Deregulated Stat3 signaling dissociates pulmonary inflammation from emphysema in gp130 mutant mice

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1Centre for Inmate Immunity and Infectious Diseases, Monash Institute of Medical Research, Monash University, Clayton; 2Departments of Medicine and Pharmacology, University of Melbourne, Parkville; 3Department of Biochemistry and Molecular Biology, Monash Micro Imaging, School of Biomedical Sciences, Monash University; and Departments of 4Pathology and 5Respiratory and Sleep Medicine, Monash Medical Centre, Clayton, Victoria, Australia

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Ruwanpura SM, McLeod L, Miller A, Jones J, Vlahos R, Ramm G, Longano A, Bardin PG, Bozinovski S, Anderson GP, Jenkins BJ. Deregulated Stat3 signaling dissociates pulmonary inflammation from emphysema in gp130 mutant mice. Am J Physiol Lung Cell Mol Physiol 302: L627–L639, 2012. First published January 20, 2012; doi:10.1152/ajplung.00285.2011.—Interleukin (IL)-6 is a potent immunomodulatory cytokine that is associated with emphysema, a major component of chronic obstructive pulmonary disease (COPD). IL-6 signaling via the gp130 coreceptor is coupled to multiple signaling pathways, especially the latent transcription factor signal transducer and activator of transcription (Stat)3. However, the pathological role of endogenous gp130-dependent Stat3 activation in emphysema is ill defined. To elucidate the role of the IL-6/gp130/Stat3 signaling axis in the cellular and molecular pathogenesis of emphysema, we employed a genetic complementation strategy using emphysematous gp130−/− mice displaying hyperactivation of endogenous Stat3 that were interbred with mice to impede Stat3 activity. Resected human lung tissue from patients with COPD and COPD-free individuals was also evaluated by immunohistochemistry. Genetic reduction of Stat3 hyperactivity in gp130−/−;Stat3+/−/mice prevented lung inflammation and excessive pro tease activity; however, emphysema still developed. In support of these findings, Stat3 activation levels in human lung tissue correlated with the extent of pulmonary inflammation but not airflow obstruction in COPD. Furthermore, COPD lung tissue displayed increased levels of IL-6 and apoptotic alveolar cells, supporting our previous observation that increased endogenous IL-6 expression in the lungs of gp130−/− mice contributes to emphysema by promoting alveolar cell apoptosis. Collectively, our data suggest that IL-6 promotes emphysema via upregulation of Stat3-independent apoptosis, whereas IL-6 induction of lung inflammation occurs via Stat3. We propose that while discrete targeting of Stat3 may alleviate pulmonary inflammation, global targeting of IL-6 potentially represents a therapeutically advantageous approach to combat COPD phenotypes where emphysema predominates.

cytokines; interleukin-6; chronic obstructive pulmonary disease; apoptosis; signal transducer and activator of transcription 1

EMPHYSEMA IS A MAJOR COMPONENT OF THE DEBILITATING DISORDER CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD), THE FIFTH MOST COMMON CAUSE OF DEATH WORLDWIDE (3). WHILE CHRONIC PULMONARY INFLAMMATION IS A TRAIT OF COPD (3), IT HAS BEEN LINKED TO THE PATHOGENESIS OF EMPHYSEMA, IT HAS EMERGED THAT THE DESTRUCTION OF LUNG PARENCHYMA IN EMPHYSEMA IS A CONSEQUENCE OF COMPLEX INTERACTIONS, ALBEIT ILL DEFINED, AT THE MOLECULAR AND CELLULAR LEVELS AMONG INFLAMMATION, EXCESSIVE APOPTOSIS, PROTEASE ACTIVITY, AND OXIDATIVE STRESS IN THE LUNG (3, 8, 30, 38).

IL-6 is a potent immunomodulatory cytokine that is associated with COPD and systemic inflammation (14, 24, 38), and its involvement in the pathogenesis of emphysema has been suggested from clinical studies demonstrating IL-6 gene polymorphisms (14) and elevated production of IL-6 in emphysema patients (5, 42). In addition, the artificially forced lung-specific overexpression of IL-6 in transgenic mice causes alveolar airspace enlargement (23). The biological actions of IL-6 are triggered by its binding to the specific IL-6 receptor α-subunit (IL-6R), which in turn associates with the signaling receptor β-subunit gp130. Ligand engagement of gp130 then leads to activation of the gp130-associated Janus kinases (JAKs), which in turn activate (by tyrosine phosphorylation) the latent transcription factors signal transducer and activator of transcription (Stat)3 and to a lesser extent Stat1, as well as the Shp2-mitogen-activated protein kinase (MAPK) and Shp2-phosphoinositol 3-kinase (PI3K)-Akt pathways (15). Although the pathological role of these gp130 signaling events in emphysema is poorly understood, considerable attention has focused on Stat3, albeit with conflicting results. For instance, transgenic mice overexpressing a tyrosine (Y) nonphosphorylatable Stat3 mutant in the respiratory epithelium develop emphysema (47), and elevated IL-6 expression and Stat3 activation as a consequence of targeted matrix metalloproteinase (MMP)-12 overexpression in the mouse lung correlates with pulmonary inflammation and emphysema (36). While these findings suggest that Stat3 can contribute to the onset of emphysema, conversely, transgenic overexpression of a hyperactive Stat3-C mutant in the respiratory epithelium leads to spontaneous pulmonary inflammation and lung adenocarcinoma without any emphysema (26), and conditional deletion of Stat3 in the respiratory epithelium fails to induce any lung pathology under steady-state conditions (22). Accordingly, there is a need to better define the involvement of the endogenous IL-6/gp130/Stat3 signaling axis in its native environment in lung disease.

