non-smokers (**Fig 5**). There was no significant increase in anti-endothelial cell auto-antibody levels in smokers compared to non-smokers.

Discussion

We have shown in patients with stable COPD, the presence of circulating antibodies against carbonyl epitopes formed on proteins as a result of exposure to chronic oxidative stress. The increased antibody titer against carbonyl-modified protein correlated with disease severity in stable COPD and was highly significant in GOLD stage III COPD. Interestingly, antibodies against carbonyl-modified protein also showed a trend toward an IgG1 phenotype. Moreover, we observed the presence of a numerous carbonyl-modified proteins in peripheral lung tissue from patients with COPD. These antigenic carbonyl adducts on self-proteins and their plasma levels have previously been shown to be associated with disease severity in stable COPD (18). Similar findings were observed in mice after chronic exposure to ozone for 6 weeks. Antibodies against carbonyl-modified protein were elevated and splenocytes isolated from ozone-exposed mice became activated in response to stimulation with carbonyl-modified protein. This was accompanied by a greater antigen-presenting cell activation (both macrophages and dendritic cells) in murine lungs as demonstrated by the increased expression of the activation markers CD80, CD86, and CD54 on these cells. Finally, we demonstrate the presence of anti-endothelial cell antibodies and activated complement localized to the endothelium of lung vessels from patients with COPD.

Carbonyl adducts, such as 4-HNE, have been observed in both the lung and systemically in muscle fibers of subjects with COPD (13, 14). Other examples of reactive carbonyl adducts include acrolein, MDA, methyl glyoxal, and numerous others. A key characteristic of this group of molecules is that they are able to covalently modify proteins nonenzymatically by targeting nucleophilic sites, such as amines or sulfhydryl groups, on proteins (19–21). Consequently, carbonyl-induced protein modifications have been shown to be markers for oxidative stress–derived tissue damage in a number of diseases (22). Protein carbonylation can also affect protein function as demonstrated by us (19, 23) and others (12). Furthermore, carbonyl-modified proteins are highly immunogenic and lead to the

formation of neoantigens in the form of carbonylated self-protein (24–26). This in turn leads to the formation of autoimmune-type responses as a result of altered self-antigens (26–28). Protein carbonylation is a heterogeneous event, and it is unlikely that only one particular carbonyl modification will impart antigenicity to a protein, as demonstrated here by the immunoreactivity toward protein modified by several different carbonylating species. Carbonylated proteins can also modulate adaptive immune responses (29, 30) as well as activate T cells and promote Th1-type responses through their uptake and presentation by antigen-presenting cells (31).

We have demonstrated that there are a large number of carbonyl-modified proteins present in the peripheral lung tissue of subjects with stable COPD (GOLD stages I and II) that could act as potential epitopes for the generation of the anti-carbonyl antibodies. HSA was used as a generic screening antigen, because there should be no immune response against it under physiological conditions. However, because of its abundant nature it is very likely to be affected by oxidative stress and hence be modified. A major modified band corresponds to the molecular weight of HSA, which has recently been reported to be the major carbonyl-modified protein in human lung tissue (32). HSA was postulated to be acting as a sacrificial, scavenger antioxidant, but prolonged exposure to oxidative stress may lead to autoantibody production. Clearly, further detailed studies will be required to ascertain the identities and function of these other carbonyl-modified proteins.

The presence of autoantibodies in COPD has been controversial. The increased prevalence of anti-elastin antibodies in a subset of patients with COPD with severe pulmonary emphysema (5) has been contradicted by more recent publications (33–36). However, autoantibodies to several unidentified epithelial cell proteins (6) and general tissue proteins (37) have also been demonstrated, as well as antibodies in the serum of patients with COPD directed against a number of peptide epitopes from a peptide array (8). In addition, a very recent publication has reported the increased presence of anti-

endothelial cell autoantibodies in COPD (9). However, none of the publications addressed how these autoantibodies might have arisen through the breakdown of immune tolerance, apart from speculating that components in cigarette smoke were somehow involved. We have demonstrated that carbonyl adducts, which are potent antigens formed as a result of exposure to chronic oxidative stress (27), such as cigarette smoking or ozone exposure, can trigger an antibody-mediated immune response. Using *in vivo* models, two groups have shown that oxidant stress and carbonyl-modified protein can lead to autoimmunity (28, 31) with specific T cell activation and corresponding antibody production, a prerequisite for any immune response. Similarly, we found that oxidant-stressed mice after chronic ozone exposure exhibited higher antibody titers against carbonyl-modified protein as well as increased activation of their splenocytes in response to stimulation with carbonyl-modified protein. Our unpublished observations also showed increased proliferation of peripheral blood mononuclear cells from subjects with COPD in response to stimulation with carbonyl-modified protein as determined by BrdU uptake, but this needs to be confirmed in a more extensive study using fluorescence-activated cell sorter analysis to determine cell specificity and the extent of proliferation. Allison and Fearon (24) demonstrated that carbonyl modified self-protein or peptides were able to break immune tolerance *in vivo*. The immune response to oxidatively, or more correctly, carbonyl-modified neoantigens is not unique to COPD. Kurien and Scofield (27) highlighted how oxidative stress in other chronic diseases associated with high oxidative stress, such as rheumatoid arthritis, systemic lupus erythematosus, scleroderma and Behçet disease, gave rise to autoimmune responses to carbonyl-modified self-antigens. Furthermore, they highlighted how these carbonyl-modified epitopes could give rise to epitope spreading, a key feature of many autoimmune diseases. Not surprisingly, we also found an anti-carbonyl antibody response in patients with severe asthma, another chronic inflammatory disease that has also been associated with the presence of oxidative stress (38). Interestingly, the response was not as widespread across those carbonyl-modified antigens tested and moreover was not as strong as that observed in subjects with GOLD III COPD. This may simply reflect the greater oxidative burden

present in the COPD lung compared with the severe asthmatic lung. Another key feature of many autoimmune diseases is the presence of antinuclear antibodies. It is interesting to note, therefore, that antibodies against adducts of 4HNE, a carbonyl known to be elevated in COPD (13), have dual specificity against both DNA and 4HNE-modified protein leading to an antinuclear antibody-type response (39).

