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The Effects of Granulocyte Colony-Stimulating Factor and Neutrophil Recruitment on the Pulmonary Chemokine Response to Intratracheal Endotoxin

Ping Zhang,*‡ Gregory J. Bagby,†‡ Jay K. Kolls,*‡ David A. Welsh,* Warren R. Summer,*‡ Jeff Andresen,§ and Steve Nelson*‡

Although G-CSF has been shown to increase neutrophil (polymorphonuclear leukocyte, PMN) recruitment into the lung during pulmonary infection, relatively little is known about the local chemokine profiles associated with this enhanced PMN delivery. We investigated the effects of G-CSF and PMN recruitment on the pulmonary chemokine response to intratracheal LPS. Rats pretreated twice daily for 2 days with an s.c. injection of G-CSF (50 μg/kg) were sacrificed at either 90 min or 4 h after intratracheal LPS (100 μg) challenge. Pulmonary recruitment of PMNs was not observed at 90 min post LPS challenge. Macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemotactant (CINC) concentrations in bronchoalveolar lavage (BAL) fluid were similar in animals pretreated with or without G-CSF at this time. G-CSF pretreatment enhanced pulmonary recruitment of PMNs (5-fold) and greatly reduced MIP-2 and CINC levels in BAL fluid at 4 h after LPS challenge. In vitro, the presence of MIP-2 and CINC after LPS stimulation of alveolar macrophages was decreased by coculturing with circulating PMNs but not G-CSF. G-CSF had no direct effect on LPS-induced MIP-2 and CINC mRNA expression by alveolar macrophages. Pulmonary recruited PMNs showed a significant increase in cell-associated MIP-2 and CINC. Cell-associated MIP-2 and CINC of circulating PMNs were markedly increased after exposure of these cells to the BAL fluid of LPS-challenged lungs. These data suggest that recruited PMNs are important cells in modulating the local chemokine response. G-CSF augments PMN recruitment and, thereby, lowers local chemokine levels, which may be one mechanism resulting in the subsidence of the host proinflammatory response. The Journal of Immunology, 2001, 165: 458–465.

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Recruitment of polymorphonuclear leukocytes (PMNs) from the peripheral circulation into the alveolar space is an essential component of the normal host defense response to bacterial infections in the lung. These recruited PMNs provide auxiliary phagocytic defenses that are critical for the eradication of bacterial pathogens in the lower respiratory tract (1, 2). Impairment of this response results in an increased susceptibility to pulmonary infections as shown in hosts with an inherited or acquired condition that impairs the ability of PMNs to migrate to infected sites (3, 4). Not surprisingly, enhancement of PMN delivery into infected tissue sites has been reported to accelerate bacterial clearance and improve survival in several animal models of infection (5–10). G-CSF, a lineage-specific hematopoietic growth factor, selectively stimulates the proliferation and maturation of bone marrow stem cells to neutrophilic cells. We have previously shown that G-CSF significantly enhances the recruitment of PMNs in response to lung infection and inflammation (5, 11, 12). PMN transendothelial migration is a complex process. The presence of chemoattractants is pivotal in guiding the migration of these immune effector cells into infected tissue sites (13, 14). Tissue sites of infection and inflammation contain a variety of inflammatory mediators including cytokines and chemokines, which are responsible for orchestrating PMN recruitment. These mediators also exert other effects within infected tissue sites to modulate the function of recruited PMNs. In addition, the recruited PMNs themselves may affect the local environment by generating and/or scavenging inflammatory mediators. At the present time, relatively little information is known about the changes in pulmonary chemokine profiles that occur during the inflammatory response and how recruited PMNs may modulate these profiles.

In this study, we investigated the effects of PMN recruitment and G-CSF on the pulmonary response of two important C-X-C chemokines, macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemotactant (CINC), in rats challenged with intratracheal LPS. The results show that G-CSF significantly increased PMN recruitment into the lung following an LPS challenge, and this response is associated with a marked reduction in soluble MIP-2 and CINC in the alveolar space. The scavenging of chemokines by recruited PMNs in infected tissue sites may serve to modulate the local host defense response.

