Mycobacterium tuberculosis 19-kDa Lipoprotein Promotes Neutrophil Activation

Clemens Neufert, Rish K. Pai, Erika H. Noss, Melvin Berger, W. Henry Boom and Clifford V. Harding

J Immunol 2001; 167:1542-1549; doi: 10.4049/jimmunol.167.3.1542
http://www.jimmunol.org/content/167/3/1542

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

References
This article cites 59 articles, 37 of which you can access for free at:
http://www.jimmunol.org/content/167/3/1542.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


Mycobacterium tuberculosis 19-kDa Lipoprotein Promotes Neutrophil Activation

Clemens Neufert,* Rish K. Pai,* Erika H. Noss,† Melvin Berger,† W. Henry Boom,‡ and Clifford V. Harding*‡

Certain microbial substances, e.g., LPS, can activate neutrophils or prime them to enhance their response to other activating agents, e.g., fMLP. We investigated the role of the Mycobacterium tuberculosis (MTB) 19-kDa lipoprotein in activation of human neutrophils. MTB 19-kDa lipoprotein initiated phenotypic changes characteristic of neutrophil activation, including down-regulation of CD62 ligand (L-selectin) and up-regulation of CD35 (CR1) and CD11b/CD18 (CR3, Mac-1). In addition, exposure of neutrophils to MTB 19-kDa lipoprotein enhanced the subsequent oxidative burst in response to fMLP as assessed by oxidation of dihydrorhodamine 123 (determined by flow cytometry). LPS also produced these effects with similar kinetics, but an oligodeoxynucleotide containing a CpG motif failed to induce any priming or activation response. Although the effects of LPS required the presence of serum, neutrophil activation by MTB 19-kDa lipoprotein occurred independently of serum factors, suggesting the involvement of different receptors and signaling mechanisms for LPS and MTB 19-kDa lipoprotein. Thus, MTB 19-kDa lipoprotein serves as a pathogen-associated molecular pattern that promotes neutrophil priming and activation. The Journal of Immunology, 2001, 167: 1542–1549.

In the course of their response to infectious agents, neutrophils undergo a process of activation that initiates or enhances microbial and inflammatory functions. Neutrophil activation includes a broad range of phenotypic and functional changes that occur in several stages. Early events induced by low levels of activating agents include modulation of surface receptors that function in neutrophil vascular adhesion and diapedesis, bind microbes or opsonins (e.g., complement receptors), and bind chemotaxants (1). Priming, or increasing responsiveness to subsequent stimuli, also occurs early in the activation process. Later changes that require higher levels of activating stimulus include the production of reactive oxygen species (ROS) and cytokines. Induction of each activation event requires a characteristic threshold stimulus level (2), and some activating agents cannot evoke all functions.

Different activating agents can promote neutrophil activation to different levels. Fixed immune complexes, PMA or fMLP directly induce the production of ROS. In contrast, many cytokines and other microbial agents, e.g., LPS, do not have the capacity to directly induce ROS, but they can induce activation-associated changes in the expression of cell surface receptors and prime neutrophils for enhanced induction of ROS by another activating stimulus. Priming occurs when one agent that does not elicit a measurable response (at the concentration used) enhances subsequent induction of that response by another activating agent or the same agent at a higher concentration. For example, priming with LPS can enhance fMLP-induced production of ROS (3–5). The mechanisms of priming are still poorly understood, but may include enhancement of intracellular signal transduction mechanisms, up-regulation of receptors to activating agents (6, 7), and/or preliminary assembly of membrane-bound and cytosolic components of NADPH oxidase (which produces ROS).

Certain steps in neutrophil activation are indicated by changes in the expression of cell surface markers, e.g., CD62 ligand (CD62L), CD35, and CD11b/CD18. CD62L (L-selectin) is highly expressed on resting neutrophils, mediates neutrophil-endothelial interaction before diapedesis, and is down-regulated during neutrophil activation and migration to extravascular sites (1, 8–12). Thus, decreased expression of CD62L can be used as a measure of neutrophil activation. CD11b/CD18, also known as Mac-1 or CR3, is an integrin that is present in secondary and tertiary granules as well as secretory vesicles of neutrophils (9). CD11b/CD18 binds iC3b and also mediates adhesion to extracellular matrix proteins, such as ICAM-1 or platelet endothelial cell adhesion molecule-1, on endothelial cells (1, 2, 11). Cell surface expression of CD11b/CD18 is increased upon activation of neutrophils (8, 13). CD35, also known as CR1, is stored in secretory vesicles (14) and binds C3b and C4b. Neutrophil activation results in degranulation (vesicle exocytosis) and increased cell surface expression of CD35 and CD11b/CD18 (13). Assessment of cell surface expression of these molecules by flow cytometry provides a measure of neutrophil activation (13, 15).

