Autoreactivity to Glucose Regulated Protein 78 Links Emphysema and Osteoporosis in Smokers

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

**Citation**

**Published Version**
doi:10.1371/journal.pone.0105066

**Accessed**
May 24, 2018 2:38:57 PM EDT

**Citable Link**
http://nrs.harvard.edu/urn-3:HUL.InstRepos:12987327

**Terms of Use**
This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

(Article begins on next page)
Autoreactivity to Glucose Regulated Protein 78 Links Emphysema and Osteoporosis in Smokers

Jessica Bon, Rehan Kahloon, Yingze Zhang, Jiamin Xue, Carl R. Fuhrman, Jiangning Tan, Mathew Burger, Daniel J. Kass, Eva Csizmadia, Leo Otterbein, Divay Chandra, Arpit Bhargava, Joseph M. Pilewski, G. David Roodman, Frank C. Sciurba, Steven R. Duncan

1 Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 2 Department of Radiology, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 3 Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, United States of America, 4 Department of Medicine, Indiana School of Medicine, Indianapolis, Indiana, United States of America

Abstract

Rationale: Emphysema and osteoporosis are epidemiologically associated diseases of cigarette smokers. The causal mechanism(s) linking these illnesses is unknown. We hypothesized autoimmune responses may be involved in both disorders.

Objectives: To discover an antigen-specific autoimmune response associated with both emphysema and osteoporosis among smokers.

Methods: Replicate nonbiased discovery assays indicated that autoimmunity to glucose regulated protein 78 (GRP78), an endoplasmic reticulum chaperone and cell surface signaling receptor, is present in many smokers. Subject assessments included spirometry, chest CT scans, dual x-ray absorptiometry, and immunoblots for anti-GRP78 IgG. Anti-GRP78 autoantibodies were isolated from patient plasma by affinity chromatography, leukocyte functions assessed by flow cytometry, and soluble metabolites and mediators measured by immunoassays.

Measurements and Main Results: Circulating anti-GRP78 IgG autoantibodies were detected in plasma specimens from 86 (32%) of the 265 smoking subjects. Anti-GRP78 autoantibodies were singularly prevalent among subjects with radiographic emphysema (OR 3.1, 95%CI 1.7–5.7, p = 0.003). Anti-GRP78 autoantibodies were also associated with osteoporosis (OR 4.7, 95%CI 1.7–13.3, p = 0.002), and increased circulating bone metabolites (p = 0.006). Among emphysematous subjects, GRP78 protein was an autoantigen of CD4 T-cells, stimulating lymphocyte proliferation (p = 0.002) and IFN-gamma production (p = 0.03). Patient-derived anti-GRP78 autoantibodies had avidities for osteoclasts and macrophages, and increased macropage NFkB phosphorylation (p = 0.005) and productions of IL-8, CCL-2, and MMP9 (p = 0.005, 0.007, 0.03, respectively).

Conclusions: Humoral and cellular GRP78 autoimmune responses in smokers have numerous biologically-relevant pro-inflammatory and other deleterious actions, and are associated with emphysema and osteoporosis. These findings may have relevance for the pathogenesis of smoking-associated diseases, and development of biomarker immunoassays and/or novel treatments for these disorders.

Introduction

Emphysema, defined as radiologic and/or histological evidence of lung parenchymal destruction, accounts for enormous worldwide morbidity and mortality [1]. Emphysema among tobacco smokers often occurs in association with chronic obstructive pulmonary disease (COPD), a complex syndrome typified by airway narrowing and inflammation, and diagnosed by the presence of expiratory airflow obstruction on spirometric testing [1–4]. Nonetheless, emphysema and COPD are by no means invariably concordant, and many patients severely afflicted by one of these lung abnormalities may have little or no evidence of the other [2–4]. In addition to directly attributable disability and premature deaths, smoking-associated lung diseases are also linked to many systemic abnormalities, including vasculopathies [5], lung cancer [6], renal dysfunction [7], and osteoporosis [8]. The abnormal and often pathological bone demineralization that occurs in smokers is particularly notable for being highly related to...
the presence and severity of emphysema, and is independent of the co-existence or magnitude of COPD per se [8].

