Anti-Chemokine Autoantibody:Chemokine Immune Complexes Activate Endothelial Cells via IgG Receptors

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Our previous studies revealed that the presence in lung fluids of anti-IL-8 autoantibody:IL-8 immune complexes is an important prognostic indicator for the development and outcome of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS). Anti-IL-8:IL-8 complexes purified from lung edema fluids trigger chemotaxis of neutrophils, induce activation of these cells, and regulate their apoptosis, all via IgG receptor, FcγRIIa. Importantly, increased levels of FcγRIIa are present in lungs of patients with ARDS, where FcγRIIa is partially associated with anti-IL-8:IL-8 complexes. In the current study, we demonstrate the ability of anti-IL-8:IL-8 complexes to promote an inflammatory phenotype of human umbilical vein endothelial cells via interaction with FcγRIIa. Human umbilical vein endothelial cells cultured in the presence of the complexes become activated, as shown by increased phosphorylation of ERK, JNK, and Akt, and augmented nuclear translocation of NF-κB. Anti-IL-8:IL-8 complexes also up-regulate expression of intracellular adhesion molecule (ICAM)-1 on the cell surface. Furthermore, we detected increased levels of ICAM-1 on lung endothelial cells from mice in which lung injury was induced by generating immune complexes in alveolar spaces. On the other hand, ICAM-1 expression was unchanged in lungs of γ chain-deficient mice, lacking receptors that interact with immune complexes. Moreover, in lung tissues from patients with ARDS, anti-IL-8:IL-8 complexes were associated with endothelial cells that expressed higher levels of ICAM-1. Our current findings implicate that anti-chemokine autoantibody:chemokine immune complexes, such as IL-8:IL-8 complexes, may contribute to pathogenesis of lung inflammation by inducing activation of endothelial cells through engagement of IgG receptors.

Keywords: chemokine; autoantibody; lung; IgG receptor

Previous studies from our laboratory revealed that anti-IL-8 autoantibody:IL-8 immune complexes (anti-IL-8:IL-8 complexes) that act through IgG receptors (specifically, FcγRIIa) may contribute to pathogenesis of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) (1–6). We have demonstrated that anti-IL-8 autoantibody:IL-8 immune complexes purified from edema fluids from patients with ALI (ICEF) trigger chemotaxis of human blood neutrophils, induce neutrophil activation, and modulate survival of these cells (4, 5). Significantly, IgG receptors (FcγRIIa) are the primary receptors responsible for mediating the biological activities of anti-IL-8:IL-8 complexes (4, 5). Moreover, our previous findings in-

CLINICAL RELEVANCE

Our findings indicate that anti-chemokine autoantibody:chemokine immune complexes, such as anti-IL-8:IL-8 complexes, may contribute to pathogenesis of lung inflammation associated with acute lung injury/acute respiratory distress syndrome by inducing activation of endothelial cells through engagement of IgG receptors.

dicating that anti-IL-8:IL-8 complexes are deposited in lungs of patients with ARDS via FcγRIIa (6). Finally, we showed that patients at risk for ARDS who had high concentrations of anti-IL-8 autoantibody:IL-8 immune complexes in their lung fluids were more likely to develop ARDS (2, 3), and the level of anti-IL-8 autoantibody:IL-8 immune complexes also correlated with mortality in patients with well established ARDS (3). The observations that are described above led us to hypothesize that anti-IL-8:IL-8 complexes that are capable of interacting with IgG receptors may contribute to the severity of the alveolar inflammation in ALI/ARDS.

The relevance of our observations related to anti-IL-8:IL-8 complexes is underscored by the fact that the experimental generation of analogous murine immune complexes (anti-KC [chemokine (CXC motif) ligand 1 (CXCL1)] autoantibody:KC immune complexes) in the lungs of mice leads to the development of severe pulmonary inflammation and injury to the lungs (7). It should be noted that although mice do not express IL-8, murine KC (CXCL1/KC) is considered to be functionally related to human IL-8 (8). Moreover, this alveolar inflammatory response is dramatically suppressed in γ chain-deficient mice that lack stimulatory receptors for IgG (FcγRs). It is these missing receptors that interact with immune complexes (7). Further, it should be stressed that γ chain-deficient mice are capable of producing anti-KC autoantibodies, and that we detected virtually the same levels of these autoantibodies in plasma of these mice as in plasma of their wild-type (WT) counterparts (7). Both groups of mice (γ chain-deficient mice and WT mice) also had similar concentrations of anti-KC:KC complexes in the lung lavage fluid. However, γ chain-deficient mice were protected from severe lung inflammation and injury due to lack of receptors that are capable of mediating activity of these complexes (7).

Furthermore, our preliminary experiments have shown that anti-IL-8 autoantibody:IL-8 immune complexes can promote an inflammatory phenotype of endothelial cells (9). It is well established that endothelial cells, because of their location, are constantly exposed to circulating mediators, and, therefore, become activated during ARDS (10–12). The endothelial cell activation has several consequences, such as releasing inflammatory mediators that recruit inflammatory cells into the vascular, interstitial, and alveolar spaces (10, 11). Furthermore, endothelial cells express intracellular adhesion molecule (ICAM)-1, which specifically interacts with CD11/CD18 integrins expressed on

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neutrophils. ICAM-1 facilitates neutrophil adhesion and trans-endothelial migration (10, 13). Interaction between ICAM-1 and CD11/CD18 is a pivotal step in the multistage process of migration of leukocytes through the endothelium (10). Understanding of pulmonary endothelial cell-leukocyte interaction is critical, because neutrophils have been implicated in the pathogenesis of ALI, as they may mediate the microvascular damage, and thus contribute to lung tissue injury (10, 14, 15).

Because several types of endothelial cells express Fc γ RIIa (16), and our preliminary results indicated that anti-IL-8:IL-8 complexes can promote an inflammatory phenotype of these cells (9), the aim of this study was to test whether Fc γ RIIa is present on the surface of human umbilical vein endothelial cells (HUVECs), and then to explore the function of Fc γ RIIa in the process of activation of HUVECs by anti-IL-8 autoantibody:IL-8 immune complexes. We also sought to establish whether anti-KC autoantibody:KC immune complexes are associated with endothelial cells in the lungs of mice with anti-KC autoantibody:KC immune complex-induced lung inflammation/injury, and whether anti-IL-8:IL-8 immune complexes are associated with endothelial cells in the lungs of patients with ARDS.

MATERIALS AND METHODS

Human Subjects

All research involving human subjects was approved by the Institutional Human Subject Committee at the University of Texas Health Center (Tyler, TX).

Cell Culture Conditions

HUVECs were purchased from Cambrex (Walkersville, MD) or Allcells (Berkeley, CA), and cultured in endothelial basal medium containing endothelial growth factor, hydrocortisone, gentamicin, amphoretic B, bovine brain extract, heparin, and FBS (EGM-MV; Cambrex). All reagents were free of endotoxin. Cells were used between passages 2 and 15, and maintained in plastic culture flasks or tissue culture dishes in a humidified atmosphere of 5% CO₂ at 37°C.

Anti-IL-8 Autoantibody:IL-8 Immune Complexes and Control Antibody

Anti-IL-8 autoantibody:IL-8 immune complexes and control antibody (CAb) were purified from plasma obtained from healthy, consenting volunteers. The complexes were also purified from edema fluids from patients with ALI.

To our knowledge, no technique exists that would allow selective purification of specific immune complexes. We and others routinely use complex preparations that are purified on a protein G column or protein A/G column (1, 4, 5, 17). Our previous studies demonstrated that immune complexes purified from subjects positive for anti-IL-8:IL-8 complexes using conventional methods had the ability to regulate function of blood neutrophils (4, 5). Importantly, removal of anti-IL-8 autoantibody:IL-8 immune complexes from samples which contain them renders these samples inactive (4). Because anti-IL-8 autoantibody:IL-8 immune complexes are the only active part of the samples of purified immune complexes, these samples were labeled anti-IL-8:IL-8 complexes. Moreover, samples obtained from healthy donors or patients with ALI who did not have anti-IL-8 autoantibody:IL-8 immune complexes did not display any activity toward neutrophils (4, 5). These samples were considered control samples, and labeled control antibody (CAb). Finally, the specific activity of anti-IL-8 autoantibody:IL-8 immune complexes toward human neutrophils was confirmed by using preformed complexes that were prepared by reacting of monoclonal anti-IL-8 antibody and recombinant human (rh) IL-8 (labeled anti-IL-8:IL-8 monoclonal antibody [mAb] complexes) (4, 5).

