Anti-KC Autoantibody:KC Complexes Cause Severe Lung Inflammation in Mice via IgG Receptors

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We have shown previously that high concentrations of IL-8 associated with anti-IL-8 autoantibodies (anti-IL-8:IL-8 complexes) are present in lung fluids from patients with the acute respiratory distress syndrome (ARDS), and correlate both with the development and outcome of ARDS. We also detected deposition of these complexes in lung tissues from patients with ARDS but not in control tissues. Moreover, we determined that IgG receptors (FcγRs) mediate activity of anti-IL-8:IL-8 complexes. In the current study, we generated anti-KC (KC = chemokine (CXC motif) ligand 1 (CXCL1)) autoantibody:KC immune complexes (KC-functional IL-8) in lungs of mice to develop a mouse model of autoimmune complex-induced lung inflammation. Both wild-type (WT) and γ-chain-deficient mice that lack receptors for immune complexes (FcγRs) were studied. First, the mice were immunized with KC to induce anti-KC autoantibodies. Then, KC was administered intratracheally to generate anti-KC:KC complexes in the lung. Presence of anti-KC:KC complexes was associated with development of severe pulmonary inflammation that was, however, dramatically suppressed in γ-chain-deficient mice. Second, because sepsis is considered the major risk factor for development of ARDS, we evaluated LPS-treated WT as well as γ-chain-deficient mice for the presence of anti-KC:KC complexes and pulmonary inflammatory responses. We detected complexes between anti-KC autoantibodies and KC in lung lavages and tissues of mice treated with LPS. Moreover, γ-chain-deficient mice that lack receptors for immune complexes were protected from LPS-induced pulmonary inflammation. Our results suggest that immune complexes containing autoantibodies contribute to development of lung inflammation in LPS-treated mice.

Keywords: chemokine; autoantibody; immune complex; endotoxin; lung

The acute respiratory distress syndrome (ARDS) affects 150,000 people each year in the United States, and the mortality of severe cases remains greater than 40% despite significant advances in treatment modalities (1, 2). In the lungs, there is an acute inflammatory response with a significant increase in both the total number of neutrophils and the proportion of neutrophils in the alveolar spaces (2). There is considerable evidence linking the number of neutrophils in the alveolar spaces to the severity of disease in most patients with ARDS (3–5). Several studies have demonstrated that high concentrations of interleukin-8 (IL-8, CXCL8), a major neutrophil activator, are present in lung fluids from patients with ARDS (6–12). However, we have

CLINICAL RELEVANCE

Our model of anti-KC:KC-induced lung inflammation can be used for testing new therapies. The observation that these complexes contribute to development of lung inflammation in LPS-treated mice is novel, and may aid in understanding the pathology of acute lung injury.

found that there is not a consistent relationship between the concentration of IL-8 and either the development or the course of ARDS (13–15). These findings contrast with prior studies, in which IL-8 concentrations were reported to predict the onset and the outcome of ARDS (6, 8–10). Other groups also have not found a relationship between IL-8 and either progression to ARDS or survival once ARDS begins (7, 11, 12). Our studies also show that a portion of the total IL-8 in lung fluids from patients with ARDS is associated with anti-IL-8 autoantibodies (anti-IL-8:IL-8 complexes) (13–15), and that the presence of anti-IL-8:IL-8 complexes in bronchoalveolar (BAL) fluids of patients with ARDS is a prognostic indicator of both the development and the outcome of ARDS (14, 15). Moreover, complexes purified from the lung fluids of patients with acute lung injury (ALI) have the ability to attract and activate human blood neutrophils, and control neutrophil survival (16, 17). Importantly, IgG receptors (FcγRIIa) that interact with immune complexes mediate activity of the anti-IL-8:IL-8 complexes (16, 17). We also demonstrated that the instillation of the purified rabbit anti-IL-8:IL-8 complexes into the lungs of rabbits stimulates an increase in lung fluid concentrations of IL-8 and neutrophils, in contrast to the instillation of a control antibody (18). Finally, we have evaluated lung tissues from patients with lung injury for the presence of anti-IL-8:IL-8 complexes by confocal microscopy. IL-8 co-stained with IgG and immune complex receptors, FcγRIIa, in lung tissues from patients with acute respiratory distress syndrome but not in control tissues, suggesting that anti-IL-8:IL-8 complexes are deposited in lungs of patients with ARDS via FcγRIIa (19). We were also able to detect anti-IL-8:IL-8 complexes bound to neutrophils present in the alveolar spaces of these patients (our unpublished information).

These studies led us to hypothesize that anti-IL-8:IL-8 complexes may contribute to the severity of the alveolar inflammation in ARDS, and that IgG receptors mediate activity of the complexes *in vivo*. Therefore, we have developed a mouse model to evaluate the contribution of anti-KC autoantibody:KC complexes generated *in situ* to lung inflammation and injury (autoimmune complex-induced lung inflammation). Although mice do not express IL-8, murine chemokine (CXC motif) ligand 1 (CXCL1) (KC) is functionally related to human IL-8 (20). We also investigated whether LPS-induced lung inflammation generates anti-KC:KC complexes in mice. Finally, we examined inflammatory responses in lungs of γ-chain-deficient mice lacking stimulatory receptors for IgG (FcγRs), which interact with immune complexes.

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MATERIALS AND METHODS

Animal Models of Lung Inflammation

All studies involving animals were approved by the Institutional Animal Care and Use Committee of the UT Health Center and the Veterans Affairs Puget Sound Health Care Systems, the University of Washington, and conform to the NIH guidelines.

BALB/c mice (Taconic, Germantown, NY) were immunized with murine KC (Peprtech Inc., Rocky Hill, NJ) conjugated to an adjuvant, purified protein derivative of tuberculin (PPD; State Serum Institute, Copenhagen, Denmark), or endotoxin-free saline-PPD mixture essentially as previously described (21, 22). Briefly, KC (2 µg) or saline was injected intraperitoneally on Days 0, 7, and 14. Because intraperitoneal immunization induces mainly a systemic antibody response, we used an intranasal administration of KC to induce an intrapulmonary immune response (22). All mice were immunized both intraperitoneally and intranasally on Day 14 (at the time of the last injection). Intranasal immunizations were performed under halothane anesthesia, and the mice received 8 µg of KC in 40 µl of endotoxin-free saline or saline alone. Blood (< 50 µl) for test samples was obtained via tail nip at 1 week after the last injection (on Day 21), and the enzyme-linked immunosorbent assay (ELISA) (for detecting anti-KC autoantibodies) was performed as routinely done in our laboratory (see next paragraph) (13). Autoantibodies against KC were detected only in plasma of KC-immunized mice. Once the presence of autoantibodies was confirmed, KC (4 µg) was administered intratracheally to generate anti-KC:KC complexes in lungs (KC-immunized/KC group). Additional KC-immunized mice received saline instead of KC (KC-immunized/Saline group). Furthermore, mice immunized with saline received either KC (Saline-immunized/KC group) or saline (Saline-immunized/Saline group). In the additional series of experiments, γ -chain-deficient BALB/c mice (Taconic) lacking functional expression of stimulatory Fc γ Rs that bind immune complexes were studied. We did not verify the presence of the null allele in the animals used for this study due to the mating format employed by Taconic. According to information provided by Taconic, all breeders are genotyped by PCR before production of newborns. This PCR assay discriminates between the wild-type (WT) and mutated (disrupted) alleles, and it can discriminate between a WT, heterozygous, or homozygous animal. Then, the colony is bred homozygote x homozygote, so all pups are also homozygous. Knockout (KO) mice were immunized with KC and had KC administered intratracheally as described above (KO/KC-immunized/KC mice). Additional KO mice were both immunized and treated with saline (KO/Saline-immunized/Saline mice).

Fourteen hours after intratracheal administration of saline or KC, mice were killed with Beuthanasia (5 µl/g, intraperitoneally; Schering-Plough Animal Health Corp., Kenilworth, NJ) and the lungs lavaged five times with 1 ml of sterile saline. One of the lobes was also fixed for histology, and a second one snap frozen in liquid nitrogen for measurement of myeloperoxidase (MPO).

