ORIGINAL ARTICLE

Proteolysis of plasminogen activator inhibitor-1 by *Yersinia pestis* remodulates the host environment to promote virulence

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Essentials

- Effect of plasminogen activator inhibitor (PAI)-1 on plague and its *Y. pestis* cleavage is unknown.
- An intranasal mouse model of infection was used to determine the role of PAI-1 in pneumonic plague.
- PAI-1 is cleaved and inactivated by the Pla protease of *Y. pestis* in the lung airspace.
- PAI-1 impacts both bacterial outgrowth and the immune response to respiratory *Y. pestis* infection.

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Summary. *Background:* The hemostatic regulator plasminogen activator inhibitor-1 (PAI-1) inactivates endogenous plasminogen activators and aids in the immune response to bacterial infection. *Yersinia pestis*, the causative agent of plague, produces the Pla protease, a virulence factor that is required during plague. However, the specific hemostatic proteins cleaved by Pla *in vivo* that contribute to pathogenesis have not yet been fully elucidated. *Objectives:* To determine whether PAI-1 is cleaved by the Pla protease during pneumonic plague, and to define the impact of PAI-1 on *Y. pestis* respiratory infection in the presence or absence of Pla. *Methods:* An intranasal mouse model of pneumonic plague was used to assess the levels of total and active PAI-1 in the lung airspace, and the impact of PAI-1 deficiency on bacterial pathogenesis, the host immune response and plasmin generation following infection with wild-type or Δ*pla Y. pestis*. *Results:* We found that *Y. pestis* cleaves and inactivates PAI-1 in the lungs in a Pla-dependent manner. The loss of PAI-1 enhances *Y. pestis* outgrowth in the absence of Pla, and is associated with increased conversion of plasminogen to plasmin. Furthermore, we found that PAI-1 regulates immune cell recruitment, cytokine production and tissue permeability during pneumonic plague. *Conclusions:* Our data demonstrate that PAI-1 is an *in vivo* target of the Pla protease in the lungs, and that PAI-1 is a key regulator of the pulmonary innate immune response. We conclude that the inactivation of PAI-1 by *Y. pestis* alters the host environment to promote virulence during pneumonic plague.

Keywords: fibrinolysis; inflammation; plague; plasminogen; pneumonia.

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Introduction

The induction of fibrinolysis is tightly regulated to maintain vascular homeostasis and to prevent uncontrolled clot lysis [1]. Tissue-bound and soluble activators, including tissue-type plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), mediate the conversion of the circulating zymogen plasminogen into its active serine protease form, plasmin. As an essential component of the fibrinolytic system, plasmin degrades fibrin clots, and has additional biological activities related to tissue remodeling, cell migration, and inflammation [2]. However, uncontrolled plasmin activity can result in bleeding disorders and tissue damage; therefore, the inhibitors α_2-antiplasmin (A2AP) and α_2-macroglobulin also contribute to the hemostatic balance and regulate fibrinolysis by binding and inactivating free plasmin [3].

To regulate the generation of plasmin, the serine protease inhibitor plasminogen activator inhibitor-1 (PAI-1) prevents the conversion of plasminogen into plasmin via binding of both t-PA and u-PA [4]. PAI-1 also has additional activities that are independent of its serpin activity, including the regulation of inflammation via immune cell

migration, binding to cell surface receptors, with subsequent activation of intracellular signaling cascades, and effects on cytokine production [5–7]. The expression and level of PAI-1 in various tissues are increased in response to infection and injury; however, PAI-1 shows a relatively short half-life in circulation, but is stabilized by binding to the host protein vitronectin [8]. Dysregulation of PAI-1 levels or activity has been implicated in numerous disease states, including cancer metastasis [9], organ fibrosis [5], atherosclerosis, and myocardial infarction [10]. PAI-1 participates in the host response to multiple microbial infections, including those caused by *Pseudomonas aeruginosa* [11], *Klebsiella pneumoniae* [12], *Streptococcus pneumoniae* [13], *Burkholderia pseudomallei* [14], and *Haemophilus influenzae* [15]. In these studies, PAI-1-deficient mice were used in models of bacterial pneumonia to demonstrate that PAI-1 is protective by inducing proinflammatory cytokine production and subsequent neutrophil recruitment to the sites of infection.

Consequently, multiple bacterial pathogens have evolved mechanisms to exploit or usurp host fibrinolysis [16–20]. *Yersinia pestis* is the causative agent of bubonic, pneumonic and septicemic plague [21]. *Y. pestis* produces the outer membrane omptin family aspartate protease known as Pla [22,23], which enhances virulence in mouse models of pneumonic and bubonic plague [24–26]. Pla is required for the outgrowth of *Y. pestis* during respiratory infection and for the development of a purulent, multifocal severe exudative bronchopneumonia that is the hallmark of pneumonic plague [27]. Pla is a potent activator of host plasminogen, and cleaves the zymogen at the same residues recognized by endogenous plasminogen activators (PAs) [23,24,28]. Other host substrates associated with the fibrinolytic system have also been identified and characterized *in vitro* as potential Pla targets, including A2AP, thrombin-activatable fibrinolysis inhibitor, and PAI-1 [29–31]. Thus, it has been hypothesized that, through the activities of Pla, *Y. pestis* enhances fibrinolysis by targeting multiple regulatory points of the host hemostatic system to aid in virulence [29,30].

Recent empirical evidence has shown that, in fact, multiple *in vitro* substrates of Pla have limited or no impact on the development of disease *in vivo*, including the group II substrate peroxiredoxin-6 and the group III substrate A2AP [32,33]. Indeed, to date only one *in vitro*-described Pla group I substrate, the apoptotic molecule Fas ligand (FasL), has been reported to contribute to disease during pneumonic plague [34]. It has been hypothesized that the inactivation of PAI-1 by Pla could serve as a mechanism by which *Y. pestis* alleviates endogenous PA inhibition *in vivo* to promote disease by enhancing plasmin generation, thus facilitating bacterial outgrowth and dissemination [30,31,35–38].

In this article, we report that the Pla protease cleaves and inactivates PAI-1 serpin activity during pneumonic plague. The loss of PAI-1 results in increased pulmonary plasmin levels and promotes the outgrowth of *Y. pestis* bacilli in the lungs. In addition, the absence of PAI-1 causes changes in the host response to *Y. pestis* infection, resulting in altered neutrophil recruitment, cytokine production, and tissue barrier permeability. Thus, our data show that PAI-1 is a group I substrate of Pla, and that the inactivation of PAI-1 by *Y. pestis* alters the host environment to facilitate the development of pneumonic plague.

