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Recombinant Human Proteoglycan-4 Mediates Interleukin-6 Response in Both Human and Mouse Endothelial Cells Induced Into a Sepsis Phenotype

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Objectives: Sepsis is a leading cause of death in the United States. Putative targets to prevent systemic inflammatory response syndrome include antagonism of toll-like receptors 2 and 4 and CD44 receptors in vascular endothelial cells. Proteoglycan-4 is a mucinous glycoprotein that interacts with CD44 and toll-like receptor 4 resulting in a blockade of the NOD-like receptor pyrin domain-containing-3 pathway. We hypothesized that endothelial cells induced into a sepsis phenotype would have less interleukin-6 expression after recombinant human proteoglycan 4 treatment in vitro.

Design: Enzyme-linked immunosorbent assay and reverse transcriptase-quantitative polymerase chain reaction to measure interleukin-6 protein and gene expression.

Setting: Research laboratory.

Subjects: Human umbilical vascular endothelial cells, human lung microvascular endothelial cells, and transgenic mouse (wild type) (*Cd44*$^{+/+}$/*Prg4*$^{+/+}$), *Cd44*$^{-/-}$ (*Cd44*^{tm1Hbg}*Prg4*$^{+/+}$), *Prg4*$^{GT/GT}$ (*Cd44*$^{+/+}$ *Prg4*$^{tm2Mawa/J}$), and double knockout (*Cd44*^{tm1Hbg} *Prg4*$^{tm2Mawa/J}$) lung microvascular endothelial cells.

Interventions: Cells were treated with 100 or 250 ng/mL lipopolysaccharide-*Escherichia coli* K12 and subsequently treated with recombinant human proteoglycan 4 after 30 minutes. Interleukin-6 levels in conditioned media were measured via enzyme-linked immunosorbent assay and gene expression was measured via reverse transcriptase-quantitative polymerase chain reaction with ΔΔ−Ct analysis. Additionally, human umbilical vascular endothelial cells and human lung microvascular endothelial cells were treated with 1:10 diluted plasma from 15 patients with sepsis in culture media. After 30 minutes, either 50 or 100 μg/mL recombinant human proteoglycan 4 was administered. Interleukin-6 protein and gene expression were assayed. Proteoglycan 4 levels were also compared between control and sepsis patient plasma.

Measurements and Main Results: Human umbilical vascular endothelial cell, human lung microvascular endothelial cell, and mouse lung microvascular endothelial cell treated with lipopolysaccharide had significantly increased interleukin-6 protein compared with controls. Recombinant human proteoglycan-4 significantly reduced interleukin-6 in human and mouse endothelial cells. Interleukin-6 gene expression was significantly increased after lipopolysaccharide treatment compared with controls. This response was reversed by 50 or 100 μg/mL recombinant human proteoglycan-4 in 80% of sepsis samples in human umbilical vascular endothelial cells and in 60–73% in human lung microvascular endothelial cells. In *Cd44*$^{-/-}$ genotypes of the mouse lung microvascular endothelial cells, recombinant human proteoglycan-4 significantly reduced interleukin-6 protein levels after lipopolysaccharide treatment, indicating that *Cd44* is not needed for recombinant human proteoglycan-4 to have an effect in a toll-like receptor 4 agonist inflammation model. Patient sepsis samples had higher plasma levels of native proteoglycan-4 than controls.

Interpretation and Conclusions: Recombinant human proteoglycan-4 is a potential adjunct therapy for sepsis patients and warrants future in vivo model studies.

Key Words: CD44; cytokines; inflammation; proteoglycan-4; sepsis; toll-like receptors

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Sepsis is the body's immune response to infection in major organs including the lung, urinary tract, blood, and skin structures. More than 1.5 million people are diagnosed with sepsis in the United States every year and roughly 250,000 of those patients die due to multiple organ failure with a mortality rate around 33% (1). Antibiotics treat the initiating cause of sepsis but effective treatments intended to blunt immune cell dysfunction are needed.

The body's immune response during sepsis is extensive and includes the up-regulation of pro-inflammatory cytokines, caspases, C-reactive protein, procalcitonin, and transcription factors (2, 3). One of the commonly used biomarkers in sepsis is the cytokine interleukin (IL)-6, which is thought to be indicative of the most severe cases of sepsis and is upregulated in inflammatory cellular pathways (4). IL-6 is a glycoprotein that is released from many different cell types during the immune reaction in response to pathogen-associated molecular patterns or damage-associated molecular patterns (5–8). Because it is noted as a severe sepsis biomarker (4, 8), we chose to focus on the IL-6 response in the current study. At present, there is still no effective treatment that is able to target and counteract the intense immune response during sepsis, making the condition difficult to treat and stabilize (9). Previously the toll-like receptor (TLR)-4 antagonist resatorvid Tak242, a cyclohexene derivative, showed promise in a phase 2 trial (NCT00143611) in patients with acute sepsis but was unable to advance to phase 3 due to iatrogenic methemoglobinemia caused by this small molecule (10).

