PAPER

# Role of Interleukin 8 in the Genesis of Acute Respiratory Distress Syndrome Through an Effect on Neutrophil Apoptosis

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**Objective:** To evaluate the role of interleukin 8 (IL-8) in the regulation of neutrophil (PMN) apoptosis in normal plasma and plasma from patients with early, fulminant acute respiratory distress syndrome (ARDS).

**Design:** Experimental study using cultured human PMNs.

**Setting:** University hospital, level I trauma center.

**Participants:** Plasma was obtained from 6 patients with early, fulminant posttraumatic ARDS (mean Injury Severity Score, 26). All samples were drawn within 24 hours after injury. Plasma was also taken from 13 healthy control subjects. These controls were also used as sources of PMNs.

**Main Outcome Measures:** Effect of early, fulminant ARDS and normal plasma on spontaneous apoptosis, CD16, and CD11-b expression in PMNs in vitro; levels of IL-8 in plasma; correlation of extracellular IL-8 concentration with rate of PMN apoptosis; and effect of IL-8 blockade on PMN apoptosis, CD16, and CD11-b expression in ARDS and normal plasma.

**Results:** Plasma from patients with early, fulminant ARDS inhibited spontaneous PMN apoptosis at 24 hours (35% ± 5% vs 54% ± 5%; $P = .01$). Neither CD16 nor CD11-b differed significantly between the 2 groups. The mean plasma level of IL-8 in patients with early, fulminant ARDS was 359 ± 161 pg/mL vs 3.0 ± 0.4 pg/mL in healthy controls ($P<.05$). Interleukin 8 inhibited apoptosis in plasma-free medium at low doses (1-50 pg/mL) but had no significant effect at higher doses (100-5000 pg/mL) ($P<.05$). Interleukin 8 blockade with monoclonal antibody suppressed apoptosis in normal plasma (28% ± 5% with monoclonal antibody vs 51% ± 5% without monoclonal antibody; $P = .008$) but not in plasma from patients with early, fulminant ARDS (29% ± 5% with monoclonal antibody vs 34% ± 6% without monoclonal antibody; $P = .67$). It had no effect on CD16 or CD11-b expression in either plasma.

**Conclusions:** Plasma from patients with early, fulminant ARDS contains soluble factors that inhibit PMN apoptosis in vitro. Low levels of IL-8 inhibit PMN apoptosis in normal plasma. Although plasma levels of IL-8 are markedly elevated in early, fulminant ARDS, IL-8 is not directly responsible for the antiapoptotic effect of plasma from patients with early, fulminant ARDS.

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THE ACUTE respiratory distress syndrome (ARDS) appears to be an inflammatory process in which activated neutrophils (PMNs) may play a role. There is evidence to suggest that PMNs may influence or initiate injury to the pulmonary alveolar-capillary membranes, leading to flooding of the alveolar spaces, disruption of normal gas exchange, and severe, refractory hypoxemia.[1] Large numbers of PMNs accumulate within the lung in the early phase of ARDS, such that PMNs constitute 80% or more of the total cells obtained by bronchoalveolar lavage (BAL), compared with approximately 3% in normal subjects. Stimulation of PMNs to secrete elastase, myeloperoxidase, and toxic oxygen metabolites leads to the observed lung injury.[1]

Elimination of PMNs through the induction of programmed cell death, or apoptosis, is thought to help regulate the inflammatory response in the lung after major trauma. Inhibition of this process may have beneficial effects by perpetuating the phagocytic function of PMNs at sites of acute inflammation. It can also, however, have a deleterious effect by causing the pathological persistence of inflammation in the lung, potentially leading to ARDS.

Many cytokines have been implicated in the modulation of PMN activity in the lung in ARDS.[2-6] Plasma and BAL

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## MATERIALS AND METHODS

All research was conducted and all patient samples were collected in accordance with the requirements of the institutional review board of University of Medicine and Dentistry of New Jersey–New Jersey Medical School, Newark.

