ORIGINAL RESEARCH

C5L2, the Second C5a Anaphylatoxin Receptor, Suppresses LPS-Induced Acute Lung Injury

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Abstract

LPS-induced lung injury in the mouse is one of the most robust experimental models used for studies of acute lung injury (ALI) and acute respiratory distress syndrome in humans. Prior clinical and experimental studies support an important role for complement activation, particularly production of C5a, in the pathophysiology of human ALI/acute respiratory distress syndrome. In the mouse model, however, the precise role of C5a and its receptors is unclear. C5L2, an enigmatic second receptor for C5a, has been characterized, and results have generated substantial debate regarding its *in vivo* function. Our previous work with human neutrophils revealed a unique role for C5L2 in negatively modulating C5a–C5a receptor (C5aR)–mediated cellular activation, in which antibody-mediated blockade of C5L2 resulted in augmented C5a-C5aR responses. Here, we demonstrate that *C5L2*$^{-/-}$ mice (BALB/c background) administered intranasal LPS exhibit significantly more airway edema and hemorrhage than do wild-type animals. Bronchoalveolar lavage fluid and lung homogenates have significantly more neutrophils and myeloperoxidase activity, as well as proinflammatory cytokines and chemokines. When a blocking antibody against the C5aR was administered before LPS administration, the increased neutrophilic infiltration and cytokine levels were reversed. Thus, our data show not only that C5a contributes significantly to LPS-induced ALI in the mouse, but also that C5L2 plays an important antiinflammatory role in this model through actions resulting at least in part from negative modulation of C5a receptor activation.

Keywords: C5L2; C5a anaphylatoxin; LPS; acute lung injury

Clinical Relevance

Mice deficient in C5L2, the second complement C5a anaphylatoxin receptor, exhibit significantly greater acute lung inflammation compared with wild-type animals after intranasal administration of LPS in a model of human acute lung injury and acute respiratory distress syndrome. Our data show that C5a is a major contributor to this injury. Furthermore, we demonstrate a significant antiinflammatory function for C5L2 *in vivo*, suggesting that maintaining or increasing its expression may have therapeutic benefit.

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) remain a significant source of morbidity and mortality in the United States, with a prevalence of almost 200,000 cases per year, causing 74,500 deaths and 3.6 million hospital days (1). The pathogenesis of ALI/ARDS involves an acute phase characterized by initial injury to the vascular endothelium and/or the alveolar epithelium, causing an increase in alveolar-capillary permeability and pulmonary edema, accumulation of neutrophils, release of proinflammatory cytokines including tumor necrosis factor-α (TNF-α), IL-1β, and IL-6, and release of toxic proteases and reactive oxygen species. Despite numerous clinical trials with various antiinflammatory agents, no pharmacologic therapy has proved beneficial in the prevention and management of ARDS (2), and mortality has remained largely unchanged (3).

The complement system, comprising >30 serum proteins activated in a cascade fashion to generate biologically active molecules, is a key player in innate immunity. Excessive activation of the complement cascade, however, can lead to severe tissue injury (4). Prior studies have suggested an important role for

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complement activation in the pathophysiology of ALI/ARDS. The activation products C3a and C5a, generated by the classical, alternative, and lectin pathways are significantly higher in the serum of patients with ARDS (5, 6). C5a in particular is a highly proinflammatory anaphylatoxin capable of activating neutrophils and numerous other inflammatory cells and has been shown to play an important role in the initial inflammatory phase of ALI/ARDS (7). In ALI associated with immune complex formation or complement activation by cobra venom factor, C5a is required for full production of the inflammatory response (8, 9). The mechanisms of C5a-dependent lung injury include activation of alveolar macrophages to produce such inflammatory cytokines as TNF-α and IL-1, neutrophil chemotaxis and activation, induction of P-selectin, and subsequent endothelial damage (8, 10, 11). In clinical studies, elevated plasma C5a is a predictor of ARDS (7).

However, in murine models of human ALI/ARDS, most commonly LPS-induced lung injury, the role of C5a and its receptors is less clear. Although it is unquestioned that LPS administration results in complement activation (12), one study suggested that it does not contribute to the pathophysiology associated with ALI/ARDS, because neither antibody blockade of the C5a receptor nor absence of C5 altered the degree of lung damage in this model (13). The same group later showed that ALI induced by either LPS or IgG-immune complexes requires the presence of the C5a-C5a receptor (C5aR) (14), corroborating our initial findings (9).

Characterization of C5L2, the second receptor for C5a, has generated much debate regarding its *in vivo* function. In 2000, C5L2 was characterized as a receptor with significant homology to the C5aR and high affinity binding to C5a and its degradation product $C5a_{desArg}$ (15, 16). In contrast to the C5aR, however, C5L2 lacks the ability to couple to signal-transducing G proteins because of an amino acid replacement of arginine by leucine in the second intracellular domain (16). As a result, initial reports indicated an inability to activate intracellular signaling, and led to the concept that C5L2 acts as a "decoy" receptor, functioning to sequester C5a and $C5a_{desArg}$ from the C5aR. Although this mechanism effectively describes some of the functions of C5L2 in transfection systems, it fails to address the caveat that in cells that express C5L2 endogenously, the receptor is largely intracellular and is unavailable for direct interaction with the ligand. Our previous work in human neutrophils revealed a unique function for C5L2 as a negative modulator of C5aR activity through sequestering β-arrestin from the C5aR; our work also revealed that its absence leads to augmented C5aR responses (17). Similar increases in inflammation and neutrophil activation have been observed *in vivo* in $C5L2^{-/-}$ mice (18). Inflammatory indices were markedly increased in $C5L2^{-/-}$ mice in a model of contact hypersensitivity (19), and diabetes when animals were placed on a high-fat/high-sucrose diet (20). In a mouse model of sepsis, blocking C5L2 resulted in increased IL-6 and TNF-α production (21).

The objective of the work presented here was to probe the role of C5L2 and its modulation of C5a-C5aR signaling in LPS-induced ALI/ARDS. We show first that C5a is a major contributing factor in LPS lung injury, and second, as in other models, that C5L2 functions to suppress C5a-C5aR responses, providing a fine-tuning role for potentially destructive actions of complement activation.