Lubricin (proteoglycan-4 [PRG4]; genebank number NM_005807) is a mucin-like 224 kDa glycoprotein originally found as a lubricating substance within the synovial fluid of diarthrodial joints (11–15). More recently, it has been found in other tissues including lung, liver, brain, heart, bladder, bone, eye, uterus, cervix, and prostate indicating that it serves a multifunctional role (16–18). Lubricin has recently been implicated as an anti-inflammatory mediator in innate immunity pathways (19–21), and CD44 (21–23) has been conceptualized to play a role in its entry into the cytoplasm thereby pointing to its role as a potential adjunct treatment in sepsis. Full-length recombinant human PRG4 (rhPRG4) has been used in limited clinical trials in xerophthalmia and no adverse effects were recorded (24, 25).

Lipopolysaccharide, a potent agonist of the TLRs, was used in this work to recapitulate inflammatory triggers in vitro in endothelial cells that are observed in sepsis (26). We used both lipopolysaccharide and plasma from culture-negative and culture-positive sepsis patients to initiate a strong IL-6 response in human umbilical vascular endothelial cells (HUVECs) and human lung microvascular endothelial cells (HLMVECs). Lipopolysaccharide was also used to treat transgenic mouse lung microvascular endothelial cells (MLMVEC) that were *Cd44* sufficient or null. After lipopolysaccharide or patient plasma treatment, cell culture samples were treated with rhPRG4 in order to determine if IL-6 gene expression and protein levels were altered. The results of the current study indicate that rhPRG4 is a potential therapeutic that can be used to reverse the inflammatory response commonly seen in sepsis. Additionally, using transgenic MLMVECs, we show that rhPRG4 reduced IL-6 levels in both the presence and absence of the CD44 receptor and endogenous Prg4, indicating that CD44 may not be required in facilitating anti-inflammatory activity in these cells, especially in regard to TLR4 ligands. Lastly, PRG4 levels in sepsis patient plasma were assayed and compared with controls.

MATERIALS AND METHODS

Patient Samples

Patient plasma samples were obtained from Sepsis, [Extracorporeal Membrane Oxygentation], and [Acute Respiratory Distress Syndrome] biobank patients at Rhode Island Hospital under institutional review board protocol number 4116-16 and used to treat both HUVEC and HLMVEC. Patient demographics are shown in **Supplementary Table 1** (Supplemental Digital Content 1, http://links.lww.com/CCX/A181).

Supplementary Materials and Methods (Supplemental Digital Content 2, http://links.lww.com/CCX/A182) provide additional information.

RESULTS

Enzyme-Linked Immunosorbent Assay IL-6 Protein Concentrations

HUVEC Culture—Lipopolysaccharide Treatment. Media from untreated control HUVECs had baseline levels of IL-6 protein of 60.6 ± 1.0 pg/mL. Lipopolysaccharide treatment increased IL-6 protein levels to 671.6 ± 53.39 pg/mL and was significantly higher from media untreated controls ($p < 0.001$) (**Fig. 1*A***). IL-6 protein levels were significantly reduced by 5–150 μg/mL rhPRG4 30 minutes after cells were treated with lipopolysaccharide. After lipopolysaccharide treatment, 5 μg/mL rhPRG4 reduced IL-6 levels 32% to 459.9 ± 75.18 pg/mL ($p < 0.01$), 10 μg/mL rhPRG4 reduced IL-6 levels 48% to 350.0 ± 22.0 pg/mL ($p < 0.001$), 25 μg/mL rhPRG4 reduced IL-6 levels 88% to 77.7 ± 2.7 pg/mL ($p < 0.001$), 50 μg/mL rhPRG4 reduced IL-6 levels 91% to 63.84 ± 1.4 pg/mL ($p < 0.001$), 100 μg/mL rhPRG4 reduced IL-6 levels 94% to 43.1 ± 1.1 pg/mL ($p < 0.001$), and 150 μg/mL rhPRG4 reduced IL-6 levels 84% to 106.7 ± 15.8 pg/mL ($p < 0.001$). Cells treated with 25 or 150 μg/mL rhPRG4 alone had IL-6 levels that were not statistically different from untreated controls.

HLMVEC Culture—Lipopolysaccharide Treatment. Media from untreated control HLMVECs had baseline levels of IL-6 that were 277.9 ± 23.3 pg/mL. Lipopolysaccharide treatment increased IL-6 levels to 2,733.5 ± 85.4 pg/mL which was significantly higher compared with media from untreated controls ($p < 0.001$) (**Fig. 1*B***). After 30 minutes of lipopolysaccharide treatment 25, 50, and 100 μg/mL rhPRG4 reduced IL-6 levels 79% to 566.8 ± 26.2, 87% to 349.0 ± 15.7, and 91% to 242.0 ± 10.3 pg/mL ($p < 0.001$ for all when compared with lipopolysaccharide). Cells treated with 150 μg/mL rhPRG4 alone had IL-6 levels that were not significantly different from untreated controls.

