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

Submitted 31 August 2011; accepted in final form 12 January 2012

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 (Stat3). 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+/−F/F 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+/−F/F:Stat3−/− mice prevented lung inflammation and excessive protease 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+/−F/F 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.

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) and has been linked to the pathogenesis of emphysema, it has emerged that the destruction of lung parenchyma in emphysema is likely 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 (Stat3) and to a lesser extent Stat1, as well as the Shp2-mitogen-activated protein kinase (MAPK) and Shp2-phosphinosotide 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 gp130+/−F/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 gp130+/−F/F mice
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 gp130F/F 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 (10-μm thick) were drawn and used for stereological examination using a computer-aided system (newCAST software, version 2.14; Visiopharm, Hørsholm, Denmark). Airway obstruction was quantified using the Image J program (NIH) by calculating the percentage of alveolar septal TUNEL-stained cells (38), and the number of p-Y-Stat3-, IL-6-, or caspase-8-stained cells per 20 fields.

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>Solitary peripheral carcinoma (no airway obstruction)</td>
<td>88.2 ± 4.2</td>
<td>76.6 ± 4.3</td>
</tr>
<tr>
<td>Solitary peripheral carcinoma (airway obstruction)</td>
<td>73.0 ± 2.3</td>
<td>57.3 ± 2.6</td>
</tr>
<tr>
<td>FEV1, % predicted post</td>
<td>0.82 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Post-BD FEV1-to-FVC ratio</td>
<td>0.73 ± 0.3</td>
<td>0.75 ± 0.2</td>
</tr>
<tr>
<td>Gold stage</td>
<td>0.62 ± 0.2</td>
<td>0.85 ± 0.1</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. CD11b/Mac-1 (M1/70) were purchased from BD Bioscience (San Jose, CA).

Mouse tissue collection. The collection of mouse lungs for stereology, histology and immunohistochemistry was performed as previously described (38).

Stereological and mean linear intercept analyses. Lung stereology was performed on methylene blue-stained lung sections using computer-assisted newCAST software (version 2.14; Visiopharm, Hørsholm, Denmark; Ref. 38). Airspace enlargement was quantified by the mean linear intercept (Lm) technique on H&E-stained lung sections. For each section, five randomly selected fields were captured at ×20 magnification in a blinded fashion using a Zeiss Axiolab Microscope (Oberkochen, Germany) and Olympus DP70 Camera (Tokyo, Japan), and images were analyzed using Image-Pro Plus 5.0 software (Media Cybernetics, Bethesda, MD). Fields with airways or blood vessels were avoided by moving one field in any one direction. Ten equally distributed horizontal lines of known length (~420 μm) were drawn in each field, and Lm was calculated as the total length of the lines divided by the number of alveolar intercepts.

Lung function analyses. The assessment of lung function on anesthetized mice was performed using the flexivent system (SCIREQ, Montreal, Canada; Refs. 6, 38).

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

Immunohistochemistry and immunofluorescence. Immunohistochemistry for the detection of B220, IL-6, and p-Y-Stat3 was performed as before (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), and 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 p-Y-Stat3-, IL-6-, or caspase-8-stained cells per 20 fields.

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 (nih.gov).

RNA isolation and gene expression analysis. For mouse lung tissue, total RNA extraction and the preparation of cDNA for quantitative RT-PCR (qPCR) expression analyses of individual genes were performed as previously described on June 20, 2017 http://ajplung.physiology.org/ Downloaded from

