C1q Deficiency Promotes Pulmonary Vascular Inflammation and Enhances the Susceptibility of the Lung Endothelium to Injury*

Received for publication, September 11, 2015, and in revised form, October 20, 2015. Published, JBC Papers in Press, October 20, 2015, DOI 10.1074/jbc.M115.690784

Dilip Shah†, Freddy Romero‡, Ying Zhu§‡§, Michelle Duong†, Jianxin Sun†, Kenneth Walsh¶, and Ross Summer††

From the †Center for Translational Medicine and Jane and Leonard Korman Lung Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, ‡Department of Respiratory and Critical Care Medicine, Changhai Hospital, Second Military Medical University, Shanghai, China, and ¶Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts 02118

The collectin proteins are innate immune molecules found in high concentrations on the epithelial and endothelial surfaces of the lung. While these proteins are known to have important anti-inflammatory actions in the airways of the lung little is known of their functional importance in the pulmonary circulation. We recently demonstrated that the circulating collectin protein adiponectin has potent anti-inflammatory effects on the lung endothelium, leading us to reason that other structurally related proteins might have similar effects. To test this hypothesis, we investigated the anti-inflammatory actions of C1q in lung endothelial homeostasis and the pulmonary vascular response to LPS or HCl injury. We show that lung endothelium from C1q-deficient (C1q−/−) mice expresses higher baseline levels of the vascular adhesion markers ICAM-1, VCAM-1, and E-selectin when compared with wild-type mice. Further, we demonstrate that these changes are associated with enhanced susceptibility of the lung to injury as evident by increased expression of adhesion markers, enhanced production of pro-inflammatory cytokines, and augmented neutrophil recruitment. Additionally, we found that C1q−/− mice also exhibited enhanced endothelial barrier dysfunction after injury as manifested by decreased expression of junctional adherens proteins and enhanced vascular leakage. Mechanistically, C1q appears to mediate its effects by inhibiting phosphorylation of p38 mitogen-activated protein kinase (MAPK) and blocking nuclear translocation of the P65 subunit of nuclear factor (NF)-κB. In summary, our findings indicate a previously unrecognized role for C1q in pulmonary vascular homeostasis and provide added support for the hypothesis that circulating collectin proteins have protective effects on the lung endothelium.

Soluble pattern recognition proteins are innate immune molecules that are highly abundant in the circulation and on select mucosal surfaces including the epithelium of the lung.

§‡ This Research was supported by funding from National Institutes of Health Grant R01HL105490. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 To whom correspondence should be addressed: Center for Translational Medicine and Jane and Leonard Korman Lung Center, Thomas Jefferson University, 1020 Locust St., JAH 368-F, Philadelphia, PA 19107. Tel: 215-503-3893; Fax: 215-955-0830; E-mail: Ross.Summer@jefferson.edu.

2 The abbreviations used are: SP, surfactant proteins; BAL, bronchoalveolar lavage; MLEC, mouse lung microvascular endothelial cell.
**Experimental Procedures**

*Animals*—This study was performed in accordance with regulations of the Institutional Animal Care and Use Committee at Thomas Jefferson University. C1q−/− mice were obtained from Marina Botto at the Imperial College School of Medicine in the United Kingdom (20). These mice have targeted deletion of the A-chain polypeptide of the C1q protein, which leads to total absence of C1q protein expression. The disrupted murine C1qa gene was backcrossed for seven generations onto the C57BL/6 strain (20). Wild-type mice of a similar genetic background (C57BL/6/J) were purchased from Charles River Laboratories (Wilmington, MA). All mice were maintained in the animal facilities at Thomas Jefferson University and only female mice eight to 10 weeks of age were used in our studies.

**Murine Model of Acute Lung Injury (ALI)—**ALI was induced by instilling 100 μl lipopolysaccride (LPS, 100 mcg) or 50 μl of HCl (0.1N) into posterior oropharyngeal space of anesthetized mice as previously described (21, 22). Control mice received i.t. placebo (normal saline). Animals were sacrificed at 4 and 24 h after LPS and 24 h after HCl administration, and bronchoalveolar lavage (BAL) fluid and lung tissues were harvested for later analyses.

**Analysis of BAL Fluid**—Bronchoalveolar lavage (BAL) was performed by cannulating the trachea with a blunt 22-gauge needle and instilling the same one ml of sterile PBS into the lung three times. Total cell count was determined with a TC20 automated cell counter (Bio-Rad Laboratories) while differential counts were performed on cells cyto-centrifuged onto glass slides (Fisher Scientific). Total protein concentration in the BAL fluid was determined using the Pierce™ BCA assay kit (Thermo Scientific, Rockford, IL) as previously described (21).

**Enzyme-linked Immunosorbent Assay**—Tumor necrosis factor-α (TNFα), Interleukin-6 (IL-6), KC (CXCL1), Macrophage-Inflammatory Protein-2 (MIP-2), and IgM were quantitated using commercially available DuoSet ELISA kits (R&D Systems) according to the manufacturer’s instructions. The relative quantity of target genes were calculated by iCycler iQ Real-Time Detection System software (version 3.0a; Bio-Rad) using the comparative threshold method (ΔΔCt) and GAPDH as endogenous control.

