Role of NF-κB in Endotoxemia-Induced Alterations of Lung Neutrophil Apoptosis

John G. Kupfner, John J. Arcaroli, Ho-Kee Yum, Steven G. Nadler, Kuang-Yao Yang and Edward Abraham

*J Immunol* 2001; 167:7044-7051; doi: 10.4049/jimmunol.167.12.7044

http://www.jimmunol.org/content/167/12/7044

**References**

This article cites 62 articles, 36 of which you can access for free at: http://www.jimmunol.org/content/167/12/7044.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Role of NF-κB in Endotoxemia-Induced Alterations of Lung Neutrophil Apoptosis

John G. Kupfner,* John J. Arcaroli,* Ho-Kee Yum,* Steven G. Nadler, † Kuang-Yao Yang,* and Edward Abraham*‡*

Acute lung injury is frequently associated with endotoxemia and characterized by the accumulation in the lungs of large numbers of neutrophils activated to produce proinflammatory mediators. In the setting of acute lung injury, the percentage of apoptotic cells among lung neutrophils is decreased. The transcriptional regulatory factor NF-κB is activated in neutrophils and other pulmonary cell populations after endotoxemia and appears to play a central role in the development of the acute inflammatory process that leads to lung injury. Because NF-κB can modulate apoptosis through increasing expression of anti-apoptotic proteins, activation of NF-κB may contribute to the alterations in lung neutrophil apoptosis associated with acute lung injury. In the present experiments, endotoxemia resulted in decreased apoptosis and increased expression of anti-apoptotic mediators among lung neutrophils. Amounts of A1, A20, and Bcl-xL, anti-apoptotic proteins whose transcription is dependent on NF-κB, were increased in lung neutrophils after endotoxemia. Inhibition of nuclear translocation of NF-κB increased the percentage of apoptotic lung neutrophils after endotoxemia, but not back to the levels found in unmanipulated animals. Although inhibition of nuclear translocation of NF-κB prevented endotoxemia-induced increases in Bcl-xL, A1, and A20 in lung neutrophils, this intervention did not prevent endotoxemia-associated elevation of Mcl-1, an anti-apoptotic protein primarily under the transcriptional regulation of CREB. These results demonstrate that mechanisms independent of NF-κB activation play an important role in modulating lung neutrophil apoptosis after endotoxemia, The Journal of Immunology, 2001, 167: 7044–7051.

Neutrophils, activated to produce cytokines and other proinflammatory mediators, play an important role in the development of acute lung injury (ALI), a frequent event associated with endotoxemia and sepsis (1). The proportion of apoptotic neutrophils in bronchoalveolar lavage fluid from patients with ALI is decreased (2, 3). In experimental models of ALI induced by endotoxemia, the percentage of apoptotic cells among lung neutrophil populations also decreases (4). Alterations in neutrophil apoptosis associated with risk factors for ALI, such as endotoxemia, may potentiate the severity of lung injury if diminished rates of apoptosis result in the prolonged presence of activated neutrophils in the lungs.

The mechanisms affecting neutrophil apoptosis in ALI remain incompletely defined. Apoptosis of peripheral blood neutrophils from normal donors is decreased when they are exposed to bronchoalveolar lavage from patients with ALI (2, 3). G-CSF and GM-CSF appear to have a role in this effect, since the anti-apoptotic effects of ALI bronchoalveolar lavage are diminished when Abs to these factors are included (2, 3, 5). At least one potential mechanism by which GM-CSF may exert its anti-apoptotic effect is by up-regulating the anti-apoptotic protein Mcl-1 through a pathway dependent on phosphatidylinositol 3-kinase (PI3-K), leading to activation of the transcriptional factor CREB (6). We have recently demonstrated activation of PI3-K and CREB among lung neutrophils in murine models of ALI (7, 8).

