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Semaphorin 7A coordinates Neutrophil response during pulmonary inflammation and sepsis

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Abstract:
Rationale: Pulmonary defense mechanisms are critical for host integrity during pneumonia and sepsis. This defense is fundamentally dependent on the activation of neutrophils during the innate immune response. Recent work has shown that Semaphorin 7A (Sema7A) holds significant impact on platelet function, yet its role on neutrophil function within the lung is not well understood. Objective: To identify the role of Sema7A during pulmonary inflammation and sepsis. Measurements and Main Results: In ARDS patients we were able to show a correlation between Sema7A and oxygenation levels. During subsequent workup we found that Sema7A binds to the neutrophil PlexinC1 receptor, increasing integrins and L-selectin on neutrophils. Sema7A prompted neutrophil chemotaxis in-vitro and the formation of platelet-neutrophil complexes in-vivo. We also observed altered adhesion and transmigration of neutrophils in Sema7A-/- animals in the lung during pulmonary inflammation. This effect resulted in increased number of neutrophils in the interstitial space of Sema7A-/- animals but reduced numbers of neutrophils in the alveolar space during pulmonary sepsis. This finding was associated with significantly worse outcome of Sema7A-/- animals in a model of pulmonary sepsis. Conclusions: Sema7A has an immunomodulatory effect in the lung affecting pulmonary sepsis and ARDS. This effect influences the response of neutrophils to external aggression and might influence patient outcome.

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Semaphorin 7A coordinates neutrophil response during pulmonary inflammation and sepsis

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ABSTRACT

Rationale: Pulmonary defense mechanisms are critical for host integrity during pneumonia and sepsis. This defense is fundamentally dependent on the activation of neutrophils during the innate immune response. Recent work has shown that Semaphorin 7A (Sema7A) holds significant impact on platelet function, yet its role on neutrophil function within the lung is not well understood.

Objective: To identify the role of Sema7A during pulmonary inflammation and sepsis.

Measurements and Main Results: In ARDS patients we were able to show a correlation between Sema7A and oxygenation levels. During subsequent workup we found that Sema7A binds to the neutrophil PlexinC1 receptor, increasing integrins and L-selectin on neutrophils. Sema7A prompted neutrophil chemotaxis in-vitro and the formation of platelet-neutrophil complexes in-vivo. We also observed altered adhesion and transmigration of neutrophils in *Sema7A*$^{-/-}$ animals in the lung during pulmonary inflammation. This effect resulted in increased number of neutrophils in the interstitial space of *Sema7A*$^{-/-}$ animals but reduced numbers of neutrophils in the alveolar space during pulmonary sepsis. This finding was associated with significantly worse outcome of *Sema7A*$^{-/-}$ animals in a model of pulmonary sepsis.

Conclusions: Sema7A has an immunomodulatory effect in the lung affecting pulmonary sepsis and ARDS. This effect influences the response of neutrophils to external aggression and might influence patient outcome.

Key Points:

1. Semaphorin 7A controls neutrophil chemotaxis in-vitro and the formation of platelet-neutrophil complexes in-vivo
2. Sema7A influences the early phase of inflammation, which is relevant to the outcome in murine experimental sepsis

INTRODUCTION

Sepsis remains one of the leading causes of death worldwide and one of the leading causes of hospitalization [1]. Pneumonia is frequently the source of pulmonary sepsis, caused by the invasion of pathogens entering the lung and through the pulmonary barrier into the human circulation. In hospitalized and ventilated patients, gram-negative bacteria are frequently the cause of pneumonia, whereas gram-positive bacteria are more common in patients with community-acquired pneumonia [2]. The therapy of pneumonia consists of treatment with supplemental oxygen if required, appropriate diagnostic testing and appropriate antiviral and antimicrobial therapy, however, this approach is not always successful, and the severity of the disease progresses. Therefore, understanding the mechanisms that control, influence or support the host response during pneumonia might help to improve the outcome of patients [3].

Neutrophils are the forefront in the defense against pathogens that challenge the lung [4]. Following activation, neutrophils tether and roll along the endothelial surface. This process is dependent on L-selectin expression [5,6]. L-selectin cleavage from the neutrophil surface is triggered by integrin engagement and is involved in neutrophil recruitment to the lung and bacterial clearance [7]. P-selectin expression and MAC-1 are also important during the recruitment of neutrophils into the lung and correlate with outcome in patients with ARDS [8,9]. Subsequently, integrins play key roles in the

transendothelial migration and activation of neutrophils. The differential context of the integrins is demonstrated by the fact that blocking antibodies against the β2 integrin CD18 result in an increased number of neutrophils in the alveolar space and a reduction in pulmonary injury. β2 integrin binds to ICAM-1 on endothelial cells, which results in increased transendothelial neutrophil migration, an essential mechanism of pulmonary host defense [10].

Recent work has shown that the neuronal guidance protein semaphorin 7A (Sema7A) is important throughout the initial stages of inflammation. During the inflammatory response, Sema7A can bind to integrin α1β1 to facilitate cytokine storm [11]. We have shown in the past that Sema7A is induced during periods of hypoxia and activates platelets through the glycoprotein Ib-IX-V (GPIb) receptor complex [12,13]. To highlight the role of Sema7A during the pulmonary immune response, we investigated the role of Sema7A during LPS-induced and *Klebsiella pneumoniae*-induced pulmonary effects and in human samples from ARDS patients.

METHODS

Animal Ethics Statement. All animal procedures conformed to the German guidelines for the use of living specimens and were approved by the Institutional Animal Care and the Regierungspräsidium Tübingen and Würzburg.

Human samples. Approval for the processing of human samples was given by the Ethics Committee (Institutional Review Board) of the University of Tübingen (approval number 156/2016BO1, Clinicaltrial.gov: NCT02692118). Informed consent was obtained from patients or legal guardians before samples were collected and processed for further analysis.

Mice. *Sema7A*$^{-/-}$ mutant mice were generated, characterized and validated as described in Pasterkamp et al. [14]. We generated the Sema7A floxed mouse line (*Sema7A*$^{loxP/loxP}$) [12], which was then crossbred with the listed Cre recombinase-positive mouse lines to generate mice with the following tissue-specific gene deletions: immune cell-specific LysMCre^{+}; megakaryocyte- and thrombocyte-specific PF4Cre^{+} and endothelial-specific Tie2Cre^{+}. As internal experimental controls, Sema7A$^{loxP/loxP}$ Cre^{-}

littermates were used. WT animals were obtained from inbreeding colonies maintained by operational researchers and animal facility staff.

Murine lung injury model with LPS inhalation. Mice were exposed to 0.5 mg/ml LPS by inhalation for 45 min, followed by a 4-h incubation to induce acute lung injury as described previously [15,16]. Additional information is provided in the Online Data Supplement.

Murine lung injury model with *Klebsiella pneumoniae* instillation. Briefly, mice were anesthetized with a 3-component fentanyl mixture applied i.p., followed by a small skin incision and exposure of the trachea. Direct tracheal instillation of 4×10^7 *Klebsiella pneumoniae* (ATCC strain 43816) in 50 µl of PBS was performed using a 30-gauge needle to minimize tracheal damage. Additional information is provided in the Online Data Supplement.

In vivo migration assay. Twenty-four hours after *Klebsiella pneumoniae* instillation, a fluorescent APC-conjugated Ly6G antibody (clone 1A8) was injected via the tail vein to label intravascular PMNs. The lungs were incubated with anti-CD45 PerCP-Cy5·5 (clone 30-F11) and anti-Ly6G PE/Cy7 (clone 1A8). The absolute cell counts in the

BALF and lungs were determined. We differentiated between interstitial PMNs (CD45-PerCP-Cy5·5$^+$; Ly6G-PE-Cy7$^+$ and Ly6G-APC$^-$) and intravascular PMNs (CD45-PerCP-Cy5·5$^+$; Ly6G-PE-Cy7$^+$ and Ly6G-APC$^+$) (all antibodies from BioLegend) by flow cytometry (FACS Canto II; BD Biosciences).

Chemotaxis Assay. For detailed information please see the Online Data Supplement.

Intravital microscopic analysis of cremaster microcirculation. Mouse cremaster preparation was performed as previously described [17]. Additional information is provided in the Online Data Supplement.

Lung intravital microscopy. Anesthesia was performed with ketamine-xylazine (100 mg/kg-16 mg/kg), and an antibody cocktail composed of 7 µg of anti-CD31-A647, 5 µg of Ly6G-A488, and 5 µg of GPIX-A546 was administered i.v. in a bolus of 100 µl. For correct microscopy of the lung, the mouse was intubated by tracheotomy, and breathing was normalized for 5 min after the instillation of 5 µg/g BW LPS (O26:B6). For murine lung microscopy, a Leica Stellaris 8 resonance microscope with a 25× objective and a resolution of 232.72 µm^2 was used. Videos were analyzed with Leica software. Additional information is provided in the Online Data Supplement.

Flow cytometry. For detailed information please see the Online Data Supplement.