Other WT BALB/c mice or γ -chain-deficient mice were injected intraperitoneally with LPS (*Escherichia coli* 0111:B4 [Sigma Chemical Co., St. Louis, MO]; 200 µl of 1 µg/g body weight). Control WT and KO mice received saline (also 200 µl). Fourteen hours after treatment with LPS or saline, the animals were killed with Beuthanasia (5 µl/g, intraperitoneally). The remaining procedures were identical to the ones described above. In some experiments mice were killed 1, 2, or 8 hours after intraperitoneal LPS administration.

In addition, lung tissue sections from mice treated with intratracheal LPS were evaluated for the presence of anti-KC autoantibody:KC complexes, and histologic changes indicative of lung injury. C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) received a dose of LPS at 1.5 µl/g (1 mg/ml of *E. coli* 0111:B4; List Biologicals, Campbell, CA) under isoflurane anesthesia. Six hours after intratracheal instillation of LPS or saline (control mice), the animals were killed with an intraperitoneal injection of pentobarbital (120 µg/g) and exsanguinated by intracardiac puncture. Then, the lungs were removed and fixed with 4% paraformaldehyde at 15 cm H₂O.

ELISA Assay for Detecting Anti-KC Autoantibodies

Blood for detecting anti-KC autoantibodies was obtained via tail nip under halothane anesthesia at 1 week after the last injection, and the

ELISA assay was performed essentially as previously described (13). Briefly, polystyrene microtiter plates (96-well; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) were coated with KC (100 ng per well) (Peprtech), and incubated overnight at 4°C. The plates were then washed three times with PBS, containing 0.05% Tween-20, and non-specific sites on the plates were blocked (overnight at 4°C) with BSA (Sigma). Then, samples were added (100 µl of serial dilutions of mouse plasma), and the plates were incubated overnight at 4°C. After washing five times with PBS, containing 0.05% Tween-20, the plates were incubated with biotinylated horse antibody against mouse immunoglobulins (Vector Laboratories Inc., Burlingame, CA) overnight at 4°C, and with horseradish peroxidase (HRP)-conjugated streptavidin for 20 minutes at the room temperature. The plates were washed (as above), and the HRP substrate, tetramethyl benzidine (Sigma), was added. The reaction was stopped with 2.0 M sulphuric acid, and absorbance measured at 450 nm. Plates coated with the coating buffer alone were used to control for nonspecific binding to plastic (control plates).

Cell Counts and Cytology in BAL Fluid

Total cell numbers were assessed using a hemacytometer. To determine cell types in BAL fluid, 5×10^5 cells were mounted on slides by cytospin centrifugation. Then, BAL fluid cells were identified and counted by differential staining microscopy using a HEMA 3 stain kit (Fisher Diagnostics, Pittsburgh, PA). In addition, digital pictures of slides were obtained using Olympus DP12 camera attached to Olympus BX41 microscope (Olympus America Inc., Center Valley, PA).

Measurement of Albumin Concentration and Total Protein Concentration

The concentration of albumin in murine lavages was determined with the Mouse Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc, Montgomery, TX), according to manufacturer's directions. The total protein concentration was measured using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL).

MPO Activity

The MPO activity in the BAL fluid was determined as previously described (23) (i.e., 10 µl of the fluid was mixed with 290 µl of 50 mM potassium phosphate buffer, pH 6.0, containing 0.167 mg/ml of o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide, and absorbance was measured at 450 nm). A standard curve was generated using purified MPO (Calbiochem, La Jolla, CA).

Detection of Anti-KC:KC Complexes by ELISA

The concentration of the complexes in the BAL fluid was measured as described (13–15). Briefly, 96-well microtiter plates were coated with antibody against KC (Peprtech). After blocking, the plates were incubated with BAL fluid samples obtained from mice. Then, the plates were washed and incubated with biotinylated horse antibody against mouse immunoglobulins (Vector Laboratories) followed by HRP-conjugated streptavidin and the substrate tetramethyl benzidine (Sigma).

Determination of Cytokine Levels

Lung tissue was homogenized in 2 to 3 ml of a buffer containing 150 mM NaCl, 2 mM PMSF, and 1% solution of protease inhibitor mixture (Sigma), then centrifuged at 2,000 rpm for 20 minutes. The resulting supernatant was collected, and stored at –70°C before analysis. Concentrations of interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) were measured using specific ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Lung Tissue MPO

MPO was extracted by homogenization of frozen lung tissue in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethyl-ammonium. The samples were homogenized on ice with an on/off technique to prevent the tip of the homogenizer from getting heated and thus denaturing MPO. The maximum time was 10 seconds on followed by a 15-second cool-off period. The homogenized specimens were frozen and thawed twice, sonicated for 10 seconds at 50 to 60 Hz after each cycle, and centrifuged at 40,000 rpm for 15 minutes at

4°C. The MPO activity was assessed in the resulting supernatants as described above (23).

Confocal Microscopy

Lung tissue sections were deparaffinized by running the tissue section slides through multiple changes of xylene, graded alcohols down to distilled water. The deparaffinized tissues were washed with PBS and incubated with blocking buffer (PBS containing 10% normal rabbit plasma and 0.5% BSA) overnight at 4°C. Next, tissue sections were treated with streptavidin blocker for 15 minutes to decrease nonspecific binding of streptavidin to tissue, and washed with PBS. To assess immune complex deposition lung tissue sections (15 μ m) were incubated with biotinylated antibody against mouse KC (Peprtech), followed by Texas Red-conjugated streptavidin (BRL, Gaithersburg, MD), and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (DAKO, Carpinteria, CA). Both types of antibodies as well as streptavidin solution were diluted in PBS containing 1% normal rabbit serum and 0.5% BSA. Control stainings were performed without any antibodies using only Texas Red-conjugated streptavidin. After washing with PBS, coverslips were mounted on tissue sections with Permount (Fisher). The sections were then evaluated using a PerkinElmer Ultra VIEW LCI confocal imaging system with Nikon TE2000-S fluorescence microscope (Nikon Instruments, Inc., Melville, NY) and a PlanApo $\times$ 60 immersion oil objective (PerkinElmer, Wellesley, MA) or Leica Microsystems TCS SP5 (Leica Microsystems Inc., Bannockburn, IL). Correlation coefficients were obtained as a measure of co-localization of objects in confocal dual-color images. The degree of interaction between KC and IgG is presented as a correlation coefficient, which reflects the overlap between green and red stains used to label these proteins, and takes into account both the intensity and location of signals generated by each stain.

To evaluate activation of signaling proteins, deparaffinized tissue sections (5 μ m) were incubated with blocking buffer (PBS containing 10% normal porcine plasma and 0.5% BSA) overnight at 4°C. After blocking, tissue sections were washed with PBS and incubated with anti-phospho ERK, anti-phospho Akt (Ser 473), or anti-phospho p-38 antibody (Cell Signaling, Beverly, MA) followed by FITC-conjugated secondary antibody (DAKO). Both types of antibodies: primary and secondary were diluted in PBS containing 1% normal porcine serum and 0.5% BSA. Control stainings were performed using only secondary antibodies. After washing with PBS, coverslips were mounted on tissue sections with Permount (Fisher), and evaluated as described above. In some experiments neutrophils were stained with biotinylated rat anti-mouse Ly-6G (Gr-1; eBioscience) and Texas Red-conjugated streptavidin, and epithelial cells were stained with rabbit anti-human surfactant protein B (Chemicon, Temecula, CA) and anti-rabbit FITC antibodies (DAKO).

Pulmonary Histopathology and Histochemistry

Lungs were fixed in ExCellPlus (AMTS, Lodi, CA), sectioned at 5 μ m, stained with hematoxylin and eosin, and photographed using Olympus DP12 camera attached to Olympus BX41 microscope (Olympus America, Inc., Center Valley, PA). Stained sections were also evaluated for the presence of alveolar exudate, infiltration of inflammatory cells, and interstitial thickening using Olympus BX41 microscope. The grading system ranged from 0 (no changes) to 2 (significant changes), and was based on previously published histopathologic criteria for evaluating the extent of lung tissue damage (24).