In the current study, we used anti-IL-8 autoantibody:IL-8 immune complexes purified from selected healthy donors or from patients with ALI (ICEF), as previously described (4, 5). We also purchased complexes purified from normal human plasma (IgG; Sigma Chemical Co., St. Louis, MO), which display similar activity to complexes purified by us

(4, 5). Furthermore, control samples (CAb) were obtained from healthy donors that did not have anti-IL-8 autoantibody:IL-8 immune complexes, as previously described (4, 5). Control samples prepared in this manner do not alter neutrophil function, as previously described (4, 5).

We also removed anti-IL-8 autoantibody:IL-8 immune complexes from samples containing these complexes, as previously described (4). Briefly, to prevent nonspecific interaction between the samples and the plate, a 1.2 M solution of sodium chloride was used as a sample diluent. The removal of anti-IL-8 autoantibody:IL-8 immune complexes was confirmed using specific ELISA assay (1), and the sample was marked as anti-IL-8:IL-8 complexes-free. In addition, complexes were formed between an anti-IL-8 mAb and rhIL-8 (anti-IL-8:IL-8 mAb; Peprotech, Rocky Hill, NJ), and also a monoclonal anti-monocyte chemotactic peptide (MCP)-1 antibody (R&D Systems, Minneapolis, MN) and rhMCP-1 (Peprotech). The monoclonal anti-IL-8 antibody was developed by Dr. Edward Leonard (National Cancer Institute, Frederick, MD) (18), and has similar properties to the anti-IL-8 autoantibody present in anti-IL-8 autoantibody:IL-8 immune complexes (1, 18). Endotoxin concentration in all samples used for HUVEC stimulation was measured in a Quantitative Chromogenic LAL Assay (BioWhittaker, Walkersville, MD), and was less than 100 pg/ml.

Expression of Fc γ RIIa Receptor on HUVECs and Its Interaction with Anti-IL-8:IL-8 Complexes

HUVECs growing on coverslips were incubated with anti-IL-8 autoantibody:IL-8 immune complexes at 4°C for 30 minutes. The cells were then washed, fixed with 100% methanol, and blocked with blocking buffer containing 10% goat serum and 0.5% BSA. Next, cells on coverslips were incubated with anti-Fc γ RIIa antibody (CD32A N-20, Santa Cruz Biotechnology, Santa Cruz, CA), followed by FITC-conjugated donkey anti-goat antibody and Texas Red (TxR)-conjugated goat anti-human IgG antibody (Santa Cruz Biotechnology). In the subsequent step, monoclonal anti-IL-8 antibody was used to recognize IL-8 in anti-IL-8:IL-8 complex followed by incubation with biotinylated horse anti-mouse IgG antibody and TxR-conjugated streptavidin. Moreover, anti-TNF- α antibody (R&D Systems) was used as a control for staining with the anti-IL-8 antibody. Finally, the cells were counterstained with Hoechst 33342 (Calbiochem, San Diego, CA). HUVECs were evaluated using PerkinElmer Ultra VIEW LCI confocal imaging system with Nikon TE2000-S fluorescence microscope (PerkinElmer, Wellesley, MA) using a PlanApo $\times 60$ immersion oil objective. Ultra VIEW Imaging Suite software (version 5.5.0.4) was used for image processing.

Cell Signaling Visualized by Confocal Microscopy

HUVECs cultured on coverslips were incubated for 5 minutes with anti-IL-8 autoantibody:IL-8 immune complexes purified from normal human plasma containing these complexes (as established using the specific ELISA assay [1–3], or CAb purified from plasma of healthy donors that do not have anti-IL-8 autoantibody:IL-8 immune complexes (based on the specific ELISA [1–3]). Cells were stimulated in medium without serum containing sodium orthovanadate (1 mM) (Sigma) at 37°C in 5% CO₂. In some experiments, HUVECs were treated with mAb against Fc γ RII (7.3; F[ab]₂; Ancell, Bayport, MN) for 30 minutes at 4°C before incubation with anti-IL-8:IL-8 complexes. mAb against Fc γ RII (7.3; F[ab]₂) was established to be equivalent to anti-Fc γ RIIa antibody (IV.3) (Medarex Corp., Princeton, NJ), as previously described (4, 5). Next, HUVECs were washed, fixed with 100% methanol, permeabilized with 0.5% (vol/vol) Triton X-100 in PBS, blocked in 5% BSA in PBS, and incubated overnight at 4°C with specific antibodies: anti-pSyk (Tyr525/526); anti-pERK (Thr202/Tyr204); anti-pAkt (Ser473); and anti-pJNK (Thr183/Tyr185) (Cell Signaling Technology, Beverly, MA). Then, the cells were incubated with FITC-conjugated swine anti-rabbit secondary antibody (Dako, Carpinteria, CA). In addition, expression of pp65 was analyzed in cells stimulated with CAb, anti-IL-8:autoantibody:IL-8 immune complexes purified from normal human plasma, complexes purified from edema fluids of patients with ALI, as well as complexes formed between a monoclonal anti-IL-8 antibody and rhIL-8 (anti-IL-8:IL-8 mAb; Peprotech). To visualize pp65 in HUVECs anti-pp65 (Ser536) antibody (Cell Signaling Technology) was used, followed by FITC-conjugated swine anti-rabbit secondary antibody (Dako). Hoechst 33342 (Calbiochem) was used to stain nuclei in these experiments.

Activation of various signaling proteins in HUVECs was observed using PerkinElmer Ultra VIEW LCI confocal imaging system with Nikon TE2000-S fluorescence microscope and PlanApo $\times 60$ immersion oil objective (numerical aperture [NA] 1.4) at room temperature. Ultra VIEW Imaging Suite software (version 5.5.0.4) was used for image processing.

ELISA Assay

Concentrations of IL-8 in culture media of HUVECs stimulated with the complexes were evaluated using a specific ELISA assay (Peprotech) according to the manufacturer's recommendations.

Western Blot

HUVECs incubated with anti-IL-8 autoantibody:IL-8 immune complexes or CAB (both purified from normal human plasma) for 5 minutes, as described above, were lysed with electrophoresis sample buffer containing 62.5 mM Tris-HCl (Fisher Scientific, Middletown, VA), 50 mM DTT (Dithiothreitol; Sigma), 2% (wt/vol) SDS, and 10% (vol/vol) glycerol (Fisher Scientific), boiled for 5 minutes, and stored at -70°C until used. Samples were loaded into SDS-PAGE gel, and separated proteins transferred to a polyvinylidene difluoride membrane (Pall Corp., Pensacola, FL). The membrane was blocked and incubated with primary and secondary antibodies. The primary antibodies used in this study were: anti-pERK (Thr202/Tyr204); anti-pAkt (Ser473); anti-pJNK (Thr183/Tyr185) (Cell Signaling Technology); and anti-Actin (I-19; Santa Cruz Biotechnology). The membrane was next incubated with peroxidase-conjugated goat anti-rabbit antibodies (Cell Signaling Technology). Bound antibodies were detected using enhanced chemiluminescence reagents (PerkinElmer Life Sciences, Inc., Boston, MA). The membrane was then exposed to X-ray film (F-GX810; Phenix, Hayward, CA). Densitometric analyses of images were performed using Quantity One software obtained from BioRad Laboratories (Hercules, CA).