**Western Blot Analysis**—Lung tissues were homogenized, and cytoplasmic and nuclear proteins were extracted separately using the Nuclear and Cytoplasmic Protein Extraction Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Protein extracts were used to detect the NF-κB p65 and histone H3; cytoplasmic extracts were used to detect IkBa, GAPDH, ICAM-1, VCAM-1, E-selectin, VE-cadherin, β-Catenin, p-Src, p-p38MAPK, p38MAPK, p-ERK, ERK, p-JNK, JNK, and CD93. Western blot analysis was performed as previously described (23). Western blot experiments were carried under the same experimental conditions and repeated twice with samples collected from different mice. Immunoblot band intensity was quantitated using the software Image J (Research Services Branch, National Institutes of Health).

**Immunohistochemistry**—Immunohistochemical staining was performed on de-paraffinized lung sections after antigen retrieval with Retrievagen A (Target Retrieval Solution; Dako, CA) and after quenching endogenous peroxidases with 3% H2O2. Tissues were subsequently blocked with 2% BSA followed by overnight incubation with primary anti-Gr1 antibody (R&D Systems, Minneapolis, MN). The following morning slides were washed with PBS and secondary antibody was applied for 1 h at room temperature. Staining was visualized using Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) followed by the addition of 3,39-diaminobenzidine (Vector Laboratories).

**Lung Endothelial Cell Isolation**—Lungs were digested into a single-cell suspension using a mouse lung dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and the gentle MACSTM dissociator as previously described (18). Endothelial cells were isolated in a two-step process: first, cells were incubated with Microbeads (Miltenyi Biotec) that bind to CD31. Using this technique, we have previously confirmed by flow cytometry that over 90% of cells in the final suspension are CD45-negative, CD31-positive (18, 24).

**Cell Culture**—Human lung microvascular endothelial cells (HMVEC) and medium were purchased from Lonza, (Walkersville, MD). Cells were maintained in 10-cm plastic dishes pre-
coated with Gelatin-Based Coating Solution. For some experiments, HMVECs were pre-incubated with C1q protein (Abcam, Cambridge, MA) for 2 h prior to LPS stimulation. At pre-specified time points after LPS, cell lysates were collected for analysis.

**In Vitro Leukocyte Adhesion Assay**—Leukocyte-endothelial interactions were assessed through using the Cytoselect Leukocyte-Endothelium Adhesion Assay kit (CBA-210; Cell Biolabs). Briefly, lung microvascular endothelial cells were cultured in 48-well gelatin coated plates for 48 h to ensure the formation of cell monolayers. After 48 h, monolayers were exposed to LPS to induce endothelial activation. Neutrophils ($5 \times 10^5$) were isolated from bone marrow of mice and labeled with leukoTracker (1 h) before being added to monolayers for 30 min. After wash-

**FIGURE 1. C1q deficiency enhances lung endothelial cell activation.** A, transcript levels for ICAM-1, VCAM-1, and E-selectin in the lungs of C1q$^{-/-}$ and wild type mice ($n = 4$, *, $p < 0.05$, and ***, $p < 0.01$ versus wild type mice) at baseline (BSL). B, Western blot analysis for ICAM-1, VCAM-1, and E-selectin in the lungs of C1q$^{-/-}$ and wild type mice at BSL. Image is representative of two different blots. Densitometry analysis shown right ($n = 8$, *, $p < 0.05$ versus wild type mice). C, Western blot analysis for ICAM-1, VCAM-1, and E-selectin in freshly isolated lung endothelial cells from C1q$^{-/-}$ and wild type mice. Densitometry analysis shown on right ($n = 6$, *, $p < 0.05$ versus wild type mice). Data are expressed as mean $\pm$ S.E. The statistical significance was assessed using a Student’s unpaired t test.

**FIGURE 2. C1q deficiency exacerbates LPS-induced lung inflammation.** A and B, total and neutrophil cell counts in the BAL fluid of C1q$^{-/-}$ and wild type mice at baseline (BSL), 4 and 24 h after it. LPS ($n = 6$ each group, *, $p < 0.05$ versus wild type mice). C, immunohistochemical staining for the granulocyte marker Gr-1 (brown stain) in the lungs of C1q$^{-/-}$ and wild type mice 24 h after it. LPS. Morphometric analysis demonstrates an increase in Gr1(+) cells in the lungs of C1q$^{-/-}$ mice 24 h after it. LPS ($n = 3$ each group, *, $p < 0.05$ versus wild type mice). Gr-1(+) cells were not readily detected in the C1q$^{-/-}$ and wild type mice at BSL (data not shown). D–E, enzyme-linked immunosorbent assay (ELISA) for TNF-$\alpha$ and IL-6 in lung homogenate from C1q$^{-/-}$ and wild type mice at BSL, 4 and 24 h after it. LPS ($n = 6$ each group; *, $p < 0.05$ versus wild type mice). F–G, ELISA for the chemokine KC and MIP-2 in lung homogenate from C1q$^{-/-}$ and wild type mice at BSL, 4 and 24 h after it. LPS ($n = 6$ each group, *, $p < 0.05$ and ***, $p < 0.01$ versus wild type mice). Data are expressed as mean $\pm$ S.E. The statistical significance was assessed using a Student’s unpaired t test and one-way analysis of variance (ANOVA) followed by Tukey post hoc analysis.
C1q Deficiency Promotes Lung Injury