In addition, immunohistochemistry (IHC) for neutrophils was performed with anti-mouse Ly-6G mAb (BD/Pharmingen, San Jose, CA) in paraffin-embedded tissue. On the day of the experiment, slide-mounted tissue sections were deparaffinized, rinsed twice with PBS for 3 minutes, and rehydrated in a series of graded ethanol. Antigen retrieval was performed using 0.05% pronase, the tissue sections washed in PBS, and then blocked with normal goat serum (Jackson Immuno Research Labs, West Grove, PA). The tissue sections were then incubated overnight with rat anti-mouse Ly-6G/C mAb or isotype-matched control IgG. This was followed by biotinylated goat anti-rat IgG antibody for 2 hours at room temperature. Endogenous peroxidases were blocked with 3% H₂O₂ in H₂O and then the tissue sections were rinsed twice with PBS and incubated with the Vector Laboratories "Elite" ABC-HP kit in a moist chamber for 90 minutes at room temperature. After two rinses with PBS, the sections were incubated with

diaminobenzidine substrate (Sigma) for 12 minutes in the dark at room temperature. The slides were counterstained with 1% methyl green for 6 minutes. Digital pictures of the slides were acquired using Nikon DXM1200F camera attached to Nikon Eclipse 80 microscope.

Statistical Analysis

Differences between groups were analyzed by a simple one-way ANOVA. The direct comparison between any two treatment groups was performed using the Student's *t* test or the nonparametric Mann-Whitney test, and the Fisher's exact test using SIGMASTAT (SPSS Science Inc., Chicago, IL). A *P* value of less than 0.05 was considered significant.

RESULTS

Murine Model of Autoimmune Complex Induced Lung Inflammation

To examine the direct contribution of the anti-IL-8:IL-8 complexes to lung inflammation we generated analogous complexes in mice. Although mice do not express IL-8, KC is closely related in function to human IL-8 (20). Thus, female BALB/c mice were immunized with KC or saline. Mice immunized with KC developed plasma autoantibodies to KC, whereas no autoantibodies were found in the plasma of mice inoculated with saline (data not shown). Next, we administered KC intratracheally to form anti-KC:KC immune complexes in the lung (KC-immunized/KC mice). Additional KC-immunized animals received saline (KC-immunized/Saline mice). Importantly, mice assigned to these two groups had similar amounts of plasma anti-KC autoantibodies. The corresponding changes in absorbance expressed as median values with 25 to 75 percentile were: 0.21 (0.16–0.37) and 0.17 (0.14–0.56) for KC-immunized/Saline and KC-immunized/KC, respectively (*P* > 0.05). KC or saline was also instilled to saline-immunized mice that did not have circulating autoantibodies against KC (Saline-immunized/KC and Saline-immunized/Saline mice, respectively).

Fourteen hours after the intratracheal instillation of saline or KC, the lungs were lavaged, and the concentrations of anti-KC:KC complexes were measured in BAL fluids. Anti-KC:KC complexes (at measurable levels [i.e., change in absorbance > 0.1]) were detected only in mice that were immunized with KC and treated with KC (KC-immunized/KC), but not in mice immunized with KC that received saline (KC-immunized/Saline) or mice immunized with saline and treated with intratracheal saline or KC (Saline-immunized/Saline and Saline-immunized/KC, respectively) (Table 1). Mice with anti-KC:KC immune complexes in lungs (KC-immunized/KC) had significantly more (*P* < 0.05) MPO (indicator of transalveolar neutrophil migration as well as neutrophil activation) in BAL fluid than other groups of mice (Table 1). Similarly, the histologic evaluation of lung tissues revealed significant changes only in KC-immunized/KC mice (*P* < 0.05, versus other groups of mice) (Table 1).

Moreover, anti-KC autoantibodies by themselves appear not to be inflammatory as evident by lack of neutrophil influx (MPO) and histologic changes in KC-immunized/Saline mice (Table 1). These mice developed plasma autoantibodies against KC due to immunization with KC. However, they did not differ from Saline-immunized/Saline mice in which autoantibodies were not induced when MPO concentrations and histologic indices were compared (Table 1).

In addition, MPO level was significantly higher in mice that were immunized with saline and had KC instilled intratracheally (Saline-immunized/KC) than in mice immunized and treated with saline (Saline-immunized/Saline) or in saline-treated mice immunized with KC (KC-immunized/Saline) (*P* < 0.05) (Table 1).

TABLE 1. ANTI-KC:KC COMPLEXES, MYELOPEROXIDASE IN BRONCHOALVEOLAR LAVAGE FLUID, AND LUNG TISSUE HISTOLOGIC CHANGES

Mice	Anti-KC:KC OD (450 nm)	MPO ($\mu\text{g/ml}$)	HC
Saline-immunized/Saline	0.00 (0.00–0.00)	0.33 ± 0.67	0.01 ± 0.03
Saline-immunized/KC	0.00 (0.00–0.06)	$1.38 \pm 0.90^{\dagger}$	0.05 ± 0.10
KC-immunized/Saline	0.00 (0.00–0.10)	0.21 ± 0.17	0.05 ± 0.06
KC-immunized/KC	$0.18 (0.13–0.35)^{*}$	$2.00 \pm 0.85^{\ddagger}$	$1.60 \pm 0.38^{\S}$
KO/KC-immunized/KC	$0.16 (0.11–0.27)^{*}$	$0.80 \pm 0.27^{\dagger}$	0.16 ± 0.17
KO/Saline-immunized/Saline	0.00 (0.00–0.00)	0.28 ± 0.21	0.02 ± 0.03

Values are median with 25 to 75 percentile or mean $\pm$ SD, and 6 to 17 mice per group were evaluated with the exception of KO/Saline-immunized/Saline mice, where 3 mice were evaluated, and specifically, for anti-KC:KC measurements 3 to 16 mice per group were used, for MPO measurements 3 to 17, and for HC analysis 3 to 8 animals per group.

Definition of abbreviations: HC, histologic changes; KO, knockout; MPO, myeloperoxidase.

* $P < 0.05$ compared with remaining groups of mice.

† $P < 0.05$ compared with Saline-immunized/Saline, KC-immunized/Saline, and KO/Saline-immunized/Saline.

‡ $P < 0.05$ compared with Saline-immunized/Saline, Saline-immunized/KC, KC-immunized/Saline, KO/KC-immunized/KC, KO/Saline-immunized/Saline.

§ $P < 0.05$ compared with remaining groups of mice.

Even though BAL fluid concentration of MPO was increased in Saline-immunized/KC mice, we observed no histologic changes in lung tissues from these mice (Table 1). Our findings agree with previously published information on function of KC in the lung, which is primarily recruitment of neutrophils without causing damage to the lung (25, 26).

One of the main objectives of the current study was to develop a model of anti-chemokine:chemokine immune complex-induced lung inflammation and evaluate pulmonary responses due to presence of these complexes. Therefore, in the subsequent parts of the article we focus on more detailed assessment of inflammatory responses in lungs of KC-immunized/KC mice, a group with anti-KC:KC complexes in BAL fluid. Moreover, these mice were compared with KO mice that lack receptors for immune complexes (*see* following section).

Mice Lacking Functional Expression of Stimulatory Fc γ Rs

Activity of immune complexes, such as anti-KC autoantibody:KC complexes, is mediated by receptors for IgG (Fc γ Rs). Mice express Fc γ RI and Fc γ RIII (27). These receptors interact with immune complexes and trigger cellular activation in the lungs (28). To study the function of IgG receptors in our model, we used γ -chain-deficient mice (lacking functional expression of both Fc γ RI and Fc γ RIII).

One group of KO mice was immunized with saline only and had saline administered intratracheally (KO/Saline-immunized/Saline). These mice did not differ from their WT counterparts (Saline-immunized/Saline mice) (Table 1), indicating that baseline parameters are similar in KO and WT mice. A second group of KO mice was immunized with KC. Importantly, these mice developed similar amounts of circulating anti-KC autoantibodies to other groups of mice immunized with KC (KC-immunized/Saline and KC-immunized/KC, respectively). The corresponding changes in absorbance expressed as median values with 25 to 75 percentile were: 0.15 (0.13–0.18), 0.21 (0.16–0.37), and 0.17 (0.14–0.56) for KO, KC-immunized/Saline, and KC-immunized/KC mice, respectively ($P > 0.05$). After the presence of autoantibodies was confirmed, KO mice had KC administered intratracheally (KO/KC-immunized/KC mice). The levels of anti-KC:KC complexes in BAL fluid of these mice were similar to those of WT mice (KC-immunized/KC) ($P > 0.05$) (Table 1).

