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Angiopoietin-like 4 Increases Pulmonary Tissue Leakiness and Damage during Influenza Pneumonia

Graphical Abstract

**Highlights**

- ANGPTL4 is upregulated by a STAT3-mediated mechanism during influenza pneumonia.
- ANGPTL4-deficient mice show reduced lung damage and accelerated lung recovery.
- Antibodies targeting ANGPTL4 reduce pulmonary tissue leakiness and damage.
- ANGPTL4 is a potential biomarker for respiratory infection and pneumonia.

**In Brief**

Li et al. show that influenza infection stimulates the expression of ANGPTL4 through a STAT3-mediated mechanism. Host ANGPTL4 enhances pulmonary tissue leakiness and exacerbates inflammation-induced lung damage. ANGPTL4 deficiency improves pulmonary tissue integrity and accelerates lung tissue recovery.

**Accession Numbers**

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Angiopoietin-like 4 Increases Pulmonary Tissue Leakiness and Damage during Influenza Pneumonia

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SUMMARY

Excessive host inflammatory responses negatively impact disease outcomes in respiratory infection. Host-pathogen interactions during the infective phase of influenza are well studied, but little is known about the host’s response during the repair stage. Here, we show that influenza infection stimulated the expression of angiopoietin-like 4 (ANGPTL4) via a direct IL6-STAT3-mediated mechanism. ANGPTL4 enhanced pulmonary tissue leakiness and exacerbated inflammation-induced lung damage. Treatment of infected mice with neutralizing anti-ANGPTL4 antibodies significantly accelerated lung recovery and improved lung tissue integrity. ANGPTL4-deficient mice also showed reduced lung damage and recovered faster from influenza infection when compared to their wild-type counterparts. Retrospective examination of human lung biopsy specimens from infection-induced pneumonia with tissue damage showed elevated expression of ANGPTL4 when compared to normal lung samples. These observations underscore the important role that ANGPTL4 plays in lung infection and damage and may facilitate future therapeutic strategies for the treatment of influenza pneumonia.

INTRODUCTION

The occurrence of annual epidemics and random global pandemics of influenza exerts a large public health burden worldwide (Mizgerd, 2006; Armstrong et al., 1999). However, designing effective vaccines and treatment options has proven challenging in view of the rapid evolution of the virus. While many aspects of host-pathogen interactions during the course of an influenza infection have been studied, there is less information on the host response during the repair stage of an infection (Mizgerd, 2008). A better understanding of the host response during the pulmonary repair phase may facilitate innovative treatment strategies. Host-specific biomarkers, indicative of the severity of lung tissue damage, could be exploited to delineate opportunities for therapeutic intervention.

Host immune responses are extremely important for containing influenza infections (Julkunen et al., 2000). Through the combined action of innate and adaptive immune responses, the infectious pathogen becomes inactivated and cleared from the body, repair processes start to resolve the tissue damage, and long-term immunity is ultimately established. However, excessive and prolonged inflammation may be detrimental to the host and contribute to the greater morbidity and mortality associated with influenza-induced inflammatory injury (Akaike et al., 1996; Monsalvo, 2010; Nicholls and Peiris, 2005; Buchweitz et al., 2007). Exaggerated inflammatory responses in the lung parenchyma can destroy alveoli, induce excessive edema, precipitate hypoxia, and cause pulmonary impairment (Narasaraju et al., 2011). Studies have documented that inflammatory injury to the lungs represents a major factor for the fatalities associated with pandemic H1N1-2009, highly pathogenic avian influenza viruses, and severe acute respiratory syndrome (SARS) coronavirus (Monsalvo, 2010; Nicholls and Peiris, 2005). Although inflammatory processes represent important therapeutic targets, anti-inflammatory therapies may also inhibit critical immune functions that mediate pathogen clearance, and they run the risk of enhancing pathogen replication and secondary infection (Uchide and Toyoda, 2011; Snelgrove et al., 2006; Aldridge et al., 2009; Ballinger and Standiford, 2010). An ideal treatment regimen should minimize the tissue damage caused by inflammation and facilitate recovery without interfering with the host’s antiviral and antibacterial responses.

Angiopoietin-like 4 (ANGPTL4) belongs to a family of angiogenic-regulating, secreted proteins that bear a high similarity to members of the angiopoietin (ANG) family. However, ANGPTL4 does not bind to ANG receptor TIE1/2, indicating that ANGPTL4 exerts its distinct functions via a different mechanism from ANG proteins (Zhu et al., 2012; Grootaert et al., 2012).
Native full-length ANGPTL4 (fANGPTL4) contains a secretory signal peptide, an N-terminal coiled-coil structure, and a C-terminal fibrinogen-like domain. ANGPTL4 undergoes proteolytic processing by proprotein convertases at the linker region, thereby releasing the N-terminal region (nANGPTL4) and the monomeric C-terminal portion (cANGPTL4) (Zhu et al., 2012; Grootaert et al., 2012). The nANGPTL4 assembles into oligomeric structures, which is important for its function as a lipoprotein lipase inhibitor (Lei et al., 2011; Dijk and Kersten, 2014). The cANGPTL4 interacts with integrin β1/5, vascular endothelial (VE)-cadherin, or claudin-5 to trigger intracellular pathways that aid wound healing and support tumor growth and metastasis (Go et al., 2010a, 2010b; Huang et al., 2011; Zhu et al., 2011). The expression of cANGPTL4 is elevated by numerous stimuli that are also involved in influenza pneumonia, including glucocorticoids, transforming growth factor β, and hypoxia-inducible factor 1α (HIF1-α) (Zhu et al., 2012; Grootaert et al., 2012). Furthermore, ANGPTL4 compromises the integrity of endothelial vascular junction by integrin signaling and disruption of intercellular VE-cadherin and claudin-5 cluster (Huang et al., 2011).

Interestingly, pulmonary edema due to vascular leakiness is a component of the fully developed viral lesion in the mouse (Harford et al., 1950). However, to our knowledge, the role of ANGPTL4 has not been studied in detail in influenza pneumonia, and study on this host response factor may open door to future intervention strategies. Thus in this study, we elucidate the role of host response protein ANGPTL4 during influenza pneumonia.

RESULTS

ANGPTL4 Expression Is Elevated in Influenza Virus-Infected Lungs

To investigate if ANGPTL4 is involved in the host response to influenza, we examined ANGPTL4 expression in mice infected with the PR8 influenza A H1N1 virus that is related to the 1918 pandemic influenza virus. The infection was performed via intratracheal inoculation with a nonlethal PR8 viral challenge that was sufficient to cause serious pulmonary damage. Viral replication was detected on bronchial structures at 3 days postinfection (dpi) and peaked at 5 dpi, as indicated by the presence of NS1 viral protein (Figure 1A). Virus was also detected in alveolar type II epithelial cells throughout the lungs by immunofluorescence staining with surfactant protein C (Figure 1B). At day 7, the viral protein expression began to decrease, and it became undetectable at 9 dpi (Figure 1A). The viral replication profile during the disease progression was further confirmed by qPCR of viral nucleoprotein RNA from infected lungs (Figure 1C). In addition, the expression of interferon-γ (IFN-γ) and interleukin-6 (IL-6), cytokines critical for innate and adaptive immunity against viral infections, peaked at 5 dpi (Figure 1C).