Neutrophils that accumulate in the lungs after endotoxemia show increased activation of the transcriptional regulatory factors NF-κB and CREB, and express cytokines as well as other proinflammatory mediators under the regulatory control of these factors (8, 9). Both NF-κB and CREB are involved in anti-apoptotic pathways that may modulate neutrophil life span in ALI (6, 10). For example, the expression of the anti-apoptotic Bcl-2 family members Bcl-2 and Mcl-1 is regulated by CREB (6, 11). In transgenic murine models, mice lacking the p65 (Rel A) subunit of NF-κB show embryonic death with massive hepatic apoptosis (12). Similar histologic findings of increased hepatic apoptosis leading to embryonic death are found in transgenic mice that are unable to activate NF-κB because of the absence of IK-β kinase (13). Among NF-κB-dependent anti-apoptotic genes are members of the Bcl-2 family (Bcl-xL, A1) (14, 15), zinc finger proteins (A20) (16–20), and anti-apoptotic members of the TNFR-associated complex (TNFR-associated factor 2 and c-inhibitor of apoptosis protein 1 and 2) (21–24).

In the present experiments, we used a specific inhibitor of nuclear translocation of NF-κB (25) to examine the role of this transcriptional factor in modulating the decreases in neutrophil apoptosis that are associated with endotoxemia-induced ALI. Although the expression of anti-apoptotic proteins under the transcriptional control of NF-κB was increased in lung neutrophils after endotoxemia, our results demonstrate that NF-κB plays a relatively minor role in affecting lung neutrophil apoptosis under these conditions.

Copyright © 2001 by The American Association of Immunologists 0022-1767/01/$02.00

1 Address correspondence and reprint requests to Dr. Edward Abraham, Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Health Sciences Center, Box C272, 4200 East Ninth Avenue, Denver, CO 80262. E-mail address: edward.abraham@uchsc.edu

2 Abbreviations used in this paper: ALI, acute lung injury; FasL, Fas ligand; PI3-K, phosphatidylinositol 3-kinase.
Materials and Methods

**Mice**

Male BALB/c mice, 8–12 wk of age, were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were kept on a 12-h light, 12-h dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board-approved protocols.

**Materials**

Isolurane was obtained from Abbott Laboratories (Chicago, IL). *Escherichia coli* 0111:B4 endotoxin, collagenase, and DNase were obtained from Sigma-Aldrich (St. Louis, MO). RPMI 1640/25 mM HEPES/glutamine was obtained from BioWhittaker (Walkersville, MD), and PBS and penicillin/streptomycin were purchased from Gemini Bioproducts (Calabasas, CA). Percoll was purchased from Amersham Biosciences (Piscataway, NJ). Bicinchoninic acid protein assay reagent was purchased from Pierce (Rockford, IL). The Annexin V-FITC apoptosis detection kit was purchased from R&D Systems (Minneapolis, MN). FITC-conjugated anti-Fas and biotin-conjugated anti-Fas ligand (FasL) Abs as well as streptavidin-PE were obtained from BD Pharmingen (Franklin Lakes, NJ). Custom mixture Abs and columns for neutrophil isolation were purchased from StemCell Technologies (Vancouver, BC, Canada).

**Interventions**

In designated experiments, mice were treated i.v. with 0.1 ml PBS (control), or 5 mg/kg BMS 205820 (25) in 0.1 ml PBS 60 min before LPS administration.

**Model of endotoxemia**

The model of endotoxemia-induced lung injury was used as reported previously (1, 26). Mice received an i.p. injection of LPS at a dose of 500 ng/kg in 0.1 ml PBS. This dose of LPS produces acute neutrophilic alveolitis, histologically consistent with acute lung injury.

**Myeloperoxidase assay**

Myeloperoxidase activity was assayed as reported previously (1). Excised lungs were frozen in liquid nitrogen, weighed, and stored at −86°C. Lungs were homogenized for 30 s in 1.5 ml 20 mM potassium phosphate (pH 7.4) and centrifuged at 4°C for 30 min at 40,000 × g. The pellet was resuspended in 1.5 ml 50 mM potassium phosphate (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide, sonicated for 90 s, incubated at 60°C for 2 h, and centrifuged. The supernatant was assayed for peroxidase activity corrected to lung weight.