Materials and methods

Reagents, bacterial strains, and culture conditions

Reagents for this study were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or VWR (Batavia, IL, USA), unless otherwise stated. *Y. pestis* strains were grown in brain heart infusion (BHI) broth or on BHI agar (VWR). For animal infections, *Y. pestis* strains were cultured in BHI at 37 °C with the addition of 2.5 mM $CaCl_2$, as previously described [26]. All experiments with select agent strains of *Y. pestis* CO92 were conducted in a Centers for Disease Control-approved BSL-3/ABSL-3 facility at Northwestern University.

Animal infections

All procedures involving animals were carried out in compliance with protocols approved by the Animal Care and Use Committee of Northwestern University. C57BL/6 mice, aged 6–8 weeks, were bred at Northwestern University or purchased from The Jackson Laboratories (Farmington, CT, USA). Breeding pairs of C57BL/6 *PAI-1*$^{-/-}$ mice were obtained from Dr Douglas Vaughan (Northwestern University). For infections, mice were age-matched and sex-matched, anesthetized with ketamine and xylazine, and infected by the intranasal route (10^4, 10^6 or 10^8 colony-forming units [CFUs]) with *Y. pestis* diluted in phosphate-buffered saline (PBS), as previously described [26,34]. Mice were killed by intraperitoneal injection of pentobarbital sodium, followed by organ removal or cervical dislocation. All animal infections were performed at least twice, and the data were combined.

To determine the bacterial burden, mice were inoculated intranasally with *Y. pestis*, and mice were killed at various times after infection. Subsequently, the lungs and spleens were removed, weighed, homogenized in sterile PBS, serially diluted, and plated onto BHI agar. To determine the survival of mice following infection, mice were inoculated intranasally with *Y. pestis* and monitored every 12 h for up to 10 days.

Histopathology

Mice were inoculated intranasally with *Y. pestis*, and at 48 h mice were killed and the lungs were inflated with

1 mL of 10% neutral buffered formalin via tracheal cannulation, removed, fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin, as previously described [32,33]. Inflamed lesions were analyzed with IMAGEJ to calculate the area of inflammation. Data represent the lesion area (mm^2) per field in two sections from three mice each at × 2.5 magnification.

Innate immune cell quantification

Mice were infected intranasally with *Y. pestis* or mock-infected with PBS, and at 48 h mice were killed and bronchoalveolar lavage (BAL) was performed, as described previously [34]. BAL fluid (BALF) was collected by pooling five 1-mL lavages with PBS per mouse; the first 1 mL of the BALF was reserved for analyses of cytokines or specific hemostatic proteins (see below). Samples were processed, stained, and analyzed by flow cytometry, as previously described [32,33].

Cytokine analysis

At 48 h after inoculation with PBS or *Y. pestis*, the levels of interleukin (IL)-12p70, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, monocyte chemoattractant protein-1 (MCP-1) and IL-6 were quantitatively established from the supernatant of the collected BALF by use of the cytometric bead array technique (BD Cytometric Bead Array Mouse Inflammation Kit; BD Biosciences, San Jose, CA, USA) as specified by the manufacturer. Data were analyzed with BD CYTOMETRIC BEAD ARRAY software.

Quantification of total, active and cleaved PAI-1 in BALF

PAI-1 levels in BALF were assessed with a total PAI-1 ELISA (Molecular Innovations, Novi, MI, USA), according to the manufacturer's instructions. Additionally, the levels of active PAI-1 in the BALF were assessed with an active PAI-1 ELISA (Molecular Innovations). For both ELISAs, the absorbance at 450 nm was measured in a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data from three independent experiments assessed in duplicate were combined. To assess PAI-1 cleavage *in vitro*, 0.15 μg of purified recombinant mouse PAI-1 (Abcam, Cambridge, VT, USA) was incubated with 1×10^7 CFUs of *Y. pestis* or *Y. pestis* Δ*pla* in PBS, and supernatants were collected after 10 min. For immunoblot analyses, purified mouse PAI-1 (Abcam) or BALF was separated by reducing SDS-PAGE, transferred to nitrocellulose, and probed with an anti-mouse PAI-1 antibody (Abcam). For *in vivo* analysis, each blot is representative of three independent experiments, and each lane represents BALF from a single mouse.

Quantification of plasminogen cleavage and plasmin activity

Plasmin activity in BALF was assessed as described previously with the chromogenic substrate D-AFK-ANSNH-iC$_4$H$_9$-2HBr (SN5; Hematologic Technologies, Essex Junction, VT, USA) [22,26]. Data are represented as the relative fluorescence at 120 min combined from two independent experiments performed in triplicate. Purified murine plasminogen (Haematologic Technologies), purified murine plasmin (Haematologic Technologies) or BALF from each of the indicated infections was separated by reducing SDS-PAGE, transferred to nitrocellulose, and probed with an anti-mouse plasmin(ogen) antibody (Haematologic Technologies), as described previously [32].

Statistics

Statistical means are plotted, and error bars represent standard errors of the mean. The Mann–Whitney *U*-test was used for bacterial load comparisons, the Kaplan–Meier test was used for comparison of survival curves, and all other experiments were analyzed by one-way ANOVA with Bonferroni's multiple comparison test where indicated, with GRAPHPAD PRISM 5.

