Title: Lung epithelial cell-derived C3 protects against pneumonia-induced lung injury

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Abstract

The complement component C3 is a fundamental plasma protein for host defense. However, recent work has demonstrated the critical importance of local C3 expression in cell survival. Here we analyzed the effects of local versus peripheral sources of C3 expression in a model of bacterial pneumonia. While mice with global C3 deficiency had severe pneumonia-induced lung injury, those deficient in liver-deficient C3 remain protected, comparable to wildtype mice. Human lung transcriptome analysis showed secretory epithelial cells are a major source of C3. Mice with a C3 gene ablation from lung epithelial cells had worse pulmonary injury compared to wild type, despite maintaining normal circulating C3 levels. Finally, in human cellular and mouse pneumonia models, we show that C3 reduces epithelial cell death mediated through the alternative pathway component Factor B. Thus, our findings suggest that a locally-derived C3-Factor B pathway protects the lung mucosal barrier.

One Sentence Summary

Lung-derived C3 mitigates severe bacterial pneumonia suggesting a novel cytoprotective role at mucosal barrier surfaces independent of circulating C3.

Main Text:

INTRODUCTION

Pathogens infect and injure mucosal surfaces resulting in a cascade of events including inflammation, tissue damage and systemic dissemination (1, 2). In the lung, this process results in severe pneumonia, a leading cause of death, even before the SARS-CoV-2 pandemic (3, 4). The outcome in pneumonia is determined by a balance between immune resistance and tissue resilience of the host (1, 5). Immune resistance is the host's ability to eradicate pathogens, whereas tissue resilience is the ability of the lung to withstand the deleterious consequences of infection and inflammation (1, 5). The complement system is a family of immune proteins that facilitate immune resistance by attacking microbes to decrease pathogen burden (6, 7). Consequently, genetic and acquired deficiencies of complement characteristically lead to recurrent bacterial infections in both children and adults (8, 9). However, there has been an increasing appreciation recently of how the system functions to promote tissue resilience in the setting of acute infections (10, 11).

A majority of the complement proteins are primarily liver-derived (12). One such protein, C3, is a central component of the complement system, as multiple pathways converge on its activation to C3a and C3b, thereby amplifying the cascade (13). The plasma concentration of C3 is approximately 1-2 mg/ml in humans, resulting it in being one of the most common proteins in the circulation after other liver-derived proteins such as albumin and α -2-macroglobulin (14). Thus, the protective effects of C3 have largely been attributed to it being sourced from the liver and present in a high concentration in the circulation. However, we and others have previously demonstrated that C3 is also produced outside of the liver by multiple immune and non-immune

cell populations (15–19). This observation has implications, especially for organs that constantly interact with pathogens and environmental toxins, such as the mucosal surface of the lung (6, 20). For example, we have demonstrated that C3 synthesized as well as internalized by lung epithelial cells protects against stress-induced cell death (16, 21). Others have demonstrated that chronic C3 upregulation in lung epithelial cells can perpetuate inflammation in SARS-CoV-2 infection (19).

It is unknown whether cell-intrinsic C3 production is relevant in vivo. It is also unclear if the protective effects of C3 are purely a result of enhancing immune resistance via its bactericidal activity, or it also contributes to tissue resilience by reducing acute lung injury (ALI). To address these questions, we utilized a model of acute *Pseudomonas aeruginosa* (*Pa*) pneumonia in mice globally deficient in C3 and report that C3 not only promotes immune resistance but also enhances tissue resilience in vivo by mitigating ALI. Using publicly available human lung datasets and a C3 reporter mouse model, we establish that multiple structural cell types in the lung produce and upregulate C3 in the setting of *Pa* infection. Building on these findings, we generated novel mouse strains and show that lung-derived C3 is functioning locally, independent of circulating (i.e., liver-derived) C3 and is necessary for reducing *Pa*-induced ALI. Using both human in vitro and mouse in vivo infection models, we show that cell-intrinsic Factor B — a component of the alternative pathway and a ligand for C3 — is a key component of tissue resilience, enhancing the ability of the lung to withstand the deleterious consequences of infection and inflammation. Our findings potentially refine the approach for therapeutically targeting the complement system in the lung with the goal of mitigating bacterial pneumonia severity.

RESULTS

C3 protects against severe bacterial pneumonia

C3-deficient mice had worse histological evidence of lung injury when compared to wildtype (WT) mice at 24 h after *Pa* infection (**Fig 1A**). They had worsened physiological function, as demonstrated by reduced dynamic compliance compared to WT mice at 24 h post-infection (**Fig 1B**), as well as a higher airway resistance (**Fig S1A**), both measured by whole body plethysmography. They also had elevated alveolar capillary-barrier permeability as demonstrated using the wet-dry ratio (**Fig S1B**). Consistent with prior reports, male C3-deficient mice had a significantly worse injury relative to female C3-deficient mice (**Fig. S1C**) (22). Hence, C3-deficient male mice were used for subsequent experiments. We next analyzed inflammatory gene expression. C3-deficient mice had an exaggerated inflammatory response, as determined by increased lung *Ccl2* and *Il1b* expression (**Fig 1C**), the proportion of bronchoalveolar lavage (BAL) Ly6C^{hi} monocytes (**Fig S1D**) and BAL CCL2 and IL-1 β levels (**Fig S1E and 1F**). The BAL levels of IL-6 and TNF- α showed a trend towards significance in C3-deficient mice compared to WT mice (**Fig S1G and S1H**). Of note, infected C3-deficient mice had lower expression of epithelial cell genes, such as *Scgb1a1* (representing secretory airway epithelial cells) and *Sftpc* (representing alveolar epithelial cells) compared to infected WT, suggesting C3 protects against epithelial cell loss (**Fig 1C**). However, C3-deficient mice also had higher lung (**Fig 1D**) and splenic bacterial counts (**Fig 1E**) at 24 h post-*Pa* infection.

One explanation for increased lung injury in C3-deficient mice is damage induced by actively proliferating bacteria. To this end, heat-killed *Pa* (HK*Pa*) were intratracheally instilled into the lungs of both C3-deficient and WT mice, and their lungs and BAL were harvested 24 h later.

Compared to WT, C3-deficient mice still retained more histologic evidence of lung injury (**Fig 1F and 1G**), characterized by an increase in alveolar (**Fig S1I**) and interstitial neutrophilia (**Fig S1J**), and septal thickening (**Fig S1K**). They also had a worse lung wet-dry ratio (**Fig 1H**) compared to WT mice. Similarly, lung inflammation was increased in the C3-deficient mice compared to WT mice, as they had an increased total BAL cell count (**Fig 1I**), and increased lung *Ccl2* and *Il1b* expression post-HKPa administration. Of note, C3-deficient mice treated with HKPa had a lower proportion of epithelial cells on flow cytometry of the lungs (**Fig 1K**), as well as lower expression of *Scgb1a1* and *Sftpc* compared to WT mice treated with HKPa (**Fig 1L**). These observations suggest increased epithelial cell loss post-infection in the setting of C3-deficiency, independent of the bacterial load.

C3 in the lungs is sourced locally

C3 increases in the BAL of both mice and humans following lung injury (23). To determine if this C3 is functional, we adapted our previously established in vitro complement assay, which considers both the activation (using LPS), and thereby, the function of the complement cascade (24–26). Functional complement activity in the serum of WT mice was intact both prior to and following 24 h post-*Pa* infection (**Fig 2A**). In comparison to serum, functional complement activity in the BAL of WT mice was upregulated several thousand-fold at 24 h post-*Pa* infection compared to BAL levels in uninfected mice (**Fig 2B**). These observations would suggest that functional complement activity is rapidly induced in an organ with low baseline activity in the first 24 h post infection. To determine the contribution of circulating C3 to that in the BAL, we administered normal mouse serum (NMS, obtained from WT mice) intraperitoneally to C3-deficient mice after intratracheal instillation of *Pa* (to induce injury) or PBS (control). NMS was

given either immediately after the *Pa* infection, and the mice were followed for 24 h prior to euthanasia, or it was given 1 h prior to euthanasia, by the time the injury had already occurred. Splenic bacterial dissemination was lower in mice administered NMS compared to C3-deficient serum (**Fig S2A**). C3 was detected in the BAL at 24 h after NMS administration only in the C3-deficient mice administered NMS who had alveolar-capillary barrier disruption in the setting of *Pa* pneumonia, not in those who had only received intratracheal PBS. (**Fig 2C**). However, C3 was not detected in the BAL at 1 h post NMS administration even if the lungs had been injured by *Pa*, despite being detected in the circulation. These observations suggest that following disruption of the alveolar-capillary barrier, circulating C3 entry into the alveolar space is delayed relative to local C3 secretion.

To quantify C3 expression and production by different cell types, we utilized C3 reporter mice with an IRES (internal ribosome entry site)-tdTomato cassette after the stop signal in the endogenous murine C3 locus, and also has the exons 37-41 sequence flanked by loxP sites enabling its conditional deletion upon the action of a Cre recombinase (15). Given that the liver is the major source of circulating C3, we bred these mice to generate an in vivo model of C3 deficiency restricted to the liver ($C3^{f/f}; Alb-Cre^{+/-}$) (**Fig 2D**). We confirmed these mice had no expression of C3 in the liver (**Fig 2E**) and had undetectable levels of circulating C3 compared to littermate controls and WT mice both pre and post *Pa* (**Fig 2F**). However, C3 was detectable (albeit in smaller amounts compared to littermate controls) in the BAL of infected mice at 24 h (**Fig 2G**).

Although there was no functional complement activity in the circulation of the $C3^{f/f}; Alb-Cre^{+/-}$ mice as compared to their littermate controls prior to infection (**Fig 2H**), BAL from $C3^{f/f}; Alb-Cre^{+/-}$ mice was still functional (**Fig 2I**). Post-infection, there was no increase in the functional complement activity in the circulation of the $C3^{f/f}; Alb-Cre^{+/-}$ mice as compared to pre-infection levels (**Fig 2H**). In comparison, the functional activity of the BAL increased at 24 h post-infection in the $C3^{f/f}; Alb-Cre^{+/-}$ mice (**Fig 2I**). Taken together, these observations suggest that although circulating C3 is functional in the lung in the setting of an acute infection, sources of C3 other than the liver also contribute to the C3 function in the lung.

Given the lack of both detectable circulating C3 in the $C3^{f/f}; Alb-Cre^{+/-}$ mice nor any functional complement activity in the serum, we hypothesized that the functional complement activity in the BAL of the $C3^{f/f}; Alb-Cre^{+/-}$ mice was generated from the lung. The presence of a thoroughly functional extra-hepatic source of C3 was corroborated by lower splenic dissemination of bacteria in the $C3^{f/f}; Alb-Cre^{+/-}$ mice as compared to the global C3-deficient mice, but higher in these mice compared to their littermate controls (**Fig 2J**). These findings would suggest that although liver-derived C3 is necessary for limiting bacterial dissemination, sources of C3 other than those from the liver also have an important role. Moreover, there was no difference in either the lung wet-dry ratio (**Fig 2K**), the BAL cell count (**Fig 2L**) or histological evidence of injury (**Fig S2B**) in mice deficient in liver-derived C3 as compared to their littermate controls at 24 h post-*Pa* infection, although it was reduced when compared to global C3-deficient mice. These observations suggest lung-derived C3 played a key role in mitigating bacterial pneumonia-induced lung injury. No differences were observed in BAL cytokine levels in mice deficient in liver-derived C3 as compared to their littermate controls at 24 h post-*Pa* infection (**Fig S2C-F**).

Non-immune cells contribute to C3 in the lung

To evaluate sources of C3 in the normal human lung, we analyzed publicly available single-cell RNASeq datasets from the Lung Gene Expression Atlas and LungMAP (27–30). Our analysis showed that, although most cells expressed C3 transcripts, major sources of C3 in the lungs are epithelial cells, fibroblasts and, to a lesser extent, monocytes/macrophages (**Fig 3A and S3A**). To identify cell-intrinsic production of C3 in the lungs in the absence of circulating C3, we profiled C3 producing non-immune cells in liver-deficient C3 mice ($C3^{f/f}; Alb-Cre^{+/-}$) infected with *Pa*, who have an IRES-Td tomato cassette after the stop signal in the endogenous murine C3 locus (C3 expression reporter mice). In these mice, C3 increased by approximately 3-fold in airway epithelial cells and Type 1 alveolar epithelial cells, and increased by nearly 4-fold from baseline in Type 2 alveolar epithelial cells, fibroblasts and endothelial cells at 24 h post-*Pa* infection (**Fig 3B and S3B**). We had previously shown that lung epithelial cells expressing SCGB1A1 (i.e., CCSP) are a major source of C3 in humans, especially in diseases associated with recurrent infections such as cystic fibrosis (16), and we were interested in how C3 from cells at the mucosal barrier surface affect the host response to pneumonia. Hence, we generated $C3^{f/f}; Scgb1a1-CreER^{T2+/-}$ mice to specifically delete C3 produced by these epithelial cells (club cell deficient-C3 mice); hereafter, termed as mice deficient in ‘lung-derived C3’ (**Fig 3C and S3C**). Successful deletion of C3 from club cells was confirmed using immunofluorescence for C3 and Cyp2f2, a club cell marker (**Fig 3D**) (31). These mice deficient in lung-derived C3 had similar levels of C3 in their circulation and BAL as their littermate controls (**Fig 3E and 3F**). They also had similar serum and BAL functional complement activity compared to their littermate controls (**Fig 3G and 3H**).