While tobacco smoking is the single greatest risk factor for the development of lung disease in industrialized societies, additional mechanisms are also important, since only a fraction of heavy smokers have severe clinical manifestations [1]. Moreover, symptomatic, pathologic, and radiographic features are highly variable among afflicted individuals, familial clustering of cases is evident, and the disorders often progress despite smoking cessation [1,4,9,10]. The pathophysiological processes that may cause or promote emphysema and its co-morbidities remain enigmatic, although systemic immunological responses, including the actions of activated monocyte-lineage phagocytes, have been implicated in these disorders [11,12].

Adaptive immune responses against a variety of autoantigens appear to be common among patients with lung disease attributable to smoking [13–15]. Unspecified autoantibodies from smokers have been shown to induce pulmonary epithelial cell cytotoxicity in vitro [13]. Immune complex and complement deposition are important mediators of autoantibody-induced tissue injury, and these abnormalities are also prevalent in the diseased lungs of smokers [13,14]. Nonetheless, most reports of autoimmunity in patients with smoking-related lung disease to date are correlative rather than mechanistic. To our knowledge, moreover, no antigen-specific immune response has yet been implicated in the systemic co-morbidities of these pulmonary disorders [5–8].

Given the systemic nature of many immunological processes [16–19], we hypothesized autoimmune responses that could contribute to lung parenchyma destruction may also be involved in pathogenesis of one or more extrapulmonary co-morbidities that are associated with the emphysema. Accordingly, we conducted investigations to identify antigen-specific autoimmune responses relevant to both emphysema and osteoporosis in smoke-exposed subjects.

Methods

Subjects

Clinical and immunological correlation studies were performed in consecutive subjects with ≥10 pack-years of cigarette smoking, ages 40–79 years old, who were recruited under auspices of the Specialized Center for Clinically Oriented Research (SCCOR) registry at the University of Pittsburgh Medical Center. Subjects with unstable cardiovascular disease, malignancies, chronic oral steroid use, or BMI >35 were excluded. Each subject completed demographic and medical history questionnaires, and chest CT scans [6–8,13]. Spirometry, lung volumes, and diffusion capacity (DLCO) were measured using standardized methods and reference equations. Healthy, never-smoked control subjects were recruited by solicitation of hospital personnel.

Ethics Statement

The protocol was approved by the University of Pittsburgh Investigational Review Board. Each subject provided written informed consent.

Emphysema Assessments

Chest CT examinations were performed with a General Electric (GE) LightSpeed VCT (64-detector) scanner at a radiation exposure of 100 mAs [6,8]. A single expert radiologist, blinded to subject identities and other characteristics, interpreted the CT images using a validated [6]. 6-point semi-quantitative visual scoring system to define emphysema severity (0 = none, 1 = trace/

minimal, 2 = mild, 3 = moderate, 4 = severe, 5 = very severe), corresponding to 0%, <10%, 10–25%, 26–50%, 51–75%, and >75% visual emphysema [6–8]. Our group has previously shown these visual emphysema scores are associated with clinically important outcomes in smokers [6–8], and the validity and comparability of these assessments with other radiographic measures of emphysema has also been established [2,3].

Blood Specimens

Plasma was obtained by centrifugation of heparinized phlebotomy specimens. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation [20]. Serum was obtained as the supernatant of clotted (non-heparinized) blood.

Dual X-Ray Absorptiometry (DXA)

DXA measurements of bone mineral density (BMD) at the hip and lumbar spine were added to subject assessments after the initiation of enrollments in the SCCOR, and were available in the last 200 smoking subjects who provided plasma for immunological studies. Bone mineral density (BMD) was measured at the hip and lumbar spine using a Hologic 4500A Discovery bone densitometer and previously detailed methods [8]. BMD is reported as a T score, the number of standard deviations from young, gender and ethnic-specific reference means. T scores ≤−2.5 defined osteoporosis [8].

