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ORIGINAL ARTICLE

PAR1 regulation of CXCL1 expression and neutrophil recruitment to the lung in mice infected with influenza A virus

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Abstract

Background: Protease-activated receptor 1 (PAR1) is expressed in various immune cells and in the lung. We showed that PAR1 plays a role in Coxsackievirus B3 infection by enhancing toll-like receptor 3-dependent interferon-β expression in cardiac fibroblasts.

Objectives: We investigated the role of PAR1 in a mouse model of influenza A virus (IAV) infection.

Methods: We used mice with either a global deficiency of PAR1, cell type-specific deficiencies of PAR1, or mutation of PAR1 at the R41 or R46 cleavage sites.

Results: PAR1-deficient mice had increased CXCL1 expression in the lung, increased neutrophil recruitment, increased protein levels in the bronchoalveolar lavage fluid, and increased mortality after IAV infection compared with control mice infected with IAV. Results from mice with cell type-specific deletion of PAR1 indicated that PAR1 expression by hematopoietic cells suppressed CXCL1 expression, whereas PAR1 expression by endothelial cells enhanced CXCL1 expression in response to IAV infection. PAR1 activation also enhanced polyinosinic:polycytodylic acid induction of interleukin-8 in a human endothelial cell line. Mutation of the R46 cleavage site of PAR1 was associated with increased CXCL1 expression in the lung in response to IAV infection, which suggested that R46 signaling suppresses CXCL1 expression.

Conclusions: These results indicate that PAR1 expression by different cell types and activation by different proteases modulates the immune response during IAV infection.

KEYWORDS
biased signaling, CXCL1, influenza A virus, neutrophils, protease-activated receptor 1

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1 | INTRODUCTION

Influenza A viruses (IAV) are responsible for annual epidemics and occasionally for pandemics.[1] IAV causes considerable morbidity and mortality particularly in the elderly population.[1] Every year, approximately 500 million people are infected with influenza viruses, which can lead to 500 000 infection-associated deaths.[1] IAV primarily infects epithelial cells (EpC) of the airways.[2,3] However, endothelial cells (EC), natural killer cells, macrophages, and dendritic cells are also susceptible to IAV infection.[2,3] The innate immune response is the first-line defense against invading pathogens, such as IAV. The host's immune system recognizes pathogen-associated molecular and damage-associated molecular patterns via different receptors. For instance, endosomal toll-like receptor (TLR) 3 detects double-stranded (ds) RNA directly from dsRNA viral genomes as well dsRNA generated during the replication of single-stranded RNA viruses.[4] IAV is an enveloped virus with a segmented single-stranded RNA genome, which has a dsRNA-like genomic state during replication. TLR3 activation leads to interferon-regulatory factor 3 and NFκB-regulated gene expression.[4] Interferon-regulatory factor 3 activation leads to expression of interferon alpha (IFN-α) and IFN-β, which subsequently induce the expression of IFN-stimulated genes with direct antiviral activities. The NFκB signaling pathway results in cytokine and chemokine expression within the infected tissue that attracts immune cells to fight the infection. One of the major chemokines produced early after IAV infection is interleukin-8 (IL-8). This chemokine plays a key role in neutrophil recruitment from the blood stream into the lung during infection.[5] Mice do not express IL-8 but instead recruit neutrophils by expressing several chemokines (CXCL1, CXCL2, and CXCL5).[6] Although neutrophils are important innate immune cells that limit IAV infection, increased numbers of neutrophils in infected tissues can be detrimental to the host by inducing tissue injury.[7,8]

Protease-activated receptors (PARs) comprise a family of four G-protein coupled receptors (PAR1-4).[9] Activation of these receptors leads to a variety of cellular responses.[9] Importantly, there is cross-talk between PARs and TLRs.[10-15] Polyinosinic:polycytidylic acid (poly I:C) is a dsRNA mimetic that is used to activate TLR3. We found that activation of PAR1 enhanced poly I:C induction of IFN-β and CXCL10 in cardiac fibroblasts and macrophages.[10,14] Interestingly, results from mice with a global PAR1 deficiency indicated that PAR1 positively regulated poly I:C induction of CXCL10 but negatively regulated poly I:C induction of CXCL1.[14] PAR1 also contributes to the innate immune response to Coxsackievirus B3 infection; $PAR1^{-/-}$ mice exhibit reduced early expression of IFN-β and CXCL10 expression and increased later inflammation and myocarditis.[10] Similarly, we observed increased levels of CXCL1 in the lungs of $PAR1^{-/-}$ mice compared with controls after IAV infection.[10] Interestingly, TLR3-deficient mice exhibit an increase in IAV virus load but an improved survival after infection because of reduced inflammation.[16]

