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Chemokine Secretion by Human Polymorphonuclear Granulocytes after Stimulation with *Mycobacterium tuberculosis* and Lipoarabinomannan

DETLEV D. RIEDEL[1] AND STEFAN H. E. KAUFMANN[1,2]*

Department of Immunology, Ulm University, 89070 Ulm, Germany,[1] and Max Planck Institute for Infection Biology, 10117 Berlin, Germany[2]

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Macrophages (MAC) and polymorphonuclear granulocytes (PNG) are professional phagocytes which perform essential functions in antibacterial defense. The intracellular bacterium *Mycobacterium tuberculosis* persists and replicates in resting macrophages. Although it is generally assumed that activated MAC are central to protection against *M. tuberculosis*, PNG may also contribute to defense. We wondered whether PNG produce proinflammatory chemokines after stimulation by *M. tuberculosis* or its major cell wall component, lipoarabinomannan (LAM). In this study, we showed that *M. tuberculosis*- and LAM-activated human PNG secrete the leukocyte attractant interleukin-8 (IL-8) and the PNG-specific chemokine GRO-α in a dose-dependent manner. Treatment of PNG with the leukotriene-B4 inhibitor MK-886 prior to stimulation with *M. tuberculosis* or LAM partially blocked IL-8 and GRO-α induction, suggesting involvement of the 5-lipoxygenase pathway in the secretion of these chemokines. We conclude that PNG contribute to early resistance to *M. tuberculosis* via chemokine secretion.

Tuberculosis is a major health threat to mankind which is responsible for almost 3 million deaths per year. The responsible pathogen, *Mycobacterium tuberculosis*, is an intracellular bacterium that survives and replicates in resting macrophages (8). Convincing evidence that activated macrophages (MAC) are responsible for control of *M. tuberculosis* has been presented (8). The highly complex cell wall of *M. tuberculosis* is rich in lipoglycans, and lipoarabinomannan (LAM), an abundant component of the mycobacterial cell wall, is a potent immunomodulator which acts on T cells and MAC (9). Polymorphonuclear granulocytes (PNG) are the first cells which, in response to microbial invasion, migrate from the blood into tissue sites, where they participate in the early inflammatory response. Their principal role in inflammation and host defense has long been thought to be restricted to phagocytosis and bacterial killing. Various stimuli activate the generation of reactive oxygen intermediates and the release of lytic enzymes with potent antimicrobial potential (4). PNG are short-lived and often regarded as terminally differentiated cells that are devoid of transcriptional activity and capable of performing little, if any, protein synthesis (4). However, in the past few years, in vitro studies have revealed that PNG produce a variety of proteins upon appropriate stimulation. Major histocompatibility complex class II expression after treatment with granulocyte-macrophage colony-stimulating factor was demonstrated (4), and a number of cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and transforming growth factor β (4), are actively produced after challenge with various stimuli. These findings suggest that PNG play an active role in inflammatory and immune processes. Although PNG have been implicated in resistance to tubercle bacilli (3), numerous results to the contrary have been reported (5).

Chemokines are potent leukocyte attractants which can be divided into two major groups according to their characteristic sequence motifs and their target cells (1). Those of the CXC type primarily act on PNG, whereas CC-type chemokines preferentially act on monocytes (MO)/MAC. IL-8 and GRO-α not only are both CXC-type chemokines; they also show marked sequence homology. Both chemokines are expressed by MO and PNG upon appropriate stimulation, and receptors for GRO-α and IL-8 have been identified on PNG and on natural killer (NK) cells (10). IL-8 and GRO-α share many biological activities, including induction of chemotaxis, exocytosis, and respiratory burst. In addition, IL-8, but not GRO-α, upregulates complement receptors 1 and 3 (10). Leukotrienes (LT) are proinflammatory lipoid compounds, derived from arachidonic acid metabolism via the 5-lipoxygenase pathway (14), which are produced by hematologic cell types. LTB_4 is a potent chemotactic and activating agent for leukocytes. It stimulates platelet aggregation, release of lysosomal enzymes, and generation of O_2 metabolites by PNG (14). LTB_4 augments IL-1 and TNF-α production of MO/MAC (7, 14). 5-Lipoxygenase is a dual-function enzyme that generates the epoxide LTA_4, which is central to LT biosynthesis. Within PNG, LTA_4 is converted to the chemoattractant LTB_4 by LTA_4 hydrolase (14). LTB_4 inhibitors, such as MK-886, inhibit LT biosynthesis in intact cells (11, 16).