Macrophages and Osteoclasts

CD14+ cells were isolated from PBMC of normal, nonsmoking humans using anti-CD14 immunomagnetic beads (Miltenyi Biotec, Auburn, CA), and differentiated in the presence of M-CSF. In brief, cells were cultured for 7–10 days in complete RPMI with 10% FCS that was also supplemented with 50 ng/ml M-CSF (R&D Systems, Minneapolis, MN), and changed every 2–3 days. Flow cytometry confirmed >98% of the cells harvested ≥day seven expressed intracellular CD68 and were CD3− and CD19−. Osteoclasts were derived from two healthy bone marrow donor volunteers. Mononuclear cells within the bone marrow aspirates were isolated by density gradient centrifugation and incubated in α-MEM with 20% FCS overnight at 37°C. Non-adherent cells were collected and cultured for 28 days in the same media supplemented with 25 ng/ml M-CSF and 30 ng/ml RANKL (R&D Systems), with frequent replenishment.

Autoantigen Discovery

The unbiased discovery assays that resulted in identification of autoreactivity to glucose regulated protein 78 (GRP78) among smokers with lung disease parallel those reported previously [21]. In brief, we hypothesized a priori that autoantigens associated with smoking-related emphysema could be identified by using patient-derived IgG antibodies to immunoprecipitate these autoantigens from cell lysates. The biologic validity of the putative autoimmunity could then be substantiated by demonstrating correlations between the presence of humoral autoreactivity to these self-proteins and disease prevalences. Additional evidence could be provided by finding concurrent T-cell autoreactivity, HLA bias, and discovering disease-relevant functional effects of the autoantibody(ies) [13–21].

Pooled circulating IgG antibodies isolated from six emphysema subjects, known to have autoantibodies on previous study [13], were used to immunoprecipitate autoantigens from cell lysates. IgG from these subjects and another preparation from six normal control specimens were isolated by protein G, and then adhered to and covalently cross-linked to protein A columns (HP
SpinTrap, GE Healthcare, Piscataway, New Jersey), per the manufacturer’s protocol.

The cell lysates were preadsorbed with the normal IgG-protein A columns, and then applied to the emphysema IgG-protein A columns. After extensive washing, the putative IgG-bound autoantigens were eluted by acidification, pH neutralized, concentrated by centrifugal size-filtration (Millipore, Bellerica, MA), and identified by two dimension 10.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were imaged by Typhoon Trio (GE Healthcare) and analyzed by Image QuantiTL software (GE Healthcare). Individual proteins were harvested by spot picking (Eitan Spot Picker, GE Healthcare), trypsinated, and sequenced by matrix-assisted laser desorption/ionization tandem time of flight mass spectrometry (MALDI-TOF/TOF) (Applied Biosystems, Carlsbad, CA).

Unpublished findings of previous investigations [13] had indicated the presence of an autoantibody with specificity for a then cryptic ~75 kDa cell antigen tended to be associated with disease manifestations among smokers, and hence discovery of potential autoantigens of this ~size was a particular interest.

Glucose regulated protein 78 (GRP78), a member of the heat shock protein 70 family, was identified in two sequential discovery assays. In addition to having an appropriate size, GRP78 seemed worthy of focus for additional study as a potential autoantigen in smokers given its myriad cellular functions [22–24] and role as an known autoantigen in other immunologic disorders [25,26].

Circulating Anti-GRP78 IgG

Immunoblots are a highly specific (“Gold Standard”) method for detection of antibodies [21]. These assays were performed using modifications of previously described methods [21].

In brief, recombinant GRP78 (rGRP78) was purchased from Prospec (Rehovot, Israel). rGRP78 was prepared as a bulk solution using modifications of previously described methods. [21]

Indirect Immunofluorescence Assays (IFA)

IgG from pooled plasma specimens of six emphysema patients known to have anti-GRP78 autoantibodies (by prior immunoblot assays) was isolated by adherence to protein G. The IgG was applied to rGRP cross-linked to an AminoLink Plus Gel Spin column (Thermo Scientific), following manufacturer protocols. After extensive washing, anti-GRP78 was eluted by acidification, pH neutralized, concentrated using 5 kDa centrifugation filters and validated for IgG characteristics and specific avidity (Figure S3 in File S1). Eleven (11) ml plasma yielded 120 mcg of anti-GRP78 IgG. The endotoxin concentration within this autoantibody preparation was below the detection threshold of the limulus amebocyte lysate assay (LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific), i.e., <0.1 EU/2 mcg of autoantibody.