PAR1 is expressed by many different cell types and can be activated by a variety of proteases.[17] Interestingly, cleavage of PAR1 at different sites can lead to different cellular responses, a phenomenon known as biased signaling.[18,19] The canonical thrombin cleavage site in PAR1 is arginine 41 (R41) in the extracellular N-terminus. Cleavage at R41 leads to proinflammatory signaling and decreased endothelial barrier function.[19] However, two studies showed that thrombin cleavage of PAR1 can also mediate anti-inflammatory signaling if the endothelial cell protein C receptor (EPCR) is occupied by protein C.[20,21] In addition, PAR1 cleavage at R41 by human FVIIa but not mouse FVIIa bound to EPCR can confer anti-inflammatory signaling.[22,23] Activated protein C (APC) cleaves PAR1 at a noncanonical site (R46) and this confers anti-inflammatory signaling and an increase in endothelial barrier function.[18,24] The Griffin laboratory generated two mouse lines expressing cleavage-resistant $PAR1^{R41Q}$ (R41Q) or $PAR1^{R46Q}$ (R46Q) that can be used to investigate biased signaling.[18] Thrombomodulin is also important for PAR1 signaling because it binds thrombin and changes its substrate specificity from fibrinogen to protein C.[25] Importantly, PAR1, EPCR, and thrombomodulin are expressed by a variety of cell types, including ECs, hematopoietic cells, fibroblasts, and EpCs.[26,27] Interestingly, APC-EPCR-PAR1 signaling was shown to reduce egress of cells from the bone marrow.[28]

In this study, we analyzed the role of PAR1 in IAV infection using global and cell type-specific PAR1-deficient mice. Furthermore, we used R41Q and R46Q mice to determine the effect of loss of R41- and R46-dependent PAR1 signaling during IAV infection.

Essentials

- PAR1 was shown to modulate antiviral responses to influenza A virus (IAV) infection.
- Early immune responses in the lung after IAV infection were analyzed in mice with global or cell-specific PAR1 deficiency as well in mice carrying point mutations in PAR1.
- Global PAR1 deficiency, PAR1 deficiency on hematopoietic cells or lack of PAR1 activation at R46 increases CXCL1 in the airspace of the lung after IAV infection.
- PAR1 expression and activation is needed to control inflammatory responses after IAV infection.

2 | METHODS

2.1 | Mice

Adult $PAR1^{-/-}$ (ΔPAR1) and wild-type (WT, $PAR1^{+/+}$) mice, maintained as cousin lines, were used for this study.[29] We generated a series of mouse lines in which PAR1 was deleted in various cell types by crossing mice with a floxed PAR1 allele ($PAR1^{fl/fl}$)[30] with various mouse lines expressing Cre in a cell type-specific manner: deletion in lung EpCs ($PAR1^{fl/fl}$;SPC^{Cre} [PAR1ΔEpC] mice[31]), deletion in hematopoietic and EC ($PAR1^{fl/fl}$;$Tie2^{Cre}$ [PAR1ΔHEC][31]), deletion in

myeloid cells (PAR1$^{fl/fl}$;LysM^{Cre} [PAR1ΔMy][31]), and deletion in ECs (PAR1$^{fl/fl}$;VECad$^{Cre\text{-}ERT2}$ [PAR1ΔEC]).[32] To activate VECad$^{Cre\text{-}ERT2}$ expression, 8-week-old PAR1$^{fl/fl}$;VECad$^{Cre\text{-}ERT2}$ mice were gavaged with 2 mg of tamoxifen (Sigma-Aldrich, St. Louis, MO) dissolved in corn oil for 5 consecutive days and then mice were used 5 days later. PAR1$^{fl/fl}$ mice treated with tamoxifen were used as controls. In addition, we used two strains of mice with either R41Q or R46Q mutations in the PAR1 gene.[18] All mouse strains were on the C57Bl/6J background. The study was performed in accordance with the guidelines of the animal care and use committee of the University of North Carolina at Chapel Hill and complies with National Institutes of Health guidelines.

2.2 | IAV infection

The mouse-adapted strain of influenza A/Puerto Rico/8/1934 was propagated at days 10–12 of embryonated eggs and the virus titer was determined using a hemagglutination unit (HAU) assay.[31,33] Mice of both sexes between 8 and 12 weeks of age were infected intranasally with 0.02 HAU in 50 µl phosphate-buffered saline as described.[15,31,33] Bronchoalveolar lavage fluid (BALF) was collected by postmortem lavage with 3 × 900 µL ice-cold phosphate-buffered saline as described previously.[15,31,33] Mice were euthanized if they had ≥ 25% loss of initial body weight, as specified in our animal protocol.[15,31,33]

2.3 | Measurement of cell counts

Total white blood cell (WBC), neutrophil, lymphocyte, and monocyte numbers were determined with a Hemavet 950 (Drew Scientific).[15,31,33] To obtain cytospins from BALF cells, 50 µl of resuspended BALF cells was spun onto poly-D-lysine–coated microscope slides (Thermo Fisher Scientific) using a StatSpin CytoFuge (HemoCue America). Cells were stained with Wright-Giemsa stain (Fisher Scientific). A 100-cell differential count (%) was performed in blinded fashion by a board-certified veterinary pathologist and the percentages were converted to absolute numbers using the total cell count obtained by Hemovet 950 (Drew Scientific).

2.4 | Real-time polymerase chain reaction

Total RNA was isolated from lung tissue and BALF cell pellets using the TRIzol (Thermo Fisher Scientific).[10,11,14,31] One microgram of total RNA was transcribed to complementary DNA (iScript RT Supermix Kit, Bio-Rad Laboratories). Expression of messenger RNA (mRNA) of selected genes were analyzed by real-time polymerase chain reaction (PCR) using SSoFast Advanced Universal Supermix in a Bio-Rad cycler (Bio-Rad Laboratories) as described elsewhere.[14,31,33] Predesigned primer-probe sets for mouse CXCL1, CXCL2, CXCL5, CCL2, CCL5, IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) were obtained from integrated DNA Technologies.

