Coagulation and fibrinolysis in tuberculosis, melioidosis and beyond
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Deficiency of protease-activated receptor-1 limits bacterial dissemination during severe Gram-negative sepsis (melioidosis)

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ABSTRACT

Protease-activated receptor-1 (PAR-1) is a G-coupled transmembrane receptor expressed by multiple cell types present in the lungs that can be activated by various proteases generated during acute inflammation. In this study we aimed to investigate the role of PAR-1 during melioidosis, a common cause of (pneumo)sepsis in Southeast Asia in a murine model of intranasal inoculation of the causative pathogen *Burkholderia (B.) pseudomallei*. Our results show that endogenous PAR-1 facilitates bacterial growth and dissemination during murine melioidosis, which is associated with increased cell influxes. However, these observations have no impact on survival.
INTRODUCTION

Melioidosis, caused by the Gram-negative bacterium *Burkholderia (B.) pseudomallei*, is a severe septic disease, characterized by pneumonia and rapid bacterial dissemination to distant body sites, with many possible disease manifestations, septic shock being the most severe\(^1\)\(^-\)\(^3\). In Southeast-Asia and Northern-Australia melioidosis is an important cause of community-acquired sepsis with a mortality rate of up to 40% despite appropriate antibiotic therapy\(^1\)\(^-\)\(^2\). Additionally, recently *B. pseudomallei* was classified as a 'Tier 1' disease agent considered to be an exceptional threat to security\(^4\). Protease-activated receptors (PARs) are G-protein-coupled receptors that carry their own ligand thereby converting an extracellular proteolytic cleavage event into an intracellular signal\(^5\)\(^-\)\(^6\). PARs, of which four subtypes have been described (PAR-1 to -4), are widely distributed throughout the airways and can be activated by a variety of proteases, including pro-inflammatory factors and proteases involved in the coagulation system\(^5\)\(^-\)\(^7\). Regulation of PAR activity by proteases is important under pathological circumstances during which these proteases are released or activated. PAR activation can play a paradoxical role as it can result in both beneficial and deleterious effects depending on the PAR subtype that is activated and on the nature of the activating trigger\(^5\)\(^-\)\(^7\). When cleaved by activated protein C (APC), PAR-1 has anti-inflammatory and barrier protective effects\(^7\)\(^-\)\(^8\), while PAR-1 exerts barrier disruptive effects when it is activated by high levels of thrombin\(^7\)\(^-\)\(^9\)\(^-\)\(^10\). Data on PAR-1 during inflammatory conditions are limited. The host response during melioidosis is characterized by upregulation of a large number of pro-inflammatory and procoagulant factors with protease activity\(^3\)\(^-\)\(^11\). In this study we investigated the role of PAR-1 in the host response during melioidosis.

METHODS

Ten weeks-old male wild-type (WT) C57BL/6 mice were compared with mice deficient for PAR-1 (PAR-1\(^{-/-}\)) on a C57BL/6 background. All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center. Melioidosis was induced by intranasal inoculation of 500 CFU of *B. pseudomallei*\(^12\)\(^-\)\(^13\). Mice were sacrificed after 24, 48 and 72 hours and survival studies were performed, during which mice were checked every 6 hours until death occurred. Samples were harvested and processed as described\(^12\)\(^-\)\(^13\). Bilateral bronchoalveolar lavage fluid (BALF) was obtained by exposing the trachea through a midline incision followed by cannulation with a sterile T-catheter and instilling and retrieving of two 0.5 mL aliquots of sterile phosphate buffered saline. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, CA). Paraffin-embedded lung sections were stained with haematoxylin and eosin and semi-quantitatively analyzed for inflammation and tissue damage as described\(^12\)\(^-\)\(^13\).
RESULTS

Deficiency of PAR-1 protected mice from bacterial growth and dissemination as reflected by decreased bacterial loads in lung and liver homogenates of PAR-1-/- mice 48 hours after infection \((P < 0.01\) and \(0.05\) versus WT mice respectively; Fig. 1 A-B). Moreover, 72 hours after infection bacterial loads in livers and BALF of PAR-1-/- mice still were lower in comparison with WT mice (for liver \(P < 0.01\); Fig. 1B, for BALF \(P < 0.05\), not shown). In addition, our results demonstrate that decreased bacterial burdens as were observed in PAR-1-/- mice did not protect from lethality \((P = 0.22\); Fig. 1C).