We (38) have recently demonstrated that gp130F/F mice homozygous for a phenylalanine (F) knockin substitution of Y757 in gp130 spontaneously develop pulmonary emphysema associated with excessive apoptosis, protease activity, and inflammation in an IL-6-dependent manner. At the molecular level, the gp130Y757F mutation abolishes binding of the gp130 signaling negative regulator suppressor of cytokine signaling (Socs)3, resulting in hyperactivation of endogenous Stat3 and, to a lesser extent, Stat1 (44). Here we report in gp130F/F mice

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that hyperactivation of the endogenous gp130/Stat3 signaling axis in the lungs is dispensable for the development of emphysema. Rather, hyperactivation of Stat3 via gp130 primarily promoted B- and T-cell pulmonary inflammation. We also observed a minor role for gp130/Stat1 signaling in contributing to the infiltration of T cells in the lungs of gp130FF mice. Importantly, in human lung tissue Stat3 activation levels also correlated with the extent of pulmonary inflammation but not airflow obstruction in COPD. Furthermore, in patients with COPD we revealed an association between increased levels of IL-6 and apoptotic alveolar cells independent of Stat3 activity. Collectively, our data unexpectedly demonstrate that inflammation and emphysema are dissociable in the context of IL-6-driven lung pathology and suggest that there is a possibility of designing IL-6/Stat3-based therapies that target pulmonary inflammation alone or together with emphysema.

**MATERIALS AND METHODS**

**Human lung samples.** Lung tissue from resection surgery for treatment of a solitary peripheral carcinoma was collected from patients with either no evidence of COPD or mild-moderate COPD (Table 1). Tissue from the subpleural parenchyma avoiding tumor-bearing areas were fixed in 10% neutral buffered formalin and embedded in paraffin for sectioning. The degree of inflammation was determined on hematoxylin and eosin (H&E)-stained sections of the neoplastic lesions. Inflammation scoring was as follows: 0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation.

**Mice.** The gp130FF, gp130FF:IL-6−/−, gp130FF:Stat3−/−, and gp130FF:Stat1−/− mice have been previously reported (9, 18, 19, 44) and were housed under specific pathogen-free conditions. Experiments were approved by the Monash University Animal Ethics Committee.

### Antibodies

Antibodies against IL-6, phospho(Y705)-Stat3, total Stat1, and actin were purchased from Cell Signaling Technology (Beverly, MA). Fluorescent-labeled antibodies to murine CD3 (clone 145-2C11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The cleaved caspase-8 antibody was purchased from Cell Signaling Technology (Danvers, MA). The cleaved caspase-8 antibody was purchased from Cell Signaling Technology (Danvers, MA). The cleaved caspase-8 antibody was purchased from Cell Signaling Technology (Danvers, MA).

### Protein extraction and immunoblotting

Protein extraction and immunoblotting. Total protein lysates were prepared from snap-frozen lung tissue and subjected to immunoblotting with the indicated antibodies as previously described (18). Proteins were visualized using the Odyssey infrared imaging system (LI-COR, Lincoln, NE) and quantified using the Image J program (National Institutes of Health).

### RNA isolation and gene expression analysis

RNA extraction and the preparation of cDNA for quantitative RT-PCR (qPCR) expression analyses of individual genes were performed as previously described (33). Sequences for mouse primers were as follows: Stat3 forward 5′-cAGAAATgTGCTCTACAAGggC-3′, Stat3 reverse 5′-GGTGGTCAGTACCTTCCATCT-3′; Sox3 forward 5′-GCGGGCACCCTTTTCTATCC-3′, Sox3 reverse 5′-TCCCCG-GACTGGGTCATGAC-3′; Stat1 forward 5′-ACAAATgTggTAgCG-3′, Stat1 reverse 5′-TGAAGAAATgCACAATACCAAC-3′; Ilp10 forward 5′-gcGGCTgTAATTTTgCCTCAT-3′, Ilp10 reverse 5′-gTCCCTCTAggCCTCCATT-3′; Sox3 forward 5′-gCCCCCTgTTgGTTgTACAC-3′, Mmp9 forward 5′-CGGACCCCCCGTGTTgTAGCA-3′, Mmp9 reverse 5′-AGGCACAGATgAGGGGCC-3′; Mmp2 forward 5′-GCTgCTATTgGGCCTCCCGC-3′, Mmp2 reverse 5′-CGTTGTgATCTTGAGCGCA-3′, Mmp2 reverse 5′-CGTTGTgATCTTGAGCGCA-3′, Mmp2 reverse 5′-CGTTGTgATCTTGAGCGCA-3′.