### NEUTROPHIL SEPARATION

Neutrophils were prepared from minimally heparinized whole blood of healthy volunteer donors (herewith referred to as *controls*). Specimens were spun at 1500 rpm for 15 minutes. Plasma was removed and kept at 4°C until cells were prepared for incubation. The PMNs were isolated by sequential separations with the use of Ficoll-Hypaque and dextran sedimentation. Osmotic lysis with 0.14-mol/L ammonium chloride was used to remove contaminating red blood cells. The cells were washed twice and resuspended in RPMI 1640 (Sigma, St Louis, Mo) at $10^6$ cells per milliliter. Purity of PMNs was assessed by flow cytometric staining for the PMN surface marker CD15; purity was typically found to be 95% or greater. Only samples that showed neutrophil viability of 85% or greater (as determined by trypan blue dye exclusion) were used for the experiments.

### PREPARATION OF PLASMA FROM PATIENTS WITH E/F ARDS AND CONTROLS

Blood was obtained from patients with clinical and radiological evidence of early posttraumatic ARDS (as defined by $Pa_{O_2}$/fraction of inspired oxygen $<$150 mm Hg, diffuse parenchymal infiltrates on chest x-ray film, pulmonary capillary wedge pressure $<$18 mm Hg, and no evidence of sepsis) and from healthy controls. The mean Injury Severity Score of the patients was 26 (range, 22-34), and the mean transfusion requirement was 12 units of packed red blood cells in their initial resuscitation (range, 4-21). The mortality rate was 67%. Samples were drawn from patients within 24 hours of injury. Plasma was prepared by spinning down minimally heparinized whole blood at 1500 rpm for 15 minutes. The plasma was decanted and either kept at 4°C for immediate use or aliquoted and stored at –80°C. Patient samples were obtained according to appropriate institutional review board protocols.

### ASSESSMENT OF CELL VIABILITY AND APOPTOSIS

#### E/F ARDS vs Control Plasma

A total of 100 000 control PMNs were suspended in 80 µL of RPMI 1640. Then 20 µL of autologous plasma or ARDS patient plasma was added to make a total of 100 µL of suspension (20% plasma), and the cells were incubated at 37°C for 24 hours. Cell viability and apoptosis were assessed with annexin V–fluorescein isothiocyanate and propidium iodide (Trevigen Inc, Gaithersburg, Md). This assay is based on the ability of annexin V to bind to phosphatidylserine molecules, which are usually confined to the inner leaflet of the cell membrane but become exposed on the cell surfaces of PMNs during early apoptosis. Uptake of propidium iodide reflects loss of membrane integrity and indicates late apoptosis or necrosis. Cells were stained with annexin V and propidium iodide for 30 minutes at room temperature in the dark. The samples were placed in ice and analyzed fresh by means of a flow cytometer (FACSCalibur; Becton Dickinson Co, San Jose, Calif) and software (CELLQuest; Becton Dickinson Co).

#### Blockade of IL-8 Activity

In some experiments, 1 µg of anti–IL-8 blocking monoclonal antibody (mAb) (R&D Systems, Minneapolis, Minn) was added to 20 µL of ARDS or control plasma, before addition to 100 000 PMNs in 80 µL of RPMI 1640. This dose of mAb is sufficient to block the activity of $10^6$ pg/mL of IL-8. Cells were incubated at 37°C for 24 hours and then stained and analyzed as previously described. Double doses of mAb (2 µg/100 µL) were used in some experiments with both ARDS and control plasma to confirm that full IL-8 blockade was achieved at the lower dose of mAb.

#### IL-8 Dose-Response Curve

A total of 100 000 donor PMNs were suspended in 95 µL of RPMI 1640, to which was added 5 µL of sterile phosphate-buffered saline (Gibco BRL, Grand Island, NY) containing varying doses of recombinant human IL-8 (R&D Systems). Final IL-8 concentrations of 1, 10, 50, 100, 500, 1000, and 5000 pg/mL were chosen because they encompass the range of levels seen in clinical samples of plasma and BAL fluid. Cells were incubated at 37°C for 24 hours and then stained and analyzed as previously described.