Expression of ICAM-1 on the Surface of HUVECs

HUVECs cultured on coverslips were incubated with anti-IL-8 autoantibody:IL-8 immune complexes purified from normal human plasma or anti-IL-8:IL-8 mAb complexes (complexes formed between a monoclonal anti-IL-8 antibody and recombinant IL-8) in medium without serum for 16 hours in 5% CO_2 at 37°C (19). In some experiments, HUVECs were preincubated with mAb against Fc γ RII (7.3; F[ab] $_2$) for 30 minutes at 4°C before stimulation with anti-IL-8:IL-8 complexes. Next, HUVECs were incubated with anti-ICAM-1 antibody (H-108; Santa Cruz Biotechnology) followed by FITC-conjugated swine anti-rabbit secondary antibody (Dako), and Hoechst 33342 (Calbiochem) to stain nuclei. HUVECs were evaluated using PerkinElmer Ultra VIEW LCI confocal imaging system with Nikon TE2000-S fluorescence microscope using PlanApo $\times 60$ immersion oil objective (NA, 1.4) at room temperature. Ultra VIEW Imaging Suite software (version 5.5.0.4) was used for image processing.

Endothelial-Neutrophil Cell Adhesion Assay

HUVECs were seeded on coverslips placed in wells of a 24-well plate at a concentration of 5×10^4 cells/well. The cells were then stimulated with medium, anti-IL-8 autoantibody:IL-8 immune complexes (purified from normal human plasma), or anti-IL-8:IL-8 mAb complexes (complexes formed between a monoclonal anti-IL-8 antibody and recombinant IL-8) for 6 hours at 37°C in 5% CO_2 . Stimulation was performed in medium without serum. Neutrophils were purified from normal human blood using Dextran and Percoll gradient, as routinely done in our laboratory (4), and the purity of neutrophil preparation was between 95 and 97%. The cells (neutrophils) were stained with cell tracker (CellTrace CFSE Cell Proliferation Kit; Molecular Probes Inc., Eugene, OR) according to the manufacturer's protocol. Neutrophils (6×10^5) in a volume of 0.1 ml were added to HUVECs and incubated for 45 minutes in 5% CO_2 at 37°C . Next, nonadherent neutrophils were removed by gentle washing, and the adherent cells lysed with 200 μl of 1 M NaOH containing 1% SDS. Fluorescent intensity of lysates was measured in a fluorescence reader, with excitation and emission wavelengths of 492 nm and 517 nm, respectively, using an FP-6500 spectrofluorometer equipped with the Spectra Manager Program (JASCO Co., Tokyo, Japan). Results are expressed as the fold increase of mean fluorescent intensity between

samples simulated with anti-IL-8 autoantibody:IL-8 immune complexes purified from human plasma or anti-IL-8:IL-8 mAb complexes and samples cultured in medium only. The attachment of neutrophils was also visualized by confocal microscopy. HUVECs were cultured in medium alone or stimulated with anti-IL-8:IL-8 mAb complexes and incubated with purified neutrophils. Anti-actin antibody (Santa Cruz Biotechnology) was used to depict the interacting cell populations.

Animal Studies

All studies involving animals were approved by Animal Research Committee of the University of Texas Health Center, and conform to National Institutes of Health guidelines. Lung tissues were obtained from mice that were previously generated and evaluated (7). The WT mice (BALB/c; Taconic Germantown, NY) were immunized with KC (functional IL-8; Peprotech) conjugated to an adjuvant, purified protein derivative of tuberculin (PPD; State Serum Institute, Copenhagen, Denmark) to induce production of anti-KC autoantibodies, as previously described (7). Once the presence of autoantibodies against KC was confirmed, KC was administered intratracheally to generate anti-KC:KC complexes in lungs (KC-immunized/KC group). The control group of mice received saline instead of KC (KC-immunized/saline group) (7). In an additional series of experiments, γ chain-deficient BALB/c mice (Taconic) lacking functional expression of stimulatory Fc γ R that bind immune complexes were studied. Knockout (KO) mice were immunized with KC and had KC administered intratracheally, as described above (KC/KC-immunized/KC) (7). At 14 hours after intratracheal administration of KC or saline, mice were killed and lungs collected for further analysis (7).

Lung tissue sections from KC-immunized/KC, KC-immunized/saline and KO/KC-immunized/KC mice were processed as previously described (7). The sections were incubated with biotinylated anti-KC antibody, followed by TxR-conjugated streptavidin, and then with FITC-conjugated rabbit anti-mouse IgG, and finally with anti-CD34 antibody used as an endothelial cell marker (Santa Cruz Biotechnology) and Alexa 647-conjugated donkey anti-goat antibody. Another set of sections was incubated with anti-KC antibody (Peprotech) and chicken anti-rabbit secondary antibody (Alexa 488; Invitrogen, Carlsbad, CA), anti-Fc γ RIII antibody (R&D Systems), and chicken anti-rat secondary antibody (Alexa 647), and finally with anti-CD34 antibody (Santa Cruz Biotechnology) and donkey anti-goat secondary antibody (Alexa 568). Furthermore, separate lung tissue sections were incubated with anti-pSyk (Tyr 525/526) antibody, followed by FITC-conjugated swine anti-rabbit antibody, and then with anti-CD34 antibody, followed by Cy3-conjugated mouse anti-goat antibody. In additional experiments, the sections were incubated with biotin-conjugated anti-mouse CD54 (ICAM-1) antibody (Cedarlane Laboratories, Burlington, NC) and with FITC-conjugated streptavidin, followed by anti-CD34 antibody and Cy3-conjugated mouse anti-goat antibody (Chemicon, Temecula, CA). Lung tissues were counterstained with Hoechst 33,342 (Calbiochem). The slides were evaluated using a PerkinElmer Ultra VIEW LCI confocal imaging system with Nikon TE2000-S fluorescence microscope, using PlanApo $\times 20$ objective and PlanApo $\times 60$ or $\times 100$ immersion oil objective (NA, 1.4) at room temperature. Ultra VIEW Imaging Suite software (version 5.5.0.4) was used for image processing.

Analysis of Human Lung Tissue Sections

Lung tissues were collected for pathological evaluation by the Department of Pathology, University of Texas Health Science Center at Tyler, and all patient specimens were deidentified and archived. The patients were hospitalized and intubated in the intensive care unit, with a clinical diagnosis of ARDS. None of the patients had a specific determined etiology, such as infection, and therefore they were taken to surgery for wedge lung biopsy in an attempt to better determine the specific etiologies for their ARDS, to institute more selective therapy. The final diagnosis for all the patients was of typical severe diffuse lung damage characteristic of ARDS.

Tissues were routinely fixed and embedded in paraffin. For the purpose of this study, we analyzed lung tissue sections from four patients that had a form of diffuse alveolar damage (DAD) (ARDS is the clinical term for DAD that refers to the pathologic description of lung injury due to a wide variety of causative agents (6, 20) and sections of normal lung tissues (from six patients). The sections were processed

as previously described (6). Briefly, lung tissue sections (15 μ M) were placed on silanized slides, dried in an oven at 60°C, and deparaffinized by running the tissue section slides through multiple changes of xylene, 100%, 95%, and 70% alcohols down to distilled water (6). All of the DAD/ARDS specimens and five of the control specimens were previously evaluated (6) for the overall lung tissue deposition of anti-IL-8:IL-8 complexes. The sections were incubated with FITC-conjugated mouse anti-human CD32a antibody (Stem Cells Technologies Inc., Tukwila, WA), then with anti-human IL-8 antibody (developed by Dr. Edward Leonard from the National Cancer Institute, Frederick, MD, which recognizes IL-8 in complex with the anti-IL-8 autoantibody [1–3]), followed by TxR-conjugated bovine anti-mouse antibody (Santa Cruz Biotechnology). Next, tissue sections were incubated with rabbit anti-human ICAM-1 antibody (Santa Cruz Biotechnology), followed by Alexa 532-conjugated goat anti-rabbit antibody (Invitrogen), and finally with goat anti-human CD34 antibody used as an endothelial cell marker (21) (Santa Cruz Biotechnology) and Alexa 647-conjugated donkey anti-goat antibody (Invitrogen). Lung tissues were counterstained with Hoechst 33342 (Calbiochem). The slides were evaluated using a PerkinElmer Ultra VIEW LCI confocal imaging system with Nikon TE2000-S fluorescence microscope using PlanApo $\times 20$ objective, and PlanApo $\times 60$ or $\times 100$ immersion oil objective (NA, 1.4) at room temperature. Ultra VIEW Imaging Suite software (version 5.5.0.4) was used for image processing.