### Immunohistochemistry and immunofluorescence

Immunohistochemistry and immunofluorescence. Immunohistochemistry for the detection of B220, IL-6, and pY-Stat3 was performed as described (33). Apoptosis was determined by the terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) technique using an ApopTag peroxidase in situ apoptosis detection kit (Millipore, Billerica, MA). Immunofluorescence for cleaved caspase-8 activity was performed as per the manufacturers instructions (Cell Signaling Technology). Stereological techniques were applied to determine the percentage of alveolar septal TUNEL-stained cells (38), and the number of pY-Stat3−, IL-6−, or caspase-8-stained cells per 20 fields.

### Flow cytometry

Flow cytometry was performed on mouse lung single cell suspensions as described (38).

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For human lung tissue, total RNA was extracted from formalin-fixed, paraffin-embedded tissue using the RNeasy FFPE kit (Qiagen) as per the manufacturers’ instructions. Briefly, the first four tissue sections (10-μm thick) were discarded from each sample block, following which 10-μm sections were then used to extract total RNA. cDNA synthesis was performed using the high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). As per the mouse gene expression analyses, qPCR was performed with SYBR Green (Invitrogen) on the 7900HT Fast RT-PCR System, and gene expres-

**Table 1. Clinical characteristics of the patients**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Healthy Smokers</th>
<th>COPD</th>
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<tbody>
<tr>
<td>NEQP</td>
<td>2.3 ± 0.3</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>FEV1, % predicted</td>
<td>82.2 ± 4.2</td>
<td>76.6 ± 4.3</td>
</tr>
<tr>
<td>Post-BD FEV1/FVC</td>
<td>73.0 ± 2.3</td>
<td>57.3 ± 2.6</td>
</tr>
<tr>
<td>Gold stage</td>
<td>0</td>
<td>1–2</td>
</tr>
</tbody>
</table>

Data are means ± SE for chronic obstructive pulmonary disease (COPD)-free healthy smokers (n = 8) and patients with COPD (n = 15). FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; BD, bronchodilator.
sion data acquisition and analyses were performed using the Sequence Detection System Version 2.3 software (Applied Biosystems). Sequences for human primers were as follows: STAT3 forward 5'-GGAGGAGGCATTCGGAAAG-3', STAT3 reverse 5'-GTCGTTGGTGTCACACAGAT-3'; SOCS3 forward 5'-GGCCACTCTTCTCAGCATC-3', SOCS3 reverse 5'-ATCTGACTGGTCCAGGAACCTC-3'; STAT1 forward 5'-TTAATCCGACCTCCATCATT-3', STAT1 reverse 5'-GAATTCTACAGAGCCCACTAT-3'; IL-6R forward 5'-AAAGCTGGGCAGGTTGGTG-3', IL-6R reverse 5'-AGCTTGTGGACAGTTGTTG-3'; IL-6 forward 5'-CTCCAGGAGCCAGCTCTGA-3', IL-6 reverse 5'-CCAGGAGAAAGCCAAGTG-3'; and ACTIN forward 5'-GATGAGATTGGCATGGCTTT-3', ACTIN reverse 5'-CACCTTCACCCTTCAAGTT-3'.

Protease expression analysis. Zymography was used to assess protease activity in lysates prepared from snap-frozen lung tissue as previously described (45).

Statistical analyses. All statistical analyses were performed using GraphPad Prism for Windows version 5.0. The normality of data was assessed using the D'Agostino and Pearson K2 normality test. Where appropriate, parametric (one-way ANOVA, Student's t-test) or nonparametric tests (Kruskal Wallis, Mann-Whitney U-test) were used to determine differences between genotypes and patient groups. A P < 0.05 was considered statistically significant. Data are expressed as the means ± SE.

RESULTS

Hyperactivation of endogenous Stat3, but not Stat1, in the lungs of emphysematous gp130F/F mice. Since deregulated activation of the gp130/Stat3 signaling axis is observed in numerous IL-6-driven disease states (2, 13, 19), we initially investigated the activation status of Stat3 in the lungs of emphysematous gp130F/F mice. Immunoblot analysis of the lungs of 6-mo-old gp130F/F and gp130+/+ mice indicated that the basal levels of tyrosine-phosphorylated Stat3 (pY-Stat3) were significantly elevated (2.5-fold; P < 0.01) in lung tissue of gp130F/F mice compared with gp130+/+ mice (Fig. 1A). In addition, the amount of pY-Stat3 in emphysema-free lungs of 6-mo-old gp130F/F;IL-6−/− mice was reduced back to wild-type levels (Fig. 1A). These findings were also supported by qPCR expression profiling of Stat3-target genes, which indicated that mRNA levels for Stat3 and Socs3 were upregulated in lungs of gp130F/F mice compared with gp130+/+ and gp130F/F;IL-6−/− mice (Fig. 1B).

In addition to Stat3, IL-6 activates the related Stat1 latent transcription factor (15), which is a potent inducer of the emphysema-associated processes apoptosis and inflammation (32). Furthermore, the expression of Stat1 is augmented in emphysema-associated processes (e.g., stomach) of airway epithelial cells from emphysematous patients with COPD (39). Although Stat1 is hyperactivated in certain organs (e.g., stomach) of gp130F/F mice (9), we were unable to detect any basal pY-Stat1 in the lungs of gp130F/F mice (Fig. 1C). While we did observe an increased expression of known Stat1-target genes Stat1 and Ip10 in the lungs of gp130F/F compared with gp130+/+ mice, these genes remained upregulated in gp130F/F;IL-6−/− mice (Fig. 1D). Therefore, these data...
suggest that gp130-dependent hyperactivation of Stat3, rather than Stat1, correlates with emphysema in gp130F/F mice.