Results

C1q Deficiency Promotes the Expression of Lung Endothelial Adhesion Markers—To assess the functional role of C1q in lung endothelial homeostasis, we examined the expression of endothelial-specific adhesion markers ICAM-1, VCAM-1, and E-selectin in the lungs of wild-type and C1q−/− mice. As shown in Fig. 1, we found that transcript and protein levels for ICAM-1, VCAM-1, and E-selectin were significantly increased in lung tissues (Fig. 1, A and B) and in freshly isolated lung endothelial cells from C1q−/− mice (Fig. 1C). Remarkably, this increase in expression of adhesion markers was not associated with enhanced immune cell infiltration into the lung as neither the quantity of cells recovered from BAL fluid nor the number of Gr-1-positive cells in tissue sections were significantly increased in C1q−/− mice (data not shown). Additionally, we found that endothelial barrier function was also maintained in C1q−/− mice as lung wet-to-dry ratio, BAL fluid protein concentration, and levels of junctional adherens proteins VE-cadherin, β-catenin, and pSrc (data not shown) were similar in the lungs of wild-type and C1q−/− mice. Taken together, these findings indicate that C1q deficiency promotes lung endothelial activation without significantly disrupting its barrier functions.

In Vitro Endothelial Permeability Assay—A transwell insert (0.4μm, 12 mm diameter, Corning) was coated with collagen for 2 h at room temperature and mouse lung microvascular endothelial cells (MLECs) were grown to confluence for a minimum of 3 days. Cells were pre-incubated with C1q protein for 2 h prior to the addition of LPS and FITC-dextran at 1 mg/ml (Molecular Probes) in the top chamber. After 1 h, sample aliquots were removed from the bottom compartment and the concentration of FITC-dextran was assessed using a fluorometer (FluorStar Optima, BMG Labtech) per manufacturer’s protocol. All experiments were performed in quintuplicate and repeated three times.

siRNA Transfection—Lung microvascular endothelial cells grown to 60% confluence were transfected with siRNA smart pool for human CD93 (Dharmacon) at a concentration of 100 nM using DharmaFECTTM 1 transfection reagent (Dharmacon) according to the manufacturer’s instructions. A non-targeting siRNA (Dharmacon) was used as a control. Cells were transfected for 24 h in serum-free medium, followed by the addition of normal endothelial culture medium for 48 h. At 72 h, cells were stimulated with LPS in the presence or absence of C1q, and cell lysates were collected for end-point determinations.

Statistical Analysis—Statistics were performed using GraphPad Prism 5.0 software. Two-group comparisons were analyzed by unpaired Student’s t test and multiple-group comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey post hoc analysis. Statistical significance was achieved when \( p < 0.05 \) at 95% confidence interval. No animals were excluded from the analysis.

C1q Deficiency Exacerbates LPS and HCl-induced Lung Inflammation and Pulmonary Vascular Injury—To determine whether baseline increases in endothelial activation enhanced the susceptibility of the lung to injury, we instilled a one-time dose of LPS or HCl into the tracheal lumen of wild-type and C1q−/− mice. These injury models were selected because of their ability to induce massive lung inflammation as well as to disrupt endothelial barrier functions (26, 27). As shown in Fig. 2 and 3, we detected a robust inflammatory response in the lungs of both wild-type and C1q−/− mice after LPS or HCl administration. However, the severity of lung inflammation was markedly increased in C1q−/− mice in both injury model systems as evidenced by enhanced neutrophil recruitment (Fig. 2, A–C and Fig. 3, A and B), increased pro-inflammatory cytokine (TNF-α and IL-6) and chemotactic factor (KC and MIP2) production (Fig. 2, D–G and Fig. 3, C–F) and increased ICAM-1, VCAM-1, and E-selectin (Fig. 4, A–C) expression. Importantly, this increase in lung inflammation after pulmonary insult could not be simply explained by an up-regulation in TLR4 expression as basal levels of this receptor were similar in the lungs of wild-type and C1q−/− mice (Fig. 4D).

Because endothelial barrier dysfunction often correlates with the severity of lung inflammation (28) we reasoned that vascular permeability might also be increased in lungs of C1q−/− mice after LPS or HCl administration. Consistent with this line of reasoning, we found that total protein concentration (Fig. 5,
A and E) as well as IgM protein concentration (Fig. 5, B and F) was increased in BAL fluid from C1q−/− mice. Further, these findings were associated with an increase in lung wet/dry weight ratio (1.10 versus 1.35, *, p < 0.05, data not shown), enhanced accumulation of peri-vascular fluid surrounding pulmonary blood vessels (Fig. 5C) and greater alterations in the expression of VE-cadherin, β-catenin, and pSrc in the lungs of C1q−/− mice (Fig. 5, D and G). Collectively, these findings indi-

C1q Deficiency Promotes Lung Injury

FIGURE 4. C1q deficiency aggravates LPS and acid-induced activation of lung endothelium. A, transcript levels for ICAM-1, VCAM-1 and E-selectin in the lungs of C1q−/− and wild type mice (n = 4; *, p < 0.05, and ***, p < 0.001 versus wild type mice) at 24 h after LPS administration. B, Western blot analysis for ICAM-1, VCAM-1, and E-selectin in the lungs of C1q−/− and wild type mice at 24 h after LPS administration. Image is representative of two different blots. Densitometry analysis shown on right (n = 8; *, p < 0.05 and **, p < 0.01 versus wild type mice). C, Western blot analysis for ICAM-1, VCAM-1, and E-selectin in the lungs of C1q−/− and wild type mice at 24 h after it. HCl aspiration. Densitometry analysis shown on right (n = 5; *, p < 0.05 and **, p < 0.01 versus wild type mice). D, Western blot analysis for TLR4 expression in the lungs of C1q−/− and wild type mice at baseline (BSL). Data are expressed as mean ± S.E. The statistical significance was assessed using a Student’s unpaired t test.

