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Peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (PIN1) regulates pulmonary effects of endotoxin and tumor necrosis factor-α in mice

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ABSTRACT

Peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (PIN1) modulates phospho-signaling by catalyzing rotation of the bond between a phosphorylated serine or threonine before proline in proteins. As depletion of PIN1 increased inflammatory protein expression in cultured endothelial cells treated with bacterial endotoxin (lipopolysaccharide, LPS) and interferon-γ, we hypothesized that PIN1 knockout would increase sensitivity to LPS-induced lung inflammation in mice. Mortality due to a high dose of LPS (30 mg/kg) was greater in knockout than wildtype mice. Lung myeloperoxidase activity, reflecting neutrophils, was increased to a 35% higher level in PIN1 knockout mouse lung, as compared with wildtype, after treatment with a sublethal dose of 3 mg LPS/kg, ip. Unexpectedly, plasma tumor necrosis factor-α (TNF) was approximately 50% less than in wildtype mice. Knockout mice, however, were more sensitive than wildtype to TNF-induced neutrophil accumulation. The neutrophil adhesion molecule, E-selectin, was also elevated in lungs of knockout mice treated with TNF, suggesting that PIN1 depletion increases endothelial sensitivity to TNF. Indeed, TNF induced more reactive oxygen species in cultured endothelial cells depleted of PIN1 with short hairpin RNA than in control cells. Collectively, the results indicate that while PIN1 normally facilitates TNF production in LPS-treated mice, it suppresses pulmonary and endothelial reactions to the cytokine. Tissue or cell-specific effects of PIN1 may affect the overall inflammatory response to LPS and other stimuli.

1. Introduction

Gram-negative bacterial endotoxin (lipopolysaccharide, LPS) is responsible for life-threatening inflammatory responses in several organ systems. Lungs are particularly sensitive to LPS where it causes edema and sequestration of neutrophils which contribute to injury [1]. Intraperitoneal (ip) injection of LPS causes a reproducible course of production of cytokines, with tumor necrosis factor-α (TNF) elevation within 2 h in mice [2]. TNF contributes significantly to neutrophil accumulation caused by LPS, as knocking out TNF receptor I, but not TNF receptor II, reduces lung neutrophils by about 50% in LPS-treated mice [3–5].

Peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (PIN1) regulates signaling by acting on proteins that are phosphorylated (p) at serine (S) or threonine (T) preceding proline (P) [6,7]. The enzyme facilitates rotation of the bond between p(S/T) and P. The conformational flexibility conferred by PIN1 impacts protein–protein interaction, subcellular localization and the susceptibility of proteins to various enzymes [6–14]. PIN1 modulates numerous signaling paths involving S/T-P phosphorylation.

Previous studies indicated that PIN1 increases expression or activity of the pro-inflammatory transcription factors AP-1, β-catenin and NFκB, suggesting that it may facilitate inflammatory gene activation [9,11,12]. However, PIN1 regulates expression and function of many proteins. For example, PIN1 antagonizes the calpain inhibitor calpastatin, such that elimination of PIN1 lowers calpain activity, leading to large increases in induction of the

Abbreviations: LPS, *E. coli* endotoxin or lipopolysaccharide; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1yl]ethanesulfonic acid; IFN, Interferon-γ; MPO, myeloperoxidase; PIN1, peptidylprolyl cis/trans isomerase, NIMA-interacting 1; PBS, phosphate-buffered saline; p, phosphorylated; −/−, PIN1 knockout; S, serine; shRNA, small hairpin RNA; T, threonine; TNF, tumor necrosis factor-α; P, proline;

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calpain substrates, inducible nitric oxide synthase and cyclooxygenase-2 in endothelial cells treated with *Escherichia coli* LPS and interferon-γ (IFN) [15–17].

The widespread role of pS/T-P in signaling likely contributes to context-dependent actions that vary due to different sets of PIN1 substrates and their phosphorylation in different cells, tissues or organisms [13,18,19]. For these reasons, consequences of inhibiting or depleting PIN1 *in vivo* are likely to be unpredictable. Despite the complexity, PIN1 may prove to be a useful target to manipulate inflammatory responses in specific conditions or in specific individuals [20].