However, the concentration of MPO in BAL fluid was decreased in KO mice ($P < 0.05$) (Table 1). There were also no appreciable histologic changes detected in KO/KC-immunized/KC mice (Table 1).

Furthermore, decreased numbers of neutrophils were present in the BAL fluid of these mice as compared with KC-immunized/KC mice ($5.1 \pm 2.8 \times 10^6$ cells/ml versus $9.8 \pm 9.1 \times 10^6$ cells/ml) (Figure 1A). KC-immunized/KC mice, on the other hand, had more neutrophils than KC-immunized/Saline mice ($9.8 \pm 9.1 \times 10^6$ cells/ml versus $0.3 \pm 0.5 \times 10^6$ cells/ml) (Figure 1A). The percentage of red blood cells was also significantly higher ($P < 0.05$) in the former group of mice compared with KC-immunized/Saline mice and KO/KC-immunized/KC mice (Figure 1B). Further, the total number of red blood cells in the BAL fluid was determined as described in MATERIALS AND METHODS. The KC-immunized/KC mice had substantially more red blood cells (indicator of alveolar hemorrhage) when compared with KC-immunized/Saline and KO/KC-immunized/KC mice ($P < 0.05$) ($31.1 \pm 22.0 \times 10^6$ cells/ml, $2.4 \pm 2.9 \times 10^6$ cells/ml, and $10.2 \pm 9.1 \times 10^6$ cells/ml, respectively). Similarly, the concentrations of albumin (indicator of pulmonary vascular and epithelial permeability) were significantly higher ($P < 0.05$) in KC-immunized/KC mice than KC-immunized/Saline mice and γ -chain-deficient mice (KO/KC-immunized/KC) (209.2 ± 39.7 $\mu\text{g/ml}$, $122.0 \pm 72.5.0$ $\mu\text{g/ml}$, and 157.4 ± 39.0 $\mu\text{g/ml}$, for KC-immunized/KC mice, KC-immunized/Saline mice, and KO/KC-immunized/KC mice, respectively). In addition, the concentrations of IL-1 β and IL-6 in lung tissue homogenates were higher ($P < 0.05$) in KC-immunized/KC mice than KC-immunized/Saline and KO/KC-immunized/KC mice (Figures 1C and 1D). Moreover, histologic analysis revealed decreased infiltration of inflammatory cells and interstitial thickening as well as absence of appreciable alveolar exudates (Figure 2) in KO/KC-immunized/KC mice.

To evaluate deposition of anti-KC:KC complexes in the lungs, tissue sections were incubated with FITC-conjugated anti-mouse IgG antibody. As shown in Figure 3A, IgG was easily detectable in specimen from KC-immunized/KC mice (*green*) indicating the presence of immune complexes associated with the lung tissue (28). The same tissue sections were also incubated with biotinylated antibody against mouse KC followed by Texas Red-conjugated streptavidin. There is substantial staining (*red*) in tissue from KC-immunized/KC mice (Figure 3A). Merging of “green” and “red” channels resulted in significant co-localization (*yellow*) in lung tissue from these mice (Figure 3A). These results suggest that a significant portion of IgG is associated with KC forming anti-KC:KC complexes in lung tissue from KC-immunized/KC mice. IgG (*green*) was not visible in tissue from KC-immunized/Saline mice or KO/KC-immunized/KC mice (Figure 3A). Staining for mouse KC (*red*) was also negligible, and, moreover, virtually no co-localization (*yellow*) was observed in these tissues (Figure 3A), indicating the absence of appreciable deposition of anti-KC:KC immune complexes in the lungs of KC-immunized/Saline, and KO/KC-immunized/KC mice (Figure 3A). In agreement with these observations correlation coefficients for co-localization between IgG and KC were 0.88, 0.06, and 0.03 for KC-immunized/KC, KC-immunized/Saline, and KO/KC-immunized/KC mice, respectively.

In addition, since our previous studies show that ERK, Akt, and p-38 pathways are evoked by anti-IL-8:IL-8 complexes in human neutrophils *in vitro* (16), we evaluated activation of these kinases in lung tissues of WT and KO mice. The phosphorylation of ERK, Akt, and p-38 was significantly increased in mice that had anti-KC:KC complexes in lungs (KC-immunized/KC) compared with KC-immunized/Saline and KO/KC-immunized/KC mice (Figure 3B), suggesting an important role for ERK, Akt,

Figure 1. Cells in bronchoalveolar lavage (BAL) fluid and cytokines in lung tissue homogenates. (A) Cytopsin of BAL fluid samples stained with Xanthene Dye and Thiazine Dye Mixture (Hema 3 Kit). (B) Percentage of erythrocytes in BAL fluids. (C) Concentrations of IL-1 β and (D) IL-6 in lung tissue homogenates. Wild-type (WT) mice were immunized with KC and treated with saline (KC-immunized/Saline group) or KC (KC-immunized/KC group) (left and middle panels in A or left and middle bars in B–D), and KO mice were immunized with KC and treated with KC (KO/KC-immunized/KC group) (right panel in A or right bars in B–D). * $P < 0.05$ versus KC-immunized/Saline and KO/KC-immunized/KC group mice. Nine to seventeen animals per group were analyzed, and typical findings are presented (A, B), or average of measurements from nine to sixteen animals (IL-1 β ; C) or six to twelve (IL-6; D) per group was presented.

Figure 2. Hematoxylin and eosin staining of lung sections. WT mice were immunized with KC and treated with saline (KC-immunized/Saline group) or KC (KC-immunized/KC group), and KO mice were immunized with KC and treated with KC (KO/KC-immunized/KC group). Nine to seventeen animals per group were analyzed, and typical findings are presented.

and p-38 pathways in lung inflammation triggered by anti-KC:KC complexes. Further, phosphorylated ERK, Akt, and p-38 were detected in inflammatory cells (neutrophils, epithelial cells, and macrophages) (data not shown). In summary, our data indicate that the inflammatory response in the lung related to formation of anti-KC:KC immune complexes is substantially down-regulated in γ -chain-deficient mice (lacking stimulatory Fc γ Rs).

LPS-Induced Lung Inflammation

Because sepsis is the most common risk factor for ARDS and quite a few cases are due to peritonitis (1, 29), we also evaluated a model of LPS-induced lung inflammation, in which LPS was administered intraperitoneally. We used this model to investigate whether administration of LPS to mice generates anti-KC:KC complexes in lungs. We also used γ -chain-deficient mice that lack receptors for immune complexes to evaluate contribution of anti-KC:KC complexes to lung inflammation/injury. Anti-KC:KC complexes were detected in the BAL fluid of mice treated with intraperitoneal LPS (WT/LPS) for 14 hours ($P < 0.05$; compared with control mice treated with saline; WT/Sal) (Table 2). KO mice treated with intraperitoneal LPS (KO/LPS) for 14 hours had amounts of the complexes similar to those of WT/LPS mice (Table 2). However, the concentration of MPO in lung tissue was significantly lower in this group of mice ($P < 0.05$) than in WT mice treated with intraperitoneal LPS (Table 2). On the other hand, there was no difference between studied

groups in the respect to MPO concentrations in lavage fluid ($P > 0.05$) (data not shown). The amount of MPO was very low, and was similar to that present in BAL fluid of KC-immunized/Saline mice ($P > 0.05$). In addition, significant histologic changes were observed only in lung tissues of WT/LPS mice ($P < 0.05$ versus WT/Sal and KO/LPS) (Table 2). It should also be stressed that KO mice injected with saline only (via intraperitoneal route) did not differ from control mice (i.e., WT/Sal mice) (Table 2), indicating that baseline parameters are similar in KO and WT mice.