Activation of neutrophils and other components of immune responses can be induced by a number of different microbial substances that can be termed pathogen-associated molecular patterns (PAMPs) (16, 17). For example, LPS directly induces certain aspects of neutrophil activation, including decreased expression of CD62L (8) and increased expression of CD11b/CD18 (8, 18), and LPS primes neutrophils for the subsequent induction of ROS by...
Bacterial lipopolysaccharides represent another class of PAMPs that affect neutrophil function. Membrane lipoproteins of *Mycoplasma fermentans* induce neutrophil secretion of IL-8 (19). Lipoproteins of various enterobacteria decrease the expression of CD62L and increase the expression of CD11b/CD18 (10). The synthetic lipopeptide Pam3Cys-Ser-(Lys)4 (20) and lipoproteins from *Treponema denticola* (21) stimulate the production of ROS and the exocytosis of specific granules. The outer surface protein A of *Borrelia burgdorferi* induces early and late neutrophil activation responses, such as increased adherence, altered expression of surface molecules, ROS production, degranulation, and IL-8 secretion (22). Thus, neutrophil activation is promoted by recognition of various types of PAMPs.

In addition to contributions by T cells and macrophages, neutrophils may play an important role in control of infection with *Mycobacterium tuberculosis* (MTB) or other related mycobacteria, e.g., *M. bovis* bacillus Calmette-Guérin (BCG). In murine models, neutrophils are recruited to sites of infection after the administration of MTB or BCG (23, 24). MTB promotes late events in neutrophil activation, including the production of ROS (25, 26) and cytokines such as IL-8, macrophage inflammatory protein-1α, and growth-related oncogene-α (27, 28). Depletion of neutrophils causes increased numbers of bacilli at sites of infection and decreased expression of IFN-γ and inducible NO synthase (23). Neutrophils can kill MTB by oxidative and nonoxidative mechanisms (29, 30). Thus, the exact role and importance of neutrophils in infection with MTB is subject to further investigation, but several lines of evidence suggest that neutrophils contribute to host responses to MTB.

Although it is clear that MTB can activate neutrophils, little is known about the components of MTB that induce neutrophil activation. MTB-derived sulfolipid-1 (31) and phenolic glycolipids (32) induce the production of ROS. Purified protein derivative of MTB can kill MTB by oxidative and nonoxidative mechanisms (29, 30). Thus, the exact role and importance of neutrophils in infection with MTB is subject to further investigation, but several lines of evidence suggest that neutrophils contribute to host responses to MTB.

**Materials and Methods**

**Reagents and materials**

*For all applications, HBSS and PBS (both from Life Technologies, Grand Island, NY) were free of calcium, magnesium, and phenol red. LPS from *Escherichia coli* 0127:B8 was obtained from Difco (Detroit, MI). CPG oligodeoxycytidinol (ODN) 2006 (TTCGTTCTTTGCGTTT GTCGGT; CPG motifs underlined) is recognized by human cells (37, 38), and was provided by the Coley Pharmaceutical Group (Wellesley, MA). Where possible, tubes, pipettes, flasks, and other materials used with neutrophils were made of sterile polystyrene or polypropylene. Glassware was autoclaved before use.*