Materials and Methods

Reagents

Recombinant human G-CSF was a gift from Amgen (sp. act. 10^8 U/mg; Thousand Oaks, CA). Cytoscreen ELISA kits for rat MIP-2 were obtained from BioSource International (Camarillo, CA). CINC, rabbit anti-rat CINC-1 Ab, and biotinylated anti-rat CINC-1 Ab were obtained from R&D...

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Systems (Minneapolis, MN). Escherichia coli (026:B6) LPS was obtained from Difco (Detroit, MI). Strep-tavadin-HRP was supplied by Jackson Im-
munoresearch (West Grove, PA). Tetramethylbenzidine was purchased from Pierce (Rockford, IL). TaqMan Gold RT-PCR kit was obtained from Perkin-Elmer Applied Biosystems (Foster City, CA). RPMI 1640 was obtained from Life Technologies (Grand Island, NY). FCS was purchased from HyClone (Logan, UT). NIM.2 density gradient reagents were obtained from Sigma (St. Louis, MO).

Animals
Male virus Ab-free (VAF) Sprague Dawley International Genetic Standard rats (Charles River Breeding Laboratories, Wilmington, MA) with a body weight of 175-200 g were maintained on a standard laboratory diet and housed in a controlled environment with a 12-h light/dark cycle. Rats were pretreated for 2 days with a s.c. injection of recombinant human G-CSF at 50 μg/kg. The cell pellet (100 μl) was resuspended in PBS. The isolated PMNs carry the AMs and were obtained from the whole blood samples by using a density gradient centrifugation to separate AMs and PMNs. The isolated PMNs was used to differentiate AMs and pulmonary recruited PMNs. Cells were stained with anti-rat CINC-1 neutralizing Ab by adding 50 μl of 0.5 μg/ml Ab to each well. The wells were sealed and incubated overnight at 4°C. After incubation, the plate was washed five times with washing buffer (PBS containing 0.05% Tween 20 and 2% BSA in washing buffer). After incubation at room temperature for 1 h, the plate was washed. Standard CINC concentrations (3.125–1000 pg/ml) and samples prepared with the same dilution buffer were then added to each well (50 μl per well). The plate was incubated at 37°C for 1 h and then washed. Biotinylated anti-rat CINC-1 Ab was added to each well (0.25 μg/ml, 50 μl per well) and the plate was incubated at 37°C for 1 h. The plate was washed, and peroxidase-conjugated streptavidin-HRP was added (100 μl per well). After incubation at room temperature for 1 h, the plate was washed before adding peroxidase substrate tetramethylbenzidine solution (150 μl per well). The mixtures were incubated at room temperature in the dark for color development. The reaction was stopped by adding 3 M sulfuric acid to each well (50 μl per well). The color developed in each well was determined spectrophotometrically at 450 nm. CINC levels are expressed as pg/ml of BAL fluid or tissue culture media or pg/10^7 PMNs.

Measurement of chemokines
MIP-2 in BAL fluid, tissue culture media, and cell lysates was measured in duplicates using the immunossay kits for rat MIP-2 and procedures supplied by the manufacturer. MIP-2 levels are expressed as pg/ml of BAL fluid or tissue culture media or pg/10^7 PMNs. CINC was determined with a specific ELISA. Wells of a 96-well tissue culture plate are coated with anti-rat CINC-1 neutralizing Ab by adding 50 μl of 0.5 μg/ml Ab to each well. The wells were sealed and incubated overnight at 4°C. After incubation at room temperature for 1 h, the plate was washed. Standard CINC concentrations (3.125–1000 pg/ml) were measured with the same dilution buffer and the samples were added to each well (50 μl per well). The plate was incubated at 37°C for 1 h and then washed. Biotinylated anti-rat CINC-1 Ab was added to each well (0.25 μg/ml, 50 μl per well) and the plate was incubated at 37°C for 1 h. The plate was washed, and peroxidase-conjugated streptavidin-HRP was added (100 μl per well). After incubation at room temperature for 1 h, the plate was washed before adding peroxidase substrate tetramethylbenzidine solution (150 μl per well). The mixtures were incubated at room temperature in the dark for color development. The reaction was stopped by adding 3 M sulfuric acid to each well (50 μl per well). The color developed in each well was determined spectrophotometrically at 450 nm. CINC levels are expressed as pg/ml of BAL fluid or tissue culture media or pg/10^7 PMNs.