Moreover, an increased number of red blood cells was present in the BAL fluid of WT/LPS mice compared with WT/Sal and KO/LPS mice ($4.2 \pm 3.3 \times 10^6$ cells/ml, $0.1 \pm 0.1 \times 10^6$ cells/ml, and $1.2 \pm 2.0 \times 10^6$ cells/ml, respectively). However, virtually no neutrophils were detected in the BAL fluid of any group of mice (Figure 4A). These results are consistent with lack of appreciable influx of neutrophils after administration of intraperitoneal LPS. On the other hand, the concentration of MPO in lung tissue was significantly higher ($P < 0.05$) in WT/LPS mice (Table 2). Our findings agree with previously published observations in mice treated with LPS via the intraperitoneal route (30–32). Virtually no neutrophils are found in BAL fluid; however, in lung tissue, neutrophils adhere to endothelium of small parenchymal vessels, suggesting that effect of intraperitoneally administered LPS is limited to intravascular sequestration of neutrophils (31, 32).

The concentration of MPO in lung tissue comprises both MPO residing in neutrophils and MPO released from the cells. The amount of lung tissue MPO is similar after LPS administration over time (32, and data not shown). However, the number of intact neutrophils in lung vasculature declines with time after LPS treatment, and maximum is reached between 0.5 and 2 hours (30). Accordingly, neutrophils were easily detectable in lungs of mice at 1 hour after injection of LPS (Figure 4B).

Further, percentage of erythrocytes was significantly higher in WT/LPS mice than WT/Sal or KO/LPS mice after 14 h ($P < 0.05$) (Figure 4C). Normal mice injected with LPS (WT/LPS) had similar albumin concentrations in lavage as compared with WT/Sal and KO/LPS mice (122.8 ± 43.7 μ g/ml, 105.8 ± 57.1 μ g/ml, and 94.8 ± 28.0 μ g/ml for WT/LPS, WT/Sal, and KO/LPS mice, respectively). Lack of a difference in albumin concentrations between WT/LPS and WT/Sal mice agrees with previously reported finding in mice treated with intraperitoneal LPS (31). However, albumin may sometimes not reflect adequately changes in permeability (33). Therefore, we measured total protein concentrations in lavage samples. We detected very modest increase in protein levels in LPS-treated mice (WT/LPS) as compared with WT/Sal and KO/LPS mice (165.9 ± 20.0 μ g/ml, 145.2 ± 58.0 μ g/ml, and 129.5 ± 42.2 μ g/ml for WT/LPS, WT/Sal, and KO/LPS, respectively) ($P < 0.05$). Interestingly, there was no change in total protein concentration in BAL fluid from WT/LPS mice at 8 hours (data not shown). These findings indicate that there is only subtle increase in vascular permeability in WT/LPS mice.

In addition, the concentration of IL-1 β in lung tissue homogenates was higher ($P < 0.05$) in WT/LPS mice than WT/Sal and KO/LPS mice (Figure 4D). Moreover, increased cellularity and interstitial thickening, and presence of alveolar exudates, were evident in WT/LPS mice but attenuated in KO/LPS mice as revealed by histologic analysis (Figure 5).

Influx of inflammatory cells, increase in concentrations of cytokines, and elevated activity of MPO are all characteristics of lung inflammation. Appearance of erythrocytes and plasma proteins in the lung lavage fluid is indicative of lung injury, whereas results of direct histopathologic evaluation of lung tissues are used to determine whether damage to the lungs actually occurred (31, 33).

Figure 3. (A) Deposition of anti-KC:KC immune complexes in the lungs, and (B) phosphorylation of extracellular signal-regulated kinase (ERK), Akt, and p-38 in lung tissues. WT mice were immunized with KC and treated with saline (KC-immunized/Saline group) or KC (KC-immunized/KC group), and KO mice were immunized with KC and treated with KC (KO/KC-immunized/KC group). Four to six (A) or four to five (B) animals per group were analyzed, and typical findings are presented.

We also examined deposition of anti-KC:KC complexes in lung tissues at 8 and 14 hours. Staining with anti-mouse IgG antibody (green) was detected only in WT mice treated with intraperitoneal LPS (WT/LPS) for 14 hours as depicted in Figure 6A, indicating presence of immune complexes associated with the lung tissue (28). The same tissue sections were also incubated with biotinylated antibody against mouse KC and Texas Red-labeled streptavidin (red) (Figure 6A). IgG co-localized with KC in lung tissues from normal mice treated with intraperitoneal LPS (WT/LPS) for 14 hours (yellow) (Figure 6A), indicating formation of complexes between of IgG and KC. These results, though unexpected, suggest that lung tissue deposition of immune complexes, such as anti-KC:KC complexes, occurs in WT/LPS mice (Figure 6A). IgG (green) was almost undetectable, as was staining for KC (red) in lung tissues from WT/Sal and KO/LPS mice (Figure 6A). Co-localization (yellow) between IgG and KC was also minimal in these tissues (Figure 6A), indicating that virtually no anti-KC:KC complex deposition was present in WT/Sal and KO/LPS mice. We calculated correlation coefficients to validate co-localization data which were 0.74, 0.02, and 0.04 for WT/LPS, WT/Sal, and KO/LPS mice, respectively. Similar results were obtained for mice treated with intraperitoneal LPS or saline for 8 hours (i.e., presence of anti-KC:KC complexes in lung tissues from animals which received LPS) (data not shown).

Intratracheal instillation of LPS induces alveolar influx of neutrophils and substantial change in permeability, and causes severe lung injury (30). We analyzed lung tissues from mice which were given LPS via intratracheal route for the presence of anti-KC:KC complexes, and we were able to detect deposition of these complexes at 6 hours after LPS treatment

TABLE 2. ANTI-KC:KC COMPLEXES IN BRONCHOALVEOLAR LAVAGE FLUID, AND LUNG TISSUE MYELOPEROXIDASE AND HISTOLOGIC CHANGES

Mice	Anti-KC:KC OD (450 nm)	MPO ($\mu\text{g}/\text{ml}$)	HC
WT/Saline	0.00 (0.00–0.03)	4.84 ± 1.56	0.00 ± 0.00
WT/LPS	0.10 (0.10–0.12)*	$7.94 \pm 3.31^\dagger$	$1.78 \pm 0.16^\ddagger$
KO/LPS	0.12 (0.03–0.43)*	3.44 ± 0.92	0.12 ± 0.19
KO/Saline	0.00 (0.00–0.00)	3.34 ± 0.68	0.01 ± 0.02

Values are median with 25 to 75 percentile or mean $\pm$ SD, and 4 to 11 mice per group were evaluated at 14 hours after intraperitoneal saline or LPS administration. For anti-KC:KC measurements 5 to 13 mice per group were used, for MPO measurements 4 to 11, and for HC analysis 5 to 6 animals per group.

For definition of abbreviations see Table 1.

* $P < 0.05$ compared with WT/Saline.

$^\dagger P < 0.05$ compared with remaining groups of mice.

$^\ddagger P < 0.05$ compared with remaining groups of mice.

Figure 4. Cells in BAL fluid and neutrophils in lung tissue, and concentrations of IL-1 β in lung tissue homogenates. (A) Cytospins of BAL fluid samples stained with Xanthene Dye and Thiazine Dye Mixture (Hema 3 Kit). WT mice were treated with saline (WT/Sal) or LPS (WT/LPS) for 14 hours (*left and middle panels*), and KO mice were treated with LPS (KO/LPS) for 14 hours (*right panel*). (B) Histochemical staining of neutrophils in lung tissue from WT/Sal and WT/LPS mice at 1 hour after treatment. (C) Percentage of erythrocytes in BAL fluids at 14 hours after treatment. (D) Concentrations of IL-1 β in lung tissue homogenates. WT mice were treated with saline (WT/Sal) or LPS (WT/LPS) for 14 hours (*left and middle bars*), and KO mice were treated with LPS (KO/LPS) for 14 hours (*right bar*). * $P < 0.05$ versus WT/Sal and KO/LPS mice. Seven to thirteen animals per group were analyzed, and typical findings are presented (A, C), or one set of lung tissues from mice was stained for neutrophils (B), or average of measurements from seven to thirteen animals per group (D) was presented.