2.5 | Measurement of mouse CXCL1 or human IL-8 protein

Levels of mouse CXCL1 protein in the lung and BALF or human IL-8 in cell media were measured by enzyme-linked immunosorbent assay as described previously.[10,14,15] Total protein in the lung and BALF was quantified by bicinchoninic acid assay.[15]

2.6 | Endothelial cell line culture and stimulation

Human umbilical vein endothelial cell–derived immortalized EA.hy926 cells were maintained as described.[34] For stimulation, cells were seeded the day before into a 24-well plate at 2.5 × 10^5 cells per well. The next day, cells were stimulated for 24 h in serum-free media with 5 µg/ml poly I:C (Sigma-Aldrich) and/or 100 µM PAR1 agonist peptide (PAR1 AP, TFLLR-NH_2, Abgent).[14]

2.7 | Statistics

GraphPad Prism 8.4 (GraphPad Software Inc) was used for statistical analysis. Data are represented as mean ± standard error of the mean. The two-tailed Student *t* test was used for two-group comparison of normally distributed data. For multiple-group comparison, normally distributed data were analyzed by two-way analysis of variance test and were Bonferroni-corrected for repeated measure over time. Survival rates were analyzed by Kaplan–Meier analysis and the log-rank test was applied to compare the survival distribution between the two groups. *P* value ≤ 0.05 was regarded as significant.

3 | RESULTS

3.1 | PAR1 regulation of CXCL1 expression in mice during IAV infection

In a previous study, we found that inflammatory mediators are significantly increased in the lungs 3 days after IAV infection.[31] Therefore, in this study we measured mRNA expression of a variety of inflammatory mediators (CXCL1, CXCL2, CXCL5, CCL2, CCL5, IL-1β, IL-6, and TNF-α) in the lungs of PAR1$^{-/-}$ and WT mice 3 days after IAV infection. Interestingly, the only significant difference we observed was an increase in the CXCL1 mRNA expression in the lungs of PAR1$^{-/-}$ mice compared with WT mice (Figure 1A and data not shown). We also observed a significant increase in CXCL1 mRNA expression in BALF cells (Figure 1B). Infected PAR1$^{-/-}$ mice also had significantly higher levels of CXCL1 protein in the lungs and BALF compared with infected WT controls (Figure 1C,D). These data

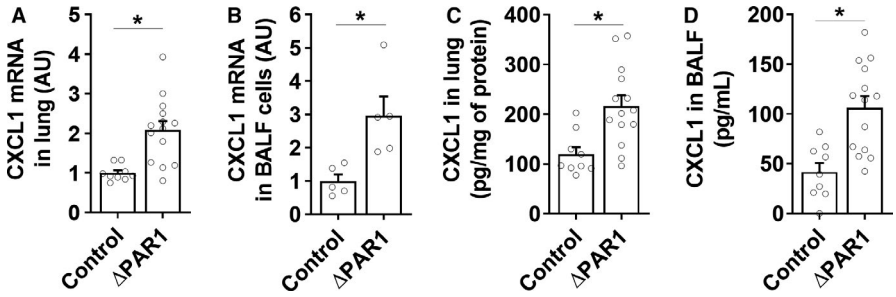

FIGURE 1 Effect of global PAR1 deficiency on CXCL1 expression in mice after influenza A virus infection. Wild-type (control) and PAR1$^{-/-}$ (ΔPAR1) mice were infected with 0.02 HAU H1N1 influenza A virus (IAV) and CXCL1 mRNA expression (A) in lungs and (B) bronchoalveolar lavage fluid (BALF) cell pellet was analyzed by real-time PCR 3 days after infection. CXCL1 protein levels in (C) lungs and (D) cell-free BALF 3 days after IAV infection were analyzed by ELISA. Data (mean ± SEM) were analyzed by Student *t* test. **P* < .05. (A: *N* = 9-14; B: *N* = 5; C: *N* = 9-14; D: *N* = 9-14). Abbreviations: ELISA, enzyme-linked immunosorbent assay; HAU, hemagglutination unit; mRNA, messenger RNA; PAR, protease-activated receptor; PCR, polymerase chain reaction; SEM, standard error of the mean