### SURFACE EXPRESSION OF CD16 AND CD11-B

Surface marker expression was determined by adding 10 µL of either fluorescein isothiocyanate–labeled anti–CD16 mAb (Becton Dickinson Co) or phycoerythrin-labeled anti–CD11-b mAb (Becton Dickinson Co) to 100 000 donor PMNs, which were then incubated in the dark at room temperature for 20 minutes. Cells were washed twice and fixed with 1% methanol-free formaldehyde (Polysciences Inc, Warrington, Pa). They were stored in the dark at 4°C until analyzed via flow cytometry within 36 hours.

### PLASMA LEVELS OF IL-8

Plasma IL-8 levels in both ARDS and control plasma samples were determined by means of a sandwich enzyme-linked immunosorbent assay kit (Endogen Inc, Woburn, Mass) as described by the vendor. This kit detects levels from 2 to 1000 pg/mL. Values greater than 1000 pg/mL were reported as such.

### DATA ANALYSIS

Mean values and SEMs were calculated for cell viability, apoptosis, CD16 and CD11-b surface expression, and plasma IL-8 concentration. The Mann-Whitney *U* test was used to analyze the differences in viability. A 1-way analysis of variance and Duncan multiple range test were used to analyze medium-dose vs low-dose and low-dose vs high-dose differences in the IL-8 dosing curve experiment. Differences in CD16 and CD11-b expression in the various groups were analyzed with the Wilcoxon sign rank test.

fluid levels of interleukin (IL) 8 are markedly increased in ARDS,[3,4] with persistent elevations correlating with a poor prognosis.[4] Few data are available, however, regarding early, fulminant (E/F) ARDS. We hypothesized that soluble factors in plasma from patients with E/F ARDS, such as IL-8, inhibit spontaneous PMN apoptosis in vitro and that dysregulation of PMN apoptosis because of these factors in vivo might be implicated in the pathogenesis of E/F ARDS after major trauma. The purpose of our study was to evaluate the effect of plasma from patients with E/F ARDS on spontaneous apoptosis of normal neutrophils in vitro and to determine whether the chemokine IL-8 played a central role in this effect.

## RESULTS

### EFFECT OF E/F ARDS VS CONTROL PLASMA ON PMN VIABILITY AND APOPTOSIS

Neutrophil viability at 24 hours was significantly higher when cells were incubated in E/F ARDS plasma than with control plasma (**Table 1**). Likewise, E/F ARDS plasma suppressed apoptosis when compared with control (Table 1). There was no significant difference in CD16 or CD11-b expression at 24 hours of incubation (**Table 2**).

### EFFECT OF IL-8 ON PMN VIABILITY AND APOPTOSIS

The addition of 1 µg of anti–IL-8 mAb to control plasma significantly increased PMN viability and suppressed apoptosis at 24 hours in culture when compared with similar plasma samples without mAb (**Table 3**). Doubling the dose of mAb did not significantly alter its effect on PMN viability and apoptosis

The addition of anti–IL-8 mAb to E/F ARDS plasma did not affect PMN viability or apoptosis (Table 3). Doubling the dose of mAb had no effect on PMN viability or apoptosis. Interleukin 8 blockade did not significantly alter CD16 or CD11-b expression after 24 hours of incubation (**Table 4**).

### IL-8 DOSING CURVE

Neutrophil viability was significantly enhanced in the presence of low concentrations of IL-8 (1, 10, and 50 pg/mL) after 24 hours vs control (0 pg/mL) and vs high concentrations (100, 500, 1000, and 5000 pg/mL) (n = 6 for all doses) ($P<.05$) (**Figure**). Likewise, apoptosis was inhibited at low doses (1-50 pg/mL) but was permitted at higher levels (100-5000 pg/mL) (Figure).

### IL-8 ENZYME-LINKED IMMUNOSORBENT ASSAY

The mean level (±SE) of IL-8 in patients with E/F ARDS was 359 ± 161 pg/mL, with a range of 41 to greater than 1000 pg/mL (n = 6), vs 3.0 ± 0.4 pg/mL in controls (range, 2-17 pg/mL) (n = 13) ($P<.05$).