Determination of chemokine mRNA expression
Total RNA was extracted from the AMs by TRizol reagent following the manufacturer’s procedure. The RNA was quantified spectrophotometrically at 260 nm. mRNA for specific chemokines was analyzed by real time quantitative RT-PCR procedure that was performed on a Gene Amp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Riboso-

minal RNA was evaluated as an internal control. Equal amounts of total RNA (2 μg) from each sample were reverse transcribed in a 100-μl reaction volume containing 1× TaqMan buffer, 5.5 mM MgCl_2, 40 U RNase inhibitor, 2.5 μM random hexamer primer, 500 μM dNTPs, 125 U mul-
tiscribe reverse transcriptase, and RNase-free water. The mixture was in-
cubated at 25°C for 10 min and then at 48°C for 30 min. The reaction was inactivated by incubating at 95°C for 5 min. PCR was then performed using a 5-μl aliquot of the RT reaction mixtures. cDNA was amplified using specific primers for the cytokines and chemokines as well as the specific probes (rat MIP-2, 5′-CTGTCAATGCTGACGACCTTAC-3′, and CINC, 5′-CCCCTGACCCCAAACCCGGTGTTA-3′) be-
nealed with a fluorophore and a quencher in a 50-μl reaction volume containing 5 mM MgCl_2, 2.5 mM dNTP, 1.25 U AmpliTaq Gold DNA polymerase, and 0.5 U AmpliErase UNG. The primers used were as follows: rat ribosomal RNA (sense, 5′-GGTTGACATTTGTGAGGTTCAAG-3′; an-
ti-sense, 5′-GTTGTTGACATTTGTGAGGTTCAAG-3′); rat MIP-2 (sense, 5′-GTTGTTGACATTTGTGAGGTTCAAG-3′; anti-sense, 5′-GTTGTTGACATTTGTGAGGTTCAAG-3′); rat CINC (sense, 5′-GTTGTTGACATTTGTGAGGTTCAAG-3′; anti-sense, 5′-GTTGTTGACATTTGTGAGGTTCAAG-3′). PCR amplification was performed using 40 thermal cycles of denaturation, annealing, and exten-
sion. Negative control PCRs without cDNA and positive ribosomal control PCRs were performed in all experiments to exclude contamination and other potential errors in the processes.

Statistical analysis
Data are given as means ± SEM of the number of experiments indicated in each figure, and table comparisons of data sets were performed with unpaired Student’s t test or one way ANOVA followed by the Student-
Newman Keuls test. Differences were considered statistically significant at p < 0.05.
**Results**

**Pulmonary recruitment of PMNs following intratracheal LPS challenge**

To determine the magnitude of PMN recruitment following intratracheal challenge, rats were challenged with either saline or LPS and then their lungs were lavaged at either 90 min or 4 h. In rats challenged with intratracheal saline, very few PMNs were recovered from the BAL fluid. G-CSF pretreatment had no effect on the number of PMNs recovered from the BAL fluid in animals challenged with intratracheal saline (0.09 ± 0.01 × 10^6/BAL vs 0.05 ± 0.03 × 10^6/BAL, *p > 0.05*). At 90 min post intratracheal LPS challenge, very few PMNs were recovered in BAL fluid in rats pretreated with either vehicle or G-CSF. Greater than 95% of the recovered cells were AMs in these animals. Intratracheal LPS induced significant PMN recruitment into the lung by 4 h after the challenge (16.27 × 10^6/BAL). G-CSF pretreatment augmented this LPS-induced pulmonary PMN recruitment ~5-fold (Fig. 1).