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Downloaded from http://ajplung.physiology.org/ by IP 10.220.33.3 on June 20, 2017
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'-TCGTTGTGTCACACAGAT-3'; SOCS3 forward 5'-GGCCACCTTTCTCAGCATCTCTC-3'; SOCS3 reverse 5'-ATCGTACTGGTCCAGGAACGT-3'; STAT1 forward 5'-GTACCCCTTGACATCTATT-3'; STAT1 reverse 5'-GAATTCAGAGCGCCAATAT-3'; IL-6R forward 5'-AAACGGCGGTGTTGTTG-3'; IL-6R reverse 5'-AGCTTGTGGCAGGTTGAG-3'; IL-6 forward 5'-CTTCAGGAGCCCAGCTCTGA-3'; IL-6 reverse 5'-CCACCAAGAAGGCAACTG-3'; and ACTIN forward 5'-GATGAGATTGGCATGGCTTT-3', ACTIN reverse 5'-CCCAGGGAGAAGGCAACTG-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 gp130<sup>F/F</sup> 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 gp130<sup>F/F</sup> mice. Immunoblot analysis of the lungs of 6-mo-old gp130<sup>F/F</sup> and gp130<sup>F/+</sup> 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 gp130<sup>F/F</sup> mice compared with gp130<sup>F/+</sup> mice (Fig. 1A). In addition, the amount of pY-Stat3 in emphysema-free lungs of 6-mo-old gp130<sup>F/F</sup>:IL-6<sup>−/−</sup> 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 the lungs of gp130<sup>F/F</sup> mice compared with gp130<sup>F/+</sup> and gp130<sup>F/F</sup>:IL-6<sup>−/−</sup> 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-free lungs of 6-mo-old gp130<sup>F/F</sup> mice (9), we were unable to detect any basal pY-Stat1 in the lungs of gp130<sup>F/F</sup> mice (Fig. 1C). While we did observe an increased expression of known Stat1-target genes Stat1 and Ip10 in the lungs of gp130<sup>F/F</sup> compared with gp130<sup>F/+</sup> mice, these genes remained upregulated in gp130<sup>F/F</sup>:IL-6<sup>−/−</sup> mice (Fig. 1D). Therefore, these data...
suggest that gp130-dependent hyperactivation of Stat3, rather than Stat1, correlates with emphysema in gp130\(^{F/F}\) mice. IL-6-driven emphysema in gp130\(^{F/F}\) mice occurs independently of Stat hyperactivation. To specifically delineate a causal role for Stat3 hyperactivity in IL-6/gp130-driven emphysema, we utilized gp130\(^{F/F}\):Stat3\(^{+/+}\) mice, which have previously been validated as an important biological model to understand the role of gp130/Stat3 hyperactivation 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 gp130\(^{F/F}\):Stat3\(^{−/−}\) mice to those comparable of gp130\(^{+/+}\) mice. However, histological evaluation of 6-mo-old gp130\(^{F/F}\): Stat3\(^{−/−}\) mice revealed emphysema characterized by enlargement of the distal air spaces and destruction of the normal alveolar architecture comparable to that of age-matched gp130\(^{F/F}\) mice (Fig. 2C). Similarly, examination of static lung compliance, lung volumes, Lm, and various stereological parameters revealed that gp130\(^{F/F}\):Stat3\(^{−/−}\) mice still manifested emphysematos changes comparable to those observed in gp130\(^{F/F}\) mice (Fig. 2, D and E, and Table 2), suggesting that the pathogenesis of emphysema in gp130\(^{F/F}\) mice is independent of Stat3.

A nonessential role for the gp130/Stat1 signaling axis in emphysema in gp130\(^{F/F}\) mice was also verified in gp130\(^{F/F}\):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 gp130\(^{F/F}\) 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 gp130\(^{F/F}\) 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 gp130\(^{F/F}\) mice (1, 8, 38). Our observations that emphysema still develops in gp130\(^{F/F}\):Stat3\(^{−/−}\) and gp130\(^{F/F}\):Stat1\(^{−/−}\) mice would therefore suggest that gp130-dependent hyperactivation of endogenous Stat3 or Stat1 does not augment apoptosis in the lungs of emphysema-
Stat3 drives lung inflammation but not emphysema

Table 2. Comparative stereological analyses of lungs from gp130<sup>+/−</sup> and gp130<sup>F/F</sup> compound mutant mice

