Anti-IL-8 autoantibody:IL-8 immune complexes suppress spontaneous apoptosis of neutrophils

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Anti-IL-8 autoantibody:IL-8 immune complexes suppress spontaneous apoptosis of neutrophils. Am J Physiol Lung Cell Mol Physiol 293: L364–L374, 2007. First published May 18, 2007; doi:10.1152/ajplung.00179.2006.—Our previous studies demonstrated that a significant fraction of interleukin-8 (IL-8) in lung fluids from patients with acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) is associated with anti-IL-8 autoantibodies (anti-IL-8:IL-8 immune complexes). Neutrophils have been implicated in the pathogenesis of ALI/ARDS, and moreover, it is well-established that apoptosis of neutrophils is delayed in patients with ALI/ARDS. The aim of this study was, therefore, to examine the role of anti-IL-8:IL-8 immune complexes in modulating spontaneous apoptosis of normal human neutrophils. Apoptosis was assessed by evaluating morphological changes, measuring enzymatic activity of caspase-3, and determining the extent of DNA degradation. We found that samples containing anti-IL-8:IL-8 immune complexes but not samples from which these complexes were removed inhibited neutrophil apoptosis. Furthermore, the former samples or effectively anti-IL-8:IL-8 complexes induced an increase in the level of antiapoptotic protein, Bcl-XL. In contrast, levels of proapoptotic proteins Bax and Bak were decreased in the same conditions. Activity of both caspase-3 and caspase-9 was also suppressed by anti-IL-8:IL-8 complex-containing samples. Finally, we established that IgG receptor, FcγRIIa, mediates antiapoptotic activity of anti-IL-8:IL-8 complexes and that the key components of the FcγRIIa signaling pathway, Src, Syk, PI3 kinase, and ERK, may be involved in regulation of neutrophil apoptosis by the complexes. These studies demonstrate for the first time that anti-IL-8:IL-8 immune complexes have the ability to prolong neutrophil life.

Elimination of inflammatory cells, such as neutrophils, by programmed cell death or apoptosis plays an important role in the resolution of inflammation. Inhibition of apoptosis could result in the continuous presence of increased numbers of inflammatory cells, an effect that would prolong the recovery process. There are several factors that can delay apoptosis of neutrophils, including cytokines, bacterial products, glucocorticoids, and certain soluble immune complexes (5, 6, 8, 9, 16), However, ability of anti-IL-8:IL-8 complexes to modulate apoptosis of neutrophils has not been evaluated before.

Neutrophils are short-lived immune cells that spontaneously undergo apoptosis in normal physiological conditions. Members of the Bcl-2 family of protein kinases are involved in regulating of neutrophil apoptosis and can be divided into two groups: antiapoptotic proteins (such as Bcl-2, Bcl-XL, Mcl-1, and A1/Bfl-1) and proapoptotic proteins (such as Bax, Bak, Bad, Bik, and Bid). The former proteins downregulate apoptosis by controlling the activity of caspases. It is also well-established that apoptotic responses in neutrophils are instigated by activation of caspase-8 or caspase-9 (the initiator caspases), and then, in turn, activation of executioner caspases, such as caspase-3, takes place, leading to apoptosis (1, 27). Moreover, since we (10) have previously determined that IgG receptor, FcγRIIa, is involved in cellular activation mediated by anti-IL-8:IL-8 complexes, the role of this receptor in modulating of neutrophil apoptosis was evaluated in this study. Engagement of FcγRIIa by anti-IL-8:IL-8 complexes led to inhibition of neutrophil apoptosis. We also identified some of the key proteins in the FcγRIIa signaling pathway [Src, Syk, PI3 kinase (PI3-K), and ERK] as well as in the apoptotic pathways (Bcl-XL, Bax, Bak, caspase-3, and caspase-9) that may mediate antiapoptotic activity of the complexes. Based on these observations, we hypothesize that anti-IL-8:IL-8 complexes present in samples purified from pulmonary edema fluids of patients with ALI may contribute to lung inflammation in ALI/ARDS by modulating survival of neutrophils.

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).

Neutrophil preparation and culture conditions. All reagents used were free of endotoxin. Blood was drawn from healthy volunteers, and neutrophils were isolated by dextran (Amersham Biosciences, Uppsala, Sweden) sedimentation and centrifugation in Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) density gradients followed by...