(Figure 6A); the correlation coefficient was 0.89. On the other hand, no complexes were present in lungs of mice that had saline administered intratracheally (data not shown). We also evaluated the tissues for histologic changes indicative of lung injury and confirmed presence of lung injury in these animals (histologic change values were 1.76 ± 0.10 for mice that received intratracheal LPS and 0.00 ± 0.00 for mice that received intratracheal saline).

Finally, we evaluated activation of ERK, Akt, and p-38 in the lungs of WT/LPS, WT/Sal, and KO/LPS mice at 14 hours. We detected significantly increased phosphorylation of ERK, Akt, and p-38 in WT/LPS mice but not in the WT/Sal or KO/

LPS mice (Figure 6B). Further, phosphorylated ERK, Akt, and p-38 were detected in inflammatory cells (neutrophils, epithelial cells, and macrophages) (data not shown).

Our results show that the γ -chain-deficient mice that lack receptors for immune complexes develop less severe pulmonary inflammation in response to LPS, indicating that immune complexes are involved in the pathogenesis of LPS induced lung inflammation. The intraperitoneal route of LPS administration produces lung inflammation without changing lung epithelial permeability to the significant extent seen in KC-immunized/KC mice. Increase in alveolar permeability is rather modest and occurs more than 8 hours after LPS treatment. On the other

Figure 5. Hematoxylin and eosin staining of lung sections. WT mice were treated with saline (WT/Sal) or LPS (WT/LPS), and KO mice were treated with LPS (KO/LPS). Seven to thirteen animals per group were analyzed, and typical findings are presented.

hand, deposition of anti-KC:KC complexes is detectable at 8 hours after intraperitoneal LPS administration. Moreover, at 8 hours anti-KC:KC complexes are present in lavage fluid, even though permeability still remains unchanged (data not shown). This strategy is well suited to study the appearance of immune complexes, such as anti-KC:KC complexes, in BAL fluids and lung tissues, where it is not necessary to differentiate between complexes formed locally and complexes coming from blood.

DISCUSSION

Although the pathophysiologic properties of autoantibody-containing immune complexes that relate to human disease (e.g., rheumatoid arthritis, diabetes, and thrombocytopenia) are well recognized (34), little is known about the possible involvement of anti-IL-8:IL-8 complexes in triggering and/or maintaining the inflammatory response in lung injury. Several key observations made by our laboratory support the likelihood that anti-IL-8:IL-8 complexes contribute to the initiation, potentiation, and severity of acute lung injury in humans. First, the presence of elevated concentrations of anti-IL-8:IL-8 complexes in lung fluids is associated with progression to ARDS (14, 15). Second, the absolute level of these complexes in the lungs is associated with mortality (14). In contrast, there is no consistent relationship between the concentration of IL-8 in BAL fluid and the course of the disease in ARDS (13–15). Moreover, we have evaluated lung tissues from patients with

lung injury for the presence of anti-IL-8:IL-8 complexes by confocal microscopy. IL-8 co-stained with IgG and immune complex receptors, FcγRIIa, in lung tissues from patients with acute respiratory distress syndrome but not in control tissues, suggesting that anti-IL-8:IL-8 complexes are deposited in lungs of patients with ARDS via FcγRIIa (19). We were also able to detect anti-IL-8:IL-8 complexes bound to neutrophils present in the alveolar spaces of these patients (our unpublished information).

We have developed a mouse model to evaluate the contribution of anti-KC:KC complexes generated *in situ* to lung inflammation and injury (autoimmune complex-induced lung inflammation). In this model autoantibodies to KC in plasma and the alveolar compartment are first induced by immunization with KC. Once the animals develop anti-KC autoantibodies, KC is administered intratracheally to generate anti-KC:KC complexes in the lung. In these animals we found increased transalveolar influx of neutrophils, increased permeability, and alveolar hemorrhage, together with histologic evidence of increased infiltration of inflammatory cells, interstitial thickening, and presence of alveolar exudate. All of these findings indicate the presence of severe pulmonary inflammation and alveolar damage.

Moreover, γ-chain-deficient mice lacking stimulatory FcγRs have substantially attenuated pulmonary inflammatory responses, suggesting that the activity of anti-KC:KC complexes is mediated by receptors for IgG (FcγRs). In support of this conclusion, we found that KO mice immunized and treated with KC had amounts of anti-KC:KC complexes in lavage fluids similar to those of WT mice, but virtually no tissue deposition when lung tissues were examined. This finding suggests that the complexes were formed in the lungs of KO mice but were not able to display activity because of lack of relevant receptors in these mice. The observation that phosphorylation of ERK, Akt, and p-38, essential components of FcγR signaling pathway, was significantly decreased in KO mice further strengthens this interpretation.

The possibility that autologous immune complexes, like anti-IL-8:IL-8 complexes or anti-KC:KC complexes in mice, may be involved in the pathogenesis of lung inflammation/injury has not been considered before. It is known that the deposition of heterologous immune complexes (reverse passive Arthus reaction) can trigger a localized inflammatory response in different tissues, including the lung (28, 35–37); however, the models of immune complex-induced alveolitis differ substantially from our model. A foreign antigen is given intravenously, and immediately after that an antibody against this antigen (usually rabbit antibody) is administered intratracheally. This leads to local formation of heterologous immune complexes, which then trigger the alveolar inflammatory response. In our model mice are immunized with murine antigen (KC) for several weeks. After autoantibodies develop, the antigen (KC) is administered intratracheally, and autologous immune complexes (anti-KC:KC complexes) form in the lung. We believe that this model mimics very well the situation observed in patients with ARDS who have anti-IL-8 autoantibody:IL-8 complexes in their lung fluids as well as deposited in lung tissue (13–15, 19).

The lung inflammation induced by heterologous immune complexes is complement dependent, and specifically C5aR plays a crucial role in initiating of the alveolar inflammation (36–38). However, a few reports suggest a more predominant role for FcγRs (39, 40), and that complement may play a secondary role in immune complex induced inflammation (40). Indeed, the most recent studies indicate that C5a is only indirectly involved in mediating activity of immune complexes, acting by regulating the expression of FcγRs (40). In our model, FcγRs are important for the development of lung inflammation,

Figure 6. (A) Deposition of anti-KC:KC immune complexes in the lungs. WT mice were treated with saline (WT/Sal) or LPS (WT/LPS) for 14 hours, or intratracheal LPS and analyzed at 6 hours, and KO mice were treated with LPS (KO/LPS) for 14 hours. (B) Phosphorylation of ERK, Akt, and p-38 in lung tissues. WT mice were treated with saline (WT/Sal) or LPS (WT/LPS) for 14 hours, and KO mice were treated with LPS (KO/LPS) for 14 hours. Four to five (A) or three to five (B) animals per group were analyzed, and typical findings are presented.

but a role for complement remains possible. Furthermore, KC receptors could also be involved in mediating the proinflammatory activity of anti-KC:KC complexes. Anti-IL-8:IL-8 complexes have the ability to bind to IL-8 receptors on human neutrophils, and both FcγRIIa and IL-8 receptors mediate chemotactic activity of the complexes, with FcγRIIa being a predominant receptor (16). Other anti-cytokine:cytokine immune complexes can also interact with receptors specific for a cytokine present in the complex (41).

Even though certain animal models, such as those involving direct pulmonary insult or dual hit models, are thought to reflect more adequately pathophysiologic changes that are characteristic of full-blown ARDS (26, 31), ALI/ARDS can occur after peritoneal sepsis. In fact, extrapulmonary ARDS is quite frequent and many cases are due to peritonitis (1, 29). Most importantly, we chose the intraperitoneal route of LPS administration to generate a relatively straightforward model of mild lung injury. Increase in alveolar permeability is rather modest and occurs more than 8 hours after LPS treatment. We also found that immune complexes, including anti-KC:KC complexes, are present in lungs of mice treated with LPS, and may contribute to lung inflammation, because inflammatory responses to LPS

are diminished in γ-chain-deficient mice. A substantial portion of IgG present in lung tissue co-localizes with KC in LPS-treated animals, indicating that the deposited immune complexes consist to a large extent of anti-KC:KC complexes. We believe that the presence of anti-KC autoantibody:KC immune complexes in BAL fluids and lung tissues of LPS mice is not a consequence of increased vascular permeability. Overall change in alveolar permeability is rather modest in these mice and occurs more than 8 hours after LPS treatment. On the other hand, deposition of anti-KC:KC complexes is already detectable at 8 hours after intraperitoneal LPS administration, and anti-KC:KC complexes are present in lavage fluid even though permeability still remains unchanged.