Our immunohistochemical analysis of lung resection samples showed evidence of complement activation around the vascular endothelium in peripheral lung from healthy smokers and patients with GOLD II COPD and not in the lungs from control nonsmokers. We also observed elevated anti-endothelial cell and anti-carbonyl-modified protein titers in smokers and subjects with GOLD II COPD compared with nonsmokers, although significance was only seen in the COPD group. However, antibody titers between the smokers and subjects with GOLD II COPD were similar and may therefore explain the similar level of complement activation. Interestingly, serum levels of C4 were negatively correlated with the degree of pulmonary emphysema in patients with COPD and chronic bronchitis (40). Other studies have reported similar findings. Miller and colleagues showed decreased levels of serum C3 and C4 proteins in patients with stable COPD compared with control subjects (41). Moreover, they found a correlation between the degree of the reduction of these serum proteins and the presence of symptoms of chronic bronchitis (41). In contrast, Marc and colleagues demonstrated that sputum levels of both C3a and C5a were significantly increased in patients with moderate to severe stable COPD compared with control healthy smokers and nonsmokers (42). Together this would suggest that as complement is sequestered toward the lung where it is activated and consumed, serum levels would decline. Combined with our data, it would suggest that the oxidant stress seen in both smokers and patients with COPD is directing an antibody-mediated autoimmune response against the endothelium. In keeping with these data, triggering an autoimmune response against lung endothelial cells in a murine model led to endothelial cell death and pulmonary emphysema (43). Similarly, Karayama and

colleagues have reported the increased presence of anti-endothelial antibodies in patients with COPD (9). We hypothesize that a similar mechanism is occurring in smokers and to a much greater extent in COPD lungs, where the chronic exposure to oxidative stress leads to carbonyl adducts being formed on lung endothelial cells. Why this should only occur on endothelial cells is not clear, and what is the identity of the carbonyl-modified endothelial-specific antigen remains to be resolved.

In conclusion, this pilot study has demonstrated for the first time the presence of antibodies against carbonyl-modified protein neoepitopes in both COPD and a murine model of chronic exposure to oxidative stress. It is also proposed that these antibody responses to carbonyl-modified protein may be targeting endothelial cells in the lung parenchyma of subjects with COPD, leading to lung destruction. We recognize that one of the important limitations of this study is the low numbers of subjects screened in each group. Therefore, it would be important to confirm our findings here in larger cohorts of control subjects and patients with COPD.

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Table 1: Patient details (Serum)

	Age (years)	M/F	Pack years	FEV₁/FVC	% pred FEV₁
Non-smokers	51± 2	8/5	N/A	0.98 ± 0.03	105 ± 4
Smokers	59± 2	14/8	28± 3	0.84 ± 0.03	86 ± 3
GOLD 1	66± 2	1/4	56±20	0.66 ± 0.01	88 ±8
GOLD 2	72 ± 2	10/2	43 ± 7	0.59 ± 0.02	62 ± 2
GOLD 3	74 ± 2	6/4	34 ± 7	0.50 ± 0.03	40 ± 1
Severe Asthma	51± 4	3/9	0	0.85 ± 0.02	70 ± 8

Data is depicted as Mean +/- SD. FEV₁/FVC ratio is post bronchodilator for subjects with COPD but not smokers or non-smokers.

Abbreviations: pred = predicted; M = male; F = female; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity.

Figure legends

Figure 1. Antibodies to carbonyl-modified protein in human serum from COPD patients, smokers and non-smokers. Human serum was screened for immunoreactivity towards carbonyl modified human serum albumin by ELISA and titres determined as detailed in material & methods. Antibody titres against human serum albumin that had been left (a) unmodified or had been modified by: (b) acrolein, (c) malonyldialdehyde (MDA), (d) 4-hydroxynonenal or (e) cigarette smoke extract are shown. The cumulative titres against all the different carbonyl-modified HSAs tested for each patient group are shown (f). Results are expressed as a box and whiskers plot and displaying the mean for each patient group. Sera from 12 patients with severe persistent asthma (SA) are used as a disease control. Statistical analysis was performed using a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post test analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to non-smokers (NS). ## $p < 0.01$, ### $p < 0.001$ compared to smokers.

Figure 2. Carbonyl-modified proteins are present in parenchymal lung tissue of COPD patients. Solubilised parenchymal lung tissue from 3 COPD patients was analysed by: (A) Western blotting for carbonyl modified proteins following DNPH-derivatisation of carbonyl-epitopes, and (B) Coomassie staining for total protein. In Lanes 1, 3 and 5, protein samples were derivatised with DNPH prior to SDS-PAGE, lanes 2, 4 and 6 were left underderivatised. Further details are available in supplementary data section.

Figure 3. Antibodies to carbonyl modified 'self' protein in mice chronically exposed to ozone. Murine serum was screened for immunoreactivity towards carbonyl modified murine serum albumin by ELISA and titres determined by ELISA as detailed in materials & methods. Murine serum from mice either acutely exposed (1 day) or chronically exposed (6 weeks) to air or ozone was screened on

ELISA plates coated with murine serum albumin that had been carbonyl-modified with malonyldialdehyde. Chronic ozone exposure results in a significant increase in antibody titre against carbonyl-modified protein. Results are expressed as the mean \pm SEM for the titre determination from 6-8 mice in each treatment group. Statistical analysis was performed using a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-test analysis. $*p < 0.05$, compared to control mice exposed to air.

Figure 4. Complement (C4d) activation in peripheral lungs of COPD and controls.

Photomicrographs showing the peripheral lung from a non-smoker (A), a healthy smoker with normal lung function (B), a patient with mild/moderate COPD (C) and the negative control (D) immunostained for identification of C4d+cells (brown). Results are representative of those from 14 non-smokers, 20 smokers with normal lung function, 16 mild/moderate COPD. A significant increase in staining between non-smokers versus healthy smokers and COPD groups was observed ($p < 0.05$ as determined using the Kruskal-Wallis test). Further methodological details are described in the online supplemental data.

Figure 5. Auto-antibodies to endothelial cells in human serum from COPD patients, smokers and non-smokers.

Human serum was screened for immunoreactivity towards human endothelial cells by ELISA and titres determined as detailed in material & methods. Plates were coated with live HUVEC cells, then treated with serum from COPD, smokers or non-smokers before detecting bound antibody. Results are expressed as the mean \pm SEM for immunoreactivity in each patient group. Statistical analysis was performed using a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-test analysis. $*p < 0.05$, compared to control non-smokers.