While PIN1 knockout reduced survival after treatment with high doses of LPS in mice of a mixed genetic background relative to wildtype mice [21], its role in the pulmonary response to sublethal LPS is unknown. Here we investigated lung inflammation in PIN1 knockout mice of a homogenous, LPS-sensitive C57Bl/6 murine strain, and in cultured C57Bl/6 endothelial cells.

2. Materials and methods

2.1. Materials

Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT). Dulbecco's minimum essential medium, F-12 Nutrient Mixture, 0.25% trypsin/1 mM EDTA, phosphate-buffered saline (PBS), 2-[4-(2-hydroxyethyl)piperazin-1yl]ethanesulfonic acid (HEPES) penicillin/streptomycin solution (200 U penicillin and 200 μg streptomycin per mL), and Tris–glycine gels and dihydroethidium were from Invitrogen Corporation (Grand Island, NY). Bradford reagent, heparin, phenylmethylsulfonyl fluoride, dithiothreitol, β-mercaptoethanol, calcium chloride, hexadecyltrimethylammonium bromide, *o*-dianisidine and *E. coli* LPS, serotype 0111:B4, were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies against E-selectin (CD62E) and TNF receptor 1 were obtained from Abcam (Cambridge, MA). Murine TNF and anti-PIN1 were from R&D Systems (Minneapolis, MN). A sandwich enzyme-linked immunoassay kit for murine TNF was obtained from BD Biosciences (San Jose, CA). Enhanced chemiluminescence reagents and triton X-100 were from Pierce (Rockford, IL). Ethylenediamine tetraacetic acid, hydrogen peroxide, methanol, sodium dodecyl sulfate, NaCl, Na_3VO_4, NaF, Tween 20, and Tris-base were obtained from Fisher Scientific (Fair Lawn, NJ). Goat anti-mouse and -rabbit antibodies conjugated with horseradish peroxidase were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA) and Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Mice and treatments

Mice bred to a homogenous C57Bl/6 background [22] were used in accordance with the Guide for the Care and Use of Laboratory Animals from the U.S. National Institutes of Health. Mice were injected ip with PBS, 3 or 30 mg LPS/kg and harvested after 2 or 6 h. Mice were treated ip with PBS or 57 μg TNF/kg in PBS for 4 h. Mice were euthanized and blood was collected via the right cardiac ventricle and plasma was isolated and stored frozen. Lungs were removed and frozen until use.

2.3. Myeloperoxidase assay

Lung myeloperoxidase (MPO) activity was measured in supernatants of samples homogenized in 50 mM potassium phosphate/5 mM EDTA/0.5% hexadecyltrimethylammonium bromide and centrifuged at 12,000×*g* for 10 min [23]. Protein was measured [24], and MPO activity was determined as the rate of increase in A460 due to oxidation of 0.5 mM *o*-dianisidine by equal amounts of supernatant protein with 1.5 mM hydrogen peroxide [23].

2.4. Western blotting

Lung samples were homogenized and sonicated in RIPA lysis buffer with protease inhibitors, and protein was again measured. After denaturing, electrophoresis and transfer to nitrocellulose, a primary antibody and horseradish peroxidase-conjugated secondary antibody with enhanced chemiluminescence was used to expose film. Digital images were produced with a transilluminating scanner. The integrated signal intensity was determined with NIH Image J software, and divided by value for α-tubulin in each sample, as described previously [2].

2.5. TNF assay

TNF was measured in plasma from blood collected 2 h after treatment with 3 mg LPS/kg by sandwich enzyme-linked immunoassay as described previously [2].

2.6. Cells

Endothelial cells were cultured from aortas of mice [25]. As described previously, cells were transduced with short hairpin RNA (shRNA) to knockdown PIN1 or with an inactive mutant sequence, and selected for stable modification [15–17].