**Effects of G-CSF treatment on the pulmonary chemokine response to intratracheal LPS challenge**

PMN recruitment into infected tissue sites is a complex process requiring the presence of chemotactants. MIP-2 and CINC are potent C-X-C chemokines accounting for the major chemotactic activity of the rat lung for PMNs (15, 16). We determined the pulmonary chemokine responses following an intratracheal challenge with LPS in rats pretreated with or without G-CSF. As shown in Table I, MIP-2 and CINC levels in BAL fluid of saline-challenged rats at 4 h after the challenge were very low and not different between vehicle- and G-CSF-pretreated rats. Intratracheal LPS induced a marked increase in MIP-2 and CINC concentrations in BAL fluid obtained at both 90 min and 4 h after the challenge. G-CSF pretreatment had no effect on the pulmonary MIP-2 and CINC responses to intratracheal LPS at 90 min after the challenge, a time before PMN entering into the alveolar compartment. In contrast, at 4 h after the challenge LPS-induced increases in MIP-2 and CINC were significantly lower in animals pretreated with G-CSF. Thus, the observed decreases in MIP-2 and CINC in G-CSF-pretreated rats was evident when PMN recruitment was increased compared with animals not given G-CSF.

**Effects of G-CSF on LPS-induced chemokine production by AMs**

Because AMs are essential for the pulmonary production of chemokines in rats with lung infection and inflammation (17, 18), we determined whether in vivo or in vitro G-CSF would affect MIP-2 and CINC production by AMs. AMs were isolated from animals pretreated with vehicle or G-CSF and stimulated with LPS in vitro in the absence and presence of G-CSF. As shown in Fig. 2, A and B, in vitro LPS induced a dose-dependent increase in MIP-2 and CINC production by cultured AMs. AMs of G-CSF-pretreated animals showed slightly higher MIP-2 and CINC production in vitro compared with each dose of LPS as compared with AMs of vehicle-pretreated animals. Adding G-CSF to the in vitro culture systems did not show any direct effect on LPS-stimulated MIP-2 and CINC production by AMs isolated from either G-CSF- or vehicle-pretreated rats. Thus, G-CSF does not have a direct effect on LPS-induced chemokine production by AMs. This suggests that the reduced chemokine concentration in BAL fluid in G-CSF-pretreated animals is not due to the direct effects of G-CSF on AMs.

**Effects of G-CSF on LPS-induced chemokine mRNA expression by AMs**

We determined MIP-2 and CINC mRNA expression by AMs by using Taqman RT-PCR methods with specific probes for MIP-2 and CINC. Data are expressed as 1/Ct value where Ct is the cycle number in which the MIP-2 or CINC signal is detected to be significantly above background. Thus the lower the Ct value or the higher the 1/Ct value, the greater the content of MIP-2 or CINC transcript in the sample. Ribosomal RNA content was used as reference RNA in each sample. As shown in Fig. 3, A and B, in vitro LPS (50 ng/ml) induced a significant increase in MIP-2 mRNA expression by cultured AMs of vehicle-pretreated rats. AMs from animals pretreated with G-CSF showed a comparatively lower expression of MIP-2 mRNA in the presence of LPS stimulation when compared with those of vehicle-pretreated rats, whereas the increase in MIP-2 mRNA expression was greater in these cells following LPS stimulation. Adding G-CSF into the in vitro culture system did not have a direct effect on LPS-stimulated MIP-2 mRNA expression by AMs of either G-CSF- or vehicle-pretreated animals. Fig. 3B shows CINC mRNA expression by cultured AMs. In vitro LPS (50 ng/ml) induced significant increases in CINC mRNA in AMs of both vehicle- and G-CSF-pretreated animals. Neither in vivo G-CSF pretreatment of rats nor adding G-CSF to the culture systems had an effect on either baseline or LPS-stimulated expression of CINC mRNA by cultured AMs.