**Preparation of MTB 19-kDa lipoprotein**

MTB and MTB 19-kDa lipoprotein were prepared as previously described (36, 39) with some modifications. MTB H37Ra (American Type Culture Collection, Manassas, VA) was grown to log phase in Middlebrook 7H9 medium (Difco) with albumin, dextrose, and catalase enrichments (Difco). MTB was harvested, and the pellet was frozen at −70°C. MTB was suspended in deionized water containing 7.5 mM EDTA, 0.7 μg/ml leupeptin (Sigma, St. Louis, MO), 0.2 mM PMSF (Sigma), 0.7 μg/ml peptatin A (Sigma), 10 μM DNase (Sigma), and 25 μM RNase A (Roche, Indianapolis, IN), the bacteria were lysed by passing them through a French press twice, and the suspension was centrifuged for 1–2 h at 100,000 × g. The supernatant was rotated at 4°C for 1 h with ice-cold 2% Triton X-114 (TX114; Sigma) in 50 mM sodium phosphate, pH 7.4 (total protein concentration, 0.5–1 mg/ml), warmed to 37°C for 15 min, and centrifuged at 37°C for 15 min at 2400 × g to separate aqueous and TX114 (detergent) layers (40). The aqueous layer was removed, and cold 50 mM phosphate buffer was added. The tube was incubated on ice until the phases merged and then was warmed and centrifuged as described above. The TX114 layer was washed three times in this manner and then precipitated by overnight incubation at −20°C with 10 volumes cold acetone. The pellet was washed once with cold 80–90% acetone, resuspended in reducing SDS-PAGE sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.7 M 2-ME, and 0.01 μg/ml bromophenol blue), boiled for 5 min, and subject to preparative 12% SDS-PAGE using a model 491 Prep Cell (Bio-Rad, Richmond, CA). Fractions were collected in elution buffer (25 mM Tris and 192 mM glycine, pH 8.3) with a flow rate of 0.1 ml/min, and the fraction containing the most bromophenol blue dye marker was designated fraction 0. Portions of the samples were analyzed by SDS-PAGE under reducing conditions and silver staining with the Bio-Rad Silver Stain Plus kit or Western analysis. For Western analysis, proteins were transferred onto a 0.2-μm pore nitrocellulose membrane (Bio-Rad) or a polyvinylidene difluoride membrane (Millipore, Bedford, MA), incubated for 1 h at room temperature in blocking buffer (5% Carnation nonfat dry milk (Nestle, Solon, OH) in PBS with 0.1% Tween 20), and probed with IT-19 mouse anti-MTB 19-kDa lipoprotein mAb (41, 42) in blocking buffer for 1 h at room temperature. Blots were washed, incubated with HRP-labeled secondary Ab (Amersham, Arlington Heights, IL) for 1 h at room temperature, and developed with Supernatex West Pico chemiluminescence kit (Pierce, Rockford, IL) or ECL Western blot detection kit (Amer sham). Fractions that contained substantial amounts of MTB 19-kDa lipoprotein and lacked significant contamination with other species by this analysis were pooled, extracted with TX114 as described above, and resuspended in 5 mM HEPES buffer, pH 7.0. Protein content was determined by a detergent-compatible protein assay (Bio-Rad). MTB 19-kDa stock preparations (30–100 μg/ml) had no detectable LPS contamination revealed by Limulus amebocyte lysate assay (E-Toxate Kit; Sigma), indicating that these preparations had <25 ng/ml LPS. Because these preparations were diluted (e.g., 1/100) for experimental use, maximum potential levels of LPS contamination under experimental conditions were <0.25 ng/ml, at least 100-fold less than the level required to activate neutrophils in our studies.

**Neutrophil purification**

Neutrophils were prepared as previously described (13) with minor modifications. Blood (10–20 ml) was harvested from healthy volunteers into siliconized tubes with heparin (20 U/ml) and transferred in samples of 5–7 ml onto 15-ml polystyrene tubes. Percoll (Amer sham Pharmacia Biotech) was diluted in PBS, and three 3-ml layers (53.4%, 62.3, and 71.2% Percoll) were introduced under the blood. The tubes were centrifuged for 12 min at 500 × g at 15°C. Neutrophils were harvested in approximately a 3-ml volume from the interface of the 62.3 and 71.2% Percoll layers and placed in 50-ml polypropylene tubes. All subsequent steps in neutrophil purification were at 4°C. To lyse erythrocytes, 20 ml saline (2 mg NaCl/ml) was added, the cells were vortexed gently and incubated on ice for 1 min, 20 ml saline (16 mg NaCl/ml) was added, the suspension was mixed, and the neutrophils were pelleted at 200 × g for 10 min. The cells were resuspended in 10 ml saline (2 mg NaCl/ml), vortexed, and combined with 20 ml HBSS. Neutrophils were washed once with HBSS and resuspended to 106 cells/ml with 10% FCS (HyClone, Logan, UT) and 0.5% penicillin-streptomycin, 10 μM HEPES, 5 × 10−4 M 2-ME, and antibiotics (D10F). For serum-free conditions, cells were resuspended at 106 cells/ml in DMEM (Life Technologies) containing 0.3 mg/ml BSA (Sigma). Cytosine preparations of cells were stained with Hema3 System (Fisher Scientific, Pittsburgh, PA) and examined microscopically. Neutrophil purity was consistently >95%. The viability of neutrophils was >99% by trypan exclusion.