Figure 1

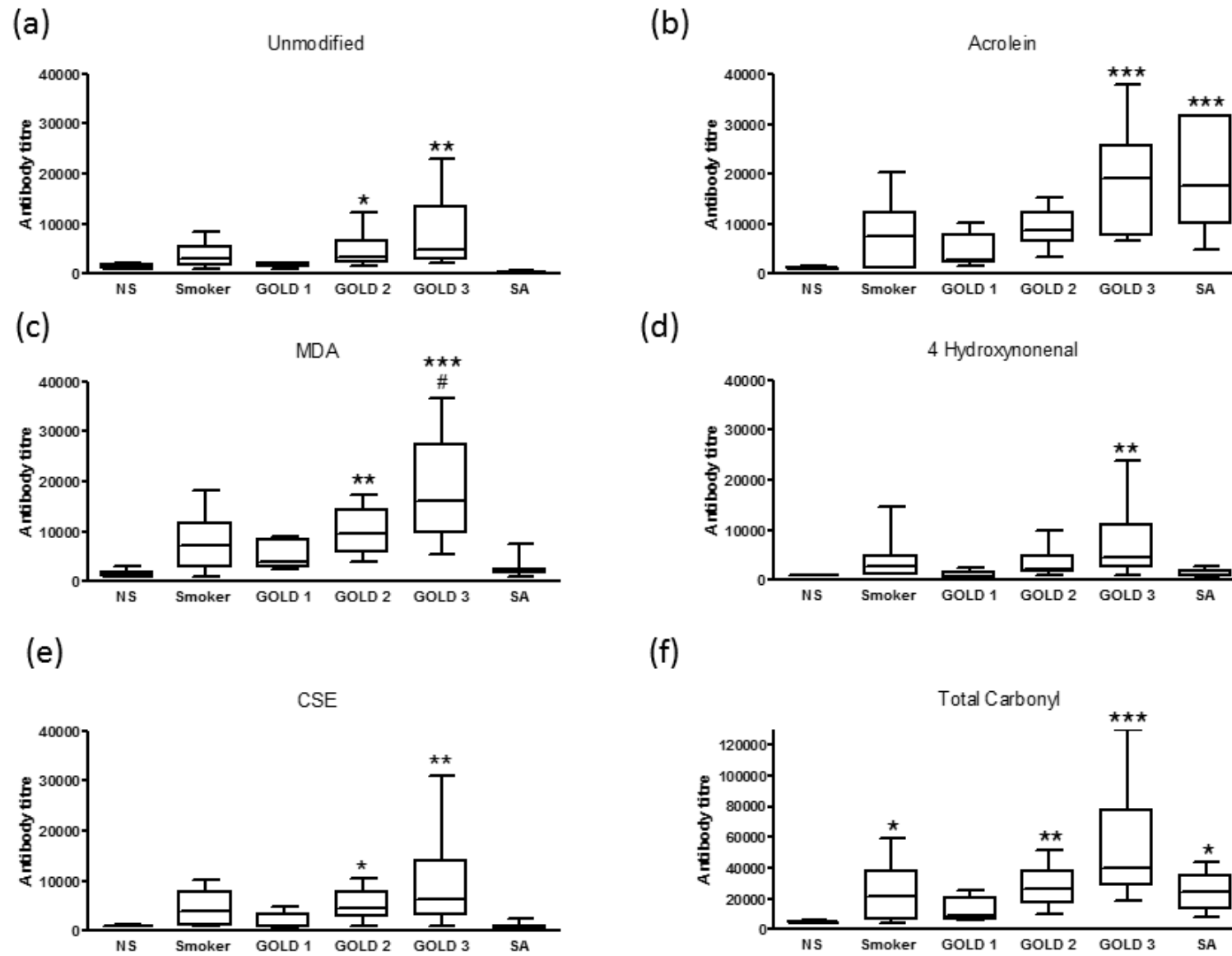


Figure 2

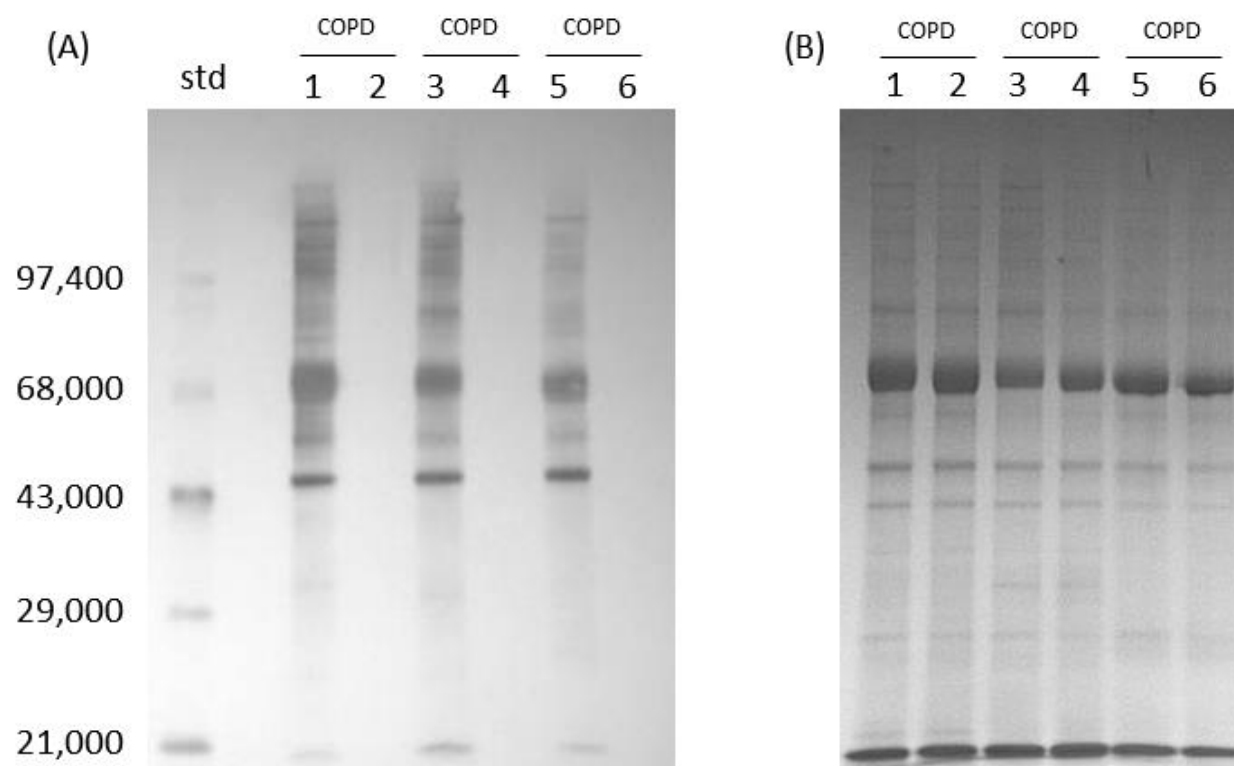


Figure 3

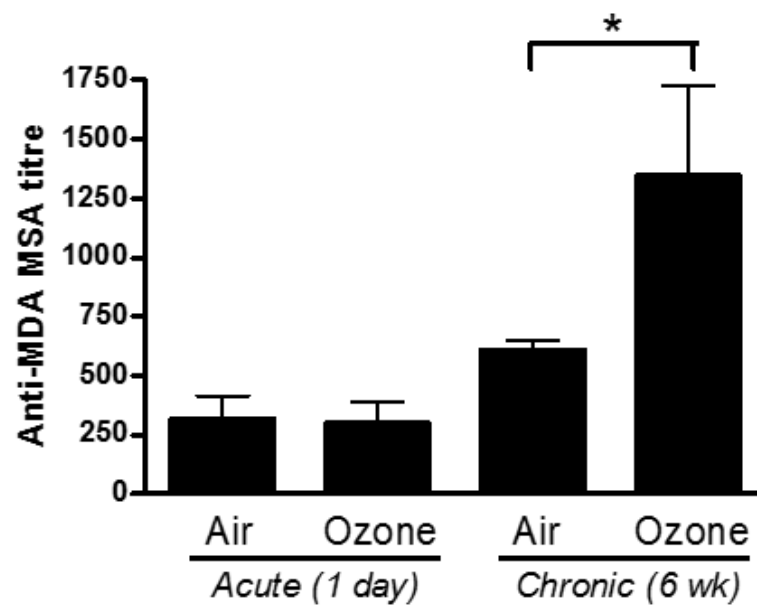
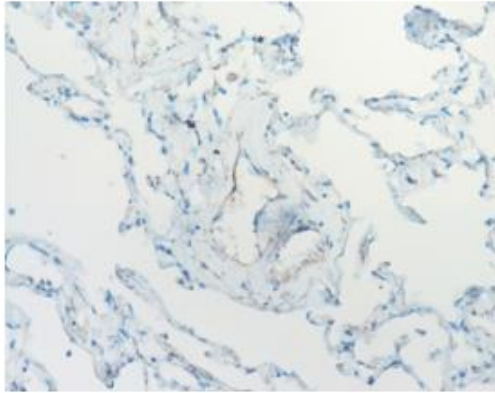
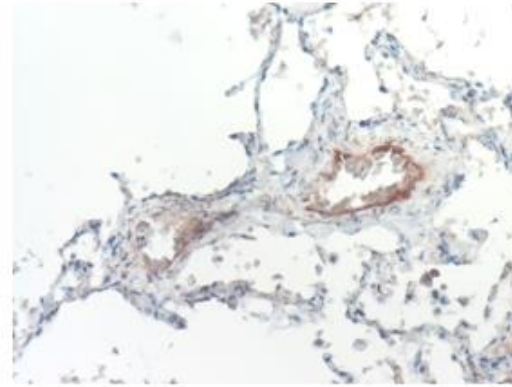


Figure 4

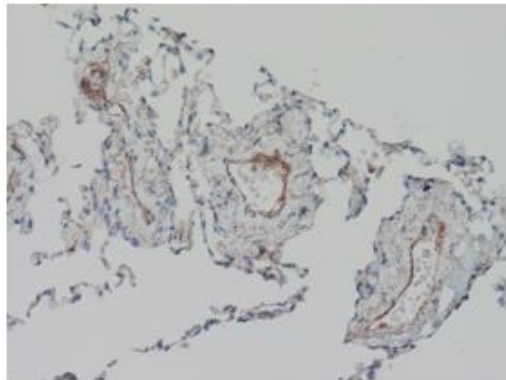
(a)



(b)



(c)



(d)

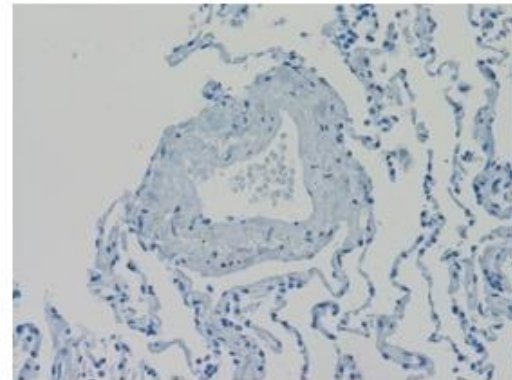
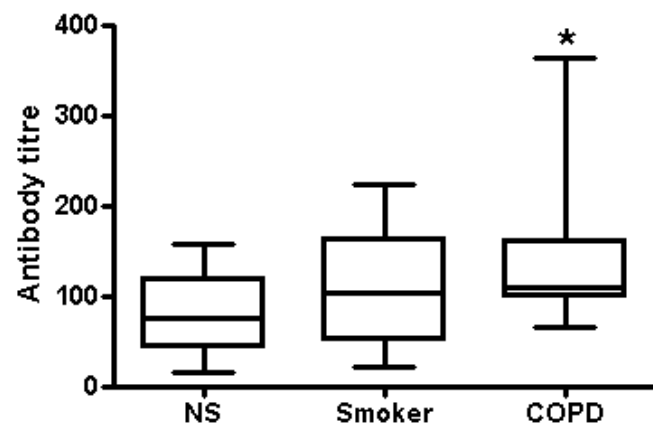


Figure 5



Online Supplementary Data

Methods:

Clinical samples

Serum: The clinical details of the subjects are summarised in Table 1. Four mls of peripheral venous blood were collected from each subject into a sterile vacuette (Greiner Bio-One, 5 mls, Z serum Sep Clot activator; <http://www.greinerbioone.com/en/row/start/>). This type of vacuette is routinely used to isolate serum for assessing autoantibody levels at the University hospital of Ferrara. The vacuette was left at room temperature for 60 minutes and then spun, at room temperature, for 5 minutes at 4000 g/min followed by serum removal. The serum was immediately stored in 500 µl aliquots in 1.5 ml Eppendorfs at –80°C until needed.