2.7. Oxidant production in endothelial cells

The endothelial cells containing or lacking PIN1 were cultured on coverglass, then rinsed 3 times with HEPES-buffered saline and then incubated in HEPES-buffered saline/0.5% FBS containing 10 μM dihydroethidium, and LPS or TNF. Nuclear fluorescence of ethidium resulting from oxidation was assessed with green excitation and red emission by microscopy and image analysis [26,27].

2.8. Data analysis

Student's *t*-test or analysis of variance with correction for multiple comparisons were used to analyze results [28].

3. Results

To better understand the role of PIN1 in the LPS-induced pulmonary inflammatory response, +/+ and PIN1 −/− mice were treated with low or high doses of LPS. A dose of 30 mg/kg killed 80% of PIN1 −/− mice but only 20% of +/+ mice after 24 h. All +/+ and PIN1 −/− mice survived a dose of 3 mg/kg. Activation and recruitment of neutrophils in response to LPS characterize the pulmonary inflammation and contribute to acute lung injury. Here, MPO activity was measured to indicate LPS-induced neutrophil accumulation in the lungs. Lung MPO activity increased in both +/+ and PIN1 −/− mice in response to LPS, although a 35% higher level was observed in −/− mice (Fig. 1). In contrast, plasma TNF, which induces neutrophil activation and pulmonary accumulation, increased to 15.5 ± 1.1 ng/mL in +/+, but only to 7.9 ± 0.4 ng/mL in −/− mice 2 h after treatment (mean ± SE, $n = 8–10$, $p < 0.05$).

Given the elevation in lung neutrophils, despite far lower plasma TNF levels in the LPS-treated −/− mice, the response to ip TNF was determined. MPO increased by 75% in lungs of +/+ mice, but there was a greater increase of 165% in PIN1 −/− mice 4 h after injection of TNF (Fig. 2). Moreover, E-selectin, which is expressed by endothelial cells and contributes to neutrophil adhesion, was greater in PIN1 −/− mice, and significantly increased by TNF

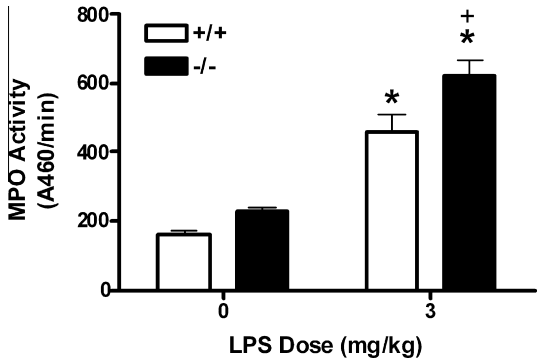

Fig. 1. LPS-induced accumulation of neutrophils in lungs of wildtype (+/+) and PIN1 knockout (−/−) mice. Mice were treated with Saline or LPS, ip. MPO activity in lung was determined 6 h after LPS treatment. Bars are the mean MPO activity (A460/min) + SE. $^{*}p < 0.05$ for comparison with 0 mg LPS/kg and $^{+}p < 0.05$ for comparison with +/+ mice treated the same way.

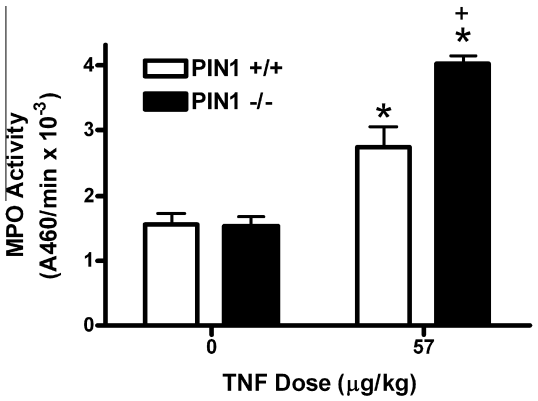

Fig. 2. Effect of TNF on accumulation of neutrophils in lungs of +/+ and −/− mice. Mice were treated with Saline or TNF, ip. MPO activity in lung was determined 4 h later. Bars are the mean activity (A460/min) + SE. $^{*}p < 0.05$ for comparison with 0 μg TNF/kg and $^{+}p < 0.05$ for comparison with +/+ mice treated the same way.