H&E staining of infected lung sections revealed that bronchial cells were damaged at 5 dpi, corresponding to the peak of viral load (Figure S1A). We observed extensive lung tissue damage that was marked by large regions of pulmonary hemorrhage and infiltration of host immune cells at 13 dpi (Figure S1A). Inflammatory cells, such as macrophages and neutrophils, infiltrated the alveolar spaces of infected lungs, causing tissue damage and bleeding (Figure S1B). Together with damaged tissue debris, the infiltrated cells formed dense cell clusters and filled up the alveolar spaces. In our model, the mice recovered from the infection, which allowed us to investigate the events that occurred during the recovery phase. Indeed, overall recovery of tissue integrity was observed at 19 dpi (Figure S1A).

Next, we determined the kinetics of ANGPTL4 mRNA and protein levels in the infected lungs by qPCR and immunoblot analyses, respectively. ANGPTL4 mRNA was significantly upregulated at day 5 and remained elevated until 9 dpi. Thereafter, its expression decreased, reaching the basal level by 17 dpi (Figure 1D). Elevated levels of ANGPTL4 protein, specifically cANGPTL4, were detected in lung tissue homogenates at day 7 and remained elevated until 19 dpi, compared to day 0 controls (Figure 1E). We also examined the spatiotemporal expression of ANGPTL4 during influenza infection by immunofluorescence staining (Figure 1F). At day 0, cANGPTL4 protein was restricted to tubular structures such as the blood vessels and bronchioles (Figure 1F). At 5 and 13 dpi, corresponding to the peaks of ANGPTL4 mRNA and protein respectively, we observed stronger cANGPTL4 staining within the inflamed regions infiltrated by immune cells (Figure 1F). To eliminate the possible interference from autofluorescence, we provided technical controls for the staining using isotype immunoglobulin G (IgG) control and Alexa Fluor 647 fluorescence antibody. Both the blood vessels and infiltrated alveolar space showed positive staining with anti-cANGPTL4 mouse monoclonal antibody as the primary antibody and anti-mouse Alexa Fluor 647 as the secondary antibody. In contrast, no staining of the blood vessel and infiltrated alveolar space at the corresponding regions in neighboring slide was observed when control IgG and anti-mouse Alexa Fluor 647 antibody were used (Figure S1C). The result confirmed the ANGPTL4 signal was specific. To confirm that the elevated cANGPTL4 expression was not virus-strain specific, we examined its expression in the lungs of mice challenged with a sublethal dose of a mouse-adapted H5N2 strain. H5N2 infection of BALB/c mice exhibited increased levels of cANGPTL4 as well as full-length ANGPTL4. The cANGPTL4 protein peaked at 10 dpi, while the expression of native ANGPTL4 protein remained elevated at 15 dpi during the recovery of lung tissues (Figures S1D and S1E). This confirmed that ANGPTL4 upregulation was not restricted to a specific strain of influenza virus or mouse. Notably, different strains of virus and mouse showed distinct ANGPTL4 mRNA and protein expression profiles.

ANGPTL4 Expression Is Regulated by a STAT3-Mediated Mechanism

As the ANGPTL4 mRNA expression profile mirrored the pattern of viral replication, we asked if viral infection could influence the expression of ANGPTL4. Dual immunofluorescence staining for NS1 viral protein and cANGPTL4 revealed that infected alveolar type II epithelial cells displayed cANGPTL4 staining, whereas the uninfected alveolar type II epithelial cells within the same lung did not exhibit cANGPTL4 staining (Figure 2A). Although Clara cells were also stained positive for ANGPTL4 (Figure S2A), we did not detect any significant ANGPTL4 mRNA level in Clara cells isolated by laser capture microdissection (Figure S2B), suggesting that the ANGPTL4 staining most likely derived from secretion from heterotypic cell types. Next,
Figure 1. Elevated cANGPTL4 mRNA and Protein Expression during Influenza Virus-Induced Pneumonia

C57BL/6J mice were infected with the PR8 virus, and lungs were harvested at the indicated days postinfection (dpi). (A) Representative immunofluorescence staining for viral protein NS1 (green) and cell nuclei (blue) of influenza-infected lungs. Scale bar, 50 μm. (B) Representative dual immunofluorescence staining of alveolar epithelial type II cells (SPC, red) and viral protein NS1 (green) showing viral infection in the alveolar space is limited to alveolar epithelial type II cells. Scale bar, 60 μm. (C) Relative mRNA expression of viral NP mRNA (means ± SEM, n = 5) and protein levels (means ± SEM, n = 4) of cytokine interleukin-6 (IL-6, blue) and interferon-γ (IFN-γ, red) in BALF as determined by Bioplex as described in Experimental Procedures. (D and E) Relative expression of ANGPTL4 mRNA (D) and protein (E) in lungs harvested at the indicated dpi. mRNA expression was normalized to that of the housekeeping gene ribosomal protein L27, which did not change under any of the studied experimental conditions (means ± SEM, n = 5). β-Tubulin served as a loading and transfer control for immunoblotting. (F) Representative H&E images and immunofluorescence-stained images of ANGPTL4 (green) counterstained with DAPI (blue) of infected lung sections. Scale bar, 1,000 μm.

All the staining pictures shown in this figure are representative images from 15 mice for each time point. See also Figure S1.
Figure 2. ANGPTL4 Expression Is Regulated by a STAT3-Mediated Mechanism

(A) Dual immunofluorescence staining of cANGPTL4 (green) and viral protein NS1 (red). Staining images are representative of five mice. Scale bar, 50 μm.

(B) Relative mRNA (left panel) and protein (middle and right panels) levels of ANGPTL4 in lungs of mice infected with a lethal dose of PR8 virus (1,000 pfu). mRNA expression was normalized to that of the housekeeping gene ribosomal protein L27, which did not change under any of the studied experimental conditions (means ± SEM, n = 3). β-Tubulin served as a loading and transfer control for immunoblotting.

(D) STAT binding site

(E) Furin Activity in BALF of Infected Mice

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we examined ANGPTL4 expression during influenza infection of mice with a lethal dose of PR8 virus (1,000 plaque-forming units [pfu]). We detected an earlier upregulation of ANGPTL4 mRNA and protein at 3 dpi, while the expression of native ANGPTL4 and cANGPTL4 remained elevated until the mice were euthanized at 7 dpi (Figure 2B).