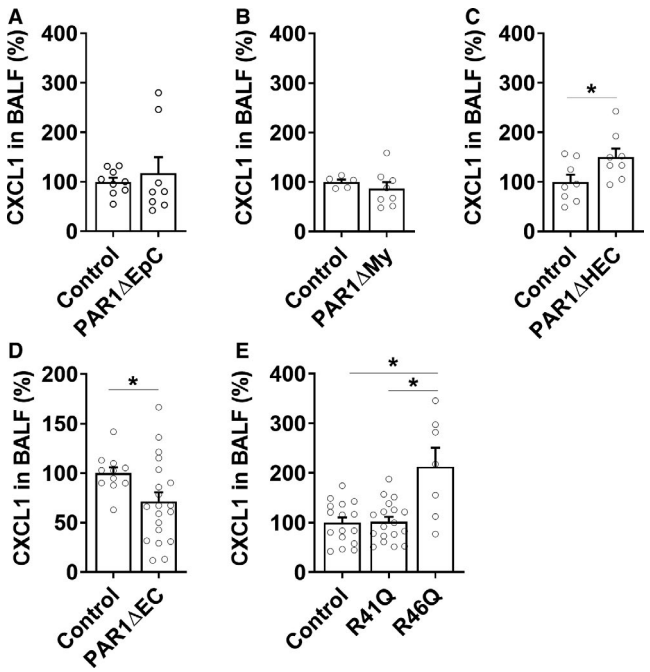

FIGURE 2 Effect of cell type-specific PAR1 deficiency or mutation of PAR1 on CXCL1 expression in mice after influenza A virus infection. Mice were infected with 0.02 HAU H1N1 influenza A virus (IAV). CXCL1 protein levels in bronchoalveolar lavage fluid (BALF) measured by ELISA in control mice (PAR1$^{fl/fl}$) or mice with cell type–specific deletion of PAR1 in (A) lung epithelial cells (PAR1ΔEpC), (B) myeloid cells (PAR1ΔMy), (C) hematopoietic and endothelial cells (PAR1ΔHEC), or (D) endothelial cells (PAR1ΔEC) 3 days after IAV infection. CXCL1 values for controls were set to 100%. (E) Wild-type (control, PAR1$^{+/+}$) and mice with PAR1 point-mutation at R41 (R41Q) or R46 (R46Q) were infected with IAV and BALF CXCL1 protein levels analyzed 3 days after IAV infection. Data (mean ± SEM) were analyzed by (A-D) Student *t* test or by (E) one-way ANOVA. * *P* < .05. (A: *N* = 8-9; C: *N* = 5-8; C: *N* = 8; D: *N* = 11-20; I: *N* = 7-18). Abbreviations: ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; HAU, hemagglutination unit; PAR, protease-activated receptor; SEM, standard error of the mean

indicate that a global deficiency of PAR1 leads to increased CXCL1 expression in the lung after IAV infection.

Next, we analyzed levels of CXCL1 protein in the BALF after IAV infection of mice lacking PAR1 in different cell types. Deletion of PAR1 in EpCs or myeloid cells did not change the level of CXCL1 expression (Figure 2A,B). Interestingly, deletion of PAR1 in hematopoietic cells and ECs was associated with increased CXCL1 expression, whereas deletion of PAR1 in ECs alone was associated with decreased CXCL1 expression after IAV infection (Figure 2C,D). These data indicate that PAR1 positively and negatively regulates CXCL1 expression in different cell types.

Finally, we analyzed the effect of mutation of PAR1 at either R41 or R46 on CXCL1 expression after IAV infection. There was no change in CXCL1 expression in IAV infected R41Q mice whereas infected R46Q mice exhibited a significant increase in CXCL1 expression compared with infected WT controls (Figure 2E). This result indicates that R46 cleavage of PAR1 suppresses CXCL1 expression during IAV infection.

3.2 | PAR1 activation enhances poly I:C induction of IL-8 expression in an endothelial cell line

Because we observed an increase in CXCL1 expression in mice with PAR1 deleted in ECs, we determined the effect of PAR1 activation of PAR1 on poly I:C induction of IL-8 in a human EC line. Importantly, PAR1 activation significantly increased poly I:C induction of IL-8 expression by EA.hy926 cells (Figure 3).

3.3 | PAR1 regulation of neutrophil recruitment to the lung in mice infected with IAV

We have shown increased levels of WBC in the lungs of mice 3 days after IAV infection.[31] Therefore, we measured the recruitment of inflammatory cells into the lungs of PAR1$^{-/-}$ and WT mice 3 days

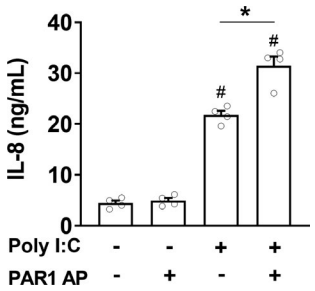

FIGURE 3 PAR1 stimulation increases IL-8 release from EA.hy926 cells. IL-8 protein levels in the media after stimulation with 5 μg/mL poly I:C and/or PAR1 agonist peptide (PAR1 AP, 100 μM) for 24 h. #$P < .05$ vs unstimulated control, *$P < .05$ ($N = 4$). Abbreviations: IL, interleukin; PAR, protease-activated receptor; poly I:C, polyinosinic:polycytidylic acid

after IAV infection. BALF from mice with a global deficiency of PAR1 had higher numbers of total WBC and neutrophils but not lymphocytes or monocytes compared with control mice after IAV infection (Figure 4). Analysis of BALF cellularity by cytospin also showed that PAR1$^{-/-}$ mice exhibited increased numbers of neutrophils in the BALF compared with controls after IAV infection (Appendix S1: Figure S1). These results indicate that PAR1 suppresses the recruitment of neutrophils after IAV infection.