In this study, we have demonstrated that *M. tuberculosis* and its major cell wall component, LAM, induce IL-8 and GRO-α in human PNG at the mRNA and protein levels. Inhibition of LTB_4 synthesis partially blocked chemokine secretion, revealing a fundamental role of LT in inflammation and PNG activation. We suggest that PNG contribute to the early host response against *M. tuberculosis* by increasing local inflammation and recruiting professional phagocytes.

MATERIALS AND METHODS

Microorganisms and LAM. *M. tuberculosis* H37Rv was a generous gift of J. K. Seidel (Forschungsinstitut Borstel, Borstel, Germany). Mycobacteria were grown in Dubos broth (Difco, Detroit, Mich.), and bacterial counts were evaluated by determining CFU on Middlebrook 7H10 agar (Difco) cultures incubated at 37°C for 3 weeks. LAM was a generous gift of P. J. Brennan (Department of Micro-

* Corresponding author. Mailing address: Max Planck Institute for Infection Biology, Monbijonstrasse 2, D-10117 Berlin, Germany. Phone: 49-30-28-026210. Fax: 49-30-28-026212. E-mail: Kaufmann@mpiib-berlin.mpg.de.

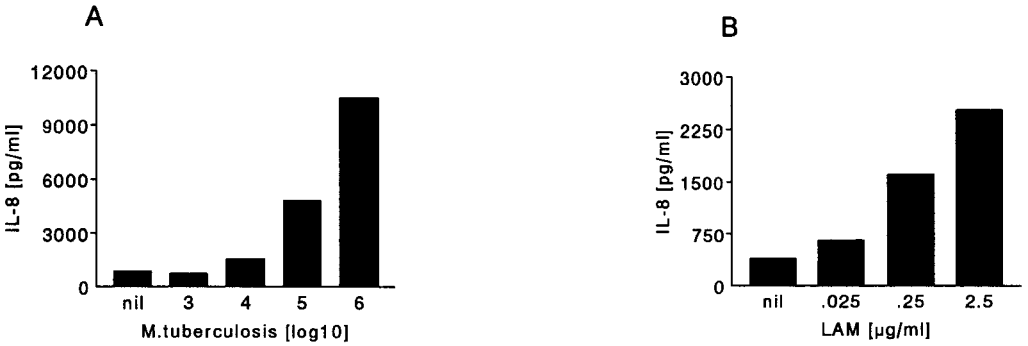

FIG. 1. *M. tuberculosis*- and LAM-induced release of IL-8 by human PNG. (A) PNG (10^6) were stimulated with various numbers of *M. tuberculosis* cells (10^6, 10^5, 10^4, or 10^3) and cultured for 4 h. Supernatants were harvested, and IL-8 was measured by ELISA. (B) PNG (10^6) were stimulated with various concentrations of LAM (2.5 μg/ml, 0.25 μg/ml, or 0.025 μg/ml). Supernatants were harvested, and IL-8 was measured by ELISA. nil, unstimulated serum control. Data represent the means of values for duplicate samples (standard deviations, <10%) from one representative experiment of three with comparable results. Viability of stimulating *M. tuberculosis* cells was revealed by plating serial dilutions on Middlebrook agar plates.

biology, Colorado State University, Fort Collins) under National Institute of Allergy and Infectious Diseases contract NO1 AI-52582. LAM was isolated from a rapidly growing *Mycobacterium* species, and the endotoxin concentration was 4.4 ng of endotoxin per mg of LAM as determined by *Limulus* amoebocyte assay (Sigma).

Preparation of human PNG. Leukocyte buffy coats obtained from healthy donors (Blutbank, Ulm, Germany) were separated over Ficoll-Hypaque 1.077 (Biochrom, Berlin, Germany). Erythrocyte-granulocyte pellets were resuspended in NH_4Cl lysis buffer to eliminate erythrocytes, and the granulocytes remaining were pelleted and washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Biochrom). The purity of the granulocyte preparation was greater than 96% as assessed by Giemsa staining. The resulting PNG were suspended in RPMI 1640 (Biochrom) supplemented with 5% pooled human type AB serum, 25 mM HEPES (Biochrom), and 2 mM L-glutamine at a density of 5×10^6 cells/ml. Cells were distributed in 0.2-ml volumes into 96-well flat-bottom plates (Greiner, Frickenhausen, Germany). PNG were incubated at 37°C under an atmosphere of 5% CO_2. Culture supernatants of both stimulated and unstimulated PNG were collected 4 h after stimulation, sterilized by filtration through 0.2-μm-pore-size filters (Millipore, Bedford, Mass.), and frozen at −20°C until assayed.