**Isolation of neutrophils**

Lung or peripheral neutrophils were purified from intrapulmonary pulmonary or bone marrow cell suspensions. To obtain the bone marrow cell suspension, the femur and tibia of a mouse were flushed with 5 ml RPMI 1640/penicillin/streptomycin, and the cells were passed through a glass-wool column. Lung neutrophils were isolated from intrapulmonary pulmonary cell suspensions, prepared as previously described by our laboratory (8, 26, 27). In brief, the chest of the mouse was opened, and the lung vascular bed was flushed with 2–3 ml of chilled (4°C) PBS injected into the right ventricle. Lungs were then excised, avoiding the paratracheal lymph nodes and thymus, and washed twice in RPMI 1640/25 mM HEPES/glutamine supplemented with penicillin/streptomycin. The excised lungs were minced finely, and the tissue pieces were placed in RPMI 1640 medium containing 5% FBS, 20 U/ml collagenase, and 1 µg/ml DNase. Following incubation for 60 min at 37°C, any remaining intact tissue was dissected by passage through a 21-gauge needle. Tissue fragments and the majority of dead cells were removed by rapid filtration through a glass-wool column, and cells were collected by centrifugation.

The cell pellets from the intrapulmonary pulmonary or bone marrow cell suspensions were resuspended in RPMI 1640/5% FCS and then incubated with 10 µl of primary Abs specific for cell surface markers F4/80, CD4, CD45R, CD5, and TER119 for 15 min at 4°C. This custom mixture (StemCell Technologies) is specific for T and B cells, RBC, monocytes, and macrophages. After a 15-min incubation, 100 µl of anti-biotin tetrameric Ab complexes were added, and the cells were incubated for 15 min at 4°C. Following this, 60 µl of colloidal magnetic dextran iron particles were added to the suspension and incubated for 15 min at 4°C. The entire cell suspension was then placed into a column, surrounded by a magnet. The T cells, B cells, RBC, monocytes, and macrophages were captured in the column, allowing the neutrophils to pass through by negative selection methods. The neutrophil suspension was then layered on 50% Percoll and centrifuged at 3000 rpm for 15 min, and the neutrophil layer was collected.

Viability, as determined by trypan blue exclusion, was consistently >98%. Neutrophil purity, as determined by Wright-stained cytopsin preparations, was >97%.

**EMSA**

Nuclear extracts were prepared as previously described (1, 8, 28, 29). Isolated neutrophils were incubated for 15 min in buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, and 10 mM KCl (pH 7.9)). After cytoplasm was removed from the nuclei by 15 passages through a 25-gauge needle, the nuclei were collected by centrifugation at 600 × g × 6 min at 4°C. The nuclear pellet was incubated on ice for 15 min in buffer C (20 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 25% glycerol), after which the extract was centrifuged at 4°C for 10 min at 12,000 × g. The supernatant was collected, divided into aliquots, and stored at −86°C. Protein concentration was determined by using the Coomassie Plus Protein Assay Reagent (Pierce) standardized to BSA according to the manufacturer’s protocol.

Activation of NF-κB was determined as described previously by our laboratory (1, 27–29). The κB DNA sequence of the Ig gene was used. Synthetic double-stranded sequences (with enhancer motifs underlined) were fill in labeled with [α-32P]dATP using Sequence DNA polymerase: κB, 5'-TTTTTCAGCTGCGAATCGACG-3' and 3'-GCTGACC CCTGAAGGCTCCTTTT-5'. DNA binding reaction mixtures of 20 µl contained 10 µg of nuclear extract, 10 mM Tris-HCl (pH 7.5), 50 mM NaF, 5 mM MgCl2, 1 mM MnCl2, 4% glycerol, 0.08 µg of poly(dI- dC)-poly(dI-dC)- and 0.7 fmol of 32P-labeled double-stranded oligonucleotide. After the samples were incubated at room temperature for 20 min, they were loaded onto a 4% polyacrylamide gel (acylamide/bisacrylamide (80:1), 2.5% glycerol in Tris-borate-EDTA) and run at 10 V/cm. Each gel was then dried and subjected to autoradiography.