HUVEC Culture—Patient Plasma Treatment. Eighty percent of sepsis patient plasma samples lowered IL-6 levels significantly following treatment with 50 μg/mL rhPRG4 ($p < 0.05$) (**Fig. 2**). As a positive control, lipopolysaccharide significantly increased IL-6 levels to 385.7 ± 18.9 pg/mL from 13.6 ± 4.0 pg/mL media levels ($p < 0.001$) which was reversed with rhPRG4 treatment 97% to 10.3 ± 2.5 pg/mL ($p < 0.001$) (data not shown). IL-6 values shown in Figure 2 were normalized by subtracting background levels of IL-6 from patient plasma.

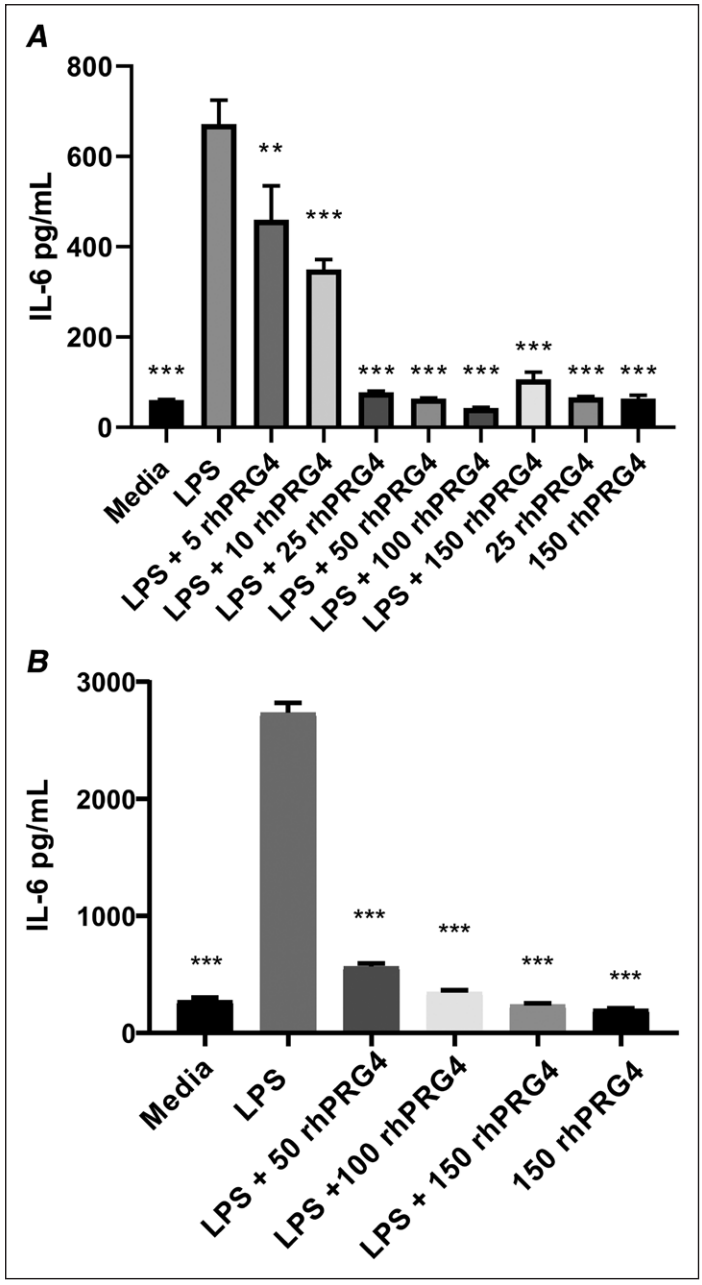

Figure 1. Concentration-dependent effect of recombinant human proteoglycan-4 (rhPRG4) on interleukin (IL)-6 production in lipopolysaccharide (LPS) stimulated human umbilical vascular endothelial cells (HUVEC) and human lung microvascular endothelial cells (HLMVEC). IL-6 protein concentrations measured via enzyme-linked immunosorbent assay after LPS followed by rhPRG4 treatment in HUVECs (**A**) and HLMVECs (**B**). Cells were treated with 250 ng/mL LPS for 30 min prior to rhPRG4 treatment for 23.5 hr. Data presented are mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; all groups compared with LPS IL-6 values using analysis of variance with Dunnett post hoc comparison. NS = not significant.