**Assay for cell surface expression of CD62L, CD11b/CD18, and CD35**

Purified neutrophils (2 × 107) were incubated in a water bath at 37°C with centrifugation of 10 ml at 800 × g for 60 min. The cells were centrifuged, and the supernatant was removed. Cells were recovered from washes by centrifugation for 10 min at

**The Journal of Immunology**
200 × g. Neutrophils were washed with HBSS containing 0.5 mM sodium azide, 0.35 mg/ml NaHCO₃, 1 mg/ml BSA, and 10⁻³ M PMSF. Cells were double stained with PE-conjugated murine anti-human CD62L (clone DR35-56, IgG1; BD Pharmingen, San Diego, CA) and CyChrome-conjugated murine anti-human CD11b (clone ICRF44, IgG1; BD Pharmingen). PE-conjugated murine IgG1 (Caltag, Burlingame, CA) and CyChrome-conjugated murine IgG1 (BD Pharmingen) were used as isotype-matched control Ab preparations. Cells were incubated with Ab for 30 min, washed twice with HBSS (with azide, NaHCO₃, BSA, and PMSF as described above), and then stained once with PBS-azide (PBS with 0.5 mg/ml azide), fixed with 1% paraformaldehyde or simply kept at 4°C in PBS-azide, and then examined by flow cytometry. CD35 was detected with murine IgG1 anti-human CD35 (clone 3D9). Mouse IgG1 (Caltag) was used as the isotype-matched control Ab. Cells were incubated for 30 min with primary Ab, washed twice with HBSS (with azide, NaHCO₃, BSA, and PMSF), incubated for 30 min with FITC-labeled F(ab)₂ goat anti-mouse secondary Ab (BioSource, Camarillo, CA), and prepared for flow cytometry as described above. Flow cytometry was performed with a FACS analyzer (BD Biosciences, San Jose, CA). Forward and side scatter properties were used to set gates for intact cells.

As observed in other studies, upon the shift from 4°C preparation conditions to 37°C experimental conditions, a background level of nonspecific neutrophil activation appeared rapidly regardless of the presence or absence of activating agents, as reflected by the somewhat decreased expression of CD62L and increased expression of CD11b/CD18 and CD35. These changes occurred within a few minutes and stabilized by 10 min at 37°C. Because specific effects of activating agents occurred after 10 min, kinetic data are displayed from the 10 min point.

Assay for oxidative burst

Incubations to induce oxidative burst were performed in a 37°C water bath with occasional gentle agitation. Purified neutrophils (5 × 10⁶ in 0.4 ml D10F) were incubated for 15 min in 5-ml polystyrene tubes with 5 μM dihydroethamine 123 (DHR; Molecular Probes, Eugene, OR). MTB 19-kDa lipoprotein or LPS was added in 0.05 ml (1 μg/ml final concentration) for 10 min to induce oxidative burst. Cells were placed on ice and immediately analyzed by flow cytometry. Events (10⁴) were acquired for analysis of intact cells, and analyzed for emitted green fluorescence (FL1-H) as previously described (43–45).

Oxidative burst was also assessed in neutrophils in whole blood (i.e., without purification of neutrophils on Percoll step gradients). Blood from healthy volunteers was drawn into heparinized syringes, and 0.15 ml was added to prewarmed polystyrene tubes containing 0.2 ml DMEM. Cells were incubated with DHR for 15 min at 37°C, with or without priming agent for 20 min, and then with FITC-MPF for 10 min as described above. Tubes were placed on ice, and erythrocytes were lysed by adding 4 ml ice-cold lysis buffer (8.3 mg/ml NH₄Cl, 1.68 mg/ml NaHCO₃, and 1 mM EDTA) and then centrifuging the cells for 5 min at 200 × g. Lysis was repeated three times. Cells were washed and immediately analyzed by flow cytometry as above.