Staining of murine platelet-neutrophil complexes. For detailed information please see the Online Data Supplement.

Human fibrinogen binding assay and human ICAM-1 binding assay. For detailed information please see the Online Data Supplement.

Regulation of adhesion receptors. For detailed information please see the Online Data Supplement.

Immunofluorescent staining of purified murine PMNs, human PMNS and lung tissue. For detailed information please see the Online Data Supplement.

ELISA. All enzyme-linked immunosorbent assay (ELISA) kits were from R&D and used the DuoSet principle. The absorbance of the developed color was measured at 450 nm in a plate reader (Tecan, Männedorf, Switzerland).

Respiratory burst assay. For detailed information please see the Online Data Supplement.

Phagocytosis assay. For detailed information please see the Online Data Supplement.

Proteomics analysis. For detailed information please see the Online Data Supplement.

Immunohistochemistry. For detailed information please see the Online Data Supplement.

H&E staining and evaluation. For detailed information please see the Online Data Supplement.

Statistical analysis. The data are presented as bar graphs using the mean ± SD. Statistical analysis was performed using Student's *t*-tests to compare two groups. When comparing several groups with each other, one-way analyses of variances and Dunnett tests were performed. For comparisons that are considered statistically significant, the p-values are displayed as $p < 0.05$ (*); $p < 0.01$ (**) and $p < 0.001$ (***).

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193 analysis.
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RESULTS

ARDS patients have increased plasma Sema7A levels. To identify a potential change of Sema7A in pulmonary inflammation or infection, we evaluated patients undergoing major surgery with ICU stay requiring mechanical ventilation and ARDS patients who presented with severe pulmonary infection **(Figure 1A, Table 1)**. The Non-ARDS patients were ventilated for about 2.5 days and showed significant impairment of organ function as determined by APACHE II and SOFA score. We measured Sema7A blood levels on the day of admission and correlated these with clinical and laboratory values. ARDS patients showed significantly increased Sema7A values associated with illness severity, such as APACHE II and SOFA scores **(Table 1)**. Patients with ARDS also showed a significant correlation between leukocyte counts and Sema7A levels, and a significant correlation between Sema7A levels and oxygenation levels **(Figure 1B)**. This points to a role of Sema7A in the inflammatory response of the lung during ARDS. Although this is of course only mild evidence, this lead us to further pursue a potential role of Sema7A in pulmonary inflammation.

Sema7A is expressed at the pulmonary immune interface and affects neutrophil migration. To evaluate the role of Sema7A in the lung further, we used *Sema7A*$^{-/-}$ mice and controls to establish a model of *Klebsiella pneumoniae*-induced pneumonia and

found significantly altered histological images with stronger changes in the interstitial and alveolar structures in the *Sema7A*$^{-/-}$ mice compared to controls (**Figure 2A, Supplemental Figure 1**). The *Sema7A*$^{-/-}$ mice showed thicker interstitial spaces, more pronounced inflammation in the alveolar septa and more pronounced tissue destruction of the lungs compared to controls. We then stained Sema7A in pulmonary tissue and on neutrophils, since these are the first line of defense at the pulmonary alveolar-capillary barrier during an external assault, and found that Sema7A was clearly expressed on neutrophils and the alveolar-capillary barrier (**Figure 2B and C, Supplemental Figure 1**). We also performed WB analysis of neutrophils to show Sema7A expression on neutrophils **(Supplemental Figure 2)**. Neutrophil migration to the site of infection is another important mechanism in the defense of the pulmonary surface; therefore, we exposed *Sema7A*$^{-/-}$ mice to i.v. LPS injection and found significantly increased cell speed, reduced numbers of stationary cells and reduced numbers of transmigrated neutrophils in *Sema7A*$^{-/-}$ animals compared to the controls in a cremaster model (**Supplemental Figure 3**). Comparison of adhesion molecules on *Sema7A*$^{-/-}$ and WT PMNs showed no difference in non-inflammatory conditions (**Supplemental Figure 4**). We transferred this analysis into the pulmonary circulation following LPS inhalation with injecting recSema7A to determine whether we could visualize the effect of Sema7A within the lung. We found an increase in the area that

was covered with neutrophils in the pulmonary circulation and an increase in the number of platelets interacting with neutrophils - platelet-neutrophil complexes (PNCs) - in recSema7A-injected animals (**Figure 2D and E)**.

Sema7A binds to PlexinC1 on neutrophils. As described in the introduction, several receptors were described as potential Sema7A target receptors in the literature. Therefore, we aimed to identify which receptor on neutrophils binds to Sema7A. We isolated neutrophils from WT animals following NaCl or LPS inhalation and examined the localization of Sema7A during inflammatory stimulation of neutrophils. An induction of Sema7A expression during stimulation was described previously, and we were able to conform this [18]. We stained for the Sema7A receptors integrin β1 (CD29), PlexinC1 and the EGF receptor, since all of these membrane proteins have been reported to be potential target receptors for Sema7A. We found a robust Sema7A signal on neutrophils after LPS inhalation, as indicated by staining for PlexinC1 and Sema7A (**Figure 3C**), whereas no association of Sema7A with CD29 or EGFR was observed (**Figure 3A and B; Supplemental Figure 5 and 6**). To further validate this finding, we isolated neutrophils from *Sema7A*$^{-/-}$ mice after recSema7A injection and LPS inhalation. Following this we found robust colocalization of Sema7A with the PlexinC1 receptor on these cells, strongly suggesting a direct binding of recSema7A to PLXNC1 but not to

another receptor (**Figure 3D**). Flowcytometry confirmed the obtained results (**Supplemental Figure 7).**

Sema7A induces neutrophil migration through PlexinC1. Chemotaxis is an essential step by which neutrophils migrate to the site of infection and/or inflammation. We used an in vitro cell migration assay with neutrophils exposed to fMLP or Sema7A or neutrophils preincubated with anti-PLXNC1 antibody before Sema7A exposure. Treatment with fMLP was used to simulate bacterial exposure of neutrophils and resulted in the attraction of neutrophils towards the highest concentration, with profound migratory velocity and distance (**Figure 3E-I**). In contrast, recSEMA7A resulted in the repulsion of neutrophils, with similar velocities and distances as the effect of fMLP. This effect could be reduced when neutrophils were preincubated with anti-PLEXINC1 antibodies before the experiment (**Figure 3E-I)**. This finding showed that neutrophil migration moved away from Sema7A and that inhibition of PlexinC1 alters this chemorepulsive–effect. Next, we examined whether Sema7A had an effect on the activation of MAC-1 by measuring fibrinogen binding. Sema7A induced the binding of fibrinogen to MAC-1, suggesting the significant influence of Sema7A on integrin activation (**Figure 3J**). In addition, we also performed an ICAM-1 binding assay and found that Sema7A significantly increased the binding of ICAM-1 to neutrophils

stimulated with Sema7A. This finding suggests that Sema7A induces a functional upregulation of key integrins involved in neutrophil migration (**Figure 3K**) and thereby significantly promotes this process.

Human neutrophil proteomics analysis confirms influence of Sema7A on integrin expression. To confirm the functional data and gain a better understanding of exactly how neutrophils react to Sema7A, we decided to stimulate neutrophils directly with recSEMA7A (Fc control respectively) for 15 min. We also exposed neutrophils to TNF-α (100 pg/ml), since TNF-α is a cardinal cytokine in the alveolar space during the early phase of ARDS and the attraction of neutrophils. We found a significant increase of the expression of PSGL-1 and ICAM-1 through Sema7A (**Figure 4A**). This was the opposite of the effect of TNF-α and resulted in a decrease in L-selectin and an increase in ICAM-1. Phosphoproteomics analysis showed that the potential pathways involved in this effect were the MAP-kinase and PTEN pathways (**Figure 4B, Supplemental Figure 8**). To confirm the obtained results, we examined some of the proteins on neutrophils through flow cytometry and found that the expression of CD11b was increased following recSEMA7A stimulation. In addition, we were able to confirm that PSGL-1 and L-selectin were reduced and that this was dependent on the Plexin C1 receptor (**Figure 4C-F, Supplemental Figure 9**).

Neutrophil- and platelet-derived Sema7A expression alters leukocyte migration during inflammation. In the next step, we sought to identify the source of Sema7A mediating the observed in-vivo effects. We pursued this in the intravital cremaster model, since the pulmonary imaging model is very complex and labour intensive and it would be almost impossible to image all the needed animals in this model.

We have previously shown that RBCs derived Sema7A is important during sterile inflammation induced by myocardial reperfusion injury. We did not find an involvement of RBC-derived Sema7A in neutrophil migration by intravital microscopy. Next, we used *LysMCre*$^{+}$*Sema7A*$^{loxP/loxP}$ animals. These mice showed significantly reduced numbers of adherent and transmigrated cells and significant differences in cell speed compared to those of littermate controls (**Figure 5A-B**). We found similar results when we examined *PF4Cre*$^{+}$*Sema7A*$^{loxP/loxP}$ mice in this model. These animals also showed reduced transmigration and adhesion properties in the microcirculation following LPS challenge (**Figure 5C-D)**. The possibility remained that endothelial-expressed Sema7A could bind to neutrophils and cause the observed results. When examining *Tie2Cre*$^{+}$*Sema7A*$^{loxP/loxP}$ animals, to our surprise, we found no contribution of endothelial Sema7A to neutrophil attachment or transmigration (**Figure 5E-F Supplemental Figure 10)**.