We next sought to elucidate the underlying mechanism of this upregulation. HIF1-α has been shown to increase the expression of ANGPTL4 in endothelial cells (Zhu et al., 2012; Grootaert et al., 2012). To investigate if HIF1-α could be responsible for the expression of ANGPTL4 mRNA, we examined the HIF1-α protein expression profile in our animal model. The peak HIF1-α protein expression was detected at 13 dpi, which did not coincide with the peak mRNA expression of ANGPTL4 (Figure S2C), suggesting HIF1-α was unlikely to be a major regulator of ANGPTL4 at the early stages of infection. However, we cannot exclude that HIF1-α may maintain or sustain ANGPTL4 expression at later stages of infection. Interrogation of GEO data sets revealed that STAT3 deficiency in pulmonary alveolar type II epithelial cells was related to lower ANGPTL4 levels (Xu et al., 2007). Interestingly, viral infection triggered host responses through IFN-γ and IL-6 pathways, which activate STAT, and displayed overlapping profiles to the ANGPTL4 mRNA expression pattern (Figure 1C). In silico analysis of the promoter of the mouse ANGPTL4 gene also revealed a putative STAT-binding site (Figure 2C). Thus, we performed chromatin immunoprecipitation (ChIP) using phospho-STAT3 (pSTAT3) antibody on the ANGPTL4 promoter in uninfected and infected lung tissues. The sequences spanning the STAT-binding site were enriched in the immunoprecipitates obtained from the virus-infected lung tissues compared to uninfected tissues (Figure 2D). pSTAT3 ChIP experiments followed by re-ChIP with p300, a STAT3 coactivator, further confirmed the existence of direct regulation of ANGPTL4 via a STAT3-mediated mechanism (Figure 2D). To further strengthen our in vivo findings, we suppressed endogenous STAT3 expression by small interfering RNA (siRNA) and examined the expression of ANGPTL4 in a human small airway epithelial cell culture exposed to IL-6. We observed a significant increase in ANGPTL4 mRNA in response to an IL-6 challenge, which was abrogated when endogenous STAT3 was knocked down by ON-TARGETPlus siRNA (Figure S2D). We confirmed that the STAT3 protein was also reduced by western blot analysis (Figure S2D).

The flANGPTL4 undergoes proteolytic processing to release cANGPTL4. Furin proprotein convertase is known to cleave flANGPTL4. Furin proprotein convertase is known to cleave ANGPTL4 (Lei et al., 2011). To determine whether furin-like activity is present and consistent with the peak of cANGPTL4 protein expression in influenza-infected lungs, we measured furin activity in the bronchoalveolar lavage fluid (BALF) of mice that were sublethally infected with PR8 virus. Furin activity in the BALF started to increase at 5 dpi, coinciding with the peak in virus titer (Figure 2E). Importantly, the furin activity profile overlapped significantly with that of cANGPTL4 protein (Figure 2E). To strengthen the role of furin in ANGPTL4 cleavage, we performed similar experiments in the presence or absence of a furin inhibitor. Furin inhibitor was added to BALF taken from 11 dpi, a time point when the furin activity was elevated. Recombinant flANGPTL4 protein with FLAG tag at the C terminus was used as exogenous substrate. We detected a reduced cANGPTL4:flANGPTL4 ratio in BALF containing furin inhibitor when compared to BALF alone (Figure S2E), confirming that furin was a major contributor of post-translational cleavage of flANGPTL4 in our animal model.

Taken together, our data demonstrated that ANGPTL4 mRNA was upregulated via a STAT3-dependent pathway during influenza pneumonia. The concomitant increase in furin activity subsequently cleaved flANGPTL4 to generate cANGPTL4, which peaked at 13 dpi, corresponding to extensive lung injury marked by large regions of pulmonary hemorrhage and infiltration of host immune cells.

**Immunoneutralization of cANGPTL4 Significantly Reduces Tissue Leakiness to Accelerate Lung Recovery**

To understand the role of cANGPTL4 in influenza pathogenesis, we investigated the in vivo effect of a neutralizing cANGPTL4 monoclonal antibody (mAb; clone 3F4F5) on the host response to influenza viral pneumonia. We employed two treatment strategies based on the stage of disease progression (Figure 3A). Virus-induced inflammation caused severe lung damage, which was observed until 13 dpi. Thereafter, tissue regeneration began to restore lung structural integrity and function. Thus, we defined the period before 13 dpi as the “damage window,” and days after 13 dpi as the “recovery window.” Daily intravenous injections of anti-ANGPTL4 mAb (10 mg/kg body weight) were administered for 5 days starting either at 6 dpi during the damage window or day 13 during the recovery period (Figure 3A). Negative control groups included mock-infected mice that received either isotype-matched control mouse IgG or anti-cANGPTL4 mAb alone. Mice infected with influenza virus and treated with control mouse IgG served as another control group. Lung tissues were harvested 24 hr after the last injection.

The anti-cANGPTL4 mAb treatment during the “damage window” did not significantly alleviate the early inflammation-induced lung damage (Figure S3A). In contrast, anti-cANGPTL4 mAb treatment during the recovery stage resulted in reduced lung damage and a significant improvement in tissue recovery compared to control groups (Figure 3B). The alveolar spaces showed a remarkable increase of noninfiltrated areas with reduced pulmonary bleeding and accelerated regeneration of alveolar type I epithelial cells (Figure 3C). To pursue this further,

(C) Schematic diagram showing the relative position of a putative STAT-binding site in the mouse ANGPTL4 gene promoter.
(D) ChIP assay was conducted using preimmune IgG or antibodies against pSTAT3 (top panel) and re-ChIP with anti-p300 (bottom panel) in infected (I) and mock-infected (N) lungs. The specific region spanning STAT3 binding site of ANGPTL4 gene was amplified using appropriate primers. A control region served as a negative control.
(E) Bar graph shows furin activity in the BALF extracts at the indicated time points after PR8 infection (means ± SEM, n = 10). Expression profile of viral NP mRNA (dark blue), ANGPTL4 mRNA (red), and protein (light blue) were plotted.

See also Figure S2.
Figure 3. Immunoneutralization of cANGPTL4 Improves Lung Tissue Recovery after Influenza Infection

(A) Schematic diagram showing administration protocols during “damage” (yellow arrows) and “recovery” (blue arrows) windows. Graph shows a summary of various events of mouse influenza infection model plotted based on earlier observations.

(B) Day 18 post infection
Mock infection + control IgG
Flu + control IgG
Flu + anti-cANGPTL4 antibody

(C) Day 18 post infection
Mock infection + control IgG
Flu + control IgG
Flu + anti-cANGPTL4 antibody

(D) HMVEC
vehicle
ZO-1
Phalloidin
Merge with DAPI

HSAEC
cANGPTL4

(E) Mock infection
Flu + anti-cANGPTL4 antibody
+control IgG

(F) Day 18 Post Infection
Mock infected
ANGPTL4

Flu infected
ANGPTL4

(G) Day 18 Post Infection
Mock infection
ANGPTL4

Flu infection
ANGPTL4

Flu infection
ANGPTL4

(legend continued on next page)
we examined the in vivo integrity of the pulmonary vasculature by in vivo imaging of the mice receiving anti-cANGLTL4 mAb during the recovery period (Figure 3E). Mice were injected intravenously with IRDye 800CW PEG contrast agent (50 nmol/kg body weight) 24 hr after the last mAb injection. The presence of infrared (IR) signal indicates the accumulation of IRDye 800CW PEG in tissue due to increased leakiness of the local tissue. As expected, infected mice treated with control IgG showed a very high IR signal in the lung compared to mock-infected mice (Figure 3E). Infected mice treated with anti-cANGLTL4 mAb showed a significantly subdued IR signal in the lungs compared with control IgG treatment, indicating that immunoneutralization of cANGLTL4 markedly abrogated tissue leakiness. Ex vivo imaging of resected mouse lungs confirmed the reduced accumulation of IRDye 800CW PEG agent following anti-cANGLTL4 mAb therapy. To more precisely define the cell types responsible for the increased tissue leakiness, we examined the cell-cell junction of human small airway epithelial cells and microvascular endothelial cells after treatment with recombinant cANGLTL4 by immunofluorescence staining with tight junction protein zonula occludens-1 (ZO-1). The cANGLTL4 protein perturbed the cell-cell boundary of primary human endothelial and epithelial cells (Figure 3D), indicating that cANGLTL4 exacerbated pulmonary bleeding and edema and could accelerate the infiltration of immune cells inside the lung to aggravate tissue damage.