Results

MTB 19-kDa lipoprotein promotes neutrophil activation, as indicated by decreased cell surface expression of CD62L and increased cell surface expression of CD11b/CD18 and CD35

MTB 19-kDa lipoprotein was purified from MTB lysate by extraction with TX114 and electrophoretic elution (see Materials and Methods). Purified neutrophils were incubated with MTB 19-kDa lipoprotein or LPS and then evaluated for expression of CD62L and CD11b/CD18 by two-color flow cytometry. Expression of CD62L was decreased upon exposure of neutrophils to MTB 19-kDa lipoprotein or LPS, but not control medium (Fig. 1). Minimum effective concentrations for decreasing CD62L expression were 520–1600 ng/ml for MTB 19-kDa lipoprotein and 32 ng/ml for LPS (Fig. 2A). Virtually complete down-regulation was achieved with 5200 ng/ml MTB 19-kDa lipoprotein or 320 ng/ml LPS (Fig. 2A). At the latter concentrations, the mean fluorescent value for CD62L staining was reduced by either agent to ~15% of the control level in the absence of the activating agents (Fig. 2A).

Down-regulation of CD62L occurred with similar kinetics after exposure to MTB 19-kDa lipoprotein or LPS, with decreased expression starting within 15 min and down-regulation largely completed by 30 min (Fig. 2B). In the same cells MTB 19-kDa lipoprotein and LPS increased the expression of CD11b/CD18 (Fig. 1). Increased CD11b/CD18 expression was induced by concentrations of MTB 19-kDa lipoprotein and LPS similar to those required for reduction of CD62L expression (Fig. 3A). Expression of CD11b/CD18 was increased ~3-fold by 5200 ng/ml MTB 19-kDa lipoprotein or 320 ng/ml LPS. Kinetic studies showed that maximum expression of CD11b/CD18 occurred after exposure to MTB 19-kDa lipoprotein or LPS for 30 min, after which CD11b/CD18 expression declined (Fig. 3B). The majority of neutrophils modulated the expression of both CD11b/CD18 and CD62L in response to LPS or MTB 19-kDa lipoprotein, but a small population of cells was less responsive (Fig. 1). MTB 19-kDa lipoprotein increased the expression of CD11b/CD18 and decreased the expression of CD62L on neutrophils obtained from three of six different donors (see below).

Neutrophil expression of CD35 was assessed with a different staining procedure and therefore evaluated in separate experiments, but the experimental conditions resembled those for the detection of CD11b/CD18 and CD62L. MTB 19-kDa lipoprotein and LPS both increased the expression of CD35 (Fig. 1). The minimum concentrations necessary to increase CD35 expression were 240–760 ng/ml for MTB 19-kDa lipoprotein and 32–100 ng/ml for LPS (Fig. 4). A 2-fold increase in CD35 expression was achieved by 2400 ng/ml MTB 19-kDa lipoprotein or 320 ng/ml LPS. Kinetic studies showed that CD35 expression began to increase within 10–15 min, and plateau expression was achieved by about 30–40 min after exposure to the activating agents. MTB 19-kDa lipoprotein increased the expression of CD35 by neutrophils obtained from three of three different donors, although other activation parameters were found to vary between different donors (discussed below).

Despite the similarity of responses to MTB 19-kDa lipoprotein and LPS, the stimulatory properties attributed to MTB 19-kDa lipoprotein and LPS increased the expression of CD11b/CD18 and decreased the expression of CD62L on neutrophils obtained from three of six different donors (see below).
MTB 19-kDa lipoprotein could not be explained by LPS contamination. MTB 19-kDa lipoprotein stock preparations contained no detectable LPS by Limulus amebocyte lysate assays, indicating LPS contamination of <25 ng/ml. Given the dilution of this stock to achieve final working concentrations of MTB 19-kDa lipoprotein (e.g., 1/100), the maximum level of LPS contamination under experimental conditions was <0.25 ng/ml, at least 100-fold less than that required to achieve LPS-mediated neutrophil activation. Furthermore, control electroelution fractions that lacked MTB 19-kDa lipoprotein did not diminish CD62L expression or increase the expression of CD35 or CD11b/CD18 (data not shown). Finally, later experiments revealed mechanistic differences in signaling by LPS and MTB 19-kDa lipoprotein (see below).