PNC formation is significantly increased by Sema7A. Platelet-neutrophil complexes are significant effectors of the immune response during the early phase of inflammation. Since we have shown that Sema7A promotes integrin activation on neutrophils, we next performed flow cytometric analysis of WT and *Sema7A*$^{-/-}$ animals 5 min after LPS inhalation. We found reduced numbers of PNCs in the blood of *Sema7A*$^{-/-}$ animals and reduced expression of platelet or neutrophil activation markers (**Figure 6A-B,** for gating strategy see **Supplemental Figure 11**). We next injected recSema7A into *Sema7A*$^{-/-}$ animals and found that following injection of 1 µg/mouse recSema7A, the formation of PNCs in the blood was restored in *Sema7A*$^{-/-}$ animals (**Figure 6C-D**). To test whether this effect could be counteracted, we injected anti-Sema7A antibodies at the start of LPS inhalation. Anti-Sema7A antibodies clearly reduced the number of PNCs in the vasculature of experimental animals compared to control IgG-treated animals (**Figure 6E-F, Supplemental Figure 12**). In contrast, the phagocytosis rate and superoxide production of PMNs were not directly affected by freely available Sema7A or Plexin C1 blockade (**Supplemental Figure 13)**. These data clearly demonstrate the PNC-inducing properties of Sema7A, which are largely mediated by integrin activation on neutrophils through Sema7A.

***Sema7A*$^{-/-}$ animals show altered pulmonary defense and reduced survival in a model of *Klebsiella pneumoniae* infection**. Bacterial invasion in the lung, the development of pneumonia and intrapulmonary inflammation are essential mechanisms during the defense against invading pathogens at the external pulmonary surface. We next examined whether Sema7A plays a role in this process. To do so, we used control and *Sema7A*$^{-/-}$ animals to establish a model of *Klebsiella pneumoniae*-induced pneumonia and evaluated pulmonary inflammation 24 h after the initiation of the experiment as well as overall survival. Sema7A did not result in altered oxidative burst or phagocytosis capacity of neutrophils (**Supplemental Figure 13)**. Next, we assessed the sequential recruitment of neutrophils into pulmonary tissue. We found that neutrophils were significantly more present in the interstitial space and significantly less present in the BALF of *Sema7A*$^{-/-}$ animals than in those of controls (**Figure 7A-C and I)**. We found a higher number of PNCs in the interstitial of the lungs of *Sema7A*$^{-/-}$ animals **(Figure 7D)**. The inflammatory cytokines Il-1β and TNF-α were increased in *Sema7A*$^{-/-}$ animals, whereas IL-6 levels were unchanged **(Figure 7E-H)**. There were more colony forming units of *Klebsiella pneumoniae* present in the lung and fewer in the blood of *Sema7A*$^{-/-}$ animals than in control animals, showing the intra-alveolar defense is impaired in the *Sema7A*$^{-/-}$ animals **(Figure 7J-M)**. When we determined the vascular permeability and the edema formed within the interstitial space we found increased

edema formation in the *Sema7A*$^{-/-}$ animals **(Figure 7N,O)**. All these findings translated into worse outcomes in the survival of *Sema7A*$^{-/-}$ animals compared to controls **(Figure 7P)**. The thickening and enlargement of the alveolar surface, the vessel walls and thus the difficulty in penetrating cell layers in the direction of the blood flow delayed the successful migration of bacteria into the blood. The infection caused by Klebsiella pneumoniae remained confined to the specific site of the lung without spreading to the bloodstream.

DISCUSSION

Defense against invading pathogens is an essential function of neutrophils that maintains the integrity and functionality of the lung. This function is important in preventing the infiltration of pathogens into the lung and eventually the human circulation. Neutrophil arrest on the endothelium and migration into the alveolar space are important early mechanisms in a multistep process. We report here that Sema7A is an important regulator of neutrophil migration to the alveolar space and that the repression of Sema7A results in altered adhesion and delayed neutrophil migration. Sema7A activates integrins on neutrophils through the PlexinC1 receptor and influences the chemotactic behavior of these cells. In vivo, this translates into a reduced immune response within the lung. As a result, Sema7A influences the early phase of inflammation, which is relevant to the outcome in murine experimental sepsis induced by pneumonia (**Figure 7Q**).

The activation of neutrophils is essential for the migration of these cells into the alveolar space, where their main task is to limit the assault on the lung from external invaders. Several adhesion receptors of the integrin class have previously been described to be important for this process notably CD11b/CD18 (MAC-1), and other adhesive membrane proteins such as PSGL-1 or L-selectin [19]. The activation of L-selectin was shown to be essential for the migration of neutrophils into the lung, to limit the extent of

pulmonary sepsis and lack of functional L-selectin resulted in susceptibility to pulmonary infection [7]. Similar effects were described for CD11b and its activation [20]. A complex interplay between CD11b, WASP and CD42c regulates the polarization of neutrophils and attachment to microtubules on the endothelial surface [21]. This interplay is then necessary for the meaningful and coordinated migration of neutrophils to the site of inflammation or infection. In addition, PSGL-1 is also an essential mediator of neutrophil attachment to the endothelium and is involved into the formation of platelet-neutrophil complexes [22,23]. This formation of PNCs can also result in obstruction of the microvasculature of the lung and thereby reduce oxygenation [24]. We have demonstrated that Sema7A triggers the functional upregulation of CD11b and downregulation of L-selectin and PSGL-1 on neutrophils and thereby modulates their chemotactic migration. As a result, neutrophils are activated, which essentially influences neutrophil chemotactic migration. When Sema7A is present, neutrophils are activated in a synchronistic manner and migrate across the alveolar-capillary barrier to reach the alveolar space and combat invading pathogens. In the absence of Sema7A, this coordinated induction does not occur, and neutrophils migrate in an uncoordinated fashion. We observed that neutrophils in *Sema7A*$^{-/-}$ animals showed altered adhesion and transmigration in response to inflammatory stimuli which translated into an altered migration pattern during bacterial infection in the lung and neutrophil arrest in the

393 interstitial space, where they cannot be sufficiently activated against invading bacteria.
394 Thus, the animals showed impaired host defense and died earlier than animals with
395 physiological Sema7A expression. This is in line with our previous results showing that
396 Sema7A aggravates pulmonary inflammation [16]. In this previous study we showed the
397 effect of Sema7A on pulmonary endothelial and epithelial cells and that Sema7A
398 enhanced cytokine production in these cells. We extended this work now and identified
399 the specific action of Sema7A on neutrophils and PNC formation. We also showed that
400 Sema7A correlated with increased pulmonary inflammation through its action on
401 myeloid derived cells [16]. In line with this, in the present study we show an increase of
402 Sema7A in patients with severe ARDS. This increased Sema7A is likely shed from
403 neutrophils or pulmonary tissue through the activation of caspases or released from
404 platelets which contain Sema7A in sufficient amount as one of their proteins [25,26]
405 PlexinC1-dependent integrin activation has not been described before in neutrophils.
406 The role of Plexin C1 during inflammation and pulmonary inflammation was evaluated
407 previously and showed a significant effect of PlexinC1 expression during mechanical
408 ventilation [27,28]. However, the fact that Sema7A has a significant effect on CD11b, L-
409 selectin and other adhesion receptors has not been previously shown. We were also
410 able to demonstrate that the pathways controlling the activation of these integrins are
411 influenced by Sema7A binding to PlexinC1. Sema7A was shown in the past to induce

cytokine storm in T-cells though a mechanism that was dependent on α1β1 integrin receptor in T-cells [11]. However, we could not confirm the binding of Sema7A to β1 integrin on neutrophils and showed that Sema7A binds to the PlexinC1 receptor instead. Previous work has demonstrated that PlexinC1 is likely involved in the migration of neutrophils and other cells, which was confirmed in models using the genetic deletion of PlexinC1 [28,29]. We now show here that this occurs by the binding of Sema7A to PlexinC1. In addition, whether the soluble form of Sema7A mediates the activation of integrins, which are essential for neutrophil migration during inflammation and infection, is unclear. Recent work has demonstrated that Sema7A is essential for a coordinated sequence of events during inflammation but also for the resolution of inflammation [30]. In accordance with this work, we also showed that Sema7A expression is important for survival during bacterial infection. Here, we used a model of *Klebsiella pneumoniae*, while Körner et al. used a model of cecal ligation and puncture, and both showed a survival benefit in animals expressing Sema7A. However, one must keep in mind that the expression of Sema7A and Sema7A target receptors is organ-specific; therefore, organ-specific immune responses to inflammatory or infectious stimuli are possible in response to this protein. We have previously shown that neutrophil migration is altered through Sema7A during myocardial infarction and in hypoxic tissue inflammation [12,13]. During hypoxia, the induction of endothelial Sema7A resulted in increased

transendothelial neutrophil migration. However, these mechanisms are not involved in the results described here. We describe a novel mechanism of integrin activation in neutrophils through direct signaling mediated by Sema7A engaging with Plexin C1.