Deletion of PAR1 in either EpC or myeloid cells did not affect levels of neutrophils, WBC, lymphocytes, and monocytes in the BALF after IAV infection (Figure 5A,B, Appendix S1: Figure S2). A deficiency of PAR1 in hematopoietic cells and ECs was associated with increased levels of neutrophils, WBC, and lymphocytes but not monocytes after IAV infection (Figure 5C, Appendix S1: Figure S2). In contrast, deletion of PAR1 in ECs only decreased neutrophils and WBC without changing lymphocytes or monocytes (Figure 5D, Appendix S1: Figure S2). These results indicate that PAR1 expression on different cell types regulates inflammatory cell recruitment to the lung after IAV infection.

Next, we measured the number of inflammatory cells in BALF in the cleavage-resistant PAR1 mutant mice. R41Q mice exhibited similar numbers of neutrophils, WBC, lymphocytes, and monocytes compared with controls after IAV infection (Figure 5E, Appendix S1: Figure S2). In contrast, a deficiency of R46-mediated signaling was associated with reduced numbers of neutrophils, WBC, and lymphocytes but not monocytes in BALF compared with controls after IAV infection (Figure 5E, Appendix S1: Figure S2). These results suggest that R46 signaling positively regulates neutrophil, WBC, and lymphocyte recruitment to the lung in mice infected with IAV.

3.4 | PAR1 regulation of protein levels in the lung in mice infected with IAV

Increased immune cell numbers are associated with increased tissue injury after IAV infection. We used levels of protein in the BALF as a marker of lung injury. We found that a global deficiency of PAR1 was associated with a significant increase in protein levels in BALF after IAV infection compared with infected controls (Figure 6A). Similarly, deletion of PAR1 in hematopoietic cells and ECs but not EpCs, myeloid cells, or ECs alone was associated with increased protein levels in BALF compared with infected controls (Figure 6B-E). These results indicate that PAR1 expression in a non-myeloid, hematopoietic cell reduces protein leakage after IAV infection. R46Q mice but not R41Q mice had decreased protein levels in BALF compared with controls after IAV infection (Figure 6F). This result suggests that R46 signaling contributes to protein leakage after IAV infection.

3.5 | Effect of PAR1 on survival after IAV infection

To investigate if PAR1 affects mortality after IAV infection, all mouse strains were infected with an IAV dose (0.02 HAU) that leads to ~ 20% mortality in control mice.[15,31,33] Mice with a global deficiency of PAR1 did not exhibit a change in weight loss but exhibited a significant increase in mortality after IAV infection compared with infected control mice (Figure 7A,B). This result indicates that PAR1 contributes to mortality after IAV infection. Deletion of PAR1 in the different cell types did not affect weight loss or mortality

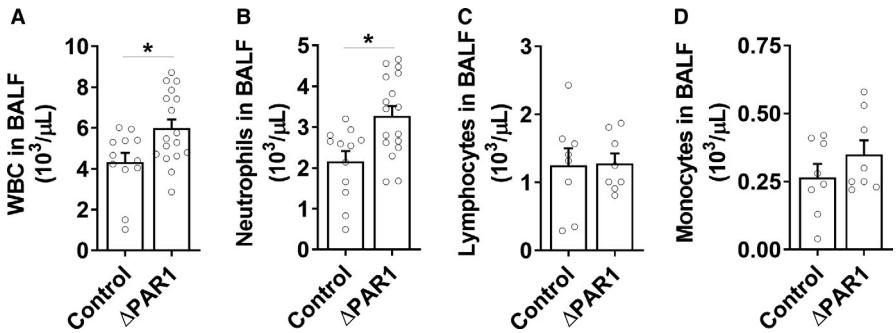

FIGURE 4 Effect of global PAR1 deficiency on immune cell recruitment to the lung after influenza A virus infection of mice. Mice with global PAR1 deficiency (ΔPAR1) or controls were infected with influenza A virus and levels of (A) total white blood cells (WBC), (B) neutrophils, (C) lymphocytes, and (D) monocytes in the bronchoalveolar lavage fluid (BALF) was measured by automated cell counting with HemoVet 3 days post infection. Data (mean ± SEM) were analyzed by Student *t* test. *$P < .05$. (A: $N = 12$-17; B: $N = 12$-17; C: $N = 8$; D: $N = 8$). Abbreviations: PAR, protease-activated receptor; p.i., ; SEM, standard error of the mean