Indirect Immunofluorescence Assays (IFA)

Plate-bound normal alveolar macrophages from BALF (n = 3) and bone marrow osteoclasts (n = 2) were pre-incubated with 0.1 bovine serum albumin and azide-free Fc receptor blocker ( Innovex Biosciences, Richmond, CA). After washing, the cells were incubated for 30 minutes with 0.5 mcg/ml of patient-derived anti-GRP78 IgG or the same concentration of pooled normal human IgG. After another washing, cells were incubated with FITC-conjugated anti-human IgG antibody (ImmuNoConcepts, Sacramento, CA) and assessed by fluorescence microscopy, as detailed previously [13,21].
Macrophage Stimulation Assays

Media was removed from concurrent, autologous macrophage cultures, and the cells were incubated with 2 mcg/ml of either anti-GRP78 or normal human IgG for 30 minutes at 37°C. Media was then replaced, and both cells and supernatant were harvested after 18 hours.

Macrophages were suspended, fixed and permeabilized, and stained with anti-NFκB Alexa Fluor 647 (Cell Signaling Technologies, Danvers, MA), which has specificity for phosphorylated Ser536 NFκB p65 subunits. Mean fluorescence intensities of the anti-GRP78-treated and normal IgG-treated macrophages were compared in >10,000 live cells, and analyzed using a BD FACSCalibur (BD Bioscience, San Jose, CA). Flow cytometry gates were established using control fluorochrome positive and negative macrophages (including isotype controls), as detailed elsewhere [20,21].

Macrophage supernatants were analyzed for mediator production using protein-suspended bead platform multiplex kits (Bio-Rad, Hercules, CA), following the manufacturer’s protocols [21].

Circulating Anti-GRP78 Antibodies

The prevalence of autoantibodies to GRP78 was greater in smokers (32.2%) than in the healthy, never-smoked controls (7.4%) (p = 0.007). The latter prevalence is similar to “false positive” rates of other disease-associated autoantibodies in healthy populations [13,16,20,21].

Clinical Associations of Anti-GRP78 Antibodies

There were no significant differences of anti-GRP78 autoantibody prevalences in the smokers with COPD (35.9%) compared to those with normal spirometry (26.5%, p = 0.11), nor a significant correlation of this autoreactivity with the severity of airflow obstruction (Figure 1A).

The presence of anti-GRP78 autoantibodies was significantly associated with the prevalence (Figure 1B) and severity of emphysema (Figure 1C). Multivariate analyses showed that adjustment for gender and severity of expiratory airflow obstruction did not alter the relationship between anti-GRP78 positivity and emphysema (OR 3.1, 95%CI = 1.6–6.1, p = 0.001).

The presence of anti-GRP78 autoantibodies was also highly associated with decreased bone mineral density and osteoporosis (Figure 2A), and remained significant after multivariate adjustment for gender, airflow obstruction severity, tobacco burden, and steroid use (OR 4.2, 95%CI = 1.5–12.2, p = 0.008). Circulating metabolites of bone turnover were also greatest in the subjects with anti-GRP78 autoantibodies (Figures 2B,2C).

Given the associations of anti-GRP78 autoreactivity with emphysema and abnormalities of bone mineralization, a post hoc analysis was performed to compare autoantibody prevalence in those subjects who have concurrent emphysema and low BMD vs. the remaining smoking cohort. Anti-GRP78 autoantibody
prevalence was significantly greater in the subjects who had both emphysema and osteopenia (50.7% vs. 25%, OR 3.1, 95%CI = 1.8–5.4, p < 0.0001), and even more so among those with both emphysema and osteoporosis (80.0% vs. 29.6%, OR 9.5, 95%CI = 2.6–34.7, p < 0.0001). A gender stratified analysis showed this relationship is strongest in males (Figure 2D).