FIGURE 5. C1q deficiency aggravates LPS and acid-induced vascular injury. A–B, total and IgM protein concentration in the BAL fluid of C1q−/− and wild type mice (n = 6 each group; *, p < 0.05 and **, p < 0.01 versus wild type mice) at baseline (BSL), 4 and 24 h after it. LPS. C, representative sections of H&E stained lungs from C1q−/− and wild type mice 24 h after it. LPS instillation. Peri-vascular edema (shown as black arrow) was increased in the lungs of C1q−/− mice, when compared with wild type mice (n = 3 each group). Bars indicate 100 μm. D, Western blot analysis for VE-cadherin, β-catenin, and pSrc in the lungs of C1q−/− and wild type mice at 24 h after it. LPS administration. Image is representative of two different blots. Densitometry analysis shown on right (n = 8; *, p < 0.05 and **, p < 0.01 versus wild type mice). E–F, total and IgM protein concentration in the BAL fluid of C1q−/− and wild type mice (n = 5 each group; *, p < 0.05 and **, p < 0.01 versus wild type mice) at BSL and 24 h after it. HCl aspiration. G, Western blot analysis for VE-cadherin, β-catenin, and pSrc in the lungs of C1q−/− and wild type mice at 24 h after HCl instillation. Densitometry analysis shown on right (n = 5; *, p < 0.05 and **, p < 0.01 versus wild type mice). Data are expressed as mean ± S.E. The statistical significance was assessed using a Student’s unpaired t test and one-way analysis of variance (ANOVA) followed by Tukey post hoc analysis.
C1q Deficiency Promotes Lung Injury

C1q Deficiency Enhances p38 MAPK Signaling in the Lung—To elucidate the mechanisms mediating the suppressive effects of C1q on endothelial adhesion marker expression we first examined levels of the MAPK proteins p38, ERK, JNK, and AKT in whole lung tissues from wild-type and C1q−/− mice. These proteins were selected because they are known to be the major signaling molecules through which pro-inflammatory insults are transmitted to the nucleus in endothelium (29–31). As shown in Fig. 6A, we found that levels of p38 MAPK were significantly increased at baseline and 24 h after LPS whereas levels of ERK, JNK, and AKT were either unaffected (AKT, ERK, data not shown) or were only increased (JNK, Fig. 6B) in the lungs of C1q−/− mice after LPS administration. Consistent with an increase in p38 MAPK signaling, we detected enhanced NF-κB activation in the lungs of C1q−/− mice at baseline and after LPS administration as manifested by both an increase in phosphorylated p65 levels in nuclear fractions as well as by a reduction in levels of the NF-κB inhibitor IκBα in cytosolic fractions (Fig. 6, C and D). Taken together, these findings suggest that C1q deficiency may promote lung endothelial activation by enhancing signaling through p38MAPK pathway.

C1q Inhibits p38 MAPK Signaling and Attenuates NF-κB Nuclear Translocation in Lung Endothelium—Next, to confirm that C1q directly inhibits p38 MAPK activation in lung endothelium we cultured endothelial cells in the presence or absence of C1q and assessed the effects on p38 MAPK protein expression. While C1q did not further reduce the already low basal levels of p38 expression in our in vitro model system (data not shown) we found that C1q markedly down-regulated p38

FIGURE 6. C1q inhibits LPS-induced NF-κB translocation in lungs. A, densitometry analysis of Western blot for p-p38MAPK/p38MAPK at baseline (BSL) and 24 h after it. LPS in the lungs of C1q−/− and wild type mice (n = 6; *, p < 0.05, **, p < 0.01 versus wild type mice). B, densitometry analysis of Western blot for p-JNK/JNK at baseline (BSL) and 24 h after it. LPS in the lungs of C1q−/− and wild type mice (n = 6, *, p < 0.05 versus wild type mice). C, densitometry analysis of Western blot for p-P65/P65 NF-κB at baseline (BSL) and 24 h after it. LPS in the lungs of C1q−/− and wild type mice (n = 6; *, p < 0.05 versus wild type mice). C, densitometry analysis of Western blot for IκB-α at baseline (BSL) and 24 h after it. LPS in the lungs of C1q−/− and wild type mice (n = 6; *, p < 0.05 versus wild type mice). Data are expressed as mean ± S.E. The statistical significance was assessed using a Student’s unpaired t test.