Epithelial (club) cell-derived C3 protects against severe bacterial pneumonia.

$C3^{fl/f}$; *Scgb1a1*-CreER^{T2/+} mice had worse acute lung injury after *Pa* infection compared to their littermate controls, as represented by measures of alveolar-capillary barrier disruption (**Fig 4A and 4B**). They also had worse histology (**Fig 4C**) and an exaggerated inflammatory response as compared to their littermate controls, as measured by BAL cytokine levels of IL-1 β , IL-6, KC and TNF- α (**Fig 4D-G**). Despite there being no significant differences in the systemic bacterial dissemination (**Fig 4H**), there was increased epithelial cell injury in these mice deficient in lung-derived C3 compared to their littermate controls, as measured by a marked increase in BAL RAGE levels (**Fig 4I**), as well as increased epithelial cell death, as measured by an increase in BAL cytokeratin-18 (CK-18) levels (**Fig 4J**).⁽³²⁾ These findings suggest that lung-derived C3 reduces *Pa*-induced lung injury despite adequate circulating (i.e., liver-derived) C3 levels.

Complement Factor B (FB) protects against stress-induced epithelial injury both in vitro and in vivo.

Upon activation, C3 is cleaved to form C3a, which binds to the C3a receptor (C3aR), and C3b fragments, which can opsonize targets but also bind to Factor B (FB) to form a C3 convertase (C3bBb) and amplify the alternative pathway of the complement cascade (33). To assess which of these putative targets may be facilitating the protective effects of C3 in the lung, we infected global C3aR-deficient (*C3aR*^{-/-}) and FB-deficient (*Cfb*^{-/-}) mice. C3aR-deficient mice were relatively protected from *Pa*-induced ALI and had a pattern of injury that was similar to the WT mice (**Fig 5A and 1A**). In comparison, FB-deficient mice had more severe injury (**Fig 5A**). Increased lung stiffness was observed at 24 h post-infection in FB-deficient mice compared to WT mice, as demonstrated by a worse dynamic compliance (**Fig 5B**) and an increased resistive

index (**Fig S4A**). There was increased alveolar-capillary barrier disruption post-infection as evidenced by an increased lung wet-dry ratio in FB-deficient mice compared to WT and C3aR-deficient mice (**Fig 5C**). FB-deficient mice had increased inflammation post-infection, as measured by *Il1b* (**Fig 5D**) and *Ccl2* (**Fig S4B**) expression, and increased number of Ly6C^{hi} monocytes in the lungs of FB-deficient mice compared to WT mice (**Fig S4C**). FB-deficient mice also had increased epithelial cell loss, measured by lung *Scgb1a1* (**Fig 5E**), *Sftpc* (**Fig S4D**) and *Cdh1* (**Fig S4E**) expression, and increased epithelial cell death compared to WT mice as measured by BAL CK-18 levels (**Fig S4F**) and confirmed using amine dye exclusion on flow cytometry (**Fig S4G**).

A similar pattern of increased lung injury in the setting of FB deficiency was observed in the setting of HKPa administration. FB-deficient mice had worse lung injury on histology (**Fig 5F**), as well as an increased wet-dry ratio as compared to WT mice (**Fig 5G**). These mice also had significantly higher expression of *Il1b* (**Fig 5H**) and *Ccl2* (**Fig S4H**) in their lungs, which paralleled our findings in C3-deficiency. Importantly, FB-deficient mice had decreased *Scgb1a1* (**Fig 5I**), *Sftpc* (**Fig S4I**) and *Cdh1* (**Fig S4J**) expression in their lungs after HKPa administration as compared to WT mice, suggesting epithelial cell loss in the setting of injury.

Given that *Pa* induces a bronchopneumonia, we sought to model these interactions between C3, C3aR and FB in primary human airway epithelial cells. We have previously reported that C3 increases in the primary human tracheobronchial epithelial cells (hTECs) with stress (16). In hTECs infected with *Pa*, FB biosynthesis increased post-infection but the levels of C3aR remained unchanged (**Fig 5J**). In line with these observations, C3 colocalized with FB in the

setting of an acute *Pa* infection; in contrast, its colocalization with the C3aR was not observed (**Fig 5K**). Hence, we subsequently sought to characterize the consequences of FB-deficiency and C3aR-deficiency in vitro in human lung epithelial cells using CRISPR-Cas9 deletion (**Fig S4K and S4L**). In an in vitro model of oxidative stress, FB-deficiency was associated with increased cell death compared to the parent clones over time (**Fig S4M**). By comparison, C3aR-deficiency was protective against H₂O₂-induced cell death, especially within the first 24 h (**Fig S4N**). Comparing RNA sequencing (RNA-Seq) of these *Pa* infected FB-deficient cells with their infected FB-sufficient parental line identified cell death as a major (central) theme, utilizing COMPBIO, an ontology-free analysis approach. (www.percayAI.com) (**Fig 5L**). ‘Cell death’ shared common biology with two other themes, ‘autocrine-paracrine signaling’ and ‘extracellular matrix (ECM) proteoglycans’ (**Fig 5M**), all of which were present in the top 5 enriched themes in the analysis between FB-deficient cells and their parent clones. These three themes shared a common signature consisting of 18 genes, and their contributions towards each theme are highlighted in the pseudoheatmap (**Fig 5M**). Correspondingly, FB-deficiency was associated with increased programmed cell death, evident by increased cleaved caspase-3 and cleaved PARP levels post-*Pa* infection (**Fig 5N**) and was paralleled by similar changes in the setting of oxidative stress (**Fig 5O**). These findings suggest that intracellular FB facilitates lung epithelial cell survival under stress by mitigating programmed cell death.

DISCUSSION

The effects of the complement system have historically been attributed to its presence in the circulation. However, our understanding of its components being synthesized by tissues and extra-hepatic organs has evolved to recognize that cells at mucosal surfaces may have an intrinsic complement system for host defense (34–38). Leveraging advances in complement biology, model systems and technologies, we demonstrate that: (1) C3 protects against acute bacterial pneumonia-induced lung injury; (2) C3 can be sourced locally, independent of that derived from the liver and present in the circulation; (3) structural cells of the lung such as epithelial cells contribute to C3 activity locally; (4) epithelial cell-derived C3 protects against severe bacterial pneumonia; and (5) this protection is potentially occurring via an intracellular complement Factor B (FB), akin to canonical complement activation that occurs extracellularly (33), but with important physiological functions necessary for cell survival. These observations put into perspective a long-standing premise that complement — an evolutionary conserved defense system — may have originated intracellularly and evolved extracellularly with the complexity of the host (39).

Liver-derived complement proteins form a necessary component of host defense, as evidenced by recurrent infections in patients with cirrhosis (9, 40). To that effect, we found that splenic dissemination of bacteria was significantly higher in the mice deficient in liver-derived C3 compared to controls, suggesting its role in enhancing immune resistance. However, there are two specific issues in this context that need consideration: First, that functional complement activity was observed in the bronchoalveolar lavage fluid of mice deficient in liver-derived C3 in the setting of infection, despite no activity in the circulation and, second, that splenic bacterial

burden in the mice deficient in liver-derived C3 was significantly lower than that observed in global C3-deficient mice. These observations would suggest that extra-hepatic sources of C3 are induced and are important in controlling bacteremic pneumonia.

We and others have previously reported that lung epithelial cells produce and secrete C3, and this secretion is apically polarized, which may facilitate host defense at the air-liquid interface (16, 19, 41, 42). Given our finding that C3 is upregulated in lung epithelial cells under stress in vitro, in the infected airways of people with cystic fibrosis, (16), we genetically depleted C3 specifically from lung epithelial cells that are known to be major sources of C3, *Scgb1a1*⁺ club cells (43). The severity of bacterial pneumonia in these mice suggests that lung epithelial cell-derived C3 is necessary for protection against lung injury despite adequate circulating C3 levels. Cell-intrinsic C3 thus affects fundamental cellular processes such as survival, which until now had primarily been demonstrated in vitro (16, 17).

C3 is cleaved to C3a and C3b upon cleavage by enzymes. C3a binds to C3aR, functions as an anaphylatoxin and also influences cellular functions such as cytokine production by T cells (44). C3b, on the other hand, is as an opsonin but also forms an enzyme called the “C3 convertase” with Factor B (C3bBb) to amplify alternative pathway activation in the circulation (13, 33). Our in vitro and in vivo findings indicating that Factor B is necessary for cellular survival, thus, aligns with the concept of an intracellular convertase that has been suggested by others (19, 45) and is now strengthened by our findings. Given that C3a-C3aR interactions have previously been shown to facilitate the differentiation of naïve CD4⁺ T cells towards a T_H1 phenotype (44), as well as glucose-induced insulin secretion in human and mouse pancreatic islet cells (17, 46), our

findings on cytoprotective role of Factor B suggests that intracellular C3 may have multiple targets and functions based on the stressor and the cell type. These observations therefore, create a precedent for complement modulating ~~certain~~ cellular processes in a stimuli-specific and cell-specific manner, and accordingly targeting therapies based on the desired effect.

There are certain limitations of our study. First, we deleted C3 from Scgb1a1⁺ cells based on our prior findings that (a) these cells are an abundant source of C3 (16), (b) C3 forms protects both epithelial and alveolar cells from infection (47), and (c) *Pa* infection induces a bronchopneumonia (48). Future work will need to address whether the protective effects of lung-derived C3 in severe bacterial pneumonia is due to the biosynthesized C3 that is stored in cells, or is secreted by neighboring lung epithelial cells and is internalized (16, 21). Second, there are sources of C3 in the lungs other than lung epithelial cells, such as monocyte/macrophages, fibroblasts and mesenchymal stem cells (15, 49–51). Future studies should determine if delivering C3 to the lung via these other sources is sufficient to mitigating lung injury in a paracrine manner. Third, our current in vivo models lack the ability to detect the specific isoform of cytoprotective C3. There is an increasing acknowledgement of a secreted form of C3 that has the signal peptide versus an intracellular cytosolic form with an alternate start site downstream of the signal peptide (17). Distinguishing these two distinct forms of C3 may have therapeutic implications if one seeks to to augment C3 within the lung. Additionally, although we have shown that C3 may be exerting its protective effect through FB (a known canonical interaction), we do not exclude non-canonical intracellular interactions, such as how C3 interacts with ATG16L1 in pancreatic β cells (17) or in gut epithelial cells, thereby targeting the intracellular bacteria towards autophagolysosomes for degradation (11). Finally, we focused on the role of C3

in acute injury due to bacterial pneumonia (i.e., within 24 h). The effects of C3 may be different based on the stressor (19, 52, 53) and the temporal course of an infection (54).

In summary, we show that in addition to the liver, lung-derived C3 provides host response to pneumonia by promoting both immune resistance and tissue resilience. Among these sources, epithelial-cell derived C3 mitigates severe bacterial pneumonia. This protection appears to occur via Factor B, which is an important component of the alternative pathway of the complement cascade (55). Our observation of an intracellular Factor B protein thus adds to the growing knowledge of how intracellular complement proteins modulate fundamental processes such as cell survival (10, 11, 16, 44, 46), and importantly, provides a putative mechanism by which lung-derived C3 offers protection. An understanding of tissue-derived and intracellular complement proteins is necessary to reconcile prior trial results of complement therapeutics, a majority of which have primarily targeted circulating complement proteins and do not necessarily penetrate inside the cell (56, 57). Thus, we are optimistic that complement therapies targeting a specific organ can be re-evaluated in terms of local protein activities, and caution against the unintentional effects of cell-permeable complement inhibitors. In this manner, we will be able to optimally harness endogenously produced immune mediators including the the complement system to better combat diseases such as pneumonia-induced lung injury.

MATERIALS AND METHODS

Materials and Methods

A table following the ARRIVE guidelines for standard reporting of animal studies is included in the Supplementary Material (**Table S1**) (58).

Study Design

Research objectives. The primary objective of the research was to assess whether C3-deficiency increases the risk of severe pneumonia occurring in the setting of *Pseudomonas aeruginosa* infection. The secondary objectives included (i) assessing whether mice deficient in liver-derived C3, and mice deficient in lung-derived C3 were at increased risk of severe pneumonia compared to their littermate controls, and (ii) whether the protective effects of C3 occurred via C3aR and/or FB.