**Intrapulmonary GRP78 Expression**

GRP78 protein was increased in emphysema specimens and predominantly localized in alveolar epithelial cells and macrophages (Figure 3A). GRP78 concentrations were also much greater in BALF from diseased lungs compared to the normal control preparations (Figure 3B).

**Anti-GRP78 Binding**

In order to explore the possibility that anti-GRP78 autoantibodies have actions relevant to emphysema and osteoporosis, we first examined macrophage and osteoclast binding of anti-GRP78 IgG isolated from patient plasma. IFA showed the anti-GRP78 autoantibodies have avidity for these cells (Figure 3C).

**Cellular Effects of Anti-GRP78 Autoantibodies**

To test for direct function-altering effects, patient-derived anti-GRP78 autoantibodies were incubated overnight with macrophages. These treatments resulted in increased macrophage NFkB activation (Figure 4A) and augmented productions of IL-8 (Figure 4B), CCL-2 (Figure 4C), and MMP9 (Figure 4D).

Table 1. Demographic and Clinical Characteristics of the Study Cohort.

<table>
<thead>
<tr>
<th>Aggregate</th>
<th>GRP78 Ab⁻</th>
<th>GRP78 Ab⁺</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>265</td>
<td>179</td>
<td>86</td>
</tr>
<tr>
<td>Age (years old)*</td>
<td>67±0.4</td>
<td>67±0.5</td>
<td>67±0.7</td>
</tr>
<tr>
<td>% male</td>
<td>58</td>
<td>63</td>
<td>47</td>
</tr>
<tr>
<td>Still smoking (%)</td>
<td>42</td>
<td>41</td>
<td>43</td>
</tr>
<tr>
<td>Pack-years*</td>
<td>63±2</td>
<td>65±2</td>
<td>59±3</td>
</tr>
<tr>
<td>ICS (%)</td>
<td>22</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>Past oral steroids (%)</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>FEV₁,% predicted*</td>
<td>77±1</td>
<td>78±2</td>
<td>74±3</td>
</tr>
<tr>
<td>FEV₁/FVC*</td>
<td>0.63±0.01</td>
<td>0.64±0.1</td>
<td>0.60±0.2</td>
</tr>
<tr>
<td>DLCO% predicted*</td>
<td>68±2</td>
<td>71±2</td>
<td>64±2</td>
</tr>
<tr>
<td>Obstructed (%)</td>
<td>63</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>Emphysema (%)</td>
<td>66</td>
<td>59</td>
<td>81</td>
</tr>
<tr>
<td>Low Bone Density (%)</td>
<td>51</td>
<td>46</td>
<td>62</td>
</tr>
<tr>
<td>Osteoporosis (%)</td>
<td>9</td>
<td>4</td>
<td>18</td>
</tr>
</tbody>
</table>

All these subjects had ≥10 pack yr smoking histories. ICS = inhaled corticosteroid use; past oral steroids = oral steroid use within the preceding six months (none were currently taking oral steroids); FEV₁,% predicted = forced expiratory volume in the first second of expiration as percentages of predicted values; FEV₁/FVC = ratio of FEV₁ to forced vital capacity; DLCO% predicted = diffusing capacity for carbon monoxide as percentages of predicted values; FEV₁/FVC = ratio of FEV₁ to forced vital capacity; DLCO% predicted = diffusing capacity for carbon monoxide as percentages of predicted values; FEV₁/FVC = ratio of FEV₁ to forced vital capacity; DLCO% predicted = diffusing capacity for carbon monoxide as percentages of predicted values; FEV₁/FVC = ratio of FEV₁ to forced vital capacity; DLCO% predicted = diffusing capacity for carbon monoxide as percentages of predicted values; FEV₁/FVC = ratio of FEV₁ to forced vital capacity; DLCO% predicted = diffusing capacity for carbon monoxide as percentages of predicted values; FEV₁/FVC = ratio of FEV₁ to forced vital capacity; DLCO% predicted = diffusing capacity for carbon monoxide as percentages of predicted values.