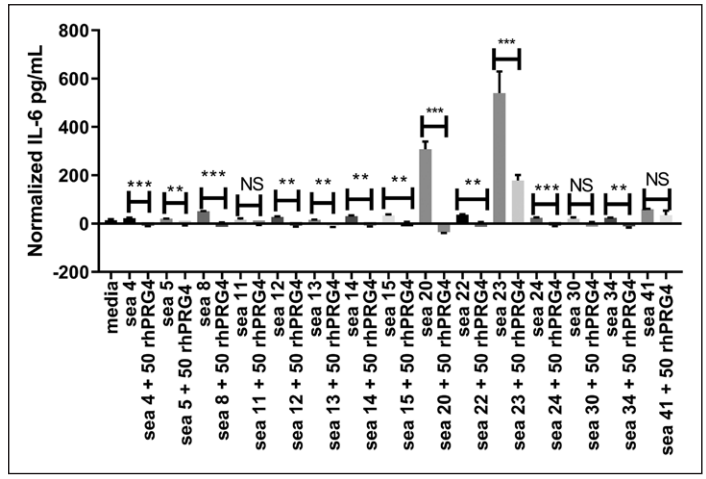

Figure 2. Patient sepsis samples used in human umbilical vascular endothelial cell (HUVEC) culture. HUVEC culture treated with 100 µL of patient plasma and 900 µL media prior to 50 µg/mL recombinant human proteoglycan-4 (rhPRG4) treatment for 23.5 hr. Data from patient samples represent normalized interleukin (IL)-6 levels corrected for native levels of IL-6. Unpaired *t* tests within patient samples were used to determine significance. Data presented are mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Treatment with 250 ng/mL lipopolysaccharide served as a positive control and resulted in an IL-6 level of 385.7 ± 18.9 pg/mL (data not shown). NS = not significant, sea = Sepsis, [Extracorporeal Membrane Oxygentation], and [Acute Respiratory Distress Syndrome].

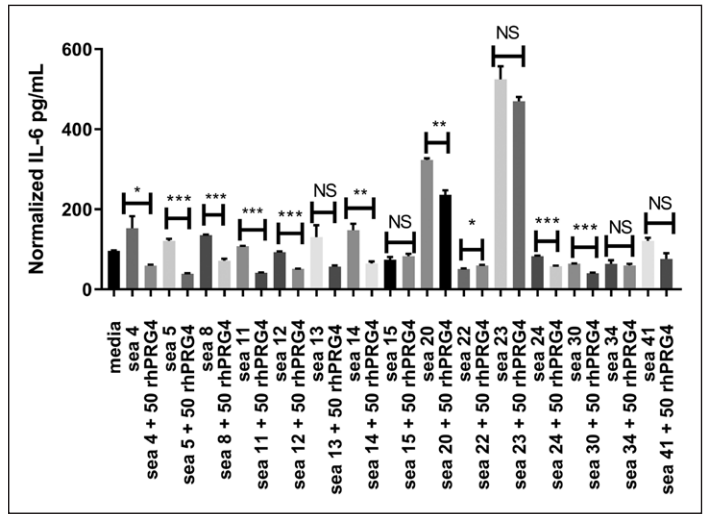

Figure 3. Patient sepsis samples used in human lung microvascular endothelial cell (HLMVEC) culture and treated with recombinant human proteoglycan-4 (rhPRG4) at a low concentration. HLMVEC culture treated with 100 µL of patient plasma and 900 µL media prior to 50 µg/mL rhPRG4 treatment for 23.5 hr. Data from patient samples represent normalized interleukin (IL)-6 levels corrected for native levels of IL-6. Unpaired *t* tests within patient samples were used to determine significance. Data presented are mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Treatment with 250 ng/mL lipopolysaccharide served as a positive control and resulted in a IL-6 level of 1,318.0 ± 14.4 pg/mL (data not shown). NS = not significant, sea = Sepsis, [Extracorporeal Membrane Oxygentation], and [Acute Respiratory Distress Syndrome].

HLMVEC Culture—Patient Plasma Treatment. Sixty percent of sepsis patient plasma samples lowered IL-6 levels significantly following treatment with 50 µg/mL rhPRG4 ($p < 0.05$) (**Fig. 3**). One sepsis patient plasma sample increased IL-6 following treatment with rhPRG4 treatment ($p < 0.05$, SEA 22). Lipopolysaccharide significantly increased IL-6 protein levels from 96.3 ± 0.8 pg/mL in media controls to 1,318.0 ± 14.4 pg/mL ($p < 0.001$) which was decreased 81% to 248.9 ± 11.7 pg/mL by 50 µg/mL rhPRG4 ($p < 0.001$) (data not shown). The IL-6 values shown in Figure 3 were normalized by subtracting background levels of IL-6 from patient plasma.

Lipopolysaccharide significantly increased IL-6 protein levels from 67.1 ± 2.6 pg/mL in media controls to 765.9 ± 12.6 pg/mL

($p < 0.001$) which was decreased 75% to 193.4 ± 2.2 pg/mL by 100 μg/mL rhPRG4 ($p < 0.001$) (data not shown). Seventy-three percent of sepsis patient plasma samples lowered IL-6 levels significantly following treatment with 100 μg/mL rhPRG4 ($p < 0.05$) (**Fig. 4**). Twenty percent of sepsis patient plasma samples increased IL-6 levels significantly following treatment with 100 μg/mL rhPRG4 (SEA 4, SEA 23, and SEA 24). The IL-6 values shown in Figure 4 were normalized by subtracting background levels of IL-6 from patient plasma.