(Fig. 3A and B). Interestingly, TNF Receptor 1 expression did not differ between saline-treated +/+ and PIN1 −/− mice, but increased in −/− mice treated with TNF (Fig. 3).

Lastly, to determine the role of PIN1 in the endothelial response to TNF, cells depleted of PIN1 via shRNA were treated with varying concentrations of TNF or LPS (Fig. 4). Dihydroethidium oxidation revealed that TNF caused the production of more oxidants in cells lacking PIN1 than in control cells. However, LPS induced reactive oxygen species similarly in knockdown and control cells. Overall, the results indicate that PIN1 knockout increased the sensitivity of mice and endothelial cells to TNF.

4. Discussion

PIN1 facilitates the expression and activity of several transcription factors that could affect inflammatory gene expression. For example, PIN1 antagonized NFκB p65 association with IkB, resulting in increased activity. PIN1 inhibited ubiquitination and degradation of p65 in murine embryonic fibroblasts, promoted the activity and expression of ß-catenin and AP-1 in cancer cell lines and NIH 3T3 cells, and supported NFκB activation by TNF in liver of wildtype mice in comparison with PIN1 −/− mice of mixed genetic background [9,11,12]. Based on these findings, we

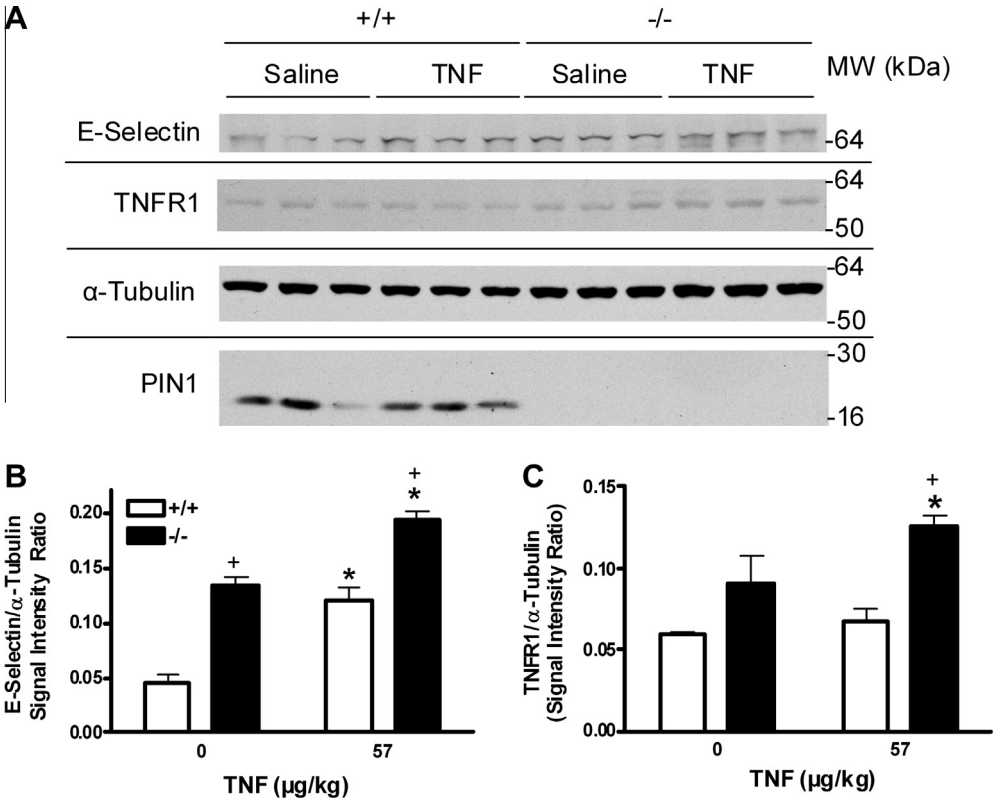