**MTB 19-kDa lipoprotein primes neutrophils for increased oxidative burst in response to fMLP**

Flow cytometry was used to detect oxidation of DHR as a measure of oxidative burst resulting from neutrophil activation (43, 44). MTB 19-kDa lipoprotein and LPS both served as priming agents to enhance the subsequent oxidative burst of purified neutrophils exposed to fMLP (Fig. 5). Priming with 70 ng/ml MTB 19-kDa lipoprotein increased the subsequent response to fMLP by ~2-fold, slightly greater than the enhancement seen with 100 ng/ml LPS (Fig. 6A). Even greater enhancement was seen with 350-1400 ng/ml MTB 19-kDa lipoprotein (Fig. 6A). In addition to these studies of neutrophil priming, we tested the ability of MTB 19-kDa lipoprotein and LPS to directly activate neutrophils. In the absence of fMLP, both MTB 19-kDa lipoprotein and LPS failed to induce oxidative burst (data not shown). Thus, studies with purified neutrophils demonstrated the ability of MTB 19-kDa lipoprotein to prime neutrophils for oxidative burst in response to fMLP.

Neutrophil oxidative burst was also evaluated in whole blood samples without neutrophil purification. Whole blood samples (still containing human serum) were incubated with DHR, priming agents, and fMLP; erythrocytes were lysed; and the samples were analyzed by flow cytometry with gating on optical scatter parameters to select neutrophils. Incubation of whole blood with MTB 19-kDa lipoprotein or LPS also resulted in priming of neutrophil response to fMLP (Fig. 6B), confirming the results with purified neutrophils.

Some differences were seen in results obtained with whole blood and purified neutrophils. In the absence of a priming agent, fMLP induced almost no oxidative burst in neutrophils in whole blood (Fig. 6B). In purified neutrophils, fMLP did induce some oxidative burst even without a priming agent (Fig. 6A), indicating that the purification procedure resulted in some degree of functional priming. Thus, the whole blood protocol may be a less perturbed system to reveal the full extent of priming effects, whereas the purified neutrophil system confirms that these effects are intrinsic to the neutrophil population. Our results, particularly with the unperturbed whole blood system, confirm that substantial oxidative burst will only occur if neutrophils are exposed to priming agents before the addition of fMLP, as seen in other studies with similar use of priming agents and fMLP (22). Another difference between purified neutrophils and whole blood lay in the relative responses of neutrophils to LPS and MTB 19-kDa lipoprotein. Whereas purified neutrophils exhibited greater priming with 70–1400 ng/ml MTB 19-kDa lipoprotein than with 100 ng/ml LPS (Fig. 6A), neutrophils...
in whole blood showed greater priming with 100 ng/ml LPS than with 1400 ng/ml MTB 19-kDa lipoprotein (Fig. 6B), possibly due to the differing roles of serum proteins in responses to these substances (see below).

Neutrophils respond to MTB 19-kDa lipoprotein, but not LPS, in serum-free medium

LPS has been shown to signal through a serum-dependent mechanism, but the role of serum components in neutrophil responses to MTB 19-kDa lipoprotein was unknown. Because previous experiments involved the incubation of neutrophils with priming or activating agents in the presence of serum, we tested the ability of MTB 19-kDa lipoprotein or LPS to induce neutrophil responses in the absence of serum. Purified neutrophils were resuspended in DMEM with 300 μg/ml BSA, incubated with MTB 19-kDa lipoprotein (760 ng/ml) or LPS (100 ng/ml). The expression of CD35 was evaluated by flow cytometry. Each experiment was performed at least four times with similar results.

FIGURE 4. MTB 19-kDa lipoprotein decreases the expression of CD35 on neutrophils. A, Purified neutrophils incubated for 40 min at 37°C with MTB 19-kDa lipoprotein or LPS at various concentrations. B, Purified neutrophils incubated for various periods with or without MTB 19-kDa lipoprotein (760 ng/ml) or LPS (100 ng/ml). The expression of CD35 was evaluated by flow cytometry. Each experiment was performed at least four times with similar results.