MLMVEC Culture—Lipopolysaccharide Treatment. In wild type MLMVECs (*Cd44*$^{+/+}$ *Prg4*$^{+/+}$), lipopolysaccharide significantly increased IL-6 protein levels to 307.5 ± 23.7 pg/mL compared with media controls at 12.8 ± 5.3 pg/mL ($p < 0.001$) (**Fig. 5*A***). However, all groups co-treated with both lipopolysaccharide and rhPRG4 had significantly reduced IL-6 levels compared with the lipopolysaccharide control group. Compared with the lipopolysaccharide only treated cells, those subsequently treated with 50, 100, and 150 μg/mL rhPRG4 had significantly reduced IL-6 levels that were lowered 26% to 227.3 ± 4.9 pg/mL ($p < 0.01$), 69% to 94.4 ± 23.3 pg/mL ($p < 0.001$), and 66% to 105.4 ± 10.1 pg/mL ($p < 0.001$).

In MLMVECs that were null for *Cd44* (*Cd44*^{tm1Hbg} *Prg4*$^{+/+}$), lipopolysaccharide treatment significantly increased IL-6 protein levels to 145.1 ± 15.1 pg/mL compared with media controls measured at 2.3 ± 0.8 pg/mL ($p < 0.001$) (**Fig. 5*B***). There was no change in IL-6 in cells treated with lipopolysaccharide and then subsequently treated with 50 μg/mL rhPRG4. However, compared with the lipopolysaccharide only group, cells subsequently treated with 100 and 150 μg/mL rhPRG4 had significantly reduced IL-6 levels that were lowered 62% to 55.4 ± 16.1 pg/mL ($p < 0.001$) and 58% to 60.9 ± 17.2 pg/mL ($p < 0.01$).

MLMVECs that were null for *Prg4* (*Cd44*$^{+/+}$ *Prg4*$^{tm2Mawa/J}$) and treated with lipopolysaccharide had significantly increased IL-6 levels measured at 237.0 ± 4.2 pg/mL compared with media controls at 8.5 ± 3.1 pg/mL ($p < 0.001$) (**Fig. 5*C***). Cells treated with lipopolysaccharide and then subsequently treated with 50 μg/mL rhPRG4 did not differ in IL-6 levels compared with lipopolysaccharide treated cells. However, compared with the lipopolysaccharide only group, cells subsequently treated with 100 and 150 μg/mL rhPRG4 had significantly reduced IL-6 levels that were lowered 49% to 120.6 ± 3.5 pg/mL and 55% to 105.5 ± 11.3 pg/mL ($p < 0.001$ for both groups).

In MLMVECs that were double knockout (*Cd44*^{tm1Hbg} *Prg4*$^{tm2Mawa/J}$), cells treated with lipopolysaccharide had significantly increased IL-6 levels measured at 167.5 ± 17.1 pg/mL in comparison to media controls at 2.5 ± 0.7 pg/mL ($p < 0.001$) (**Fig. 5*D***). Cells treated with lipopolysaccharide and subsequently treated with 50, 100, and 150 μg/mL rhPRG4 had significantly reduced IL-6 compared with lipopolysaccharide only treated cells that were lowered 32% to 113.0 ± 3.1 pg/mL ($p < 0.01$), 78% to 36.2 ± 10.7 pg/mL ($p < 0.001$), and 80% to 33.5 ± 2.4 pg/mL ($p < 0.001$).

Supplementary Figure 1 (Supplemental Digital Content 3, http://links.lww.com/CCX/A183; **legend**, Supplemental Digital Content 7, http://links.lww.com/CCX/A187), **Supplementary Figure 2** (Supplemental Digital Content 4, http://links.lww.com/CCX/A184; legend, Supplemental Digital Content 7, http://links.lww.com/CCX/A187), **Supplementary Figure 3** (Supplemental Digital Content 5, http://links.lww.com/CCX/A185; legend, Supplemental Digital Content 7, http://links.lww.com/CCX/A187), **Supplementary Figure 4** (Supplemental Digital Content 6, http://links.lww.com/CCX/A186; legend, Supplemental Digital Content 7, http://links.lww.com/CCX/A187), and **Supplementary Results** (Supplemental Digital Content 8, http://links.lww.com/CCX/A188) provide additional information.