Research subjects or units of investigation. All animal studies were conducted on protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Washington University School of Medicine (WUSM). Mice (8–12 weeks of age) on a C57BL/6 background were used for all experiments. Commercially available mice (Jackson Laboratories, Bar Harbor, ME) purchased included: wildtype (WT, 000664) and B6.Cg-*Speer6-psI*^{Tg(Alb-cre)21Mgn}/J (Alb-Cre, 003574) mice. C3-deficient, C3aR-deficient and Cfb-deficient mice were maintained in colonies at WUSM (55, 59, 60). Scgb1a1-CreER^{T2+/-} mice were generated by breeding an Scgb1a1-CreERTM (B6N.129S6(Cg)-Scgb1a1^{tm1(cre/ERT)Blh}/J, strain # 016225) to an Ai9 mouse (B6.Cg-*Gt(ROSA)26Sor*^{tm9(CAG-tdTomato)Hze}/J, strain# 007909). C3^{f/f} IRES-tdTomato mice were obtained from Dr. Claudia Kemper at the National Institutes of Health (15), and bred

with the Alb-Cre mice to create mice deficient in liver-derived C3, and with the Scgb1a1-CreER^{T2+/-} mice to generate mice deficient in lung-derived C3.

Human tissue use has been reviewed by the Institutional Review Board of Washington University. Primary human tracheobronchial epithelial cells (hTEC) and BEAS-2B lung epithelial cells (CRL-9609, ATCC, Manassas, VA) were used for in vitro experiments. hTECs were isolated from the surgical excess of tracheae and proximal bronchi of lungs donated for transplantation. Tissues from deceased individuals are exempt from regulation by HHS regulation 45 CFR Part 46. The method for cell culture, including media composition, has been described in detail previously (16). All cell cultures were maintained at 37 °C in 5% CO₂.

C3aR- and FB-deficient BEAS-2B cells were generated by CRISPR nuclease-induced targeted double strand break at the Genome Engineering Center at WUSM. *C3aR* only has one coding exon; hence, the gene was knocked out by using two gRNAs to delete the whole open reading frame (**Fig S4G**). *CFB* was disrupted by introducing out-of-frame indels in all alleles so that the mRNA would be degraded by nonsense mediated decay (**Fig S4H**). These cells were confirmed to be mycoplasma-negative prior to experimentation and were compared to their parent clones in all experiments.

Experimental design. All data are reported from controlled laboratory experiments. Mice were infected with *Pseudomonas aeruginosa* (*Pa*) M57-15, a clinically relevant strain obtained from the sputum of a patient with cystic fibrosis (61). The mice were infected with *Pa* for 24 h before euthanasia for blood, bronchoalveolar lavage fluid and tissue harvest. Lungs were harvested either for the wet-dry ratio, BAL collection, flow cytometry, bacterial colony counts, or as a

whole organ for fixation in 10% formalin before submission for histology. Spleens were harvested for colony counts. BAL was collected from the mouse lungs using 0.5-1 ml PBS with 1X Halt Protease Inhibitor Cocktail (diluted from 100X; #78429, Thermo Fisher). After collection, the BAL was spun down at 500 g for 5 min before the supernatant was collected and stored at -80 °C for further experiments.

For serum reconstitution experiments, normal mouse serum (NMS, Complement Technology, Inc.) was administered to C3-deficient mice at a dose of 10 µL/g body weight intraperitoneally at the time points noted in the figure legend.

For in vitro experiments, the same *Pa* was used at different multiplicity of infection (MOI) and for different periods of time as indicated in the figure legends. Hydrogen peroxide (H₂O₂, H1009, Sigma, St. Louis, MO) was freshly prepared to a concentration of 1000 µM and used for in vitro experiments.

Sample size. Mouse experiments included at least 5 mice in each group unless animal death occurred during the procedure. Each experiment was repeated at least twice. The effect was not attributed to the differences between the groups, unless specified. Primary cell culture experiments included at least three different donors. RNA sequencing experiments from BEAS-2B cells included 3 samples in each group.

Replicates. Each experiment was performed at least twice, in most cases, by two independent authors.

Blinding. Investigators who assessed, measured and quantified the results were blinded to the intervention.

Experimental Procedures

Genotyping. To confirm the genotypes, tips of the tails of the mice were collected before weaning. Oligomers for PCR amplification were designed to yield products of unique size to identify deleted sequences from *C3*, *C3aR*, and *Cfb* as previously published (62, 63). PCR reaction for the digested tail samples is as follows: Step 1 at 94 °C for 4 min, Step 2: 94 °C for 1 min, Step 3: 68 °C for 1 min, Step 4: 72 °C for 1 min, Steps 2-4 repeated for 30 cycles, followed by Step 5 at 72 °C for 5 min, and reaction is concluded at 4 °C until samples are retrieved. Primers used are listed in **Table S3**. The band are visualized with gel electrophoresis using 2-3% agarose (#16500500, Thermo Fisher) gel with SYBR Safe dye (#S33102, Thermo Fisher).

Bacterial infection. *Pa* (M57-15 strain) was cultured on plates with LB broth with agar (#L7025-500TAB, Sigma-Aldrich) overnight at 37 °C from frozen stock. Liquid inocula for the mice infections were prepared by inoculating *Pa* from the plates in LB broth (#L3522, Sigma-Aldrich) overnight at 37 °C and keeping in a rotating shaker at 220 rpm for 16 h (logarithmic phase of infection). Subsequently, the bacteria were recovered by centrifugation at 3000 g for 6 min at 4 °C, resuspended in 10 ml sterile PBS, centrifuged again and resuspended a final time in 10 ml PBS. Several dilutions (neat, 1:2, 1:5, 1:10) were prepared in PBS and the absorbance was measured at 380 nm (Epoch Microplate Spectrophotometer, BioTek) to calculate the amount needed to make the required dose using the bacterial growth standard curve.

For in vivo experiments, all mice were infected at a dose of 2×10^5 CFU/g of body weight, with the bacteria being in their logarithmic phase of growth at 16-18 h post-incubation in previously autoclaved Luria broth, and were euthanized at 24 h. To prepare heat-killed *Pa* (HK*Pa*), the

stock solution was incubated at 100 °C for 30 min and frozen at -80 °C till use. First, mice were anesthetized with a cocktail of ketamine (100mg/kg) and xylazine (10mg/kg), intraperitoneally. They were orotracheally intubated utilizing a fiber-optic cable connected to a light source (BioLite Intubation System, BIO- MI-KIT, Braintree Scientific, Braintree, MA to facilitate insertion of a Surflo 20-gauge catheter (# SR-OX2025CA, Terumo Medical Products, Somerset, NJ) within the trachea. A 2×10^5 CFU/ μ L suspension of bacteria (or equivalent dose of HKPa) in sterile PBS was introduced into the catheter at a volume of 1 μ L per gram of body weight. After 24 h, euthanasia was carried out using avertin (2% working solution of avertin is prepared from 100% avertin, which is made by mixing 1 g of 2,2,2-tribromoethyl alcohol (#T48402, Sigma-Aldrich) with 1 ml of tert-amyl alcohol (#240486, Sigma-Aldrich)),

For in vitro infections, the bacterial cultures were suspended in DMEM (Dulbecco's Modified Eagle's Medium, #10-017-CV, Corning, NY) supplemented with 10% heat-inactivated fetal bovine serum (#26140079, Thermo Fisher) containing no antibiotics. Pa infection was carried out for different time points at the indicated multiplicities of infection (MOIs) as described in the figure legends.

Outcome Measures

Assessment of functional complement activity in the serum and BAL. We adapted a previously published assay in the serum (25). For its use with BAL, ELISA plates (96-well flat-bottom, #3855, Thermo Fisher) were coated with LPS (2 μ g/well in 100 μ L; #L2762, Sigma-Aldrich) overnight at 4 °C. After washing three times with a solution of 0.05% Tween 20 in PBS, samples (serum 1:10, BAL 1:5) in Mg²⁺-EGTA buffer were added 50 μ L/well, and

incubated at 37 °C for 1 h. The plates were washed again three times and 100μL/well goat anti-mouse C3 (#55463, MP Biomedicals) diluted in 1% bovine serum albumin (BSA, #A7906, Sigma) in PBS (1:4000 in 1% BSA/PBS) was added for 1 h. After another three washes, the samples were incubated with 100μL/well HRP-conjugated donkey anti-goat IgG antibody (1:2000 in 1% BSA/PBS) (#705-035-147, Jackson ImmunoResearch Laboratories) for 1 h. After three washes, TMB Color Substrate (#DY999, R&D Systems) was added 100μL/well and incubated at room temperature (RT) for 10 min. The reaction was stopped by the addition of 50μL/well of 2N H₂SO₄ (#DY994, R&D Systems), and the OD of the samples were measured at 450 nm (Epoch Microplate Spectrophotometer, BioTek).

Assessment of acute lung injury (ALI). ALI was assessed using multiple domains (64). To assess alveolar-capillary barrier disruption, we used the lung wet-dry ratio and/or BAL protein levels. To assess lung inflammation, we used flow cytometry, BAL cytokine level measurements by ELISA, BAL cell counts, or transcriptional expression of certain inflammatory genes (e.g., *Ccl2*, *Il1β*) measured by qRT-PCR. To assess physiological dysfunction, we performed small animal whole body plethysmography. To assess histological evidence of injury, we obtained images at 20x magnification. To assess for epithelial cell death, we measured death using flow cytometry, BAL RAGE levels using an ELISA, or qRT-PCR expression of certain epithelial cell genes (e.g., *Scgb1a1*, *Sftpc*, *Cdh1*).

For the wet-dry ratio, the weight of the “wet” lungs was immediately measured right after euthanasia (#AB54-S, Mettler Toledo, Columbus, OH), and the value obtained was divided by their “dry” weight that was measured after 24 h in an 65 °C oven.

To assess physiological dysfunction, whole body phethysmography was performed. At 24 h post-infection, the mice were anesthetized with the same ketamine/xylazine cocktail as mentioned above and their tracheae were exposed and cannulated with a Surflo 20-gauge catheter (#SR-OX2025CA, Terumo Medical Products). The mice were connected by the cannula to the Buxco FinePointe Resistance and Compliance Station (Data Sciences International, Inc, St. Paul, MN) and their responses to inhaled methacholine (#A2251, Sigma Aldrich) were recorded in a dose-response study, starting from control PBS to increasing doses of nebulized methacholine (0.625, 1.25, 2.5, 5, 10, and 20 mg/ml in PBS), performing deep breaths. Changes in airway resistance (RI) and dynamic compliance (Cdyn) were recorded.

To assess systemic bacterial burden, spleens were harvested into 0.9 ml sterile PBS and homogenized in microtubes (#72.694.006, Sarstedt AG & Co, Nümbrecht, Germany) using stainless steel beads (11079132ss, Biospec, Bartlesville, OK), on a bead beater. The homogenate was serially diluted, plated on LB agar, and incubated at 37 °C overnight prior to colony counting. To assess local bacterial burden, lungs were harvested into 0.8 ml sterile PBS and processed similarly. The total volume of both the spleen and lung homogenate was 1 ml.

Flow cytometry. To obtain a single cell suspension mouse lungs were digested using collagenase A (10103586001, Roche, 1.5 mg/ml), DNase I (D4527, Sigma, 0.1 mg/ml), 5% fetal bovine serum, 10 mM HEPES buffer (25-060-CI, Corning, Durham, NC) and PBS. Specifically, after perfusing the right atrium with 10 ml of 1X PBS, the lungs were inflated with 1 ml of the abovementioned digest solution, cut into 3-4 mm pieces using scissors, and gently vortexed in 2 ml of the digest solution. The digest was incubated at 37 °C for 40 min in a shaker incubator at

190 rpm, while gently vortexing the samples every 5-7 min. After adding 10 ml of ice-cold PBS, the digest was vortexed for 30 seconds, filtered through a 70 μ M strainer (431751, Corning) and spun at 500 g for 10 min at 4 °C. After discarding the supernatant, the cells were resuspended in 2 ml ACK Lysing buffer (A10492-01, Gibco) and incubated at RT for 4-7 min, after which they were resuspended in 10 ml of ice-cold 1% BSA in PBS and centrifuged again at 500 g for 10 min at 4° C. 1-2 x 10⁶ cells were added to a 96-well plate for staining using the antibodies listed in **Table S2**. Data was acquired on an Attune Nxt (14 color) laser (Thermo Scientific) and analyzed using FlowJo v10.8.0 (Becton, Dickinson and Company; Ashland, OR).

Quantitative reverse-transcriptase polymerase chain reaction. mRNA extraction was performed using the RNeasy Plus Mini kit (#74134, QIAGEN, Germantown, MD) per the manufacturer's protocol and cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (#4368814, Thermo Scientific) according to manufacturer's recommendations. cDNA sample was diluted by 5-fold following cDNA synthesis and real-time PCR of each gene was carried out in duplicate on a Roche LightCycler 480 real-time PCR instrument. Each PCR reaction contained 2 μ L of cDNA template, 0.6 μ L of forward and reverse primers (with the final concentration of 0.5 μ M/well), 4 μ L of the SYBR Green master mix (A46109, Applied Biosystems), and 5.4 μ L of RT-PCR grade H₂O. All primers were ordered from Integrated DNA Technologies (**Table S4**). The PCR program was run as follows: an initial pre-incubation step at 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 10 seconds, annealing and elongation at 60 °C for 20 seconds, and the fluorescence reading at 72 °C for 1 second. Ct values were read and exported from the LightCycler 480 software, and the

commonly used $2(-\Delta\Delta Ct)$ method was used to calculate relative gene expression using *Gapdh* or *Hprt1* as housekeeping gene(65). Data are shown are adjusted to the housekeeping gene.