Results

PAI-1 is cleaved and inactivated in the lungs by the Pla protease

To determine whether the Pla protease of *Y. pestis* inactivates PAI-1 *in vivo*, we inoculated wild-type C57BL/6 mice via the intranasal route with PBS (mock) or with equivalent doses (10^4 CFUs) of *Y. pestis* strain CO92 or an isogenic mutant of *Y. pestis* CO92 lacking Pla [26]. We found that the levels of total PAI-1 were significantly increased in the lungs in response to *Y. pestis* infection as compared with uninfected mice (Fig. 1A). The levels of PAI-1 in the lungs of wild-type *Y. pestis*-infected mice were also increased as compared with those in *Y. pestis* Δ*pla*-infected mice, most likely because of the difference in bacterial load between the strains at 48 h after infection ('output CFU') (Fig. 1A). Therefore, to assess the impact of Pla on PAI-1 levels when bacterial loads were equivalent, we increased the inoculum ('input CFU') of *Y. pestis* Δ*pla* to 10^8, so that the output CFU at 48 h was matched to the output CFU of the wild-type strain [32–34]. We found no difference in the levels of total PAI-1 in the lungs at 48 h when output CFUs were equivalent (Fig. 1A). We also assessed the levels of active PAI-1 present in the same BALF by using an active PAI-1-specific ELISA. In contrast to total PAI-1, there was a significant decrease in the level of active PAI-1 in wild-type *Y. pestis*-infected mice as compared with Δ*pla*-infected mice, when output CFUs were matched (Fig. 1B).
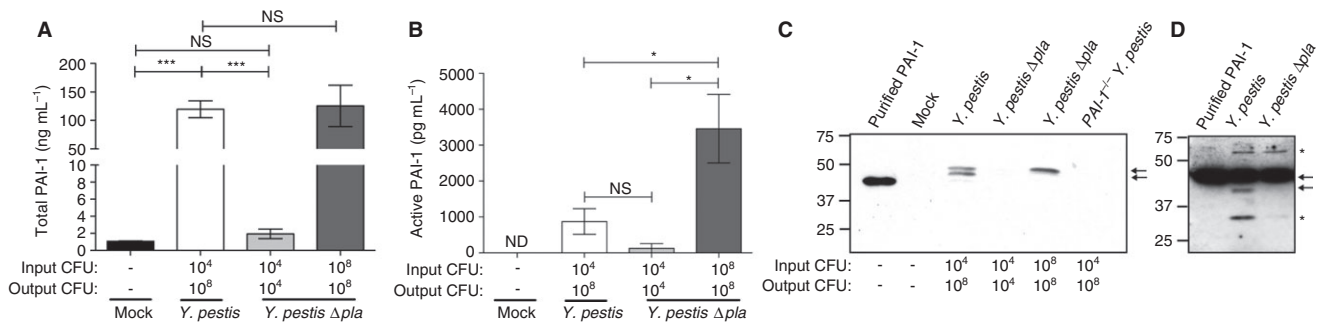
Fig. 1. Plasminogen activator inhibitor-1 (PAI-1) is cleaved and inactivated by the Pla protease *in vivo*. (A, B) Quantification of (A) total PAI-1 and (B) active PAI-1 by ELISA recovered by bronchoalveolar lavage (BAL) of C57BL/6 mouse lungs 48 h after inoculation with phosphate-buffered saline (mock), 10^4 colony-forming units (CFUs) of *Yersinia pestis*, 10^4 CFUs of *Y. pestis Δpla*, or 10^8 CFUs of *Y. pestis Δpla*. Data from two independent experiments are combined ($n = 10$ for each group); error bars represent standard errors of the mean (SEMs), and significance was determined by one-way ANOVA with Bonferroni's multiple comparison (*$P \leq 0.05$; ***$P \leq 0.001$). (C, D) Immunoblot analysis of PAI-1 recovered by BAL (C) or PAI-1 cleaved *in vitro* (D). Blots are representative of three independent experiments. Arrows represent full-length PAI-1 protein and cleavage product; asterisks indicate non-specific bands. Numbers to the left of the blot indicate molecular masses in kilodaltons. Note that purified, recombinant PAI-1 migrates at a lower molecular mass than PAI-1 isolated from mouse BAL fluid, owing to the lack of post-translational modifications on the protein. The input CFU and output CFU are included below each figure to denote the number of bacteria inoculated and the number at the time of assessment, respectively. ND, not detectable; NS, not significant.

To determine whether the cleavage of PAI-1 by Pla could be observed *in vivo*, we analyzed the same BALF by immunoblotting with an antibody against total PAI-1. Although PAI-1 protein was not detectable in the lung airspaces of uninfected mice or mice infected with 10^4 CFUs of *Y. pestis Δpla*, we observed a single species at ~ 47 kDa, corresponding to full-length, glycosylated PAI-1 [7], in the lungs of mice infected with 10^8 CFUs of *Y. pestis Δpla* (Fig. 1C), consistent with the ELISA data in Fig. 1A. Infection with wild-type *Y. pestis* also resulted in detectable full-length PAI-1; however, we also observed a second, slightly faster migrating band that was not present in any other condition (Fig. 1C). Incubation of recombinant mouse PAI-1 with *Y. pestis in vitro* showed a similar Pla-dependent cleavage product of PAI-1 (Fig. 1D, lower arrow), consistent with the characterized cleavage of human PAI-1 by Pla *in vitro* [29]. This suggests that Pla directly cleaves PAI-1 in the lungs. From these data, we conclude that the presence of *Y. pestis* bacteria in the lungs increases the levels of PAI-1 in the airspace, but that PAI-1 is cleaved and inactivated in a Pla-dependent manner during pneumonic plague.

PAI-1 restricts the proliferation of Y. pestis in the absence of Pla

As Pla contributes to the proliferation of *Y. pestis* in the lungs [26], and Pla inactivates PAI-1 during pneumonic plague, we hypothesized that the loss of PAI-1 would partially compensate for the absence of Pla. To test this, wild-type or *PAI-1*$^{-/-}$ mice were infected intranasally, and CFUs in the lungs and spleens were enumerated. We found no difference in the number of CFUs in the lungs at any point between wild-type and *PAI-1*$^{-/-}$ mice after infection with wild-type *Y. pestis* (Fig. 2A). On the other

hand, we observed a 100–1000-fold increase in the number of CFUs of the Δpla strain in the lungs of *PAI-1*$^{-/-}$ mice as compared with wild-type mice at both 48 h and 72 h after infection (Fig. 2A). However, this enhanced outgrowth in the lungs did not enhance bacterial dissemination, as determined by measuring the number of CFUs in the spleen (Fig. 2B). Finally, we tested the impact of PAI-1 deficiency on the survival of mice following intranasal infection, and observed no difference in the survival of *PAI-1*$^{-/-}$ mice as compared with wild-type controls after infection with either wild-type *Y. pestis* or the Δpla mutant (Fig. 3). These data indicate that PAI-1 cleavage contributes to bacterial outgrowth in the lungs but not during systemic infection.