Materials and Methods

Animals

The generation and characterization of $C5L2^{-/-}$ mice have been described previously (18). Animals were backcrossed through at least 10 generations to the BALB/c background and maintained in the Boston Children's Hospital Animal Facility. All experiments used $C5L2^{-/-}$ and wild-type mice at 8–12 weeks of age. Studies were conducted in accordance with the Institutional Animal Care and Use Committee of Boston Children's Hospital.

Antibodies and Reagents

LPS was derived from *Escherichia coli*, O111:B4, and purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal rat antimurine C5aR (clone 20/70, low endotoxin) was obtained from Bio-Rad Antibodies (Hercules, CA). Isotype control antibody, rat IgG2bκ (clone R35–38), was purchased from BD Pharmingen (Franklin Lakes, NJ).

LPS-Induced ALI

Mice were lightly anesthetized with isoflurane, and LPS was administered intranasally at 2 mg/kg. Sham-treated animals were given PBS alone. For antibody blockade of the C5aR, the animals were given 30 μg intranasally 30 minutes before LPS. At 2, 6, and 24 hours later, the animals were killed by pentobarbital overdose, and tissues were harvested for subsequent analysis. Assessment of lung mechanics was performed 48 hours after LPS administration. For some analyses, results from sham-treated wild-type and $C5L2^{-/-}$ mice were combined because baseline parameters of inflammation in these animals are not different from those of wild-type animals (18, 19, 22–24).

Histopathology

After sacrifice, the lungs were fixed, and tissues were imbedded in paraffin, sectioned, and stained with hematoxylin and eosin, as described previously (25).

Evaluation of Pulmonary Edema

Lung wet-to-dry weights and extravasation of Evans blue dye were determined as described previously (9, 25). Evans blue content (mg dye/g lung) was determined by extrapolation from a standard curve.

Bronchoalveolar Lavage and Protein Determination

Immediately after sacrifice, lungs were cannulated and lavaged three times with 0.8 ml PBS. The bronchoalveolar lavage (BAL) fluid was assessed for total protein, myeloperoxidase (MPO), and hemoglobin and cytokine levels, as described (25). After red blood cell lysis, the remaining BAL cells were resuspended in PBS for quantitation and differential analysis.

Assessment of MPO and Cytokines

BAL fluids and lung homogenates were assessed for hemoglobin and MPO content as described previously (18). ELISA for TNF-α, and IL-6 were used according to the manufacturer's protocols (BD OptEIA, BD Biosciences, San Diego, CA).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

RNA was isolated from lungs (RNeasy; QIAGEN, Hilden, Germany) and used to determine relative gene expression by the ΔΔCT method as described previously

(19). Primers were designed following recommendations from the LifeTechnologies Primer Design Tool (ThermoFisher Scientific, Waltham, MA). Targets span at least one intron where possible, and amplification efficiency was determined at 92–100%. Primer sequences are tabulated in Table 1.

Lung Mechanics

Animals were anesthetized by intraperitoneal injection with pentobarbital, intubated, and connected to a FlexiVent (SCIREQ, Montreal, QC, Canada) system for measurements of pulmonary mechanics. Mice were ventilated with a tidal volume of 0.25 ml and a positive end-expiratory pressure of 3 cm of H_2O, at 150 breaths per minute. The following parameters were determined: total lung capacity, airway resistance, dynamic compliance, and elastance. Pressure–volume relationships were determined after inflation of the lungs to 30 cm H_2O.

Statistical Evaluation

Data are expressed as the mean ± SEM and were evaluated for significance by analysis of variance or *t* test, as appropriate (Prism Software; GraphPad, San Diego, CA). Differences were considered significant for $P < 0.05$.

Results

C5L2$^{-/-}$ Mice Exhibit Increased Lung Hemorrhage, Edema, and Neutrophil Influx after Intranasal LPS Administration Compared with Wild-Type Mice

In the model of ALI associated with LPS administration in mice, previous work has shown that abundant alveolar hemorrhage increased vascular permeability and neutrophil infiltration (14, 26, 27). LPS is known to activate the complement pathway, generating C5a, which may contribute at least in part to this injury (14). Because our previous studies indicated a negative modulatory role for C5L2 on C5aR-mediated activity both *in vivo* and in isolated cells (17, 18), we tested the responses of *C5L2*$^{-/-}$ mice to LPS-induced lung injury as well. Both wild-type and *C5L2*$^{-/-}$ animals exhibited significantly increased BAL fluid, total protein, and hemoglobin at 24 hours after LPS administration. In *C5L2*$^{-/-}$ mice, the total protein was >50% greater than in LPS-treated wild-type animals, and the hemoglobin level was increased by 100% (Figures 1A and 1B).

To assess increases in edema associated with LPS-induced lung injury, we determined wet-to-dry weight ratios as well as extravasation of Evans blue dye (Figures 1C and 1D). Although both mouse strains revealed significant increases in wet-to-dry weight ratios compared with PBS-treated control animals, the increase for LPS-treated *C5L2*$^{-/-}$ mice was ∼50% greater than for wild-type animals. Similarly, the increase in Evans blue extravasation after LPS administration was ∼100% greater for *C5L2*$^{-/-}$ animals compared with wild-type mice. Histologic comparison of hematoxylin and eosin–stained lung sections corroborated these findings (Figure 1E). At 24 hours after LPS administration, both wild-type and *C5L2*$^{-/-}$ mice had developed neutrophilic infiltrates, interstitial thickening, and hemorrhage. In wild-type animals, these changes included ∼30% of the lobe, whereas in *C5L2*$^{-/-}$ mice, the injury appeared more severe and extended throughout the entire lobe.

Prior studies have shown that intranasal administration of LPS results in maximal BAL fluid neutrophil content 24 hours after administration, with the maximal macrophage and total inflammatory cell influx occurring at 48 hours (28). We quantified the cellular content of BAL fluid as a function of time at 6 and 24 hours after LPS administration and found that both *C5L2*$^{-/-}$ and wild-type mice developed significant increases in total cells and neutrophils as early as 6 hours after LPS (Figures 2A and 2B). The BAL fluid from *C5L2*$^{-/-}$ animals contained twice as many total cells and 4-fold increased neutrophil numbers, compared with wild-type mice. At 24 hours, *C5L2*$^{-/-}$ mice had ∼4-fold greater BAL fluid (total cells) compared with wild-type animals, >90% of which were neutrophils (Figures 2C and 2D). At 48 hours, there was no longer a significant difference in the total BAL fluid cells between *C5L2*$^{-/-}$ and wild-type animals (data not shown).