In summary, we have shown that Sema7A directly triggers the functional upregulation of integrins in neutrophils and thereby modulates their adhesion and migration, which to a significant extent determines outcomes during pulmonary infection. Data in human ARDS patients corroborate this mechanism of Sema7A-mediated promotion of neutrophil migration.

Figure Legends

Figure 1: Clinical Sema7A values correlate with leukocyte count and clinical oxygenation values in ARDS patients. Demographic data and samples of patients undergoing elective surgery with postoperative ventilation and ICU stays and patients admitted to the ICU for ARDS with severe pulmonary inflammation that were matched with propensity score. **A)** Demographic data, ICU scores, and laboratory values for both patient groups are presented as means ± standard deviations, with values compared using the Wilcoxon Rank-Sum Test. **B)** The correlation of various laboratory values, ventilation parameters, and oxygenation values with serum levels of Sema7A is depicted. Pearson's r and the lower and upper limits of the 95% confidence interval are shown. Significant correlations are highlighted in red.

Figure 2: Semaphorin 7A is required for neutrophil adhesion and migration during inflammation. A) Histological cross-sections and magnifications of lung tissue from WT and *Sema7A*$^{-/-}$ mice 24 h after the instillation of 4×10^7 *Klebsiella pneumoniae* cells (scale bar = 200µm). **B)** Immunofluorescence (IF) staining of Sema7A (green) and vWF (red) in endothelial cells of murine lung tissue and nuclear staining with DAPI (blue; scale bar = 20µm. **C)** IF staining showing Sema7A expression (green) on the surface of human CD45-marked PMNs, (red) treated with NaCl or fMLP for 15 min (scale bar = 10µm). **D)** Representative videos of PNCs in murine lungs after LPS instillation with

additional recombinant Sema7A or IgG_{2A} Fc (controls) treatment after 30min (scale bar = 30µm). **E)** Total neutrophil area coverage, total and adhesive platelet area coverage and the fractions of PNCs formed in the lung, as determined via intravital confocal microscopic analysis of the lung in WT mice instilled with 5 µg/g BW LPS, with or without additional treatment with recombinant Sema7A (the data are the mean ± SD). *p < 0.05, **p < 0.01 and ***p < 0.001 as indicated.

Figure 3: Sema7A binds to neutrophil PlexinC1 and influences neutrophil chemotaxis. Stained neutrophils isolated from saline (NaCl)- or LPS-treated WT and *Sema7A*$^{-/-}$ mice 4 h after incubation. **A)** Expression of Sema7A (red) and CD29 (green) on PMNs harvested from WT mice treated with NaCl or LPS (scale bar = 10µm). **B)** Expression of Sema7A (red) and EGFR (green) on PMNs harvested from WT mice treated with NaCl or LPS. No protein colocalization was visible in the merged pictures in either condition (scale bar = 10µm). **C)** Expression of Sema7A (red) and PlexinC1 (green) on PMNs harvested from WT mice treated with NaCl or LPS. Sema7A expression is highly increased in LPS-treated mice, and the merged pictures show a strong interaction between Sema7A and PlexinC1 (scale bar = 10µm). **D)** Surface PMN expression of Sema7A (red) and PlexinC1 (green) in *Sema7A*$^{-/-}$ mice after the injection

of exogenous recombinant Sema7A or IgG-Fc (control) after LPS or NaCl (controls) inhalation. Strong binding of exogenous Sema7A to *Sema7A*$^{-/-}$ PMNs was observed in LPS inhalation group. Multiple acquisitions of stained cells were analyzed from independently performed triplicate experiments (scale bar = 10µm). **E)** human PMNs were subjected to different stimuli in bidirectional chemotactic chambers. Acquired time lapse videos over a 3 h period were analyzed. Representative plots of PMN chemotactic tracks towards NaCl (control; red), fMLP (green), recombinant human SEMA7A (recSema7A; blue) or recSema7A together with antibodies against human PlexinC1 (anti-PLEXINC1; gray). **F-I)** Comparison of the chemotaxis parameters FMI (forward migration index), Euclidean distance under the aspect of the direction, PMN velocity and accumulated PMN distance. **J)** PMN binding affinity was indicated by APC-labeled fibrinogen on the surface of Ly6G-positive PMNs, as analyzed by FACS. The EDTA group was the internal negative control to measure the baseline autofluorescence, the untreated group was the fibrinogen negative control, TNF-α was used as a potent PMN stimulator, and treatment of PMNs with recombinant SEMA7A prior to APC-labeled fibrinogen represented the fibrinogen binding target group of interest. The fibrinogen-APC MFI was normalized and is displayed as a percentage. **K)** PMN binding affinity was indicated by PerCP-labeled ICAM-1 on the surface of Ly6G-positive PMNs by FACS. The untreated group was the ICAM-1 negative control, TNF-α

was used as a potent PMN stimulator, and treatment of PMNs with recombinant SEMA7A prior to PerCP-labeled ICAM-1 represented the ICAM-1 binding target group of interest. CD11b antibody treatment was used as a control for the inactivation of ICAM-1 binding. The ICAM-1 PerCP MFI was normalized and is displayed as a percentage. In (**F - K**), all group comparisons were performed by unpaired two-tailed Student's t-tests (the data are the mean ± SD) *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as indicated.

Figure 4: Essential neutrophil integrins are influenced by SEMA7A. Human PMNs were incubated with NaCl, 10 ng/ml TNFα or 2 µg/ml recSEMA7A for 15 min prior to proteomics analysis The acquired raw data were analyzed following normalization. To analyze the samples, a one-factorial linear model was fitted with LIMMA, resulting in a two-sided t-test or F-test based on moderated statistics. All presented p values were adjusted for multiple analyses by controlling the false discovery rate according to Benjamini and Hochberg. Proteins were defined as differential when $|logFC| > 0.5$ and an adjusted p value < 0.05 from triplicate experiments. **A)** Expression of neutrophil surface lectin proteins and membrane integrin proteins from harvested samples. **B)** Expression of intracellular neutrophil Rho/Ras GTPases, mitogen-associated kinases,

adaptor proteins and detoxifying enzymes. **C)** PMN surface expression of CD11b after 15 min of incubation with recSEMA7A or TNFα. Measurement was performed by FACS, and the MFI (PE) was normalized to the highest measured value. **D)** PMN surface expression of PSGL-1 after 15 min of incubation with recSEMA7A or TNFα. **E)** PMN surface expression of CD11a after 15 min of incubation with recSEMA7A or TNFα. **F)** PMN surface expression of CD62L after 15 min of incubation with recSEMA7A or TNFα. Measurement was performed by FACS, and the MFI (BV510) was normalized to the highest measured value. Multiple cells were analyzed from independently performed experiments in triplicate. Group comparisons were performed by unpaired two-tailed Student's t-tests (the data are the mean ± SD). *p < 0.05, **p < 0.01 and ***p < 0.001 as indicated.

Figure 5: Tissue-specific expression of Sema7A controls neutrophil migration in response to inflammation. Intravital microscopic analysis of murine cremaster tissue after i.v. LPS stimulation shows the role of Sema7A expression in different cells during inflammation. **A)** Representative video-images of the microvasculature of *LysMCre*$^{+}$*Sema7A*$^{loxP/loxP}$ mice and littermate controls exposed to LPS for 15 min compared to the baseline control (0 min; scale bar = 50µm). **B)** Cell speed,

539 transmigration, transmigration distance and stationary PMNs in
540 *LysMCre*$^{+}$*Sema7A*$^{loxP/loxP}$ and littermate controls were analyzed by intravital microscopy
541 after exposure to LPS for 15 min and compared to the baseline control (0 min). **C)**
542 Representative video-images of the microvasculature of *PF4Cre*$^{+}$*Sema7A*$^{loxP/loxP}$ mice
543 and littermate controls exposed to LPS for 15 min compared to the baseline control (0
544 min; scale bar = 50µm). **D)** Cell speed, transmigration, transmigration distance and
545 stationary PMNs of *PF4Cre*$^{+}$*Sema7A*$^{loxP/loxP}$ mice and littermate controls was analyzed
546 by intravital microscopy after exposure to LPS for 15 min and compared to the baseline
547 control (0 min; scale bar = 50µm). **E)** Representative video-images of the
548 microvasculature of *Tie2Cre*$^{+}$*Sema7A*$^{loxP/loxP}$ mice and littermate controls exposed to
549 LPS for 15 min and compared to the baseline control (0 min). **F)** Cell speed,
550 transmigration, transmigration distance and stationary PMNs in *Tie2Cre*$^{+}$*Sema7A*$^{loxP/loxP}$
551 and littermate controls was analyzed by intravital microscopy after exposure to LPS for
552 15 min and compared to the baseline control (0 min). Triplicate experiments were
553 performed, and multiple cells were tracked for 15 to 20 min after LPS incubation over
554 periods of 10 sec at 90 fps. From the acquired videos, cells were tracked manually, and
555 relevant group comparisons were performed by unpaired two-tailed Student's t-tests
556 (the data are the mean ± SD). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as indicated,
557 (arrows mark Platelet-Neutrophil Complexes = PNCs)

