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Peripheral Blood Neutrophil Activation Patterns Are Associated with Pulmonary Inflammatory Responses to Lipopolysaccharide in Humans

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Increased nuclear accumulation of NF-κB in LPS-stimulated peripheral blood neutrophils has been shown to be associated with more severe clinical course in patients with infection associated acute lung injury. Such observations suggest that differences in neutrophil response may contribute to the pulmonary inflammation induced by bacterial infection. To examine this question, we sequentially measured LPS-induced DNA binding of NF-κB in neutrophils collected from healthy humans on at least three occasions, each separated by at least 2 wk, and then determined pulmonary inflammatory responses after instillation of LPS into the lungs. Consistent patterns of peripheral blood neutrophil responses, as determined by LPS-induced NF-κB DNA binding, were present in volunteers, with a >80-fold difference between individuals in the mean area under the curve for NF-κB activation. The number of neutrophils recovered from bronchoalveolar lavage after exposure to pulmonary LPS was significantly correlated with NF-κB activation in peripheral blood neutrophils obtained over the pre-LPS exposure period (r = 0.65, p = 0.009). DNA binding of NF-κB in pulmonary neutrophils also was associated with the mean NF-κB area under the curve for LPS-stimulated peripheral blood neutrophils (r = 0.63, p = 0.01). Bronchoalveolar lavage levels of IL-6 and TNFRII were significantly correlated with peripheral blood neutrophil activation patterns (r = 0.75, p = 0.001 for IL-6; and r = 0.48, p = 0.049 for TNFRII. These results demonstrate that stable patterns in the response of peripheral blood neutrophils to LPS exist in the human population and correlate with inflammatory response following direct exposure to LPS in the lung. *The Journal of Immunology, 2006, 176: 7753–7760.

Acute lung injury (ALI) is a frequent complication of severe infection and is characterized by the rapid development of pulmonary inflammation, with accumulation of large numbers of neutrophils in the lungs, endothelial and epithelial dysfunction, and increased pulmonary levels of proinflammatory cytokines and chemokines (1). Neutrophils appear to play an important role in the development of ALI. In experimental models, the severity of ALI is decreased when neutrophils are eliminated before exposure to LPS or other pathophysiologic insults that target the lung (2–6). Similarly, in neutropenic patients, worsening pulmonary function and the development of ALI often occur as neutrophil counts return toward normal levels (7, 8).

The transcriptional regulatory factor NF-κB participates in modulating the expression of many proinflammatory mediators associated with ALI, and increased activation of NF-κB has been demonstrated to be present in the alveolar macrophages of patients with ALI, compared with healthy controls (9, 10). NF-κB normally resides in the cytoplasm, bound to IκB proteins, such as IκB-α. Upon cellular activation resulting from TLR engagement with bacterial products such as LPS, IκB-α is phosphorylated, ubiquitinated, and degraded in the proteasome, allowing NF-κB to move into the nucleus where it binds to specific regulatory sequences in the promoters of genes for cytokines, chemokines, adhesion molecules, and other mediators involved in the induction of an acute inflammatory process (11).

In clinical studies of patients with infection-induced ALI or sepsis, elevations of nuclear concentrations of NF-κB in PBMC and in neutrophils are associated with worse outcome, including prolonged requirements for ventilatory support and mortality (12–14). A greater degree of nuclear accumulation of NF-κB in response to LPS stimulation among peripheral blood neutrophils collected within 24 h of the development of ALI predicted a more severe clinical course, as characterized by increased time on mechanical ventilation (14). In experimental models, inhibition of nuclear translocation of NF-κB decreased the severity of lung injury and inflammation, indicating that activation of NF-κB is likely to play an important role in the development of ALI (15, 16).