IL-6-driven emphysema in gp130F/F mice occurs independently of stat hyperactivation. To specifically delineate a causal role for Stat3 hyperactivity in IL-6/gp130-driven emphysema, we utilized gp130F/F:Stat3−/+ mice, which have previously been validated as an important biological model to understand the role of gp130/Stat3 hyperactation in the pathogenesis of various disease states (8, 16). Immunoblot (Fig. 2A) and qPCR (Fig. 2B) analyses confirmed that heterozygous genetic ablation of Stat3 reduced the levels of basal pY-Stat3 and Stat3-target gene expression, respectively, in the lungs of 6-mo-old gp130F/F:Stat3−/+ mice to those comparable of gp130+/+ mice. However, histological evaluation of 6-mo-old gp130F/F:Stat3−/+ mouse lungs revealed emphysema characterized by enlargement of the distal air spaces and destruction of the normal alveolar architecture comparable to that of age-matched gp130F/F mice (Fig. 2C). Similarly, examination of static lung compliance, lung volumes, Lm, and various stereological parameters revealed that gp130F/F:Stat3−/+ mice still manifested emphysematous changes comparable to those observed in gp130F/F mice (Fig. 2, D and E, and Table 2), suggesting that the pathogenesis of emphysema in gp130F/F mice is independent of Stat3.

A nonessential role for the gp130/Stat1 signaling axis in emphysema in gp130F/F mice was also verified in gp130F/F:Stat1−/− mice (9), whereby the lungs of these mice also showed emphysematous morphological changes (Fig. 2C and Table 2), increased lung volumes, and altered respiratory mechanics (Fig. 2, D and E) comparable to those observed in gp130F/F mice. Taken together, these data indicate that IL-6/gp130-driven emphysema occurs independently of either Stat3 or Stat1 hyperactivation.

Stat3 hyperactivation upregulates protease activity, but not apoptosis, in the lungs of gp130F/F mice. Apoptosis is a key process associated with the development of human emphysema and several animal models of the disease, including IL-6/gp130-driven emphysema in gp130F/F mice (1, 8, 38). Our observations that emphysema still develops in gp130F/F:Stat3−/+ and gp130F/F:Stat1−/− mice would therefore suggest that gp130-dependent hyperactivation of endogenous Stat3 or Stat1 does not augment apoptosis in the lungs of emphysema-

Fig. 2. IL-6-driven emphysema in gp130F/F mice occurs independently of Stat hyperactivation. A) Immunoblot analyses were performed on lung tissue lysates from 6-mo-old gp130+/+ (+/+), gp130F/F (F/F) and gp130F/F:Stat3−/+ (F/F:S3−/+ mice using the indicated antibodies. Each lane represents tissue from an individual mouse. Densitometry quantification for pY-Stat3 was performed on samples belonging to the indicated genotypes, and relative expression was determined against actin. **P < 0.01. B) qPCR expression analyses of the Stat3 and Socs3 Stat3 target genes were performed on lung cDNA from 6-mo-old mice of the indicated genotypes. Expression data are normalized against IRS and are presented from n = 4 mice per genotype as the means ± SE. *P < 0.05, **P < 0.01. C) representative methylene blue stained cross sections of lungs from +/+ F/F, F/F:S3−/+, and gp130F/F:Stat1−/− mice aged 6 mo. Arrows indicate enlarged air spaces. Scale bars = 100 μm. Static compliance (D) and lung volume (E) of 6-mo-old mice of the indicated genotypes. Data are presented from n = 5 mice per genotype as the means ± SE per body weight. *P < 0.05, **P < 0.01, and ***P < 0.001.
tous gp130F/F mice. To test this notion, we assessed the extent of apoptotic TUNEL-stained cells in the lungs of these compound mutant mice at 6 mo of age, which we (38) have previously reported to primarily comprise alveolar epithelial (septal) cells rather than endothelial or interstitial inflammatory cells. Indeed, increased numbers of TUNEL-stained alveolar septal cells were detected in the lungs of gp130F/F, gp130F/F; Stat3−/−, and gp130F/F; Stat1−/− mice compared with gp130+/+ mice (Fig. 3B). To further delineate the specific apoptotic pathways associated with gp130-driven emphysema in gp130F/F mice, we next used immunofluorescence to detect downstream proteins belonging to two main apoptotic pathways; intrinsic (activated caspase-9) and extrinsic (activated caspase-8). As shown in Fig. 3, C and D, we observed increased numbers of activated caspase-8 immunoreactive cells in lung sections from emphysematous gp130F/F, gp130F/F; Stat3−/− and gp130F/F; Stat1−/− mice compared with

Table 2. Comparative stereological analyses of lungs from gp130+/+ and gp130F/F compound mutant mice