Lung tissue: Fifty subjects undergoing lung resection surgery for a solitary peripheral neoplasm were recruited. Fourteen subjects were lifelong non-smokers with normal lung function. Twenty were smokers with normal lung function and sixteen subjects were smokers with COPD (Table 2). All former smokers had stopped smoking for more than one year. All subjects did not undergo preoperative chemotherapy and/or radiotherapy and had not been treated with bronchodilators, theophylline, antibiotics, antioxidants and/or glucocorticoids in the month prior to surgery. Lung tissue processing was performed as previously described (18). Two to four randomly selected tissue blocks were taken from the subpleural parenchyma of the lobe obtained at surgery, avoiding areas grossly invaded by tumour. Samples were fixed in 4% formaldehyde in phosphate-buffered saline at pH 7.2 and, after dehydration, embedded in paraffin wax. Serial sections 4µm thick were first cut and stained with haematoxylin-eosin (H&E) in order to visualize the morphology and to exclude the presence of microscopically evident tumour infiltration. Tissue specimens were then cut for immunohistochemical analysis and were placed on charged slides as previously reported (18).

Animals and treatments

Ozone exposure of mice: Pathogen-free, 6-8 week old male BALB/c mice (Harlan, UK) were housed within 'maximiser' filter-topped cages (Maximiser, Theseus caging system Inc., Hazelton, PA, USA). Mice were exposed to ozone produced by an Ozoniser (Model 500 Sander Ozoniser, Germany), mixed with air for 3 hours at a concentration 2.5 parts per million (ppm) in a sealed Perspex container. Ozone concentration was continuously monitored with an ozone probe (ATi Technologies, Ashton-U-Lyne, UK). Ozone exposure was carried out in 2 groups: (i) Single exposure of 3 hours (ii) two exposures (every 3 days) per week over 6 weeks (20). Control animals were exposed to air over the equivalent period. Twenty-four hours after the last exposure, the mice were culled and the lungs, spleens and blood removed. Blood was collected into sterile 1.5ml ependorf tubes and allowed to clot at 4°C followed by centrifugation at 12000rpm. The serum supernatant was collected and stored at -80°C until needed.

Isolation of spleen and lymph node cells: Spleens or draining lung lymph nodes were removed from mice and a cell suspension prepared by mashing and filtering through cell strainers. The cells were then treated with red cell lysing solution (Sigma-Aldrich) before washing and resuspended in RPMI-1640 culture medium supplemented with 10% heat-inactivated FBS and 1% (v/v) penicillin-streptomycin.

Isolation of dendritic cells and lung macrophages: Cells were purified by positive selection from cell suspensions of lymph nodes or enzyme digested lung tissue. For the latter, harvested lungs were cut into small pieces before incubating with enzyme digestion buffer containing 1mg/ml collagenase type III (Worthington Biochemicals) and 0.5mg/ml bovine pancreatic DNase I (Sigma-Aldrich). Digested lung tissue was ground and filtered through cell strainers. After washing the collected cells, the cell suspension was layered over a Nycoprep 1.077A density gradient (Axis-Shield). Following centrifugation at 1800rpm (no brake), cells at the interphase layer were collected and washed before further purified by positive selection using pan dendritic cell (DC) or macrophage microbeads (Miltenyi Biotec).

Antigen preparation and assessment of carbonyl adduct formation

Various carbonylated-protein antigens were prepared. Human serum albumin (Sigma; cat# A3782) at 1mg/ml in phosphate buffered saline was modified with either 50mM acrolein, 1mM 4-hydroxynonenol, 10mM malonyldialdehyde (MDA) or 10% (v/v) cigarette smoke condensate for 24 hours at 37°C. Stock solutions of all carbonyls were initially made and then diluted down to the final concentration required in the protein solution to be modified. To obtain a stock solution of MDA, 162 µl of 100% (v/v) bis-MDA was added to 200 µl of 2M HCl and left to incubate at room temperature for 15 minutes. It was then neutralised with 4.8ml of 0.1M phosphate buffer (pH 6.4) to give a stock of 0.2 M MDA. A stock solution of 100% (v/v) cigarette smoke condensate was prepared as previously described (26). After modification the protein solutions were then extensively dialysed against PBS for over 24 hours at 4°C with several changes of dialysis buffer. Protein concentration was then assessed using the Pierce (UK) BCA protein assay and carbonyl content determined as previously described (22).

ELISA for anti-carbonyl protein adducts in serum

96-well immunoplates (Maxisorb Nunc, Roskilde, Norway) were coated with either 100 µl unmodified HSA/MSA or carbonyl-modified HSA/MSA in carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, USA) at 1 µg/ml for 3 hours at 4°C. After washing three times with (PBST) phosphate buffer saline containing 0.05% (v/v) Tween 20, the plates were blocked with 200 µl of PBST containing 1% bovine serum albumin for 2 hours at 37°C. The plates were again washed three times with PBST and after all subsequent incubation steps. Next serial dilutions of the serum samples (50 µl/well) in blocking buffer were aliquoted out and left to incubate for 16 hours at 4°C. The presence of specific antibodies to the coating antigen was detected with 50 µl/well of a 4000 fold dilution of peroxidase-labelled goat anti-human IgG polyclonal antibody in blocking buffer for 1.5 hours at 37°C. Plates

were then developed with 50 µl/well of TMB substrate solution (Thermo Fisher Scientific, Rockford, USA) for 15 minutes at room temperature in the dark. Colour development was stopped by addition of 50µl of 2M H₂SO₄ and absorbance read with a Rosys Anthos HT2 microplate reader (Anthos-Labtec, Eugendorf, Austria) set to 450nm and corrected at 550nm. Each ELISA plate screened had the same control serum sample in quadruplicate, diluted 1000 fold in blocking buffer, so as to allow the data to be normalised between plates.

Determination of carbonyl specific serum immunoglobulin class and isotype

96-well immunoplates (Maxisorb Nunc, Roskilde, Norway) were coated with either 100ul unmodified HSA or MDA-modified HSA in carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, USA) at 0.5 µg/ml for 3 hours at 37°C. After washing three times with (PBST) phosphate buffer saline containing 0.05% (v/v) Tween 20, the plates were blocked as previously described. The plate was washed again three times with PBST and after all subsequent incubation steps. Serum samples pre-diluted to 1:1000 with blocking buffer, added in duplicate and left to incubate 16 hours at 4°C. Next, 100µl/well peroxidase labelled monoclonal anti-human IgG (1-4) or IgM (Sigma-Aldrich, St. Louis, USA) at 1000 fold dilution in blocking buffer was incubated for 3 hours at 37°C. Plates were then developed as described earlier.