Whereas such findings have correlated the extent of nuclear translocation of NF-κB in the setting of ALI to severity of pulmonary inflammation and worse clinical outcomes, it has not been...
possible to determine whether the variability in NF-κB results from a preexistent proinflammatory phenotype among neutrophils and other cells contributing to the innate immune response. Stable patterns of heterogeneity in NF-κB activation could directly contribute to the extent of inflammation in the lungs and other organs through affecting the expression of proinflammatory mediators whose expression is regulated by NF-κB. However, an alternate hypothesis for the range of NF-κB responses is that the extent of NF-κB activation simply reflects the severity of the inflammatory state accompanying ALI. To examine this issue, we measured patterns of NF-κB activation in LPS-stimulated peripheral blood neutrophils collected on multiple occasions and established that the inflammatory potential of the neutrophil remained stable over time in a normal human population. We then used a model of LPS-induced lung inflammation to determine whether the degree of neutrophil activation seen in vitro during stable periods of good health correlated with the intensity of pulmonary inflammation produced by LPS exposure. Our findings show that the relative extent of LPS-induced NF-κB activation in neutrophils is associated with the severity of subsequent LPS-induced airway inflammation.

Materials and Methods

Subjects

Subjects eligible for this study met the following criteria: 18–40 years of age, male or female (1); nonsmoker (2); no active medical problems (3); and no concurrent medications (4), including aspirin or nonsteroidal anti-inflammatory drugs. Women taking oral contraceptives were not excluded. Exclusions included pregnancy; lactation; history of recent clinically significant asthma; allergies to both trimethoprim/sulfamethoxazole and penicillin (or amoxicillin); allergy to lidocaine or related compounds; allergy to opiates or benzodiazepines used for sedation during bronchoscopy; history of asthma or history of exercise-induced wheezing; signs of any acute illness on the day of LPS instillation; abnormalities on screening laboratory tests, electrocardiogram, chest radiograph, or pulmonary function tests; and any personal or family history of bleeding disorders.

Isolation and culture of human neutrophils

Peripheral blood was obtained at least three times from volunteers at intervals separated by at least 2 wk. Neutrophils (purity >98%) were isolated by plasma-Percoll gradients after dextran sedimentation of erythrocytes (17). Neutrophils were resuspended in RPMI 1640 medium, 5% FCS (Invitrogen Life Technologies) at a final concentration of 5 × 10⁶ cells/ml and cultured with phenol re-extracted 100 ng/ml LPS (O111:B4; Sigma-Aldrich) for 15, 30, or 60 min, as described previously (17, 18). A baseline, unstimulated sample of neutrophils also was prepared.

Model of LPS-induced pulmonary inflammation

The procedure for pulmonary LPS administration has been described previously (17, 19). All subjects were admitted to the General Clinical Research Center at the University of Colorado Hospital (Denver, CO). Approval for this study was obtained from the Colorado Multiple Institutional Review Board, and informed consent was provided according to the Declaration of Helsinki. The volunteers were premedicated with one double-strength trimethoprim/sulfamethoxazole tablet 12 h and 1 h before bronchoscopy. Subjects with a history of sulfa allergy were given two doses of oral amoxicillin (300 mg), separated by 8 h. At the time of the first bronchoscopy, 10 ml of saline was instilled into a lung subsegment (either the right middle lobe or lingula, followed by instillation of the test dose of reference LPS (O113; National Institutes of Health, Bethesda, MD) (19, 20) 4 ng/kg in 10 ml of saline into the contralateral lung. The subjects were randomized to left or right lungs for LPS or saline instillation. Following reconstitution of the LPS, a quantitative Limulus amebocyte lysate test (Cambrex) was done to verify the proper reconstitution and dosage of LPS. A second bronchoscopy was performed 16 h after the initial bronchoscopy. At the time of the second bronchoscopy, both the LPS and saline-instilled subsegments were lavaged with 150 ml of normal saline.

A pulmonary symptom scale (dyspnea, cough, wheezing, and chest pain), as described previously (17), was obtained every 8 h during the admission period and every 12 h for 72 h following the second bronchoscopy. All subjects were observed for at least 4 h after the second bronchoscopy. Although subjects were contacted by phone 1 wk after completing the study to further assess safety, no subject stated any ongoing medical complaints.