We corroborated the cell counts by evaluating MPO levels in BAL cells at these time points. We also determined MPO levels in cell-free BAL fluid to evaluate the extent of neutrophil activation and degranulation. Consistent with the direct cell counts, both *C5L2*$^{-/-}$ and wild-type mice revealed significantly elevated BAL-cell MPO levels relative to sham mice (Figure 3). *C5L2*$^{-/-}$ mice exhibited significantly greater MPO than did wild-type animals 6 hours after LPS (Figure 3A). At 24 hours, *C5L2*$^{-/-}$ animals showed significantly increased MPO in both the BAL fluid (total cells) and cell-free supernatant (Figures 3B and 3C). No difference was observed in the MPO levels in the BAL fluid of the two mouse strains after intranasal administration of PBS.

Table 1. Primer Sequences

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
IL-6	CCG GAG AGG AGA CTT CAC AG	CAG AAT TGC CAT TGC ACA AC
TNF-α	TGG CCT CCC TCT CAT CAG TT	TCC ACT TGG TGG TTT GCT AC
CXCL2 (MIP-2α)	TCC AGA GCT TGA GTG TGA CG	AGG CAC ATC AGG TAC GAT CC
CCL20 (MIP-3α)	CCT TGC TTT GGC ATG GGT ACT	GCA ACA GTC GTA GTT GCT TGC T
SOCS	ATG GTC ACC CAC AGC AAG TTT C	CGC CCC CAG AAT AGA TGT AGT AAG
IRG-1	AGT TTT CTG GCC TCG ACC TG	AGA GGG AGG GTG GAA TCT CT
IL-23a	CCA GCG GGA CAT ATG AAT CT	AGG CTC CCC TTT GAA GAT GT
RPS29	ACG GTC TGA TCC GCA AAT AC	ATC CAT TCA AGG TCG CTT AGT C

Definition of abbreviations: CCL20, chemokine (C-C motif) ligand 20; CXCL2, chemokine (C-X-C motif) ligand 2; IRG-1, immunoresponsive gene 1; MIP, macrophage inflammatory protein; RPS29, ribosomal protein S29; SOCS, suppressor of cytokine signaling protein.