Our results indicate that changes in alveolar permeability occurring in WT/LPS mice are relatively mild. We detected very modest increase in protein levels in LPS-treated mice (WT/LPS) as compared with saline controls and KO mice (WT/Sal and KO/LPS, respectively). Even though percentages of erythrocytes are similar in WT/LPS mice and KC-immunized/KC mice (animals that were immunized with KC and had KC instilled intratracheally), the number of erythrocytes was much smaller in WT/LPS mice compared with KC-immunized/KC

mice ($4.2 \pm 3.3 \times 10^6$ cells/ml and $31.1 \pm 22.0 \times 10^6$ cells/ml, respectively).

Importance of anti-KC:KC complexes in LPS-induced lung inflammation was supported by experiments with mice treated with intratracheal LPS. Intratracheal instillation of LPS induces alveolar influx of neutrophils and substantial change in permeability, and causes severe lung injury (30). We analyzed lung tissues from mice that were given LPS via intratracheal route for the presence of anti-KC:KC complexes, and we were able to detect deposition of anti-KC:KC complexes. Our findings support the hypothesis that anti-KC:KC complexes play a role in pathogenesis of lung inflammation and injury. In agreement with this concept, we showed deposition of anti-IL-8:IL-8 complexes in lung tissues from patients with ARDS but not control tissues (19). (IL-8 is functionally related to KC in mice that do not express IL-8 [20].)

Furthermore, KO mice (i.e., γ -chain-deficient mice lacking stimulatory Fc γ Rs) were protected from LPS-induced lung inflammation. There was virtually no lung tissue deposition of anti-KC:KC complexes (which were, however, present in lavage fluid in quantities similar to those detected in WT/LPS mice). These findings indicate that anti-KC:KC complexes were formed in the lungs of KO mice but did not contribute to the inflammatory response in the lung because of lack of relevant receptors in these mice. The activity of anti-KC:KC complexes is mediated by receptors for IgG (Fc γ Rs) what is evident from studies conducted on KC-treated mice and was confirmed using KO mice treated with intratracheal KC.

Our findings suggest for the first time that there is a possible link between LPS-induced lung inflammation and autoimmune responses. A recent study showing ability of LPS to induce a relapse of autoimmune encephalomyelitis in normal mice supports this hypothesis (42). Further, LPS is capable of inducing autoantibody production in mice (43). In agreement with this finding, we detected production of anti-KC autoantibodies by splenocytes from mice that received LPS, upon stimulation with LPS, but not by splenocytes from saline-treated mice (data not shown). It has been postulated that LPS could stimulate proliferation and differentiation of B cells by bridging the B cell receptor with Toll-like receptors (44). Further, antibody-forming cells are capable of migrating to the lung from the spleen (45). Taking into consideration the presence of cells producing anti-KC autoantibodies in the lungs of mice injected intraperitoneally with LPS, and also the release of KC in response to LPS treatment it is logical that anti-KC:KC complexes will be formed.

Our studies demonstrate that anti-KC:KC complexes contribute to lung inflammation and ultimately may cause lung injury. Excessive activation of various signaling pathways by the complexes (e.g., Akt, ERK, p-38) will lead to release of unrestrained amounts of proinflammatory mediators, recruitment, and activation of abundant inflammatory cells, and tissue injury within the lung. The clinical relevance of these events is underscored by the observation that activation of specific signaling proteins, such as Akt, is related to survival in patients with ARDS (46).

Activity of immune complexes is mediated by receptors for IgG (Fc γ Rs), and mice express two types of stimulatory Fc γ Rs, Fc γ RI and Fc γ RIII (27, 28). Therefore, to study the function of IgG receptors we used mice lacking functional expression of both Fc γ RI and Fc γ RIII (γ -chain-deficient mice). Our results demonstrate that anti-KC autoantibodies develop normally in KO mice, and anti-KC autoantibody:KC complexes are present in lavage fluid in quantities similar to those detected in WT mice. This is true for both mouse models characterized in the current study (i.e., LPS- and anti-KC:KC complex-induced lung

inflammation). On the other hand, our data indicate that the inflammatory response in the lung, related to formation of anti-KC:KC immune complexes, is substantially downregulated in γ -chain-deficient mice (lacking stimulatory Fc γ Rs). This is consistent with the fact that activity of immune complexes, such as anti-KC:KC complexes, is mediated by Fc γ Rs (28). Our *in vitro* studies showing that anti-IL-8 autoantibody:IL-8 complexes display their pro-inflammatory activity by binding to Fc γ Rs (specifically Fc γ RIIIa) also support this concept (16). In addition, γ -chain-deficient mice are protected from alveolar inflammation induced by initiating of reverse passive Arthus reaction in the lung (intravenous injection of ovalbumin followed by intratracheal administration of a rabbit antibody against chicken egg albumin to form anti-ovalbumin:ovalbumin complexes in lungs of these animals) (28, 39). Moreover, no lung tissue deposition of the anti-KC:KC complexes was detected in KO mice in either model (i.e., LPS- or anti-KC:KC complex-induced lung inflammation). These observations are in agreement with data derived from a parallel comparison of BALB/c mice with systemic autoimmunity induced by administration of mercury and γ -chain-deficient mice also treated with mercury (47). Deposits containing IgG were not present in kidneys and spleens obtained from KO mice, indicating that Fc γ Rs are required for the formation of tissue deposits of immune complexes in autoimmune disease (47). Similar conclusions can be reached on the basis of our findings—that is, absence of anti-KC:KC immune complexes in lung tissues of KO mice.

In summary, we showed that anti-chemokine:chemokine immune complexes containing autoantibodies can induce severe lung inflammation in mice. This finding supports our prior observations describing proinflammatory activity of anti-IL-8:IL-8 complexes purified from lung fluids obtained from patients with acute lung injury (16). Our model of chemokine-autoimmune complex-triggered lung inflammation is ideal for studying the function of such complexes *in vivo*, and can be used to test of new therapeutic interventions. The observation that anti-KC:KC complexes are deposited in lungs of LPS-treated mice, and may contribute to development of lung inflammation in these animals, is novel, and provides a mechanism to understand some of the pathologic features of acute lung injury.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD. Incidence and outcome of acute lung injury. *N Engl J Med* 2005;353:1685–1693.
2. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–1349.
3. Weiland JE, Davis WB, Holter JF, Mohammed JR, Dorinsky PM, Gadek JE. Lung neutrophils in the adult respiratory distress syndrome: clinical and pathological significance. *Am Rev Respir Dis* 1986;133:218–225.
4. Martin TR, Pistorese BP, Hudson LD, Maunder RJ. The function of lung and blood neutrophils in patients with the adult respiratory distress syndrome: implications for the pathogenesis of lung infections. *Am Rev Respir Dis* 1991;144:254–262.
5. Gadek JE. Adverse effects of neutrophils on the lung. *Am J Med* 1992;92:27S–31S.
6. Miller EJ, Cohen AB, Nagao S, Griffith D, Maunder RJ, Martin TR, Weiner-Kronish JP, Sticherling M, Christophers E, Matthay MA. Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am Rev Respir Dis* 1992;146:427–432.
7. Jorens PG, Van Damme J, De Backer W, Bossaert L, De Jongh RF, Herman AG, Rampart M. Interleukin-8 (IL-8) in the bronchoalveolar