Enzyme-linked immunosorbent assays (ELISA). For assessing complement activity in the mouse serum and BAL, we adapted the LPS assay as reported in a prior section. Serum was obtained before and after infection from the same mice; however, BAL was obtained from different mice in the uninfected and infected groups, as the BAL procedure is terminal.

For the BAL protein levels (i.e., BCA assay), fresh or thawed BAL samples were diluted (1:10 to 1:50 in PBS) to fall within the range of the recommended standard curve of the assay, before the total protein level was quantified using Pierce™ BCA Protein Assay Kit (#23227, Thermo Fisher) according to provided protocol.

To assess cytokine levels in the mouse serum and BAL, we used the a multianalyte assay (LEGENDplex Mouse Inflammation Panel; #740446, BioLegend, San Diego, CA) according to the provided protocol.

To assess epithelial cell injury in the mouse BAL, the Mouse RAGE DuoSet ELISA (#DY1179, R&D Systems, Minneapolis, MN) was used according to the provided protocol. The BAL samples were either diluted 1:2 or 1:4 based on known protein levels, but in a single run, the dilutions were the same. To assess epithelial cell death in the mouse BAL, the Mouse Cytokeratin 18 ELISA (#ab243678, Abcam, Cambridge, United Kingdom) was used according to the provided protocol. The BAL samples were diluted 1:2 for this assay.

Immunoblotting. Samples obtained from mice were diluted in PBS at a ratio of 1:10 for plasma and 2:10 for BAL. Laemmli sample buffer (1610747, Bio-Rad, Hercules, CA) was added to each sample and boiled for 5 min at 100 °C in a heat block for complete denaturation of protein. To prepare cell lysates for immunoblotting, cells were washed in ice-cold PBS and lysed using lysis buffer (9803, Cell Signaling Technology, Danvers, MA) supplemented with Halt Protease Inhibitor Cocktail (78429, Thermo Scientific, Waltham, MA) for 20 min on ice. Lysates were cleared by centrifugation (14,000 g) for 10 min and cooked in SDS-denaturing Laemmli sample buffer for 5 min. Cell lysates were separated on SDS-PAGE (Novex™ WedgeWell™ 4 to 20%, XP04202BOX, Thermo Fisher) in 1X Tris-Glycine-SDS buffer (BP1341-4, Fisher Scientific, Waltham, MA) and transferred using electrophoresis to nitrocellulose membranes by using the iBlot 2 Gel Transfer Device (Thermo Scientific, iBlot 2 NC Regular Stacks, IB23001, Invitrogen, Walham, MA). Membranes were blocked for 1 h at RT in 5% (w/v) nonfat dry milk in TBST (20mM Tris-HCl (pH 7.5), 150mM NaCl and 0.1% Tween 20) and incubated at 4 °C overnight with the antibodies in 5% non-fat dairy milk (w/v) in TBS-T (listed in **Table S2**). For beta actin staining, the membrane was stripped and reprobed. The stripping buffer was prepared by mixing 0.7 ml of β -mercaptoethanol, 2 g SDS and 2.5 ml of 1 M Tris-HCl (pH 6.8) in 100 ml of deionized water. The membrane was incubated for 30 min at 55 °C on a shaker in stripping buffer with gentle agitation followed by washing with TBS-T. The membrane was then blocked and detection with antibody was carried out as described above.

Immunostaining. Cells were plated on cover slips in 24 well plates at a seeding density of 10^5 cells/well overnight prior to the experiment. After treating with *Pa*, cells were fixed by directly adding fixative solution (10% neutral buffered formalin, prepared by mixing 100 ml

formaldehyde (37-40%), 900 ml distilled water, sodium phosphate (monobasic) 4 g/L and sodium phosphate (dibasic, anhydrous) 6.5 g/L) equal in amount to the medium present in the well. After removing the media and fixative solution after 2 min, 500 μ l of fixative solution was again added to the cells and incubation for 20 min at RT. The solution was aspirated and cells were then washed twice with PBS. Cells were permeabilized by adding 0.05% Triton-X 100 (T8787, Sigma, St. Louis, MO) in PBS for 5 min at RT, followed by washing twice with PBS. Cells were blocked by adding 2% bovine serum albumin (9048-46-8, Santa Cruz Biotechnology) in PBS for 30 min at RT followed by washing twice with PBS. After blocking, cells were incubated with primary antibody in 5% donkey serum in 2% BSA in PBS at 4 °C overnight. Cells were subsequently washed thrice with PBS and incubated with secondary antibody at a dilution of 1:200 in 2% BSA for 60 min at RT in the dark. After washing with PBS thrice, the cover slips were mounted using VECTASHIELD Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA). After drying for a few hours, cells were imaged using Zeiss LSM880 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) at 62X oil magnification.

Immunofluorescence staining of the lungs was performed on paraffin sections. Prior to staining, paraffin embedded lungs tissue sections were deparaffinized using xylene, followed by decreasing concentrations of ethanol. Specifically, slides were dipped in the following sequence for 3 minutes in each solution: 100% xylene, followed by 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol followed by washing in MilliQ water for hydration and heat-treated with Ag unmasking solution (#H3300, Citrate-Based, Vector Laboratories). Immunostaining was performed with different primary antibodies overnight at 4 °C (**Table S2**). The next day, sections

were incubated using Alexa Fluor-conjugated secondary antibodies (**Table S2**) and counterstained using VECTASHIELD Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA). After drying for a few hours, cells were imaged using Zeiss LSM880 laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY) at 63X oil magnification.

Live-cell imaging. Cell death was analyzed using an IncuCyte Live Cell Imager (Sartorius, Goettingen, Germany) at the Genome Engineering and iPSC Center at Washington University. The ratio of SYTOX Green Nucleic Acid Stain (1:10,000, S7020, Invitrogen) and SYTO 24 Green Fluorescent Nucleic Acid Stain (1:1000, S7559, Invitrogen) was reported over time, averaged over 4 independent fields in each well of a 96-well plate, with 4 technical replicates per time point.

RNA extraction and sequencing. RNA was isolated from lung epithelial cells by using the RNeasy plus Mini Kit (74134, QIAGEN). The starting number of cells used for extraction was about 8×10^6 . Cells were washed twice with ice-cold PBS, scraped in 1 ml of PBS and centrifuged at 3000 x g for 5 min at 4 °C. The pellet was homogenized using the kit reagents and processed as per the insert. Isolated RNA was sent to Genome Technology Access Center at the McDonnell Genome Institute (GTAC@MGI), WUSM for RNA sequencing. Poly-A selection was used for library preparation and sample processing. A target of at least 30 million reads per library utilizing NovaSeq S4, 2x150 reads was accomplished. Dual indexing during library construction was used to uniquely identify each sample.

Analysis of RNA Sequencing data from BEAS-2B cells. Differentially expressed genes (1.6 fold, adjusted p value <0.05) were further analyzed by a novel knowledge engine called ‘COMprehensive Multi-omics Platform for Biological InterpretatiOn’ (COMP BIO V2.0) (66). COMP BIO performs a literature analysis to identify relevant processes and pathways represented by differentially expressed, or otherwise related, biological entities (genes, proteins, miRNA’s, or metabolites) or their synonyms using Pubmed abstracts. Knowledge is extracted in an ontology-free manner and maintained in a Persistent Contextual Memory Model (PCMM) that can be aggregated, normalized and computed across from any stored dimension. Conditional probability analysis is utilized to compute the statistical enrichment of contextually associated biological concepts (processes/pathways) over those that occur by random sampling. These related concepts built from the input entities are further clustered into higher-level themes (e.g., biological pathways/processes, cell types and structures, etc.).

Genes, concepts, and theme enrichment is calculated using a multi-component function referred to as the Normalized Enrichment Score (NES) which reflects both the rarity of the concept event associated with an entity list, as well as its degree of overall enrichment. Entity-concept scores above 10.0, 100.0, and 1,000.0 are labeled as moderate, marked, or high in level of enrichment above random. Theme scores are then computed from their constituent entity-concept NES scores. As such, theme enrichment scores above 10,000, 20,000, and 30,000 are identified as having moderated, marked and high levels of entity-concept enrichment, respectively.

Concepts are biologically relevant words or names that have been collected from a multitude of sources of biological language (text) or entities. Within COMP BIO themes, concepts and their

relationships are computed in a fully automated fashion. The themes generated by COMPBIO can be annotated with a summary level description of the biological processes and pathways that they represent. This can be achieved either by semi-automated annotation or human assessment. Semi-automated annotation occurs through a comparison of, up to, the 10 most central concepts of each theme with pathways and processes in common knowledge bases such as GO, Reactome, and several others. The most closely associated entries are computed and presented, with score, for annotation of theme. However, as COMPBIO is not restricted to these knowledge bases in its biological discovery process, some themes can only be annotated with human assessment. In these cases, the process is similar in that the annotation is typically a summary description of the biology described by the most central concepts to the theme.

Single cell RNA sequencing analysis (scRNASeq) from LungMAP. The single cell reference program for human lungs was used on the Lung Gene Expression Atlas (LGEA, LungMAP Phase 2) web portal (27–30), available at:

https://research.cchmc.org/pbge/lunggens/tools/lung_at_glance.html?tab=reference.

Cell ontologies are obtained from the NHLBI Molecular Atlas of Lung Development Program Consortium (67). The LungMAP scRNASeq reference is associated with the Lung CellCards resource (30). This initial reference integrated 259k cells from 72 donors from five published (68–72) and one unpublished single cell RNA-seq cohort. It consists of non-diseased adult and pediatric healthy lung single-cell 10x Genomics captures (v2 and v3).

Statistical Analysis

Data are presented as scatter plots showing individual data points, and dispersion is shown by mean $\pm$ standard deviation. The unpaired t test was used for comparing two groups, assuming equal variance and the ordinary one-way ANOVA test with multiple comparisons testing was used for more than two groups. A p value of < 0.05 was considered significant, although values > 0.05 have been reported when there was evidence of a trend. Prism v9.3.0 (GraphPad, San Diego, CA) was used for statistical analysis.

SUPPLEMENTARY MATERIALS

Supplementary Figure 1. C3 protects against severe bacterial pneumonia.

Supplementary Figure 2. Deficiency in liver-derived C3 did not increase the predisposition to *Pseudomonas aeruginosa* (Pa)-induced acute lung injury.

Supplementary Figure 3. C3 in the lungs is produced locally.

Supplementary Figure 4. Complement Factor B (FB) protects against stress-induced epithelial injury both in vitro and in vivo.

Supplementary Table S1. ARRIVE Guidelines checklist for animal research.

Supplementary Table S2. Antibodies used for experiments.

Supplementary Table S3. Primers used for genotyping of mice.

Supplementary Table S4. Primers used for qRT-PCR of mouse lungs.

Table S1. ARRIVE Guidelines checklist for animal research.

	Item	Section/line number, or reason for not reporting
1.	Study Design	A general description of all strains used are provided in the section “Research subjects or units of investigation” under Methods. The groups being compared, including control groups, number of animals and the experimental units are reported in the Figure Legends for each figure.
2.	Sample Size	A general description of sample size has been provided in the section “Sample size.” under Methods. The number of animals and the experimental units are reported in the Figure Legends for each figure.
3.	Inclusion and Exclusion Criteria	For <i>C3^{-/-}</i> mice, males were used as reported in Figure S1. For <i>C3aR^{-/-}</i> and <i>FB^{-/-}</i> mice, there was no effect of sex on the phenotype and hence, animals of both sexes were used.
4.	Randomisation	No specific randomization schema was used. However, to minimize potential confounders, mice were infected and euthanized alternately (e.g., Group 1 mouse 1, Group 2 mouse 1, Group 1 mouse 2, Group 2 mouse 2...)
5.	Blinding	The person collecting the data (i.e. outcome assessment) for each experiment was blinded to the group allocation.
6.	Outcome Measures	Listed under the “Outcome Measures” section in the Methods.
7.	Statistical methods	Listed under the “Statistical Analysis” section in the Methods.
8.	Experimental Animals	Species-appropriate details have been provided under “Research subjects or units of investigation” in the Methods section.
9.	Experimental Procedures	Detailed procedures to facilitate replication, including rationale, have been described under the section “Experimental Procedures” in the Methods section.
10.	Results	All figures have been represented as scatter plots with individual data points, and range is represented as mean $\pm$ standard deviation.