**Effects of PMNs on LPS-induced chemokines in the media of AM cultures**

G-CSF caused a significant increase in the recruitment of PMNs into the alveolar space in response to intratracheal LPS. Because G-CSF did not suppress AM chemokine expression directly, we investigated whether the PMNs themselves may modulate the LPS-induced chemokine responses by AMs. AMs isolated from normal rats were cocultured with different ratios of either circulating or pulmonary recruited PMNs. As shown in Table II, adding circulating PMNs to AM cultures significantly decreased MIP-2 and CINC concentrations in the culture media at 6 h following LPS stimulation. This decrease in MIP-2 and CINC concentrations in the culture media was PMN dose dependent. Circulating PMNs isolated from the vehicle-pretreated rats showed a greater ability to reduce chemokines in the AM culture media compared with circulating PMNs isolated from G-CSF-pretreated animals. In contrast to circulating PMNs, pulmonary recruited PMNs isolated...
from G-CSF-pretreated rats at 4 h following intratracheal challenge with LPS had no effect on MIP-2 and CINC concentrations in the media of AM cultures in the presence of LPS stimulation (Table III). These data indicate that after recruitment into an inflammatory site, the ability of PMNs to modulate chemokine concentrations is decreased.

**Effects of PMN-conditioned media on LPS-induced chemokine production and chemokine mRNA expression by AMs**

To understand the mechanisms whereby PMNs reduce MIP-2 and CINC in AM cultures, circulating PMNs isolated from either G-CSF- or vehicle-pretreated rats were cultured with LPS (50 ng/ml) for 6 h to generate conditioned media. The conditioned media of PMNs were added to the AM cultures to evaluate the effects of PMN-released factors on AM chemokine responses to in vitro LPS stimulation. As shown in Fig. 4, A and B, LPS-induced MIP-2 and CINC production by AMs tended to be lower when AMs were cultured with conditioned media of PMNs isolated from rats pre-treated with or without G-CSF. However, these reductions in chemokine production did not attain statistical significance.

The effects of PMN-conditioned media on MIP-2 and CINC mRNA expression by AMs were also determined by Taqman RT-PCR techniques. As shown in Fig. 5, A and B, conditioned media of circulating PMNs isolated from rats pretreated with vehicle showed a statistically significant inhibition on LPS-induced MIP-2 but not CINC mRNA expression by cultured AMs. Conditioned media of PMNs from rats pretreated with G-CSF showed a statistically significant inhibition of both LPS-induced MIP-2 and CINC mRNA expressions by cultured AMs.

**PMN-associated chemokines**

Both MIP-2 and CINC have been shown to bind to PMNs (19). Therefore, we determined cell-associated chemokine content after

**FIGURE 2.** MIP-2 and CINC concentration in the supernatant of AM cultures. Values are means ± SEM, n = 6 in each group.

**FIGURE 3.** Effects of G-CSF on MIP-2 and CINC mRNA expression by cultured AMs. Values are means ± SEM, n = 3 in each group. V, AMs of vehicle-treated rats; V/G, AMs of vehicle-treated rats cultured with in vitro G-CSF; G, AMs of G-CSF-treated rats; G/G, AMs of G-CSF-treated rats cultured with in vitro G-CSF; Control, without LPS stimulation; LPS, LPS stimulation. Columns with different letters (a–e) are statistically different (p < 0.05).

| Chemokine | Time   | G-CSF/PBS | Vehicle/PBS |
|-----------|--------|-----------|-------------|
| MIP-2     | 455 ± 86 | 423 ± 55  |             |
| CINC      | 3487 ± 1145* | 4306 ± 1145* |         |
incubating PMNs with BAL fluid. Following a 30-min incubation with BAL fluid from animals challenged with intratracheal LPS, the circulating PMNs of vehicle-treated rats showed a significant increase in cell-associated MIP-2 (6634 pg/10⁷ cells, p < 0.05) and CINC (956 pg/10⁷ cells, p < 0.05) compared with PMNs incubated with control BAL fluid. The chemokine binding activity of circulating PMNs isolated from G-CSF-treated rats was lower (for MIP-2, 3548 pg/10⁷ cells vs 585 pg/10⁷ cells, p < 0.05; for CINC, 492 pg/10⁷ cells vs 36 pg/10⁷ cells, p < 0.05) compared with circulating PMNs isolated from the vehicle-treated animal (Fig. 6, A and B).