PAI-1 enhances lung pathology during Y. pestis infection

To determine the impact of PAI-1 on the host response to pneumonic plague, we evaluated the overall histopathology of infected lungs in the presence or absence of PAI-1. This analysis revealed a noticeable reduction in the gross size of the inflammatory lesions in *PAI-1*$^{-/-}$ mice as compared with wild-type mice after infection with wild-type *Y. pestis* (Fig. 4A; Fig. S1). This was confirmed by the quantification of lesion size by IMAGEJ analysis (Fig. 4B). Furthermore, we observed more intact lung architecture throughout the lesions of *PAI-1*$^{-/-}$ mice than in the lesions of wild-type mice (Fig. 4A). Consistent with these observations, the levels of serum albumin present in the lung airspaces of *PAI-1*$^{-/-}$ mice were significantly reduced as compared with wild-type mice (Fig. 4C), suggesting that the presence of PAI-1 affects tissue barrier integrity during infection. However, wild-type and *PAI-1*$^{-/-}$ mice infected with *Y. pestis Δpla* had equivalent levels of albumin in the lung airspace (Fig. 4C).
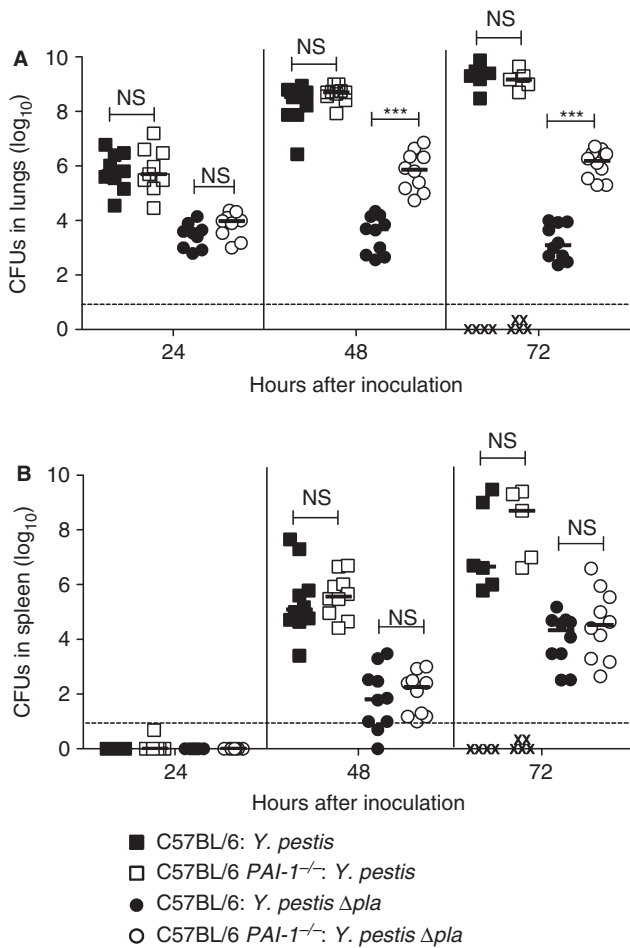
Fig. 2. Plasminogen activator inhibitor-1 (PAI-1) restricts the outgrowth of *Yersinia pestis* in the absence of Pla. (A, B) Bacterial burden in the (A) lungs and (B) spleens of C57BL/6 or C57BL/6 $PAI-1^{-/-}$ mice infected with 10^4 colony-forming units (CFUs) of *Y. pestis* or *Y. pestis* Δpla at the indicated times. Each point represents the numbers of bacteria recovered from a single mouse. An 'X' indicates a mouse that succumbed to the infection, the median number of CFUs is denoted by a solid line, and the dashed line indicates the limit of detection. Data from two independent experiments are combined ($n = 10$ for each group). The Mann–Whitney U-test was used for comparison of bacterial burden measurements (***$P \leq 0.001$). NS, not significant.

PAI-1 promotes neutrophil recruitment to the lungs during pneumonic plague

PAI-1 has been shown to contribute to immune cell recruitment in the lungs during bacterial pneumonia [11,14]. We found that the lungs of wild-type mice infected with *Y. pestis* contained significantly more immune cells than those of $PAI-1^{-/-}$ (Fig. 5A). Wild-type mice infected with *Y. pestis* Δpla showed a significant reduction in the total number of immune cells recruited to the lungs as compared with those infected with wild-type *Y. pestis*. However, PAI-1 deficiency did not alter the total number of immune cells present in the airspaces of mice infected with *Y. pestis* Δpla as compared with the
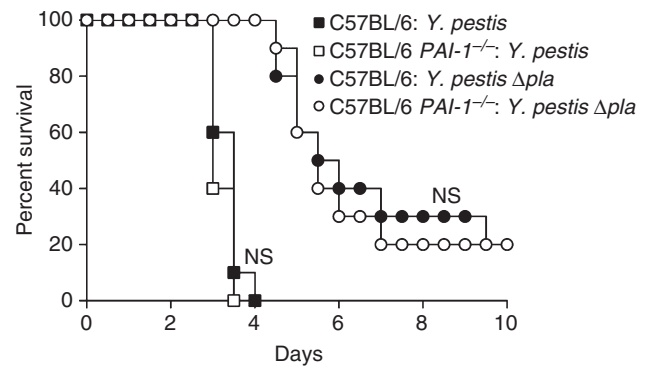

Fig. 3. Plasminogen activator inhibitor-1 (PAI-1) is dispensable for survival during *Yersinia pestis* respiratory infection. Survival over time of C57BL/6 or C57BL/6 $PAI-1^{-/-}$ mice infected with 10^4 colony-forming units of *Y. pestis* or *Y. pestis* Δpla. Data from two independent experiments are combined ($n = 20$ for each group). The Kaplan–Meier test was used to determine the significance of differences in survival rates between wild-type and $PAI-1^{-/-}$ mice. NS, not significant.

wild-type (Fig. 5A). We found that, of these immune cells, neutrophils were the only population to show a significant reduction in recruitment to the lungs of *Y. pestis*-infected $PAI-1^{-/-}$ mice as compared with wild-type mice (Fig. 5B–G).