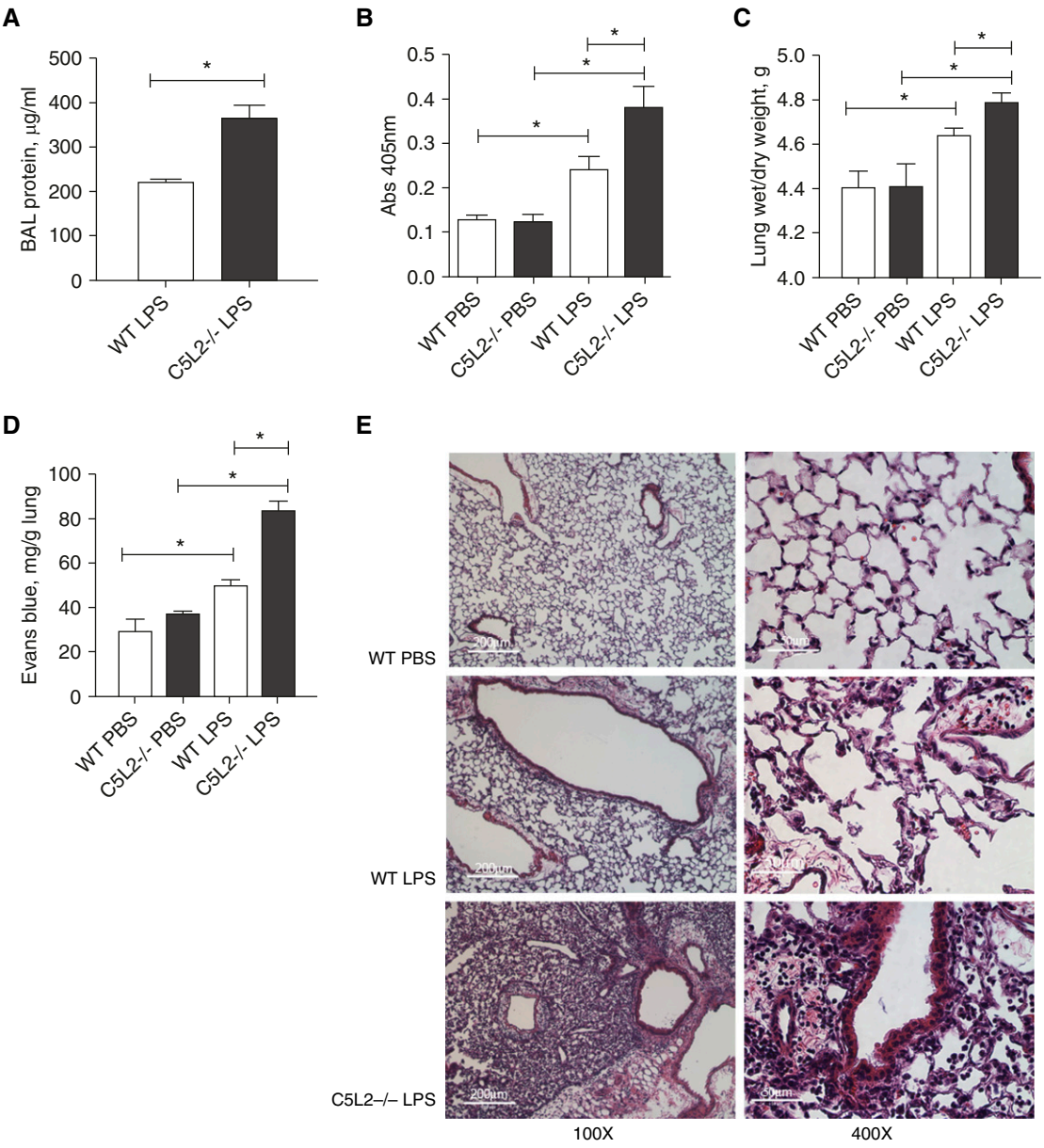

Figure 1. Lung injury associated with intranasal LPS is greater in *C5L2*$^{-/-}$ mice compared with wild-type (WT) animals. (*A*) Total protein in bronchoalveolar lavage (BAL) fluid 24 hours after LPS administration was assessed by bicinchoninic acid assay (protein in BAL from sham-treated animals was below the limit of detection). (*B*) Hemoglobin content was determined by measuring the absorbance (Abs) at 405 nm. (*C*) Lung edema was assessed by comparing the wet-to-dry weight ratios. (*D*) LPS-induced increases in vascular permeability were determined by measuring Evans blue extravasation into the lungs. Results are presented as the mean ± SEM of four mice per group, representative of five independent experiments. *$P < 0.05$. (*E*) Microscopic evaluation of hematoxylin and eosin–stained lung sections reveals neutrophilic infiltrates in alveoli, interstitial thickening, and intraalveolar hemorrhage at 24 hours after LPS administration to WT and *C5L2*$^{-/-}$ mice. In WT animals, the injury includes ~30% of the lobes; in *C5L2*$^{-/-}$ mice, the injury is much more severe and extends throughout 100% of the lobe. *Left panels*, original magnification ×100 (*scale bars*, 200 μm); *right panels*, ×400 (*scale bars*, 50 μm); representative of three mice per group.

LPS-Treated *C5L2*$^{-/-}$ Mice Exhibit Increased Proinflammatory Cytokines and Chemokines Compared with Wild-Type Animals

ALI associated with LPS administration is characterized by increases in BAL fluid, IL-6, and TNF-α, which are believed to be derived from lung macrophages and dependent on nuclear factor-κB activation (27). Consistent with this, we found a significant elevation in both cytokines 24 hours after LPS administration in the BAL fluid of both wild-type and *C5L2*$^{-/-}$ mice (Figure 4). Consistent with other measures of inflammation, BAL fluid from *C5L2*$^{-/-}$ animals contained approximately twice the IL-6 and TNF-α levels observed in wild-type mice at this time point.

The increased cytokine levels were associated with increased gene expression

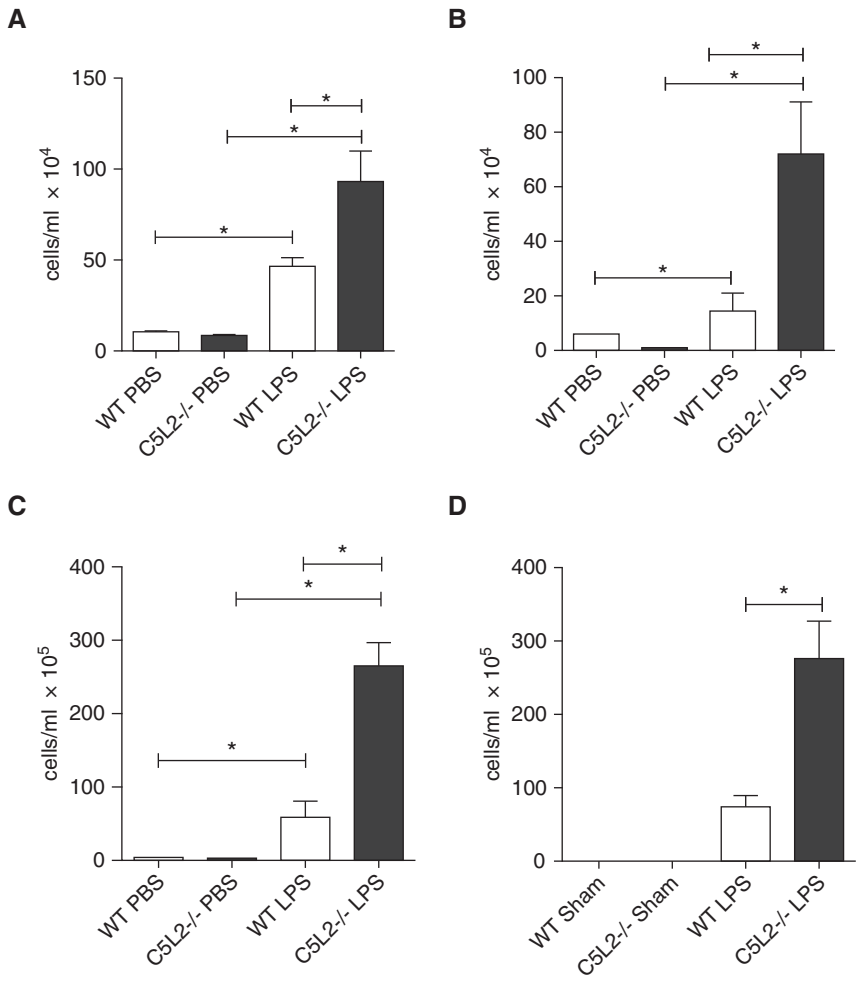

Figure 2. LPS-treated *C5L2*$^{-/-}$ mice exhibit significantly greater influx of total cells and neutrophils compared with WT animals. (*A*) Six hours after intranasal LPS administration, both *C5L2*$^{-/-}$ and WT mice demonstrate significantly increased BAL fluid total cells and (*B*) neutrophils compared with PBS–treated control animals. The total cell content of *C5L2*$^{-/-}$ animals was elevated 2-fold compared with that of WT mice; the neutrophil content was increased 4-fold. (*C*) At 24 hours after LPS, the total cell and (*D*) neutrophil content of *C5L2*$^{-/-}$ mouse BAL fluid was increased ~4-fold compared with WT animals. At 24 hours, no BAL neutrophils were observed in PBS-treated mice. Results are presented as the mean ± SEM of five mice per group, representative of three independent experiments. *$P < 0.05$.

of IL-6 and TNF-α in the lungs in both *C5L2*$^{-/-}$ and wild-type mice as early as 2 hours after LPS administration (Figures 5A and 5B). As in the case of the influx of inflammatory cells, cytokine gene expression was significantly greater for *C5L2*$^{-/-}$ animals compared with wild-type mice. We also examined expression levels of IL-23, and the chemokines MIP-2α (CXCL-2) and MIP-3α (CCL20) and observed elevated gene expression in both mouse strains after LPS treatment (Figures 5C–5E). Similar to the results for IL-6 and TNF-α, *C5L2* deficiency results in significant increases compared with the findings in wild-type animals. Gene expression of suppressor of cytokine signaling proteins (SOCS), which inhibits the JAK-STAT signaling pathway, and immunoresponsive gene 1 (IRG-1) were also more highly expressed in LPS-treated *C5L2*$^{-/-}$ mice (Figures 5F and 5G). Both gene products are associated with suppression of or recovery from Toll-like receptor responses (29, 30), and, although somewhat counterintuitive, deficiency of C5L2 appears to amplify both the pro- and the antiinflammatory arm of LPS-mediated injury. Thus, although proinflammatory processes are elevated by C5a after LPS exposure in *C5L2*$^{-/-}$ mice, these processes, in turn, result in elevated production of suppressive processes.