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Immune complexes modulate neutrophil apoptosis

L365

hypotonic lysis as previously described (7). Purity of neutrophil preparations was between 95–97%. Freshly isolated neutrophils were suspended in either RPMI 1640 medium (Cellgro Diagnostics, Herndon, VA) supplemented with 10% BSA (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY), and 2 mM L-glutamine (Gibco) or HBSS with Ca²⁺/Mg²⁺ and incubated in humidified 5% CO₂ atmosphere at 37°C for 18 h, 6 h, or 5 min. Samples containing anti-IL-8:IL-8 complexes (IC) and control antibodies (CAb) were purified on the protein A/G column from human plasma obtained from selected healthy donors or from lung fluids from patients with ALI as previously described (10). It should be noted that 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 (10, 13, 30). Samples utilized in this procedure are obtained from donors/patients that have immune complexes of interest (10, 13, 30). Autoantibody preparations are also purified using this procedure (17). Control samples are obtained from donors/patients who lack immune complexes or autoantibodies that are being studied. All of the control samples used in the current study were obtained from healthy donors or patients with ALI that did not have appreciable levels of the complexes and are, therefore, free of anti-IL-8:IL-8 complexes. Importantly, control samples prepared in this manner do not alter neutrophil function (10). To create additional controls, we removed anti-IL-8:IL-8 complexes from the samples containing these complexes that were purified on protein A/G column by adsorption onto an ELISA plate coated with anti-IL-8 antibody. This antibody recognizes anti-IL-8:IL-8 complexes and is used routinely as coating antibody in the ELISA assay for detecting the complexes (10, 13). To prevent nonspecific interaction between the samples and the plate, 1.2 M solution of NaCl was used as a sample diluent. The removal of anti-IL-8:IL-8 complexes was confirmed using the specific ELISA assay (10, 13), and these samples were marked as anti-IL-8:IL-8 free ICEF. In addition, in some experiments, purified IC (IgG) purchased from Sigma were used. Furthermore, complexes were formed between a monoclonal anti-IL-8 antibody and recombinant human IL-8 (rhIL-8; PeproTech, Rocky Hill, NJ) and also a monoclonal anti-monocyte chemotactic peptide-1 (MCP-1) antibody (R&D Systems) and recombinant human MCP-1 (rhMCP-1; R&D Systems or PeproTech). The monoclonal anti-IL-8 antibody was developed by Dr. Edward Leonard (National Cancer Institute, Frederick, MD; Ref. 29) and has similar properties to the anti-IL-8 autoantibody (12, 13, 29). Endotoxin concentration in IC and CAb preparations was measured in a quantitative chromogenic Limulus amebocyte lysate (LAL) assay (BioWhittaker, Walkersville, MD) and was less than 100 pg/ml.

Pretreatment of neutrophils with various inhibitors. Neutrophils were suspended in cold HBSS with Ca²⁺/Mg²⁺ and pretreated with pharmacological inhibitors of different components of the FcyRIIA signaling cascade for 30 min on ice in the dark. The following inhibitors were used: LY-294002 (5 μM; PI3-K inhibitor), U-0126 (10 μM; MAP ERK kinase-1/2 (MEK-1/2) inhibitor; Cell Signaling, Beverly, MA), PP2 (20 μM; Src tyrosine kinase family inhibitor), and piceatannol (20 μM; Syk inhibitor; Calbiochem, La Jolla, CA). In some experiments, the cells were preincubated with a specific MAb against FcyRIIA [IV.3; F(ab'); Medarex, West Lebanon, NH] or a MAb against FcyRII (7.3; F(ab'); Ancell, Bayport, MN) established to be equivalent to the IV.3 antibody (10).

Morphological analysis of neutrophil apoptosis. Neutrophils were cytocentrifuged, stained with Hema3 stain set (Fisher Scientific, Middletown, VA), and counted under light microscopy (×400) to determine the proportion of cells showing characteristic apoptotic morphology. Apoptotic neutrophils were clearly identifiable as cells with condensed and dark stained nuclei. At least 800 cells were counted per slide.