Cell-associated MIP-2 of circulating PMNs isolated from rats challenged with intratracheal LPS 4 h earlier was similar to the levels found with circulating PMNs isolated from naive rats (414 ± 58 and 100 ± 2 pg/pg/10⁷ cells, respectively), and G-CSF treatment had no effect on cell-associated MIP-2 of circulating PMNs in intratracheal LPS-challenged rats (Fig. 7A). Pulmonary recruited PMNs showed a marked increase in cell-associated MIP-2 (15,220 ± 1,740 vs 414 ± 58 pg/10⁷ cells, p < 0.05) compared with PMNs remaining in the blood. This increase in cell-associated MIP-2 of the recruited PMNs was lower in G-CSF-treated animals (11,593 ± 1,250 vs 158 ± 16 pg/10⁷ cells, p < 0.05). In contrast to MIP-2, cell-associated CINC of circulating PMNs isolated from rats challenged with intratracheal LPS 4 h earlier was significantly increased when compared with those of circulating PMNs isolated from native rats (928 ± 30 vs 36 ± 3 pg/pg/10⁷ cells, p < 0.05) (Fig. 7B). Pulmonary recruited PMNs showed a similar increase in cell-associated CINC (1089 ± 102 pg/10⁷) compared with circulating PMNs in these animals. Likewise, G-CSF pretreatment attenuated this increase in cell-associated CINC of both circulating (525 ± 50 pg/10⁷) and pulmonary recruited PMNs (248 ± 57 pg/10⁷) in rats challenged with intratracheal LPS for 4 h. These data suggest that binding of chemokines to PMNs as these cells are recruited from the peripheral circulation into the alveolar space is, in part, responsible for decreasing chemokine concentrations within the lower respiratory tract.

**Discussion**

G-CSF pretreatment significantly enhanced the recruitment of PMNs into the lungs of rats challenged with intratracheal LPS. The mechanism(s) resulting in this response appears to be complex and most likely involves multiple effects of this hemopoietic growth factor. G-CSF stimulates PMN release from the bone marrow and increases the number of PMNs in the peripheral circulation (20). In this study, rats pretreated with G-CSF had a 7-fold or greater increase in the number of circulating PMNs (15.13 ± 2.16 × 10⁶/ml vs 2.09 ± 0.31 × 10⁶/ml, p < 0.01) compared with vehicle-treated controls. This increase in circulating PMN counts is likely to be an important factor contributing to the increased recruitment of neutrophils into the lung in response to pulmonary infection and inflammation. In addition, G-CSF up-regulates adhesion molecule CD11b/c expression on circulating PMNs (11, 12, 21, 22), suggesting that G-CSF may also prime PMNs for adhesion to endothelial cells. The firm attachment of PMNs to the endothelium mediated by β₂ integrins is known to be a critical step for PMN transendothelial migration (23). Little is known concerning the effect of G-CSF on the chemotactractors that guide the migration of PMNs from the vasculature into the infected tissue site. MIP-2 and

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**Table II. Effects of circulating PMNs on LPS-induced chemokine production by AMs**

|                  | MIP-2          | CINC          |
|------------------|----------------|---------------|
|                  | Vehicle        | G-CSF         | Vehicle        | G-CSF         |
| AMs only         | 9441 ± 1013    | 8898 ± 1266   | 1851 ± 217     | 1819 ± 211    |
| AMs + 10 × PMNs  | 1507 ± 184*    | 3715 ± 339*   | 471 ± 93*      | 901 ± 127*    |
| AMs + 20 × PMNs  | 575 ± 212*     | 1890 ± 254*   | 109 ± 11*      | 466 ± 61*     |

* Data are means ± SEM (pg/ml) of five experiments in each group. AMs were cultured for 6 h in the presence of LPS (50 ng/ml). G-CSF, circulating PMNs isolated from G-CSF-treated rats; Vehicle, circulating PMNs isolated from vehicle-treated rats. *, p < 0.05 when compared to AMs only.