**Table 1. Normal PMN Viability and Apoptosis After 24 Hours of Incubation in Plasma From Patients With ARDS and Normal Subjects***

| | Mean ± SEM, % | |
|---|---|---|
| Group (No.) | Viability | Apoptosis |
| Control (10) | 41 ± 4.2† | 54 ± 4.8‡ |
| ARDS (6) | 63 ± 5.3† | 35 ± 5.5‡ |

*PMN indicates neutrophil; ARDS, acute respiratory distress syndrome.
†P = .005 by Mann-Whitney U test.
‡P = .01 by Mann-Whitney U test.

**Table 2. Normal PMN Expression of CD16 and CD11-b After 24 Hours of Incubation in Plasma From Patients With ARDS and Normal Subjects***

| | Mean ± SEM Fluorescence, % | |
|---|---|---|
| Group (No.) | CD16 | CD11-b |
| Control (8) | 35.6 ± 5.6† | 275.4 ± 68.6‡ |
| ARDS (6) | 54.4 ± 15.4† | 296.4 ± 68.5‡ |

*PMN indicates neutrophil; ARDS, acute respiratory distress syndrome.
†P = .18 by Mann-Whitney U test.
‡P = .67 by Mann-Whitney U test.

**Table 3. Normal PMN Viability and Apoptosis After 24 Hours of Incubation in Plasma From Patients With ARDS and Normal Subjects With and Without Blocking Anti–IL-8 Monoclonal Antibody***

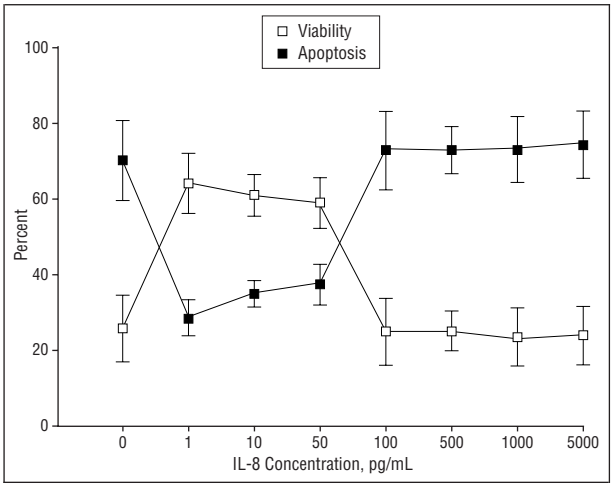
| | Mean ± SEM, % | |
|---|---|---|
| Group (No.) | Viability | Apoptosis |
| Control | | |
| Without anti–IL-8 (10) | 43 ± 5.5† | 51 ± 5.4† |
| With anti–IL-8 (10) | 71 ± 4.7† | 28 ± 4.6† |
| ARDS | | |
| Without anti–IL-8 (6) | 63 ± 5.6‡ | 34 ± 5.7‡ |
| With anti–IL-8 (6) | 67 ± 4.8‡ | 29 ± 4.8‡ |

*PMN indicates neutrophil; ARDS, acute respiratory distress syndrome; and IL-8, interleukin 8.
†P = .005 (viability) and P = .008 (apoptosis) by Wilcoxon sign rank test.
‡P = .67 (viability) and P = .68 (apoptosis) by Wilcoxon sign rank test.

**Table 4. Normal PMN Expression of CD16 and CD11-b After 24 Hours of Incubation in ARDS and Normal Plasma With and Without Blocking Anti–IL-8 Monoclonal Antibody***

| | Mean ± SEM Fluorescence, % | |
|---|---|---|
| Group (No.) | CD16 | CD11-b |
| Control | | |
| Without anti–IL-8 (4) | 38 ± 5† | 193 ± 101† |
| With anti–IL-8 (4) | 44 ± 10† | 359 ± 109† |
| ARDS | | |
| Without anti–IL-8 (4) | 46 ± 10‡ | 313 ± 65‡ |
| With anti–IL-8 (4) | 37 ± 15‡ | 417 ± 58‡ |

*PMN indicates neutrophil; ARDS, acute respiratory distress syndrome; and IL-8, interleukin 8.
†P = .29 (CD16) and P = .47 (CD11-b) by Wilcoxon sign rank test.
‡P = .45 (CD16) and P = .43 (CD11-b) by Wilcoxon sign rank test.