C5L2$^{-/-}$ Mice Exhibit Significant Alterations in Lung Mechanics Compared with Wild-Type Animals After LPS

To determine whether the changes in inflammatory parameters observed after LPS administration to *C5L2*$^{-/-}$ and wild-type mice correlated with changes in lung physiology, we examined a number of mechanical parameters of the animals using a technique of forced oscillation to determine pressure and volume signals (31). Consistent with literature reports indicating little or no alteration in lung physiology despite increased inflammatory cell infiltrates and cytokine production (32, 33), our findings indicated that wild-type mice at 48 hours after LPS administration exhibited no significant change in total lung volume, resistance, or elastic recoil, although dynamic compliance was reduced (Figures 6A–6D). In contrast, *C5L2*$^{-/-}$ mice revealed significant differences from wild-type animals in all these parameters, in a pattern consistent with restrictive lung disease. Furthermore, LPS-treated *C5L2*$^{-/-}$ mice always exhibited pressure–volume loops that were shifted down and to the right compared with LPS-treated wild-type animals or PBS-treated mice of either strain, supporting the observed decrease in compliance (Figure 6D). Changes in lung physiology were not apparent at 24 hours after LPS administration. These results are consistent with enhanced C5a-C5aR–mediated responses in the absence of C5L2, and the concomitant increases in inflammatory parameters caused by absent damping effects that we observed in human polymorphonuclear leukocytes (17).

Elevated BAL Fluid Inflammation and Cytokines Observed in C5L2-Deficient Mice Are Reversed by Anti-C5aR Blockade

To confirm this hypothesis, we questioned whether increased LPS mediated inflammation results from unopposed C5a signaling through the C5aR in the *C5L2*$^{-/-}$ mice by testing the ability of a blocking antibody against the receptor to inhibit responses. As we demonstrated in a model of contact hypersensitivity (19), *C5L2*$^{-/-}$ mice treated with anti-C5aR mAb exhibited significantly reduced BAL fluid neutrophilic inflammation, whereas wild-type animals

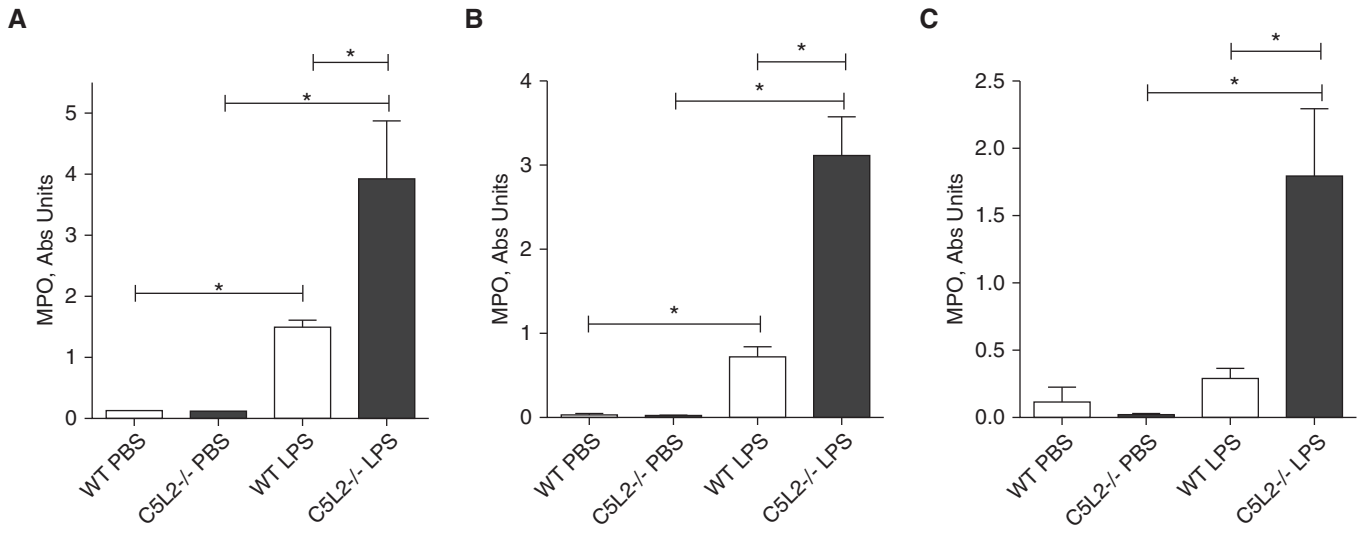

Figure 3. Myeloperoxidase (MPO) content of BAL from LPS-treated *C5L2*$^{-/-}$ mice is elevated relative to WT animals. (*A*) Six and (*B*) 24 hours after intranasal LPS, both *C5L2*$^{-/-}$ and WT mice exhibit significantly increased BAL-cell MPO. (*C*) At 24 hours after LPS, the cell-free MPO level for *CL2*$^{-/-}$ animals was elevated ~6-fold compared with that of WT mice, reflecting increased polymorphonuclear leukocyte activation. Results are presented as the mean ± SEM of five mice per group, representative of four independent experiments. *$P < 0.05$.

were relatively unaffected (Figures 7A–7C). The total BAL fluid cell number, as well as the MPO content of both BAL fluid cell homogenates and cell-free supernatant from *C5L2*$^{-/-}$ mice, all returned to levels not significantly different from the values observed for wild-type animals. In addition, the heightened BAL fluid TNF-α and IL-6 associated with *C5L2* deficiency after LPS administration were returned to wild-type levels by antibody blockade of the C5aR (Figures 7D and 7E).