Isolation of neutrophils recovered by bronchoalveolar lavage (BAL)

The cells in BAL fluid (BALF) were pelleted, resuspended in 10 ml of PBS with 1 mM EDTA and 2% FCS, then applied to a siliconized glass wool column. After washing, the cells were again pelleted by centrifugation and resuspended in PBS with 1 mM EDTA and 2% FCS at 50 × 10⁶ cells/ml. Anti-HLA-DR Ab and anti-human glycophorin A tetramer (StemCell Technologies) were added to a final concentration of 1 μg/ml. After incubation at 4°C for 20 minutes, 60 μl/ml colloidal magnetic dextran iron particles was added to the suspension and incubated for 20 min at 4°C. The entire cell suspension was then placed into a column surrounded by a magnet, followed by washing of the column with at least 12 sample volumes of PBS with 1 mM EDTA and 2% FCS. The cells in the eluate were pelleted by centrifugation and resuspended in 2 ml RPMI 1640 medium with 2% heat-inactivated platelet-poor plasma. Neutrophil purity was determined for each sample and was consistently >98%.

EMSAs

Nuclear extracts were prepared as described previously (14). Isolated neutrophils were incubated for 15 min in buffer A (10 mM N-2-hydroxyethylpiperezine-N’-ethane sulfonic acid (pH 7.9), 1.5 mM magnesium chloride, 10 mM potassium chloride (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 for 6 min at 4°C. The nuclear pellet was incubated on ice for 15 min in buffer C (20 mM N-2-hydroxyethylpiperezine-N’-ethane sulfonic acid (pH 7.9), 0.42 M sodium chloride, 1.5 mM magnesium chloride, 0.2 mM EDTA, 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.

Nuclear extracts (5 μg) were incubated at room temperature for 15 min in 20 μl of reaction buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM

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magnesium chloride, 0.5 mM EDTA, 0.5 mM DTT, 50 mM sodium chloride, and 4% glycerol, with 32P end-labeled, double-stranded oligonucleotide probe specific for the β site, 5′-GCCATGGOGGAATCCCCG AAGTCC-3′ (Geneka Biotechnology) and 1 μg of poly(dI:dC). The complexes were resolved on 5% polyacrylamide gels in Tris-HCl (pH 8.0)/borate/EDTA buffer at 10 V/cm. Dried gels were exposed with Kodak Biomax MS film for 1–24 h at −70°C. Specificity of binding was confirmed by incubation with a 100-fold excess of unlabeled κB and cAMP response element oligonucleotides, with confirmation of elimination of the NF-κB band with the cold κB oligonucleotide, but not the cold cAMP response element oligonucleotide. Supershifts, using Abs to the p50 and p65 subunits of NF-κB, were performed, as described previously (21, 22), to demonstrate that the band identified for densitometry analysis consisted of the p50-p65 NF-κB heterodimer. Samples from all time points for the LPS-stimulated neutrophils (i.e., 0, 15, 30, and 60 min), as well as 5 μg of a nuclear extract obtained from LPS-stimulated RAW 264.7 cells to allow for standardization, were all performed on the same gel. Densitometry was performed using an imaging system and analysis software (Bio-Rad).

Inflammatory markers
Concentrations of BAL inflammatory markers (IL-1α, IL-1β, IL-6, IL-8, G-CSF, IFN-γ, MCP-1, MIP-1β, TNF-α, TNFRI, TNFRII, and IL-1Ra) were measured using ELISA or ECL assays, as described previously (17).

TLR4 genotyping
Genomic DNA was prepared from isolated PBMC using a DNA isolation kit (QiAamp DNA Blood Mini kit; Qiagen). Subsequent allele-specific PCR amplification for the TLR4 alleles Asp299Gly and Thr399Ile was performed according to a previously described protocol (23, 24).