ELISA for anti-endothelial cell autoantibodies

A confluent human umbilical vein endothelial cell monolayer was established by seeding gelatine pre-coated 96 well flat bottomed microtitre plates with 2 x 10⁴ cells/well and culturing the cells for approximately 2 days. Non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in Hanks Balanced Salt Solution (HBSS) for 1h at 37°C. Serial dilutions of test sera were prepared in blocking buffer and 50µl aliquots were added to each well and incubated for 2h at room temperature. The wells were washed 3 times with HBSS and incubated with 100µl per well horseradish peroxidase-

conjugated anti-human IgG diluted 1/1000 in blocking buffer for 1h at room temperature. Following 3 more washes with HBSS, 100µl/well *o*-Phenylenediamine dihydrochloride (OPD) substrate was added and plates were incubated for 20min at room temperature in the dark. The enzyme reaction was stopped by the addition of 100µl/well 2M H₂SO₄ and optical density was measured at 490nm using an ELISA reader (BioTek, Winooski, VT). The monolayer was monitored throughout the assay to ensure that cells remained well attached and confluent.

Western blot for carbonyl-modified proteins

Frozen lung tissue was ground to a powder under liquid nitrogen and then lysed in RIPA buffer containing 2% 2-mercaptoethanol. Carbonyl groups were derivatised to DNP-hydrazone by reacting with DNPH or control using buffer only according to manufactures protocol in the OxyBlot Protein Oxidation Detection Kit (Millipore). Equal amounts of protein were then separated on duplicate 10% Bis/Tris gels. Derivatised carbonyl groups were then detected following Western transfer according to manufacturer's protocol. Total protein was visualised using the coomassie G-250 based dye, GelCode Blue Stain (Thermo Scientific).

Assessment of IL-2 secretion from murine splenocytes

Isolated splenocytes were cultured with either unmodified or carbonyl-modified MSA proteins at 0, 50 and 200µg/ml in RPMI-1640 culture medium supplemented with 10% heat-inactivated FBS and 1% (v/v) penicillin-streptomycin, to determine cytokine responses. After 5 days of culture with either unmodified or carbonyl-modified MSA, culture supernatant were harvested and assessed for murine IL-2 using an ELISA kit from R&D Systems following the manufacturer's protocol. Results were expressed in pg/ml of cytokine concentrations.

Immunohistochemistry for C4d in human peripheral lung tissue

Immunohistochemical staining was performed as previously described (18). After deparaffinization and rehydration to expose the immunoreactive epitopes of C4d, the sections to be stained, immersed in target retrieval solution pH 9.0 (Dakocytomation, code S2367), were incubated in a microwave oven (model NN S200W; Panasonic, Milano, Italy) on high power for 40 min. Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide (H₂O₂) in phosphate-buffered saline (PBS) followed by washing in PBS. Cell membranes were permeabilised adding 0.1% saponin to the PBS. Non-specific labeling was blocked by coating with blocking serum (5% normal goat serum) for 20 minutes at room temperature. After washing in PBS the sections were incubated for 1 hour at room temperature with rabbit anti human C4d (Biomedica code BI-RC4D) at dilution of 1:100 of a 200 µg/ml solution.

This antibody has been selected because C4d is an inactive fragment of C4b but maintains a reactive thioester group that forms a strong covalent bond with nearby structures, this antibody has been previously validated for its use in paraffin-embedded tissues (43). For the negative control slides normal rabbit non-specific immunoglobulins (Santa Cruz Biotechnology) were used at the same protein concentration as the primary antibody. Control slides were included in each staining run using human normal tonsils and heart rejection biopsies (kindly provided by Dr Margaret Burke) as a positive control for all the immunostaining performed. After repeated washing steps with PBS, the sections were subsequently incubated with goat anti-rabbit biotinylated antibody (Vector ABC Kit, Vector Laboratories; www.vectorlabs.com) for 30 minutes at room temperature. After further washing the sections were subsequently incubated with ABC reagent (Vector ABC Kit, Vector Laboratories) for 30 minutes at room temperature. Slides were then incubated with chromogen-fast diaminobenzidine (DAB) as chromogenic substance. After which they were counterstained in haematoxylin and mounted on permanent mounting medium.

Grading C4d immunostaining on formalin-fixed paraffin-embedded lung tissue

There were three categories of deposition which were recognized: low, intermediate, and high based on the number of positive staining vessels in five high power field (HPF, 400x) fields. The numbers were arbitrarily chosen according to Magro et al (44) . A low category was assigned in cases showing positivity amid <10 vessels per 5 HPF, an intermediate category in those cases showing positive staining of 10–15 vessels per 5 HPF, and a high category in those cases manifesting positive staining of >15 vessels per 5 HPF.

Flow cytometry analysis

Isolated lung macrophages and dendritic cells were stained for surface markers and expression analysed by flow cytometry using a FACSCanto II (BD Biosciences). Fluorescent-conjugated antibodies to mouse CD11c, CD80, CD86, CD54 (all from BD Biosciences) and F4/80 (Caltag-Medsystems Ltd) were used to characterize macrophage and DC activation. Acquired data was analysed using FACSDiva software (BD Biosciences).

Table E1: Patient details (lung tissue)

	Age (years)	M/F	Pack years	FEV₁/FVC	% pred FEV₁
Non-smokers	68 ± 3	4/10	NA	0.80 ± 0.02	104 ± 4
Smokers	64 ± 2	17/3	28 ± 4	0.78 ± 0.01	100 ± 4
COPD	67 ± 2	14/2	45 ± 6	0.59 ± 0.02	69 ± 5

Data is depicted as Mean +/- SD. FEV₁/FVC ratio is post bronchodilator for subjects with COPD but not smokers or non-smokers.

Abbreviations: pred = predicted; M = male; F = female; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity.

Supplementary Figure legends:

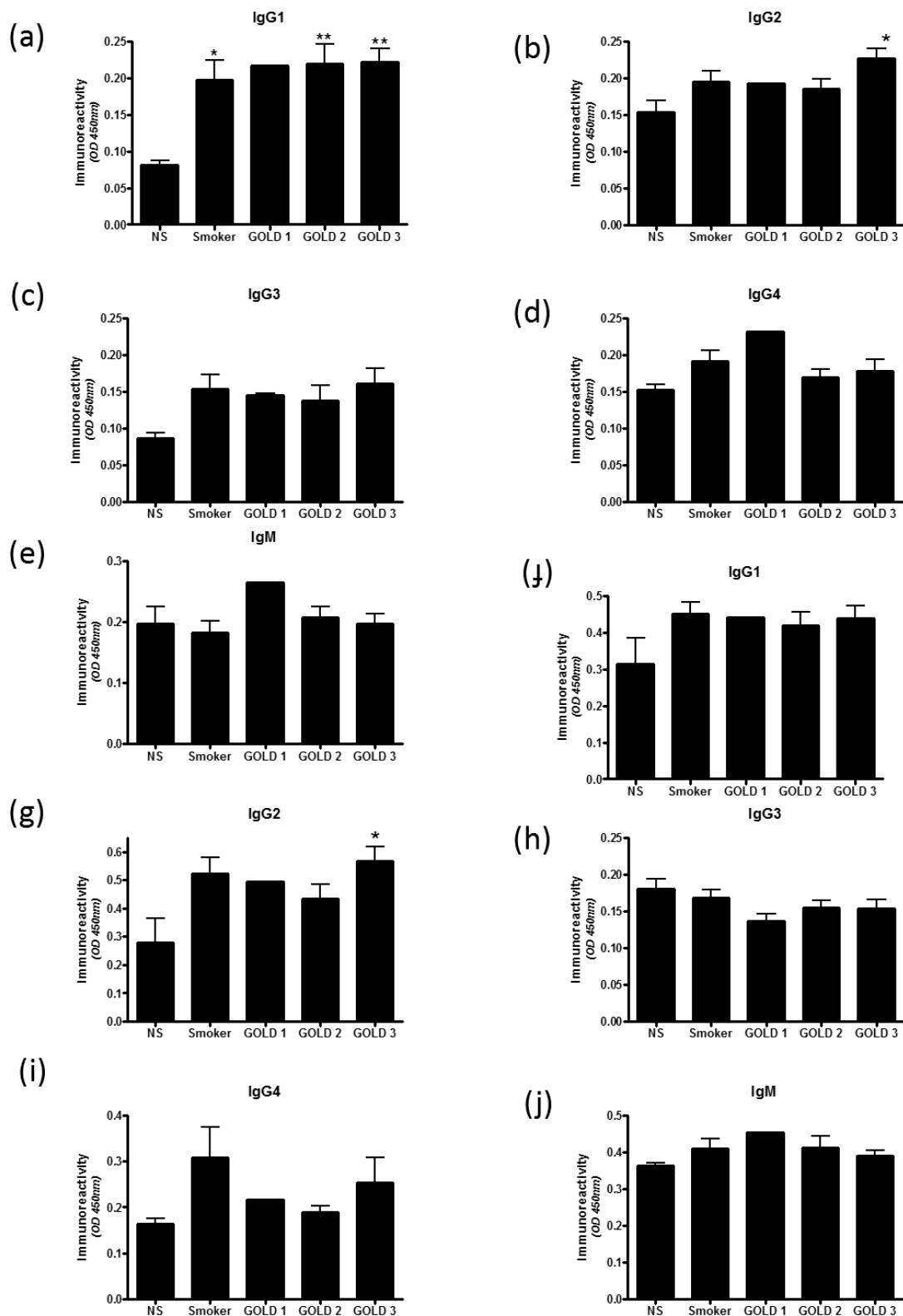
Figure 1. Isotype-specific immunoreactivity against carbonyl-modified and unmodified ‘self’ protein in serum from COPD patients. Serum from non-smokers (NS), smokers and GOLD 1, 2 and 3 COPD patients were assessed for isotype specific immunoreactivity; **(a, f)** IgG1, **(b, g)** IgG2, **(c, h)** IgG3, **(d, i)** IgG4 and **(e, j)** IgM, against malonyldialdehyde (MDA)-modified **(a-e)** versus unmodified human serum albumin **(f-j)** as a surrogate ‘self’ protein. All serum samples were tested at a 1:1000 dilution and detection of bound isotype specific antibodies were as described in materials & methods. IgG1 antibody responses were significantly increased against carbonyl-modified protein in COPD patients and smokers compared to non-smokers (NS). IgG2 antibody responses against both modified **(b)** and unmodified **(g)** HSA were significantly increased only in GOLD 3 COPD patients. Results are expressed as the mean \pm SEM for immunoreactivity in each patient group. Statistical analysis was performed using a non-parametric Kruskal-Wallis test with Dunn’s multiple comparison post-test analysis. $*p<0.05$, $**p<0.01$, compared to control non-smokers.

Figure 2. Carbonyl-modified protein preferentially activates splenocytes from mice chronically exposed to ozone. Splenocytes from air/chronic ozone exposed mice incubated with either MDA-modified or unmodified MSA were assessed by ELISA for Murine IL-2 secretion. Chronic ozone exposure results in a significant preferential increase of IL-2 secretion from ozone exposed splenocytes treated with MDA-modified MSA only. Results are expressed as the mean \pm SEM from 4 determinations of 6 mice in each treatment group. Filled bars are splenocytes from mice exposed to ozone for 6 weeks. Open bars from mice exposed to air only for 6 weeks. $^{\#}p<0.05$, compared to control unstimulated splenocytes from mice exposed to ozone. Statistical analysis was performed using a non-parametric Kruskal-Wallis test with Dunn’s multiple comparison post-test analysis. $*p<0.05$, $**p<0.01$, compared to MDA-MSA stimulated splenocytes from mice exposed to air.