Discussion

Here, we demonstrate that LPS-mediated ALI in BALB/c mice is dependent at least in part on actions of the C5a anaphylatoxin. Evidence is further provided that C5L2, the second receptor for C5a, plays an important antiinflammatory role through inhibition of C5aR signaling, because its deficiency leads to increases in all parameters of inflammation, as well as to changes in lung function. This work therefore underscores the importance of complement activation in LPS-induced lung injury, a concept that has been questioned in recent literature (13). We also corroborate our previous finding of the role of C5L2 in negatively modulating C5a-C5aR activities in models of contact dermatitis and immune complex lung injury (18, 19). In addition, the suppressive role of C5L2 has been demonstrated *in vivo* in cecal ligation and puncture, as well as in cultured human colonic epithelial cells and peripheral blood neutrophils (17, 21, 34).

Intranasal administration of LPS in mice is one of the most commonly used experimental models for human ALI/ARDS, mimicking many features of the human disease, including neutrophilic infiltration into BAL fluid, expression of inflammatory cytokines, decreased lung compliance, and increased permeability of alveolar capillary membranes (26). Although it is well accepted that the mouse model has significant limitations, in part because of differences in endotoxin sensitivity and mechanisms of the host defense, it remains useful in large measure because of the ease of manipulating gene expression. Thus, the model facilitates studies of the role of gene products for which inhibitors are not available, and it is useful for indicating targets appropriate for more extensive studies in humans.

It is well known that LPS can activate the complement system via both classical

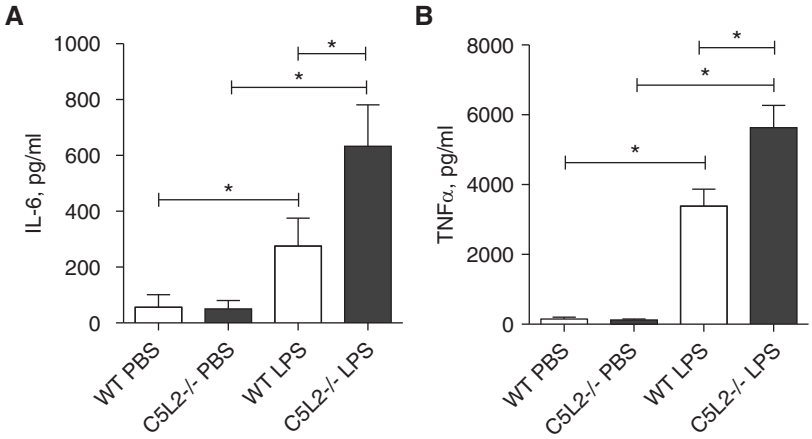

Figure 4. Cytokine levels in LPS-treated *C5L2*$^{-/-}$ mice are elevated compared with those in LPS-treated WT animals. At 24 hours after intranasal LPS administration, *C5L2*$^{-/-}$ mice demonstrate significantly more BAL fluid (*A*) IL-6 and (*B*) TNF-α. Results are presented as the mean ± SEM of five to eight mice per group, representative of three independent experiments. *$P < 0.05$.