Statistics

Differences between groups were evaluated by a simple one-way ANOVA or *t* test when appropriate. All pair-wise multiple comparisons were performed using the Fisher's least significant differences method. In addition, the Spearman method was used to determine whether there is a correlation between presence of ICAM-1 and occurrence of anti-IL-8:IL-8 complexes on endothelial cells (CD34-positive cells). A *P* value of less than 0.05 was considered significant. All statistics were performed using SigmaStat (SPSS Science, Chicago, IL).

RESULTS

Anti-IL-8:IL-8 Complexes Interact with Fc γ RIIa on Human Endothelial Cells

In this study, we tested the function of Fc γ RIIa in the process of activation of HUVECs by anti-IL-8:IL-8 complexes. Anti-IL-8 autoantibody:IL-8 immune complexes were purified from plasma of healthy donors or edema fluids from patients with ALI (ICEF) (1, 4, 5). Control samples (CAb) were purified from plasma of healthy donors who lack anti-IL-8:IL-8 immune complexes and are, therefore, free of anti-IL-8:IL-8 complexes. Importantly, our previous studies showed that control samples prepared in this manner, either from normal plasma or edema fluids from patients with ALI, did not alter neutrophil function (4, 5). It also should be noted that all of the *in vitro* experiments were done in the absence of serum or other sources of complement (5).

Fc γ RIIa is present on the surface of several types of endothelial cells (16). However, there are also reports showing lack of expression of this receptor on HUVEC surface (16, 22). Thus, we first set out to determine whether HUVECs express Fc γ RIIa. As shown in Figures 1A and 1B, expression of Fc γ RIIa was detected on the surface of HUVECs. We then employed confocal microscopy techniques to assess interaction of anti-IL-8:IL-8 complexes with Fc γ RIIa on HUVECs. Fluorophores that were selected for this analysis have well separated excitation and emission spectra (23). In addition, to minimize cross-talk between channels, band pass filters were used, the images were acquired in sequential manner, and background fluorescence eliminated. Using anti-IL-8 antibody, which recognizes IL-8 present in the anti-IL-8 autoantibody:IL-8 immune complex (1, 18), we found that substantial levels of IL-8 (red) colocalized with Fc γ RIIa (green) on the surface of HUVECs

(colocalization is shown in yellow) (Figure 1A). We also used anti-TNF- α antibody as the specificity (negative) control, and did not detect any TNF- α associated with cells (Figure 1B). Our findings indicate that IL-8 present in the anti-IL-8 autoantibody:IL-8 immune complex is detectable on the surface of the HUVECs, as only IL-8 associated with the anti-IL-8 autoantibodies and not “free” IL-8 can interact with Fc γ RIIa (5). To further validate our observations, we used antibody against human IgG, an antibody portion of the anti-IL-8 autoantibody:IL-8 immune complex. We found that the IgG portion of the complex (magenta) also colocalizes with Fc γ RIIa (green) on HUVECs (co-localization between IgG (magenta) and Fc γ RIIa [green] is shown as white) (Figure 1C). In summary, our results suggest that anti-IL-8 autoantibody:IL-8 immune complexes have the ability to interact with Fc γ RIIa on the surface of HUVECs.

To confirm these findings, we analyzed activation of Syk-tyrosine kinase, one of the upstream components of the Fc γ RIIa signaling pathway (24). It is known that phosphorylation of an immunoreceptor tyrosine-based activation motif by members of Src tyrosine kinase family (Src, a homolog of v-src sarcoma [Shmidt-Ruppin A-2] viral oncogene) initiates the Fc γ RIIa signaling cascade. Next, the phosphorylated immunoreceptor tyrosine-based activation motif binds to and activates Syk-tyrosine kinase (24).

Therefore, we evaluated activation of Syk kinase in HUVECs treated with the anti-IL-8:IL-8 immune complexes and control samples using confocal microscopy. To determine differences between treatments, positively stained cells were counted, and appropriate figures depict both confocal images and mean numbers of positively stained HUVECs. Activation (phosphorylation) of Syk kinase was present only in HUVECs stimulated with anti-IL-8 autoantibody:IL-8 immune complexes purified from normal human plasma (*P* < 0.001) (Figure 1D). There was no phosphorylation of Syk (lack of fluorescence) in cells treated with medium or CAb. In agreement with our previous findings (4), removing of anti-IL-8 autoantibody:IL-8 immune complexes from samples containing these complexes caused the loss of their ability to induce activation of Syk in HUVECs (anti-IL-8:IL-8 complexes-free samples in Figure 1D). Finally, activation of Syk kinase was significantly inhibited after blocking of Fc γ RIIa on HUVECs with specific antibodies (Figure 1D). Moreover, immune complexes formed between anti-MCP-1 antibody and MCP-1 (anti-MCP-1:MCP-1 complexes) were incapable of inducing activation of Syk (Figure 1D). Effect of these complexes on the HUVECs was comparable to that of medium alone (Figure 1D). In summary, our observations indicate that anti-IL-8 autoantibody:IL-8 immune complexes are responsible for activating Syk in HUVECs, the kinase that initiates Fc γ RIIa signaling pathway.

Characterization of Downstream Components of the Fc γ RIIa Signaling Pathway

We have previously identified some of the key proteins of the Fc γ RIIa signaling cascade that is activated by anti-IL-8 autoantibody:IL-8 immune complexes (4, 5). Our earlier results suggest that engagement of Fc γ RIIa by anti-IL-8 autoantibody:IL-8 immune complexes triggers activation of Syk, Akt, and mitogen-activated protein kinases (i.e., ERK and p38) (5). These signaling proteins are implicated in regulation of respiratory burst, degranulation, chemotaxis, and apoptosis of human blood neutrophils (4, 5).

In the next part of the current study, we evaluated signaling events that mediate downstream consequences of the interaction of the anti-IL-8 autoantibody:IL-8 immune complexes with

Figure 1. Detection of binding of anti-IL-8 autoantibody:IL-8 immune complexes to Fc γ RIIa on human umbilical vein endothelial cells (HUVECs) using laser confocal microscopy. (A) Images depicting Fc γ RIIa on HUVECs (green) and IL-8 on the surface of cells (red) from the same field of view were overlaid to show colocalization demonstrated as yellow. (B) Images depicting Fc γ RIIa on HUVECs (green) and lack of staining for TNF- α on the surface of cells (red) from the same field of view were overlaid to show absence of colocalization (yellow). (C) Images depicting Fc γ RIIa on HUVECs (green) and IgG on the surface of cells (assigned as a magenta pseudocolor [41]) from the same field of view were overlaid to show colocalization as white. Nuclear staining was performed using Hoechst 33342 (blue). Experiments were repeated twice, and a representative result is shown. (D) Effect of anti-IL-8 autoantibody:IL-8 immune complexes on Syk activation in HUVECs. Examination of level of pSyk in HUVECs and percentage of activated (positive) cells incubated with medium, or anti-IL-8:IL-8 complexes purified from normal human plasma, or control antibody (CAb), or samples free of anti-IL-8:IL-8 complexes (anti-IL-8:IL-8 complexes free), or antibody against Fc γ RIIa and anti-IL-8:IL-8 complexes, and medium or anti-MCP-1:MCP-1 complexes. Phosphorylated form of Syk (green) is visualized in the cytoplasm of HUVECs using laser confocal microscopy. Representative data from three experiments are shown.