**Western blot analysis**

Whole-cell extracts from lung neutrophils were denatured in ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 1 mM Na3 vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 300 µM ρ-nitrophenyl phosphate, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin, pH 7.3) for 15 min. The protein concentration of each sample was assayed using the bicinchoninic acid protein assay kit standardized to BSA according to the manufacturer’s protocol. Then, 70 µg of protein was loaded on a 10% Tris-HCl SDS-polyacrylamide gel. Protein was electrotransferred to a nitrocellulose membrane and blocked with 5% nonfat dry milk and 20 mM TBS with 0.1% Tween 20. After blocking, the membrane was incubated overnight at 4°C with a rabbit polyclonal-specific primary Ab: anti-A20 (Imgenex, San Diego, CA) and anti-A1, anti-Bcl-2, anti-Bcl-xL, or anti-Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA) using a dilution of 1/1000, followed by HRP-coupled secondary Ab at a dilution of 1/2000. After washing five times, bands were detected using ECL Western blotting detection reagents (Amersham Biosciences). Densitometry was performed using a chemiluminescence system and analysis software (Bio-Rad, Hercules, CA).

**Gene array analysis**

RNA was isolated from neutrophils using an RNAeasy kit according to the manufacturer’s protocol. Briefly, neutrophils were lysed and placed on ice for 10 min in 300 µl of a buffer containing 25% guanidinium thiocyanate and 1% 2-ME. Samples were incubated with proteinase K at 55°C for 10 min, centrifuged, washed, incubated with DNase for 15 min at room temperature, and washed again. RNA was eluted from the membrane in 40 µl of RNAse-free water, and the quantity of RNA was determined by the ratio of A260 to A280. A range of 1.9–2.0 was considered to be adequately pure.

Total RNA (20 µg) was converted to cDNA using the Superscript Choice System (Life Technologies, Gaithersburg, MD). An oligo(dT) primer containing a T7 RNA polymerase promoter (Genset, Kents Store, VA, USA) was used. After second-strand synthesis, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol, and cDNA was recovered by ethanol precipitation. In vitro transcription was performed to generate biotin-labeled cRNA using an RNA Transcript Labeling kit (Enzo, Farmingdale, NY), and 3.3 µl of cDNA template was transcripted in the presence of a mixture of biotin-labeled ribonucleotides. Biotin-labeled cRNA was purified using an RNeasy affinity column (Qiagen, Valencia, CA). To ensure optimal hybridization to the oligonucleotide array, the cRNA was fragmented. Fragmentation was performed such that the cRNA fragments were between 55 and 200 bases in length by incubating the cRNA at 94°C for 35 min in fragmentation buffer. The sample was then added to a hybridization solution containing 100 mM MES (Sigma), 1 M NaCl, and 20 mM EDTA in the presence of 0.01% Tween 20. The final
concentration of the fragmented cRNA was 0.05 μg/μl. Hybridization was performed by incubating 200 μl of the sample to the Affymetrix GeneChip Mu11KsB A and Mu11KsB B (Affymetrix, Santa Clara, CA). Hybridization occurred at 45°C for 16 h using a GeneChip Hybridization Oven 640 (Affymetrix). After hybridization, the hybridization solutions were removed, and the arrays were washed and stained with streptavidin-PE using a GeneChip Fluidics Station 400 (Affymetrix). Arrays were read at a resolution of 6 μm using a HP Gene Array Scanner (Affymetrix). Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described previously (30–32). Briefly, each gene is represented by the use of ~20 perfectly matched and mismatched control probes. The mismatched probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals. The GeneChip Suite program determines the intensity of expression for all genes on the array and provides pairwise comparison between chips.

Flow cytometry

Annexin V assays were performed using the manufacturer’s protocol (R&D Systems). Briefly, intraparenchymal pulmonary cell populations were collected and counted, and 5 × 10^5 cells were resuspended in the binding buffer. Propidium iodide- and fluorescein-conjugated annexin V were added, and the reaction was stopped after 15 min. To determine expression of Fas and FasL on lung neutrophils, anti-murine biotin-conjugated anti-FasL and FITC-conjugated anti-Fas Abs (10 μg/ml) were added, and the reaction was stopped after 15 min. The cells were then washed with sterile PBS three times, followed by incubation with streptavidin-PE for 30 min at 4°C. The cells were then washed with sterile PBS three times, followed by incubation with streptavidin-PE for 30 min at 4°C. The cells were then washed with sterile PBS three times, followed by incubation with streptavidin-PE for 30 min.