Western blot technique. The cells were lysed in electrophoresis sample buffer containing 62.5 mM Tris·HCl (Fisher Scientific), 50

Fig. 1. Inhibition of spontaneous apoptosis of neutrophils by anti-IL-8:IL-8 complexes (IC). A: morphological features of cells cultured for 18 h in the presence of medium (right, top) or control antibody (left, bottom) or samples containing anti-IL-8:IL-8 complexes (right, bottom). Freshly isolated neutrophils are shown in left, top. Neutrophils undergoing apoptosis are indicated by arrows. Representative data from 6 (control antibody) to 11 (medium and complexes) experiments are shown. Images were obtained using a Nikon Eclipse TS100 microscope (×100) and processed with Nikon ACT-1/2.12 software (Nikon Instruments, Lewisville, TX). B: percentages of apoptotic cells were calculated. Results from all experiments are presented as means ± SD. C: cell death detection ELISA. Results (absorbance at 405 nm) from 4 experiments are presented as means ± SD. *P < 0.001.
Fig. 2. Effect of anti-IL-8:IL-8 complexes on caspase-3 activity. A: caspase-3 activity was determined fluorometrically by monitoring cleavage of a fluorescent-labeled substrate (Ac-DEVD-AFC) in cell lysates prepared from neutrophils cultured for 18 h. B: decreased level of caspase-3 in neutrophils after stimulation with samples containing anti-IL-8:IL-8 complexes. The whole cell lysates were subjected to SDS-PAGE. Right: Western blot analysis was performed using an antibody specific for cleaved caspase-3 (Asp175). Levels of cleaved caspase-3 were quantified by densitometric scanning of immunoblots. Representative data from 1 of 3 independent experiments are shown. C: concentration-dependent inhibition of activity of caspase-3 by the complexes. In all cases (A–C), analyzed samples contained similar amounts of protein as confirmed by Western blotting using antibodies against actin (right). *P < 0.001.
mM dithiothreitol (DTT; Sigma), 2% (wt/vol) SDS (Fisher Scientific), and 10% (vol/vol) glycerol (Fisher Scientific), boiled for 5 min, and stored at −70°C until used. Samples were loaded into a SDS-PAGE gel, and separated proteins were transferred to a polyvinylidene difluoride membrane (Pall, Pensacola, FL). Membrane was blocked and incubated with a primary antibody. The primary antibodies used in this study were anti-actin (I-19) from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-cleaved caspase-3 (Asp175) from Cell Signaling. The membrane was next incubated with peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, Cell Signaling) followed by a substrate (chemiluminescence reagent; ECL, PerkinElmer Life Sciences, Boston, MA). Then, the membrane was exposed to X-ray film F-GX810 (Phenix, Hayward, CA). Densitometric analysis of the images was carried out using the Quantity One software obtained from Bio-Rad (Hercules, CA).

Fluorometric assay for caspase-3 activity. Activity of caspase-3 was tested in neutrophil lysates by measuring of the release of fluorescent 7-amido-4-trifluoromethylcoumarin (AFC) moiety from substrate acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC; Sigma). The excitation and emission wavelengths of AFC are 400 and 505 nm, respectively. Neutrophils (4 × 10⁶) were collected by centrifugation and incubated with 0.4 ml of lysis buffer (50 mM HEPES, 5 mM CHAPS [3-[3(cholamidopropyl)dimethylammonio]-1-propanesulfonate], 5 mM DTT, pH 7.4) on ice for 15–20 min. Then, 50 µl of the lysate was added to 200 µl of solution containing 8 mM Ac-DEVD-AFC, 20 mM HEPES, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA, pH 7.4 and incubated at 20–25°C in the dark for 90 min. Cleavage of Ac-DEVD-AFC was quantified fluorometrically.

Laser confocal microscopy analysis of neutrophils. Neutrophils were mounted on slides by cytospin centrifugation, fixed with 4% (wt/vol) buffered paraformaldehyde (pH 7.4) for 30–60 min, washed three times with PBS, and further fixed with 100% methanol for 5–10 min (−20°C). Then, the cells were blocked, permeabilized in PBS/5% BSA with 0.1% (wt/vol) saponin at room temperature for 1 h, and incubated overnight with anti-cleaved caspase-3 or anti-cleaved caspase-9 antibody (18-h stimulation, 1:250 dilution), anti-Bak, anti-Bax, or anti-Bcl-XL antibody (6-h stimulation, 1:125 dilution), or...
anti-pSyk or anti-pSrc (5-min stimulation, 1:125 dilution). After three washes with PBS/BSA, the samples were further blocked for 30 min before staining with FITC- or Texas red-conjugated secondary antibodies for 1 h in the dark at room temperature. All blocking steps as well as incubation with antibodies required the inclusion of 0.1% saponin. In some experiments, the cells were counter-stained with Hoechst 33342 (10 μg/ml) for 45 min at room temperature before they were air-dried and mounted. The cells were evaluated using PerkinElmer UltraVIEW LCI confocal imaging system with Nikon TE2000-S fluorescence microscope and Plan Apo ×60 immersion oil objective.