Table S2. Antibodies used for experiments

Antibody	Supplier	Catalog #	Dilution
Immunoblotting			
Goat anti-C3	MP Biomedicals	55444	1:3000
Goat anti-Factor B	Complement Technology, Inc.	A235	1:3000
Rabbit anti-caspase 3	Abcam	ab13847	1:1000
Rabbit anti-PARP	Cell Signaling Technology	9542	1:1000
Mouse anti-beta actin	Santa Cruz Biotechnology	sc-47778	1:2000
Anti-goat IgG, HRP-linked	Thermo Fisher	31402	1:4000
Anti-mouse IgG, HRP-linked	Cell Signaling Technology	7076	1:4000
Anti-rabbit IgG, HRP-linked	Cell Signaling Technology	7074	1:4000
Immunofluorescence			
Chicken Anti-C3 (human)	Sigma	GW20073	1:100
Rabbit anti-C3aR (human)	Invitrogen	PA5-29979	1:100
Goat Anti-Factor B (human)	Quidel	A311	1:100
Rabbit anti-C3 (mouse)	Antibody Verify	AAS72600	1:50
Rabbit anti-Scgb1a	Seven Hills Bioreagents	WRAB-3950	1:100
Rabbit anti-Prosurfactant Protein C (proSP-C)	Millipore Sigma	AB3786	1:100
Mouse anti-Cyp2f2	Santa Cruz	sc-374540	1:100
Goat anti-Chicken IgY (H+L) Secondary, Alexa Fluor 647	Thermo Fisher	A-21449	1:300
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary, Alexa Fluor 555	Thermo Fisher	A-21432	1:300
Donkey anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary, Alexa Fluor 488	Thermo Fisher	A-21206	1:300
Donkey anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary, Alexa Fluor 555	Thermo Fisher	A-31572	1:300
Goat anti-Mouse IgG (H+L), Cross-Adsorbed Secondary, Alexa Fluor 647	Thermo Fisher	A-21235	1:300
Flow Cytometry			
BV711 anti-mouse CD45	Biolegend	103147	1:200
APC/Cy7 anti-mouse CD31	Biolegend	102440	1:100
BV510 anti-mouse CD326 (EpCAM)	Biolegend	118231	1:100
APC anti-mouse CD104	Biolegend	123612	1:100
PerCP/Cy5.5 anti-mouse PDPN (podoplanin)	Biolegend	127422	1:100
PE-Cy7 anti-mouse Ly-6A/E (Sca-1)	eBioscience	25-5981-81	1:100
Zombie (Zombie Violet Fixable Viability Kit)	Biolegend	423114	1:400

Table S3. Primers used for genotyping of mice.

Allele	Sequences	Product
<i>C3^{fl}</i>	5`-AGTCAGAAAACCTCACGGTCCATCGACTAG-3`, 5`-TGCAAAGTCCCCAACCTCAGACCTAG-3`, 5`-CCTAGCACAAGAGACAGAGACAGCCTAGC-3`	3 primers KI product 259-bp WT product 197-bp
<i>Alb-Cre^{+/-}</i>	WT 5`- TGCAAACATCACATGCACAC-3`, common 5`- TTGGCCCCCTTACCATAACTG-3`, mutant 5`- GAAGCAGAAGCTTAGGAAGATGG-3`	3 primers, KI product 390-bp, WT product 350-bp
<i>Scgblal-CreER^{T2+/-}</i>	5`- GCATTACCGGTTCGATGCAACGAGGATGAG -3` 5`- GAGTGAACGAACCTGGTTCGAAATCAGTGCG -3`	2 primers, KI product 408-bp

Table S4. Primers used for qRT-PCR of mouse lungs.

Transcript	Sequences	Reference
<i>Ccl2</i>	Forward: 5`-AGGTCCCTGTCATGCTTCTG-3` Reverse: 5`-TCTGGACCCATTCTTCTTG-3`	(73)
<i>Ccl7</i>	Forward 5`-TGAAAACCCCAACTCCAAAG-3` Reverse 5`-CATTCCTTAGGCGTGACCAT-3`	(73)
<i>Il1β</i>	Forward 5`-GCAACTGTTCTGAACTCAACT-3` Reverse 5`-ATCTTTTGGGGTCCGTCAACT-3`	(74)
<i>Scgblal</i>	Forward 5`-ATCAGAGTCTGGTTATGT-3` Reverse 5`-ATCCACCAGTCTCTTCAG-3`	(75)
<i>Sftpc</i>	Forward 5`-CAGACACCATCGCTACCTTTTCC-3` Reverse 5`-AGAATCGGACTCGGAACCAGTATC-3`	(75)
<i>Cdh1</i>	Forward 5`-GAGAACGGTGGTCAAAGAGC-3` Reverse 5`-GCTGGCTCAAATCAAAGTCC-3`	(76)
<i>Gapdh</i>	Forward 5` -AATGGTGAAGGTCGGTGTG-3` Reverse 5` - GTGGAGTCATACTGGAACATGTAG-3`	(77)
<i>Hprt1</i>	Forward 5` -CACAGGACTAGAACACCTGC-3` Reverse 5`-GCTGGTGAAAAGGACCTCT-3`	(78)

REFERENCES AND NOTES

1. L. J. Quinton, A. J. Walkey, J. P. Mizgerd, Integrative Physiology of Pneumonia. *Physiol Rev* **98**, 1417–1464 (2018).
2. A. Torres, C. Cilloniz, M. S. Niederman, R. Menéndez, J. D. Chalmers, R. G. Wunderink, T. van der Poll, Pneumonia. *Nat Rev Dis Primers* **7**, 25 (2021).
3. B. T. Thompson, R. C. Chambers, K. D. Liu, Acute Respiratory Distress Syndrome. *N Engl J Med* **377**, 1904–1905 (2017).
4. M. A. Matthay, R. L. Zemans, G. A. Zimmerman, Y. M. Arabi, J. R. Beitler, A. Mercat, M. Herridge, A. G. Randolph, C. S. Calfee, Acute respiratory distress syndrome. *Nat Rev Dis Primers* **5**, 18 (2019).
5. C. S. Dela Cruz, S. E. Evans, M. I. Restrepo, N. Dean, A. Torres, I. Amara-Elori, S. Awasthi, E. Caler, B. Cao, J. D. Chalmers, J. Chastre, T. S. Cohen, A. H. Cohen, K. Crothers, Y. P. Di, M. E. Egan, C. Feldman, S. Gautam, E. S. Halstead, S. Herold, B. E. Jones, C. Luna, M. S. Niederman, R. Mendez, R. Menendez, J. P. Mizgerd, R. Nusrat, J. Ramirez, Y. Shindo, G. Waterer, S. M. Yeligar, R. G. Wunderink, Understanding the Host in the Management of Pneumonia. An Official American Thoracic Society Workshop Report. *Ann Am Thorac Soc* **18**, 1087–1097 (2021).
6. S. K. Sahu, D. H. Kulkarni, A. N. Ozanturk, L. Ma, H. S. Kulkarni, Emerging roles of the complement system in host-pathogen interactions. *Trends Microbiol* , S0966-842X(21)00213–4 (2021).
7. W. Bain, H. Li, R. van der Geest, S. R. Moore, T. F. Olonisakin, B. Ahn, E. Papke, K. Moghbeli, R. DeSensi, S. Rapport, M. Saul, M. Hulver, Z. Xiong, R. K. Mallampalli, P. Ray, A. Morris, L. Ma, Y. Doi, Y. Zhang, G. D. Kitsios, H. S. Kulkarni, B. J. McVerry, V. P. Ferreira, M. Nouraie, J. S. Lee, Increased Alternative Complement Pathway Function and Improved Survival during Critical Illness. *Am J Respir Crit Care Med* **202**, 230–240 (2020).
8. Y. Okura, I. Kobayashi, M. Yamada, S. Sasaki, Y. Yamada, I. Kamioka, R. Kanai, Y. Takahashi, T. Ariga, Clinical characteristics and genotype-phenotype correlations in C3 deficiency. *J. Allergy Clin. Immunol.* **137**, 640-644.e1 (2016).
9. A. Albillos, R. Martin-Mateos, S. Van der Merwe, R. Wiest, R. Jalan, M. Álvarez-Mon, Cirrhosis-associated immune dysfunction. *Nat Rev Gastroenterol Hepatol* (2021), doi:10.1038/s41575-021-00520-7.
10. J. C. H. Tam, S. R. Bidgood, W. A. McEwan, L. C. James, Intracellular sensing of complement C3 activates cell autonomous immunity. *Science* **345**, 1256070 (2014).
11. M. T. Sorbara, E. G. Foerster, J. Tsalikis, M. Abdel-Nour, J. Mangiapane, I. Sirluck-Schroeder, I. Tattoli, R. van Dalen, D. E. Isenman, J. R. Rohde, S. E. Girardin, D. J. Philpott, Complement C3 Drives Autophagy-Dependent Restriction of Cyto-invasive Bacteria. *Cell Host Microbe* **23**, 644-652.e5 (2018).

12. M. Torisu, T. Yokoyama, P. F. Kohler, A. L. Durst, G. Martineau, G. Schroter, H. Amemiya, C. G. Groth, T. E. Starzl, Serum complement after orthotopic transplantation of the human liver. *Clin. Exp. Immunol.* **12**, 21–30 (1972).
13. B. J. C. Janssen, A. Christodoulidou, A. McCarthy, J. D. Lambris, P. Gros, Structure of C3b reveals conformational changes that underlie complement activity. *Nature* **444**, 213–216 (2006).
14. I. Charlie-Silva, A. Klein, J. M. M. Gomes, E. J. R. Prado, A. C. Moraes, S. F. Eto, D. C. Fernandes, J. J. Fagliari, J. D. C. Junior, C. Lima, M. Lopes-Ferreira, K. Conceição, W. G. Manrique, M. A. A. Belo, Acute-phase proteins during inflammatory reaction by bacterial infection: Fish-model. *Sci Rep* **9**, 4776 (2019).
15. M. Kolev, E. E. West, N. Kunz, D. Chauss, E. A. Moseman, J. Rahman, T. Freiwald, M. L. Balmer, J. Lötscher, S. Dimeloe, E. C. Rosser, L. R. Wedderburn, K. D. Mayer-Barber, A. Bohrer, P. Lavender, A. Cope, L. Wang, M. J. Kaplan, N. M. Moutsopoulos, D. McGavern, S. M. Holland, C. Hess, M. Kazemian, B. Afzali, C. Kemper, Diapedesis-Induced Integrin Signaling via LFA-1 Facilitates Tissue Immunity by Inducing Intrinsic Complement C3 Expression in Immune Cells. *Immunity* **52**, 513-527.e8 (2020).
16. H. S. Kulkarni, M. L. Elvington, Y.-C. Perng, M. K. Liszewski, D. E. Byers, C. Farkouh, R. D. Yusef, D. J. Lenschow, S. L. Brody, J. P. Atkinson, Intracellular C3 Protects Human Airway Epithelial Cells from Stress-associated Cell Death. *Am. J. Respir. Cell Mol. Biol.* **60**, 144–157 (2019).
17. B. C. King, K. Kulak, U. Krus, R. Rosberg, E. Golec, K. Wozniak, M. F. Gomez, E. Zhang, D. J. O'Connell, E. Renström, A. M. Blom, Complement Component C3 Is Highly Expressed in Human Pancreatic Islets and Prevents β Cell Death via ATG16L1 Interaction and Autophagy Regulation. *Cell Metab* **29**, 202-210.e6 (2019).
18. J. Friščić, M. Böttcher, C. Reinwald, H. Bruns, B. Wirth, S.-J. Popp, K. I. Walker, J. A. Ackermann, X. Chen, J. Turner, H. Zhu, L. Seyler, M. Euler, P. Kirchner, R. Krüger, A. B. Ekici, T. Major, O. Aust, D. Weidner, A. Fischer, F. T. Andes, Z. Stanojevic, V. Trajkovic, M. Herrmann, A. Korb-Pap, I. Wank, A. Hess, J. Winter, V. Wixler, J. Distler, G. Steiner, H. P. Kiener, B. Frey, L. Kling, K. Raza, S. Frey, A. Kleyer, T. Bäuerle, T. R. Hughes, A. Grüneboom, U. Steffen, G. Krönke, A. P. Croft, A. Filer, J. Köhl, K. Klein, C. D. Buckley, G. Schett, D. Mougiakakos, M. H. Hoffmann, The complement system drives local inflammatory tissue priming by metabolic reprogramming of synovial fibroblasts. *Immunity* **54**, 1002-1021.e10 (2021).
19. B. Yan, T. Freiwald, D. Chauss, L. Wang, E. West, C. Mirabelli, C. J. Zhang, E.-M. Nichols, N. Malik, R. Gregory, M. Bantscheff, S. Ghidelli-Disse, M. Kolev, T. Frum, J. R. Spence, J. Z. Sexton, K. D. Alysandratos, D. N. Kotton, S. Pittaluga, J. Bibby, N. Niyonzima, M. R. Olson, S. Kordasti, D. Portilla, C. E. Wobus, A. Laurence, M. S. Lionakis, C. Kemper, B. Afzali, M. Kazemian, SARS-CoV-2 drives JAK1/2-dependent local complement hyperactivation. *Sci Immunol* **6** (2021), doi:10.1126/sciimmunol.abg0833.