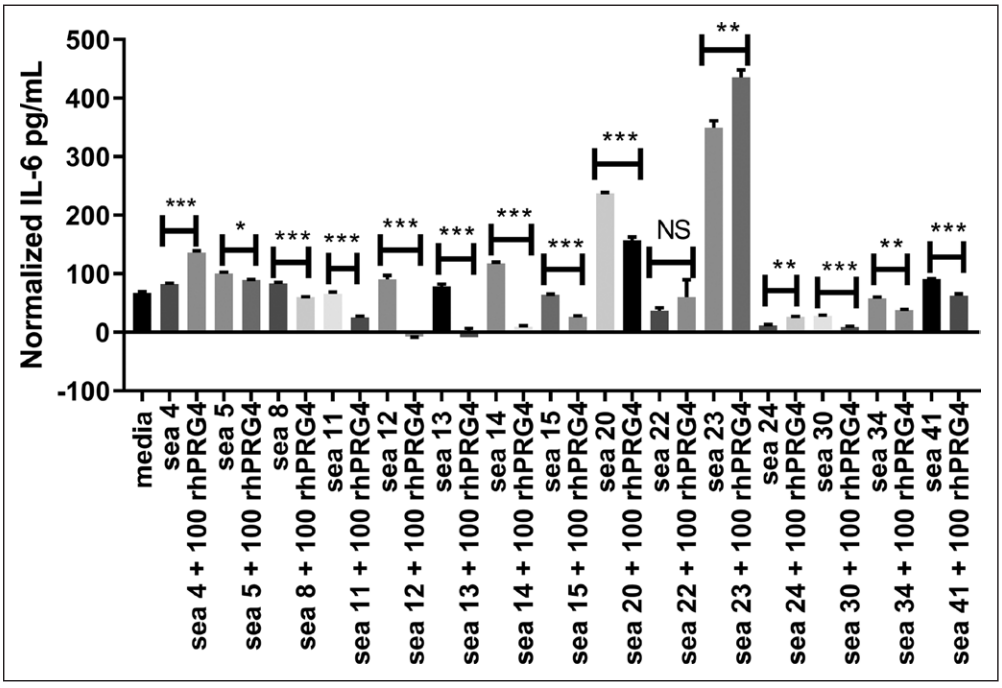

Figure 4. Patient sepsis samples used in human lung microvascular endothelial cell (HLMVEC) culture and treated with recombinant human proteoglycan-4 (rhPRG4) at a high concentration. HLMVEC culture treated with 100 μL of patient plasma and 900 μL media prior to 100 μg/mL rhPRG4 treatment for 23.5 hr. Data from patient samples represent normalized interleukin (IL)-6 levels corrected for native levels of IL-6. Unpaired *t* tests within patient samples were used to determine significance. Data presented are mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Treatment with 250 ng/mL lipopolysaccharide served as a positive control and resulted in an IL-6 level of 765.9 ± 12.6 pg/mL (data not shown). NS = not significant, sea = Sepsis, [Extracorporeal Membrane Oxygentation], and [Acute Respiratory Distress Syndrome].

DISCUSSION

The TLR family includes many members each playing interrelated roles in host defense mechanisms during the immune response (27). Lipopolysaccharide is a strong agonist of the TLRs and upon binding, results in an inflammatory cascade within the cell, leading to the release of cytokines and chemokines, including IL-6 (28, 29). The majority of the culture-positive clinical isolates used