FIGURE 7. Treatment with C1q down-regulates p38MAPK expression and suppresses NFκB signaling. A, Western blot and densitometry analysis for p-p38MAPK/P38MAPK in lung microvascular cells stimulated with LPS (1 μg/ml) in the presence or absence of either C1q (10 μg/ml) or the MAPK inhibitor SB203580 (10 μM). B and C, Western blot and densitometry analysis for NFκB p-P65/IκBα-P65 or IκB-α/GAPDH in lung microvascular cells stimulated with LPS in the presence or absence of either C1q or the MAPK inhibitor SB203580. All experiments were repeated for three times, *, p < 0.05, **, p < 0.01, and *** p < 0.001 versus control cell. Data are expressed as mean ± S.E. The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey post hoc analysis.
expression in response to LPS stimulation (Fig. 7A). Moreover, this decrease in p38 expression associated with a down-regulation in the translocation of p65 to the nucleus as well as caused an up-regulation in IκB expression (Fig. 7B, C). Additionally, we found that these results could be fully recapitulated by exposing cells to a p38 MAPK inhibitor SB203580 (Fig. 7A–C), suggesting that C1q may indeed mediate its effects through inhibiting p38 MAPK activation.

C1q Attenuates LPS-induced Endothelial Inflammation and Barrier Dysfunction through Suppressing p38MAPK Activation—Next, to confirm that C1q directly suppresses endothelial activation and enhances barrier functions, we cultured lung microvascular endothelial cells with or without C1q, and assessed the response to LPS stimulation. As shown in Fig. 8, we found that pre-treatment with C1q markedly attenuates LPS-induced ICAM1, VCAM1, and E-selection expression (Fig. 8A). Moreover, we show that down-regulation of these adhesion markers associate with reduced neutrophil-endothelial interactions as measured by the number of leukocytes bound to endothelium in culture (Fig. 8B). Additionally, we found that C1q also enhanced the expression of VE-cadherin and β-catenin while also downregulating phosphorylation of Src (Fig. 8C). Consistent with these changes in the expression of junctional adherens proteins we found that endothelial barrier function was enhanced by C1q pre-treatment as evident by a decrease in FITC-Dextran leaking through LPS-exposed endothelial monolayers (Fig. 8D). Importantly, the effects of C1q on adhesion markers and endothelial permeability were reproduced by treating cells with a p38 MAPK inhibitor, suggesting that C1q might mediate its effects through similar mechanisms.

CD93 Expression Is Required for C1q-induced Suppression of Lung Endothelial Activation—Because CD93 is believed to be the major receptor by which C1q activates cell signaling pathways we first sought to confirm its expression in whole lung tissues and cultured lung endothelium. We detected high levels of protein expression in the lung and in cultured lung endothelium and, interestingly, levels were not significantly affected by LPS stimulation (data not shown). Next, to elucidate the functional role of CD93 in cultured lung endothelium we performed siRNA knockdown of this gene and examined the effects on the expression of adhesion markers and junctional adherens proteins. Results from these studies indicated that CD93 deficiency effectively prevents C1q mediated suppression of E-selectin and ICAM-1 expression and blocks the up-regulation of endothelial adhesion marker expression (VE-cadherin) in response to LPS stimulation (Fig. 9, B and C). Taken together, these findings indicate that CD93 expression is required for mediating the vascular protective effects of C1q in lung endothelium.

Discussion

In this study, we tested the hypothesis that the circulating innate immune protein C1q plays an important role in regulating pulmonary vascular homeostasis. We have shown that targeted deletion of C1q in mice alters pulmonary vascular homeostasis by augmenting the expression of endothelial adhesion markers. Also, we have demonstrated that these baseline
C1q Deficiency Promotes Lung Injury

Reminiscent of findings in this study, targeted deletion of the pulmonary collectins SP-A and SP-D has been shown to promote airway inflammation (1, 34) by enhancing alveolar macrophage activation (10, 15). Interestingly, the type of immune modulatory response induced by these proteins is influenced by the binding orientation of SP-A or SP-D on alveolar macrophages. For example, binding of the globular head of SP-A and SP-D to the signal inhibitory regulatory protein-α on the surface of alveolar macrophages initiates signaling pathways which suppress cell activation (35, 36), whereas interactions between the collagen tail and calreticulin/CD91 on macrophages enhances pro-inflammatory responses (11, 15). Although our study did not determine whether the binding orientation of C1q also dictates the type of inflammatory response by the lung endothelium we presume this information will be important if C1q is ever going to be exploited as an anti-inflammatory therapy.

While the up-regulation of endothelial adhesion markers is most often accompanied by the influx of immune cells into tissues (37), we did not observe an increase in cellular infiltration in the lungs of C1q−/− mice. We hypothesize this might relate to the fact that baseline increases in chemotactic factors (e.g. MIP2 or KC) (38) were not observed in the lungs of C1q−/− mice. That said, increased cellular infiltration was observed in the lungs of C1q−/− mice after LPS or HCl administration, implying that the lung endothelium is poised for facilitating immune cell entry when leukocyte chemotactic factors are present.

Another important finding in this study is the observation that C1q enhances lung endothelial barrier function after LPS or HCl instillation. Further, we found that C1q mediates these effects by blocking the degradation of key junctional adherens proteins in the lung endothelium. However, we did not observe changes in the expression of various junctional adherens proteins in the lungs of C1q−/− mice at baseline, suggesting that while C1q ameliorates agonist-induced degradation of junctional proteins it is not required controlling their turnover under homeostatic conditions.