Stimulation of PNG by *M. tuberculosis* and LAM. PNG were stimulated with various numbers of *M. tuberculosis* cells (10^3 to 10^6) or different concentrations of LAM (0.025, 0.25, and 2.5 μg/ml) as indicated in the figures.

Determination of cytokine release. An enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Minneapolis, Minn.) for IL-8 and GRO-α was done according to the protocol supplied by the manufacturer.

PCR analysis. To determine the relative level of IL-8 or GRO-α mRNA transcription, both stimulated and unstimulated cells were lysed by the addition of guanidine isothiocyanate. The following RNA extraction was performed with the RNeasy kit (Qiagene, Hilden, Germany). Total cellular RNA was used for reverse transcriptase-PCR (RT-PCR; Stratagene, La Jolla, Calif.). Briefly, total RNA was transcribed by using Moloney murine leukemia virus RT (40 U/ml) in the presence of oligo(dT) and deoxynucleoside triphosphates (Pharmacia, Uppsala, Sweden). The resulting cDNA was utilized to probe for IL-8 and GRO-α gene sequences in PCR reactions in the presence of an internal standard, β-actin, to be coamplified for 15, 20, and 25 cycles. Specific primers for IL-8 and β-actin (both from Stratagene), as well as GRO-α (sense, TAGCCACACTCAAGAAT GGGCGGAAAGCTTGC; antisense, TGGCCATTTGCTTGGATCCGCCAG CCT), were used. Five milliliters of the relevant RT reaction mixture was amplified in the presence of 0.5 mM (final concentration) 5′ and 3′ primers, 0.2 mM deoxynucleotides, and 2.5 U of *Taq* polymerase (Gibco, Paisley, Scotland) in 10× PCR buffer. The PCR was done in a Perkin-Elmer thermal cycler with cycles consisting of 60 s of denaturation at 94°C, 60 s of annealing at 56°C (60°C for β-actin), and 60 s of extension at 72°C. The reaction products were analyzed on a 1.5% agarose gel (Gibco) and made visible by ethidium bromide staining.

RESULTS

***M. tuberculosis* and LAM stimulate IL-8 and GRO-α secretion by PNG.** Freshly isolated PNG (10^6) were treated with different numbers of *M. tuberculosis* (10^6 to 10^3) cells for 4 h. Culture supernatants were collected, and IL-8 concentrations were determined by ELISA. As shown in Fig. 1A, *M. tuberculosis*-stimulated human PNG released the leukocyte attractant IL-8. This release of IL-8 critically depended on the ratio of PNG to *M. tuberculosis* cells. At a ratio of 1:1, PNG responded with a 10- to 50-fold increase in IL-8 release. At lower mycobacterial numbers, the amount of IL-8 decreased, reaching background levels at a ratio of 1,000:1. We were interested in determining whether the major mycobacterial cell wall component of *M. tuberculosis*, LAM, stimulated PNG. LAM was shown to possess IL-8-inducing capacity, causing a 6- to 10-fold increase in IL-8 (Fig. 1B). The existence of an IL-8-inducing capacity of *M. tuberculosis*- or LAM-stimulated human PNG was supported by the finding of an increase in mRNA production as revealed by semiquantitative PCR (Fig. 2). RT-PCR data corresponded with those from the protein secretion studies. GRO-α, a chemokine which recruits and activates human PNG, was also induced after PNG stimulation with *M. tuberculosis* and LAM (Fig. 3). Mycobacterial treatment stimulated up to a 12-fold increase of GRO-α in a concentration-dependent manner (Fig. 3A). Similarly, stimulation of PNG by LAM induced a concentration-dependent release of GRO-α (Fig.