20. H. S. Kulkarni, M. K. Liszewski, S. L. Brody, J. P. Atkinson, The complement system in the airway epithelium: An overlooked host defense mechanism and therapeutic target? *J Allergy Clin Immunol* **141**, 1582-1586.e1 (2018).
21. M. Elvington, M. K. Liszewski, P. Bertram, H. S. Kulkarni, J. P. Atkinson, A C3(H2O) recycling pathway is a component of the intracellular complement system. *J. Clin. Invest.* **127**, 970–981 (2017).
22. Z. Zeng, B. G. J. Surewaard, C. H. Y. Wong, C. Guettler, B. Petri, R. Burkhard, M. Wyss, H. Le Moual, R. Devinney, G. C. Thompson, J. Blackwood, A. R. Joffe, K. D. McCoy, C. N. Jenne, P. Kubes, Sex-hormone-driven innate antibodies protect females and infants against EPEC infection. *Nat Immunol* **19**, 1100–1111 (2018).
23. M. S. Bolger, D. S. Ross, H. Jiang, M. M. Frank, A. J. Ghio, D. A. Schwartz, J. R. Wright, Complement levels and activity in the normal and LPS-injured lung. *Am. J. Physiol. Lung Cell Mol. Physiol.* **292**, L748-759 (2007).
24. Y. Kimura, T. Miwa, L. Zhou, W.-C. Song, Activator-specific requirement of properdin in the initiation and amplification of the alternative pathway complement. *Blood* **111**, 732–740 (2008).
25. P. Bertram, A. M. Akk, H. Zhou, L. M. Mitchell, C. T. N. Pham, D. E. Hourcade, Anti-mouse properdin TSR 5/6 monoclonal antibodies block complement alternative pathway-dependent pathogenesis. *Monoclon Antib Immunodiagn Immunother* **34**, 1–6 (2015).
26. X. Wu, I. Hutson, A. M. Akk, S. Mascharak, C. T. N. Pham, D. E. Hourcade, R. Brown, J. P. Atkinson, C. A. Harris, Contribution of Adipose-Derived Factor D/Adipsin to Complement Alternative Pathway Activation: Lessons from Lipodystrophy. *J. Immunol.* **200**, 2786–2797 (2018).
27. Y. Du, M. Guo, J. A. Whitsett, Y. Xu, “LungGENS”: a web-based tool for mapping single-cell gene expression in the developing lung. *Thorax* **70**, 1092–1094 (2015).
28. Y. Du, J. A. Kitzmiller, A. Sridharan, A. K. Perl, J. P. Bridges, R. S. Misra, G. S. Pryhuber, T. J. Mariani, S. Bhattacharya, M. Guo, S. S. Potter, P. Dexheimer, B. Aronow, A. H. Jobe, J. A. Whitsett, Y. Xu, Lung Gene Expression Analysis (LGEA): an integrative web portal for comprehensive gene expression data analysis in lung development. *Thorax* **72**, 481–484 (2017).
29. Y. Du, W. Ouyang, J. A. Kitzmiller, M. Guo, S. Zhao, J. A. Whitsett, Y. Xu, Lung Gene Expression Analysis Web Portal Version 3: Lung-at-a-Glance. *Am J Respir Cell Mol Biol* **64**, 146–149 (2021).
30. X. Sun, A.-K. Perl, R. Li, S. M. Bell, E. Sajti, V. V. Kalinichenko, T. V. Kalin, R. S. Misra, H. Deshmukh, G. Clair, J. Kyle, L. E. Crotty Alexander, J. M. Silva, J. A. Kitzmiller, K. A. Wikenheiser-Brokamp, G. Deutsch, M. Guo, Y. Du, M. P. Morley, M. J. Valdez, H. V. Yu, K. Jin, E. E. Bardes, J. A. Zepp, T. Neithamer, M. C. Basil, W. J. Zacharias, J. Verheyden, R. Young, G. Bandyopadhyay, S. Lin, C. Ansong, J. Adkins, N. Salomonis, B. J. Aronow, Y. Xu,

- G. Pryhuber, J. Whitsett, E. E. Morrissey, NHLBI LungMAP Consortium, A census of the lung: CellCards from LungMAP. *Dev Cell* , S1534-5807(21)00892–3 (2021).
31. L. S. Van Winkle, A. R. Buckpitt, C. G. Plopper, Maintenance of differentiated murine Clara cells in microdissected airway cultures. *Am J Respir Cell Mol Biol* **14**, 586–598 (1996).
32. B. M. Henry, I. Cheruiyot, S. W. Benoit, F. Sanchis-Gomar, G. Lippi, J. Benoit, Cytokeratin 18 cell death assays as biomarkers for quantification of apoptosis and necrosis in COVID-19: a prospective, observational study. *J Clin Pathol* , jclinpath-2020-207242 (2021).
33. F. J. Milder, L. Gomes, A. Schouten, B. J. C. Janssen, E. G. Huizinga, R. A. Romijn, W. Hemrika, A. Roos, M. R. Daha, P. Gros, Factor B structure provides insights into activation of the central protease of the complement system. *Nat. Struct. Mol. Biol.* **14**, 224–228 (2007).
34. C. Hess, C. Kemper, Complement-Mediated Regulation of Metabolism and Basic Cellular Processes. *Immunity* **45**, 240–254 (2016).
35. L. T. Roumenina, M. V. Daugan, F. Petitprez, C. Sautès-Fridman, W. H. Fridman, Context-dependent roles of complement in cancer. *Nat. Rev. Cancer* (2019), doi:10.1038/s41568-019-0210-0.
36. B. C. King, A. M. Blom, Complement in metabolic disease: metaflammation and a two-edged sword. *Semin Immunopathol* **43**, 829–841 (2021).
37. A. Sünderhauf, K. Skibbe, S. Preisker, K. Ebbert, A. Verschoor, C. M. Karsten, C. Kemper, M. Huber-Lang, M. Basic, A. Bleich, J. Büning, K. Fellermann, C. Sina, S. Derer, Regulation of epithelial cell expressed C3 in the intestine - Relevance for the pathophysiology of inflammatory bowel disease? *Mol. Immunol.* **90**, 227–238 (2017).
38. Y. Laumonnier, C. M. Karsten, J. Köhl, Novel insights into the expression pattern of anaphylatoxin receptors in mice and men. *Mol Immunol* **89**, 44–58 (2017).
39. M. Elvington, M. K. Liszewski, J. P. Atkinson, Evolution of the complement system: from defense of the single cell to guardian of the intravascular space. *Immunol. Rev.* **274**, 9–15 (2016).
40. C. Homann, K. Varming, K. Høgåsen, T. E. Mollnes, N. Graudal, A. C. Thomsen, P. Garred, Acquired C3 deficiency in patients with alcoholic cirrhosis predisposes to infection and increased mortality. *Gut* **40**, 544–549 (1997).
41. D. K. Pillai, B.-J. V. Sankoorikal, E. Johnson, A. N. Seneviratne, J. Zurko, K. J. Brown, Y. Hathout, M. C. Rose, Directional secretomes reflect polarity-specific functions in an in vitro model of human bronchial epithelium. *Am. J. Respir. Cell Mol. Biol.* **50**, 292–300 (2014).
42. J. R. Peters-Hall, K. J. Brown, D. K. Pillai, A. Tomney, L. M. Garvin, X. Wu, M. C. Rose, Quantitative proteomics reveals an altered cystic fibrosis in vitro bronchial epithelial secretome. *Am. J. Respir. Cell Mol. Biol.* **53**, 22–32 (2015).

43. E. L. Rawlins, T. Okubo, Y. Xue, D. M. Brass, R. L. Auten, H. Hasegawa, F. Wang, B. L. M. Hogan, The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. *Cell Stem Cell* **4**, 525–534 (2009).
44. M. K. Liszewski, M. Kolev, G. Le Friec, M. Leung, P. G. Bertram, A. F. Fara, M. Subias, M. C. Pickering, C. Drouet, S. Meri, T. P. Arstila, P. T. Pekkarinen, M. Ma, A. Cope, T. Reinheckel, S. Rodriguez de Cordoba, B. Afzali, J. P. Atkinson, C. Kemper, Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. *Immunity* **39**, 1143–1157 (2013).
45. N. Niyonzima, J. Rahman, N. Kunz, E. E. West, T. Freiwald, J. V. Desai, N. S. Merle, A. Gidon, B. Sporsheim, M. S. Lionakis, K. Evensen, B. Lindberg, K. Skagen, M. Skjelland, P. Singh, M. Haug, M. M. Ruseva, M. Kolev, J. Bibby, O. Marshall, B. O'Brien, N. Deeks, B. Afzali, R. J. Clark, T. M. Woodruff, M. Pryor, Z.-H. Yang, A. T. Remaley, T. E. Mollnes, S. M. Hewitt, B. Yan, M. Kazemian, M. G. Kiss, C. J. Binder, B. Halvorsen, T. Espevik, C. Kemper, Mitochondrial C5aR1 activity in macrophages controls IL-1 β production underlying sterile inflammation. *Sci Immunol* **6**, eabf2489 (2021).
46. P. Atanes, I. Ruz-Maldonado, A. Pingitore, R. Hawkes, B. Liu, M. Zhao, G. C. Huang, S. J. Persaud, S. Amisten, C3aR and C5aR1 act as key regulators of human and mouse β -cell function. *Cell Mol Life Sci* **75**, 715–726 (2018).
47. J. I. Moliva, M. V. S. Rajaram, S. Sidiki, S. J. Sasindran, E. Guirado, X. J. Pan, S.-H. Wang, P. Ross, W. P. Lafuse, L. S. Schlesinger, J. Turner, J. B. Torrelles, Molecular composition of the alveolar lining fluid in the aging lung. *Age (Dordr)* **36**, 9633 (2014).
48. K. Bielen, B. 's Jongers, S. Malhotra-Kumar, P. G. Jorens, H. Goossens, S. Kumar-Singh, Animal models of hospital-acquired pneumonia: current practices and future perspectives. *Ann Transl Med* **5**, 132 (2017).
49. Z. Zheng, Y.-N. Li, S. Jia, M. Zhu, L. Cao, M. Tao, J. Jiang, S. Zhan, Y. Chen, P.-J. Gao, W. Hu, Y. Wang, C. Shao, Y. Shi, Lung mesenchymal stromal cells influenced by Th2 cytokines mobilize neutrophils and facilitate metastasis by producing complement C3. *Nat Commun* **12**, 6202 (2021).
50. S. Kumar, M. G. Frid, H. Zhang, M. Li, S. Riddle, R. D. Brown, S. C. Yadav, M. K. Roy, M. E. Dzieciatkowska, A. D'Alessandro, K. C. Hansen, K. R. Stenmark, Complement-containing small extracellular vesicles from adventitial fibroblasts induce proinflammatory and metabolic reprogramming in macrophages. *JCI Insight* **6**, e148382 (2021).
51. F. Bastaert, S. Kheir, V. Saint-Criq, B. Villeret, P. M.-C. Dang, J. El-Benna, J.-C. Sirard, R. Voulhoux, J.-M. Sallenave, Pseudomonas aeruginosa LasB Subverts Alveolar Macrophage Activity by Interfering With Bacterial Killing Through Downregulation of Innate Immune Defense, Reactive Oxygen Species Generation, and Complement Activation. *Front Immunol* **9**, 1675 (2018).
52. L. Ma, S. K. Sahu, M. Cano, V. Kuppuswamy, J. Bajwa, J. McPhatter, A. Pine, M. L. Meizlish, G. Goshua, C.-H. Chang, H. Zhang, C. Price, P. Bahel, H. Rinder, T. Lei, A. Day, D.

Reynolds, X. Wu, R. Schriefer, A. M. Rauseo, C. W. Goss, J. A. O'Halloran, R. M. Presti, A. H. Kim, A. E. Gelman, C. S. Dela Cruz, A. I. Lee, P. A. Mudd, H. J. Chun, J. P. Atkinson, H. S. Kulkarni, Increased complement activation is a distinctive feature of severe SARS-CoV-2 infection. *Sci. Immunol.* **6**, eabh2259 (2021).

53. T. Okamoto, S. K. Mathai, C. E. Hennessy, L. A. Hancock, A. D. Walts, A. L. Stefanski, K. K. Brown, D. A. Lynch, G. P. Cosgrove, S. D. Groshong, C. D. Cool, M. I. Schwarz, N. K. Banda, J. M. Thurman, I. V. Yang, V. M. Holers, D. A. Schwartz, The relationship between complement C3 expression and the MUC5B genotype in pulmonary fibrosis. *Am. J. Physiol. Lung Cell Mol. Physiol.* (2018), doi:10.1152/ajplung.00395.2017.

54. P. S. Hair, L. A. Sass, T. Vazifedan, T. A. Shah, N. K. Krishna, K. M. Cunnion, Complement effectors, C5a and C3a, in cystic fibrosis lung fluid correlate with disease severity. *PLoS ONE* **12**, e0173257 (2017).

55. M. Matsumoto, W. Fukuda, A. Circolo, J. Goellner, J. Strauss-Schoenberger, X. Wang, S. Fujita, T. Hidvegi, D. D. Chaplin, H. R. Colten, Abrogation of the alternative complement pathway by targeted deletion of murine factor B. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8720–8725 (1997).