DNA fragmentation assay. DNA fragmentation was measured using an ELISA kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions.

Statistical analysis. Differences between groups were analyzed by a simple one-way ANOVA. The direct comparison between any two treatment groups was performed using Student’s t-test or nonparametric Mann-Whitney test when the data sets were not normally distributed. Results are presented as means ± SD. All statistics were performed with SPSS SigmaStat software.

RESULTS

Anti-IL-8:IL-8 complexes suppress spontaneous apoptosis of human blood neutrophils. Our preliminary data indicated that complexes between IL-8 and anti-IL-8 autoantibodies may regulate neutrophil apoptosis (11). Based on these findings (11), we studied the effect of samples containing anti-IL-8:IL-8 immune complexes purified from normal human plasma on spontaneous apoptosis of human blood neutrophils obtained from healthy volunteers. Control antibodies were prepared in a similar way to anti-IL-8:IL-8 complexes and contained the same amount of IgG but did not contain anti-IL-8:IL-8 complexes as determined by the specific ELISA (10, 13). Apoptosis was evaluated by assessing morphological changes, i.e., dense condensation of chromatin in the form of either a single nucleus or nuclear fragments not connected by strands as previously described (24). Neutrophils were cultured for 18 h in the presence or absence of either samples containing anti-
IL-8:IL-8 complexes or the control antibody (samples devoid of anti-IL-8:IL-8 complexes). This time of incubation was found to be optimal (24). Then, cytospin slides were obtained, and cells showing dense condensation of chromatin or apoptotic bodies were classified as undergoing apoptosis. In addition, at least 800 neutrophils were evaluated on each slide. The ability of the complexes to inhibit neutrophil apoptosis is shown in Fig. 1, A and B. Figure 1A depicts morphological changes in neutrophils undergoing apoptosis, which were clearly less frequent when the cells were treated with samples containing anti-IL-8:IL-8 complexes. A significant reduction in the percentage of spontaneous apoptosis was observed in the presence of samples containing anti-IL-8:IL-8 complexes (P < 0.001; Fig. 1B). In contrast, the control antibody (samples devoid of anti-IL-8:IL-8 complexes) had no effect (Fig. 1, A and B). These findings were confirmed by evaluating DNA fragmentation, which is considered another hallmark of apoptosis (Fig. 1C). Diminished fragmentation was observed upon treatment of neutrophils with samples containing anti-IL-8:IL-8 complexes (P < 0.001) but not control antibody (samples devoid of anti-IL-8:IL-8 complexes; Fig. 1C). Only minimal DNA fragmentation was present in samples obtained from freshly isolated cells (data not shown).

**Activity of caspase-3 is downregulated by anti-IL-8:IL-8 complexes.** Caspase-3, an effector caspase, plays a critical role in the initiation of spontaneous apoptosis of neutrophils (27). Activation of caspase-3 was investigated by measuring its enzymatic activity (Fig. 2A) and by detection of the cleaved form in Western blot (Fig. 2B). Enzymatic activity of caspase-3 was significantly decreased (P < 0.001; Fig. 2A), and less of the active form of caspase-3 was detected in the presence of samples containing anti-IL-8:IL-8 complexes (Fig. 2B). Control antibody (samples devoid of anti-IL-8:IL-8 complexes), on the other hand, had no effect on degree of caspase-3 activation (Fig. 2, A and B). Furthermore, there was a concentration-dependent relation between different dilutions of the samples containing anti-IL-8:IL-8 complexes and activity of caspase-3 (Fig. 2C).