Although this study focused exclusively on the pulmonary circulation we presume that C1q also has important anti-inflammatory actions on the systemic endothelium. This hypothesis is supported by several lines of evidence including clinical observations demonstrating that low plasma C1q levels are associated with the development of systemic vasculitis (39, 40) as well as by experimental studies in mice showing that endothelial barrier dysfunction is exacerbated in systemic blood vessels of C1q−/− mice during sepsis (41). Together, these support the notion that C1q has important anti-inflammatory actions in both the systemic and pulmonary circulations.

While C1q is largely confined to the circulation under baseline conditions it has been shown to readily move into the lung during the plasma leak caused by inflammation (4). Thus, it has been proposed that while C1q does not play a major role in homeostatic processes in the lung, it may have an important role in the response to pulmonary inflammation. As such, we speculate that loss of anti-inflammatory and opsonizing activities of C1q within the parenchyma of the lung might have contributed to enhanced cytokine production and increased neu-

We previously reported that mice deficient in adiponectin develop a spontaneous lung phenotype characterized by increased endothelial E-selectin expression (18, 19). These findings, along with results from our current study, indicate that adiponectin and C1q are functionally redundant proteins that inhibit endothelial activation. We presume this functional redundancy is needed to maintain the lung endothelium in a quiescent state while it is continuously exposed to pro-inflammatory substances from the circulation (e.g. lipids, cytokines) (32, 33). This hypothesis might also help to explain why adiponectin and C1q are present in such unusually high concentrations in the circulation as high levels would be needed to adequately blanket the entirety of the lung’s endothelium (5).

changes are associated with enhanced susceptibility of the lung endothelium to injury. Moreover, our mechanistic studies indicate that the ability of C1q to reduce endothelial activation and enhance barrier functions is mediated, at least in part, by its ability to engage CD93 and block p38 MAPK-induced NF-κB signaling.

We previously reported that mice deficient in adiponectin develop a spontaneous lung phenotype characterized by increased endothelial E-selectin expression (18, 19). These findings, along with results from our current study, indicate that adiponectin and C1q are functionally redundant proteins that inhibit endothelial activation. We presume this functional redundancy is needed to maintain the lung endothelium in a quiescent state while it is continuously exposed to pro-inflammatory substances from the circulation (e.g. lipids, cytokines) (32, 33). This hypothesis might also help to explain why adiponectin and C1q are present in such unusually high concentrations in the circulation as high levels would be needed to adequately blanket the entirety of the lung’s endothelium (5).
C1q Deficiency Promotes Lung Injury

trophil accumulation in C1q−/− mice after LPS or HCl administration. Alternatively, it is possible that the increase in plasma leak and the greater influx of serum factors into the lungs of C1q−/− mice might have also contributed to enhanced lung inflammation after LPS or HCl instillation.

Lastly, our study suggests that C1q inhibits NF-κB signaling in the lung endothelium by suppressing p38 MAPK activation. Further, we present in vitro evidence to suggest that these effects are dependent on expression of CD93. Importantly, these findings are consistent with our previous work demonstrating that adiponectin also suppresses NF-κB signaling in the lung endothelium (42). While we have yet to test whether adiponectin mediates its effects through similar mechanisms we are intrigued by recent reports showing that C1q and adiponectin form complexes within the circulation of obese individuals (32, 43). We hypothesize that this interaction may reduce the ability of C1q and adiponectin to bind the lung endothelium and suppress its activation.

In conclusion, this study describes a previously unrecognized role for C1q in the suppression of lung endothelial activation and in protecting the lung from injury. These findings provide further support for the concept that circulating innate immune proteins play an important role in regulating pulmonary vascular homeostasis, and suggest that exploiting the signaling pathways used by these proteins might be effective for limiting the onset or severity of inflammatory vascular diseases in the lung.

Author Contributions—Conceived and designed the experiments: D. S. and R. S. Performed the experiments: D. S., F. R., Y. Z., and M. D. Analyzed the data: D. S. Wrote the manuscript: D. S. and R. S. Edited the manuscript: J. S. and K. W. Supervised the entire project: R. S.

Acknowledgment—We thank Dr. Marina Botto (Imperial College School of Medicine in the United Kingdom) for providing C1qa−/− mice.