Figure 6: Activation of neutrophils and platelet-neutrophil complex formation is Sema7A dependent. Murine blood was collected from WT and *Sema7A*$^{-/-}$ mice after LPS inhalation and analyzed by flow cytometry. **A)** Representative color dot blots of platelet-neutrophil complexes (PNCs; Ly6G^{+}/CD42b^{+} events) in WT and *Sema7A*$^{-/-}$ blood from NaCl (control) or LPS-inhaled mice. **B)** PNC formation, platelet effector GPIIb/IIIa expression (antibody clone JON/A MFI), PMN activity marker CD11b (MFI) expression and platelet activity marker CD42b (MFI) expression were assessed by flow cytometry in the mice described in (A). **C)** Representative dot blots of platelet-neutrophil complexes (PNCs; Ly6G^{+}/CD42b^{+} events) in the blood of WT and *Sema7A*$^{-/-}$ mice treated with recombinant Sema7A (recSema7A) after NaCl (control) or LPS inhalation. **D)** PNC formation, platelet effector GPIIb/IIIa expression (antibody clone JON/A MFI), PMN activity marker CD11b (MFI) expression and platelet activity marker CD42b (MFI) expression were assessed by flow cytometry in the mice described in (C). **E)** Representative dot blots of platelet-neutrophil complexes (PNCs; Ly6G^{+}/CD42b^{+} events) in the blood of WT mice that were untreated or treated with IgG or the Sema7A blocking antibody (anti-Sema7A) after LPS inhalation compared to mice without conditioning (Sham). **F)** PNC formation, platelet effector GPIIb/IIIa expression (antibody clone

JON/A MFI), PMN activity marker CD11b (MFI) expression and platelet activity marker CD42b (MFI) expression was assessed by flow cytometry in the mice described in (E). Group comparisons were performed by unpaired two-tailed Student's t-tests (the data are the mean ± SD). *p < 0.05, **p < 0.01 and ***p < 0.001 as indicated.

Figure 7: Sema7A is crucial for pulmonary defense against Klebsiella-induced pneumonia. In a murine model of bacterial-induced lung injury, 4×10^7 gram-negative *Klebsiella pneumoniae* was administered by intratracheal instillation directly into the lungs of WT and *Sema7A*$^{-/-}$ mice. Measurements of PMN counts **A)** on the endothelial surface **B)** in the interstitial space and **C)** in the BAL and **D)** PNC numbers per tissue section (magnification 1000×) 24 h after Klebsiella instillation in histological lung sections of WT and *Sema7A*$^{-/-}$ mice (n=3 / group on 3 different layers). The proinflammatory cytokines **E)** TNF-α **F)** IL-6 **G)** IL-1β and **H)** Myeloperoxidase activity within the BAL of WT and *Sema7A*$^{-/-}$ mice. **I)** Histological sections demonstrating the quantity of Klebsiella, the alveolar inflammation (H&E staining) and PNC debris (PNC specific staining) 24 h after instillation (scale bar = 50µm; magnification 1000×). Colony Forming Units in **J)** BALF and **K)** blood taken 24 h after Klebsiella instillation and incubated on nutrient agar plates for 24 h. **L)** Representative

images of cultured bacteria and **M)** counts per tissue sections of *Klebsiella pneumoniae*. **N)** Representative images of H&E stained sections focusing on lung tissue injury 24 h after Klebsiella instillation. (scale bar = 50µm; magnification 1000×). **O)** Thickness of alveolar wall in tissue sections of WT and *Sema7A*$^{-/-}$ mice 24 h following *Klebsiella pneumoniae* instillation (n=3 / group; 10 random fields of view / mouse) **P)** Survival curves of WT and *Sema7A*$^{-/-}$ animals after the instillation of 4×10^7 *Klebsiella pneumoniae* cells (n≥6/group). Group comparisons were performed by unpaired two-tailed Student's t-tests, the data are the mean ± SD. For statistical comparisons of survival, the Gehan-Breslow-Wilcoxon test and the Log-rank (Mantel-Cox) test were performed. *p < 0.05, **p < 0.01 and ***p < 0.001 as indicated **Q)** Schematic drawing of the role of Sema7a in pulmonary infection and defense. In pulmonary hemostasis Sema7A is expressed on neutrophils and other tissues (left). During pulmonary infection Sema7A gains pathophysiological importance. Sema7A binds to Plexin C1, activates neutrophils and increases the expression of integrins and L-selectin on their surface. This is important for a coordinated immunological response and the defense of the lung

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Conflict of Interest

The authors have no conflict of interest to report.

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	Non-ARDS (n=28)	**ARDS (n=14)**	**p-Values**
Demographic Data			
Age – yr (mean ± SD)	63 ± 12	57 ± 13	0.1718
Male sex – no. (%)	17 (60.7)	10 (71.4)	0.7337
ICU length of stay [d]	2.5	20.0	**<0.0001**
Hospital mortality (%)	1 (3.6)	3 (21.4%)	0.1000
ICU scores			
SAPSII	42 ± 9	46 ± 11	0.2020
APACHE II	20 ± 4	26 ± 4	**<0,0001**
SOFA	8 ± 3	10 ± 4	**0.0386**
Laboratory values			
SEMA7A (ng/ml)	3.3 ± 3.2	4.3 ± 2.5	**0.0423**
Bilirubin [mg/dl]	2.043 ± 4.042	1.293 ± 1.244	0.5057
Creatine kinase [IU/l]	370 ± 412	2193 ± 5162	0.0979
Creatine kinase MB-Isoform	45 ± 29	53 ± 64	0.6705
INR	1.12 ± 0.11	1.25 ± 0.47	0.2363
Creatinine [mg/dl]	0.88 ± 0.34	1.6 ± 1.77	**0.0084**

Lactate dehydrogenase [U/l]	277 ± 127	634 ± 594	**0.0082**
Leukocytes [x10^9/l]	13301 ± 3975	13744 ± 7279	0.8090
Thrombocytes [x10^6/l]	216 ± 93	162 ± 84	0.0809
Hemoglobin [g/dl]	10.9 ± 1.8	9.2 ± 2.1	**0.0144**
Arterial blood gas analysis			
pH	7.40 ± 0.06	7.36 ± 0.09	0.1641
paO2 [mmHg]	155 ± 45	92 ± 21	**<0.0001**
paO2/F_iO_2 [mmHg]	322 ± 104	168 ± 71	**<0.0001**
pCO2 [mmHg]	39 ± 5	46 ± 9	**0.0037**
Lactate peak (on admission day) [mmol/l]	1.98 ± 1.8	2.7 ± 2.4	0.3469