**Anti-IL-8:IL-8 complexes purified from patients with ALI and monoclonal anti-IL-8:IL-8 complexes also suppress neutrophil apoptosis.** We also evaluated the ability of complexes purified from lung fluids from patients with ALI (ICEF), CAb purified from pulmonary edema fluids from patients with ALI, or antibody against FcγRIIa and ICEF. Bak and Bax (green) are visualized in the cytoplasm of cells. Representative data from 3 experiments are shown. However, the graphs depict averages from all experiments. *P < 0.001.
from lung fluids from patients with ALI) significantly suppressed spontaneous apoptosis of neutrophils ($P < 0.001$) as well as enzymatic activity of caspase-3 ($P < 0.01$; Fig. 3, A and B). ICEF samples from which anti-IL-8:IL-8 complexes were removed (anti-IL-8:IL-8 free ICEF) as well as CAb (samples devoid of anti-IL-8:IL-8 complexes) purified from lung fluids from patients with ALI had no effect on neutrophil apoptosis (Fig. 3). Appearance of cleaved caspase-3 was also monitored by confocal microscopy using specific antibodies that recognize only the active form of the enzyme. To determine differences between treatments, positively stained cells were counted. Figure 3C depicts confocal images and mean numbers of positively stained neutrophils. ICEF (anti-IL-8: IL-8 complex-containing samples purified from edema fluids of patients with ALI) blocked the occurrence of the cleaved form of caspase-3 ($P < 0.001$), whereas processing of this caspase was not affected by the anti-IL-8:IL-8 free ICEF or the control antibody (samples devoid of anti-IL-8:IL-8 complexes) purified from edema fluids of patients with ALI (Fig. 3C). During spontaneous apoptosis of neutrophils, activation of caspase-3 is proceeded by activation of an initiator caspase, caspase-9 (27). Cleavage of caspase-9 was inhibited by anti-IL-8:IL-8 complex-containing samples purified from edema fluids of patients with ALI (ICEF) ($P < 0.001$) but not by the anti-IL-8:IL-8 free ICEF or the control antibody (samples devoid of anti-IL-8:IL-8 complexes); both the control antibody and anti-IL-8:IL-8 free ICEF were purified from edema fluids of patients with ALI (Fig. 3D).

As shown in Fig. 4, anti-IL-8:IL-8 MAb complexes displayed activity similar to ICEF. Spontaneous apoptosis (Fig. 4A), enzymatic activity of caspase-3 (Fig. 4B), and cleavage of both caspase-3 and caspase-9 (Fig. 4, C and D) was inhibited by anti-IL-8:IL-8 MAb complexes ($P < 0.001$). In addition, neither CAb (samples devoid of anti-IL-8:IL-8 complexes) purified from normal human plasma nor complexes formed between anti-MCP-1 MAb and rhMCP-1 affected apoptosis of neutrophils (Fig. 4).

IgG receptor, FcγRIIa, mediates antiapoptotic activity of anti-IL-8:IL-8 complexes. Our previous studies (10) demonstrated that IgG receptor, FcγRIIa, is a predominant receptor involved in cellular activation mediated by anti-IL-8:IL-8 complexes. Therefore, we evaluated involvement of this receptor in inhibition of spontaneous neutrophil apoptosis by samples containing anti-IL-8:IL-8 complexes. Blocking of FcγRIIa with specific antibodies resulted in the reversal of antiapoptotic activity of the complexes (Figs. 3 and 4). As shown in Fig. 3A, the percentage of apoptotic cells was not changed when neutrophils were cultured with samples containing anti-IL-8:IL-8 complexes purified from edema fluids from patients with ALI (ICEF) in the presence of the antibody against the receptor and was similar to that measured in cells cultured in medium alone. Blocking of FcγRIIa resulted also in complete suppression of activity of caspase-3 triggered by ICEF ($P < 0.001$; Fig. 3B). Moreover, levels of cleaved forms of caspase-3 and caspase-9 in cells treated with both ICEF and anti-FcγRIIa antibody were similar to those observed in cells treated with medium alone (Fig. 3, C and D). Finally, antibody against FcγRIIa blocked activity of anti-IL-8:IL-8 MAb complexes ($P < 0.001$; Fig. 4). These results indicate that engagement of FcγRIIa by anti-IL-8:IL-8 complexes leads to suppression of neutrophil apoptosis.

**Effect of anti-IL-8:IL-8 complexes on the expression of proteins from the Bcl-2 family.** Neutrophil apoptosis is regulated by proteins belonging to the Bcl-2 family. Some of the members of this family are antiapoptotic, such as Bcl-XL, and others are proapoptotic, e.g., Bak and Bax (1, 27). Level of Bcl-XL was significantly increased in the presence of samples containing anti-IL-8:IL-8 complexes purified from edema fluids from patients with ALI (ICEF) ($P < 0.001$) but was not altered in the presence of CAb (samples devoid of anti-IL-8:IL-8 complexes; also purified from edema fluids from patients with ALI; Fig. 5). In addition, blocking of FcγRIIa resulted in complete suppression of activity of ICEF ($P < 0.001$; Fig. 5). On the other hand, the levels of proapoptotic proteins, Bak and Bax, were reduced in samples treated with ICEF ($P < 0.001$; Fig. 6, A and B). Moreover, antibody against FcγRIIa reversed suppression of these proteins by ICEF (Fig. 6, C and D). These results indicate that engagement of FcγRIIa by anti-IL-8:IL-8 complexes leads to suppression of neutrophil apoptosis.