Fc γ RIIa on HUVECs. To analyze activation of the components of Fc γ RIIa signaling cascade (i.e., ERK, Akt, and JNK), Western blotting technique was employed. Figure 2A shows a significant increase in phosphorylation of analyzed proteins in HUVECs in response to the stimulation with anti-IL-8:IL-8 complexes. There were the 2.4-, 2.2-, and 1.5-fold increases in

pERK, pAkt, and pJNK levels, respectively, in relation to medium only (histograms in Figure 2A). On the other hand, CAb had no effect on activation of ERK, Akt, or JNK in HUVECs. Because CAb showed no effect on activation of analyzed signaling proteins in HUVECs, in subsequent experiments we used only anti-IL-8:IL-8 complexes. We also con-

Figure 2. Activation of ERK, Akt, and JNK in HUVECs stimulated with anti-IL-8 autoantibody:IL-8 immune complexes. (A) Western blot analysis of whole lysates of HUVECs incubated with medium, or anti-IL-8:IL-8 complexes purified from normal human plasma, or CAb purified from normal human plasma using specific antibodies against pERK, pAkt, pJNK, and actin. The histograms depict the fold increase in pERK, pAkt, and pJNK level compared with medium. Representative data from one of three experiments are shown. (B) Examination of level of pERK, pAkt, and pJNK in HUVECs and percentage of activated (positive) cells incubated with medium, or anti-IL-8:IL-8 complexes purified from normal human plasma, or antibody against FcγRIIa and anti-IL-8:IL-8 complexes determined by laser confocal microscopy. Representative data from three experiments are shown.

firmed our observations that anti-IL-8 autoantibody:IL-8 immune complexes purified from normal human plasma activate ERK, Akt, and JNK in HUVECs employing confocal microscopy technique ($P < 0.001$) (Figure 2B). In addition, we showed that phosphorylation of ERK, Akt, and JNK can be inhibited by blocking of FcγRIIIa on the cells stimulated with anti-IL-8:IL-8 complexes (Figure 2B).

Next, we evaluated activation of the transcription factor NF-κB. The most prevalent activated form of NF-κB is a heterodimer of the subunit p65 connected to either subunit p50 or p52. Phosphorylated p65 (Ser536) migrates from cytoplasm to the nucleus, where it activates a variety of NF-κB target genes (25–27). Importantly, NF-κB is one of the main mediators implicated in the development and progression of ALI. It is involved in transcriptional regulation of proinflammatory cytokines, persistent elevation of which in the lungs is associated with increased mortality from ALI (15, 28).

Activation (phosphorylation) of p65 and its translocation to the nucleus was detected in HUVECs that were stimulated with anti-IL-8 autoantibody:IL-8 immune complexes purified from normal human plasma ($P < 0.001$) (Figure 3A). In contrast, there was no detectable p65 in cells treated with CA or medium. Blocking of FcγRIIIa on HUVECs before the stimulation with anti-IL-8:IL-8 complexes caused inhibition of p65 activation, suggesting that NF-κB is a part of the FcγRIIIa signaling pathway initiated by anti-IL-8 autoantibody:IL-8 immune complexes.

We also tested the ability of complexes formed between monoclonal anti-IL-8 antibody and rhIL-8 (anti-IL-8:IL-8 mAb complexes) to regulate phosphorylation of NF-κB. It should be stressed that the anti-IL-8 antibody used to prepare these complexes has similar properties to the anti-IL-8 autoantibody present in anti-IL-8 autoantibody:IL-8 immune complexes (1, 18). Our previous studies demonstrate that anti-IL-8:IL-8 mAb complexes can modulate neutrophil apoptosis and display similar proinflammatory activity toward these cells as anti-IL-8:IL-8 complexes purified from normal human plasma or patients with ALI (4, 5). Figure 3B shows that anti-IL-8:IL-8 mAb complexes stimulate phosphorylation of p65 in HUVECs ($P < 0.001$), and that blocking of FcγRIIIa on HUVECs inhibits activation of p65.

Moreover, we evaluated the ability of anti-IL-8 autoantibody:IL-8 immune complexes purified from lung fluids from patients with ALI (ICEF) to stimulate activation of NF-κB in HUVECs. Our previous findings indicate that ICEFs have similar properties to anti-IL-8 autoantibody:IL-8 immune complexes purified from normal human plasma, and display proinflammatory activity toward human neutrophils as well as regulate survival of these cells (4, 5). In this study, we show that ICEFs induce phosphorylation and considerable translocation to the nucleus of p65 in HUVECs ($P < 0.001$). Importantly, activation of p65 is inhibited by blocking of FcγRIIIa on HUVECs (Figure 3B).

Finally, we evaluated production of IL-8 in HUVECs stimulated with anti-IL-8:IL-8 mAb complexes. As shown in Figure 3C, there was a significant increase ($P < 0.001$) in the amount of IL-8 that was released by cells incubated with the complexes.

Effect of Anti-IL-8:IL-8 Complexes on Surface Level Expression of ICAM-1 on Human Endothelial Cells

Because endothelial cells, including HUVECs, express ICAM-1 (19), which facilitates neutrophil adhesion (10, 13), and neutrophil-endothelial interaction plays an important role in the development of inflammatory injury in ALI/ARDS, we hypothesized that anti-IL-8 autoantibody:IL-8 immune complexes may change the surface level of ICAM-1 affecting neutrophil adhesion to HUVECs.

In agreement with our hypothesis, we observed significant up-regulation of ICAM-1 on the surface of HUVECs after 16 hours of stimulation with anti-IL-8 autoantibody:IL-8 immune complexes purified from normal human plasma ($P < 0.001$ for the difference in the intensity of fluorescence between treatments) (Figure 4A). Blocking of FcγRIIIa substantially inhibited the amount of ICAM-1 on the cell surface (Figure 4A). Furthermore, we detected an increased level of ICAM-1 on the surface of cells treated with anti-IL-8:IL-8 mAb complexes ($P < 0.001$ for the difference in the intensity of fluorescence between treatments), which was significantly down-regulated by blocking of FcγRIIIa (Figure 4B).

Next, we evaluated physiological consequences of an increased level of ICAM-1 on the surface of HUVECs by studying the ability of neutrophils to adhere to stimulated HUVECs. Therefore, we measured adhesion of human neutrophils (stained with cell tracker [CellTrace CFSE Cell Proliferation Kit]) to HUVECs stimulated for 6 hours with anti-IL-8:IL-8 complexes or anti-IL-8:IL-8 mAb complexes. Our results indicate that significantly more neutrophils adhered to HUVECs that were stimulated with anti-IL-8:IL-8 complexes or anti-IL-8:IL-8 mAb complexes than to cells stimulated with medium alone (2.9 [± 0.43]- or 2.4 [± 0.27]-fold increase, respectively). Moreover, attachment of neutrophils to HUVECs pretreated with anti-IL-8:IL-8 mAb complexes was also evaluated using laser confocal microscopy. As shown in Figure 4C, there was a substantial rise in numbers of neutrophils associated with the stimulated cells.

FcγRs Control Endothelial Cell Activation in Lungs of Mice with Immune Complex-Induced Lung Injury

Although mice do not express IL-8, murine KC (CXCL1/KC) is functionally equivalent to human IL-8 (8). Previously, we have developed a mouse model to evaluate the contribution of anti-KC:KC complexes generated *in situ* to lung inflammation and injury (immune complex-induced lung inflammation). In this model, autoantibodies to KC (in plasma and the alveolar compartment) are first induced by immunization with KC, and then KC is administered intratracheally to generate anti-KC:KC complexes in the lung (KC-immunized/KC mice) (7). Consequently, anti-KC autoantibody:KC immune complexes were present in lung lavage fluid from KC-immunized/KC mice. We also observed deposition of anti-KC autoantibody:KC immune complexes in lung tissues obtained from KC-immunized/KC mice. Furthermore, we found increased transalveolar influx of neutrophils, increased permeability, and alveolar hemorrhage, together with histological evidence of increased infiltration of inflammatory cells, interstitial thickening, and presence of alveolar exudate in these animals. All of these findings indicate the presence of severe pulmonary inflammation and alveolar damage. Moreover, γ chain-deficient mice (lacking stimulatory FcγRs that bind immune complexes) that were immunized with KC developed anti-KC autoantibodies, and then were treated with KC (KO/KC-immunized/KC mice), had substantially attenuated pulmonary inflammatory responses, suggesting that the activity of anti-KC:KC complexes is mediated by receptors for IgG (FcγRs) (7). Similarly, no lung tissue deposition of anti-KC:KC complexes was detected in KO mice (KO/KC-immunized/KC mice), as these mice lack receptors for immune complexes (7). It should be noted that, although mice do not express FcγRIIIa, there are stimulatory FcγRs present in these animals (i.e., FcγRI, FcγRIII, and FcγRIV) (29). It is unlikely that FcγRIV plays a major role in our model, because it primarily binds IgG2a and IgG2b, whereas anti-KC:KC complexes are of the IgG1 subclass (our unpublished observations). On the other hand, murine FcγRIII, which is abundant in the lung, interacts with immune complexes and triggers cellular activation in the lungs that