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![FIGURE 4](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/) Effects of PMN-conditioned media on LPS-induced MIP-2 and CINC production by AMs. Values are means ± SEM, n = 4 in each group. Control, without LPS stimulation; LPS, LPS stimulation; LPS/CM-V, LPS stimulation in the presence of conditioned media of PMNs from vehicle-treated rats; LPS/CM-G, LPS stimulation in the presence of conditioned media of PMNs from G-CSF-treated rats. *, p < 0.05 compared with control group.

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**Table III. Effects of pulmonary-recruited PMNs on LPS-induced chemokine production by AMs**

|                  | n  | MIP-2          | CINC          |
|------------------|----|----------------|---------------|
|                  |    | 8823 ± 1109    | 1385 ± 165    |
| AMs only         | 5  | 9842 ± 1195    | 1299 ± 194    |
| AMs + 10 × PMNs  | 5  | 9143 ± 1358    | 1119 ± 141    |
| AMs + 20 × PMNs  | 4  | 861* 901       | 109 ± 11*     |
CINC, two members from the C-X-C chemokine family, have been shown to be responsible for the major chemotactic activity for PMNs in the alveolar space in rats (15, 16). In this study, we determined the effects of G-CSF pretreatment on the expression of these chemokines in the lung following intratracheal challenge with LPS. Interestingly, LPS-induced increases in MIP-2 and CINC in the alveolar space were significantly lower in rats pretreated with G-CSF. Because these chemokines exert their effects on PMNs through surface receptors and the number of PMNs in the alveolar compartment was increased at this time in rats pretreated with G-CSF, we explored the possibility that the increased number of PMNs in G-CSF-treated rats were responsible for this decrease in chemokines. Pulmonary recruitment of PMNs typically begins ~2 h following intratracheal LPS challenge. The results of this study show that the chemokine response to LPS in the lung was not decreased by G-CSF pretreatment at a time (90 min following intratracheal LPS) before PMN entering the alveolar space.

AMs, the resident phagocytes of the lung, produce chemokines in response to infectious stimuli (24, 25). In addition, monocyte/macrophages have been reported to possess G-CSF receptors (26). We further explored the effects of G-CSF on LPS-induced MIP-2 and CINC production by AMs. The results show that the production of both MIP-2 and CINC by AMs isolated from either vehicle- or G-CSF-pretreated animals was unaffected by adding G-CSF into the in vitro culture system. Similarly, LPS-induced MIP-2 and CINC mRNA expression by this macrophage population was not suppressed by G-CSF. AMs of G-CSF-pretreated rats exhibited a higher, not lower, MIP-2 and CINC production in response to in vitro LPS compared with AMs from vehicle-pretreated animals. In vitro LPS-induced MIP-2 mRNA expression by AMs of G-CSF-pretreated animals was also higher in comparison to those of vehicle-pretreated controls. Thus, G-CSF does not appear to be directly responsible for the lower chemokine values observed following intratracheal LPS challenge despite reports suggesting an immunosuppressive role for G-CSF (27). The mechanisms underlying the up-regulated chemokine response of AMs from G-CSF-pretreated animals observed in these experiments remain unclear.

To investigate the role of PMNs in attenuating the LPS-induced chemokine response in the alveolar space, AMs isolated from naive rats were cocultured with different ratios of circulating or pulmonary recruited PMNs. The results show that circulating PMNs isolated from either vehicle- or G-CSF-treated rats incubated with BAL fluid of rats challenged with intratracheal PBS; G/PBS, circulating PMNs of G-CSF-treated rats incubated with BAL fluid of rats challenged with intratracheal PBS; V/LPS, circulating PMNs of vehicle-treated rats incubated with BAL fluid of rats challenged with intratracheal LPS; G/LPS, circulating PMNs of G-CSF-treated rats incubated with BAL fluid of rats challenged with intratracheal LPS. Columns with different letters (a–c) are statistically different (p < 0.05).
MIP-2 or CINC concentrations in the media of the coculture system following in vitro LPS stimulation. One possible explanation for this is that the capacity of these recruited PMNs to modulate chemokines has been exhausted during their migration into the alveolar space and exposure to chemokines in the process.