Table 1. Demographic data and co-morbidities

A

B

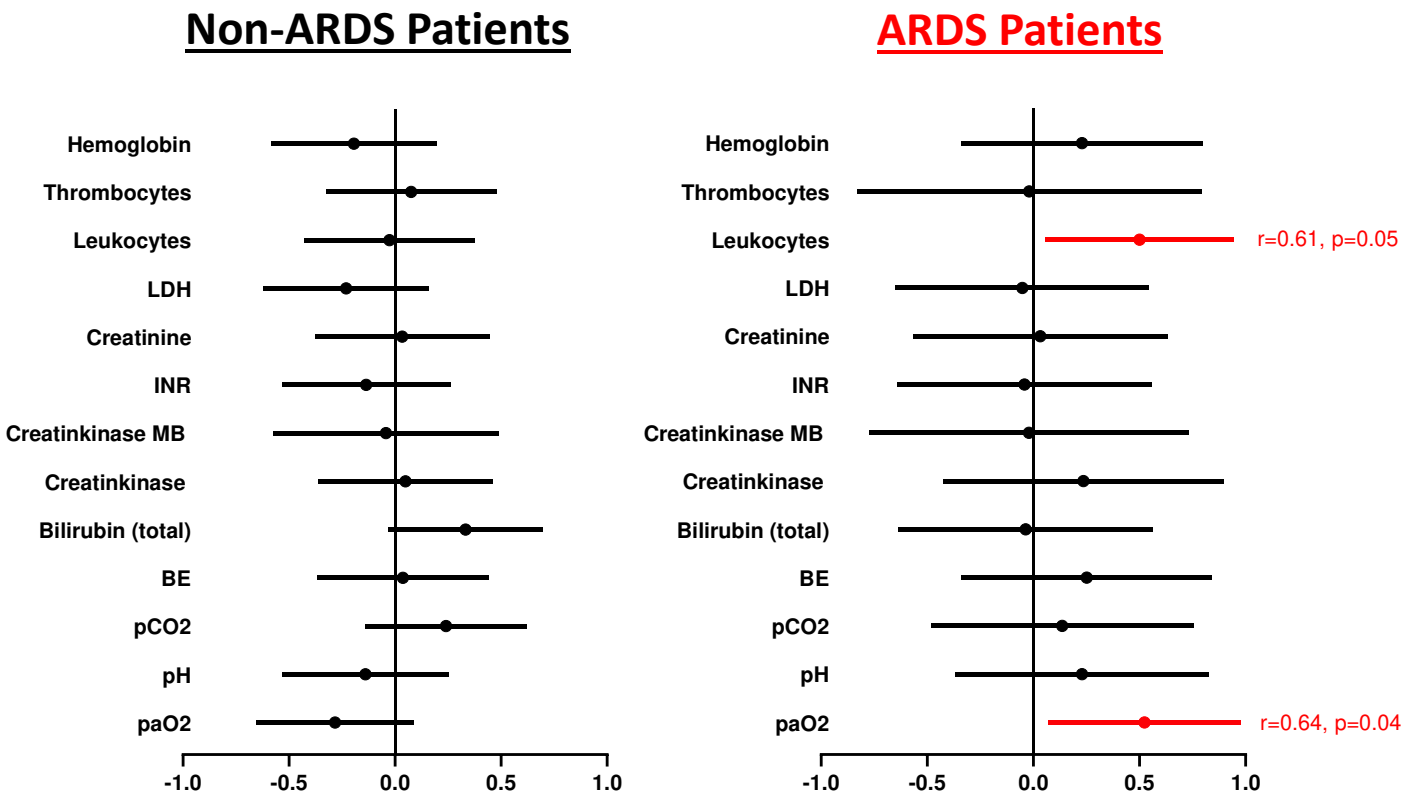


Figure 1

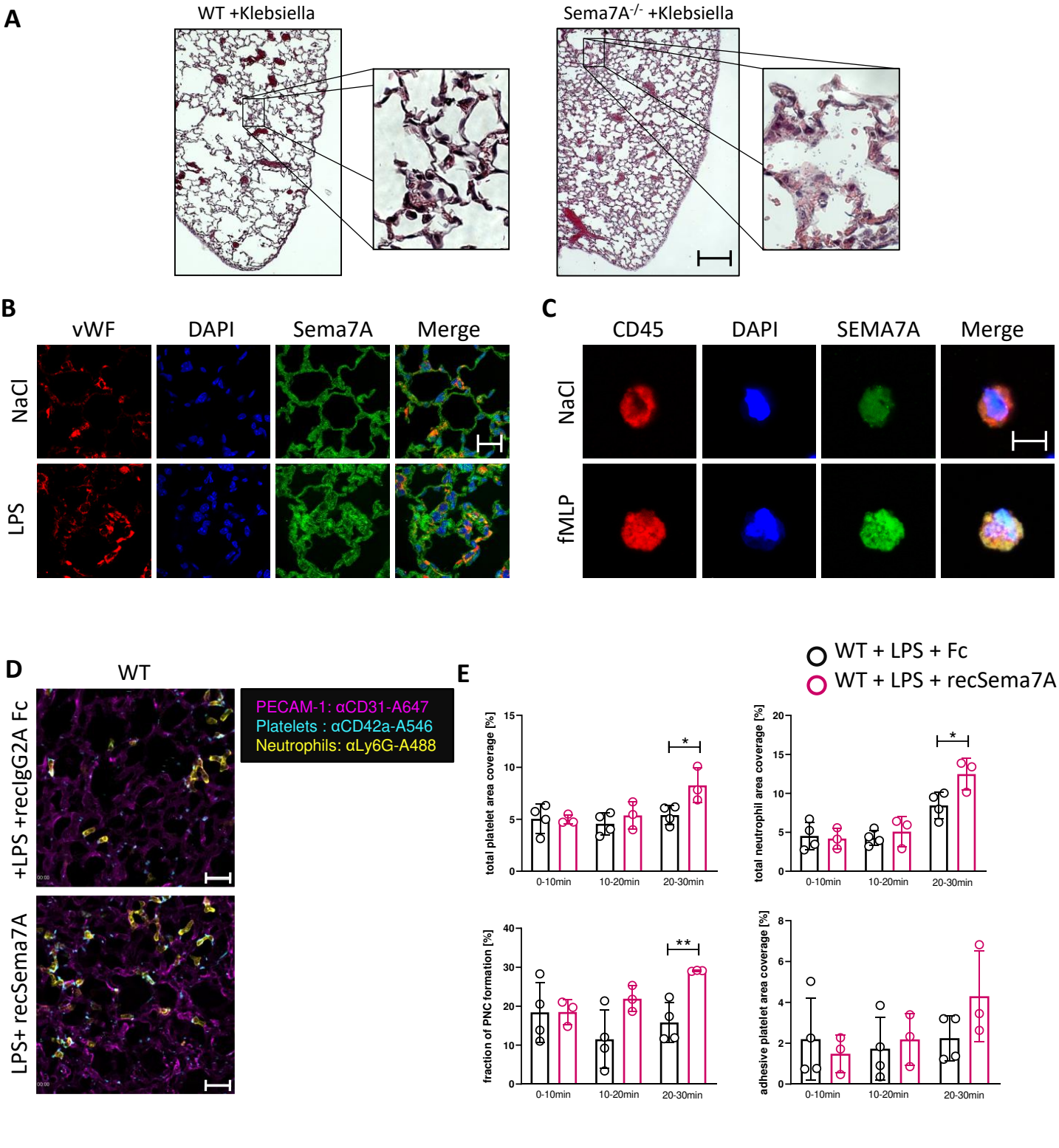


Figure 2