Figure 3. Effect of anti-IL-8 autoantibody:IL-8 immune complexes on NF- κ B (pp65) activation in HUVECs. (A) Examination of level of pp65 in HUVECs and percentage of activated (positive) cells incubated with medium, or CAb, or anti-IL-8:IL-8 complexes purified from normal human plasma, or antibody against Fc γ RIIa and anti-IL-8:IL-8 complexes purified from normal human plasma. (B) Examination of level of pp65 in HUVECs and percentage of activated (positive) cells incubated with medium, or anti-IL-8:IL-8 monoclonal antibody (mAb) complexes, or antibody against Fc γ RIIa and anti-IL-8:IL-8 mAb complexes, or anti-IL-8:IL-8 complexes from acute lung injury (ALI) edema fluids (ICEF), or antibody against Fc γ RIIa and ICEF. Phosphorylated form of p65 is visualized in HUVECs using laser confocal microscopy. Representative data from three experiments are shown. (C) IL-8 ELISA assay. Concentrations of IL-8 were measured in supernatants of HUVECs cultured in the medium alone, or in the presence of anti-IL-8:IL-8 mAb complexes.

Figure 4. Effect of anti-IL-8 autoantibody:IL-8 immune complexes on intracellular adhesion molecule (ICAM)-1 expression on the surface of HUVECs. (A) Expression of surface level of ICAM-1 in HUVECs (green) stimulated with medium, or anti-IL-8:IL-8 complexes purified from normal human plasma, or preincubated with antibody against FcγRIIa and stimulated with anti-IL-8:IL-8 complexes was detected using laser confocal microscopy. Nuclear staining was performed using Hoechst 33342 (blue). Representative data from three experiments are shown. (B) Expression of surface level of ICAM-1 in HUVECs (green) stimulated with medium, or anti-IL-8:IL-8 mAb complexes, or preincubated with antibody against FcγRIIa and stimulated with anti-IL-8:IL-8 mAb complexes was detected using laser confocal microscopy. Nuclear staining was performed using Hoechst 33342 (blue). Representative data from three experiments are shown. (C) Adhesion of neutrophils to HUVECs. HUVECs were cultured in medium alone or stimulated with anti-IL-8:IL-8 mAb complexes and incubated with purified neutrophils. Anti-actin antibody (red) was used to depict the interacting cell populations (arrows). H, HUVECs. Enlarged image depicts one HUVEC with attached neutrophils.

leads to lung injury (30, 31). Moreover, anti-KC:KC immune complexes preferentially bind to this receptor (our unpublished observations).

In this study, we evaluated endothelial cells in lung tissues obtained from KC-immunized/KC mice, KO/KC-immunized/KC mice, and control mice (KC-immunized/saline mice) that had saline administered intratracheally instead of KC (7). To visualize deposition of anti-KC:KC immune complexes in the lung, tissue sections were incubated with FITC-conjugated anti-mouse IgG antibody. As shown in Figure 5A, panel II, IgG was easily detectable in specimen from KC-immunized/KC mice (green), indicating the presence of immune complexes associated with the lung tissue (7), and specifically with endothelial cells (CD34 was used as a marker of endothelial cells [32]). The same tissue sections were also incubated with biotinylated antibody against mouse KC, followed by TxR-conjugated streptavidin. There is substantial staining (red) in tissue from KC-immunized/KC mouse. Merging of “green” and “red” channels resulted in significant colocalization (yellow) (Figure 5A, panel II). These findings suggest that a significant portion of IgG is associated with KC forming anti-KC:KC complexes, and that

these complexes are associated with endothelial cells in lungs of KC-immunized/KC mice (Figure 5A, panel II). IgG (green) was not visible in tissues from KC-immunized/saline mice (Figure 5A, panel I) and KO/KC-immunized/KC mice (Figure 5A, panel III). Staining for mouse KC (red) was also negligible, and, moreover, virtually no colocalization (yellow) was observed in these tissues, indicating the absence of appreciable presence of anti-KC:KC immune complexes associated with endothelial cells in the lungs of KC-immunized/saline (Figure 5A, panel I) and KO/KC-immunized/KC mice (Figure 5A, panel III).

Murine FcγRIII is considered an equivalent of human FcγRIIa (29). Therefore, we performed additional experiments to evaluate binding of anti-KC:KC complexes to FcγRIII (Figure 5B). We analyzed colocalization between KC (green) and FcγRIII (magenta), because only KC associated with anti-KC autoantibodies will have the ability to interact with FcγRIII. In agreement with the data presented in Figure 5A, there is substantial staining (green) in tissue from KC-immunized/KC mice. We also detected enhanced expression of FcγRIII in this tissue, and a significant colocalization (white) between “green” and “magenta” signals (Figure 5B, panel II). These observa-

Figure 5. Deposition of anti-KC:KC immune complexes in the lungs of mice (A), and interaction of the complexes with Fc γ RIII (B). Nuclei are stained blue. (A, Panel I) KC-immunized/saline. Left panels from the top ($\times 20$): IgG (green)/KC (red); CD34 (magenta). Right top panels ($\times 60$): IgG (green); KC (red). Right middle panel ($\times 60$): colocalization of IgG (green) and KC (red). Right bottom panel ($\times 60$): CD34 (magenta). (A, Panel II) KC-immunized/KC. Left top panels ($\times 20$): IgG (green)/KC (red); CD34 (magenta). Right top panels ($\times 60$): IgG (green); KC (red). Right middle panel ($\times 60$): Colocalization (yellow) of IgG (green) and KC (red). Right bottom panel ($\times 60$): CD34 (magenta). To the far right are: enlarged image of colocalization of IgG and KC (depicting anti-KC:KC complexes) (yellow), merge of IgG, KC, CD34 (anti-KC:KC complexes on endothelial cells), and enlarged image of CD34. (A, Panel II) Knockout (KO)/KC-immunized/KC. Left panels from the top ($\times 20$): IgG (green)/KC (red); CD34 (magenta). Top right panels ($\times 60$): IgG (green); KC (red). Right middle panel ($\times 60$): Colocalization of IgG (green) and KC (red). Right bottom panel ($\times 60$): CD34 (magenta). (B, Panel I) KC-immunized/Saline. Left panels from the top ($\times 20$): KC (green)/Fc γ RIII (magenta); CD34 (red). Top right panels ($\times 60$): KC (green); Fc γ RIII (magenta). Right middle panel ($\times 60$): Colocalization of KC (green) and Fc γ RIII (magenta). Right bottom panel ($\times 60$): CD34 (red). (B, Panel II) KC-immunized/KC. Left top panels ($\times 20$): KC (green)/Fc γ RIII (magenta); CD34 (red). Right top panels ($\times 60$): KC (green); Fc γ RIII (magenta). Right middle panel ($\times 60$): Colocalization of KC (green) and Fc γ RIII (magenta). Right bottom panel ($\times 60$): CD34 (red). To the far right are: enlarged image of colocalization of KC and Fc γ RIII (depicting the association of anti-KC:KC complexes with Fc γ RIII) (white) and enlarged image of CD34. In each case, five animals per group were analyzed and typical findings are presented. Acquisition settings for the images were UV/488/567/647 and specific parameters for the fluorophores were FITC (green) excitation (Ex) at 488 nm, emission (Em) at 525 nm, Texas Red (TxR; red) Ex at 567 nm, Em at 600 nm; Alexa 647 (magenta) Ex at 647 nm and Em at 700 nm.