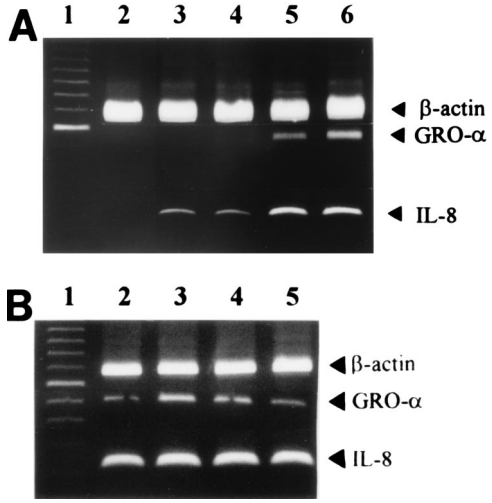

FIG. 2. IL-8 and GRO-α RNA expression after stimulation with *M. tuberculosis* and LAM. PNG (10^6) were incubated with various numbers of *M. tuberculosis* cells or stimulated by different concentrations of LAM for 4 h and lysed; then RNA was extracted, and RT-PCR was done as described in Materials and Methods. IL-8, GRO-α, and β-actin RNAs were amplified for 25 cycles. (A) *M. tuberculosis* infection. Lanes: 1, marker; lane 2, control; 3 to 6, 10^3, 10^4, 10^5, and 10^6 *M. tuberculosis* cells, respectively. (B) LAM stimulation. Lanes: 1, marker; 2, control; 3 to 5, 2.5, 0.25, and 0.025 μg of LAM/ml, respectively.

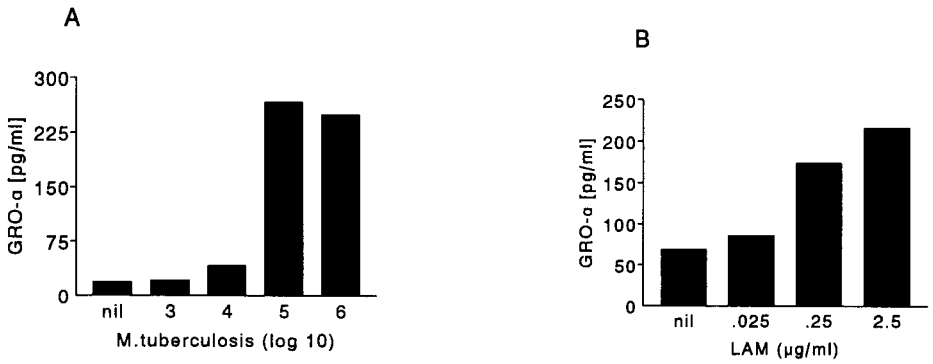

FIG. 3. *M. tuberculosis*- and LAM-stimulated GRO-α production in human PNG. PNG were stimulated as described in the legend to Fig. 1. GRO-α concentrations were determined by ELISA. (A) *M. tuberculosis* stimulation; (B) LAM stimulation. For further details, see the legend to Fig. 1. Data represent the means of values for duplicate samples (standard deviations, <10%) from one representative experiment of three with comparable results.

3B). Stimulation of human PNG with the same concentration (2.5 μg/ml) of lipopolysaccharide (LPS) led to even a higher level of chemokine production (15,000 pg/ml for IL-8 and 550 pg/ml for Gro-α). In contrast, stimulation with 10 pg of LPS/ml did not cause PNG-mediated chemokine secretion (data not shown). These data exclude the possibility that LPS contamination is responsible for the effects observed with LAM. Evaluation of GRO-α induction by semiquantitative PCR revealed an increase in GRO-α PCR fragment production (Fig. 2). PCR data (Fig. 2) correspond to the increasing protein secretion determined by ELISA (Fig. 1), demonstrating increased PCR cDNA fragment production for IL-8 and GRO-α after stimulation with *M. tuberculosis* or LAM. Differences in the appearance of the brightness of the PCR fragments seem to be caused by variations in donor cells. LAM was preincubated with polymyxin B (5 μg/ml) to neutralize potentially contaminating endotoxin and then added to the cells. Polymyxin B treatment did not affect chemokine production (data not shown).

Interference of MK-886 with *M. tuberculosis*- and LAM-induced chemokine secretion by PNG. The LTB_4 inhibitor MK-886 blocks the activity of 5-lipoxygenase-activating protein (11, 16). Consequently, cells fail to produce any LT because LTB_4 is unavailable as a substrate. Treatment of PNG with the leukotriene-B_4 inhibitor MK-886 prior to stimulation with *M. tuberculosis* or LAM partially blocked IL-8 and GRO-α secretion (Table 1). Inhibition was concentration dependent, with maximal inhibitions of 75% for GRO-α and 60% for IL-8 at 50 μg of MK-886/ml (both with *M. tuberculosis*). The reduction of GRO-α was more pronounced than that of IL-8. In the presence of 5 or 50 μg of inhibitor/ml, cell survival was 83 or 70%, respectively, whereas higher concentrations caused a more dramatic loss of cell viability, as assessed by Trypan blue exclusion (data not shown). Thus, even taking the unspecific cytotoxicity of MK-886 into account, specific inhibition of chemokine secretion by MK-886 still occurred.