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**Fig. 7.** Examination of level of pSrc (A) and pSyk (B) in neutrophils after 5-min stimulation with samples containing anti-IL-8:IL-8 immune complexes purified from pulmonary edema fluids from patients with ALI (ICEF). CAb purified from pulmonary edema fluids from patients with ALI or with ICEF after preincubation with Src inhibitor (PP2) or Syk inhibitor (piceatannol, Pic) by laser confocal microscopy. Representative data from 3 experiments are shown. However, the graph depicts averages from all experiments. *$P < 0.001$.**
activity of ICEF ($P < 0.001$; Fig. 6). These results suggest that the mechanism that allows anti-IL-8:IL-8 complexes to suppress apoptosis may be associated with the upregulation of Bel-XL and downregulation of Bak and Bax.

Contribution of major components of the FcγRIIa signaling pathway to antiapoptotic activity of anti-IL-8:IL-8 complexes. We (10) have previously identified some of the key proteins of the FcγRIIa signaling cascade that are activated by anti-IL-8:IL-8 complexes. Our (10) results suggest that phosphorylation of a protein from Src tyrosine kinase family as well as phosphorylation of Syk, ERK, and PI3-K mediate the respiratory burst, neutrophil degranulation, and chemotaxis induced by anti-IL-8:IL-8 complexes. FcγRIIa signaling cascade is initiated upon phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) by members of the Src tyrosine kinase family. Next, the phosphorylated ITAM binds to and activates Syk-tyrosine kinase that subsequently activates several effector pathways (3, 4). We examined phosphorylation of Src (Fig. 7 A) and Syk (Fig. 7 B) in the presence or absence of anti-IL-8:IL-8 complex-containing samples (purified from edema fluids from patients with ALI/ICEF) or control antibody (samples devoid of anti-IL-8:IL-8 complexes) purified from edema fluids from patients with ALI. In addition, pharmacological inhibitors of Src (PP2) and Syk (piceatannol) were used. Neutrophils were incubated with anti-pSrc or anti-pSyk antibody followed by FITC-conjugated secondary antibody, and images were analyzed by confocal microscopy. ICEF induced activation of Src ($P < 0.001$), whereas control antibody (samples devoid of anti-IL-8:IL-8 complexes) had no appreciable effect on Src phosphorylation (Fig. 7 A). Furthermore, PP2 suppressed activity of ICEF (Fig. 7 A). Phosphorylation of Syk was also induced by ICEF ($P < 0.001$) but not control antibody (samples devoid of anti-IL-8:IL-8 complexes) and was inhibited by piceatannol (Fig. 7 B). These findings are in agreement with our previous studies (10) showing reversal of activity of the complexes in the presence of PP2 or piceatannol.

Fig. 8. Effect of inhibitors of key elements of the FcγRIIa signaling pathway on antiapoptotic activity of samples containing anti-IL-8:IL-8 complexes purified from normal human plasma (IC) on human neutrophils. A: effect on spontaneous apoptosis. Percentages of apoptotic cells were calculated. Experiments were repeated at least 3 times, and results from all experiments are presented as means ± SD. B: effect of inhibitors on caspase-3 activity. *$P < 0.001$. C: effect on amount of cleaved caspase-3 detected in Western blot (right). The graph shows densitometric analysis of the gel. Representative data from 1 of 5 experiments are shown. In A and B, analyzed samples contained similar amounts of protein as confirmed by Western blotting using antibodies against actin. Medium, untreated neutrophils undergoing spontaneous apoptosis (control). Inhibitors: U-0126, MAP ERK kinase-1/2 (MEK-1/2) inhibitor; LY-294002, PI3 kinase (PI3-K) inhibitor; piceatannol, Syk inhibitor; PP2, Src tyrosine kinase family inhibitor.
Next, we examined the effect of inhibitors of ERK, PI3-K, Syk, and Src on antiapoptotic activity of anti-IL-8:IL-8 complex-containing samples (purified from normal human plasma; Fig. 8). All of the inhibitors neutralized activity of the complexes. This result was true for spontaneous apoptosis based on morphological changes in neutrophils (Fig. 8A) as well as activity and cleavage (Fig. 8B and C) of caspase-3. Our findings indicate involvement of key components of the FcγRIIa signaling pathway in regulating activity of anti-IL-8:IL-8 complexes in regard to neutrophil apoptosis (summarized in Fig. 9).