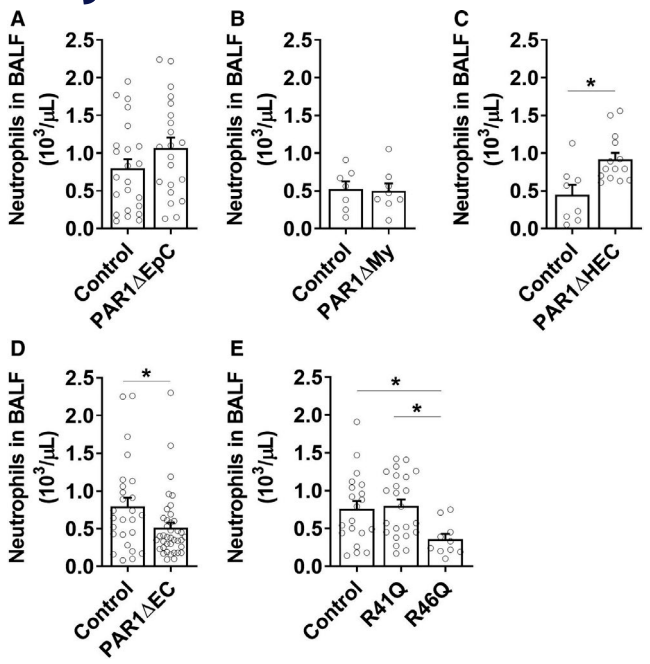

FIGURE 5 Effect of cell type-specific PAR1 deficiency or mutation of PAR1 on neutrophil recruitment to the lung after influenza A virus infection of mice. Mice with cell type-specific PAR1 deficiency or controls were infected with influenza A virus and levels of total white blood cell (WBC), neutrophils, lymphocytes, and monocytes in the bronchoalveolar lavage fluid (BALF) was measured by automated cell counting with HemoVet 3 days post infection. Data from mice with PAR1 deficiency in (A) lung epithelial cells (PAR1ΔEpC), (B) myeloid cells (PAR1ΔMy), (C) hematopoietic and endothelial cells (PAR1ΔHEC), (D) endothelial cells (PAR1ΔEC), or (E) mice with PAR1 point-mutation at R41 (R41Q) or R46 (R46Q) is shown. Data (mean ± SEM) were analyzed by (A-D) Student *t* test or (E) by one-way ANOVA. **P* < .05. (A: *N* = 21-23; B: *N* = 7-8; C: N = 8-14; D: N = 26-39; E: N = 10-24). Abbreviations: ANOVA, analysis of variance; PAR, protease-activated receptor; p.i., ; SEM, standard error of the mean

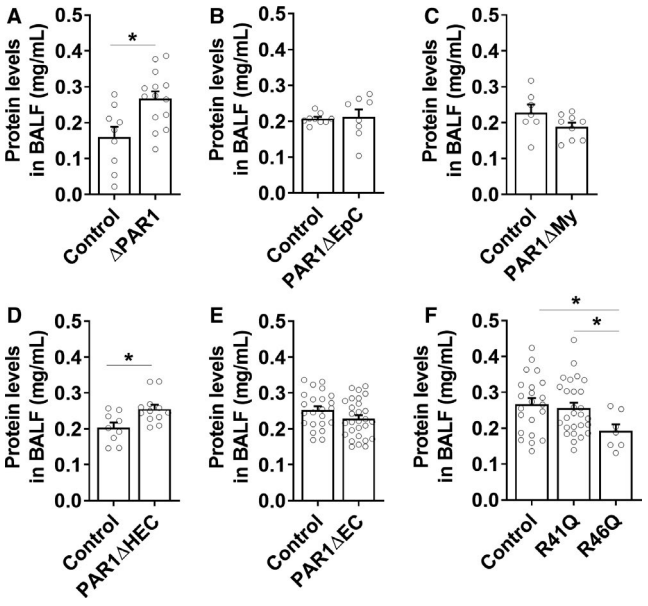

FIGURE 6 Effect of cell type-specific PAR1 deficiency or mutation on protein levels in mice after influenza A virus infection. (A) Mice with global PAR1 deficiency (ΔPAR1) mice or (B) PAR1 deficiency in lung epithelial cells (PAR1ΔEpC), (C) myeloid cells (PAR1ΔMy), (D) hematopoietic and endothelial cells (PAR1ΔHEC), (E) endothelial cells (PAR1ΔEC), or (F) mice with PAR1 point-mutation at R41 (R41Q) or R46 (R46Q) and controls were infected with influenza A (IAV). Protein levels in bronchoalveolar lavage fluid (BALF), as marker for tissue lung injury, was quantified 3 days after infection. Data (mean ± SEM) were analyzed by (A-E) Student *t* test or (F) by one-way ANOVA. **P* < .05. (A: *N* = 9-14; B: *N* = 8; C: *N* = 7-9; D: *N* = 9-12; E: N = 23-28; F: N = 7-27). Abbreviations: ANOVA, analysis of variance; PAR, protease-activated receptor; SEM, standard error of the mean

(Figure 7C-J). Similarly, mice expressing mutant versions of PAR1 (R41Q or R46Q) exhibited similar weight loss and survival after IAV infection compared with control mice (Figure 7K,L).

4 | DISCUSSION

Our study indicates that PAR1 plays a role in the innate immune response in mice infected with IAV. A global deficiency of PAR1 was associated with increased CXCL1 expression in the lungs of mice infected with IAV compared with controls. Similarly, in a previous study, we had found that a global deficiency of PAR1 was associated with increased CXLC1 expression in the spleen in response to intraperitoneal injection of poly I:C.[10,14] Deletion of PAR1 in hematopoietic cells and ECs, but not myeloid cells, and mutation of R46 led to increased CXCL1 expression after IAV infection. These data suggest that PAR1 activation via cleavage at R46 suppresses CXCL1 expression in a non-myeloid, hematopoietic cell during IAV infection. Currently, we do not know the specific cell type that expresses PAR1 and responds to IAV infection by expressing CXCL1. We have shown that PAR1 activation reduced poly I:C induction of CXCL1 in splenocytes.[14] TLR3-dependent induction of CXCL1 expression is primarily regulated by NFκB.[35,36] Importantly, APC activation of PAR1 has been shown to inhibit NFκB activation and TNF-α expression by monocytes.[24] In addition, we found that PAR1 activation suppressed LPS activation of NFκB in murine embryonic fibroblasts (data not shown). Taken together, these data suggest that APC activation of PAR1 via cleavage at R46 suppresses NFκB activation and CXCL1 expression in a hematopoietic cell type during IAV infection (Figure 8).