To further strengthen our above observation, we examined the effect of influenza infection on wild-type (ANGLTL4+/+) and ANGLTL4 mutant mice (knockout ANGLTL4−/− and heterozygous ANGLTL4+/−). We observed lung damage in ANGLTL4−/− at 11 dpi, albeit at reduced severity when compared to influenza-infected ANGLTL4+/+ (Figure S3B). Consistent with above observation with neutralizing anti-cANGLTL4, infected ANGLTL4−/− mice showed rapid lung tissue recovery at 18 dpi compared with ANGLTL4+/+ (Figure 3F) and accelerated regeneration of alveolar type I epithelial cells (Figure 3G). We also studied the effect of ANGLTL4 gene dosage on lung tissue damage and recovery using ANGLTL4+/− mice. Similar to cANGLTL4 antibody-treated ANGLTL4+/−/− mice, significant improvement in tissue recovery was observed in PR8-infected ANGLTL4+/− mice when compared with ANGLTL4+/+/− mice at 18 dpi, but no significant difference in tissue damage was observed at 11 dpi (Figures S3C and S3D). We also confirmed the observations by immunoblotting of podoplanin (PDNP) as a marker of lung damage as well as scoring of lung tissue damage by trained pathologist (Figures S3E and S3F).

To explore the biological impact of anti-cANGLTL4 mAb therapy on infected lungs, we performed microarray gene expression analyses of lungs of mice treated with either anti-cANGLTL4 antibody or control IgG from 13 to 17 dpi. Data analyses revealed that mAb therapy resulted in differences in numerous major physiological activities, including angiogenesis, lung tissue development, inflammatory responses, and extracellular matrix and endopeptidase activities (Figures 4A and S4). Genes involved in the development of lung alveoli, respiratory tubes, and blood vessels were identified in the anti-cANGLTL4 mAb-treated infected mice, indicating earlier tissue regeneration. The mAb treatment also dampened the inflammation-related tissue damage, consistent with the pathological phenotype. Taken together, our findings indicate that the functional neutralization of cANGLTL4 during the tissue recovery stage promoted lung tissue recovery and was associated with improved tissue integrity.

cANGLTL4 Expression Is Enhanced in Human Lung Biopsy Specimens of Patients with Infection-Induced Pneumonia

To underscore the clinical relevance of ANGLTL4 in lung infection and lung damage, we performed a retrospective immunofluorescence examination of ANGLTL4 expression in 40 archived human lung biopsy specimens from patients either without pneumonia or with pneumonia induced by various infections as approved by the institutional review board of the National University Hospital (reference number 2012/00661). The staining and microscopic imaging techniques were performed simultaneously under the same conditions, allowing the signal intensity to serve as a semiquantitative measure of the cANGLTL4 expression level. In the ten pneumonia samples that were symptomatic or microbe positive, we found brightly stained structures (Figure 4B; Table S2). These structures included the thickened layers around tubular structures as well as intensively stained structures in the damaged alveolar regions that appeared to be collapsed membrane-like structures (Figure 4B; Table S2). These structures were abundant in regions with dense infiltration or collapsed alveolar spaces. The nonpneumonia samples showed either very weak staining or staining restricted to a thin layer along the tubular structures, consistent with our observations in healthy mouse lung tissues (Figures 4B and 1F; Table S2). The obstructive pneumonia sample that was not caused by infection did not show positive staining of cANGLTL4, which is consistent with our proposed mechanism whereby...
an infection-induced STAT pathway contributes to ANGPTL4 upregulation. These findings suggest that cANGPTL4 may be a potential biomarker for respiratory infection and pneumonia. Clearly, further validation with a larger patient cohort that includes human virally induced ARDS cases and other bodily fluids such as blood and sputum will be necessary.

**DISCUSSION**

One of the hallmarks of influenza pneumonia is the aggravated inflammatory host response accompanied by pulmonary edema and associated acute lung injury (Mizgerd, 2008). In this study, we showed that influenza infection elevated the expression of the host protein ANGPTL4 via direct transcriptional regulation by STAT3. The spatiotemporal expression of ANGPTL4 coincided with the inflammation phase of influenza infection, localized to lung regions with elevated immune cell infiltration and tissue damage. Similarly, our analysis of human clinical infection-associated pneumonia samples showed higher levels of ANGPTL4 compared to uncomplicated human lung sections. Notably, influenza infection of ANGPTL4-knockout mice and immunoneutralization of ANGPTL4 in wild-type mice showed significantly improved pulmonary tissue integrity and accelerated recovery from inflammation-induced tissue damage.

Recent high-throughput RNA sequencing of formalin-fixed, paraffin-embedded autopsy lung tissue samples from the 1918 and 2009 influenza pandemics revealed that ANGPTL4 mRNA was one of the most significantly upregulated genes in both samples (Xiao et al., 2013). Our interrogation of microarray data from influenza-infected mouse lungs also consistently detected elevated ANGPTL4 expression (Pommerenke et al., 2012). These observations underscore the importance of ANGPTL4 in response to pneumonia. However, its role in infected lungs remained unclear. From our influenza mouse model, we observed that the ANGPTL4 mRNA expression profile mirrored the pattern of viral replication. Probing further, we showed that IL-6-activated STAT3 directly regulated the expression of ANGPTL4. This is consistent with microarray analysis showing that pulmonary alveolar type II epithelial cells deficient in STAT3 have lower ANGPTL4 mRNA levels compared to their wild-type counterparts (Xu et al., 2007).

Furin or furin-like proprotein convertases (PCs) play multiple roles in host response to influenza infection. Toll-like receptor 7 (TLR7) triggers antiviral immune responses by recognizing viral single-stranded RNA in endosomes. hTLR7 is proteolytically processed by furin-like PCs, and the C-terminal fragment of hTLR7 selectively accumulates in endocytic compartments.
TLR7 processing was required for its functional response to TLR7 agonists such as R837 or influenza virus (Hipp et al., 2013). Interestingly, we observed that the peak expression of cANGPTL4 protein at 13 dpi was likely attributed to the increase in activity of furin PCs that preceded and overlapped with cANGPTL4 protein expression. Recent work showed that two novel peptidomimetic furin inhibitors inhibit hemagglutinin cleavage and viral propagation of a highly pathogenic avian H7N1 influenza virus strain in vitro (Becker et al., 2012). The in vivo effect of such furin inhibitors, which conceivably prevent the proteolytic cleavage of hANGPTL4 to release cANGPTL4, on lung tissue recovery remains to be explored.