PAI-1 contributes to the regulation of cytokine production during Y. pestis respiratory infection

The hemostatic system is tightly linked to the inflammatory response and the production of cytokines [6,39]. Therefore, we investigated the effects of PAI-1 deficiency on cytokine levels, and found that the levels of IL-12p70, TNF-α and MCP-1 were unchanged in $PAI-1^{-/-}$ mice infected with *Y. pestis* as compared with wild-type mice (Fig. 6A,C,D). However, we did observe a significant decrease in the levels of both IFN-γ and IL-6 (Fig. 6B,E). In contrast, in $PAI-1^{-/-}$ mice infected with *Y. pestis* Δpla, we detected significant increases in the levels of IFN-γ, TNF-α and IL-6 in the lung airspaces as compared with wild-type mice (Fig. 6B,D,E). These results indicate that PAI-1 enhances the inflammatory response during wild-type *Y. pestis* infection, whereas it acts a suppressor of inflammatory cytokine production during *Y. pestis* Δpla infection.

PAI-1 restricts plasminogen activation in the lungs of Y. pestis Δpla-infected mice

We have previously shown that *Y. pestis* generates high levels of plasmin in the lungs in a Pla-dependent manner [32]. Therefore, we hypothesized that the inactivation of PAI-1 by Pla would result in increased plasmin levels during infection. The level of plasmin is robustly increased in the lungs of mice infected with wild-type *Y. pestis* as compared with both mock-infected and *Y. pestis* Δpla-infected
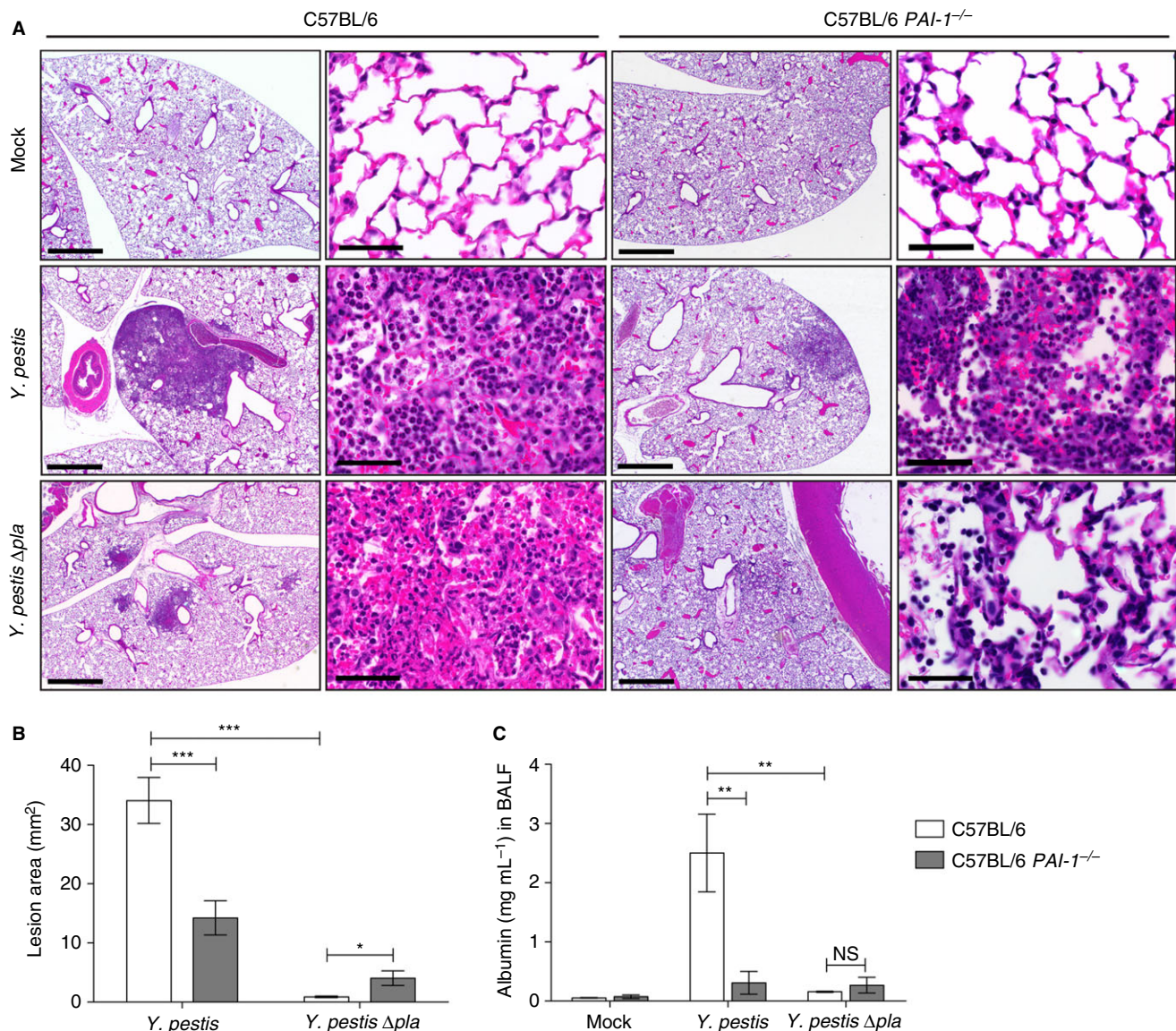
Fig. 4. Plasminogen activator inhibitor-1 (PAI-1) enhances lung pathology during *Yersinia pestis* infection. (A) Sections of formalin-fixed lungs stained with hematoxylin and eosin from C57BL/6 or C57BL/6 *PAI-1$^{-/-}$* mice inoculated with phosphate-buffered saline (mock), 10^4 colony-forming units (CFUs) of *Y. pestis*, or 10^4 CFUs of *Y. pestis Δpla*. Representative images of inflammatory lesions are shown (*n* = 3). For each infection, the scale bar of the left image (× 2.5 magnification) represents 100 μm, and the scale bar of the right image (× 63 magnification) represents 50 μm. (B) Inflamed lesions were analyzed with IMAGEJ to calculate the area of inflammation. Data represent the lesion area (mm^2) per field in two sections from three mice, each at × 2.5 magnification. (C) Quantification by ELISA of albumin levels in the bronchoalveolar lavage fluid (BALF) of C57BL/6 or C57BL/6 *PAI-1$^{-/-}$* mice infected with 10^4 CFU of *Y. pestis* or *Y. pestis Δpla*, or mock-infected. Data from two independent experiments are combined (*n* = 10 for each group), and error bars represent standard errors of the mean (SEMs). One-way ANOVA with Bonferroni's multiple comparison test was used to determine significance (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). NS, not significant.