**Figure 1. Anti-GRP78 autoantibodies, airflow obstruction, and emphysema.**

A) Anti-GRP78 autoantibody prevalence was not significantly associated with COPD severity (GOLD stages) [40]. Numbers within columns denote subject n. B) Anti-GRP78 autoantibodies are significantly associated with emphysema prevalence (%) in the smoking subjects. OR = odds ratio; CI = confidence interval. C) Emphysema scores per CT were also significantly greater among those subjects with anti-GRP78 autoantibodies.

doi:10.1371/journal.pone.0105066.t001

doi:10.1371/journal.pone.0105066.g001
CD4 T-cell Autoreactivity

Disease-associated autoantibody responses are also accompanied by concurrent T-cell responses to the autoantigen(s) [15,16,20,21]. Addition of heat-denatured rGRP78 protein to PBMC cultures from smokers induced proliferations of their CD4 T-cells, unlike effects of denatured ESP (Figure 5A). Moreover, the magnitude of the GRP78-triggered proliferative responses was greatest among the CD4 T-cells from emphysematous smokers (Figure 5B). CD4 T-cell IFN-gamma production was also uniquely increased in rGRP78-supplemented cultures (Figure 5C) and, again, especially so in the preparations from subjects with emphysema (Figure 5D). rGRP78 does not induce proliferation or IFN-gamma production in CD4 T-cells from normal nonsmoking subjects (data not shown) nor IPF patients [21].

Discussion

Autoimmunity is a frequent complication of numerous, varied, primary injury responses [15–18]. The microbiome within smoking-damaged lungs is postulated to be highly immunogenic and, hence, a likely predisposition for the development of autoimmunity [13–15]. Neoantigens generated by reactive constituents within tobacco smoke can also provoke autoimmune responses [14]. Most “secondary” autoimmune responses are benign and clinically irrelevant. In some cases they are highly pathogenic and injurious, however, as exemplified by carditis associated with microbial...
infections, neurological syndromes linked to malignancies, and myriad other tissue-specific autoimmune disorders [16–18,26].

The adaptive immune responses to GRP78 in smokers have several features of classical autoimmunity [15–20]. The biological plausibility of a particular autoimmune response is conditional on the presence of the corresponding autoantigen in the target organ(s), and GRP78 is highly expressed in lungs, especially in the disease specimens (Figures 3A,3B). HLA haplotypes are major (and often the strongest) known genetic determinants of autoimmune susceptibilities. Finding distinct HLA alleles are over- or under-represented (conferring predilection or protection, respectively) within a defined disease population is a hallmark of autoimmune disorders [16,21]. DRB1*15 appears to “protect” smokers against the production of disease-associated anti-GRP78 autoantibodies. This result is the obverse of analogous studies in IPF patients, in whom DRB1*15 is over-represented among those with autoantibodies against heat shock protein 70 (HSP70), a stress response protein with considerable sequence homology to GRP78 [21]. These disparate findings are thus indicative of biologically-distinct, antigen- and disease-specific autoimmune responses, rather than being a generalized epiphenomenon of “lung disease”.

Most compellingly, the increased prevalence of anti-GRP78 autoantibodies in emphysematous smokers is a defining criteria of “abnormal” autoreactivity [15–20]. The pathogenicity of anti-GRP78 IgG in smokers is at least implied by the stringent, independent, and overlapping associations of this specific autoantibody with concurrent emphysema, osteoporosis, and increased bone turnover. Furthermore, other findings here showing that patient-derived anti-GRP78 autoantibodies activate monocyte-lineage phagocytes, and enhance their productions of injurious mediators that are implicated in the genesis of emphysema and osteoporosis are direct evidences of deleterious autoantibody effects [11,12,27–29].

In particular, anti-GRP78 IgG treatments increased cellular elaborations of IL-8 and CCL2 (aka MCP-1), which are potent pro-inflammatory chemoattractants of neutrophils and monocytes/macrophages, respectively. Both of these mediators also stimulate osteoclastogenesis, and are increased among patients with osteoporosis and emphysema [11,12]. Incubations with anti-GRP78 also increased macrophage production of MMP9, a Type IV collagenase produced by macrophages and osteoclasts that promotes enzymatic breakdown of extracellular matrix. MMP9 has an imputed causal or contributing role in lung parenchyma destruction, pulmonary metastases, and bone resorption [27–29].