Previous work has shown that cANGPTL4 can affect the paracelluar permeability of blood vessels in cancer (Huang et al., 2011; Guo et al., 2014). In vivo and ex vivo imaging of infected mice treated with neutralizing anti-cANGPTL4 mAb revealed diminished pulmonary tissue leakiness compared to isotype control IgG treatment. The direct consequence was reduced pulmonary edema and immune cell-infiltrated lung regions. The overall lung tissue integrity was improved and the alveolar space appeared to be well recovered for normal function as supported by microarray analysis. Administration of anti-cANGPTL4 mAb during the damage window, when cANGPTL4 protein levels were low, showed no observable difference compared to control. Thus, targeting cANGPTL4 to modulate tissue leakiness along with its collateral benefits is a promising approach toward the development of therapy for influenza treatment, specifically lung recovery. Our finding that host protein ANGPTL4 participates in pulmonary leakiness and lung injury responses during influenza pneumonia is an important step toward a better understanding of influenza pathogenesis and how it can be manipulated to reduce the burden of pneumonia.

EXPERIMENTAL PROCEDURES

Mice, Viruses, and Infections
Female 8-12-week-old C57BL/6J mice and BALB/c mice were purchased from the Biological Resource Centre, Biopolis, Singapore. Wild-type mice and mice heterozygous for ANGPTL4 (C57/B6 background) were obtained from the Mutant Mouse Regional Resource Center (MMRRC), an NIH-funded strain repository, and were donated to the MMRRC by Genentech. ANGPTL4 KO mice were identified using RT-PCR quantification of ANGPTL4 expression. Recent work showed that two

REFERENCES


Antibody Treatment of Mice
Anti-cANGPTL4 mAb (clone 3F4F5) was produced using hybridoma as described previously (Goh et al., 2010a, 2010b; Zhu et al., 2011). Mice were intraperitoneally injected daily with the antibody in 200 µl saline at a dose of 10 mg/kg body weight on 6–10 dpi (harvested on day 11) or on 13–17 dpi (harvested on day 18). More details on the experimental procedures can be found in Supplemental Experimental Procedures.

In Vivo Imaging of Mice
Mice were mock infected with heat-inactivated influenza virus, infected with influenza virus and injected with control IgG, or infected with influenza virus and treated with anti-cANGPTL4 antibody on 13–17 dpi as described above. At the day of lung harvesting, mice were injected through the tail vein with IRDye 800CW PEG contrast agent (Li-Cor 926-50401) and subjected to in vivo imaging under anesthesia, using the Li-Cor MousePOD in vivo imaging facility. The IR fluorescent-tagged PEG800 was used to detect tissue leakiness when the tissue exhibited abnormally high paracelluar permeability. Following imaging, the lungs of the mice were harvested and reimmersed, and quantification of fluorescence signal was done according to the manufacturer’s instructions.

ACCESSION NUMBERS
Microarray data have been submitted to the GEO database under accession number GSE58647.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.01.011.

AUTHOR CONTRIBUTIONS

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vates the prosurvival intracellular O2(-):H2O2 ratio and confers anoikis resis-

Angiopoietin-like 4 Increases Pulmonary Tissue Leakiness and Damage during Influenza Pneumonia

Liang Li, Han Chung Chong, Say Yong Ng, Ka Wai Kwok, Ziqiang Teo, Eddie Han Pin Tan, Chee Chong Choo, Ju Ee Seet, Hyung Won Choi, Martin Lindsay Buist, Vincent Tak Kwong Chow, and Nguan Soon Tan
SUPPLEMENTAL INFORMATION

Li et al., Figure S1
Mild H3N2 infection

Day post infection 0 5 10 15 kDa
fANGPTL4
cANGPTL4
β-tubulin

p<0.01
p<0.05
p<0.05

Relative protein expression
(ANGPTL4/β-tubulin)

Day post infection

Supplemental Figure A

Supplemental Figure B

Supplemental Figure C

Supplemental Figure D

Supplemental Figure E
Li et al., Figure S4

- membrane organization (1)
- defense response (52)
- defense response to bacterium (29)
- serine-type endopeptidase inhibitor activity (31)
- peptidase inhibitor activity (31)
- membrane invagination (1)
- cellular response to reactive oxygen species (2)
- endopeptidase inhibitor activity (8)
- extracellular region (258)
- hydrogen peroxide metabolic process (2)
- morphogenesis of an epithelium (2)
- tube development (2)
- inflammatory response (32)
- response to cytokine stimulus (12)
- melanosome (3)
- response to hormone stimulus (6)
- response to peptide hormone stimulus (10)
- lung development (27)
- antioxidant activity (7)
- response to reactive oxygen species (5)
- oxidoreductase activity, acting on peroxide as acceptor (1)
- tube morphogenesis (1)
- response to bacterium (3)
- vasculature development (3)
- blood vessel development (6)
- endocytosis (27)
- cytoplasmic vesicle (55)
- extracellular matrix organization (15)
- extracellular matrix (21)
- extracellular matrix binding (8)
- positive regulation of cell adhesion (5)
- respiratory tube development (1)
- basement membrane (17)
- response to wounding (2)
- cellular response to oxidative stress (2)
- cellular response to hydrogen peroxide (3)
- membrane–bounded vesicle (5)
- vacuole (3)
- hydrogen peroxide catabolic process (7)
- response to hydrogen peroxide (5)
- blood vessel morphogenesis (6)
- positive regulation of cell–substrate adhesion (11)
- lysosome (49)
- regulation of cell adhesion (2)
- lytic vacuole (1)
- lung alveolus development (10)
- response to organic substance (7)
- cytoplasmic membrane–bounded vesicle (8)
- vesicle (9)
- peroxidase activity (9)
- enzyme inhibitor activity (8)
- vesicle–mediated transport (28)
- angiogenesis (35)
- response to oxidative stress (24)
SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Pulmonary damage during sub-lethal PR8 influenza infection of C57BL/6J mice, and upregulation of ANGPTL4 in sublethal H3N2 influenza virus infection of BALB/c mice, related to Figure 1.

(A-B) Representative H&E images of lungs to show tissue damage progression at the indicated time points after influenza infection on mice. Scale bar = 100 µm (A); images to show macrophage, neutrophil, and lymphocyte infiltration at the indicated time points. Scale bar = 30 µm (B). Each picture is representative for 15 mice.

(C) Immunofluorescence staining of ANGPTL4 on mouse lung sections from PR8-infected C57 mice at 13 dpi using either control or anti-cANGPTL4 IgG primary antibodies, and indicated Alexa647 (red) and Alexa488 (green) conjugated secondary antibodies. H&E staining was used to show the tissue morphology on the same slide after taking picture for fluorescence staining. Each image is representative of 5 mice.

(D) Histopathologic analyses depicting lung samples of mice subjected to H3N2 infection and fatal PR8 infection at different time-points post-infection. Pictures are representative for 3 mice for each time point. Scale bar = 100 µm.