Next, we investigated the inflammatory response in the lungs. Our results show that 72 hours after infection bacterial loads were determined in lung homogenates (A), and liver homogenates (B). Mortality did not differ between WT mice and PAR-1-/- mice (C). Cellular influx (D), numbers of neutrophils (E) and total protein content (F) in broncholaveolar lavage fluid (BALF) are shown for infected WT and PAR-1-/- mice. Lung histopathology scores did not differ between WT and PAR-1-/- mice (G): both WT (H) and PAR-1-/- mice (I) showed comparative inflammatory infiltrates in the lungs characterized by interstitial inflammation together with necrosis, endothelialitis, bronchitis, edema, thrombi and pleuritis 48 hours after inoculation (H&E staining, original magnification x40). Data are expressed as box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Grey boxes/ black dots represent WT mice, white boxes/ white dots represent PAR-1-/- mice \((n = 7-8\) mice per group for each time point, in survival studies \(n = 17-18\) mice per group). \(*P < 0.05\) and \(**P < 0.01\) for WT versus PAR-1-/- mice (Mann-Whitney \(U\) test). For survival studies mortality was assessed every 6 hours. Comparison between groups was done by Kaplan-Meier analysis followed by log rank tests.
PAR-1 in melioidosis

Infection total leukocyte counts were decreased in BALF of PAR-1−/− mice when compared to WT mice (P < 0.05; Fig.1D), which was caused by lower numbers of neutrophils (P < 0.05; Fig.1E). PAR-1 deficiency did not alter lung damage, as indicated by similar protein levels in BALF (Fig.1F) and similar lung pathology scores (Fig.1G-1) in PAR-1−/− and WT mice. In addition, apart from significantly lower levels of MCP-1 in plasma of PAR-1−/− mice 24 hours after infection (P < 0.01, not shown), levels of cytokines in lung homogenates, plasma and BALF of WT and PAR-1−/− mice did not differ at any time point (data not shown).

DISCUSSION

Experimental models of melioidosis have shown that after intranasal infection B. pseudomallei rapidly causes severe pneumonia and dissemination of bacteria to distant body sites12, 13. Our data are in line with previous studies showing that during experimental pneumococcal pneumonia PAR-1 deficiency was associated with lower bacterial loads in the lungs and livers compared to WT mice14. Next, in order to find out whether differences in bacterial growth would impact on lethality, we performed a survival study, showing no differences between both study groups. Our results are in contrast with observations during pneumococcal pneumonia and murine influenza A (H1N1) infection, in which PAR-1−/− mice had a significantly delayed mortality14, 15. Moreover, in a model of polymicrobial sepsis induced by cecal ligation and puncture (CLP), survival was improved after administration of a PAR-1 antagonist, indicating that functional PAR-1 enhances lethality16. In the same study it was shown that PAR-1−/− mice had a better survival after a LD90 endotoxin challenge16, which differed from earlier data that failed to show beneficial effects for PAR-1-deficiency on mortality after endotoxin administration17, 18. Finally, Kaneider et al. failed to show any survival benefits for PAR-1−/− mice after CLP19. However, these authors did show by a pepducin-based approach that early administration of a PAR-1 antagonist lead to a better survival, whereas, interestingly, late administration (after 4 hours) of a PAR-1 agonist had the same effects on survival, indicating that PAR-1 switched from being a harmful receptor into a beneficial receptor during the progression of polymicrobial sepsis in mice19.

In our model of experimental melioidosis, bacterial growth and dissemination is accompanied by a strong pro-inflammatory host response consisting of marked neutrophil recruitment into the lungs, release of pro-inflammatory cytokines and serious lung damage12, 13. We investigated whether PAR-1 deficiency impacted on this response. Together, these results indicate that PAR-1 plays a limited role in the inflammatory response during experimental melioidosis. Our data should be compared with earlier studies on the role of PAR-1 in the host response to pulmonary infection and inflammation. During pneumococcal pneumonia PAR-1 contributed to lung inflammation as reflected by attenuated neutrophil recruitment, lower pulmonary levels of pro-inflammatory cytokines and lower mean lung pathology scores in PAR-1−/− mice relative to WT mice14. In line, PAR-1−/− mice
were protected from bleomycin-induced lung inflammation and fibrosis, as was demonstrated by significantly lower inflammatory cell recruitment and total protein in BALF and decreased levels of lung MCP-1 in comparison to WT controls\(^20\). Moreover, during Coxsackie B virus infection PAR-1\(^{-/-}\) mice expressed reduced levels of IFN-\(\beta\) and chemokine (C-X-C motif) ligand (CXCL)-10 during the early phase of infection compared with WT mice that resulted in higher viral loads at day 8 after infection\(^21\). Additionally, PAR-1\(^{-/-}\) mice also had decreased CXCL10 expression and increased viral levels in the lung after influenza A infection compared to WT mice\(^21\). On the other hand, however, after endotoxin challenge no differences in cytokine responses were seen in PAR-1\(^{-/-}\) mice when compared to WT mice\(^{17,18}\), illustrating the different effects of PAR-1 during various inflammatory challenges. Evidently, the net effect of PAR-1 stimulation during inflammatory conditions largely depends on the nature and dynamics of the stimulating agents and the timing of release of these mediators. PAR-1 could be an interesting target in the development of new drugs against sepsis; however, much more detailed research has to be done to further unravel its effects during infections by different causative pathogens. In conclusion, we here demonstrate that PAR-1 deficiency has a limited detrimental role in the inflammatory response during experimental melioidosis, although it facilitates bacterial dissemination from the lungs to the liver. These data further expand the knowledge on the host immune response during severe (pneumo)sepsis caused by \textit{B. pseudomallei}.

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