Results
Patterns of NF-κB activation in peripheral blood neutrophils over time
Nuclear translocation of NF-κB was determined on at least three separate occasions in LPS-stimulated peripheral blood neutrophils from 48 healthy volunteers, who had no history of allergy, respiratory symptoms, or use of any medications, except for oral contraceptives (Table I). Each determination of neutrophil activation was performed at least 2 wk from the previous measurement.

As shown in Fig. 1, there was >80-fold difference among volunteers for nuclear accumulation of NF-κB, as determined by mean AUC during the 60 min after LPS exposure. Whereas variable activation of NF-κB was present for neutrophils isolated at the sequential blood draws, a consistent response to LPS was found for....
most volunteers. For example, the highest individual measurement of AUC for the five volunteers with the lowest mean AUC (i.e., volunteers V066, V025, V024, V008, and V039) was less than the lowest AUC value for the five volunteers with the highest mean AUC (i.e., V018, V016, V053, V037, and V058). Similarly, when the volunteers were divided into quartiles based on the mean values of AUC for LPS-induced NF-κB translocation, the mean of each quartile was significantly different from the mean of every other quartile using a Tukey-Kramer test (p < 0.05) (Fig. 2). The mean difference between the quartiles of volunteers with the lowest and highest mean NF-κB responses was 60.02, with confidence limits of 50.63 and 69.41. Representative EMSA results from volunteers in each of the quartiles of LPS response are shown in Fig. 3.

For each individual, there was no significant relationship between peripheral blood total white cell counts or neutrophil numbers and LPS-induced NF-κB DNA binding. Similarly, there was no association between resting nuclear levels of NF-κB in unstimulated neutrophils and the AUC for DNA binding of NF-κB after LPS stimulation (Fig. 4).

The TLR4 299/399 polymorphism has been described to be associated with diminished LPS response of PBMC, as well as a decrease in bronchial reactivity to LPS (25, 26). Among the volunteers included in this study, V008, V026, V057, and V059 were found to be homozygous for the TLR4 299/399 mutation. There was no apparent relationship between the TLR4 299/399 genotype and LPS-induced NF-κB response.

**FIGURE 4.** Lack of relationship between nuclear NF-κB levels in unstimulated neutrophils and LPS-induced NF-κB activation. Baseline nuclear NF-κB levels were determined by EMSA, standardized to that present in nuclear extracts from RAW 264.7 cells applied to all gels. Volunteers were ranked on the x-axis by LPS-induced NF-κB AUC values. No significant relationship (p = 0.79) was found between baseline and stimulated NF-κB nuclear values.

**FIGURE 5.** Nuclear concentrations of NF-κB in BALF neutrophils that migrated to the airspaces after pulmonary LPS exposure are correlated with LPS-induced activation in peripheral blood neutrophils. NF-κB levels in neutrophils isolated from BALF obtained 16 h after LPS instillation were determined by EMSA, standardized to that present in nuclear extracts from RAW 264.7 cells applied to all gels.
Pulmonary responses to LPS exposure correlate with in vitro patterns of neutrophil activation

Among the 48 volunteers who had at least three measurements of LPS-induced DNA binding of NF-κB, 15 were subjected to pulmonary instillation of LPS. We divided the peripheral blood neutrophil NF-κB response to LPS into three groups (low to high). The first five subjects in each group who consented to instillation of LPS into the lung were studied. There were no unexpected adverse events due to the study procedures, and each subject given LPS received a second bronchoscopy for collection of BAL 16 h after LPS administration. There were no significant differences in peripheral blood total white blood cell or neutrophil counts between the samples obtained before and after pulmonary instillation of LPS.

The level of nuclear translocation of NF-κB among neutrophils isolated from BAL from the LPS-exposed lung was significantly correlated ($r = 0.72, p = 0.004$) with the mean NF-κB AUC for LPS-stimulated peripheral blood neutrophils (Fig. 5). Similarly, there was significant association ($r = 0.65, p = 0.009$) between the number of neutrophils that accumulated in the airspaces of a subject and the mean level of NF-κB activation in peripheral blood neutrophils of the same subject (Fig. 6).