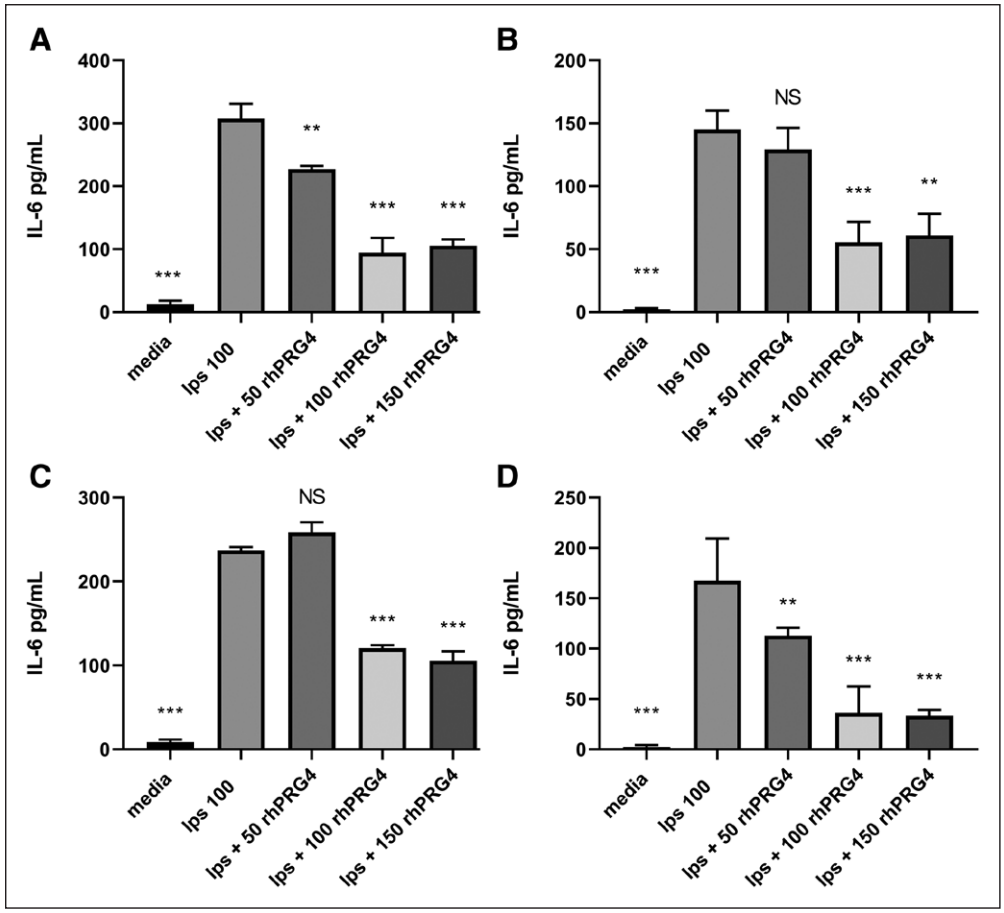

Figure 5. Interleukin (IL)-6 levels from transgenic mouse lung microvascular endothelial cells after lipopolysaccharide (LPS) and recombinant human proteoglycan-4 (rhPRG4) treatments. IL-6 protein concentrations measured via enzyme-linked immunosorbent assay after 100 ng/mL LPS and rhPRG4 treatment in wild-type (*Cd44*$^{+/+}$ *Prg4*$^{+/+}$) (**A**), *Cd44* null (*Cd44*^{tm1Hbg} *Prg4*$^{+/+}$) (**B**), *Prg4* null (*Cd44*$^{+/+}$ *Prg4*$^{tm2Mawa/J}$) (**C**), and double knockout (DKO) (*Cd44*^{tm1Hbg} *Prg4*$^{tm2Mawa/J}$) (**D**). Cells were treated with LPS for 30 min prior to rhPRG4 treatment for 23.5 hr. Data presented are mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; all groups compared with LPS IL-6 values using analysis of variance with Dunnett post hoc comparison. NS = not significant.

in this study contained lipopolysaccharide by virtue of the presence of *Escherichia coli*. The glycoprotein IL-6 is released from many cell types, including endothelial cells (30), as a result of tissue injury or pathogen invasion. It is a ubiquitous biomarker of severe sepsis in both neonates and adults (4, 6–8, 31–35). Therefore, we chose to evaluate IL-6 protein and gene expression in the current study as a representation of a sepsis-like response by mouse and human endothelial cells. Endothelial cells were chosen because their function is highly affected during sepsis (36). Barrier function, signal transduction, vasoregulation, and blood coagulation are all affected by endothelial cells due to their intimal location in blood vessels (36). Dyscrasias in endothelial cells caused by sepsis plays a major role in blood vessel permeability, acidosis, and coagulopathy. Macrophages were not studied in this investigation as we have already shown that NOD-like receptor pyrin domain-containing-3 (NLRP3) is inhibited by rhPRG4 in a human leukemia monocytic cell line THP-1 cells (21).

The lipopolysaccharide used in the current study was the *E. coli* K12-ultrapure variety which only activates TLR4 receptors. Levels of IL-6 protein and gene expression significantly increased in HUVECs, HLMVECs, and MLMVECs after cells were treated with lipopolysaccharide in the current study. Furthermore, the results of the current study show that even though lipopolysaccharide elicited a significant increase of IL-6 protein levels and gene expression in HUVECs, HLMVECs, and MLMVECs these levels were reversed by rhPRG4 in a concentration-dependent manner. Although this study is the first of its kind to use endothelial cells treated with rhPRG4, the results indicate that rhPRG4 has strong anti-inflammatory properties consistent with similar studies in other cell types, pointing to an anti-inflammatory role of lubricin (19–23). These studies indicate that PRG4 has two biological mechanisms of action within the cell in order to counteract inflammation.

Both native human PRG4 (nhPRG4) and rhPRG4 were found to bind to and act as an antagonist to TLR2 and TLR4 receptors in the human embryonic kidney-293 reporter cell line which was verified using enzyme-linked immunosorbent assay, flow cytometry, and immunoprecipitation (19). nhPRG4 was also able to block activation of both TLR2 and TLR4 after the agonists synthetic triacylated lipoprotein and lipopolysaccharide were used, further supporting the role of PRG4 as an anti-inflammatory biologic (19). These results fall in line with the results of our current study in that rhPRG4 competes with lipopolysaccharide in a concentration-dependent manner in order to prevent and reverse inflammation because the addition of rhPRG4 in our experiments occurred "after" cells were exposed to lipopolysaccharide. In using MLMVECs that were null for *Cd44*, there remained a reduction in IL-6 following rhPRG4 treatment, indicating that its anti-inflammatory properties are not dependent on an interaction with CD44. Overall, endothelial cells that were Cd44 null (*Cd44*^{tm1Hbg}*Prg4*$^{+/+}$ and *Cd44*^{tm1Hbg}*Prg4*$^{GT/GT}$) showed a net lower level of IL-6 upon exposure to lipopolysaccharide indicating that animals sufficient for *Cd44* may have greater inflammatory effects from an induced cellular sepsis response. We also observed in Figure 5 that rhPRG4 is more effective than the endothelial cell's native Prg4 in lowering IL-6.