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>F/F</th>
<th>F/F:S3−/+</th>
<th>F/F:S1−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vv (par/lung), %</td>
<td>86.4 ± 0.7</td>
<td>88.9 ± 1.2*</td>
<td>89.0 ± 0.3*</td>
<td>84.4 ± 1.8</td>
</tr>
<tr>
<td>Vv (air/lung), cm³</td>
<td>58.4 ± 5.1</td>
<td>82.2 ± 2.6*</td>
<td>80.2 ± 3.5*</td>
<td>71.1 ± 4.9*</td>
</tr>
<tr>
<td>Vv (sep/lung), %</td>
<td>23.9 ± 1.1</td>
<td>16.1 ± 0.9***</td>
<td>14.5 ± 1.2**</td>
<td>18.6 ± 1.4*</td>
</tr>
<tr>
<td>V (sep/lung), cm³</td>
<td>20.2 ± 2.0</td>
<td>16.7 ± 1.1*</td>
<td>14.3 ± 0.8*</td>
<td>17.1 ± 1.7</td>
</tr>
<tr>
<td>Sv (sep/lung), l/cm</td>
<td>639 ± 36</td>
<td>538 ± 17*</td>
<td>531 ± 52</td>
<td>548 ± 41</td>
</tr>
<tr>
<td>S (sep/lung), cm²</td>
<td>535 ± 49</td>
<td>560 ± 33</td>
<td>531 ± 58</td>
<td>498 ± 40</td>
</tr>
<tr>
<td>Lm, μm</td>
<td>23.9 ± 0.4</td>
<td>44.7 ± 2.5***</td>
<td>41.8 ± 2.5***</td>
<td>41.4 ± 0.3***</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 5–6 mice aged 6 mo/genotype. Genotypes are as follows: gp130+/+ (+/+), gp130F/F (F/F), gp130F/F; Stat3−/− (F/F:S3−/+), and gp130F/F; Stat1−/− (F/F:S1−/−) mice. Vv, volume fraction; par, parenchyma; air, air space; sep, septal tissue; Sv, surface density; S, surface area; Lm, mean linear intercept. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. age-matched +/+ mice.

Fig. 3. Lungs of gp130F/F mice undergo apoptosis via the caspase-8 pathway. A: Representative photomicrographs showing terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL)-stained cells in lung cross sections from 6-mo-old gp130+/+ (+/+), gp130F/F (F/F), gp130F/F; Stat3−/− (F/F:S3−/+), and gp130F/F; Stat1−/− (F/F:S1−/−) mice. B: stereological quantification of the percentage of TUNEL-stained cells in the lungs of 6-mo-old mice of the indicated genotypes. Data from ≥3 mice per genotype are expressed as means ± SE. *P < 0.05. C: representative caspase-8 immunostained (in red) cross sections of lungs from 6 mo mice of the indicated genotypes. Scale bars = 10 μm. D: quantification of the number of caspase-8 immunoreactive cells in the lungs of 6 mo mice per 20 fields (at ×40 magnification). Data are expressed as means ± SE from ≥3 mice per genotype. *P < 0.05 and **P < 0.01.
gp130\textsuperscript{+/+} mice at 6 mo of age. By contrast, no immunoreactive activated caspase-9 staining was observed in the lungs among the various mouse genotypes (data not shown). Collectively, these data suggest that alveolar cells undergo apoptosis via the caspase-8-dependent apoptotic pathway in all emphysematous mice.

The excessive pulmonary gelatinase activity of MMP-2 and MMP-9 previously observed in gp130\textsuperscript{F/F} mice did not correlate with IL-6/gp130-driven emphysema but may be implicated in inflammation (38). We therefore investigated whether the pulmonary activity of these MMPs was promoted by Stat1 and/or Stat3 hyperactivation. Gelatinase zymography of lung lysates from 6-mo-old mice demonstrated that MMP-9 protease activity was reduced to wild-type levels in F/F mice (Fig. 4A). In contrast, lung MMP-2 activity was comparable among all genotypes. Expression analyses of Mmp-2, Mmp-9, and Timp-1 mRNA expression levels remained elevated in the lungs of gp130\textsuperscript{F/F}:Stat1\textsuperscript{−/−} mice (Fig. 4A). These observations correlated with increased (gp130\textsuperscript{F/F}:Stat1\textsuperscript{−/−}) and decreased (gp130\textsuperscript{F/F}:Stat3\textsuperscript{−/−}) lung mRNA levels for Mmp-9 compared with gp130\textsuperscript{F/F} mice at 6 mo (Fig. 4B) and are consistent with previous reports (7) on the contrasting regulatory actions of Stat1 and Stat3 on Mmp-9 gene expression. A similar gene expression profile was also observed for Mmp-12 (Fig. 4C). The augmented Mmp-9 activity was not a consequence of the impaired mRNA expression of the negative regulator tissue inhibitor MMP-1 (Timp-1; Fig. 4D). In contrast, lung MMP-2 activity was comparable among all genotypes (Fig. 4A), and Mmp-2 mRNA expression levels remained elevated in the lungs of gp130\textsuperscript{F/F}, gp130\textsuperscript{F/F}:Stat3\textsuperscript{−/−}, and gp130\textsuperscript{F/F}:Stat1\textsuperscript{−/−} mice compared with gp130\textsuperscript{+/+} mice (Fig. 4E). Therefore, these data demonstrate a specific link between lung expression and activity of MMP-9 and Stat3.