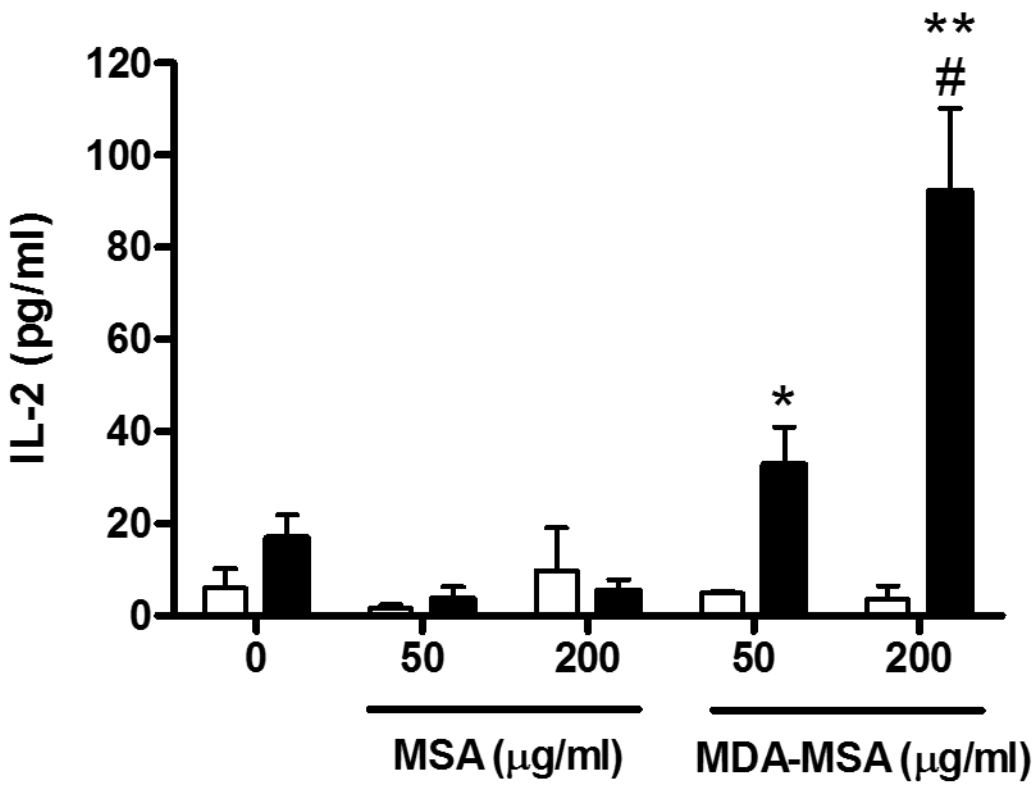
Figure 3. Cellular activation markers in lung antigen-presenting cells after ozone. CD11c⁺; F4/80⁺ macrophages (**a-f**) and CD11c⁺; F4/80⁻ dendritic cells (**g-l**) were purified from mice lungs that had either been exposed to air (**a-c, g-i**) or ozone (**d-e, j-l**) for 6 weeks. Purified cells were assessed for expression of activation markers CD80 (**a, d, g, j**), CD86 (**b, e, h, k**) and CD54 (**c, f, i, l**) by FACS. Ozone exposure causes increased expression in all three markers of antigen presenting cell activation in both macrophages and dendritic cells. FACS plots are representative of the experiment repeated 3 times with 6-8 mice in each treatment group (air versus ozone).

Figure 4. Cellular activation markers in dendritic cells from the draining lung lymph nodes of ozone-exposed mice. CD11c⁺; F4/80⁻ dendritic cells were purified from the draining lung lymph nodes of mice that had either been exposed to air (**a, c**) or ozone (**b, d**) for 6 weeks. Purified cells were assessed for expression of the activation markers CD80 (**a, b**) and CD86 (**c, d**) by FACS. Ozone exposure causes a large increase in expression for both CD80 and CD86 on dendritic cells isolated from the draining lung lymph nodes. FACS plots are representative of the experiment repeated 3 times with 6-8 mice in each treatment group (air versus ozone),

Supplemental data: Fig E1



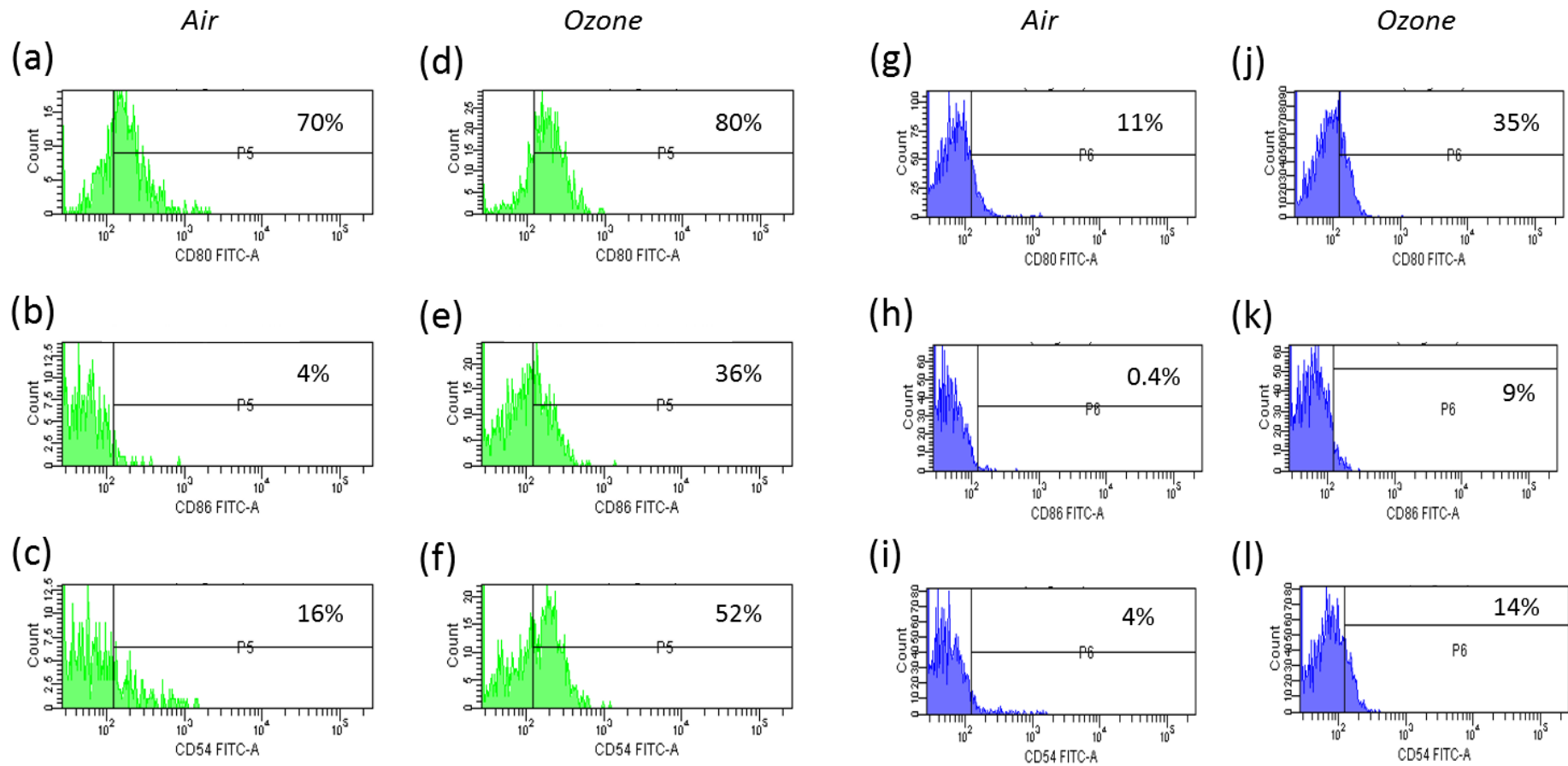
Supplemental data: Fig E2



Supplemental data: Fig E3

Macrophages (CD11c⁺; F4/80⁺)

Dendritic cells (CD11c⁺; F4/80⁻)



Supplemental data: Fig. E4