(E) Mice were challenged with a low dose of H3N2 virus, and lungs were harvested at 0, 5, 10, and 15 dpi. Both flANGPTL4 and cANGPTL4 protein levels were detected by Western blot and quantified using β-tubulin protein for normalization. Results are calculated from 3 mice for each time point (means±s.e.m).

Figure S2. STAT3 upregulates ANGPTL4 mRNA in type II alveolar epithelial, related to Figure 2.

(A) Dual immunofluorescence staining of CC10 (red) to show the distribution and morphology of Clara cells and of cANGPTL4 (green) to show the location of cANGPTL4 protein on mouse
lung sections harvested at 0, 5, 13 and 17 dpi. Scale bar = 40 µm. Each picture is representative of 15 mice.

**B** Clara cells did not express ANGPTL4 mRNA. Left panel: Representative H&E images of before-and-after laser capture microdissection of Clara cells from mouse lung sections of day 0, 5, and 13 post PR8 infection. Right panel: Relative mRNA expression of ANGPTL4 in AECII and Clara cells as determined by real-time PCR. No significant ANGPTL4 expression was detected in the RNA harvested from dissected Clara cells. Scale bar = 100 µm. Each picture is representative of 3 mice. Results for qPCR are calculated from 3 mice for each time point (means±s.e.m).

**C** HIF1-α protein expression profile in PR8-infected mouse lung. Left panel: Representative immunoblot showing HIF1-α protein expression profile in PR8-infected mouse lung samples at 3, 5, 13 and 17 dpi. β-tubulin served as a loading and transfer control. Right panel: Graph shows the protein expression profile of HIF1-α and ANGPTL4 mRNA level (red). The peak of HIF1-α protein expression (at day 13 post infection) did not coincide with the peak ANGPTL4 mRNA expression (at day 5 post infection) (means±s.e.m, n=3).

**D** IL-6 mediated upregulation of ANGPTL4 is dependent on STAT3. Left panel: Immunoblot analysis of STAT3 in human small airway epithelial cells transfected with either scrambled siRNA or ON-TARGETPlus siRNA against STAT3. Middle panel: Graph shows relative expression of STAT3 protein in scrambled and STAT3 siRNA cells. Right panel: Relative ANGPTL4 mRNA in scrambled and STAT3 siRNA cells treated with either vehicle or IL-6. The mRNA expression was normalized to that of the housekeeping gene ribosomal protein L27, which did not change under any of the studied experimental conditions (means±s.e.m, n=3).

**E** Furin was a major contributor of post-translational cleavage of flANGPTL4. Top panel: Representative immunoblot of C-terminal FLAG-tagged ANGPTL4 incubated with BALF extracted from lungs at 11 dpi in the presence of vehicle or furin inhibitor. Inhibition of furin
activity significantly reduced ANGPTL4 cleavage. Bottom panel: Graph shows the ratio of cANGPTL4 to flANGPTL4 in the two indicated samples. (means±s.e.m, n=3).

**Figure S3. ANGPTL4 deficiency reduces lung damage, related to Figure 3.**

(A-D) H&E stained images of lung sections from C57BL/6J mice infected with a sublethal dose of the PR8 virus and injected with anti-cANGPTL4 antibody (A); from ANGPTL4−/− knockout and wild-type ANGPTL4+/+ mice (B) and from ANGPTL4+/− heterozygous and wild-type ANGPTL4+/+ mice infected with a sublethal dose of the PR8 virus (C-D). Neutralising cANGPTL4 was done from 6 to 10 dpi. Lungs were harvested at either 11 dpi (A-C) or 18 dpi (D). Each picture is representative of 5 mice for each experimental group. Scale bar = 100 µm.

(E) Immunoblot of PDPN in indicated PR8-infected mouse lung samples at 11 and 18 dpi. β-tubulin served as a loading and transfer control for immunoblotting. Values below the band represent fold change compared to cognate control IgG. Each blot is representative of 3 mice.

(F) Graph shows percentage of damaged area in lung tissue sections from indicated treatments described in (A-D). (means±s.e.m, n=5).

**Figure S4. Heat maps show gene expression profiles of influenza-infected lungs left untreated or treated with anti-cANGPTL4 antibody (I vs A), related to Figure 4.** Genes are clustered according to the following biological gene functions. The colour spectrum from blue to red depicts the fold-change in LOG form from −1.0 to 1.0. Data was submitted to GEO under accession number GSE58647.
<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Species</th>
<th>Forward Primer Sequence (5' to 3')</th>
<th>Reverse Primer Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL27</td>
<td>Human/Mouse</td>
<td>CGCAAAGCTGTCATCGTG</td>
<td>CGCAAAGCTGTCATCGTG</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Human</td>
<td>TGGTTTGGCACCTGCAGCCATTC</td>
<td>TGCTGCCATGGGCTGGATCAAC</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Mouse</td>
<td>TCCAACGCCACCCACTTAC</td>
<td>TGAAGTCATCTCACAGTTGACCA</td>
</tr>
<tr>
<td>H1N1 NP</td>
<td>Mouse</td>
<td>GGGTGAGAATGGACGAAAAA</td>
<td>GCTCCAGTGAATTCGGAAAG</td>
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<td>pSTAT3 Binding Site</td>
<td>Mouse</td>
<td>GTCAATTTGCCCTAAGGGTC</td>
<td>GTCTCTGGCTTGCTCCCCTCTC</td>
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<tr>
<td>pSTAT3 Negative Control</td>
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<td>TTGTACGGGTTGGTTGAGCC</td>
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<tr>
<td>STAT3</td>
<td>Human</td>
<td>ATCACGCCTTCTACAGACTGC</td>
<td>CATCCTGGAGATTCTCTACACT</td>
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Table S2: List of Clinical Sample Background Information, related to Figure 4