Pulmonary inflammation in gp130\textsuperscript{F/F} mice is primarily driven by endogenous Stat3 hyperactivation. In addition to emphysema, another feature of the lungs of gp130\textsuperscript{F/F} mice is the spontaneous development of pulmonary inflammation predominantly comprising B220\textsuperscript{+} B cells as well as CD3\textsuperscript{+} T cells (Fig. 5, A and D; Ref. 38), the numbers of which are also increased in patients with COPD (12, 34). Considering that Stat3 and Stat1 can display proinflammatory activities (13, 25) and promote the tissue infiltration of B- and T-lineage cells (2, 29, 31, 40), we therefore investigated whether elevated gp130-dependent Stat3 and/or Stat1 signaling in the lung promoted inflammation. Histological evaluation of lung sections of 6-mo-old mice revealed that the focal inflammatory infiltrates characteristic of gp130\textsuperscript{F/F} mouse lungs were absent in gp130\textsuperscript{F/F}:Stat3\textsuperscript{−/−} mice, whereas such infiltrates were present in gp130\textsuperscript{F/F}:Stat1\textsuperscript{−/−} mice (Fig. 5A). Immunohistochemistry with the B220 antibody confirmed that these inflammatory infiltrates were mainly B cells (Fig. 5B). Consistent with these observations, flow cytometry performed on whole lung cell suspensions confirmed that the increased proportion of B220\textsuperscript{+} B cells in lungs of gp130\textsuperscript{F/F} mice was reduced to a greater extent in the lungs of gp130\textsuperscript{F/F}:Stat3\textsuperscript{−/−} mice but not gp130\textsuperscript{F/F}:Stat1\textsuperscript{−/−} mice (Fig. 5C and Table 3). Notably, the increased proportion of CD3\textsuperscript{+} T cell infiltrates in the lungs of gp130\textsuperscript{F/F} mice was similarly reduced in either gp130\textsuperscript{F/F}:Stat3\textsuperscript{−/−} or gp130\textsuperscript{F/F}:Stat1\textsuperscript{−/−} mouse lungs (Fig. 5D and Table 3), consistent with a regulatory role for both Stat1 and Stat3 in promoting T-cell infiltration (29, 31, 40). By contrast, the proportions of Mac-1\textsuperscript{+} and Mac-1\textsuperscript{+}Gr-1\textsuperscript{+} myeloid cells in the lungs of mice were comparable among the genotypes (Fig. 5E; Table 3). Therefore, these data indicate that endogenous gp130-dependent Stat3 hy-

![Image](https://example.com/image1.png)

**Fig. 4.** Stat3 hyperactivation upregulates protease activity in the lungs of gp130\textsuperscript{F/F} mice. A: zymography of gelatinase activity was performed on lung tissue lysates from 6-mo-old gp130\textsuperscript{+/+}, gp130\textsuperscript{F/F}, gp130\textsuperscript{F/F}:Stat3\textsuperscript{−/−}, and gp130\textsuperscript{F/F}:Stat1\textsuperscript{−/−} mice. The 90 and 60-kDa bands correspond to matrix metalloprotease (MMP)-9 and MMP-2, respectively. Each lane represents an individual mouse lung lystate. qPCR expression analyses of Mmp-9 (B), Mmp-12 (C), Timp-1 (D), and Mmp-2 (E) were performed on lung cDNA from 6-mo-old mice of the indicated genotypes. Expression data are shown from ≥4 mice per genotype following normalization for 18S expression and are presented from triplicate analysis as the means ± SE. *p < 0.05 and **p < 0.01.
peractivation primarily promotes the accumulation of both B- and T-inflammatory cells in the lungs of gp130\textsuperscript{F/F} mice.

In human disease, increased Stat3 activation is associated with pulmonary inflammation, whereas increased IL-6 expression and alveolar cell apoptosis are features of COPD. To further support our findings from gp130\textsuperscript{F/F} mice that Stat3 hyperactivation drives pulmonary inflammation but not emphysema, we performed immunohistochemistry for pY-Stat3 on human lung tissue from patients with COPD and, as a control, COPD-free healthy smokers. Stereological and histopathological analyses of H&E-stained lung tissue sections led to the classification of these lungs as displaying either no-mild inflammation (Fig. 6, E and F). Notably, increased numbers of pY-Stat3 stained alveolar cells were only observed in lung tissues displaying moderate inflammation, regardless of the COPD status (Figs. 7, A–D, and 8A and Table 4). Consistent with these data, the expression of Stat3 target genes SOCS3 and STAT3 was elevated, albeit not significantly, in patients with COPD and COPD-free healthy smoker controls displaying moderate inflammation compared with their counterparts displaying no-mild inflammation (Fig. 8B). By contrast, the gene expression of STAT1, a target of hyperactivation correlates with lung inflammation rather than emphysema.
itself, in all patient groups was undetectable by qPCR. Collectively, these data suggest an association between Stat3 activation levels and pulmonary inflammation, but not emphysema, in a clinical setting.