Surprisingly, deletion of PAR1 in ECs alone led to decreased CXCL1 expression in the lungs of mice infected with IAV, which suggested that PAR1 positively regulates CXCL1 expression in ECs. Indeed, we found that PAR1 activation enhanced poly I:C induction of IL-8 in EA.hy926 cells. In other studies, we showed that PAR1 enhancement of poly I:C induction of IL-8 expression was mediated by cross-activation of PAR2 (manuscript in preparation).[37] Importantly, PAR2 activation has been shown to enhance poly I:C activation of IL-8 in human epithelial cell lines.[12] A previous study showed that thrombin induction of phosphoinositide hydrolysis in human ECs was mediated by a PAR1/PAR2 complex.[38] Moreover, we found that

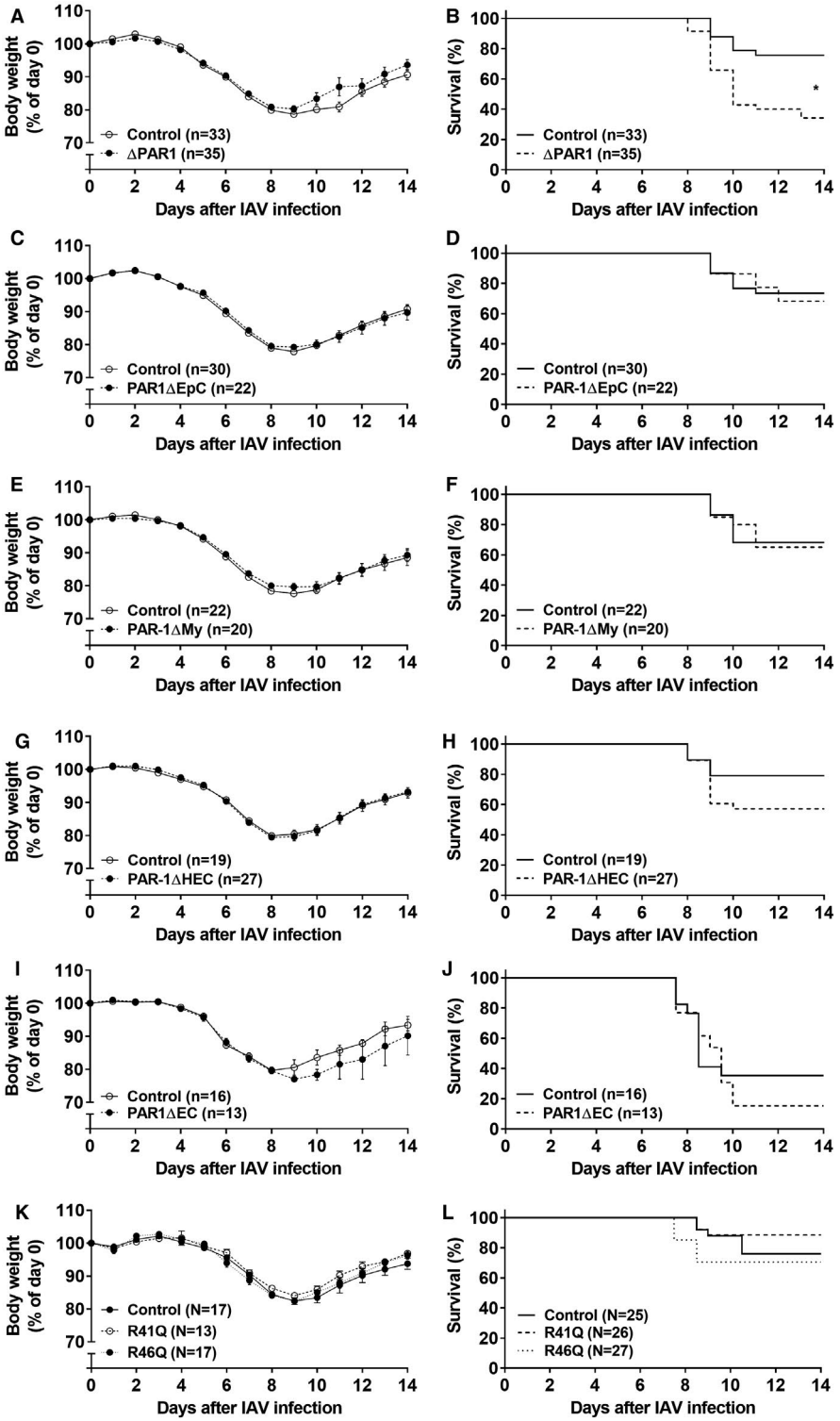

FIGURE 7 Effect of PAR1 deficiency or mutation of PAR1 on weight loss and mortality after influenza A virus infection. Mice of different genotypes were infected with 0.02 HAU IAV and (A, C, E, G, I) changes in body weights and (B, D, F, H, J) survival were measured over 14 days after infection. Results with mice with (A, B) global PAR1 deficiency (ΔPAR1) or (C, D) mice with PAR1 deficiency in lung epithelial cells (PAR1ΔEpC), (E, F) myeloid cells (PAR1ΔMy), (G, H) hematopoietic and endothelial cells (PAR1ΔHEC), (I, J) endothelial cells (PAR1ΔEC), or (K, L) mice with PAR1 point-mutation at R41 (R41Q) or R46 (R46Q) are shown. Body weights before infection were set to 100% and did not significantly differ between genotypes. Data (mean ± SEM) and calculated survival were analyzed by (A, C, E, G, I, K) two-way ANOVA and (B, D, F, H, J, L) log-rank test. *$P < .05$. Abbreviations: ANOVA, analysis of variance; PAR, protease-activated receptor; SEM, standard error of the mean

thrombin induction of plasminogen activator inhibitor 1 expression in 4T1 murine breast cancer cells required both PAR1 and PAR2.[39] These studies suggest that thrombin activation of a PAR1-PAR2 complex on ECs enhances CXCL1 expression during IAV infection (Figure 8).

A global deficiency of PAR1 and deletion of PAR1 in hematopoietic cells and ECs was associated with increased numbers of neutrophils in the lungs after IAV infection. These two transgenic lines also exhibited increased CXCL1 expression, suggesting that the increase in CXCL1 expression may be responsible for the increased recruitment of neutrophils into the lung after IAV infection. Conversely, deletion of PAR1 in ECs was associated with decreased levels of CXCL1 and neutrophils after IAV infection. R46 mice had increased levels of CXCL1 but decreased levels of neutrophils in the lungs after IAV infection. Therefore, levels of CXCL1 and neutrophils do not correlate in all the mouse lines suggesting that other

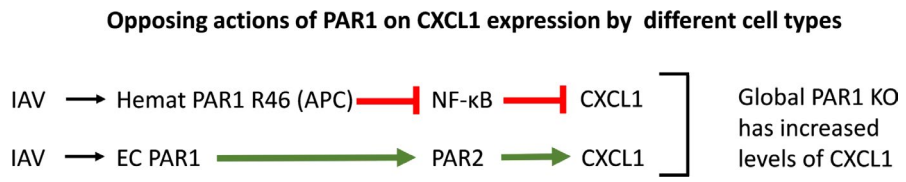