*Neutrophil viability and apoptosis after 24 hours of incubation in RPMI 1640 with varying concentrations of interleukin (IL) 8. n = 6 for each dose.* P<*.005 for each data point. Bars represent SEM for each data point.*

## COMMENT

Termination of PMN-mediated inflammatory processes such as ARDS may be effected through PMN apoptosis. Apoptosis is a $Ca^{2+}$-dependent process of regulated cellular death, mediated by a family of intracellular cysteine proteases.[7] Induction of PMN apoptosis with the subsequent rapid phagocytosis of the PMNs by alveolar macrophages provides the lung with a mechanism by which it can remove PMNs from an area of inflammation, minimizing damage to the surrounding tissue. Normal PMNs survive for less than 24 hours in the circulation before undergoing apoptosis, but apoptosis is delayed in PMNs migrating into inflammatory foci.[7] This inhibition is mediated through cytokines such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, and IL-8.[7] Inhibition of PMN apoptosis and prolongation of PMN survival have been identified in circulating PMNs isolated from patients with severe burns.[8] This dysregulation of apoptosis may enhance the tendency of these patients to develop ARDS. The mechanisms by which PMN apoptosis is blocked after injury are unknown. It is probably in part inhibited by soluble factors. Plasma from patients with systemic inflammatory response syndrome suppresses apoptosis of normal PMN apoptosis.[7] Similarly, BAL fluid from patients with ARDS inhibits normal PMN apoptosis when compared with control lavage fluid. This effect can be blocked by immunodepleting BAL fluid of G-CSF and GM-CSF.[9] Interleukin 8 has also been shown to delay spontaneous and tumor necrosis factor α–induced apoptosis of PMNs, albeit at concentrations much higher than those found in plasma or BAL fluid from patients with ARDS.[10]

Several studies have attempted to correlate circulating and BAL fluid cytokine levels with risk of ARDS, systemic inflammatory response syndrome, or multiple organ failure. Levels of G-CSF, GM-CSF, IL-8, IL-6, epithelial-derived neutrophil attractant-78 (ENA-78), and growth-related protein α (Gro-α) in BAL fluid are all significantly elevated in patients with ARDS after trauma, intra-abdominal sepsis, or pneumonia.[2-6] Survival after ARDS is typically associated with a rapid reduction in BAL fluid cytokine levels, whereas persistent elevations correlate with a poor outcome.[3] Neutrophil concentrations in BAL fluid consistently correlate with levels of IL-8, presumably because of its chemotactic activity.[4] The data regarding circulating levels of these cytokines are, however, less clear. Chollet-Martin et al[11] reported uniformly elevated plasma IL-8 levels in 12 patients with ARDS, although these levels did not correlate with survival or the presence of shock. Schutte et al[12] demonstrated consistently increased levels of circulating IL-6 in a similar patient population, but inconsistent elevations in plasma IL-8 levels. Donnelly et al,[2] however, showed no difference in plasma concentrations of IL-8 in a group of patients with ARDS of mixed cause, compared with similar patients who did not progress to ARDS. Marty et al[13] reported a mixed group of intensive care unit patients in whom elevated plasma levels of IL-8 correlated with mortality in septic but not nonseptic patients. None of these studies consisted solely of trauma patients. Most recently, however, Nast-Kolb et al[14] examined a population of multiply injured patients (Injury Severity Score, >18) and confirmed that persistently high circulating levels of IL-6 and IL-8 correlated with risk of multiple organ failure and death.