Table 4. Stereological immunohistochemistry and histology analysis of lungs from patients with COPD and COPD-free healthy smokers

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>pY-Stat3</th>
<th>IL-6</th>
<th>TUNEL</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy smokers (no-mild inflammation)</td>
<td>10.0 ± 3.3</td>
<td>4.6 ± 0.6</td>
<td>1.8 ± 0.8</td>
<td>5.0 ± 2.0</td>
</tr>
<tr>
<td>Healthy smokers (moderate inflammation)</td>
<td>26.9 ± 3.8*</td>
<td>7.4 ± 4.4</td>
<td>0.9 ± 0.3</td>
<td>26.3 ± 2.4</td>
</tr>
<tr>
<td>COPD (no-mild inflammation)</td>
<td>10.3 ± 2.2</td>
<td>30.9 ± 8.2**</td>
<td>6.2 ± 0.4*</td>
<td>5.0 ± 2.2</td>
</tr>
<tr>
<td>COPD (moderate inflammation)</td>
<td>42.7 ± 6.7</td>
<td>39.2 ± 5.7</td>
<td>10.2 ± 2.6</td>
<td>40.6 ± 3.6</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 4–9/patient group. TUNEL, terminal deoxyribonucleotidyl transferase dUTP-mediated nick-end labeling. Inflammation scoring is as follows: 0–19, no-mild; 20–70, moderate; 71–100, severe. *P < 0.05 and **P < 0.01 vs. COPD-free healthy smokers (no-mild inflammation).
nohistochemical staining for IL-6 expression and TUNEL assays for the assessment of apoptosis in lung tissue from patients with COPD and COPD-free individuals. Indeed, we observed increased numbers of immunoreactive IL-6-stained cells and TUNEL-stained cells in the lungs of patients with COPD compared with COPD-free healthy smokers, with staining predominantly localized to alveolar cells (Figs. 7, A–D, and 8A and Table 4). The increased expression of the IL-6 gene in the lungs of patients with COPD was also independently verified by qPCR (Fig. 8B). By contrast, the gene expression levels of IL-6R were comparable among the patient groups (Fig. 8B). Importantly, these data are consistent with our findings in gp130F/F mice and therefore support a pathological role for IL-6-driven apoptosis in the pathogenesis of human emphysema.

DISCUSSION

In this study, we provide strong evidence in the gp130F/F mouse model that endogenous gp130-dependent (via IL-6) Stat3 hyperactivation in the lung primarily drives chronic inflammation. Importantly, a positive correlation between increased Stat3 activation and pulmonary inflammation was also verified in human lung tissue irrespective of the presence or absence of COPD, and these findings are consistent with the observation that some smokers with excessive pulmonary inflammation do not present with COPD (3). In addition, in patients with COPD a modest up-regulation of Stat3-dependent genes was previously observed only in those with a history of smoking (37), who would be predicted to have marked inflammation compared with patients with COPD who were non-smokers, which further supports the notion that Stat3 hyperactivity in the lung primarily promotes inflammation. In the lungs of gp130F/F mice, we also observed that increased expression and protease activity of MMP family members were associated with inflammation in an IL-6/Stat3-dependent manner, which contrasted the IL-6-driven, but Stat3-independent, lung epithelial cell apoptosis and emphysema. While this suggests that excessive IL-6/Stat3-regulated MMP-associated protease ac-
activity is not linked to emphysema in this mouse model, it is possible that other proteases (and/or anti-proteases for that matter) regulated by IL-6 independently of Stat3 may play a potential role in IL-6-driven apoptosis and emphysema. Such a notion is worth pursuing further experimentally since proteases/anti-proteases, the regulation of which by IL-6 signaling pathways remains obscure, can influence lung epithelial apoptosis associated with emphysema (28, 35, 43, 49).

Another key finding of this current study that endogenous pulmonary Stat3 hyperactivation in gp130F/F mice does not promote emphysema was supported by the lack of any correlation in human lungs between increased Stat3 activity and emphysema. Such a notion is worth pursuing further experimentally since proteases/anti-proteases, the regulation of which by IL-6 signaling pathways remains obscure, can influence lung epithelial apoptosis associated with emphysema (28, 35, 43, 49).

Not surprisingly then, the role of Stat3 in the pathogenesis of emphysema has been studied in various mouse models artificially engineered to either suppress or augment Stat3 activity, albeit with conflicting findings. For instance, conditional deletion of Stat3 in respiratory epithelial cells failed to induce any lung pathology under steady-state conditions (22). By contrast, transgenic mice overexpressing a “dominant negative” Stat3-Y705F mutant in the respiratory epithelium caused pulmonary emphysema (47). However, interpretation of the latter study should proceed with caution in light of recent evidence that the nonphosphorylatable Stat3-Y705F mutant is not a true dominant negative but rather constitutes a novel transcription factor complex with nonphosphorylated NF-κB to activate a distinct subset of genes, many of which are proinflammatory (46). More recently, overexpression of MMP12 in mouse respiratory epithelial cells spontaneously