FIGURE 8 Cell-specific actions of PAR1 on CXCL1 bronchoalveolar lavage fluid (BALF) expression in influenza A virus infection. During influenza A virus (IAV) infection, activation of PAR1 at R46 on a hematopoietic cell (Hemat) leads to reduced NF-κB–dependent CXCL1 expression in the BALF. Activation of endothelial cell (EC) PAR1 enhances CXCL1 expression in the BALF via a PAR1-PAR2 heterodimer after IAV infection. Global PAR1 deficiency (PAR1 KO) in mice results in increased BALF CXCL1 expression after IAV infection. Abbreviation: PAR, protease-activated receptor

chemokines, such as CXCL2 and CXCL5, are involved in the recruitment of neutrophils to the lung during IAV infection.

Exaggerated inflammatory responses during IAV infection are typically associated with severe disease. Neutrophils are among the immune cells that can drive this excessive and detrimental inflammation.[40] IAV infection causes loss of EpC, local inflammation, and EC activation, all of which lead to endothelial and epithelial permeability causing leakage of plasma proteins into alveolae. These effects are caused either directly by the virus or by the recruited immune cells. We found that tissue injury measured as protein levels in BALF was associated with increased BALF cellularity in $PAR1^{-/-}$ and mice with a PAR1 deletion in hematopoietic and EC after IAV infection, which may be due, in part, to increased levels of neutrophils. In contrast, R46 mice had decreased neutrophils and decreased cell injury. Based on the EC barrier protective role of R46, one would have expected that R46 mice would have increased protein leakage. $PAR1^{-/-}$ mice exhibited increased mortality compared to controls, whereas deletion of PAR1 in different cell types or mutation of PAR1 did not affect survival. At present, we do not know the factors that cause the increased mortality of $PAR1^{-/-}$ mice.

Our studies indicate that PAR1 modulates the immune response to IAV infection. The role of PAR1 is complex. PAR1 negatively regulates CXCL1 expression by hematopoietic cells, possibly via APC cleavage at R46, whereas it positively regulates CXCL1 expression in ECs, possibly via cross-activation of PAR2. Further studies are required to elucidate the role of PAR1 in the innated immune response.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Silvio Antoniak, Kohei Tatsumi, Clare M. Schmedes, Grant J. Egnatz, Alyson C. Auriemma, Vanthana Bharath, and Tracy Stokol performed the study and collected data. Silvio Antoniak and Nigel Mackman conceived and designed the study. Melinda A. Beck, John H. Griffin, and Joseph S. Palumbo provided essential material. Silvio Antoniak and Nigel Mackman wrote the paper in consultation with Tracy Stokol. Nigel Mackman provided funding and supervised the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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