<table>
<thead>
<tr>
<th></th>
<th>+/-</th>
<th>F/F</th>
<th>F/F:S3&lt;sup&gt;−+/−&lt;/sup&gt;</th>
<th>F/F:S1&lt;sup&gt;−+/−&lt;/sup&gt;</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/par), %</td>
<td>69.8 ± 1.3</td>
<td>79.3 ± 1.9***</td>
<td>80.3 ± 1.2**</td>
<td>77.5 ± 1.4**</td>
</tr>
<tr>
<td>V (air/lung), cm&lt;sup&gt;3&lt;/sup&gt;</td>
<td>58.4 ± 3.1</td>
<td>82.2 ± 2.6*</td>
<td>80.2 ± 3.5*</td>
<td>71.1 ± 4.9*</td>
</tr>
<tr>
<td>Vv (air/par), %</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 (air/lung), cm&lt;sup&gt;3&lt;/sup&gt;</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/par), 1/cm</td>
<td>639 ± 36</td>
<td>538 ± 17*</td>
<td>531 ± 52</td>
<td>538 ± 41</td>
</tr>
<tr>
<td>Vv (sep/par), %</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<sup>+/−</sup> (+/+), gp130<sup>F/F</sup> (F/F), gp130<sup>F/F</sup>:Stat3<sup>−+/−</sup> (F/F:S3<sup>−+/−</sup>), and gp130<sup>F/F</sup>:Stat1<sup>−+/−</sup> (F/F:S1<sup>−+/−</sup>) 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.

tous gp130<sup>F/F</sup> 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 gp130<sup>F/F</sup>, gp130<sup>F/F</sup>:Stat3<sup>−+/−</sup>, and gp130<sup>F/F</sup>:Stat1<sup>−+/−</sup> mice compared with gp130<sup>+/+</sup> controls (Fig. 3A). Furthermore, stereological quantification revealed that TUNEL staining in lungs of gp130<sup>F/F</sup>:Stat3<sup>−+/−</sup> and gp130<sup>F/F</sup>:Stat1<sup>−+/−</sup> mice remained significantly elevated compared with gp130<sup>+/+</sup> mice (Fig. 3B). To further delineate the specific apoptotic pathways associated with gp130-driven emphysema in gp130<sup>F/F</sup> 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 gp130<sup>F/F</sup>, gp130<sup>F/F</sup>:Stat3<sup>−+/−</sup> and gp130<sup>F/F</sup>:Stat1<sup>−+/−</sup> mice compared with
gp130^{F/F} 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^{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 gp130^{F/F}:Stat3^{−/−} mice, whereas MMP-9 activity remained elevated in the lungs of gp130^{F/F}:Stat1^{−/−} mice (Fig. 4A). These observations correlated with increased (gp130^{F/F}:Stat1^{−/−}) and decreased (gp130^{F/F}:Stat3^{−/−}) lung mRNA levels for Mmp-9 compared with gp130^{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^{F/F}, gp130^{F/F}:Stat3^{−/−}, and gp130^{F/F}:Stat1^{−/−} mice compared with gp130^{+/+} mice (Fig. 4E). Therefore, these data demonstrate a specific link between lung expression and activity of Mmp-9 and Stat3.

Pulmonary inflammation in gp130^{F/F} mice is primarily driven by endogenous Stat3 hyperactivation. In addition to emphysema, another feature of the lungs of gp130^{F/F} mice is the spontaneous development of pulmonary inflammation predominantly comprising B220⁺ B cells as well as CD3⁺ 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^{F/F} mouse lungs were absent in gp130^{F/F}:Stat3^{−/−} mice, whereas such infiltrates were present in gp130^{F/F}:Stat1^{−/−} mouse (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⁺ B cells in lungs of gp130^{F/F} mice was reduced to a greater extent in the lungs of gp130^{F/F}:Stat3^{−/−} mice but not gp130^{F/F}:Stat1^{−/−} mice (Fig. 5C and Table 3). Notably, the increased proportion of CD3⁺ T cell infiltrates in the lungs of gp130^{F/F} mice was similarly reduced in either gp130^{F/F}:Stat3^{−/−} or gp130^{F/F}:Stat1^{−/−} 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⁺ and Mac-1⁺Gr-1⁺ 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-
perfusion primarily promotes the accumulation of both B- and T-inflammatory cells in the lungs of gp130	extsuperscript{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	extsuperscript{F/F} mice that Stat3 hyperactivation correlates with lung inflammation rather than 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 or moderate lung inflammation (Fig. 6 and Table 4). Histopathological evaluation of lung sections indicated that the predominant inflammatory cells present were plasma cells, with neutrophils and lymphocytes also observed (Fig. 6, E and F).