Most data on inflammatory responses after trauma have been obtained from patients with the late septic complications of injury. Our study is important in that the patients recruited represent a subpopulation of severely traumatized patients with fulminant ARDS developing early after injury in the absence of sepsis but with a high mortality (67% [4/6]). Our data demonstrate that soluble antiapoptotic factors exist in E/F ARDS plasma that inhibit spontaneous apoptosis in normal PMNs in vitro as assessed by staining with annexin V and propidium iodide. Plasma from patients with E/F ARDS does not appear to reduce surface expression of CD16, which has previously been used as an indirect marker of apoptosis. Shedding of CD16 may be a terminal change of apoptosis that our assay did not detect at 24 hours of incubation. Plasma from patients with ARDS did not significantly increase CD11-b expression, suggesting that in this model, E/F ARDS plasma prolongs PMN survival without causing cellular activation. We have yet to correlate this finding with other markers of PMN activation, such as enhanced respiratory burst activity. Jiminez and his group[7] found that plasma from patients with systemic inflammatory response syndrome inhibited apoptosis of normal PMNs when compared with control plasma. They also showed that PMNs from patients with systemic inflammatory response syndrome, when incubated in autologous plasma, showed delayed apoptosis and enhanced respiratory burst activity when compared with cells isolated from healthy controls or from patients who had undergone elective surgery and incubated in their own plasma.[7] Expression of CD11-b was not statistically different in the 3 groups, in agreement with our data. It is still unknown whether PMNs isolated from patients with ARDS, systemic inflammatory response syndrome, or multiple organ failure exhibit an intrinsic resistance to spontaneous apoptosis separate from the effects of soluble antiapoptotic factors in the plasma of these patients.

Our findings suggest that suppression of PMN apoptosis in E/F ARDS plasma is *not* primarily mediated by

### Statement of Clinical Relevance

It is now widely accepted that recruitment and subsequent activation of PMNs within the lung are important causative factors in the pathogenesis of ARDS. Proinflammatory cytokines such as IL-8 stimulate both PMN chemotaxis and activation. High levels of these cytokines have been documented in both the plasma and lungs of patients with ARDS.

Cytokines also help to regulate PMN activity within inflammatory foci by mediating the normal elimination of PMNs through the induction of apoptosis (programmed cell death). Alterations in cytokine production within the circulation and the lung can lead to a dysregulation of apoptosis. This in turn can induce abnormal persistence of PMNs at sites of inflammation and exaggerated PMN generation of reactive oxygen intermediates and proteases. Under these conditions, the beneficial antimicrobial activity of PMNs becomes replaced by extensive PMN-mediated damage of lung tissue, the hallmark of early ARDS.

If alterations in the normal cytokine milieu of the plasma and lung are responsible for inducing excessive stimulation and impairing apoptotic elimination of PMNs within the lung after major injury, therapies aimed at restoring the normal equilibrium of cytokine activity may help to prevent or treat ARDS. Our study aimed to demonstrate that plasma of patients with ARDS after major trauma inhibited the apoptosis of normal PMNs in vitro. Furthermore, since treatment with anti–IL-8 antibodies reduces acute lung injury in animal models, we wished to see whether neutralization of IL-8 activity in ARDS plasma might work by restoring normal patterns of PMN apoptosis.

IL-8, since our dose-response results show that the mean IL-8 level seen in E/F ARDS plasma (359 ± 161 pg/mL) should have no effect on PMN apoptosis. Blockade of IL-8 activity inhibits PMN apoptosis in control plasma, even though plasma levels of IL-8 in healthy individuals are low (3 ± 0.4 pg/mL). Inhibition of IL-8 activity in E/F ARDS plasma has no effect on PMN apoptosis, again refuting the hypothesis that high plasma levels of IL-8 inhibit PMN apoptosis in E/F ARDS.