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Reason for operation (normal samples)/Pathologic findings (abnormal samples) and relevant clinical information where available</th>
<th>Normal/Abnormal (N/A)</th>
<th>cANGPTL4 staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>67</td>
<td>Lung cancer</td>
<td>N</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>64</td>
<td>Lung cancer</td>
<td>N</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>65</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on tubular structure</td>
</tr>
<tr>
<td>M</td>
<td>70</td>
<td>Lung cancer</td>
<td>N</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>73</td>
<td>Metastatic renal cell carcinoma</td>
<td>N</td>
<td>Weak Staining</td>
</tr>
<tr>
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<td>44</td>
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<td>N</td>
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</tr>
<tr>
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<td>21</td>
<td>Congenital abnormality</td>
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</tr>
<tr>
<td>F</td>
<td>63</td>
<td>Lung cancer</td>
<td>N</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>67</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on tubular structure</td>
</tr>
<tr>
<td>M</td>
<td>40</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on RBC</td>
</tr>
<tr>
<td>M</td>
<td>58</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on RBC</td>
</tr>
<tr>
<td>M</td>
<td>45</td>
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<td>Weak Staining</td>
</tr>
<tr>
<td>F</td>
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<td>Lung cancer</td>
<td>N</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>44</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on tubular structure</td>
</tr>
<tr>
<td>F</td>
<td>77</td>
<td>Lung cancer</td>
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<td>Staining on tubular structure</td>
</tr>
<tr>
<td>F</td>
<td>44</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on tubular structure</td>
</tr>
<tr>
<td>F</td>
<td>79</td>
<td>Lung cancer</td>
<td>N</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>56</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on RBC</td>
</tr>
<tr>
<td>F</td>
<td>78</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on RBC</td>
</tr>
<tr>
<td>M</td>
<td>72</td>
<td>Lung cancer</td>
<td>N</td>
<td>Staining on tubular structure</td>
</tr>
<tr>
<td>M</td>
<td>64</td>
<td>Tuberculosis</td>
<td>A</td>
<td>Bright staining on tissue</td>
</tr>
<tr>
<td>M</td>
<td>51</td>
<td>Actinomycosis (patient has diabetes mellitus)</td>
<td>A</td>
<td>Bright staining on tissue</td>
</tr>
<tr>
<td>M</td>
<td>41</td>
<td>Lung abscess (known intravenous drug use)</td>
<td>A</td>
<td>Bright staining on tissue</td>
</tr>
<tr>
<td>F</td>
<td>65</td>
<td>Organising pneumonia. No specific organisms identified.</td>
<td>A</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>F</td>
<td>29</td>
<td>Fungal infection (bone marrow transplant patient)</td>
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<td>Weak Staining</td>
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<tr>
<td>F</td>
<td>35</td>
<td>Obstructive pneumonia (endobronchial leiomyoma)</td>
<td>A</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>40</td>
<td>Lung abscess (bacterial), patient has diabetes mellitus</td>
<td>A</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>53</td>
<td>Cryptococcosis (patient previously had chemotherapy for cancer)</td>
<td>A</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>M</td>
<td>63</td>
<td>Bulla with Asperillus (patient with chronic obstructive pulmonary disease)</td>
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<td>Bright staining on tissue</td>
</tr>
<tr>
<td>M</td>
<td>61</td>
<td>Lung abscess (bacterial), patient has diabetes mellitus</td>
<td>A</td>
<td>Bright staining on tissue</td>
</tr>
<tr>
<td>M</td>
<td>51</td>
<td>Organising pneumonia. No specific organisms identified.</td>
<td>A</td>
<td>Weak Staining</td>
</tr>
<tr>
<td>F</td>
<td>30</td>
<td>Tuberculosis (diagnosed during follow-up for lymphoma 8 years previously,</td>
<td>A</td>
<td>Weak Staining</td>
</tr>
<tr>
<td></td>
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<td>---</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>50</td>
<td>Lung abscess (amebic)</td>
<td>A</td>
<td>Bright staining on tissue</td>
</tr>
<tr>
<td>M</td>
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<td>Tuberculosis</td>
<td>A</td>
<td>Bright staining on tissue</td>
</tr>
<tr>
<td>M</td>
<td>53</td>
<td>Tuberculosis (patient on chemotherapy for cancer)</td>
<td>A</td>
<td>Bright staining on tissue</td>
</tr>
<tr>
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<td>49</td>
<td>Cryptococcosis</td>
<td>A</td>
<td>Weak Staining</td>
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<tr>
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<td>Bronchiectasis. Previous tuberculosis.</td>
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</tr>
<tr>
<td>F</td>
<td>56</td>
<td>Tuberculosis</td>
<td>A</td>
<td>Weak Staining</td>
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<tr>
<td>M</td>
<td>89</td>
<td>Fungal infection</td>
<td>A</td>
<td>Bright staining on tissue</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies. Primary antibodies used for the following targets were purchased from commercial sources as stated: influenza NS1 (sc130568), SPC (sc13979), CC10 (sc9772), β-tubulin (sc58886) (Santa Cruz), HIF-1α (ab113642), STAT3 (ab7966) (Abcam), Pdpn (AF3244, R&D Systems), pSTAT3 (9145, Cell Signalling), p300 (05-257, Millipore), FLAG (F7425, Sigma) and ZO-1 (40-2200, Zymed). Anti-mouse cANGPTL4 antibody and anti-human cANGPTL4 antibody were produced in-house as described previously (Goh et al., 2010; Zhu et al., 2011).

Immunofluorescent staining of lung sections. Harvested mouse lungs were fixed overnight in 10% neutral buffered formalin solution (Sigma Aldrich), followed by embedding in paraffin with tissue processor (Leica). Sections (5 μm thick) on SuperFrost™ Plus coated slides (Thermal Scientific) were de-waxed with xylene and rehydrated in water. Antigen retrieval was processed with proteinase K (20 mg/ml in 50 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 37°C for 30 min or incubated with 10 mM sodium citrate buffer, pH 6.0 at 95°C for 20 min. The sections were incubated with the appropriate antibody overnight at 4°C, and stained with secondary antibody for 1 h at room temperature. The slides were mounted with anti-fade reagent with DAPI (Invitrogen) and then scanned by high-resolution MIRAX MIDI system or Observer Z1 fluorescence microscopy (Carl Zeiss). Technical controls were performed using sections from the same tissue stained with either the target primary antibody (i.e. anti-cANGPTL4 antibody) or mouse control IgG, followed by secondary detection with either Alexa 647 or Alexa 488 (Invitrogen) conjugated secondary antibody. Sections were counterstained with DAPI and mounted. After imaging, the same sections were washed with PBS and stained with hematoxylin and eosin (H&E) for imaging again to reveal the corresponding tissue morphology.
Quantitative real-time RT-PCR. Total RNA was extracted by the Qiagen RNeasy mini kit and treated with DNaseI (Qiagen). RNA concentration was measured by the ND-1000 spectrophotometer (NanoDrop Technologies), and 1 µg of RNA was reverse transcribed using iScript reverse transcriptase (Bio-Rad). PCR was performed with the Bio-Rad CFX-96 real-time system using KAPA Sybr fast qPCR master mix according to the manufacturer's instructions (KAPA Biosystems). The primers used are listed in the Table S1.

Immunoblotting. Lung tissues were lysed with M-PER Mammalian Extraction Buffer (Pierce) and the protein concentration was determined by the Bio-Rad Protein Assay kit. Five µg of protein were heated in 2x Laemmlli sample buffer, separated by SDS-PAGE and transferred to low fluorescent PVDF membranes (Millipore). Immunoblotting was performed as previously described (Chong et al., 2014).

Bronchoalveolar Lavage Fluid (BALF) Analysis for Furin Activity and Cytokines. Using a 3 mL syringe and 23G needle, BALF was taken from mice by injecting and retracting 1 mL of PBS into the lung of mice through trachea at 0, 3, 5, 7, 9, 11, 13, 15, 17, and 19 dpi. BALF was kept at -80 ºC before use. Furin activity was measured using BALF incubated together with fluorogenic furin convertase substrate for 5 hours at 37 ºC (Enza Life Sciences, ALX-260-040-M005) (Cork et al., 2012). For furin inhibitor assay, BALF from 11 dpi was used to evaluate the capability for cleavage of flANGPTL4 protein. Recombinant C-terminal FLAG-tagged flANGPTL4 (MyBioSource, MBS636187) was added to BALF treated with or without furin inhibitor (Calbiochem, 344930) and incubated at 37 ºC for 12 h. Post-translational cleavage of flANGPTL4 to cANGPTL4 was analysed by immunoblotting with anti-FLAG antibody. Cytokine concentrations were measured from BALF using Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad, M60-009RDPD) following manufacturer’s instructions.
ChIP and Re-ChIP. In vivo ChIP was carried out as previously described, except that anti-phospho-STAT3 antibody was used (Chong et al., 2009). The putative pSTAT3-binding site in the mouse ANGPTL4 promoter was based on reported STAT-binding sites (Seidel et al., 1995; Ehret et al., 2001). The primers used for ChIP are shown in the table below.