FIGURE 5. MTB 19-kDa lipoprotein primes neutrophils for oxidative burst in response to fMLP. Purified neutrophils were incubated with DHR (5 μM) for 15 min at 37°C and then exposed to MTB 19-kDa lipoprotein (1400 ng/ml), LPS (100 ng/ml), or control medium for 20 min at 37°C. Subsequently, fMLP (10^{-6} M) was added for 10 min at 37°C. Cells were analyzed immediately by flow cytometry. These results are representative of five independent experiments.

Variation in responses to MTB 19-kDa lipoprotein and other PAMPS

Significant variation was observed in the ability of cells from different donors to respond to MTB 19-kDa lipoprotein (Table I). Of seven donors, four provided neutrophils that responded to MTB 19-kDa lipoprotein. Four different activation parameters were evaluated (increase in CD35, increase in CD11b/CD18, decrease in CD62L, and increase in DHR oxidation). Cells that responded to MTB 19-kDa lipoprotein in one of these parameters were responsive in all other parameters tested, whereas others were unresponsive in all parameters tested (Table I; some donors were not evaluated for all parameters). Neutrophils from a given donor provided consistent results when obtained on different days, indicating that the degree of responsiveness was a stable genetic or acquired trait and was not an acute effect of a transient subclinical illness. In contrast to the variability observed with MTB 19-kDa lipoprotein, Table I shows that neutrophils from all donors consistently responded to LPS in all activation parameters (although LPS-nonresponsive donors have been reported in other types of studies; Ref. 46). Neutrophils from five of five donors failed to respond in any activation parameters to CpG ODN 2006 (used at a 1–10 μg/ml final concentration for priming or direct activation), indicating that human neutrophils do not respond to this particular PAMP. Thus, responses to MTB 19-kDa lipoprotein were observed consistently in neutrophils from a subset of donors, whereas neutrophils from other donors consistently failed to respond to this lipoprotein, even though they were responsive to LPS.
Differences between these agents.

Lipoprotein, also implies distinct steps in receptor signaling that some donors responded to LPS, but not to MTB 19-kDa lipoprotein. Furthermore, the finding that MTB 19-kDa lipoprotein produced several changes characteristic of neutrophil activation by increasing cell surface expression of CD35 and CD11b/CD18 and decreasing expression of CD62L. In addition, MTB 19-kDa lipoprotein primes neutrophils for increased oxidative burst in response to fMLP, but does not induce oxidative burst by itself. In contrast to LPS and MTB 19-kDa lipoprotein, CpG ODN 2006 did not prime neutrophils for increased oxidative burst in response to fMLP. DHR was added to purified neutrophils (A) or whole blood (B) for 15 min at 37°C. The cells were then exposed to control medium, MTB 19-kDa lipoprotein, or LPS for a 20-min priming incubation at 37°C. DMEM or fMLP (10^{-6} M) was then added for 10 min at 37°C. Purified neutrophils were analyzed immediately by flow cytometry. Whole blood was processed to lyse RBC and then was analyzed by flow cytometry. These results are representative of at least four independent experiments.

**FIGURE 6.** MTB 19-kDa lipoprotein primes neutrophils for oxidative burst in response to fMLP. DHR was added to purified neutrophils (A) or whole blood (B) for 15 min at 37°C. The cells were then exposed to control medium, MTB 19-kDa lipoprotein, or LPS for a 20-min priming incubation at 37°C. DMEM or fMLP (10^{-6} M) was then added for 10 min at 37°C. Purified neutrophils were analyzed immediately by flow cytometry. Whole blood was processed to lyse RBC and then was analyzed by flow cytometry. These results are representative of at least four independent experiments.

**FIGURE 7.** MTB 19-kDa lipoprotein can signal through a serum-independent pathway. Purified neutrophils were incubated for 40 min at 37°C with MTB 19-kDa lipoprotein (B and D) or LPS (A and C) in the presence or the absence of serum. Cells were double stained for CD62L (A and B) and CD11b/CD18 (C and D) and were analyzed by flow cytometry with gating for intact cells. These results are representative of two independent experiments.