References
1. Sano, H., and Kuroki, Y. (2005) The lung collectins, SP-A and SP-D, modulate pulmonary innate immunity. Mol. Immunol. 42, 279–287
2. Han, S., and Mallampalli, R. K. (2015) The role of surfactant in lung disease and host defense against pulmonary infections. Ann. Am. Thorac. Soc. 12, 765–774
3. Yamada, M., Oritani, K., Kaisho, T., Ishikawa, J., Yoshida, H., Takahashi, I., Kawamoto, S., Ishida, N., Ujiie, H., Masae, H., Botto, M., Tomiyama, Y., and Matsuzawa, Y. (2004) Complement C1q regulates LPS-induced cytokine production in bone marrow-derived dendritic cells. Eur. J. Immunol. 34, 221–230
4. Kever, J. S., Riley, D. J., and Leung, K. (1986) C1q content of serum and lung lavage fluid of rats exposed to toxic levels of oxygen. Immunol. Invest. 15, 473–480
5. Scherer, P. E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H. F. (1995) A novel serum protein similar to C1q, produced exclusively in adipocytes. J. Biol. Chem. 270, 26746–26749
6. Eiden, D. P. (2010) Mannose-binding lectin deficiency and respiratory tract infection. J. Innate Immun. 2, 114–122
7. Holmskov, U., Malhotra, R., Sim, R. B., and Jenedus, J. C. (1994) Collectins: collagenous C-type lectins of the innate immune defense system. Immunol. Today 15, 67–74
8. Hansen, S., and Holmskov, U. (1998) Structural aspects of collectins and receptors for collectins. Immunobiology 199, 165–189
9. Takahashi, H., Sano, H., Chiba, H., and Kuroki, Y. (2006) Pulmonary surfactant proteins A and D: innate immune functions and biomarkers for lung diseases. Curr. Pharm. Des. 12, 589–598
10. Sano, H., Kuronuma, K., Kudo, K., Mitsuwa, H., Sato, M., Murakami, S., and Kuroki, Y. (2006) Regulation of inflammation and bacterial clearance by lung collectins. Respiratory 11, S46–50
11. Vandervier, R. W., Ogden, C. A., Fadok, V. A., Hoffmann, P. R., Brown, K. K., Botto, M., Walport, M. J., Fisher, J. H., Henson, P. M., and Greene, K. E. (2002) Role of surfactant proteins A, D and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. J. Immunol. 169, 3978–3986
12. Takemura, Y., Ouchi, N., Shibata, R., Aprahamian, T., Kirber, M. T., Sumner, R. S., Kihara, S., and Walsh, K. (2007) Adiponectin modulates inflammatory reactions via calreticulin receptor-dependent clearance of early apoptotic bodies. J. Clin. Invest. 117, 375–386
13. Fraser, D. A., Bohlinson, S. S., Jasinskiene, N., Rawal, N., Palmarini, G., Ruiz, S., Rochford, R., and Tenner, A. J. (2006) C1q and MBL, components of the innate immune system, influence monocyte cytokine expression. J. Leukoc. Biol. 80, 107–116
14. Hazan, A. (2008) Protective role of the lung collectins surfactant protein A and surfactant protein D in airway inflammation. J. Allergy Clin. Immunol. 122, 861–879; quiz 880–861
15. Gardai, S. J., Xiao, Y. Q., Dickinson, M., Nick, J. A., Voelker, D. R., Greene, K. E., and Henson, P. M. (2003) By binding SIRPa or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. Cell 115, 13–23
16. Litwack, M. L., and Palaniyar, N. (2010) Review: soluble innate immune pattern-recognition proteins for clearing dying cells and cellular components: implications on exacerbating or resolving inflammation. Innate Immun. 16, 191–200
17. Chang, W. C., White, M. R., Moyo, P., McClean, S., Thiel, S., Hartshorn, K. L., and Takahashi, K. (2010) Lack of the pattern recognition molecule mannose-binding lectin increases susceptibility to influenza A virus infection. BMC Immunol. 11, 64
18. Kontter, J. M., Parker, J. L., Baez, E., Li, S. Z., Ranscht, B., Denzel, M., Little, F. F., Nakamura, K., Ouchi, N., Fine, A., Walsh, K., and Sumner, R. S. (2012) Adiponectin attenuates lipopolysaccharide-induced acute lung injury through suppression of endothelial cell activation. J. Immunol. 188, 854–863
19. Summer, R., Fiack, C. A., Ikeda, Y., Sato, K., Dwyer, D., Ouchi, N., Fine, A., Faber, H. W., and Walsh, K. (2009) Adiponectin deficiency: a model of pulmonary hypertension associated with pulmonary vascular disease. Am. J. Physiol. Lung Cell Mol Physiol 297, L432–438
20. Botto, M., Dell’Agnola, C., Bygrave, A. E., Thompson, E. M., Cook, H. T., Petry, F., Loos, M., Pandolfi, P. P., and Walport, M. J. (1998) Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat. Genet. 19, 56–59
21. Shah, D., Romero, F., Stafstrom, W., Duong, M., and Summer, R. (2014) Extracellular ATP mediates the late phase of neutrophil recruitment to the lung in murine models of acute lung injury. Am. J. Physiol. Lung Cell Mol Physiol. 306, L152–161
22. Zarbock, A., Singbartl, K., and Ley, K. (2006) Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. J. Clin. Invest. 116, 3211–3219
23. Romero, F., Shah, D., Duong, M., Penn, R. B., Fessler, M. B., Maden, J., Stafstrom, W., Kawuru, M., Lu, B., Kallen, C. B., Walsh, K., and Sumner, R. (2014) A Pneumocyte-macrophage Paracrine Lipid Axis Drives the Lung Toward Fibrosis. Am. J. Respir. Cell Mol Biol. 5, 74–86
24. Shah, D., Romero, F., Duong, M., Wang, N., Pauldy, B., Suratt, B. T., Kallen, C. B., Sun, J., Zhu, Y., Walsh, K., and Sumner, R. (2015) Obesity-induced adipokine imbalance impairs mouse pulmonary vascular endothelial function and primes the lung for injury. Sci. Rep. 5, 11362
25. Chavakis, T., Keiper, T., Matz-Westphal, R., Hessemer, K., Sachs, U. J., Navroth, P. P., Preisner, K. T., and Santos, S. (2004) The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. J. Biol. Chem. 279, 55602–55608
26. Matute-Bello, G., Frevert, C. W., and Martin, T. R. (2008) Animal models of acute lung injury. Am. J. Physiol. Lung Cell Mol. Physiol. 295, L379–399
27. Wang, H. M., Bodenstein, M., and Markstaller, K. (2008) Overview of the pathology of three widely used animal models of acute lung injury. *Eur. Surg. Res.* **40**, 305–316