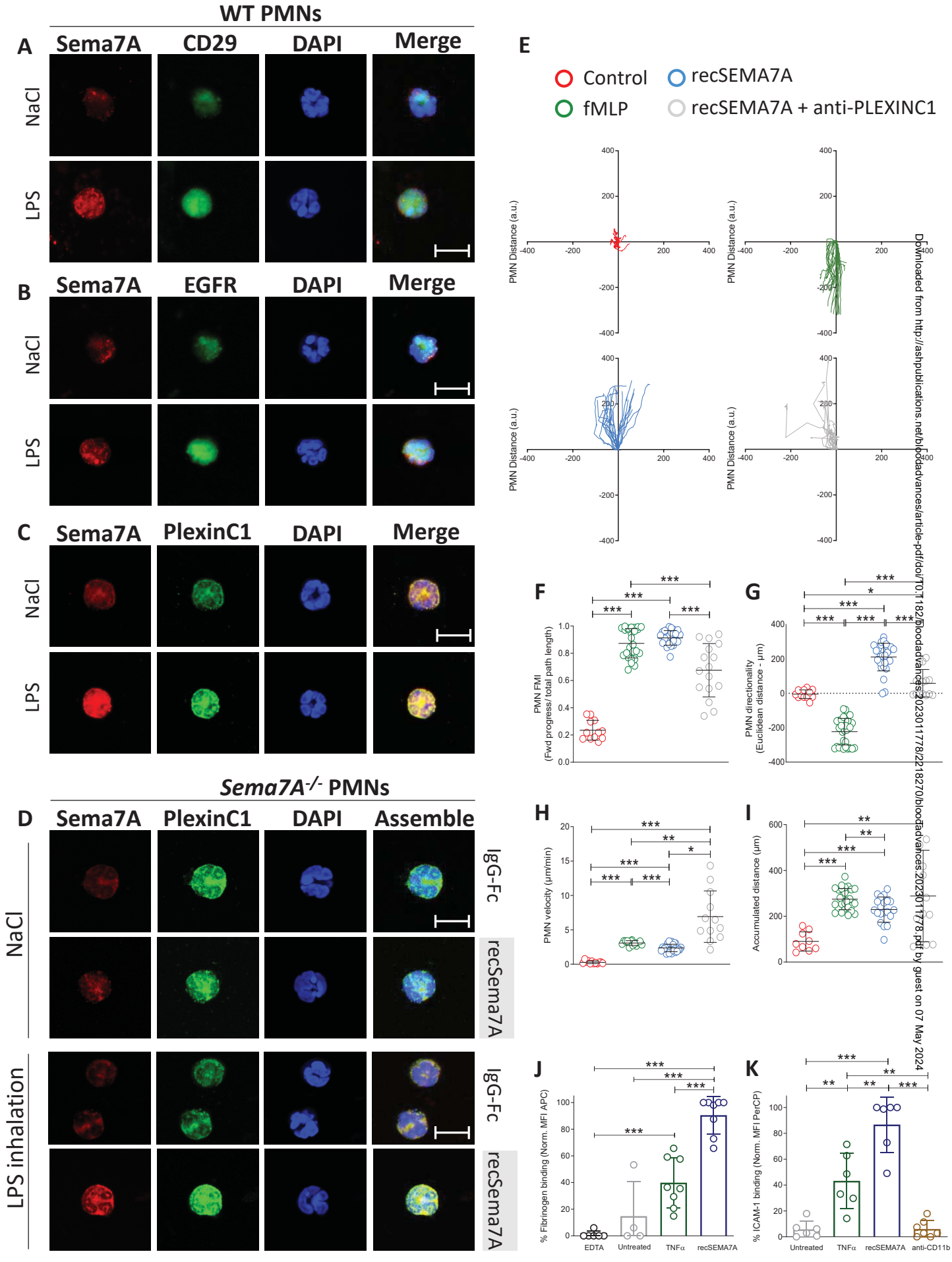
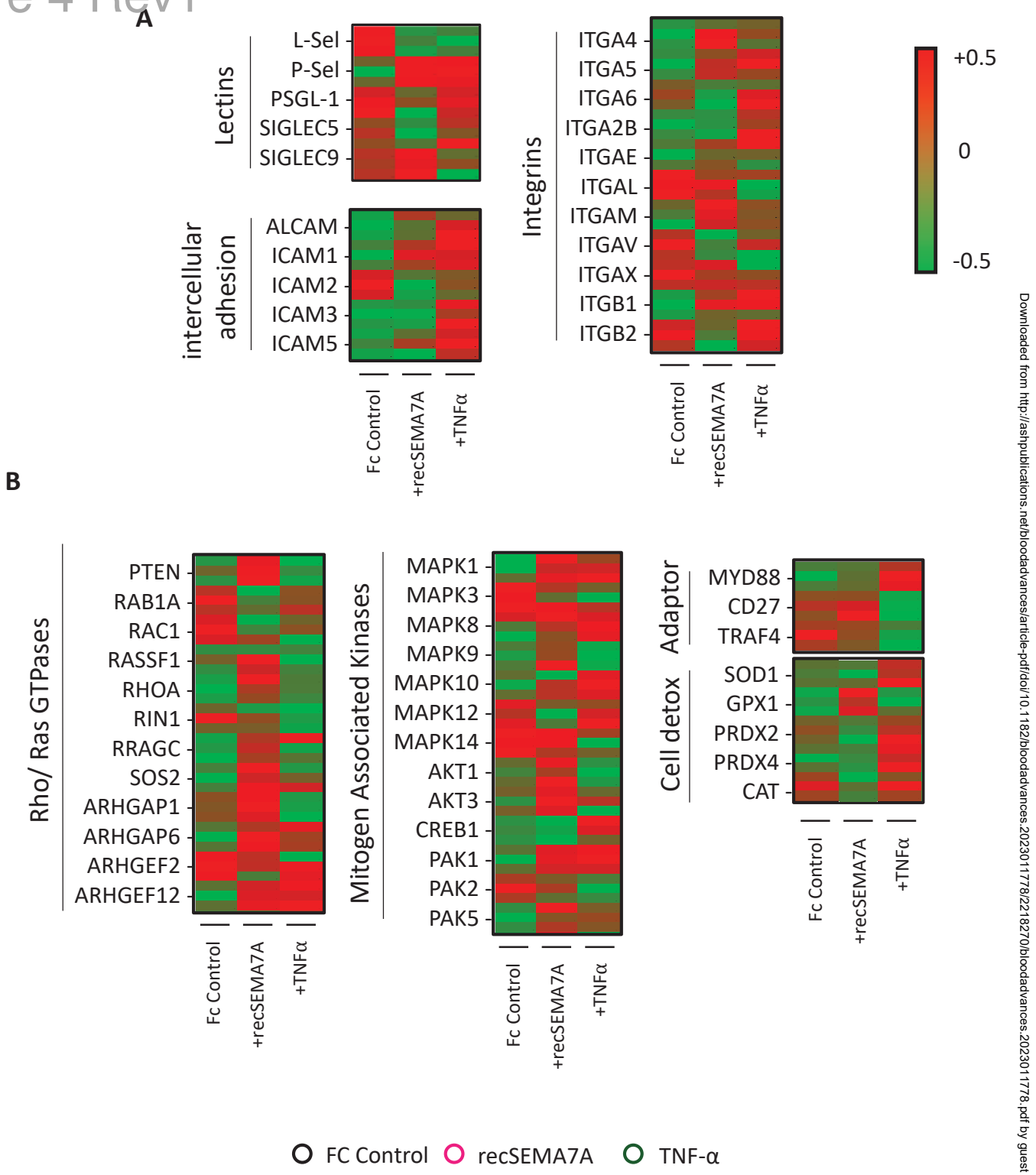


Figure 3



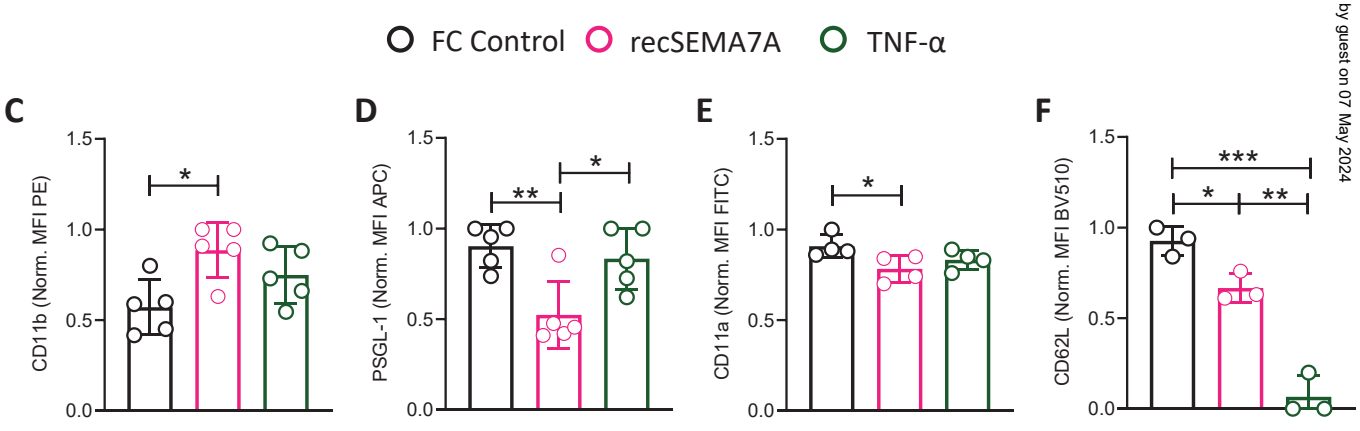
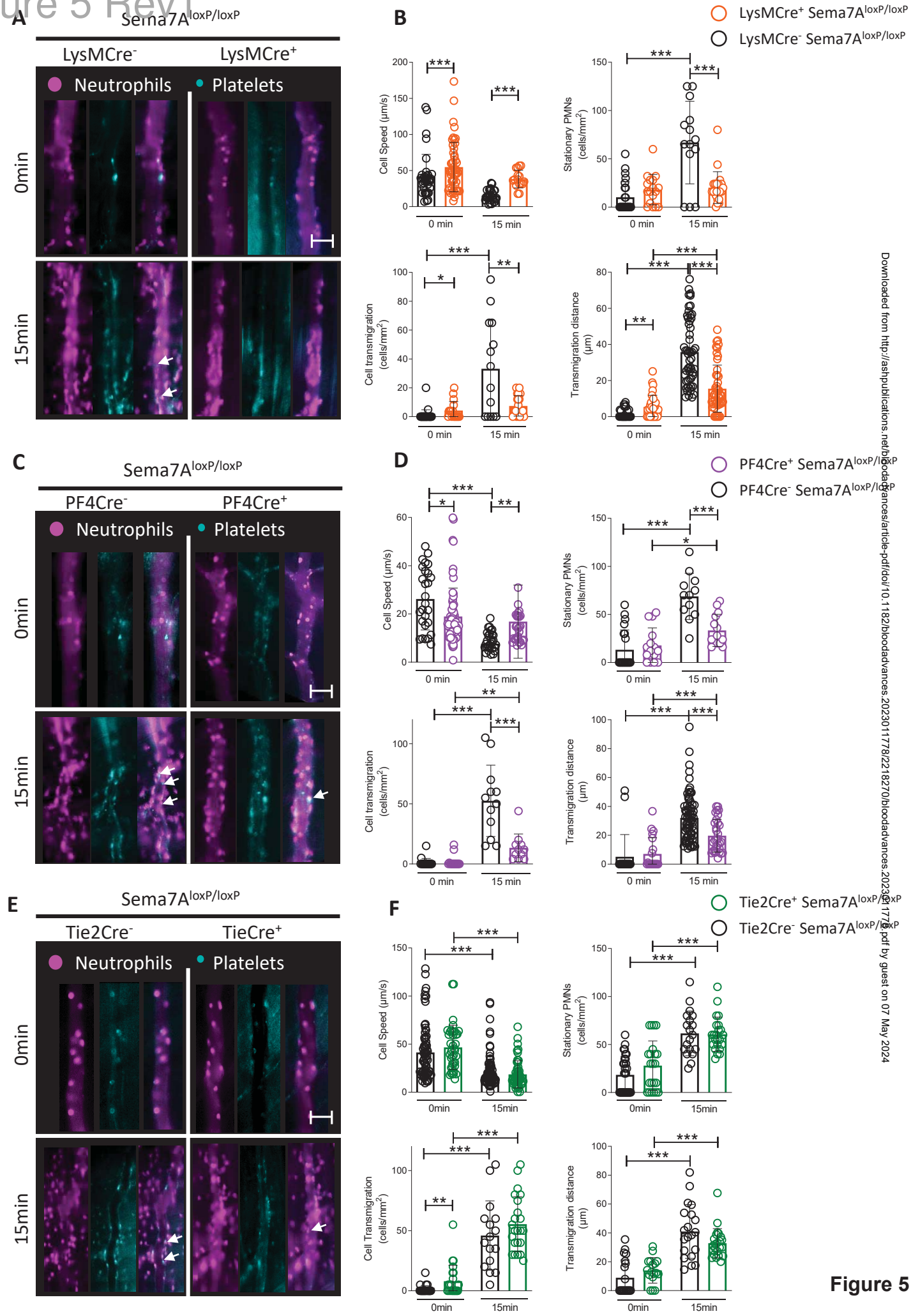