- lavage fluid from patients with adult respiratory distress syndrome (ARDS) and patients at risk for ARDS. *Cytokine* 1992;4:592–597.
8. Cholle Martin S, Montravers P, Gibert C, Elbim C, Desmonts JM, Fagon JY, Gougerot-Pocidalo MA. High levels of interleukin-8 in the blood and alveolar spaces of patients with pneumonia and adult respiratory distress syndrome. *Infect Immun* 1993;61:4553–4559.
 9. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC, Grant IS, Pollok AJ, Haslett C. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 1993;341:643–647.
 10. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. *Chest* 1995;108:1303–1314.
 11. Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maunder RJ, Kunkel SL, Walz A, Hudson LD, Martin TR. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1996;154:602–611.
 12. Martin TR, Goodman RB. The role of chemokines in the pathophysiology of the acute respiratory distress syndrome (ARDS). In: Hebert CA, editor. *Chemokines in disease*. Totowa, NJ: Humana Press; 1999. pp. 81–110.
 13. Kurdowska A, Miller EJ, Noble JM, Baughman RP, Matthay MA, Brelsford WG, Cohen AB. Anti-interleukin-8 autoantibodies in alveolar fluid from patients with the adult respiratory distress syndrome. *J Immunol* 1996;157:2699–2706.
 14. Kurdowska A, Noble JM, Steinberg KP, Ruzinski J, Hudson LD, Martin TR. Anti-interleukin-8 autoantibody: interleukin-8 complexes in the acute respiratory distress syndrome: relationship between the complexes and clinical disease activity. *Am J Respir Crit Care Med* 2001;163:463–468.
 15. Kurdowska A, Noble JM, Grant IS, Robertson R, Haslett C, Donnelly SC. Anti-interleukin-8 autoantibodies in patients at risk for the acute respiratory distress syndrome. *Crit Care Med* 2002;30:2335–2337.
 16. Krupa A, Kato H, Matthay MA, Kurdowska A. Proinflammatory activity of anti-IL-8 autoantibody: IL-8 complexes in alveolar edema fluid from patients with acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2004;286:1105–1113.
 17. Fudala R, Krupa A, Matthay MA, Allen TC, Kurdowska AK. Anti-IL-8 autoantibody: IL-8 immune complexes suppress spontaneous apoptosis of neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2007;293:364–374.
 18. Kurdowska A, Miller EJ, Alden SM, Noble JM, Harmon C. Anti-interleukin-8 (IL-8) autoantibody is an important modulator of IL-8 function in the lung [abstract]. *FASEB J* 2000;14:A137.7.
 19. Allen TC, Fudala R, Nash S, Kurdowska A. Anti-interleukin-8 autoantibody: interleukin-8 immune complexes visualized by laser confocal microscopy in injured lung: co-localization with FcγRIIa in lung tissues from patients with acute respiratory distress syndrome. *Arch Pathol Lab Med* 2007;131:452–456.
 20. Bozic CR, Kolakowski LF, Gerard NP, Garcia-Rodriguez C, von Uexkull-Guldenband C, Conklyn MJ, Breslow R, Showell HJ, Gerard C. Expression and biologic characterization of the murine chemokine KC. *J Immunol* 1995;154:6048–6057.
 21. Svenson M, Hansen MB, Thomsen AR, Diamant M, Nansen A, Rieneck K, Otterness IG, Bendtzen K. Cytokine vaccination: neutralising IL-1α autoantibodies induced by immunisation with homologous IL-1α. *J Immunol Methods* 2000;236:1–8.
 22. Saunders NB, Shoemaker DR, Brandt BL, Moran EE, Larsen T, Zollinger WD. Immunogenicity of intranasally administered meningococcal native outer membrane vesicles in mice. *Infect Immun* 1999;67:113–119.
 23. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982;78:206–209.
 24. Matute-Bello G, Frevert CW, Liles WC, Nakamura M, Ruzinski JT, Ballman K, Vong VA, Vathanaiprida C, Martin TR. Fas/Fas ligand system mediates epithelial injury, but not pulmonary host defenses, in response to inhaled bacteria. *Infect Immun* 2001;69:5768–5776.
 25. Lira SA. Genetic approaches to study chemokine function. *J Leukoc Biol* 1996;59:45–52.
 26. Nemzek JA, Call DR, Ebong SJ, Newcomb DE, Bolgos GL, Remick DG. Immunopathology of a two-hit murine model of acid aspiration lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L512–L520.
 27. Daeron M. Fc receptor biology. *Annu Rev Immunol* 1997;15:203–234.
 28. Ravetch JV, Bolland S. IgG Fc receptors. *Annu Rev Immunol* 2001;19:275–290.
 29. Pelosi P, D'Onofrio D, Chiumello D, Paolo S, Chiara G, Capelozzi VL, Barbas CSV, Chiaranda M, Gattinoni L. Pulmonary and extrapulmonary acute respiratory distress syndrome are different. *Eur Respir J* 2003;22:485–56S.
 30. Hirano S. Migratory responses of PMN after intraperitoneal and intratracheal administration of lipopolysaccharide. *Am J Physiol* 1996;270:836–845.
 31. Zhou Z, Kozlowski J, Schuster DP. Physiologic, biochemical, and imaging characterization of acute lung injury in mice. *Am J Respir Crit Care Med* 2005;172:344–351.
 32. Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, Kubes P. Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* 2003;111:1011–1020.
 33. Doyle IR, Nicholas TE, Bersten AD. Partitioning lung and plasma proteins: circulating surfactant proteins as biomarkers of alveolar capillary permeability. *Clin Exp Pharmacol Physiol* 1999;26:185–197.
 34. Plotz PH. The autoantibody repertoire: searching for order. *Nat Rev Immunol* 2003;3:73–78.
 35. Johnson KJ, Ward PA. Acute immunologic pulmonary alveolitis. *J Clin Invest* 1974;54:349–357.
 36. Mulligan MS, Yeh CG, Rudolph AR, Ward PA. Protective effects of soluble CR1 in complement- and neutrophil-mediated tissue injury. *J Immunol* 1992;148:1479–1485.
 37. Mulligan MS, Schmid E, Beck-Schimmer B, Till GO, Friedl HP, Brauer RB, Hugli TE, Miyasaka M, Warner RL, Johnson KJ, *et al.* Requirement and role of C5a in acute lung inflammatory injury in rats. *J Clin Invest* 1996;98:503–512.
 38. Guo RF, Ward PA. Mediators and regulation of neutrophil accumulation in inflammatory responses in lung: insights from the IgG immune complex model. *Free Radic Biol Med* 2002;33:303–310.
 39. Clynes R, Maizes JS, Guinamard R, Ono M, Takai T, Ravetch JV. Modulation of immune complex-induced inflammation in vivo by the coordinate expression of activation and inhibitory Fc receptors. *J Exp Med* 1999;189:179–185.
 40. Shushakova N, Skokowa J, Schulman J, Baumann U, Zwirner J, Schmidt RE, Gessner JE. C5a anaphylatoxin is a major regulator of activating versus inhibitory FcγRs in immune complex-induced lung disease. *J Clin Invest* 2002;110:1823–1830.
 41. Revoltella RP. Natural and therapeutically-induced antibodies to cytokines. *Biotherapy* 1998;10:321–331.
 42. Nogai A, Siffrin V, Bonhagen K, Pfueller CF, Hohnstein T, Volkmer-Engert R, Bruck W, Stadelmann C, Kamradt T. Lipopolysaccharide injection induces relapses of experimental autoimmune encephalomyelitis in nontransgenic mice via bystander activation of autoreactive CD4+ cells. *J Immunol* 2005;175:959–966.
 43. Dziarski R. Preferential induction of autoantibody secretion in polyclonal activation by peptidoglycan and lipopolysaccharide. II. *In vivo* studies. *J Immunol* 1982;128:1026–1030.
 44. Viau M, Zouali M. B-lymphocytes, innate immunity, and autoimmunity. *Clin Immunol* 2005;114:17–26.
 45. Bice DE, King-Herbert AP, Morris MJ, Hanna N, Haley PJ. Inflammation and recruitment of immune cells into the lung. *Reg Immunol* 1989;2:376–384.
 46. Yang KY, Arcaroli JJ, Abraham E. Early alterations in neutrophil activation are associated with outcome in acute lung injury. *Am J Respir Crit Care Med* 2003;167:1567–1574.
 47. Martinsson K, Hultman P. The role of Fc-receptors in murine mercury-induced systemic autoimmunity. *Clin Exp Immunol* 2006;144:309–318.