**DISCUSSION**

The aim of the present study was to determine whether anti-IL-8:IL-8 complexes have the ability to prolong neutrophil life and identify specific pathways that may mediate antiapoptotic activity of the complexes. It was previously reported that soluble immune complexes prepared by reacting human IgG or ovalbumin with specific rabbit antibodies are capable of inhibiting spontaneous apoptosis of neutrophils (8). However, endotoxin contamination was not evaluated in these samples, which makes the data presented in the paper difficult to interpret, especially that endotoxin can affect neutrophil apoptosis (5, 8).

Our findings indicate that anti-IL-8:IL-8 complex-containing samples purified from edema fluids obtained from patients with ALI have the ability to prolong neutrophil life. Anti-IL-8:IL-8 complexes seem to be responsible for the antiapoptotic activity of these samples. First, removal of anti-IL-8:IL-8 complexes renders them inactive. Second, control antibodies purified from edema fluids from patients with ALI that were devoid of the complexes had no effect on neutrophil apoptosis. Moreover, anti-IL-8:IL-8 MAAb immune complexes (complexes formed between a monoclonal anti-IL-8 antibody and rhIL-8) display antiapoptotic activity towards neutrophils in a similar manner to purified samples. These preformed complexes serve as model for anti-IL-8 autoantibody:IL-8 complexes, and we (10) have previously reported that they do not differ from purified counterparts in their ability to activate neutrophils and trigger chemotaxis of these cells. Importantly, the monoclonal anti-IL-8 antibody used to form these complexes has similar properties to anti-IL-8 autoantibody (12, 13). Finally, preformed anti-MCP-1:MCP-1 complexes do not have any effect on neutrophil apoptosis even in concentrations up to 50× higher than effective concentrations of anti-IL-8:IL-8 MAAb complexes. Anti-MCP-1:MCP-1 complexes substantially differ from anti-IL-8:IL-8 complexes in their ability to affect neutrophil function. They do not activate neutrophils (10), and they trigger neutrophil chemotaxis at concentrations 10× higher than anti-IL-8:IL-8 complexes through a different receptor (FcγRIIb) than anti-IL-8:IL-8 complexes (FcγRIIa; Ref. 10). The latter should be emphasized since the activity of preformed and purified anti-IL-8:IL-8 complexes can be suppressed by blocking FcγRIIa (10). In summary, the results of our studies indicate for the first time that anti-IL-8:IL-8 complexes may be involved in the regulating of neutrophil apoptosis. This is an important finding since it is known that apoptosis of neutrophils is altered in patients with ARDS, and, furthermore, there is limited information regarding regulation of this process (20).

Anti-IL-8:IL-8 complex-containing samples significantly suppressed spontaneous apoptosis of neutrophils as well as enzymatic activity of caspase-3 and caspase-9. In addition, our results indicate that level of antiapoptotic protein (Bcl-XL) is increased in human neutrophils incubated with these samples, whereas levels of proapoptotic proteins (Bax, Bak) are decreased.

Since our previous studies (10) demonstrated that IgG receptor, FcγRIIa, is the receptor involved in cellular activation of neutrophils triggered by anti-IL-8:IL-8 complexes, we explored the role of this receptor in the regulating of neutrophil apoptosis. Blockade of FcγRIIa abrogated antiapoptotic activity of the complexes. This observation indicates that engagement of FcγRIIa by anti-IL-8:IL-8 complexes leads to extension of neutrophil life span. Furthermore, antiapoptotic activity of the samples was abolished in the presence of pharmacological inhibitors of crucial signaling proteins of the FcγRIIa cascade (U-0126, MEK-1/2 inhibitor; LY-294002, PI3-K inhibitor; piceatannol, Syk inhibitor; and PP2, Src tyrosine kinase family inhibitor). Our findings suggest that these key components of the FcγRIIa signaling pathway (Src, Syk, PI3-K, and
ERK) may be involved in regulation of neutrophil apoptosis by anti-IL-8:IL-8 complexes. It is known that members of the Src tyrosine kinase family initiate the FcγRIIa signaling cascade. Subsequently, Syk tyrosine kinase is activated. Then, Syk activates a number of effector pathways including PI3-K pathway (3, 4). PI3-K generates a survival signal through activating Akt, which, in turn, alters expression and/or activity of other proteins involved in regulation of neutrophil apoptosis such as members of the Bcl-2 family of protein kinases. Other components of the FcγRIIa signaling pathway are also involved in regulating neutrophil apoptosis, and in particular, ERK was reported to inhibit neutrophil apoptosis (Fig. 9; Ref. 1).