mice; dose-matched *Δpla* infection results in a higher level of total plasminogen but little or no plasmin (Fig. 7A). In *PAI-1$^{-/-}$* mice infected with wild-type *Y. pestis*, we found that the presence of Pla also increased the conversion of plasminogen to plasmin, but not to the same extent as in wild-type mice, most likely because of reduced immune cell recruitment and tissue damage (Figs. 4C and 5B). In *PAI-1$^{-/-}$* mice, we observed an increase in the level of plasmin in the airspace as compared with wild-type mice

after infection with *Y. pestis Δpla* (Fig. 7A). When the inoculum of the *Y. pestis Δpla* strain is increased so that the output CFU is equivalent to that of a *Y. pestis* infection, we found that the lack of PAI-1 enhances the conversion of plasminogen to plasmin (Fig. 7A). To confirm these observations, we assessed the levels of active plasmin via cleavage of a plasmin-specific chromogenic substrate, and found that the data mimicked those obtained with immunoblotting (Fig. 7B). Although the differences
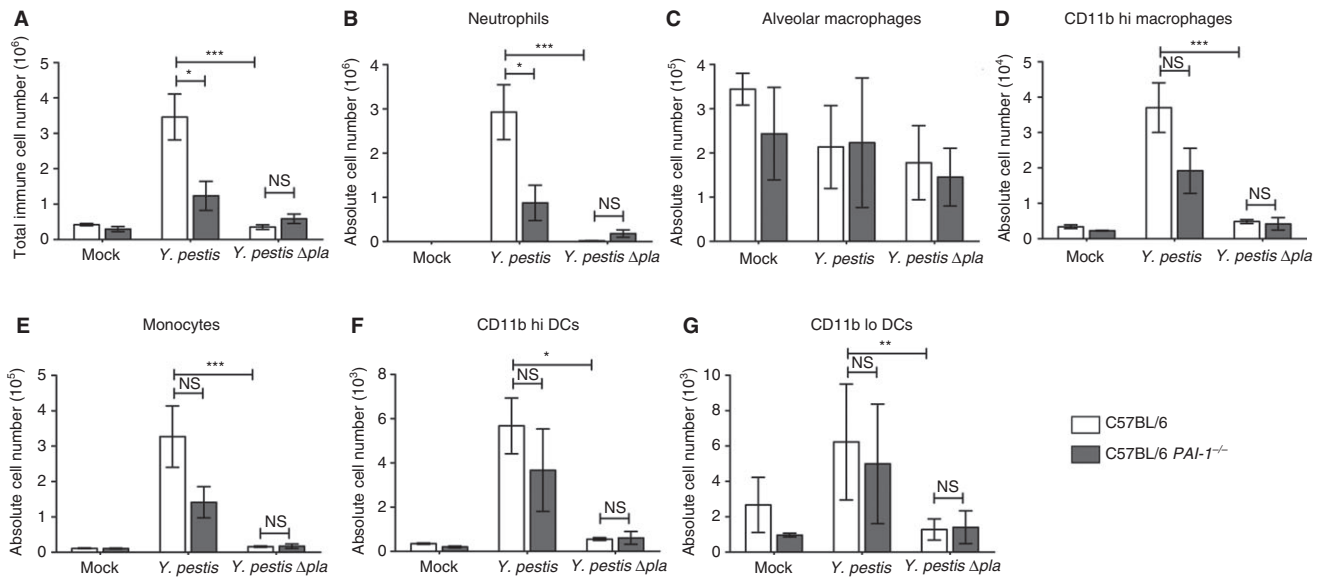
Fig. 5. Plasminogen activator inhibitor-1 (PAI-1) promotes neutrophil recruitment during *Yersinia pestis* infection. C57BL/6 or C57BL/6 *PAI-1*$^{-/-}$ mice were inoculated with phosphate-buffered saline (mock), 10^4 colony-forming units (CFUs) of *Y. pestis*, or 10^4 CFUs of *Y. pestis* Δ*pla*, and after 48 h bronchoalveolar lavage was performed to recover immune cells. (A) The total numbers of cells recruited to the lungs were determined by counting on a hemacytometer. (B–G) Flow cytometry was performed to determine the absolute numbers of (B) neutrophils, (C) alveolar macrophages, (D) CD11b hi macrophages, (E) monocytes, (F) CD11b hi dendritic cells (DCs) and (G) CD11b lo DCs recruited to the lungs. Data from two or three independent experiments are combined (*n* = 10–15 for each group); error bars represent standard errors of the mean. One-way ANOVA with Bonferroni's multiple comparison test was used to determine significance (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). NS, not significant.

in the levels of active plasmin in the airspaces did not reach statistical significance on comparison of *Y. pestis* Δ*pla* infection of wild-type mice and *PAI-1*$^{-/-}$ mice infected with 10^4 CFUs, the effects of PAI-1 and Pla on active plasmin generation were clear on comparison of *Y. pestis* Δ*pla* dose-matched samples in wild-type and *PAI-1*$^{-/-}$ mice (Fig. 7B). These data confirm that, when bacterial loads are equivalent and hemostatic proteins have access to the lung airspace, PAI-1 restricts endogenous plasminogen activation, and that Pla can subsequently overcome this effect.

Discussion

In vitro, the Pla protease of *Y. pestis* has been shown to inactivate multiple regulators of host hemostasis while simultaneously converting plasminogen to plasmin [24,29,30]. Thus, it has been widely hypothesized that Pla contributes to virulence not only by generating plasmin but also by circumventing the activity of endogenous inhibitors of fibrinolysis [30,31,35–38]. We recently found that, in fact, Pla does not cleave A2AP *in vivo*, and nor does A2AP have an effect on either bacterial outgrowth or disease progression in a mouse model of pneumonic plague [32]. This study suggests that plasmin is excessively generated during a *Y. pestis* infection to an extent that A2AP cannot overcome [32].