Table 3. Flow cytometric analyses of immune cell infiltrates in the lungs of gp130	extsuperscript{+/+} and gp130	extsuperscript{F/F} compound mutant mice

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>F/F</th>
<th>F/F:S3	extsuperscript{+/−}</th>
<th>F/F:S1	extsuperscript{−/−}</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220, %</td>
<td>8.8 ± 1.7</td>
<td>24.9 ± 1.7*</td>
<td>11.7 ± 1.2*</td>
<td>18.7 ± 2.0</td>
</tr>
<tr>
<td>CD3 +</td>
<td>10.9 ± 3.7</td>
<td>24.6 ± 2.5*</td>
<td>14.1 ± 3.9</td>
<td>13.8 ± 0.9</td>
</tr>
<tr>
<td>Mac+Gr1 +</td>
<td>2.0 ± 0.8</td>
<td>1.5 ± 0.4</td>
<td>1.5 ± 0.5</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Mac +, %</td>
<td>5.1 ± 0.3</td>
<td>5.3 ± 1.1</td>
<td>7.0 ± 0.2</td>
<td>6.9 ± 0.8</td>
</tr>
</tbody>
</table>

Data are expressed as the means ± SE; n = 4 mice aged 6 mo/genotype. Genotypes are as follows: gp130	extsuperscript{+/+} (+/+), gp130	extsuperscript{F/F} (F/F), gp130	extsuperscript{F/F}:Stat3	extsuperscript{−/−} (F/F:S3	extsuperscript{−/−}), and gp130	extsuperscript{F/F}:Stat1	extsuperscript{−/−} (F/F:S1	extsuperscript{−/−}) mice. *P < 0.05 and **P < 0.01 vs. age-matched +/+ mice. *P < 0.05 vs. age-matched F/F mice.
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.

In the gp130F/F mouse model, we have also recently revealed that IL-6-driven pulmonary emphysema correlates with enhanced alveolar cell apoptosis (38). To validate these findings in the onset of human disease, we performed both immunohistochemistry and histology analysis of lungs from patients with COPD and COPD-free healthy smokers.

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>Number of Positively Stained Cells/20 Fields</th>
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<tbody>
<tr>
<td></td>
<td>pY-Stat3</td>
</tr>
<tr>
<td>Healthy smokers (no-mild inflammation)</td>
<td>10.0 ± 3.3</td>
</tr>
<tr>
<td>Healthy smokers (moderate inflammation)</td>
<td>26.9 ± 3.8*</td>
</tr>
<tr>
<td>COPD (no-mild inflammation)</td>
<td>10.3 ± 2.2</td>
</tr>
<tr>
<td>COPD (moderate inflammation)</td>
<td>42.7 ± 6.7</td>
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Data are means ± SE; n = 4–9/patient group. TUNEL, terminal deoxynucleotidyl 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).
nullhistochemical 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 gp130\(^{−/−}\) mice does not promote emphysema was supported by the lack of any correlation in human lung tissue 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).

Another key finding of this current study that endogenous pulmonary Stat3 hyperactivation in gp130\(^{−/−}\) mice does not promote emphysema was supported by the lack of any correlation in human lung tissue between increased Stat3 activation and COPD. In this respect, it is worth noting that Stat3 can modulate inflammatory, apoptotic, proteolytic, and oxidative stress processes (10, 41), each of which either individually or collectively in the lung can induce emphysematous alterations (3). 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
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.

ACKNOWLEDGMENTS

We thank E. Vidacs and M. Thompson for expert technical assistance.

GRANTS

This work was supported in part by a research grant from the National Health and Medical Research Council (NHMRC), Australia (to B. J. Jenkins and P. G. Bardin), as well as the Operational Infrastructure Support Program by the Victorian Government of Australia. S. M. Ruwanpura is supported by a NHMRC Postdoctoral Training Fellowship. B. J. Jenkins is supported by both a Senior Medical Research Fellowship awarded by the Sylvia and Charles Viertel Charitable Foundation, and a Monash University Fellowship.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00285.2011 • www.ajplung.org