To investigate the mechanisms underlying the effects of PMNs on LPS-induced chemokine production by AMs, circulating PMNs isolated from either vehicle- or G-CSF-treated rats were cultured with LPS, and the conditioned media were collected at 6 h. Several studies have previously shown that in vitro LPS stimulates peripheral blood PMNs to produce various mediators (28, 29). After these conditioned media were added into the AM cultures, LPS-induced MIP-2 and CINC production in the supernatant of the AM cultures tended to be reduced, but statistical significance was not reached. LPS-induced MIP-2 mRNA expression by AMs was inhibited by conditioned media of PMNs isolated from both vehicle- and G-CSF-treated rats. LPS-induced CINC mRNA expression by AMs was also inhibited by the conditioned medium of PMNs isolated from G-CSF-treated animals. These results suggest that certain soluble substances released from PMNs can themselves inhibit LPS-induced chemokine expression by AMs. The nature of these inhibitory substances remains to be further defined.

Because the inhibitory effects of PMN-conditioned media on AM chemokine production were relatively small compared with the in vivo suppression of LPS-induced pulmonary chemokine response in animals pretreated with G-CSF and the in vitro inhibition of circulating PMNs on LPS-induced chemokine production in the supernatant of AM cultures, it appears that other mechanisms are involved. To explore this possibility further, the capacity of PMNs to bind MIP-2 and CINC in the BAL fluid of LPS-challenged lungs was determined. The results show that a brief exposure of circulating PMNs to the BAL fluid of rats challenged with intratracheal LPS resulted in a marked increase in cell-associated MIP-2 and CINC in these PMNs. Pulmonary recruited PMNs isolated from either vehicle- or G-CSF-pretreated rats at 4 h after intratracheal LPS challenge showed a dramatic increase in cell-associated MIP-2 in comparison to their counterparts remaining in the peripheral circulation and the circulating PMNs from naive control rats. This likely explains why pulmonary recruited PMNs were not effective at decreasing MIP-2 and CINC concentrations in the media of LPS-stimulated AMs. Neither circulating nor recruited PMNs appear to be active producers of MIP-2 or CINC in response to LPS. In this study, circulating PMNs and pulmonary recruited PMNs were cultured in vitro for 6 h in the presence of LPS (50 ng/ml). Only trace amounts of MIP-2 and CINC could be detected in the supernatant of the cultures.

PMNs are key components of the pulmonary host defense system against invading pathogens (1, 2). G-CSF augments the pulmonary recruitment of PMNs, which has been shown to decrease the morbidity and mortality of respiratory infections in several animal models (5, 30, 31). However, PMNs have also been implicated as mediators of tissue injury in a variety of inflammatory disorders (32, 33). Thus, inflammation evoked by the host response to infections should be restrained within a certain magnitude and duration. Clinical observations of patients with acute respiratory distress syndrome (ARDS) and experimental studies on different animal models of lung injury have shown that sustained increases in pulmonary chemokines including IL-8, MIP-2, and CINC is a risk factor for the development of acute lung injury (17, 34–37). Suppression of the ELR+ C-X-C chemokine (including MIP-2 and CINC) response has been shown to be associated with a significant decrease in lung injury irrespective of PMN influx in the lung in a rat model of hepatic ischemia and reperfusion (38). Although the inciting infection triggers the inflammatory cascade which serves to initiate an immune response, scavenging of soluble chemokines by the responding PMNs may be important in modulating the local host response. With the reduction of chemokines in the alveolar space, chemotactic activity for PMNs would be diminished. This would then limit the influx of PMNs into the tissue sites and signal the resolution of the inflammatory response.

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