Figure 6. Activation of endothelial cells in lungs of mice. (A) Phosphorylation of Syk in lungs of mice. pSyk, green; nuclei, blue; staining with Hoechst 33342; CD34, red, endothelial cells marker. (A, Panel I) KC-immunized/saline. Left panels from the top ($\times 20$): pSyk; CD34. Right top panels ($\times 60$): pSyk; CD34. Right bottom panel ($\times 60$): Merge of pSyk (green), nuclei (blue), and CD34 (red). (A, Panel II) KC-immunized/KC. Left panels from the top ($\times 20$): pSyk; CD34. Right top panels ($\times 60$): pSyk; CD34. Right bottom panel ($\times 60$): Merge of pSyk (green), nuclei (blue), and CD34 (red). Next to this panel, there is an enlarged image depicting the presence of pSyk (green) in endothelial cells (CD34, red). (A, Panel III) KO/KC-immunized/KC. Left panels from the top ($\times 20$): pSyk; CD34. Right top panels ($\times 60$): pSyk; CD34. Right bottom panel ($\times 60$): Merge of pSyk (green), nuclei (blue), and CD34 (red). In each case, five animals per group were analyzed and typical findings are presented. (B) Expression of ICAM-1 in lungs of mice. ICAM-1, green; nuclei, blue; staining with Hoechst 33342; CD34, red, endothelial cell marker. (B, Panel I) KC-immunized/Saline mice. Left panels from the top ($\times 20$): ICAM-1; CD34. Right top panels ($\times 60$): ICAM-1; CD34. Right bottom panel ($\times 60$): Merge of ICAM-1 (green), nuclei (blue), and CD34 (red). (B, Panel II) KC-immunized/KC. Left panels from the top ($\times 20$): ICAM-1; CD34. Right top panels ($\times 60$): ICAM-1; CD34. Right bottom panel ($\times 60$): Merge of ICAM-1 (green), nuclei (blue), and CD34 (red). Next to this panel, there is an enlarged image depicting the presence of ICAM-1 (green) on the surface on endothelial cells (CD34, red). (B, Panel III) KO/KC-immunized/KC. Left panels from the top ($\times 20$): ICAM-1; CD34. Right top panels ($\times 60$): ICAM-1; CD34. Right bottom panel ($\times 60$): Merge of ICAM-1 (green), nuclei (blue), and CD34 (red). In each case, five animals per group were analyzed and typical findings are presented. Acquisition settings for the images were UV/488/567 and specific parameters for the fluorophores were FITC (green) Ex at 488 nm, Em at 525 nm; TxR (red) Ex at 567 nm, Em at 600 nm.

tions indicate that anti-KC:KC complexes are associated with Fc γ RIII present on the endothelial cells (CD34 [endothelial marker], red) in lungs of KC-immunized/KC mice (Figure 5B, panel II). Fc γ RIII (magenta) was also detected on endothelial cells (CD34, red) in lung tissues from KC-immunized/saline mice (Figure 5B, panel I). However, staining for mouse KC (green) was negligible, confirming the results presented in Figure 5A. Consequently, there was no appreciable colocalization (white), which would suggest the lack of interaction between anti-

KC:KC complexes and Fc γ RIII, and, by association, would also indicate that no detectable amounts of the complexes were present in these tissues. All these findings agree again with the data shown in Figure 5A, where practically no anti-KC:KC immune complexes were detected in proximity of endothelial cells in lung tissues from KC-immunized/saline mice (Figure 5B, panel I).

The findings that anti-KC autoantibody:KC immune complexes are present in KC-immunized/KC mice, and, importantly, KO/KC-immunized/KC mice are devoid of these complexes,

Figure 7. Deposition of anti-IL-8:IL-8 immune complexes via Fc γ RIIa and expression of CD34 and ICAM-1 in normal lung tissues (A) and tissues from patients with acute respiratory distress syndrome (ARDS) (B). Nuclei are stained blue. (A) Left panels from the top ($\times 20$): Fc γ RIIa (green)/IL-8 (red); CD34 (magenta); ICAM-1 (light blue). Right top small panels ($\times 60$): Fc γ RIIa (green); IL-8 (red). Big panel ($\times 60$): Co-localization of Fc γ RIIa (green) and IL-8 (red). Right bottom small panels ($\times 60$): CD34 (magenta); ICAM-1 (light blue). Big panel ($\times 60$): Merge of CD34 (magenta) and ICAM-1 (light blue). (B) Left panels from the top ($\times 20$): Fc γ RIIa (green)/IL-8 (red); CD34 (magenta); ICAM-1 (light blue). Right top small panels ($\times 60$): Fc γ RIIa (green); IL-8 (red). Big panel ($\times 60$): Colocalization of Fc γ RIIa (green) and IL-8 (red) with CD34 (magenta). Right bottom small panels ($\times 60$): CD34 (magenta); ICAM-1 ($\times 60$) (light blue). Big panel ($\times 60$): Merge of CD34 (magenta) and ICAM-1 (light blue). To the far right are: enlarged image of colocalization of Fc γ RIIa and IL-8 (depicting anti-IL-8:IL-8 complexes) (yellow) with nuclei stained blue merged with CD34 (anti-IL-8:IL-8 complexes on endothelial cells), and merge of CD34, ICAM-1, and nuclei (enlarged) (ICAM-1 on endothelial cells). Four to six patients per group were analyzed, and typical findings are presented. Acquisition settings for the images were UV/567/488/647/514, and specific parameters for the fluorophores were TxR (red) Ex at 567 nm and Em at 600 nm, FITC (green) Ex at 488 nm, Em at 525 nm, Alexa 647 (magenta) Ex at 647 nm and Em at 700 nm, Alexa 532 (light blue) Ex at 514 nm, Em at 575 nm. (C) Presence of ICAM-1 on the surface of endothelial cells was associated with positive staining of these cells for anti-IL-8:IL-8 immune complexes in lung tissue from patients with ARDS ($P < 0.001$; $r = 0.6$). A total of 629 endothelial cells (CD34-positive cells) were analyzed, from which 20.7% were negative for both ICAM-1 and the complexes, 9.5% were positive for the complexes only, 7% for ICAM-1 only, and 62.8% for both.

indicate that Fc γ R_s mediate activity of anti-KC autoantibody:KC immune complexes. To test the concept that binding of the complexes to endothelial cells occurs via Fc γ R_s further, we evaluated expression of Syk, the kinase, which initiates Fc γ R signaling pathways (24). As shown in Figure 6A, significant phosphorylation (activation) of Syk was observed in endothelial cells of KC-immunized/KC mice only (Figure 6A, panel II) (CD34 was used as a marker of endothelial cells [32]). This observation is consistent with the idea that the interaction between anti-KC autoantibody:KC immune complexes and Fc γ R_s leads to activation of these receptors on endothelial cells.

Next, we analyzed expression of ICAM-1 on endothelial cells in lung tissues of all three groups of mice. As shown in Figure 6B, there was an increased expression of ICAM-1 on lung endothelial cells in KC-immunized/KC mice (panel II) in comparison to KO and control mice (KO/KC-immunized/KC mice [panel III] and KC-immunized/saline mice [panel I], respectively). The finding that levels of ICAM-1 were similar in KO and control mice suggests that the increase in ICAM-1 expression may be induced by interaction between Fc γ R_s present on endothelial cells and anti-KC autoantibody:KC immune complexes (CD34 was used as a marker of endothelial cells [32]).