Inflammatory cascades within many cell types, including endothelial cells, are also activated via activation of the CD44 receptor. If the inflammatory cascades are activated within the cell, the NLRP3 inflammasome becomes activated and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) nuclear translocation occurs, both of which drive cytokine release from the cell (21, 22, 37–40). Previous studies indicate that rhPRG4 blocks NF-κB translocation and prevents activation of the NLRP3 inflammasome after it is

internalized in the cell (22). The results from that study indicated that aside from the well-known agonist, hyaluronic acid, rhPRG4 is also a ligand for the CD44 receptor and binds with a higher affinity than HA (22). However, rhPRG4 still showed anti-inflammatory properties in endothelial cells null for *Cd44*. Therefore, it is possible that rhPRG4 exerted its effects via a different mechanism, such as inactivation of TLR4 and possible cellular internalization via other receptor-mediated and nonreceptor mediated pathways.

Based upon the results of the current study and previous studies, it appears that rhPRG4 may be useful as an adjunct therapeutic for a variety of disorders with an immune reaction, including sepsis by either antagonizing TLR2 and TLR4, as was recently reviewed (41). Although a CD44 dependent mechanism in endothelial cells was not observed, this cell surface receptor may still be involved in other immune cells such as macrophages (21). TLR2 and TLR4 have been antagonized in the past in clinical studies using small molecules (10) which appeared promising but eluded translation. PRG4 is normally present in the serum in low levels as it is expressed by hepatocytes and many other organ systems (16–18, 42). In our study, sepsis patients showed higher overall PRG4 levels in plasma in comparison to control patients which indicate that PRG4 may act as an antagonist in the sepsis response in humans (Supplementary Results, Supplemental Digital Content 8, http://links.lww.com/CCX/A188). However, there was no within-subject correlation between IL-6 and PRG4 levels in sepsis patient plasma which could be due to the timing of plasma collection and sepsis severity. In a mouse sepsis model, an organ-based proteomics study (43) indicated that both protein and transcript levels of *Prg4* were upregulated in the liver suggesting it may act as an antagonist of the sepsis response in a murine model as well.

When endothelial cells were treated with plasma from septic patients, the results of IL-6 protein levels and gene expression (Supplementary Results, Supplemental Digital Content 8, http://links.lww.com/CCX/A188) did not share the same magnitude of change as the results from cells treated with only lipopolysaccharide. This may have been due to our RNA collection timepoint at 24 hours post lipopolysaccharide and rhPRG4 treatment or due to the array of inflammatory factors in the septic plasma. Plasma from septic patients can contain either gram-positive and negative bacterial components and a variety of cytokines. These components can further amplify inflammation as measured in our assays via interaction with tumor necrosis factor receptor, CD44, and other targets which may explain why rhPRG4 did not decrease IL-6 protein and gene expression in all cell samples treated with septic patient plasma (44–49). Furthermore, it is also possible that gene expression analysis should have been performed at an earlier timepoint than 24 hours post-treatment with patient plasma due to the latency between upregulated gene expression and subsequent protein release. For example, it has been reported that peak cytokine release occurs between 2 and 6 hours post lipopolysaccharide administration in macrophages and liver tissue (50–52). Unfortunately, due to limitations on patient plasma availability, only one timepoint was used for both protein and RNA analysis in our study.

As far as limitations of our study, we believe this study is relevant to the early stages of sepsis that are lipopolysaccharide dependent. Sepsis is a complicated disease process that takes time to evolve; as it does, a biomolecule like PRG4 theoretically becomes less effective. Ongoing work on additional patient samples will be conducted in order to study endothelial cell gene expression at earlier sepsis timepoints post lipopolysaccharide and rhPRG4 treatment in vitro. We are presently also using an in vivo mouse model to test the effects of rhPRG4 in an lipopolysaccharide-induced sepsis model.

CONCLUSIONS

Based upon the data presented in the current study, innate immune cellular responses of IL-6 from HUVECs, HLMVECs, and MLMVECs can be reversed by treatment with rhPRG4. Therefore, we believe that rhPRG4 is deserving of additional study as a potential adjunct therapeutic for sepsis patients.

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Supplemental digital content is available for this article. Direct URL citations appear in the HTML and PDF versions of this article on the journal's website (http://journals.lww.com/ccejournal).

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