56. S. Keshavjee, R. D. Davis, M. R. Zamora, M. de Perrot, G. A. Patterson, A randomized, placebo-controlled trial of complement inhibition in ischemia-reperfusion injury after lung transplantation in human beings. *J Thorac Cardiovasc Surg* **129**, 423–428 (2005).

57. D. C. Mastellos, B. G. P. Pires da Silva, B. A. L. Fonseca, N. P. Fonseca, M. Auxiliadora-Martins, S. Mastaglio, A. Ruggeri, M. Sironi, P. Radermacher, A. Chrysanthopoulou, P. Skendros, K. Ritis, I. Manfra, S. Iacobelli, M. Huber-Lang, B. Nilsson, D. Yancopoulou, E. S. Connolly, C. Garlanda, F. Ciceri, A. M. Risitano, R. T. Calado, J. D. Lambris, Complement C3 vs C5 inhibition in severe COVID-19: Early clinical findings reveal differential biological efficacy. *Clin Immunol* **220**, 108598 (2020).

58. N. Percie du Sert, V. Hurst, A. Ahluwalia, S. Alam, M. T. Avey, M. Baker, W. J. Browne, A. Clark, I. C. Cuthill, U. Dirnagl, M. Emerson, P. Garner, S. T. Holgate, D. W. Howells, N. A. Karp, S. E. Lazic, K. Lidster, C. J. MacCallum, M. Macleod, E. J. Pearl, O. H. Petersen, F. Rawle, P. Reynolds, K. Rooney, E. S. Sena, S. D. Silberberg, T. Steckler, H. Würbel, The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *J Physiol* **598**, 3793–3801 (2020).

59. A. Circolo, G. Garnier, W. Fukuda, X. Wang, T. Hidvegi, A. J. Szalai, D. E. Briles, J. E. Volanakis, R. A. Wetsel, H. R. Colten, Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA. *Immunopharmacology* **42**, 135–149 (1999).

60. J. Kildsgaard, T. J. Hollmann, K. W. Matthews, K. Bian, F. Murad, R. A. Wetsel, Cutting edge: targeted disruption of the C3a receptor gene demonstrates a novel protective anti-inflammatory role for C3a in endotoxin-shock. *J Immunol* **165**, 5406–5409 (2000).

61. A. Heeckeren, R. Walenga, M. W. Konstan, T. Bonfield, P. B. Davis, T. Ferkol, Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with *Pseudomonas aeruginosa*. *J Clin Invest* **100**, 2810–2815 (1997).
62. L. Zou, Y. Feng, Y. Li, M. Zhang, C. Chen, J. Cai, Y. Gong, L. Wang, J. M. Thurman, X. Wu, J. P. Atkinson, W. Chao, Complement factor B is the downstream effector of TLRs and plays an important role in a mouse model of severe sepsis. *J. Immunol.* **191**, 5625–5635 (2013).
63. M. P. Triebwasser, X. Wu, P. Bertram, D. E. Hourcade, D. M. Nelson, J. P. Atkinson, Timing and mechanism of conceptus demise in a complement regulatory membrane protein deficient mouse. *Am. J. Reprod. Immunol.* , e12997 (2018).
64. H. S. Kulkarni, J. S. Lee, J. A. Bastarache, W. M. Kuebler, G. P. Downey, G. M. Albaiceta, W. A. Altemeier, A. Artigas, J. H. T. Bates, C. S. Calfee, C. S. Dela Cruz, R. P. Dickson, J. A. Englert, J. I. Everitt, M. B. Fessler, A. E. Gelman, K. M. Gowdy, S. D. Groshong, S. Herold, R. J. Homer, J. C. Horowitz, C. C. W. Hsia, K. Kurahashi, V. E. Laubach, M. R. Looney, R. Lucas, N. S. Mangalmurti, A. M. Manicone, T. R. Martin, S. Matalon, M. A. Matthay, D. F. McAuley, S. A. McGrath-Morrow, J. P. Mizgerd, S. A. Montgomery, B. B. Moore, A. Noël, C. E. Perlman, J. P. Reilly, E. P. Schmidt, S. J. Skerrett, T. L. Suber, C. Summers, B. T. Suratt, M. Takata, R. Tudor, S. Uhlig, M. Witzernath, R. L. Zemans, G. Matute-Bello, Update on the Features and Measurements of Experimental Acute Lung Injury in Animals: An Official American Thoracic Society Workshop Report. *Am J Respir Cell Mol Biol* **66**, e1–e14 (2022).
65. K. J. Livak, T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods* **25**, 402–408 (2001).
66. P. Kelly, B. Amadi, K. Chandwe, E. Besa, K. Zyambo, M. Chama, P. I. Tarr, N. Shaikh, I. M. Ndao, C. Storer, R. Head, Gene expression profiles compared in environmental and malnutrition enteropathy in Zambian children and adults. *EBioMedicine* **70**, 103509 (2021).
67. H. Pan, G. H. Deutsch, S. E. Wert, Ontology Subcommittee, NHLBI Molecular Atlas of Lung Development Program Consortium, Comprehensive anatomic ontologies for lung development: A comparison of alveolar formation and maturation within mouse and human lung. *J Biomed Semantics* **10**, 18 (2019).
68. M. Deprez, L.-E. Zaragosi, M. Truchi, C. Becavin, S. Ruiz García, M.-J. Arguel, M. Plaisant, V. Magnone, K. Lebrigand, S. Abelanet, F. Brau, A. Paquet, D. Pe’er, C.-H. Marquette, S. Leroy, P. Barbry, A Single-Cell Atlas of the Human Healthy Airways. *Am J Respir Crit Care Med* **202**, 1636–1645 (2020).
69. K. C. Goldfarbmuren, N. D. Jackson, S. P. Sajuthi, N. Dyjack, K. S. Li, C. L. Rios, E. G. Plender, M. T. Montgomery, J. L. Everman, P. E. Bratcher, E. K. Vladar, M. A. Seibold, Dissecting the cellular specificity of smoking effects and reconstructing lineages in the human airway epithelium. *Nat Commun* **11**, 2485 (2020).
70. P. A. Reyfman, J. M. Walter, N. Joshi, K. R. Anekalla, A. C. McQuattie-Pimentel, S. Chiu, R. Fernandez, M. Akbarpour, C.-I. Chen, Z. Ren, R. Verma, H. Abdala-Valencia, K. Nam, M. Chi, S. Han, F. J. Gonzalez-Gonzalez, S. Soberanes, S. Watanabe, K. J. N. Williams, A. S.

Flozak, T. T. Nicholson, V. K. Morgan, D. R. Winter, M. Hinchcliff, C. L. Hrusch, R. D. Guzy, C. A. Bonham, A. I. Sperling, R. Bag, R. B. Hamanaka, G. M. Mutlu, A. V. Yeldandi, S. A. Marshall, A. Shilatifard, L. A. N. Amaral, H. Perlman, J. I. Sznajder, A. C. Argento, C. T. Gillespie, J. Dematte, M. Jain, B. D. Singer, K. M. Ridge, A. P. Lam, A. Bharat, S. M. Bhorade, C. J. Gottardi, G. R. S. Budinger, A. V. Misharin, Single-Cell Transcriptomic Analysis of Human Lung Provides Insights into the Pathobiology of Pulmonary Fibrosis. *Am J Respir Crit Care Med* **199**, 1517–1536 (2019).

71. T. S. Adams, J. C. Schupp, S. Poli, E. A. Ayaub, N. Neumark, F. Ahangari, S. G. Chu, B. A. Raby, G. DeIuliis, M. Januszyk, Q. Duan, H. A. Arnett, A. Siddiqui, G. R. Washko, R. Homer, X. Yan, I. O. Rosas, N. Kaminski, Single-cell RNA-seq reveals ectopic and aberrant lung-resident cell populations in idiopathic pulmonary fibrosis. *Sci Adv* **6**, eaba1983 (2020).

72. A. C. Habermann, A. J. Gutierrez, L. T. Bui, S. L. Yahn, N. I. Winters, C. L. Calvi, L. Peter, M.-I. Chung, C. J. Taylor, C. Jetter, L. Raju, J. Roberson, G. Ding, L. Wood, J. M. S. Sucre, B. W. Richmond, A. P. Serezani, W. J. McDonnell, S. B. Mallal, M. J. Bacchetta, J. E. Loyd, C. M. Shaver, L. B. Ware, R. Bremner, R. Walia, T. S. Blackwell, N. E. Banovich, J. A. Kropski, Single-cell RNA sequencing reveals profibrotic roles of distinct epithelial and mesenchymal lineages in pulmonary fibrosis. *Sci Adv* **6**, eaba1972 (2020).

73. S. V. Bardina, D. Michlmayr, K. W. Hoffman, C. J. Obara, J. Sum, I. F. Charo, W. Lu, A. G. Pletnev, J. K. Lim, Differential Roles of Chemokines CCL2 and CCL7 in Monocytosis and Leukocyte Migration during West Nile Virus Infection. *J Immunol* **195**, 4306–4318 (2015).

74. H.-M. Hsiao, R. Fernandez, S. Tanaka, W. Li, J. H. Spahn, S. Chiu, M. Akbarpour, D. Ruiz-Perez, Q. Wu, C. Turam, D. Scozzi, T. Takahashi, H. P. Luehmann, V. Puri, G. R. S. Budinger, A. S. Krupnick, A. V. Misharin, K. J. Lavine, Y. Liu, A. E. Gelman, A. Bharat, D. Kreisel, Spleen-derived classical monocytes mediate lung ischemia-reperfusion injury through IL-1 β . *J Clin Invest* **128**, 2833–2847 (2018).

75. Y. Cao, T. Vo, G. Millien, J.-B. Tagne, D. Kotton, R. J. Mason, M. C. Williams, M. I. Ramirez, Epigenetic mechanisms modulate thyroid transcription factor 1-mediated transcription of the surfactant protein B gene. *J Biol Chem* **285**, 2152–2164 (2010).

76. Y. Zhang, S. Yokoyama, J. C. Herriges, Z. Zhang, R. E. Young, J. M. Verheyden, X. Sun, E3 ubiquitin ligase RFWF2 controls lung branching through protein-level regulation of ETV transcription factors. *Proc Natl Acad Sci U S A* **113**, 7557–7562 (2016).

77. J. L. Proenca-Modena, R. Sesti-Costa, A. K. Pinto, J. M. Richner, H. M. Lazear, T. Lucas, J. L. Hyde, M. S. Diamond, Oropouche virus infection and pathogenesis are restricted by MAVS, IRF-3, IRF-7, and type I interferon signaling pathways in nonmyeloid cells. *J Virol* **89**, 4720–4737 (2015).

78. M. S. Kuefner, K. Pham, J. R. Redd, E. J. Stephenson, I. Harvey, X. Deng, D. Bridges, E. Boilard, M. B. Elam, E. A. Park, Secretory phospholipase A2 group IIA modulates insulin sensitivity and metabolism. *J Lipid Res* **58**, 1822–1833 (2017).

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Figure and Table Captions.

Figure 1. C3 protects against severe bacterial pneumonia. (A-E) Global C3-deficient ($C3^{-/-}$) and wildtype (WT) B6 mice were euthanized 24 h after infection with *Pseudomonas aeruginosa* (*Pa*). (A) Representative images from n=5 mice/group at 4x magnification; an area of 20x magnification shows both the airways and alveolar space. (B) To assess physiological dysfunction, small animal whole body plethysmography was performed using increasing concentrations of methacholine from 0.625-20 mg/ml (x-axis). Dynamic compliance (in ml/cm H₂O) represented on the Y-axis. (C) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) measurements for *Ccl2*, *Il1b*, *Scgb1a1* (an airway club cell marker) and *Sftpc* (an alveolar type II cell marker), all normalized to *Gapdh*. (D-E) Colony forming units (CFU) in the harvested (D) lungs and (E) spleens. X-axis is on a logarithmic scale. (F-L) $C3^{-/-}$ and WT mice were euthanized 24 h after infection with heat-killed *Pa* (HK*Pa*). (F) Representative histology images at 20x magnification (n=5-8/group). Differences in (G), lung injury scores, (H) lung wet-dry ratios, (I) bronchoalveolar lavage (BAL) cell count, (J) qRT-PCR for *Ccl2* and *Il1b*, (K) proportion of epithelial cells (gated as CD45⁻CD31⁻CD326⁺) from digested mouse lungs, and (L) qRT-PCR for *Scgb1a1* and *Sftpc*. J and L are normalized to *Hprt1*. Distribution of data represented as scatter plots showing individual data points, and bars representing mean $\pm$ standard deviation. ^ p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 using unpaired two-tailed t-test.