Moreover, these varied effects occurred after only limited (18 hour) macrophage exposures to subphysiological concentrations (2 μg/ml) of the anti-GRP78 autoantibody. It seems possible that even greater effects might result from incubations with autoantibody concentrations found in vivo (~11 μg/ml), and for longer periods. The cumulative effects of increased inflammation and protease activity in target tissues over many months-to-years may be especially deleterious, and emphysema and osteoporosis

![Figure 4. Cellular effects of autoantibodies to GRP78 on macrophages.](doi:10.1371/journal.pone.0105066.g004)
Antigen- (or autoantigen-) stimulated CD4 T-cells have been identified as a "Gold Standard" of autoimmune disease [16]. The specific mechanism(s) by which anti-GRP78 autoantibodies affect phagocytes is yet unknown, and is a focus of ongoing investigations in our laboratories. In addition to intracellular actions as an endoplasmic reticulum protein transporter and scavenger, and importance in the unfolded protein response, cell surface and extracellular GRP78 mediate anti-inflammatory and pro-resolatory effects [22-24]. Anti-GRP78 might counter these actions by decreasing the concentrations or bioavailability of immunomodulatory extracellular GRP78, and/or by activating signal transduction pathways after cross-linking cell surface GRP78. Anti-GRP78 autoreactivity is notably prevalent among smokers who are most at-risk for these interrelated disorders. Nonetheless, these findings need additional corroboration and refinement before the adoption of autoimmune assays into the clinical management of smoking-associated lung disease [13–15]. The present studies show anti-GRP78 autoreactivity is notably prevalent among smoking patients.

The in situ pathogenicity of GRP78 autoimmunity in smokers is also strongly supported by finding this stress response protein is an autoantigen of CD4 T-cells in these subjects, especially among those with emphysema. T-cells are inert to anatomically accessible autoantigen of CD4 T-cells in these subjects, especially among smokers [1]. The specific mechanism(s) by which anti-GRP78 autoantibodies affect phagocytes is yet unknown, and is a focus of ongoing investigations in our laboratories. In addition to intracellular actions as an endoplasmic reticulum protein transporter and scavenger, and importance in the unfolded protein response, cell surface and extracellular GRP78 mediate anti-inflammatory and pro-resolatory effects [22-24]. Anti-GRP78 might counter these actions by decreasing the concentrations or bioavailability of immunomodulatory extracellular GRP78, and/or by activating signal transduction pathways after cross-linking cell surface GRP78. Anti-GRP78 autoreactivity is notably prevalent among smokers who are most at-risk for these interrelated disorders. Nonetheless, these findings need additional corroboration and refinement before the adoption of autoimmune assays into the clinical management of smoking patients.

In addition, many immune diseases, and antibody-mediated lung disorders in particular, are refractory to treatment with nonspecific immunosuppressive regimens (e.g., glucocorticoids). Conversely, however, specific therapies that directly target pathological autoimmune processes, including autoantibody removal or interruption of autoantibody production per se, more often have clinical efficacy [34–39]. The most important ultimate result of the present study and related reports may be to draw attention to the potential for novel treatments, mechanistically-focused at critical stages of autoimmune cascades, to prevent or reduce damage from autoimmunity.

**Figure 5.** CD4 T-cell autoreactivity to GRP78. A) Addition of GRP78 to PBMC cultures increased CD4 T-cell proliferation (BrdU uptake) relative to media controls (no added protein) (n = 47), unlike addition of elastin split products (ESP). P, denotes alpha level corrected for multiple comparisons. B) GRP78-induced CD4 T-cell proliferation was greatest among cultures from smokers with emphysema (n = 34). Specific indices (SI) of proliferation were calculated as %CD4 T-cells incorporating BrdU in GRP78-supplemented cultures minus incorporation in concurrent media controls. C) GRP78 also increased percentages of CD4 T-cells that produced IFN-gamma again, unlike effects of ESP. D) IFN-gamma production was also greatest in the CD4 T-cells from the emphysematous smokers. Specific indices (SI) were calculated as %CD4 T-cells producing IFN-gamma production in GRP78-supplemented cultures minus that of concurrent controls.