Statistical analysis

To limit variability and provide appropriate controls, for each experimental condition the entire group of animals was prepared and studied at the same time. For each experimental condition, mice in all groups had the same condition the entire group of animals was prepared and studied at the same time. For each group, one-way ANOVA and the Tukey-Kramer multiple comparisons test (for multiple groups) or Student’s t test (for comparisons between two groups) was used. A value of p < 0.05 was considered to be significant.

Results

Neutrophil apoptosis following LPS exposure

Endotoxin administration resulted in a decreased percentage of apoptotic neutrophils in the lungs, as determined by annexin V staining (Fig. 1). In unmanipulated mice, ~40% of the neutrophils in the lungs were apoptotic. One hour after endotoxin administration, the percentage of apoptotic lung neutrophils decreased by >75% compared with controls, and then remained significantly decreased 4 h after endotoxemia. By 24 h after endotoxin administration, the percentage of apoptotic lung neutrophils had returned to baseline levels.

Gene array analysis of mediators of apoptosis

We used gene array analysis to examine the effects of endotoxemia on the expression of genes involved in the apoptotic response. As shown in Table I, endotoxemia primarily resulted in increased expression of anti-apoptotic genes in lung neutrophils, while the expression of pro-apoptotic mediators, with the exception of Fas and FasL, was either decreased or did not change. More than 100-fold increases were found in the expression of the anti-apoptotic Bcl-2 family member A1 and the zinc finger protein A20. Among the Bcl-2 family, mRNA levels for anti-apoptotic members, including Bcl-2, Bcl-xL, and Mcl-1, were increased, while amounts of mRNA for Bcl-2 family members with pro-apoptotic properties, such as Bad and Bid, were decreased.

Table 1. Analysis by gene array of endotoxemia-induced alterations of apoptosis-related genes in lung neutrophils

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>McI-1</td>
<td>+2</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>+2.2</td>
</tr>
<tr>
<td>AI</td>
<td>+113.6</td>
</tr>
<tr>
<td>A20</td>
<td>+140</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>+2</td>
</tr>
<tr>
<td>Bcl-w</td>
<td>NC</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>NC</td>
</tr>
<tr>
<td>G-CSF</td>
<td>+4.4</td>
</tr>
<tr>
<td>G-CSF receptor</td>
<td>+2.7</td>
</tr>
<tr>
<td>Caspase 1</td>
<td>NC</td>
</tr>
<tr>
<td>Caspase 2</td>
<td>−3.2</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>NC</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>NC</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>NC</td>
</tr>
<tr>
<td>BAD</td>
<td>−1.4</td>
</tr>
<tr>
<td>BAK</td>
<td>NC</td>
</tr>
<tr>
<td>Bax</td>
<td>NC</td>
</tr>
<tr>
<td>BID</td>
<td>−2.8</td>
</tr>
<tr>
<td>Fas</td>
<td>+6</td>
</tr>
<tr>
<td>FasL</td>
<td>+7</td>
</tr>
</tbody>
</table>

The relative fold change (+ or −) in each gene in endotoxemia-elicited lung neutrophils, as compared to levels in peripheral blood neutrophils, is shown. The results presented are combined from two array analyses, using separate groups of mice.

b NC, No change.
Expression of proteins involved in apoptosis

Although the gene array experiments (Table I) demonstrated increased expression of anti-apoptotic genes in endotoxin-elicited lung neutrophils, mRNA levels of pro-apoptotic mediators, such as Fas and FasL, were also elevated under these conditions. To determine the functional significance of the observed increases in the expression of genes involved in apoptosis, we examined the levels of these proteins among lung neutrophils after endotoxin administration.