Figure 2. C3 in the lungs is sourced locally. Lipopolysaccharide (LPS)-induced complement activation in (A) mouse serum, and (B) BAL assessed in both uninfected and infected WT mice. Results expressed as % activity of concurrently run uninfected WT B6 mouse sera and BAL

respectively. (C) Infected $C3^{-/-}$ mice were administered normal mouse serum (NMS) or phosphate-buffered saline (PBS) intraperitoneally either 1 h or 24 h before euthanasia (i.e., at the time of infection). (D) PCR products from genomic DNA separated by agarose gel electrophoresis used to identify mice deficient in liver-derived C3 and their littermate controls. (E) Livers from $C3^{f/f}$; $Alb-Cre^{+/-}$ and $C3^{f/f}$; $Alb-Cre^{-/-}$ mice analyzed using flow cytometry using negative selection (CD45 excluded immune cells). C3 expression assessed using TdTomato reporter. Immunoblots of reduced (F) serum and (G) BAL from mice drawn pre-and post-*Pa* infection. To assess functional activity in liver-deficient C3 mice, LPS-induced complement activation in (H) mouse serum, and (I) BAL was assessed. Comparison of (J) spleen colony forming units (CFU, x-axis is on a logarithmic scale), (K) lung wet-dry ratios, and (L) BAL immune cell counts post-infection. Distribution of data represented as scatter plots showing individual data points, and bars representing mean $\pm$ standard deviation. ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using unpaired two-tailed t-test.

Figure 3. Non-immune cells contribute to C3 in the lung. (A) Human lung expression of C3 (ENSG00000125730) using the LungMAP Single Cell Reference (v1) in the Lung Gene Expression Atlas (LGEA) Web Portal (LungMAP Phase 2). (B) Changes in C3 expression on flow cytometry in cells obtained from lungs of non-infected (Control) and infected (*Pa*) mice deficient in liver-derived C3 ($C3^{f/f}$; $Alb-Cre^{+/-}$, n=5/group). (C) Schematic for tamoxifen induction of *Cre* recombinase followed by infection. (D) Confocal microscopy imaging of C3 (green, FITC equivalent 488 nm laser) and Cyp2f2 (pink, Cy5 equivalent 640 nm laser) in lungs of $C3^{f/f}$; $Scgb1a1-CreER^{T2+/-}$ mice, as compared to littermate controls that have normal C3 and Cyp2f2 expression. Cyp2f2 was used as the club cell marker instead of *Scgb1a1*, as antibodies to both C3 and *Scgb1a1* are of the same species (**Table S2**, 63x oil magnification, representative of

n=4/group, bar = 10 μ M). Individual inserts show single colors. Immunoblots of reduced (E) serum and (F) BAL from lung-deficient C3 mice and littermate controls. Mice deficient in liver-derived C3 and their littermate controls have been used as a comparison for the serum immunoblots, and C3^{-/-} mice have been used to compare BAL. (G) LPS-induced complement activation in mouse serum. (H) The same assay performed on BAL post-infection. Distribution of data represented as scatter plots showing individual data points, and bars representing mean $\pm$ standard deviation. ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using unpaired two-tailed t-test.

Figure 4. Epithelial cell-derived C3 protects against severe bacterial pneumonia. Mice deficient in epithelial club cell-derived C3 (lung-derived C3, C3^{f/f}; *Scgblal*-CreER^{T2+/-} mice) had worse alveolar-capillary barrier dysfunction at 24 h post-*Pa* infection compared to their littermate controls, as represented by (A) lung wet-dry ratio, and (B) BAL protein. (C) Representative lung histology images of mice deficient in lung-derived C3 and their littermates at 24 h post-infection (20x magnification, n=5/group). (D-G) Comparison of lung inflammation in mice deficient in lung-derived C3 versus their littermates, using cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), keratinocyte chemoattractant (KC, CXCL1) and tumor necrosis factor (TNF- α) in the BAL. (H) Comparison of colony forming units (CFU) in the spleens obtained from mice deficient in lung-derived C3 and their littermates at 24 h post-infection. (I) Comparison of epithelial cell injury using the levels of receptor for advanced glycation end products (RAGE) in the BAL of mice deficient in lung-derived C3 and their littermates. (J) Comparison of epithelial cell death using the levels of cytokeratin-18 (CK-18) in the BAL. Distribution of data represented as scatter plots showing individual data points, and bars

representing mean $\pm$ standard deviation. ns, not significant, * $p < 0.05$, ** $p < 0.01$ using unpaired two-tailed t-test.

Figure 5. Complement Factor B (FB) protects against stress-induced epithelial injury both in vitro and in vivo. (A-D) Global Factor B-deficient (*Cfb*^{-/-}) and C3a receptor-deficient (*C3aR*^{-/-}) B6 mice euthanized 24 h after *Pa* infection. (A) Representative images (n=5/group, 20x). These images should be compared to Fig 1A as the lungs were obtained at the same time. (B) Dynamic compliance (y axis, methacholine dose, x axis). (C) Lung wet-dry ratio. (D) *Il1b* expression, normalized to *Hprt1*. (E) *Scgb1a1* expression normalized to *Gapdh*. (F-I) *Cfb*^{-/-} and WT mice administered HK*Pa* and euthanized 24 h after infection. (F) Representative images (n=5/group, 20x) with differences in lung injury scores. These images should be compared to Fig 1F as the lungs were obtained at the same time. Comparison of (G) lung wet-dry ratios, and qRT-PCR for (H) *Il1b*, and (I) *Scgb1a1*, both normalized to *Hprt1*. (J) Immunoblots of FB and C3aR expression in primary human tracheobronchial epithelial cells (hTECs) infected with *Pa* (MOI 5). Representative of n=3 donors. Molecular weights in brackets. (K) Immunostaining for C3, C3aR, and FB in uninfected and infected hTECs using confocal microscopy at 63x (oil) magnification. Phalloidin used to visualize F-actin and identify individual cells. Representative of n=3 donors. (L) A bargraph with the top 5 significant themes plotted by their enrichment scores on RNASeq analysis using the COMPBIO, when comparing infected human BEAS-2B cells (*Pa*, MOI 5, harvested at 4 h) rendered deficient in FB using CRISPR and their FB-sufficient parent clones (the number of genes mapping to these themes are indicated in parentheses. All 5 have $p < 0.001$, which indicates the chance of seeing the theme (genes and concepts) in a random set of genes of the input size). (M) Interrelationship between the three

central themes, namely Cell death, ECM proteoglycans and Autocrine-paracrine Signaling.

Shared genes between themes are indicated in parenthesis. The top 10 central concepts and genes for the cell death theme are shown in the accompanying table. The pseudo heatmap shows the 18 shared genes common to all three themes and their contribution in each theme. The numeric values indicate the enrichment score of the genes. (N) Immunoblot analysis of caspase-3, PARP and β -actin in FB-deficient human cells and their parent clones (*CFB*^{+/+}) after *Pa* infection. Arrows denote cleaved fragments. (O) Similar immunoblot of cleaved PARP in FB-deficient cells and parent clones treated with H₂O₂ (250 μ M). All qRT-PCR data normalized to *Hprt1*. Distribution of data represented as scatter plots showing individual data points, and bars representing mean $\pm$ standard deviation. ^ p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001 using unpaired two-tailed t-test for two group comparisons, and ordinary one-way analysis of variance (ANOVA) with multiple comparisons testing for more than two groups.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. C3 protects against severe bacterial pneumonia. (A) To assess physiological dysfunction in C3-deficient mice ($C3^{-/-}$) and wildtype (WT) mice, small animal whole body plethysmography was performed using increasing concentrations of methacholine from 0.625-20 mg/ml (x-axis) at 24 h post-*Pa* infection. Airway resistance (invasive resistance, RI, in cmH₂O.s/ml, y axis). Data points and error bars represent mean $\pm$ standard deviation (n=4-6/group). Alveolar-capillary barrier disruption assayed by the lung wet-dry ratio in (B) $C3^{-/-}$ and WT mice, and (C) C3-deficient male ($\sigma C3^{-/-}$) versus C3-deficient female ($\phi C3^{-/-}$) mice post-*Pa*. Lung inflammation, determined as (D) the proportion of lung Ly6C^{hi} monocytes (n=4/group), and BAL cytokines such as (E) chemokine ligand 2 (CCL2), (F) interleukin-1 β (IL-1 β), (G) interleukin-6 (IL-6), and (H) tumor necrosis factor (TNF- α). (I-M) Histology assessed using individual components of a lung injury score. Distribution of data represented as scatter plots showing individual data points, and bars representing mean $\pm$ standard deviation. ns, not significant, * $p < 0.05$, ** $p < 0.01$ using a unpaired two-tailed t-test, except for (B) and (D-H), a one-tailed t-test was used given the null hypothesis was that C3-deficient mice had worse lung injury than WT mice, based on the initial findings.

Supplementary Figure 2. Deficiency in liver-derived C3 does not increase the predisposition to *Pseudomonas aeruginosa* (*Pa*)-induced acute lung injury.

(A) Spleen CFU comparing $C3^{-/-}$ mice receiving $C3^{-/-}$ serum versus $C3^{-/-}$ mice receiving normal mouse serum (NMS or C3-sufficient serum) immediately after *Pa* infection, 24 h prior to euthanasia. (B) Representative lung histology images of mice deficient in liver-derived C3 and their littermates at

24 h post-infection (n=4/group). (C) To assess lung inflammation, we compared the levels of cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), keratinocyte chemoattractant (KC, i.e., CXCL1) and tumor necrosis factor (TNF- α) in the BAL of mice deficient in liver-derived C3 versus their littermates. Distribution of data represented as scatter plots showing individual data points, and bars representing mean $\pm$ standard deviation. ns, not significant using a unpaired two-tailed t-test.

Supplementary Figure 3. C3 in the lungs is produced locally. (A) C3 (ENSG00000125730) expression in query cells and annotations projected onto the reference UMAP on the LGEA web portal, using the LungMAP Single Cell Reference (v1). (B) Gating strategy for flow-cytometric analysis of cells derived from the lungs of mice deficient in liver-derived C3. (C) Agarose gel electrophoresis of PCR products used to identify genotypes of mice used in experiments. PCR products were imaged using SYBR green and size indicated by notation on the right. Gel showing PCR products from tails of $C3^{f/f}$; *Scgblal*-CreER^{T2+/-} mice (i.e., mice deficient in lung-derived C3), and their littermate controls ($C3^{f/+}$; *Scgblal*-CreER^{T2+/-} and $C3^{f/f}$; *Scgblal*-CreER^{T2-/-} mice). Abbreviations: AF1: alveolar fibroblast 1; AF2: alveolar fibroblast 2; aMac: alveolar macrophage; ASMC: airway smooth muscle cell; AT1: Type 1 alveolar epithelial cells, AT2: Type 2 alveolar epithelial cells; Cap1: capillary cell 1; Cap2: capillary cell 2; abbreviations for two capillary subsets; DC: dendritic cells; iMac: interstitial macrophages; mDC1: myeloid type 1 dendritic cell; maDC: mature human dendritic cell; mDC2: myeloid type 2 dendritic cell; MEC: myoepithelial cells; NK: NK cells; pDC: plasmacytoid dendritic cells; PDPN: podoplanin; pMac: PNEC: pulmonary neuroendocrine cell; SCMF: Secondary crest myofibroblast cell; VSMC: vascular smooth muscle cell.

Supplementary Figure 4. Complement Factor B (FB) protects against stress-induced

epithelial injury in vitro and in vivo. (A) Airway resistance (y-axis) measured using whole body plethysmography post *Pa*-infection. Data points and error bars represent mean $\pm$ standard deviation (n=3-5/group). Lung inflammation post-infection assessed using (B) qRT-PCR of *Ccl2*, and (C) proportion of Ly6C^{hi} monocytes using mass cytometry. Epithelial cell loss assessed using qRT-PCR of (D) *Sftpc*, and (E) *Cdh1* (cadherin), and death assessed by (E) BAL CK-18, and (F) epithelial cells (CD45⁻CD31⁻CD326⁺Zombie⁺) on flow cytometry. (H) Lung *Ccl2* expression to assess inflammation post-HK*Pa*. Lung (I) *Sftpc* and (J) *Cdh1* expression to assess epithelial cell loss post-HK*Pa*. Schematic for CRISPR-induced CRISPR-induced (K) FB (*CFB*) deletion, and (L) C3aR (*C3aR1*) deletion in BEAS-2B cells. Quantification of SYTOX⁺:SYTO24⁺ cells on live cell imaging (Incucyte) over time when (M) *CFB*^{-/-} cells and their parent clones (*CFB*^{+/+}), and (N) *C3aR*^{-/-} cells and their parent clones (*C3aR*^{+/+}) were treated with hydrogen peroxide (H₂O₂). B and D normalized to *Gapdh*. C, I and J normalized to *Hprt1*. Distribution of data represented as scatter plots showing individual data points, and bars representing mean $\pm$ standard deviation. * p < 0.05, ** p < 0.01, *** p < 0.001 using unpaired two-tailed t-test for two group comparisons, and ordinary one-way analysis of variance (ANOVA) with multiple comparisons testing for more than two groups.

FIGURE 1

FIGURE 2

FIGURE 3

A

Human Lungs (LGEA Web Portal)

Expression distribution

B

C

D

E

G

F

FIGURE 4

FIGURE 5