There are presumably other factors present in E/F ARDS plasma that suppress PMN apoptosis. Candidate antiapoptotic factors include G-CSF, GM-CSF, Gro-α, IL-6, and ENA-78.[5,9] Growth-related protein α, ENA-78, and IL-8 are all C-X-C chemokines that can bind to CXC RI and RII receptors on the surface of PMNs.[15] Elevations in BAL fluid levels of Gro-α, IL-6, and ENA-78 are all associated with ARDS,[4] with Gro-α levels exceeding IL-8 levels by more than 2.4 times.[6] Recent work has shown that the suppressive effect of ARDS BAL fluid on PMN apoptosis is reversed with immunodepletion of G-CSF and GM-CSF.[9] Since IL-8, in conjunction with IL-1, down-regulates the surface expression of both CXC RI and RII receptors on normal PMNs in vitro,[16] it is possible that blocking IL-8 activity in normal plasma suppresses PMN apoptosis by altering CXC RI and RII expression, thereby modulating the activity of other C-X-C chemokines, such as Gro-α and ENA-78. This down-regulation of CXC RI and RII only occurs at low levels of IL-8, explaining why IL-8 blockade in ARDS plasma (ie, at high IL-8 levels) has no effect on PMN apoptosis. We are presently analyzing the effects of IL-8 blockade on PMN CXC RI and CXC RII expression in both normal and ARDS plasma. Modulation of C-X-C chemokine activity through IL-8–mediated alterations in CXC RI and RII receptor function on PMNs may disrupt the complex equilibrium existing between proapoptotic and antiapoptotic factors in plasma, leading to a pathological dysregulation of PMN apoptosis and the onset of E/F ARDS.

In conclusion, we have demonstrated that plasma from patients with E/F ARDS suppresses apoptosis and that multiple agents are probably involved in this process. Our data suggest that IL-8, although present in high levels in E/F ARDS plasma, is not directly responsible for this suppressive effect. Interleukin 8 may act indirectly by modulating the effects of other C-X-C chemokines on PMN apoptosis. Low levels of IL-8 inhibit PMN apoptosis in normal plasma, perhaps by down-regulating CXC RI and RII receptor expression on PMNs. More studies are needed to determine the exact role of C-X-C chemokines in the modulation of PMN apoptosis under normal and pathological conditions. These data may facilitate appropriate strategies to manipulate PMN apoptosis as a basis for the prevention and treatment of E/F ARDS after major trauma.

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## DISCUSSION

**Ronald V. Maier, MD, Seattle, Wash:** I congratulate the authors on their attempts to elucidate this extremely complex issue of apoptosis dysregulation during inflammatory conditions. Apoptosis, as was mentioned earlier, is considered a normal process, and inhibition will prolong the survival of the neutrophil. Since neutrophils are linked to multiple inflammatory conditions including multiple organ failure, inhibition of apoptosis may contribute to the organ injury and failure.

The authors have confirmed prior work done by our president, Dr Rotstein, who demonstrated that plasma from inflammatory conditions does inhibit apoptosis in neutrophils. Others have shown that inhibiting GM-CSF and G-CSF in bronchoalveolar lavage fluid appears to identify the mediator(s) of this inhibition of apoptosis. Eliminating or blocking these 2 cytokines did prevent the change in apoptosis, which is opposite to the current data. Interestingly, the authors show that IL-8 is markedly elevated in the plasma, but inhibiting it did not reverse the dysregulation.

Since the patients in the ARDS group had, on average, 12 units of blood, did the authors merely measure the levels of IL-8 in bank blood? Were they transfusing enormous levels of IL-8? Was the IL-8 measured under these conditions actually functional IL-8 or only immunologic IL-8? Are there data to support whether this was a functional cytokine under these conditions?

In the normal plasma, there was a significant effect of low-level IL-8 causing an increased apoptosis rate. Is it possible that these low levels of IL-8 under normal conditions maintain the high apoptotic rate that is normal, and that when you block it, or increase the levels of IL-8, the response is to actually increase survival and preserve neutrophil function and therefore apoptosis goes down?

Thirdly, what do they know about their blocking antibody? Does it block IL-8 function? Does it block binding of the IL-8 to both IL-8 receptors or to only one preferentially? Is the difference in their results at high and low levels of IL-8 merely the selective binding of both receptor 1 and receptor 2 for the C-X-C cytokines? Does this explain the difference of high and low levels of IL-8?

Lastly, a hypothetical question. Granulocyte colony-stimulating factor, which is known to prolong neutrophil survival, has actually been shown in clinical studies to enhance patient survival in pneumonia and sepsis. One could argue that inhibition of apoptosis is actually beneficial for the host. Therefore, why are the authors pursuing in this setting that prolongation of neutrophil survival and blocking apoptosis is bad? Thank you.