Increases in A1, A20, Mcl-1, and Bcl-xL, but not Bcl-2, were present in lung neutrophils within 1 h after the administration of endotoxin (Fig. 2). The levels of A1, A20, Mcl-1, and Bcl-xL, compared with baseline conditions, continued to be significantly elevated 4 h after endotoxemia, but the magnitude of increase was less than that present 1 h after endotoxin administration. By 24 h after endotoxemia, only Mcl-1 showed a significant increase in lung neutrophils compared with baseline conditions. There was no change in Bcl-2 levels at any postendotoxemia time point. Similarly, flow cytometry showed no alteration in Fas or FasL expression on lung neutrophils at any of the postendotoxemia time points that were examined.

Role of NF-κB in endotoxemia-induced alterations of lung neutrophil apoptosis

In previous studies (1, 8, 33), we found that the transcriptional factor NF-κB was activated in lung neutrophils after endotoxemia. NF-κB is important in the regulation of expression of anti-apoptotic proteins, including members of the Bcl-2 family, such as A1, Bcl-2, Bcl-xL, as well as the zinc finger protein A20 (14, 15, 18, 20, 34–36). As shown in the above experiments, levels of these

---

**FIGURE 2.** Alterations in anti-apoptotic proteins in lung neutrophils after endotoxemia. Representative Western gels are shown in A. Mean alterations for each of the anti-apoptotic mediators, compared with control levels, are presented in B. Combined results from between three and six individual experiments at each time point after endotoxin administration are shown. *, p < 0.05 compared with levels in neutrophils from control, unmanipulated mice.
anti-apoptotic mediators was increased in endotoxemia-induced lung neutrophils, suggesting that activation of NF-κB might play an important role in modulating lung neutrophil apoptosis in this setting.

To explore the role of NF-κB activation in lung neutrophil apoptosis after endotoxemia, an inhibitor of NF-κB nuclear translocation (BMS 205820) was used (25). Administration of BMS 205820 prevented endotoxin-induced translocation of NF-κB to the nucleus of lung neutrophils (Fig. 3). There were no significant changes in the numbers of neutrophils accumulating in the lungs of BMS 205820-treated animals (after endotoxemia, myeloperoxidase levels were 13.1 ± 4.1 U/g lung in control mice and 9.7 ± 2.1 U/g lung in mice pretreated with BMS 205820). Inhibition of endotoxemia-induced NF-κB activation in BMS 205820-treated mice resulted in a significant increase in the percentage of apoptotic lung neutrophils compared with that present after endotoxemia (Fig. 4). However, the level of apoptosis in endotoxin-elicited lung neutrophils, despite inhibition of NF-κB translocation, still remained significantly less than that in lung neutrophils from control, unmanipulated mice.

**Effects of inhibition of nuclear translocation of NF-κB on expression of anti-apoptotic proteins in lung neutrophils**

The above results (Fig. 4), showing that inhibition of NF-κB translocation incompletely corrected endotoxemia-induced decreases in lung neutrophil apoptosis, indicated that alterations in apoptosis after endotoxemia are not due purely to NF-κB-dependent regulatory events. To explore this issue further, we examined the expression of anti-apoptotic proteins in lung neutrophils from animals treated with BMS 205820.

Inhibition of the nuclear translocation of NF-κB by administration of BMS 205820 prevented endotoxin-induced increases in Bcl-xL, A1, and A20 (Fig. 5). However, levels of Mcl-1 remained increased in mice treated with BMS 205820 before endotoxemia compared with those found in neutrophils from unmanipulated animals. There were no alterations in Bcl-2 after endotoxin administration in mice either treated with BMS 205820 or left untreated.

**Discussion**

In the present experiments, gene array analysis showed that endotoxemia-associated decreases in lung neutrophil apoptosis were accompanied by elevations in the expression of anti-apoptotic mediators, including G-CSF, members of the Bcl-2 family, and the zinc finger protein A20. The greatest increases in gene expression among lung neutrophils were found for the anti-apoptotic proteins A1 and A20, where endotoxin administration resulted in RNA levels that were >100-fold greater than control values. Transcription of A1 and A20 is primarily under the regulatory control of NF-κB (15, 16). Previous studies have demonstrated that exposure of neutrophils to endotoxin resulted in enhanced nuclear translocation of NF-κB and production of immunoregulatory cytokines, such as macrophage inflammatory protein 2 and TNF-α, whose transcription is dependent on NF-κB (1, 8, 36, 37). Additionally, endotoxemia-induced activation of NF-κB in neutrophils and other pulmonary cell populations has been shown to play a major role in the development of acute inflammatory lung injury (1, 38). Increased NF-κB activation, with associated increased transcription of NF-κB-dependent anti-apoptotic proteins, therefore appeared to be a plausible mechanism for the decrease in neutrophil apoptosis associated with endotoxemia-induced acute lung injury.