Previous studies, including our own, had shown increases in cytokines, including IL-6, IL-8, and MCP-1, as well as soluble cytokine receptors, such as TNFRI and TNFRII, and IL-1Ra in BALF obtained at 16 h after LPS exposure, the time point used in this study (17, 19). Among these, mean IL-6, TNFRII, and IL-1Ra levels were consistently $>1000$ pg/ml. Although other cytokines, such as TNF-α and IL-1β, were found to be elevated at earlier time points after pulmonary LPS administration (19), they were not increased at the 16 h time point. Despite these previous findings, a panel of cytokines was examined in the BAL to detect any possible relationship with underlying neutrophil phenotypes. Nine cytokines (IL-1α, IL-1β, IL-6, IL-8, G-CSF, IFN-γ, MCP-1, MIP-1β, and TNF-α) as well as TNFRI,
TNFRII, and IL-1Ra were measured. However, concentrations of IL-8, TNF-α, and MCP-1 were below the level of detection in the BALF.

There was a highly significant association between BALF levels of IL-6, a late-appearing cytokine, and mean NF-κB activation in peripheral blood neutrophils, collected from healthy volunteers over prolonged sampling intervals. The differences in mean values for nuclear translocation of NF-κB between the highest and lowest responders in the population examined were >80-fold. When the subjects were divided into quartiles, there was a difference of 60-fold. Significant differences in mean values for nuclear translocation of NF-κB between the highest and lowest responders in the population were found. Only one volunteer in the lowest response quartile was found to have this polymorphism, and neutrophils from other volunteers with the TLR4 299/399 polymorphism were found to have a range of responses to LPS, without any evidence that this genetic characteristic was associated with diminished neutrophil response to LPS. The absence of the TLR4 299/399 polymorphism in the other volunteers whose neutrophils demonstrated low response to LPS, coupled with the lack of any discontinuity in the spectrum of neutrophil profiles, suggests that complex genetic traits, independent of TLR4 or other single polymorphisms are responsible for large differences in neutrophil response within the human population.

Measurements of neutrophil response to LPS were obtained at intervals separated by at least 2 wk, with the total period between the first and last blood draw for determining activation profiles extending from 6 wk to almost 2 years. Such stability in neutrophil phenotypes, as determined under unstimulated and LPS-stimulated conditions, suggest that in vivo inflammatory responses in which neutrophils participate also would show substantial variability. Furthermore, such variability might be related to the underlying neutrophil profiles. We found that significant correlation does indeed exist between peripheral blood neutrophil phenotypes and pulmonary inflammatory responses induced by direct exposure of the airspace to LPS, a relevant bacterial product involved in Gram-negative infection. In particular, the accumulation of neutrophils into alveoli and airspaces, and the intensity of the inflammatory response, as measured by BALF IL-6 and TNFRII levels were significantly and directly associated with peripheral blood neutrophil activation determined under in vitro conditions. Because we measured pulmonary inflammatory responses 16 h after LPS instillation, late-appearing indicators of inflammation (19, 28), such as IL-6 and TNFRII, were increased in BAL. It is likely that other proinflammatory mediators would have shown similar relationships with neutrophil phenotypes if lung responses had been examined at earlier time points after exposure to LPS.

Our observations suggest that the degree of pulmonary injury induced by severe infection, especially due to Gram-negative organisms that have LPS as a cell wall component, is associated with underlying neutrophil phenotypes. In particular, less severe lung injury in response to Gram-negative pneumonia or infection at other sites would be associated with a lower level of neutrophil activation induced by LPS. In contrast, patients whose neutrophils have a more proinflammatory phenotype would be more likely to have a greater degree of pulmonary inflammation and more severe ALI. Because cell signaling events downstream of TLR2 and the related IL-1R family are largely homologous to those initiated by TLR4 (29, 30), and because the differences in neutrophil proinflammatory phenotypes are unlikely to be due to polymorphisms in TLR4, it is probable that underlying peripheral blood neutrophil profiles also would predict responses to bacterial products that interact with TLR2, such as peptidoglycan or lipoteichoic acid derived from Gram-positive bacteria, or proinflammatory cytokines, such as IL-1α or IL-1β, that interact with the IL-1R.