doi:10.1371/journal.pone.0105066.g005

**GRP78 Autoreactivity in Smokers**

Protein and typically very injurious actions, including elaboration of numerous mediators that recruit and activate diverse leukocyte and somatic effector cells [21]. A finding of T-cell autoreactivity is very unlikely to be a benign epiphenomenon [16,19]. Increased production of IFN-gamma in particular (Figures 5C,5D) is believed to be an important factor in the pathogenesis of smoking-associated lung disease [11]. Furthermore, the GRP78 reactivity of CD4 T-cells in emphysematous subjects cannot be simply attributable to global, nonspecific hyperactivity, since elastin split products (ESP) were inert (Figures 5A and 5C). Although ESP was previously reported to be an autoantigen of emphysema [30], we have been unable to replicate those findings, and similarly negative studies have been reported by other investigators [31–33].

GRP78 is also a frequent antigen of other autoimmune syndromes [25,26]. GRP78 expression and extracellular export are up-regulated by varied stresses, including viral infections and smoke inhalation [22–24]. Ongoing immunologic responses that were initially directed at other antigenic peptides may generalize to co-associated chaperone proteins, such as GRP78, by a bystander mechanism and/or by epitope spread [23]. Subsequent injury (or infection) could cause further up-regulation of the newly antigenic GRP78, potentially creating a positive feedback loop that promotes immune responses and disease progression.

The findings of GRP78 autoreactivity here lend support to an evolving paradigm of autoimmune pathogenesis in smoking-associated lung diseases [13–15]. The present studies show anti-GRP78 responses in smokers are manifest by activation of autoantigen-specific T-cells, a process that leads to numerous deleterious consequences [19], and anti-GRP78 IgG autoantibodies directly exert pro-inflammatory effects. Even if these pathogenic actions are completely discounted, the multiple strong clinical-immunological associations here indicate assays for specific autoimmune responses may be useful to identify smokers who are most at-risk for these interrelated disorders. Nonetheless, these findings need additional corroboration and refinement before the adoption of autoimmune assays into the clinical management of smoking patients.
methodological details. Left panel: 75 kDa molecular weight marker (MW) and adjacent plasma specimen negative for anti-GRP78 (Lane A). Right panel: 75 kDa molecular weight marker (MW) and subject plasma specimen positive for anti-GRP78 IgG (Lane B). Note: GRP78 migrates on 12% Bis–Tris gels as though it were slightly smaller than its expected 78 kDa size. Figure S2, Detection of GRP78 Protein in Lung Specimens. Control immunoblots. Lane A.) rGRP78 detected with mouse anti-human GRP78 monoclonal antibody (R and D Systems) at 1:500 dilution. The secondary antibody was chicken anti-mouse IgG at 1:4000 dilution. Lane B.) GRP78 in a human CD14^+ derived macrophage lysate (29 μg total protein) was also detected using this anti-human GRP78 monoclonal antibody. MW.) 75 kDa molecular weight marker. Figure S3, Patient-Derived Anti-GRP78 Autoantibody Characterizations. Validations of patient-derived anti-GRP78 autoantibodies. Left panel: Evaluations of the patient-derived anti-GRP78 on SDS gels showed protein bands typical for IgG, i.e., 23 kDa light chains and 50 kDa heavy chains. Right panel: The avidity of the isolated, patient-derived autoantibody for GRP78 was confirmed by rGRP78 immunoblot. Lane A.) 75 kDa molecular weight marker; Lane B.) rGRP78. The patient-derived anti-GRP78 IgG was used here at a concentration of 1 μg/ml. (DOCX)

Author Contributions
Conceived and designed the experiments: JB SRD. Performed the experiments: RK YZ JX CF EC LO AB GDR SRD JT MB DJK. Analyzed the data: JB DC SR. Contributed reagents/materials/analysis tools: JP FS DJK. Wrote the paper: JB SRD. Edited the manuscript: JB DC FS RK YZ JX CF EC LO AB GDR SRD JT MB DJK.

References