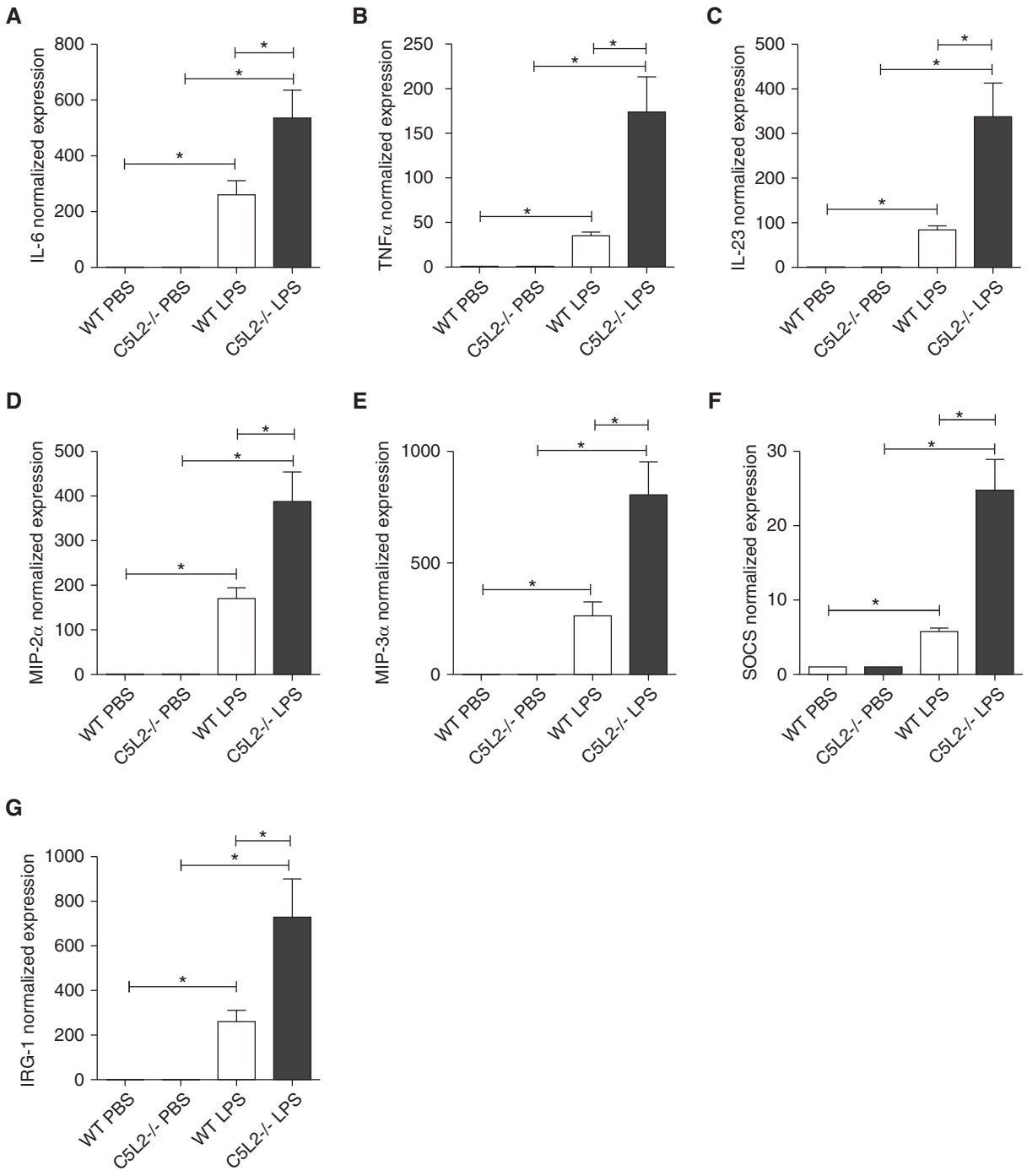

Figure 5. Gene expression of cytokines, chemokines, and Toll-like receptor suppressive proteins are elevated in LPS-treated *C5L2*$^{-/-}$ mice compared with those in WT animals. At 2 hours after intranasal LPS administration, both WT and *C5L2*$^{-/-}$ mice demonstrate significantly more lung gene expression of proinflammatory markers compared with PBS-treated mice of either strain. Levels for LPS-treated *C5L2*$^{-/-}$ animals were all elevated relative to those of LPS-treated WT mice. (*A*) IL-6, (*B*) TNF-α, (*C*) IL-23, (*D*) MIP-2α, (*E*) MIP-3α, (*F*) suppressor of cytokine signaling proteins (SOCS), and (*G*) immunoresponsive gene 1 (IRG-1). Results are presented as the mean ± SEM of 6–10 mice per group, representative of two independent experiments. *$P < 0.05$. MIP, macrophage inflammatory protein.

and alternative pathways (12, 35, 36). The bacterial endotoxin LPS is known to be present in BAL fluid from patients with ARDS and is a potent activator of the innate immune system (37). Patients with ARDS also show evidence of complement activation, and the degree of activation correlates with the severity of disease, as well as with clinical outcome (7, 38, 39). Rittirsch and colleagues (13), however, found no detectable C5a in murine BAL fluids after intratracheal LPS

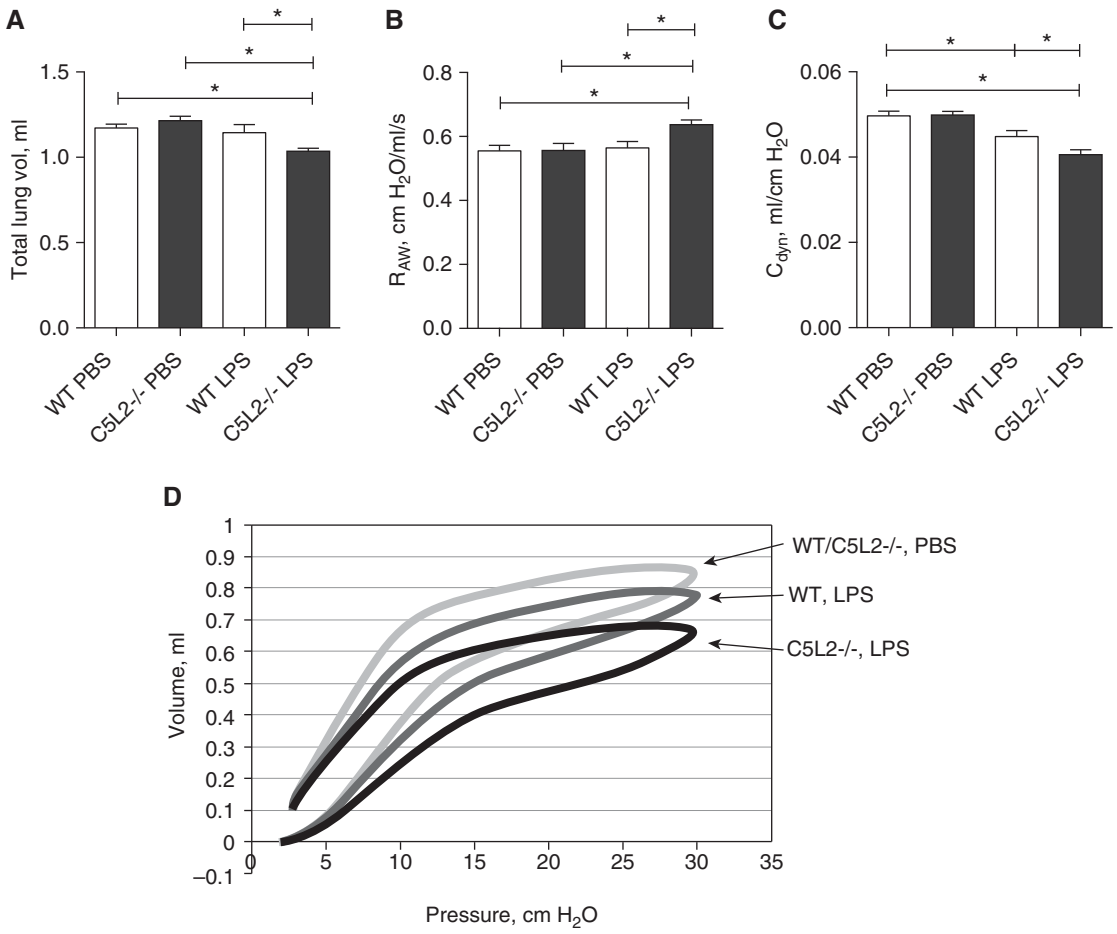

Figure 6. *C5L2*$^{-/-}$ mice exhibit more severe impairment of lung physiological parameters compared with WT animals after intranasal LPS. At 48 hours after intranasal LPS or PBS administration, (*A*) total lung volume, (*B*) airway resistance (R_{AW}), (*C*) dynamic compliance (C_{dyn}), and (*D*) pressure–volume relationships were assessed. *C5L2*$^{-/-}$ mice demonstrate significant reduction in total lung volume and compliance, increased airway resistance, and a down- and rightward shifted pressure–volume loop compared with WT animals. Results are presented as the mean ± SEM of five to six mice per group, representative of four independent experiments. *$P < 0.05$. Representative pressure–volume loops of five to six mice from each group are shown in *D*.

administration, and showed that injury was fully expressed in *C5*$^{-/-}$ mice or animals given anti-C5a antibody. The same group later demonstrated that mice deficient in either *C5aR* or *C5L2* are protected from intratracheal LPS (14). The discrepancy between the reports has not been resolved adequately. Our results concur with the finding of protection from LPS injury in the absence of the C5aR. However, in our studies, *C5L2*$^{-/-}$ mice exhibit more severe lung injury rather than less, and the reason for this difference is not clear.