The agent used to inhibit NF-κB translocation, BMS 2035820, exerts this function through using nuclear localization signal peptides that alter the interaction of NF-κB with karyopherin α and block its transport into the nucleus. In a previous report (25), BMS 2035820 appeared to be relatively selective for NF-κB, since no effect on Oct-1 DNA binding or NFAT-1 nuclear localization was found. Global nuclear protein levels were not affected by treatment of cells with BMS 2035820, suggesting that activation-induced nuclear translocation of NF-κB is more sensitive to inhibition by this nuclear localization signal peptide than other transcription factors.

Effective inhibition of nuclear translocation of NF-κB prevented endotoxemia-induced increases in levels of NF-κB-dependent anti-apoptotic mediators, including A1, A20, and Bcl-xL, but did not restore lung neutrophil apoptosis to baseline levels. Such results show that the decreases in apoptosis that occur in lung neutrophils after endotoxemia are not solely a result of NF-κB activation, but, rather, must reflect anti-apoptotic mechanisms not dependent on NF-κB.

There are several NF-κB-independent mechanisms that may contribute to alterations in lung neutrophil apoptosis after endotoxemia. Mcl-1 is an anti-apoptotic member of the Bcl-2 family...
associated with neutrophil survival (6, 39, 40). Experimental models of Mcl-1 overexpression have demonstrated that this protein protects cells against apoptotic death (41). Protection against Bax-mediated cell death occurred when Mcl-1 was overexpressed in a yeast two-hybrid system (40, 42). Mcl-1 coimmunoprecipitates with Bax in human neutrophils, suggesting that interactions between Bax and Mcl-1 may be involved in modulating cellular apoptotic balance in the same manner as do other members of the Bcl-2 family, such as the anti-apoptotic proteins Bcl-xL and Bcl-2, that also bind to Bax and prevent its pro-apoptotic activity (40, 43).

In our studies, levels of Mcl-1 were increased in lung neutrophils after endotoxin administration and remained elevated when nuclear translocation of NF-κB was inhibited. Although previous studies (40–43) have shown that Mcl-1 has potent anti-apoptotic effects in neutrophils, this is the first report that suggests such an effect under acute inflammatory conditions in vivo. Activation of

**FIGURE 5.** Effects of blocking nuclear translocation of NF-κB with BMS 205820 on LPS-induced changes in anti-apoptotic proteins in lung neutrophils. Cell extracts were obtained from peripheral neutrophils of mice that were unmanipulated (Control) or from lung neutrophils harvested 1 h after LPS administration in mice either pretreated with BMS 205820 before LPS administration (LPS plus BMS 205820) or given only the PBS vehicle before LPS administration (LPS). Representative gels are shown in A. Combined results from between three and six separate experiments, showing mean alterations for each of the anti-apoptotic mediators, compared with control levels are presented in B. *, p < 0.05 compared with levels in neutrophils from control, unmanipulated mice.
Mcl-1 appears to be primarily regulated through a transcription factor complex containing CREB (6, 44). We have previously (8, 37) demonstrated that endotoxemia results in rapid increases in the transcriptionally active, serine 133 phosphorylated form of CREB in lung neutrophils, providing a mechanism for NF-κB-independent up-regulation of Mcl-1 in this setting.