Figure 4



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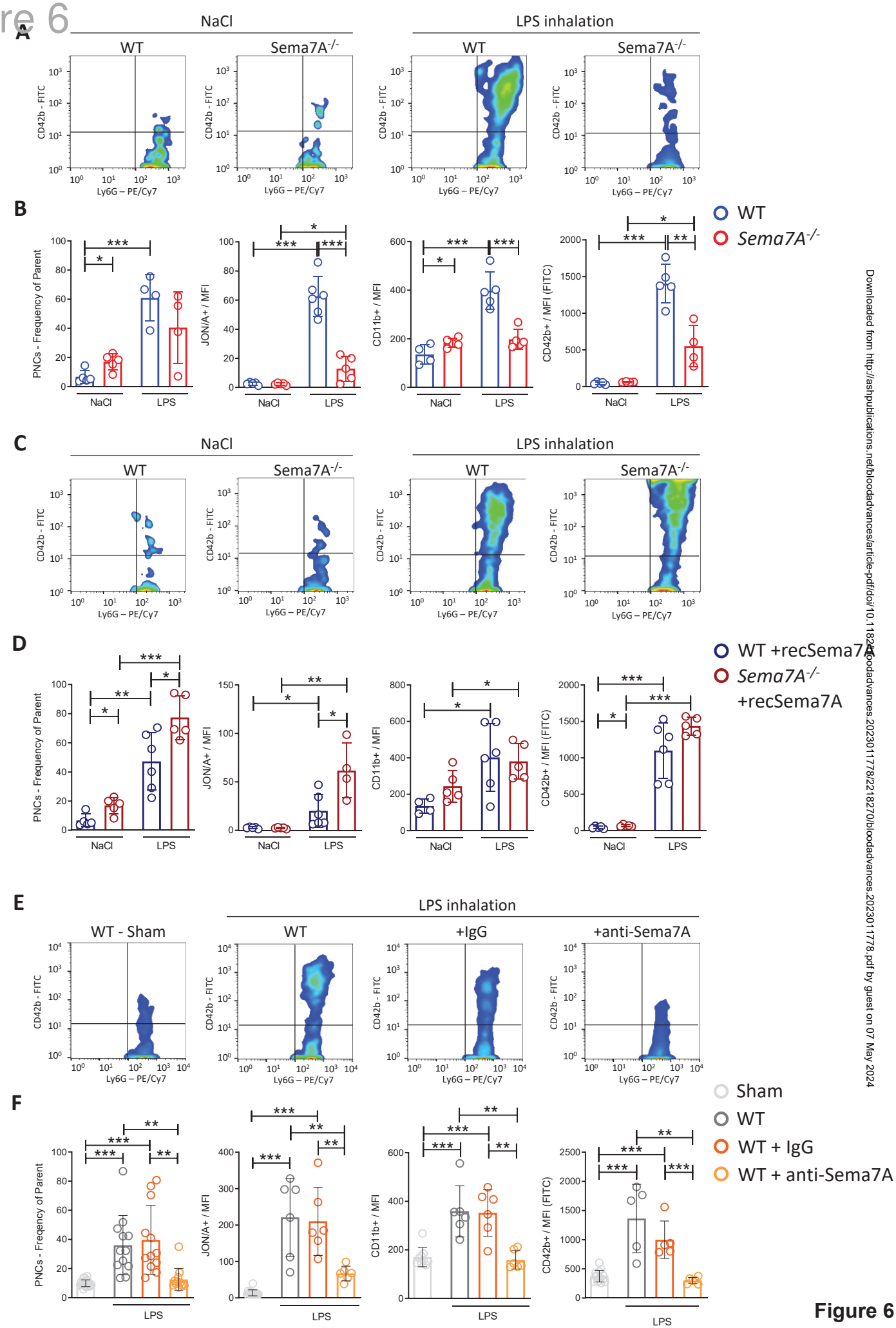

Figure 6

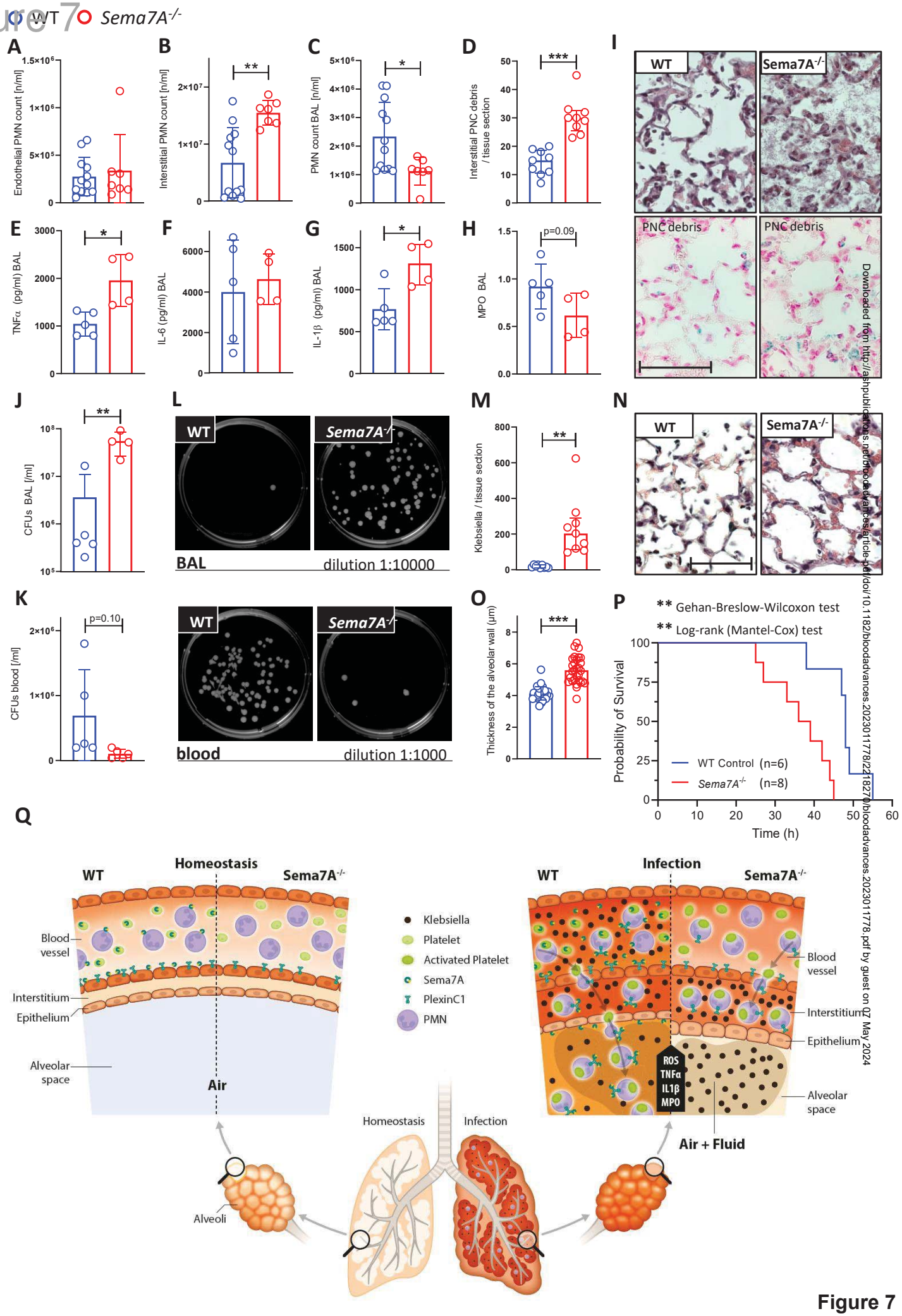

Figure 7