Fig. 8. Stat3 hyperactivation associates with inflammation regardless of COPD status, while increased IL-6 expression and apoptosis are related in human COPD. A: stereological analyses of the number of pY-Stat3-, IL-6-, and TUNEL-stained cells in human lungs per 20 fields (×40 magnification) patients with COPD and COPD-free controls with no-mild or moderate (mod) inflammation. Data are expressed as means ± SE. *P < 0.05 and **P < 0.01. B: qPCR expression analyses of SOCS3, STAT3, IL-6, and IL-6R were performed on lung cDNA from the same groups of patients with COPD and COPD-free controls. Expression data are presented following normalization for ACTIN expression, and are presented from triplicate analysis as the means ± SE. *P < 0.05.
induced pulmonary inflammation and emphysema, which progressed to lung adenocarcinoma that was coincident with increased IL-6 expression and Stat3 activation (36), although a casual role for the IL-6/Stat3 axis in disease was not investigated. Nonetheless, this latter observation is more consistent with the finding that transgenic overexpression of a hyperactive Stat3-C mutant in the respiratory epithelium leads to spontaneous pulmonary inflammation and lung adenocarcinoma but not emphysema (26). Notably, a role for Stat3 in human lung cancer rather than emphysema is also supported by recent clinical data revealing that a subset of Stat3 target genes is elevated in lung adenocarcinoma but not in COPD tissue (37). Taken together, these data support our current findings that gp130-dependent hyperactivation of endogenous Stat3 does not induce emphysema but rather promotes pulmonary inflammation. Furthermore, we propose that our gp130F/F model provides a robust genetic tool to examine whether endogenous gp130-dependent Stat3 hyperactivation enhances the susceptibility to lung carcinogenesis, which is the subject of ongoing investigations in our laboratory.

Another key finding of this study was that in human COPD tissue independent of the extent of pulmonary inflammation, both increased IL-6 expression and augmented alveolar cell apoptosis were observed. These novel observations validate our previous work (38) demonstrating that increased IL-6 expression in the lungs of gp130F/F mice promotes emphysema characterized by increased numbers of apoptotic alveolar septal cells. In this regard, we also note that lung cell death in this model is not a consequence of excessive autophagy, since electron microscopy revealed that autophagosomes/autophagic vacuoles were similarly barely detectable in lung sections of both gp130F/F and gp130+/+ mice (data not shown). Collectively, these findings address a fundamental gap in our understanding of the cellular events by which IL-6 has been linked to the pathogenesis of COPD (5, 14, 42). Indeed, our current data demonstrating that the extent of lung apoptosis (Fig. 3) and emphysema (Fig. 2 and Table 2) are comparable in gp130F/F and gp130+/+;Stat3−/− mice despite the differential inflammatory cell content in the lungs of these 2 genotypes, and alveolar epithelial septal cells (aka type II pneumocytes) are the TUNEL-positive lung cell type undergoing apoptosis (Fig. 3A), further suggest that alveolar septal cells rather than infiltrating inflammatory/immune cells contribute to the emphysema phenotype of gp130F/F mice. Our study did not show any relationship between smoking pack year history and apoptosis in patients with COPD and COPD-free individuals, thus excluding the possibility that the increase in apoptosis of structural cells was only related to cigarette smoking, rather than being specifically associated with increased IL-6 levels. While other studies have previously reported a positive correlation between apoptosis and human emphysema either by histological examination of lung tissues (17, 48) or in bronchoalveolar lavage fluid (16), the level of IL-6 in these patients was not investigated. Clearly, further studies are now needed to clarify the full extent by which apoptotic pathways and gene networks are deregulated by elevated IL-6 production in human emphysema.

In summary, our current data led us to propose that the discrete therapeutic targeting of Stat3 may prevent or alleviate pulmonary inflammation and its sequelae. On this note, given that Stat3 is implicated in inflammation-associated lung carcinogenesis (26, 37) and numerous pulmonary chronic inflammatory diseases such as pneumonia, bronchitis, and asthma (11, 21), Stat3 activation via gp130 may have considerable translational potential as a biomarker and/or therapeutic target for these lung diseases. Furthermore, based on our previous findings in gp130F/F mice (38) and those presented here in human COPD of an association between increased IL-6 expression and apoptosis in the lung, we predict that targeting of IL-6 would be therapeutically advantageous in COPD phenotypes where apoptosis-associated emphysema predominates. In this scenario, it is important to consider that the diverse patho-physiological portfolio of IL-6 is largely explained by its ability to initiate 2 modes of signaling: “ classical” signaling via interaction with its membrane-bound IL-6Rα subunit, and “trans-signaling” via a naturally occurring soluble IL-6Rα that is proteolytically cleaved from the cell surface (20). Considering the emerging evidence that classical signaling is important for maintaining homeostatic immune responses, whereas trans-signaling is responsible for the pathogenesis of an increasing number of IL-6-driven disease states (e.g., inflammatory bowel disease, colon cancer, and arthritis; Refs. 4, 27, 33), as part of our ongoing studies, it will be important to identify which mode of signaling promotes IL-6-driven lung disease. Such knowledge for the treatment of specific lung pathologies (i.e., emphysema) will provide a critical advantage in determining the potential therapeutic benefits of selectively targeting IL-6 trans-signaling with new generation antagonists (13, 27, 33) rather than “global” IL-6 signaling (i.e., trans-signaling and classical signaling) with existing nondiscriminate antibodies against IL-6 or the IL-6R.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