Here, we investigated the contribution of the Pla substrate PAI-1 to the development of primary pneumonic plague. Our data clearly demonstrate that murine PAI-1 is cleaved and inactivated by *Y. pestis* in a Pla-dependent manner during pneumonic plague. The cleavage of human PAI-1 by Pla occurs between residues Arg346 and Met347 within the reactive center loop of PAI-1, removing ~ 20 residues from the C-terminus of the protein [29]. Although this proteolysis event inhibits the serpin activity of PAI-1, a biologically active but serpin-defective form of PAI-1 may remain. Some of the serpin-independent functions attributed to PAI-1 include the regulation of immune cell migration, binding to cell surface receptors, and the activation of intracellular signaling cascades [5–7]. Thus, Pla-cleaved PAI-1 may still contribute to the host response to plague. Alternatively, there may be sufficient uncleaved PAI-1 present in the lungs that retains full activity, thereby limiting the impact of Pla via PAI-1 inhibition (providing an explanation for the differences between wild-type and *PAI-1*$^{-/-}$ mice following *Y. pestis* infection). In addition, although Pla can cleave both human and mouse PAI-1, differences between the species may result in different consequences for the host during infection.

We report that PAI-1 restricts the outgrowth of *Y. pestis* Δ*pla* in the lungs. These results demonstrate that PAI-1 serves as a protective host defense mechanism for *Y. pestis* during pneumonic plague in the absence of Pla. Interestingly, bacterial proliferation in the lungs in the absence of PAI-1 does not correspond with enhanced dissemination or the outcome of disease (i.e. survival),
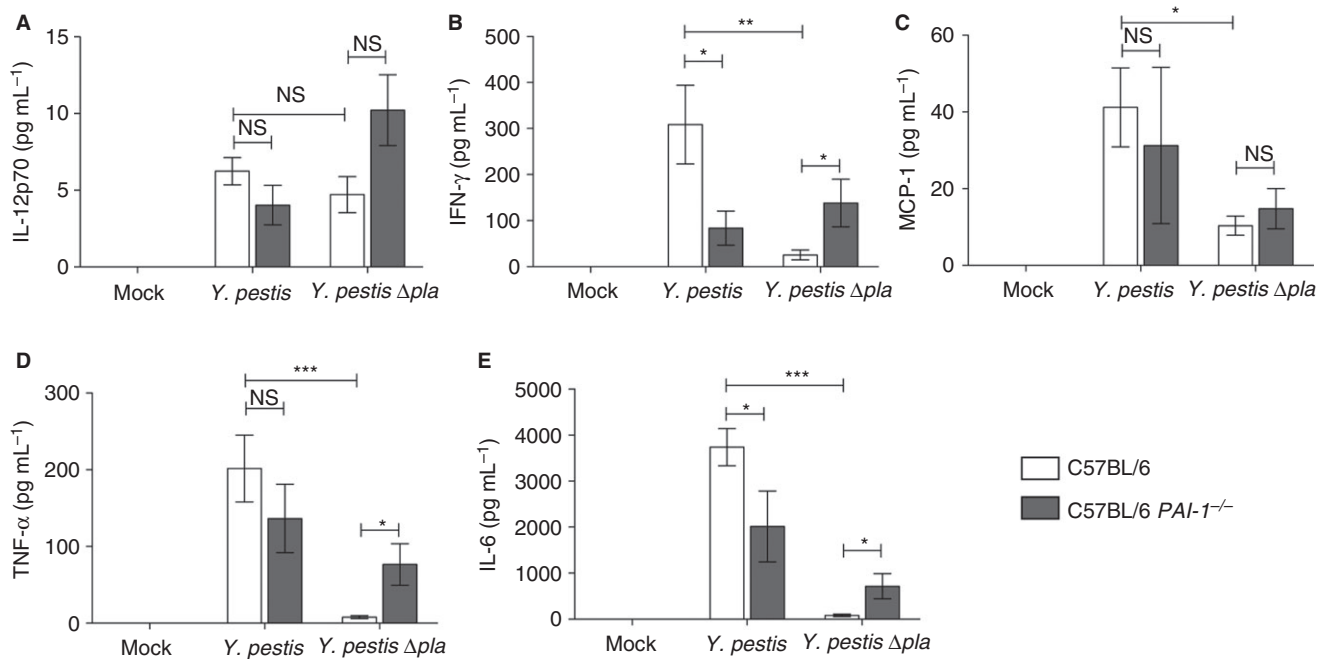
Fig. 6. Plasminogen activator inhibitor-1 (PAI-1) regulates cytokine production during pneumonic plague. Forty-eight hours after inoculation with phosphate-buffered saline (mock), 10^4 colony-forming units (CFUs) of *Yersinia pestis*, or 10^4 CFUs of *Y. pestis* Δ*pla*, the levels of the indicated cytokines present in the bronchoalveolar lavage fluid of C57BL/6 or C57BL/6 *PAI-1*$^{-/-}$ mice were assessed by cytometric bead array. Data from two or three independent experiments are combined ($n = 10$–15 for each group); error bars represent standard errors of the mean. One-way ANOVA with Bonferroni's multiple comparison test was used to determine significance (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). IFN, interferon; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NS, not significant; TNF, tumor necrosis factor.

suggesting additional activities of Pla that are independent of PAI-1 cleavage.

PAI-1 has been shown to affect both immune cell recruitment and cytokine production via its regulation of the hemostatic system and through mechanisms that are independent of its serpin activity [5,6]. Here, we demonstrate that PAI-1 deficiency impacts the immune response to both *Y. pestis* and *Y. pestis* Δ*pla* infection. During a wild-type *Y. pestis* infection, the absence of PAI-1 significantly decreases neutrophil recruitment to the lungs. This is consistent with a wide array of literature demonstrating the critical role of PAI-1 in the recruitment of neutrophils to the pulmonary compartment following infection with other respiratory pathogens [11–15]. However, during these infections, the absence of PAI-1 increases bacterial outgrowth, which is in contrast to our findings with wild-type *Y. pestis*, but consistent with Δ*pla* infection. This suggests that *Y. pestis*, which encodes a direct PA, must still overcome the inhibitory effects of PAI-1, and requires additional endogenous plasminogen activation, at least in the lungs. Furthermore, the control and clearance of multiple bacterial pathogens is highly dependent on the presence of neutrophils [40–42], whereas *Y. pestis* outgrowth is unchanged following depletion of neutrophils [43]. This provides another potential explanation for the differences in the phenotypes observed in *PAI-1*$^{-/-}$ mice with different pathogens. The significant reduction in the number of neutrophils recruited to the lungs in the absence of PAI-1 probably explains the lack of damage to tissue barrier integrity, as pneumonic plague is primarily a host-driven disease mediated through neutrophils [43].