Despite clear evidence of antiinflammatory roles for C5L2, the function of this second receptor remains controversial. Rittirsch and colleagues (40) demonstrated a proinflammatory role for C5L2 in cecal ligation and puncture in mice, and further showed that C5L2 promotes release of the proinflammatory mediator high mobility group box 1 from circulating leukocytes. In septic humans, expression of C5L2 was positively correlated with prognosis, consistent with an antiinflammatory function (41). Gao and colleagues (21) showed that rats subjected to cecal ligation and puncture exhibited increased plasma levels of IL-6, in accordance with our findings. Chen and colleagues (22), using a distinct line of *C5L2*$^{-/-}$ mice, concluded that this receptor is necessary for C5a signaling *in vitro* and postulated that it acts as a positive modulator for C5a-C5aR signaling. Simultaneously, they showed that *C5L2*$^{-/-}$ mice exhibited increased mortality and higher IL-1β serum levels after intraperitoneal LPS. These discrepancies concerning the role of C5L2 on the basis of differing knockout strains and disease models remain puzzling and unresolved. It is possible that the receptor can mediate G protein independent signaling both positively or negatively, depending on the cell type, the animal model, and the disease state.

Measurement of lung physiologic parameters 48 hours after LPS administration revealed a significant reduction in dynamic compliance in wild-type mice compared with PBS-treated control mice of either strain. *C5L2*$^{-/-}$ mice demonstrated significantly greater impairment relative to both PBS-treated control animals and LPS-treated wild-type mice, exhibiting decreased total lung

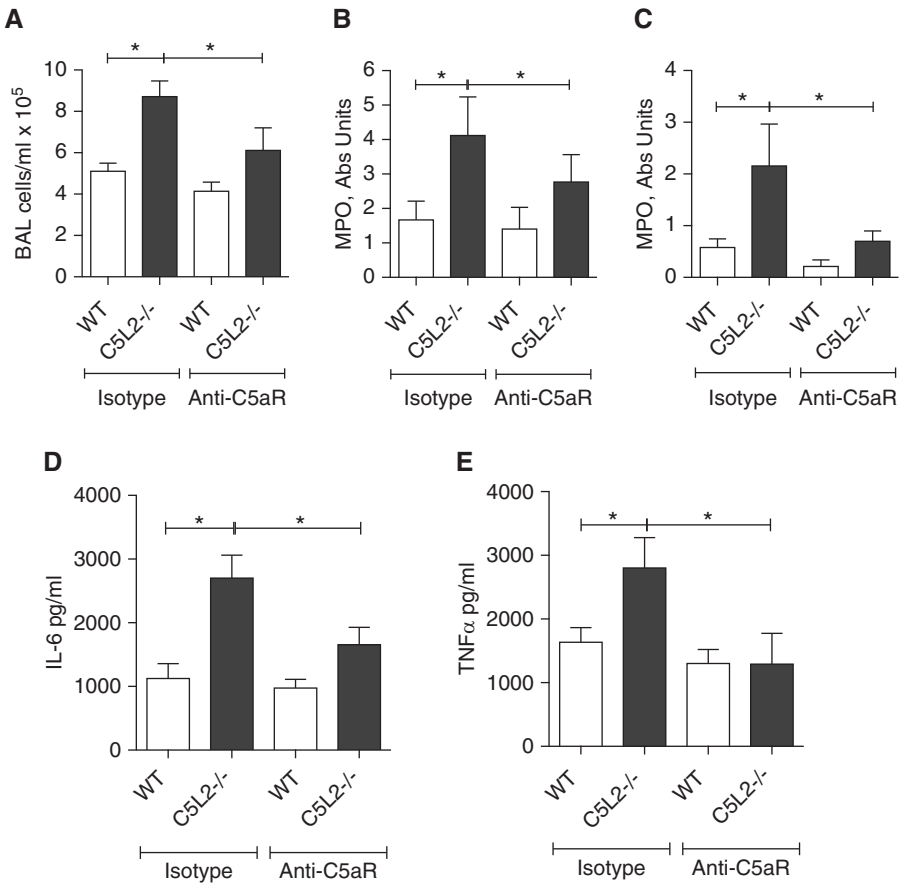

Figure 7. LPS-induced elevation in inflammatory parameters in $C5L2^{-/-}$ mice are reversed by anti-C5aR treatment. Mice were given anti-C5aR monoclonal antibody or isotype control IgG 30 minutes before intranasal LPS, and 24 hours later, BAL fluid was assessed for (*A*) total cells, (*B*) MPO, (*C*) cell-free MPO, (*D*) IL-6, and (*E*) TNF-α. The increases in $C5L2^{-/-}$ mice were significantly reduced by anti-C5aR. Results are presented as the mean ± SEM of 12–15 mice per group, representative of two independent experiments. $^*P < 0.05$.

capacity, increased resistance and elastance, and decreased compliance. These findings corresponded to the finding of increased BAL fluid neutrophils and inflammatory cytokines in LPS-treated $C5L2^{-/-}$ mice relative to wild-type animals. However, although the differences in neutrophilic inflammation between the two strains peaked at 24 hours after LPS, the differences in physiologic parameters were apparent only at 48 hours. Prior studies have demonstrated mixed results regarding changes in lung function associated with LPS-induced ALI. Several investigators found no change in lung physiology after LPS administration (32, 33), whereas others reported impairment in certain parameters such as tissue elastance, but no effect in others including lung capacity (42, 43). Håkansson and colleagues (28), in contrast, demonstrated a temporal relationship between inflammatory cell influx and changes in parameters of lung function resulting from LPS, corroborating our finding of impaired lung function measureable at 48 hours. Interestingly, these authors attribute differences between their results and other studies that failed to demonstrate changes in lung mechanics to the use of LPS derived from *Pseudomonas aeruginosa* delivered by inhalation (28). To our knowledge, the current report is the first to demonstrate significant changes in murine lung function using *E. coli*–derived LPS.

Conclusions

The current study demonstrates the important role of C5a in LPS-induced ALI and provides additional *in vivo* support for the concept that C5L2 acts as a negative modulator of C5a-C5aR signal transduction. More endeavors are required to truly delineate the complex and intriguing mechanisms by which C5L2 regulates inflammation. Nonetheless, our results suggest that complement receptors are promising targets in clinical settings, because antagonizing C5aR or overexpressing C5L2 may lead to decreased inflammatory injury. This is an especially tantalizing finding because expression of C5L2 is positively correlated with the survival of humans with ARDS (41). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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