Other groups also studied immune receptors and apoptosis of myelocytic cells, but these reports differ substantially from our findings (25, 28). Suttman et al. (28) observed inhibition of spontaneous apoptosis of neutrophils obtained from healthy volunteers by Mycobacterium bovis bacillus Calmette-Guérin (BCG). BCG also upregulated the surface expression of FcγRII on these cells. The authors, however, did not study function of FcγRII in relation to neutrophil apoptosis. It was also reported that human osteoclast-associated receptor (hOSCAR), an immune receptor associated with the Fc receptor γ-chain (FcγRy), has the ability to regulate apoptosis of monocyte-derived dendritic cells (25). Even though the γ-chain is also associated with FcγRIIa (4), hOSCAR and FcγRIIIa are two different receptors (4, 25). Nevertheless, engagement of either receptor activated ERK and PI3-K pathways in dendritic cells in case of hOSCAR (25) or neutrophils in case of FcγRIIa involvement. In addition, cross-linking of hOSCAR induced expression of Bcl-2 and Bcl-X₇ in dendritic cells (25). Increased level of Bcl-X₇ was also observed as a result of interaction between anti-IL-8:IL-8 complexes and FcγRIIa. Bcl-2 was not studied because neutrophils do not express this protein (1).

Our findings suggest that anti-IL-8:IL-8 complexes present in samples purified from normal human plasma or lung edema fluids obtained from patients with ALI are capable of modulating neutrophil apoptosis. Specifically, anti-IL-8:IL-8 complex delay spontaneous apoptosis of neutrophils through interaction with FcγRIIa. Significance of these observations remains to be established. However, it should be noted that occurrence of anti-IL-8:IL-8 complexes is quite high since they are present in 55–70% of normal human plasmas (13, 29). Although we have not studied specifically the frequency of plasma anti-IL-8 autoantibody:IL-8 complexes in patients with ARDS, we evaluated eight ARDS plasmas for the presence of the complexes. All these plasmas contained detectable levels of anti-IL-8:IL-8 complexes (13). In addition, our previous studies (14, 15) demonstrated that patients at risk for ARDS who had high concentrations of anti-IL-8:IL-8 complexes in their lung fluids were more likely to develop ARDS. The level of anti-IL-8:IL-8 complexes also correlated with mortality in patients with ARDS (13, 15). Moreover, we showed that anti-IL-8:IL-8 complexes present in lung edema fluids from patients with ALI possess proinflammatory activity in vitro and act through IgG receptors, FcγRIIa, that are expressed on human neutrophils (10). On the other hand, presence of other autoantibodies or immune complexes in patients with ARDS cannot be precluded. Antiphospholipid autoantibodies, for example, have been detected in lung fluids from patients with ARDS (18). This study together with our previous (13–15, 18) and current findings suggest that autoimmune component can play a role in pathogenesis of ARDS. To test this hypothesis, we used confocal microscopy technique to evaluate lung tissues from patients with lung injury and control lung tissues for the presence of anti-IL-8:IL-8 complexes (2). IL-8 costained with IgG and FcγRIIa (immune complex receptors) in lung tissues from patients with ARDS but not in control tissues. These observations suggest that anti-IL-8:IL-8 complexes are deposited in lungs of patients with ARDS via FcγRIIa (2). We were also able to detect anti-IL-8:IL-8 complexes bound to neutrophils present in the alveolar spaces of ARDS patients (R. Fudala and A. K. Kurdowska, unpublished observations).

Since lung deposition of immune complexes causes severe lung inflammation leading to injury in animal models (3), anti-IL-8:IL-8 immune complexes could potentially contribute to pathogenesis of ARDS. However, the actual diagnostic and therapeutic significance of our observations remains to be established.

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