28. Müller-Redetzky, H. C., Suttorp, N., and Witzenrath, M. (2014) Dynamics of pulmonary endothelial barrier function in acute inflammation: mechanisms and therapeutic perspectives. *Cell Tissue Res.* **355**, 657–673

29. Liu, S., Feng, G., Wang, G. L., and Liu, G. J. (2008) p38MAPK inhibition attenuates LPS-induced acute lung injury involvement of NF-κB pathway. *Eur. J. Pharmacol.* **584**, 159–165

30. Yan, W., Zhao, K., Jiang, Y., Huang, Q., Wang, J., Kan, W., and Wang, S. (2002) Role of p38 MAPK in ICAM-1 expression of vascular endothelial cells induced by lipopolysaccharide. *Shock* **17**, 433–438

31. Asaduzzaman, M., Wang, Y., and Thorlacius, H. (2008) Critical role of p38 mitogen-activated protein kinase signaling in septic lung injury. *Crit. Care Med.* **36**, 482–488

32. Nakatsuji, H., Kobayashi, H., Kishida, K., Nakagawa, T., Takahashi, S., Tanaka, H., Akamatsu, S., Funahashi, T., and Shimomura, I. (2013) Binding of adiponectin and C1q in human serum, and clinical significance of the measurement of C1q-adiponectin / total adiponectin ratio. *Metabolism* **62**, 109–120

33. Hirata, A., Kishida, K., Kobayashi, H., Nakatsuji, H., Funahashi, T., and Shimomura, I. (2013) Correlation between serum C1q-adiponectin / total adiponectin ratio and polyvascular lesions detected by vascular ultrasonography in Japanese type 2 diabetics. *Metabolism* **62**, 376–385

34. Winkler, C., Atochina-Vasserman, E. N., Holz, O., Beers, M. F., Erpenbeck, V. J., Krug, N., Roepcke, S., Lauer, G., Elmlinger, M., and Hohlfeld, J. M. (2011) Comprehensive characterisation of pulmonary and serum surfactant protein D in COPD. *Respir Res* **12**, 29

35. Reid, K. B., Clark, H., and Palaniyar, N. (2005) Surfactant and lung inflammation. *Thorax* **60**, 620–622

36. Wright, J. R. (2005) Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol* **5**, 58–68

37. Williams, M. R., and Luscinskas, F. W. (2011) Leukocyte rolling and adhesion via ICAM-1 signals to endothelial permeability. Focus on “Leukocyte rolling and adhesion both contribute to regulation of microvascular permeability to albumin via ligation of ICAM-1”. *Am. J. Physiol. Cell Physiol.* **301**, C777–779

38. Olson, T. S., and Ley, K. (2002) Chemokines and chemokine receptors in leukocyte trafficking. *Am. J. Physiol. Regul. Integr Comp. Physiol.* **283**, R7–28

39. Melboucy-Belkhir, S., Glitho, S., Caux, F., Mekinian, A., and Fain, O. (2014) Hypocomplementemic urticarial vasculitis syndrome. *Rev. Prat.* **64**, 25

40. Wisnieski, J. J., Baer, A. N., Christensen, J., Cupps, T. R., Flagg, D. N., Jones, J. V., Katzenstein, P. L., McFadden, E. R., McMillen, J. J., and Pick, M. A. (1995) Hypocomplementemic urticarial vasculitis syndrome. Clinical and serologic findings in 18 patients. *Medicine* **74**, 24–41

41. Dahlke, K., Wranne, C. D., Sommerfeld, O., Sosdorff, M., Recknelag, P., Sachse, S., Winter, S. W., Klos, A., Stahl, G. L., Ma, Y. X., Claus, R. A., Reinhart, K., Bauer, M., and Riedemann, N. C. (2011) Distinct different contributions of the alternative and classical complement activation pathway for the innate host response during sepsis. *J. Immunol.* **186**, 3066–3075

42. Ohashi, K., Parker, J. L., Ouchi, A., Higuchi, A., Vita, J. A., Gokce, N., Pedersen, A. A., Kalthoff, C., Tullin, S., Sams, A., Summer, R., and Walsh, K. (2010) Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J. Biol. Chem.* **285**, 6153–6160

43. Hong, E. S., Lim, C., Choi, H. Y., Ku, E. J., Kim, K. M., Moon, J. H., Lim, S., Park, K. S., Jang, H. C., and Choi, S. H. (2015) The amount of C1q-adiponectin complex is higher in the serum and the complex localizes to perivascular areas of fat tissues and the intimal-medial layer of blood vessels of coronary artery disease patients. *Cardiovasc. Diabetol.* **14**, 50