Microarray analysis. Infected mice were treated with either control IgG or anti-cANGPTL4 antibody on 13 to 17 dpi as described above. Lungs were harvested at day 12 (for mice with infection alone), or days 14, 16 and 18 (for infected mice treated with either control IgG or anti-cANGPTL4 antibody). RNA was extracted from the lungs with Trizol following the manufacturer’s protocol. Further sample processing of the RNA was carried out using Applause® WT-Amp ST System (NuGEN), and microarray experiments were performed on GeneChip® Mouse Gene 1.0 ST arrays according to the manufacturer’s instructions.

In vitro permeability assay. Human microvascular endothelial cells (HMVEC) and human small airway epithelial cells (HSAEC) were purchased from ATCC and cultured following manufacturer’s instructions. After reaching confluence, cells were treated with either recombinant cANGPTL4 protein (6 µg/mL) or vehicle for 6 h before fixation with 4% paraformaldehyde in PBS solution. Fixed cells were treated with 0.1% Triton X-100 in PBS solution and stained with ZO-1 antibody, followed by phalloidin-Alexa 594 (Invitrogen, A12381), anti-rabbit Alexa 488, and finally counterstained with DAPI (Huang et al., 2011). Imaging was carried out using Zeiss LSM 710 confocal microscopy.

Laser capture microdissection (LCM). Samples consisted of infected lungs at three reference time-points, i.e. mock infection (day 0); peak of viral replication (day 5); and peak of edema (day 13). LCM was performed with the PALM Microbeam III (Carl Zeiss) as previously described
RNA samples from LCM were isolated using RecoverAll Total Nucleic Acid Isolation kit (Life Technologies) and amplified as cDNA with Full Spectrum Complete Transcriptome RNA Amplification Kit (System Biosciences). qPCR was performed as described above.

**Suppression by RNA interference (siRNA).** HSAEC cells were cultured in 6-well plate and transfected with either STAT3 siRNA (ON-TARGETplus SMARTpool, L-003544-00-0005) or scramble siRNA (Dharmacon) using DharmaFect 1 as described by manufacturer. After 48 h, cells were treated with either recombinant IL6 (20 ng/mL) or vehicle for 12 h before harvesting for RNA and protein analysis.

**Lung Damage Scoring.** The extent of lung damage from H&E stained lung sections was scored by a trained histophatologist (J.E. Seet, National University Hospital, National University Health System, Singapore) in a double-blinded fashion using scoring criteria as previously described (Matute-Bello et al., 2001). Percentage of significant lung damage, alveolar haemorrhage, alveolar infiltrates, fibrin and alveolar septal congestion were examined. With the whole lung considered as 100%, percentages of significantly damaged lung regions were defined to meet at least 2 criteria of alveolar haemorrhage, alveolar infiltrates, fibrin and alveolar septal congestion.

**Microarray data processing.** To obtain mRNA expression data, the raw data (.CEL) were processed using the Affy package and mogene10stv1cdf / mogene10sttranscriptcluster.db libraries in Bioconductor (http://www.bioconductor.org, with R 3.0.2). All gene level annotation data (such as gene IDs, chromosomal location, Gene Ontology membership) were mapped using the bioconductor utilities. This process generated intensity measurement for 34,760 transcript clusters (probesets) at all four time-points in both control and infected samples. Since some
transcript clusters mapped to the same genes, intensity data were averaged for a unique gene ID (Entrez ID), resulting in a processed dataset with 8 samples and 20,694 genes.

**Mixture model and statistical significance.** To identify differentially expressed genes during the time course between infected and uninfected cells, the ratios of intensities between the two conditions were calculated at each time-point for each of the 20,694 genes. Statistical significance was then calculated in the form of the posterior probability of differential expression given the ratio. To do this, the distribution of intensities and their ratios were examined visually, and it was observed that the data for day 16 was significantly different from the other three days in both conditions. Hence, the data for day 16 was removed for subsequent analysis. To compute the statistical significance of differential expression, the distribution of intensity ratios for all genes were modeled as a mixture of three distributions, each component representing those genes underexpressed in infected cells, equal expression in both cells, and overexpressed in infected cells, respectively: 

\[ f(r) = \pi_{-1} f_{down}(r) + \pi_0 f_{down}(r) + \pi_1 f_{up}(r). \]

Using the estimated mixture model \( f(r) \), the posterior probability of differential expression (either under- or over-expression) can be computed using Bayes rule. For a gene with ratio \( r \), the probability of upregulation is computed as \( pp_1(r) = \pi_1 f_{up}(r)/f(r) \), and likewise for downregulation (probability of downregulation was assigned a negative sign in the reported table). The posterior probability of differential expression of gene \( k \) was denoted by \( pp_k \). Using these scores, we also computed the Bayesian false discovery rates (FDR) in selecting all genes above a probability threshold \( x \) as 

\[ FDR(x) = \frac{\sum_{\{pp_i>x\}}(1-pp_i)/\sum_{\{pp_i>x\}}}{\sum_{\{pp_i>x\}}}. \]

Setting \( x = 0.8 \), the corresponding FDR was 4.99%.

**Patient biopsy samples.** Archived paraffin blocks from 20 patients with ‘normal’ non-inflamed lungs and 20 patients with pneumonia were selected by a pulmonary pathologist. The patient
backgrounds and pathologic findings were recorded for each sample. The ‘normal’ samples were obtained from lung resections for non-infectious/inflammatory causes, most commonly lung cancer. The samples were taken away from the tumour, and the pathologist verified that no cancer or significant inflammatory process was present in the given tissue block. Cases of lung resections performed for various inflammatory or infectious diseases (‘abnormal’ samples) were retrieved from the archives of the Pathology Department. Examples of such cases included lung abscess or empyema (mainly bacterial), infection causing hemoptysis (e.g. tuberculosis, mycetoma), and infection masquerading as lung mass and excised due to the possibility of cancer and/or obstructive pneumonitis. Details of the samples are attached in Table S2. All the slides were processed together under the same conditions for both anti-cANGPTL4 and H&E staining. Images were taken within the regions identified by the pathologist to ensure the findings reflected representative patterns in pneumonia and normal lung regions. Anti-cANGPTL4 staining and microscopic imaging were performed as described above. This study was approved by the Institutional Review Board of the National University Hospital (reference number 2012/00661).

**Statistical Analysis.** Statistical analysis was performed using two-tailed Mann-Whitney test using SPSS v.19 software (IBM Corporation, USA). A p value <0.05 was considered significant. For *in vivo* intensity measurement in Figure 3E, statistical analysis was performed using single-factor ANOVA method. A p value <0.05 was considered significant.
SUPPLEMENTAL REFERENCES