DISCUSSION

In this study, we have shown that PNG respond to *M. tuberculosis* and its major cell wall component, LAM, by secreting the chemokines IL-8 and GRO-α in vitro. Our data suggest the contribution of chemokines produced during the host response to tuberculosis. Not only *M. tuberculosis* but, more importantly, LAM stimulated production of the chemokines IL-8 and GRO-α by PNG. Consistent with findings obtained in other systems (3, 4, 13), our data emphasize that PNG are actively synthesizing cells and suggest that these cells participate in the early host response to *M. tuberculosis*. Similarly, activation of human PNG by *M. tuberculosis* sulfolipids has been reported to stimulate superoxide generation (18). We assume that two mechanisms underlie chemokine induction in PNG, the first one being dependent on, and the second one being independent of, phagocytosis. Consistent with this assumption, PNG produce IL-8 after phagocytosis of heat-killed yeast (2) and after stimulation by LPS at 100 ng/ml (6). We also detected endotoxin (2.5 μg/ml)-induced chemokine production by PNG but found no chemokine induction with low doses of LPS (10 pg/ml). Involvement of contaminating LPS in LAM-induced chemokine secretion in our experiments, therefore, can be excluded. LAM and LPS possibly activate PNG through the same receptor, since cytokine induction in the human monocytoid cell line THP-1 by LAM and LPS was inhibitable by a monoclonal antibody to CD14 (20). However, they differ in that LAM appears to require an additional signalling receptor component restricted to hemopoietic cells (15). Thus, common receptor components for LAM and LPS may be expressed on PNG, including the CD14 signalling system.

IL-8 and GRO-α are CXC-type chemokines which are chemotactic for PNG but not MO/MAC. GRO-α activates PNG and NK cells (1, 10), whereas IL-8 is chemotactic for T lymphocytes in addition, although at a later time point (17). *M. tuberculosis*-infected MAC synthesize various proinflammatory

TABLE 1. Inhibition of chemokine release by the LTB_4 inhibitor MK-886[a]

Inducer (inhibitor)	Chemokine concn, pg/ml (% inhibition)[b]	
	IL-8	GRO-α
M. tuberculosis (none)	11,900	250
M. tuberculosis (MK-886, 5 μg/ml)	8,120 (30)[c]	125 (50)[c]
M. tuberculosis (MK-886, 50 μg/ml)	4,820 (60)[c]	62 (75)[c]
LAM (none)	2,050	66
LAM (MK-886, 5 μg/ml)	1,130 (45)	39 (40)[c]
LAM (MK-886, 50 μg/ml)	1,230 (40)[c]	34 (48)[c]

[a] PNG were treated with increasing concentrations of the LTB_4 inhibitor MK-886 (5 or 50 μg/ml) for 5 min; cells were incubated with *M. tuberculosis* (10^6 cells) or stimulated by LAM (2.5 μg/ml) for 4 h. Supernatants were collected and tested for IL-8 and GRO-α by ELISA; parentheses denote the percentage of MK-886-mediated inhibition. Data represent the means of values for duplicate specimens (standard deviations, <10%) for one representative experiment of three with comparable results.

[b] Unless otherwise noted, $P < 0.05$ (Student's *t* test).

[c] $P < 0.01$ (Student's *t* test).

cytokines, including TNF-α and IL-8 (19), and present mycobacterial antigens to T lymphocytes (8). Upon stimulation by an appropriate antigen, T cells produce an array of cytokines, including gamma interferon (IFN-γ) and IL-2. IFN-γ is the major MAC-activating cytokine which causes NO production and subsequent inactivation of *M. tuberculosis*, at least in the experimental mouse model (8). Although the mechanisms underlying tuberculosis control in humans are still enigmatic, evidence suggesting that similar mechanisms are involved is accumulating. In particular, activation of inducible nitric oxide synthase has been detected in human alveolar MAC from tuberculosis patients (12). Furthermore, successful resolution of tuberculosis in an immunocompromised patient was achieved by adjunctive therapy with IFN-γ and granulocyte colony-stimulating factor (13). The latter cytokine is known to act on PNG.

LT are important inflammatory molecules with potent chemotactic activity for PNG (14). Moreover, they increase production of the monokines TNF-α and IL-1β and stimulate exocytosis in MAC (7, 14). Because PNG activation results in LTB_4 synthesis, we tried to block eicosanoid production by PNG with MK-886. Our experiments revealed that the LTB_4 inhibitor MK-886 interfered with chemokine release by LAM- or *M. tuberculosis*-treated PNG, with the effects on LTB_4 depending on the inhibitor concentration. Our results are not only in agreement with the involvement of LTB_4 in monokine production (14); they also emphasize the importance of LT in inflammation.

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