Presence of Anti-IL-8:IL-8 Complexes Associated with Endothelial Cells in Lung Tissues from Patients with ARDS

We have previously reported deposition of anti-IL-8:IL-8 complexes via Fc γ RIIa in lung tissues obtained from patients with ARDS, but did not specify with which cells the complexes interacted (6). In this study, lung tissues from patients with ARDS and control tissues were evaluated for the presence of anti-IL-8:IL-8 complexes associated with endothelial cells. Anti-IL-8:IL-8 complexes can be detected by evaluating colocalization between IL-8 and Fc γ RIIa, a specific receptor for anti-IL-8:IL-8 complexes, as only IL-8 associated with anti-IL-8 autoantibodies has the ability to interact with Fc γ RIIa (4–6). CD34 (magenta) was used as an endothelial cell marker (21). IL-8 (red) colocalized with Fc γ RIIa (green) in ARDS tissue (yellow), indicating interaction of anti-IL-8:IL-8 complexes with endothelial cells in ARDS lung tissue (Figure 7B). Fc γ RIIa (green) was also detected in normal tissue, whereas staining for IL-8 (red) was minimal, as was colocalization (yellow) between Fc γ RIIa and IL-8 (Figure 7A). Moreover, we observed elevated expression of ICAM-1 on endothelial cells that also showed positive staining for anti-IL-8:IL-8 immune complexes in lung tissues from patients with ARDS (Figure 7B). Presence of ICAM-1 on the cell surface was in fact associated with positive staining for the complexes ($P < 0.001$; $r = 0.6$). As shown in Figure 7C, we evaluated 629 endothelial cells (CD34-positive cells), from which 20.7% were negative for both ICAM-1 and the complexes, 9.5% were positive for the complexes only, 7% for ICAM-1 only, and 62.8% for both.

Presence of anti-IL-8 autoantibody:IL-8 immune complexes bound to Fc γ RIIa on the surface of activated endothelial cells indicates that these complexes may interact with endothelial cells in lungs of patients with ARDS, and possibly exhibit their proinflammatory activity, adding to severity of the inflammatory response in these patients. Our data showing that complexes purified from edema fluids of patients with ALI have the ability to induce endothelial cell activation by the engagement of Fc γ RIIa, and that analogous murine complexes behave in a similar way, make this scenario even more plausible.

In summary, the results of our current studies implicate anti-chemokine autoantibody:chemokine immune complexes as triggers of pathophysiological alternations in endothelial cell function that may lead to the development of proinflammatory phenotype of these cells.

DISCUSSION

Anti-IL-8 autoantibody:IL-8 immune complexes are present in 55–70% of normal human plasmas (1, 18). We have reported that lung fluids of patients with ALI/ARDS contain high concentrations of these complexes, and our previous studies indicate that anti-IL-8 autoantibody:IL-8 immune complexes could contribute to pathogenesis of ALI/ARDS (1–3). Importantly, our group discovered that anti-IL-8 autoantibody:IL-8 immune complexes are deposited in the lungs of patients with ARDS via Fc γ RIIa (6). Moreover, anti-IL-8 autoantibody:IL-8 immune complexes purified from normal human plasma or from edema fluids from patients with ALI, and finally formed between recombinant IL-8 and monoclonal anti-IL-8 antibody, trigger chemotaxis of human blood neutrophils, induce neutrophil activation, and modulate survival of these cells (4, 5). Significantly, activity of these complexes is mediated by IgG receptors—specifically, Fc γ RIIa (4, 5).

There is limited information regarding the functional effects of soluble immune complexes on endothelial cells and, importantly, anti-IL-8:IL-8 complexes have never been studied before. Preliminary studies from our laboratory revealed that anti-IL-8:IL-8

complexes have the ability to promote an inflammatory phenotype of endothelial cells (9). Moreover, because the level of Fc γ RIIa is increased in lungs of patients with ARDS, and proinflammatory activity of anti-IL-8:IL-8 complexes is controlled by this receptor (6), the goal of the current study was to determine whether HUVECs express Fc γ RIIa, and to test its role in the process of activation of endothelial cells triggered by anti-IL-8 autoantibody:IL-8 immune complexes. We found that anti-IL-8:IL-8 complexes interact with Fc γ RIIa on HUVECs and induce activation of signaling proteins (Syk, ERK, Akt, and JNK) in the Fc γ RIIa cascade. Importantly, the role of Fc γ RIIa in mediating activity of the complexes was confirmed by blocking of this receptor with specific antibodies that inhibited activation (phosphorylation) of analyzed signaling proteins.

NF- κ B has been implicated in the development and progression of ALI, and increased levels of NF- κ B were detected in neutrophils that accumulated in the lungs and airways of patients with ALI (28). We found that binding of anti-IL-8 autoantibody:IL-8 immune complexes to Fc γ RIIa on HUVECs results in activation of NF- κ B subunit, p65. Phosphorylation of p65 was inhibited by blocking of Fc γ RIIa. To the best of our knowledge, soluble immune complexes, such as anti-IL-8 autoantibody:IL-8 immune complexes, have not been extensively studied in relation to evoking of downstream signals in the Fc γ RIIa cascade in endothelial cells. There are few reports addressing the role of NF- κ B in the Fc γ RIIa signaling cascade (33–35). Furthermore, Alonso and colleagues (36) demonstrated activation of NF- κ B in response to stimulation of Fc γ Rs on human monocyte/macrophage cell line (THP-1) by insoluble aggregates of human IgG; however, the authors did not distinguish between the subclasses of Fc γ Rs.

In the current study, we observed that binding of anti-IL-8 autoantibody:IL-8 immune complexes to HUVECs causes the increase in surface level of adhesion molecule ICAM-1. This effect was also mediated by Fc γ RIIa. Up-regulation of ICAM-1 affected the recruitment of neutrophils to HUVECs that was 2.4- to 2.9-fold higher than spontaneous adhesion of these cells to nonstimulated HUVECs. Furthermore, increased expression of ICAM-1 was detected on endothelial cells in lungs of mice with lung injury caused by deposition of anti-KC:KC immune complexes (murine KC [CXCL1/KC] is a functional equivalent of human IL-8) (8). Furthermore, even more importantly, expression of ICAM-1 remained unchanged in mice deficient in IgG receptors capable of interacting with anti-KC:KC immune complexes.

In our model of anti-chemokine autoantibody:chemokine (anti-KC:KC) immune complex-induced lung injury, IgG receptors (Fc γ Rs) are essential for lung tissue deposition of anti-KC:KC complexes and, by association, for maintaining the increased expression of ICAM-1 (Ref. 7 and this study). Accordingly, ICAM-1 levels are normal in mice deficient in Fc γ Rs, in which we did not detect deposition of anti-KC:KC complexes in lungs (Ref. 7 and this study). There have been several studies describing the presence of ICAM-1 on endothelial cells in IgG immune complex-mediated lung injury in rats (37–39). The level of ICAM-1 expression is regulated by complement in these animals (39). The animal model used in these studies differs substantially from our model. It is based on the local formation of heterologous immune complexes, which then trigger the alveolar inflammatory response (reverse passive Arthus reaction). A foreign antigen is given intravenously, whereas an antibody against this antigen is administered intratracheally. We developed a model in which 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. This model mimics very well the situation observed

in patients with ARDS who have anti-IL-8 autoantibody complexes in their lungs (1–3, 7).

In conclusion, we found that anti-IL-8:IL-8 complexes are capable of activating endothelial (HUVEC) cells and inducing an increase in expression of ICAM-1 in these cells. Both of these activities are mediated by FcγRIIa. Analogous anti-chemokine autoantibody:chemokine immune complexes are essential for triggering enhanced expression of ICAM-1 via murine FcγRs. Anti-IL-8:IL-8 immune complexes are also found in lungs of patients with ARDS, and are associated with lung endothelial cells by interacting with endothelial cell FcγRIIa. Moreover, these complexes are deposited on endothelial cells that express increased levels of ICAM-1. Tsokos (40) also observed enhanced expression of ICAM-1 on pulmonary endothelium in sepsis-induced ALI employing immunohistochemical analysis of human lung tissues. It is likely that anti-IL-8 autoantibody:IL-8 immune complexes, by engaging FcγRIIa present on lung endothelial cells, could contribute to pathophysiological changes observed in these cells during the course of ARDS.

Our findings are important in relation to inflammatory responses associated with ALI/ARDS because up-regulated expression of adhesion molecules on the surface of activated endothelial cells facilitates neutrophil adherence leading to accumulation of these cells in lungs (12). It is known that influx of neutrophils may initiate or amplify lung injury contributing to edema formation and causing fibrosis (10, 12). By activating of endothelial cells, anti-IL-8 autoantibody:IL-8 immune complexes may facilitate neutrophil migration in patients with ALI/ARDS. These results implicate a novel mechanism by which anti-IL-8 autoantibody:IL-8 immune complexes may contribute to the pathogenesis of lung inflammation in ALI/ARDS.

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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