PAI-1 also impacts cytokine production in response to *Y. pestis*, probably as a consequence of its role in regulating neutrophil recruitment. Wild-type mice infected with *Y. pestis* show increased levels of the cytokines IFN-γ and IL-6 in the lung compartment as compared with *PAI-1*$^{-/-}$ mice. Thus, in a wild-type *Y. pestis* infection, the reduction in polymorphonuclear leukocyte infiltration in *PAI-1*$^{-/-}$ mice may contribute to the diminished levels of these cytokines. In contrast, there are minimal levels of proinflammatory cytokines in the airspaces of wild-type mice infected with the Δ*pla* strain, as Pla is absolutely required for the transition to the proinflammatory phase of pneumonic plague [26,44]. However, PAI-1 deficiency can restore part of this transition, as the levels of IFN-γ, TNF-α and IL-6 are all significantly increased, a phenomenon that is also seen in A2AP-deficient mice infected with *Y. pestis* Δ*pla* [32].

These seemingly paradoxical results suggest that Pla probably contributes to other aspects of the innate immune response beyond PAI-1 cleavage (such as is observed with the Pla-mediated inactivation of FasL) [34]. In addition, it has been repeatedly demonstrated that PAI-1 can have both positive and negative effects on
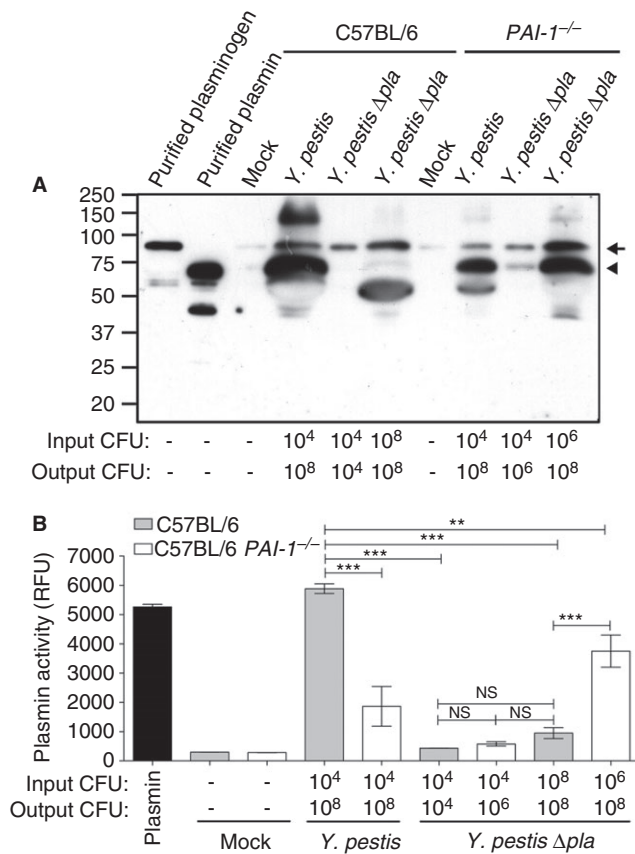
Fig. 7. Plasminogen activator inhibitor-1 (PAI-1) restricts endogenous plasmin(ogen) activation in the lungs of *Yersinia pestis* Δ*pla*-infected mice. (A) Immunoblot analysis of plasmin(ogen) recovered by bronchoalveolar lavage (BAL) from C57BL/6 or C57BL/6 *PAI-1*$^{-/-}$ mice inoculated with phosphate-buffered saline (mock) or the indicated strains. The arrow indicates plasmin(ogen), and the arrowhead indicates plasmin. Numbers to the left of the blot indicate molecular masses in kilodaltons. The blot is representative of three independent experiments. (B) Quantification of active plasmin levels present in the BAL fluid as described above or purified active murine plasmin. Data are presented as relative fluorescence units (RFUs) at 120 min, and are combined from two independent experiments plated in triplicate; error bars represent standard errors of the mean. One-way ANOVA with Bonferroni's multiple comparison test was used to determine significance (*$P \leq 0.05$, ***$P \leq 0.01$). CFU, colony-forming unit; NS, not significant.

inflammation, depending on experimental conditions, cell types studied, and other factors [45]. A study on bubonic plague demonstrated that PAI-1 and fibrin deficiency have similar phenotypes regarding innate immune cell responses, thus highlighting the importance of fibrinolysis during *Y. pestis* infection and inflammation [46]. However, that study used a strain of *Y. pestis* engineered to produce a more stimulatory form of lipopolysaccharide, and administration was performed through a different route, thus potentially explaining the differences between our studies.

These data, combined with our previous work, allow us to formulate a hypothesis on the mechanisms by which the Pla protease disrupts the regulation of fibrinolysis during pneumonic plague. In a wild-type *Y. pestis* infection, the Pla protease potently converts plasminogen into active plasmin, while at the same time disrupting the serpin activity of PAI-1, thus enhancing endogenous plasminogen activation. When these activities are combined, the excess plasmin generated during infection cannot be overcome by the inhibitory effects of A2AP [32]. In contrast, during a *Y. pestis* Δ*pla* infection, the absence of either A2AP or PAI-1 increases the levels of free plasmin present in the lung airspace; however, this is not sufficient for the full virulence of *Y. pestis* in the lungs (because of either limited plasmin abundance or other activities associated with Pla). The loss of PAI-1 results in 2–3 logs greater bacterial outgrowth in the absence of Pla, but, ultimately, neither A2AP nor PAI-1 deficiency alters the outcome of infection. This is supported by data demonstrating that, once the bacteria have invaded the bloodstream, the Pla protease is no longer required for infection, as mice succumb to sepsis. Further studies on the role of plasmin during pneumonic plague will elucidate the contributions of plasminogen activation versus other activities of PAI-1 during *Y. pestis* infection, and the impact of Pla on these processes.

Addendum

Acknowledgements

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Additional histologic sections of formalin-fixed lungs stained with H&E from C57BL/6 or C57BL/6 $PAI-1^{-/-}$ mice inoculated with 10^4 CFUs of *Y. pestis* or *Y. pestis* Δpla.

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