Fig. 3. Effect of PIN1 knockout and TNF on E-selectin and TNFR1 in lungs of wildtype and knockout mice. Mice were treated as in Fig. 2, then protein was extracted from lungs and subjected to Western blotting (A) for E-selectin (67 kDa), TNFR1 (55 kDa), α-Tubulin (52 kDa) and PIN1 (18 kDa), and image analysis (B, C). $^{*}p < 0.05$ for comparison with 0 μg TNF/kg and $^{+}p < 0.05$ for comparison with +/+ mice treated the same way.

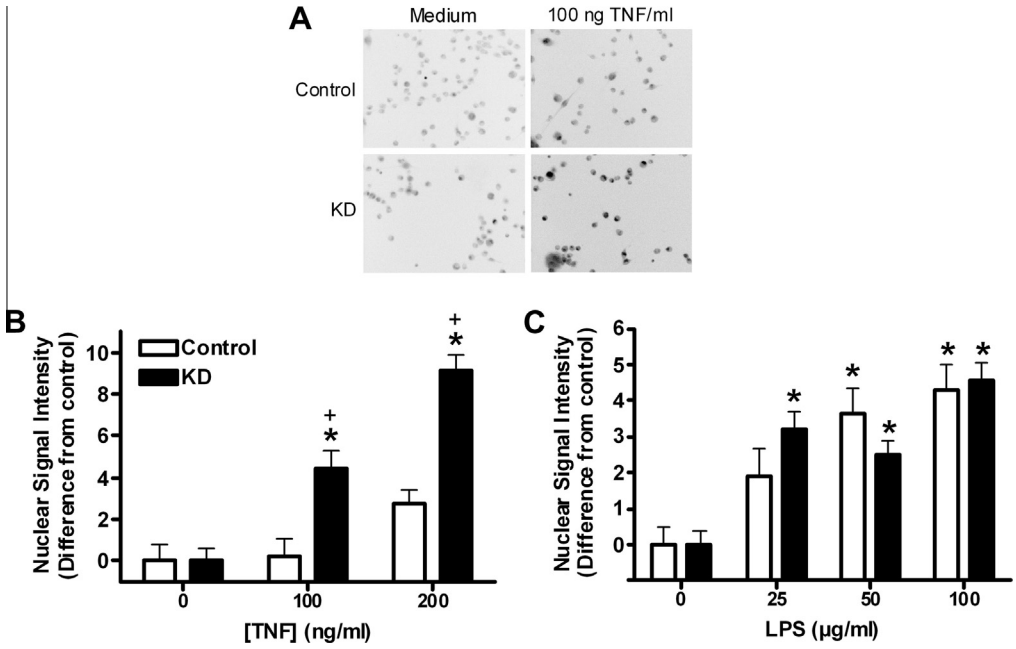

Fig. 4. Effect of PIN1 depletion on TNF and LPS-induced reactive oxygen species in endothelial cells. Cells harboring Control or PIN1 shRNA (KD) were grown on coverslips and treated with the indicated concentrations of TNF (representative images (A) and analysis (B)) or LPS (C) for 30 min or 2 h, respectively, in the presence of dihydroethidium. Bars are the mean difference in nuclear signal intensity between TNF or LPS-treated and medium-treated (0) cells + SE of the difference. $^{*}p < 0.05$ for comparison with 0 TNF or LPS, and $^{+}p < 0.05$ for comparison with +/+ mice treated the same way.

expected that PIN1 depletion would reduce LPS-induced lung inflammation.

More C57Bl/6 PIN1 −/− mice were killed within 24 h of a high (30 mg/kg) dose of LPS than +/+ mice, however. This is consistent with another study showing that PIN1 knockout reduced survival in mice of mixed genetic background 100 h after treatment with 10 mg/kg of LPS (*E. coli* 0111:B4). Interestingly, the authors qualitatively indicated that lung injury was more severe in a PIN1 −/− mouse that survived 100 h, compared with surviving wildtype mice [21].