**Dr Goodman:** In no particular order, we didn't actually measure the level of IL-8 in the bank blood, in our blood bank given to these patients. It is a very interesting idea, and perhaps if there are high levels of IL-8 in bank blood, this effect may be more of a function of massive blood transfusion rather than ischemia reperfusion and the induction of ARDS. In our study, we are looking at IL-8 levels based on an immunological methodology. We haven't looked at a functional assay of IL-8 in this setting.

In terms of low-level IL-8 inhibition of normal apoptosis, there is evidence that IL-8, in combination with IL-1, can actually down-regulate IL-8 receptor expression, both the CXC RI and the CXC RII expression on normal neutrophils. And so perhaps low levels of IL-8 act to modulate apoptosis by interfering with the binding of other C-X-C chemokines to the IL-1 and IL-2 receptors.

To add to that, we have just started to look at CXC RI and CXC RII receptor expression in normal neutrophils incubated in both normal and ARDS plasma, and we found that the ARDS plasma causes an up-regulation of the CXC RII receptor on neutrophils. And if you double-stain the neutrophils to look at annexin staining and expression of the R-2 receptor, it appears that the cells that have a high level of the CXC RII after incubation in ARDS plasma are those cells that are nonapoptotic. So perhaps up-regulation of the receptor is having a protective effect in these cells.

And we are also looking at the levels of other C-X-C chemokines in the plasma of these early, fulminant ARDS patients, and we are beginning to show elevated levels of Gro-α in these patients, and we are going to look at whether these elevations of Gro-α are involved in mediating the antiapoptotic effect of the plasma and may also be involved in up-regulation of the CXC RII expression of the nonapoptotic neutrophils.

Lastly, in terms of the philosophical question on why proceed with this research, there is no real direct evidence to show that a delay in apoptosis directly leads to ARDS. Anything I've seen in the literature is somewhat obscure. Ischemic retinopathy in rats produced by retinal artery occlusion and then reperfusion leads to damage of the retina, and this seems to be an apoptotic phenomenon, and if you inhibit apoptosis, you can modulate the degree of severity of this ischemia reperfusion injury. And if you consider that early, fulminant ARDS in these patients is an ischemia reperfusion injury, then that may be indirect evidence that apoptosis or a delay in apoptosis is involved in mediating ischemia reperfusion injury in various tissues and organs.

**Ori D. Rotstein, MD, Toronto, Ontario:** Dr Watson, working with Dr Marshall at the University of Toronto, showed in an in vitro model of transendothelial migration that he saw a differential response depending on whether or not the neutrophils were activated before they were induced to transmigrate; that is, if they were activated and induced to transmigrate they showed delayed apoptosis; if they were normal neutrophils, unactivated and induced to transmigrate, there was either no effect or they showed an increase in apoptosis.

In your studies you used normal neutrophils and exposed them to the serum or to IL-8. Have you looked at cells that were taken from patients who may have undergone some sort of similar trauma or injury or operation who did not have ARDS to discern whether IL-8 or maybe the ARDS serum might have a different effect on the cells than the ones you saw here on perfectly normal neutrophils?

**Dr Goodman:** We haven't as yet done that. We have plans to take plasma and/or cells from people undergoing major elective surgery to see whether the surgical stress mimics the stress of ARDS. We actually did also look at CD11-b expression in these cells exposed to ARDS plasma, thinking that if there is an inhibition apoptosis, maybe there is an up-regulation of CD11-b expression, and we found actually no difference between ARDS plasma and control plasma; the rates of CD11-b expression were the same in both plasmas.

**Basil A. Pruitt, Jr, MD, San Antonio, Tex:** Dr Goodman, these patients received an average of 12 units of blood and up to 22. Did they also receive a lot of crystalloid? If so, what did body fluid balance turn out to be? And is this just a reflection of hyponatremia and cell swelling?

**Dr Goodman:** In addition to the packed red cell transfusion they did receive a lot of crystalloid, at least initially as they came into the emergency room. I don't have the exact numbers. They also received FFP and platelets. This is all data on the trauma registry, so it would be interesting to go back to the trauma registry and look at exactly how much crystalloid they got and perhaps correlate this effect with serum electrolyte changes, such as hyponatremia.