Although we did not find up-regulation of GM-CSF expression in lung neutrophils, previous studies have shown that pulmonary levels of GM-CSF are increased in the setting of acute lung injury and contribute to decreased neutrophil apoptosis (2, 3, 45). Because transcription of GM-CSF is not dependent on NF-κB (46), inhibition of NF-κB activation, as performed in our experiments, would not affect the production of GM-CSF by nonneutrophil pulmonary cell populations. GM-CSF increases Mcl-1 in neutrophils through cooperative interactions between signaling pathways involving PI3-K and Janus kinase/STAT (43). We (7) and others (47) demonstrated that exposure of neutrophils to LPS results in activation of PI3-K and the downstream kinase, Akt. We recently found that PI3-K and Akt are activated in lung neutrophils after endotoxin administration (7). In addition to increasing Mcl-1 via a CREB-dependent mechanism (6), Akt is involved in at least three other anti-apoptotic pathways that may play a role in modulating neutrophil survival (48, 49). Akt activation leads to phosphorylation of the pro-apoptotic protein Bad, thereby preventing Bad from binding to and inactivating Bcl-xL (49). Akt also decreases apoptosis through phosphorylating caspase 9 and inhibiting protease activity of this death caspase (50). Although Akt can enhance nuclear translocation of NF-κB and expression of NF-κB-dependent anti-apoptotic genes through activating IκB kinases (51), our results showing minimal alterations in neutrophil apoptosis when NF-κB translocation is inhibited argue against this being a major mechanism in affecting lung neutrophil survival after endotoxemia.

In the present experiments, the expression of G-CSF and the G-CSF receptor was increased in lung neutrophils after endotoxemia. Previous studies have shown that endotoxin exposure increases G-CSF release by pulmonary cell populations, including alveolar macrophages, fibroblasts, monocytes, and endothelial cells (52-55). G-CSF levels are elevated in bronchoalveolar lavage specimens from patients with acute lung injury and contribute to reductions in neutrophil apoptosis in this setting (2, 3). Correlations between neutrophil accumulation in the lungs and bronchoalveolar lavage levels of G-CSF as well as between bronchoalveolar lavage levels of G-CSF and poor survival in patients with acute lung injury suggest that G-CSF may exacerbate acute neutrophil-driven pulmonary inflammation (45). Because NF-κB does not appear to have a major role in regulating the transcription of G-CSF (56), endotoxin-induced increases in pulmonary levels of G-CSF coupled with up-regulation of G-CSF receptors on lung neutrophils would be capable of diminishing neutrophil apoptosis even under conditions where nuclear translocation of NF-κB is inhibited.

In addition to the intracellular anti-apoptotic proteins and growth factors examined in the present studies, endotoxia results in the release of other anti-apoptotic mediators that may affect lung neutrophils and whose regulation is not solely dependent on NF-κB. For example, glucocorticoids circulate in increased concentrations during endotoxemia and exert potent anti-apoptotic effects on neutrophils (57, 58). Plasma catecholamine concentrations also rise after endotoxia (59). Increases in cytosolic free calcium as a result of enhanced adrenergic response can decrease neutrophil apoptosis (60). Furthermore, it is possible that inhibition of nuclear translocation of NF-κB, as performed in these experiments, may indirectly affect the number of apoptotic neutrophils in the lungs through altering neutrophil traffic patterns.

Although our studies did not find any significant alterations in the total number of endotoxia-elicted lung neutrophils after treatment with BMS 205820, the percentage of apoptotic lung neutrophils could still be altered if the relative numbers of immature neutrophils in the lungs were altered by such therapy. Such a mechanism seems unlikely since levels of Mcl-1, an anti-apoptotic peptide induced in mature neutrophils (40, 43, 61), remain elevated in lung neutrophils from BMS 205820-treated mice.

Acute lung injury is characterized by the prolonged presence of activated neutrophils in the lungs. In this setting, therapies that increase neutrophil apoptosis may provide synergistic benefit with those that decrease neutrophil activation. Previous studies (38, 62) have found that inhibition of nuclear translocation of NF-κB decreases the expression of NF-κB-dependent proinflammatory mediators and diminishes the severity of endotoxemia-induced acute lung injury. The present results, showing that mechanisms distinct from NF-κB activation are involved in modulating neutrophil apoptosis after endotoxemia, suggest that additional benefit might be achieved through NF-κB-independent approaches that enhance neutrophil clearance from the lungs.

References


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.


Mcl-1 in differentiating U937 cells.