The impact of PIN1 knockout on lung inflammation and plasma TNF induced by a low, sublethal dose of LPS (3 mg/kg) was assessed in order to study them in the absence of mortality. TNF was measured as it is a major mediator of pulmonary neutrophil accumulation [3–5,29]. Neutrophil accumulation was slightly, but significantly, more pronounced in −/− mouse lung (Fig. 1), but plasma TNF was only about half the level of wildtype. Interestingly, PIN1 knockout did not affect induction of TNF mRNA in peritoneal macrophages taken from mice of mixed genetic background that were treated with LPS *in vitro* [21]. Furthermore, Barberi et al. found that ip injection of 15 mg LPS/kg induced similar plasma TNF after 3 h in the C57Bl/6 PIN1 −/− and +/+ mice that were used here [30]. Taken together, PIN1 deletion does not appear to increase TNF production preceding LPS-induced lung inflammation. Since pulmonary neutrophil accumulation was increased by LPS in −/− mice, it was possible that they might be much more sensitive to TNF. This appears to be the case since TNF injected ip caused greater accumulation of neutrophils in PIN1 −/− mice compared with wildtype (Fig. 2).

TNF affects vascular endothelium as part of inflammatory activation. It induces E-selectin in isolated perfused lungs and elevates hydrogen peroxide production by endothelial cells [31]. Here, E-selectin was increased in lungs of PIN1 −/−, mice compared with +/+ mice and was increased to a higher level by TNF (Fig. 3). The fact that expression of E-selectin is normally confined to activated endothelial cells [31–33] suggests that the lung endothelium was sensitized by PIN1 knockout. Supporting this notion, endothelial cell culture studies demonstrated that depletion of PIN1 with shRNA increased production of reactive oxygen species in response to TNF, but not LPS (Fig. 4).

TNF Receptor I is the main receptor that stimulates neutrophil adherence to endothelial cells and pulmonary neutrophil sequestration in LPS-treated mice [3,5,29]. We expected that PIN1 knockout might increase this receptor in lung, but it was not altered in untreated mice. However, TNF enhanced TNF Receptor I expression in knockout mouse lung, which might further promote inflammation after treatment. It is also possible that PIN1 knockout increases sensitivity of mice, as well as endothelial and non-endothelial cells, to TNF receptor activation.

Others indicated that PIN1 is required for, rather than a suppressor of induction of reactive oxygen species via NADPH oxidase in TNF-treated neutrophils [34]. PIN1 associated with the p47phox subunit of NADPH oxidase, and oxidant production was reduced by the PIN1 inhibitor, juglone. However, juglone can affect other enzymes, including protein kinase C, which stimulates NADPH oxidase activation in neutrophils [34–39]. PIN1 can also enhance oxidant defense by stimulating peroxiredoxin activity by association with the enzyme. As a result, hydrogen peroxide production was elevated in PIN1 −/− mouse embryonic fibroblasts [40].

The effect of PIN1 on TNF-induced oxidants in endothelial or other cells could depend on phosphorylation level, relative PIN1-sensitivty and the inherent balance between counter-acting systems, such as NADPH oxidase and peroxiredoxin, in different cell types. Furthermore, PIN1 modulates synthesis, degradation, interactions and function of numerous phosphoproteins [7,8,15–17], and there are many potential S/T-P motifs in proteins in the TNF signaling path [41,42]. The effect of PIN1 on TNF signaling, and on production and removal of reactive oxygen species in endothelial cells is yet to be determined.

In conclusion, while PIN1 depletion reduced TNF production in response to LPS, sensitivity of mice to TNF-induced lung inflammation and the sensitivity of endothelial cells to TNF were increased. PIN1 may normally limit the sensitivity of lungs of C57Bl/6 mice to inflammatory actions of TNF, while facilitating production of

the cytokine in response to LPS. The results suggest that manipulation of PIN1 has cell or tissue-specific effects in mice.

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