Previous studies in which peripheral blood neutrophils or mononuclear cells were obtained after admission of patients to the intensive care unit reported that increased nuclear accumulation of NF-κB was associated with worse clinical outcome from sepsis and ALI (12–14). However, it was unclear from those patient studies whether the greater accumulation of nuclear NF-κB was due to innate, genetically determined characteristics that then contributed to subsequent clinical course or if such patterns of NF-κB activation were only a reflection of greater in vivo inflammatory responses.

**FIGURE 8.** TNFRII levels in BALF after pulmonary LPS exposure are significantly correlated with LPS-induced activation of peripheral blood neutrophils.
that produced more organ damage and severity of illness. The results from the present experiments suggest that it is the underlying cellular profile that primarily determines clinical outcome. Proinflammatory phenotypes, as characterized by increased nuclear translocation of NF-κB in response to bacterial products, such as LPS, or mediators, such as cytokines, chemokines, and complement proteins. Of note, in the present study, the level of NF-κB activity among neutrophils that migrated into the airspaces after LPS challenge was significantly associated with that found in peripheral blood neutrophils after stimulation with LPS. Such results demonstrate that in vivo neutrophil phenotypes reflect those observed in vitro after exposure to a relevant bacterial stimulus.

Translocation of NF-κB from the cytoplasm to the nucleus is dependent on multiple cellular events involving activation of TLR/IL-1R-associated kinases and leading to phosphorylation, ubiquitination, and degradation of IkB-α (11, 29, 31, 32). In situations where a single polymorphism is responsible for alterations in NF-κB signaling, a distinct and recognizable phenotype different from the normal human population has been reported (33–36). However, in the present study, a continuous range of neutrophil responses to LPS was found, suggesting that genetically determined alterations in several steps in this intracellular cascade are likely to contribute to the broad range of NF-κB activation demonstrated to be present in the human population. Such complex haplotypes would be expected to reflect LPS responsiveness in other cellular populations, such as alveolar macrophages and PBMC. This assumption is supported by data showing that stable responses, in terms of cytokine release, are present in unpurified populations of peripheral blood leukocytes from an unselected population of human volunteers after stimulation with LPS (37).

Exposure of the lungs to LPS is unlikely to affect neutrophils directly. Rather, it is probable that alterations in the response of other pulmonary cell populations, similar to those found in neutrophils, resulted in the observed range of LPS-induced airway inflammation. Instillation of LPS into the airspaces is likely to produce activation of alveolar macrophages and epithelial cells, resulting in the subsequent release of cytokines and chemokines that then induce neutrophil migration into the lungs. O’Grady and colleagues (19) found that BAL levels of TNF-α, IL-8, MIP-1α, and MIP-1β were increased within 2 h of pulmonary LPS instillation to humans. Each of these cytokines and chemokines is chemotactic for neutrophils. Additional studies will be necessary to determine whether early alterations in airway inflammatory mediators correlate with peripheral blood neutrophil phenotypes and subsequent accumulation of neutrophils in the alveolar space.

The existence of stable neutrophil phenotypes that are associated with pulmonary inflammatory responses may have important diagnostic and therapeutic implications. The finding that there is heterogeneity in neutrophil responses to bacterial products, such as LPS, that participate in host responses to infection indicates that therapies for established ALI and other sepsis-associated organ dysfunction may need to be individualized on the basis of the specific pathways activated in each patient. In addition, early determination of neutrophil response patterns may allow patients at risk for developing more severe ALI to be identified before pulmonary dysfunction becomes manifest, allowing institution of appropriate anti-inflammatory therapies, especially since the intracellular alterations contributing to such stable neutrophil profiles are characterized.

Disclosures
The authors have no financial conflict of interest.

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