**Discussion**

A number of bacterial substances, i.e., PAMPs, are recognized by pattern recognition receptors, e.g., Toll-like receptors (TLRs) (16, 17). LPS signals via TLR4 (47–49), CpG DNA signals via TLR9 (50), and bacterial lipoproteins (including MTB 19-kDa lipoprotein) signal via TLR2 (34–36, 51). Other components of mycobacteria may also signal via TLR2 (52, 53). We now demonstrate that MTB 19-kDa can promote neutrophil activation by increasing cell surface expression of CD35 and CD11b/CD18 and decreasing expression of CD62L. In addition, MTB 19-kDa lipoprotein primes neutrophils for increased oxidative burst in response to fMLP, but does not induce oxidative burst by itself. In contrast to LPS and MTB 19-kDa lipoprotein, CpG ODN 2006 did not prime or activate neutrophils in any parameter. Thus, MTB 19-kDa lipoprotein produced several changes characteristic of neutrophil priming and activation that are seen with some (but not all) PAMPs.

Despite the functional similarity of priming/activation by MTB 19-kDa lipoprotein and LPS, these agents must use different signaling mechanisms. Modulation of the expression of cell surface receptors (CD11b/CD18 and CD62L) was serum dependent for LPS, but serum independent for MTB 19-kDa lipoprotein. This observation indicates that the effects of MTB 19-kDa lipoprotein in our experiments were not explained by contaminating LPS, and it shows that the effects of MTB 19-kDa lipoprotein did not involve the function of serum components that contribute to LPS function, e.g., LPS binding protein or soluble CD14. Furthermore, the finding that some donors responded to LPS, but not to MTB 19-kDa lipoprotein, also implies distinct steps in receptor signaling that differ between these agents.

Although the experiments presented here did not directly test the roles of different TLRs, our results suggest that both TLR2 and TLR4 can contribute to neutrophil priming. In other systems LPS is known to signal via TLR4 (16, 17, 47), and it is likely that the effects of LPS on neutrophil priming are mediated by TLR4. In contrast, studies in other systems have shown that MTB 19-kDa lipoprotein signals via TLR2 (34–36), and it is likely that the effects of this lipoprotein on neutrophil priming are also mediated via TLR2. CpG DNA signals via TLR9 in other systems (50), but failed to prime or activate neutrophils in our studies. Taken together, these studies suggest that neutrophil priming can result from signaling via TLR4 or TLR2, but not TLR9 (which may not be expressed by neutrophils).

We observed variation in the ability of neutrophils from different donors to respond to MTB 19-kDa lipoprotein. Although neutrophils from all donors were responsive to LPS, neutrophils from a significant subset (three of seven donors) were consistently unresponsive to MTB 19-kDa lipoprotein. The physiological basis of variation in responses to MTB 19-kDa lipoprotein is unclear, but one possibility is that nonresponsive cells may lack functional TLR2 or TLR6 (which has been shown to participate with TLR2) in responses to some PAMPs) (54, 55). The impact of this variation on host defense is unclear (all donors were healthy volunteers). Experimental tools to fully address these issues in human neutrophils are currently lacking, but future studies to characterize the expression and function of TLRs will clarify the mechanisms by which PAMPs mediate priming or activation of neutrophils.

It is interesting to speculate about the impact of variation in neutrophil responses to MTB 19-kDa lipoprotein between different donors. Cells from different individuals may have different sensitivities for responding to this particular PAMP. However, MTB may be recognized by multiple TLRs (e.g., TLR4 and TLR2) (35, 36, 52, 53) as well as other pattern recognition receptors (e.g., for polysaccharides or opsonins) and the redundancy of multiple recognition pathways may allow responses to MTB when one of these
pathways is deficient. Despite this redundancy, TLR2 and its ligands appear to play important roles in recognition of MTB (35, 36, 52, 53), and MTB 19-kDa lipoprotein appears to be very important for recognition of MTB by neutrophils (this paper) and other cells (34–36). These observations suggest that MTB 19-kDa lipoprotein plays an important role in neutrophil activation and other innate immune responses during infection with MTB.

MTB 19-kDa lipoprotein is expressed by MTB and some other slow-growing, pathogenic mycobacteria (e.g., M. bovis BCG and M. avium-intracellulare). It is not expressed by fast-growing non-pathogenic mycobacteria (e.g., M. smegmatis or M. vaccae) or other bacteria. Its gene encodes a protein of 159 aa, but a leader peptide of 21 aa is removed post-translationally to expose an N-terminal cysteine, which is then converted to N-acyl-S-diacylglycerol-cysteine. The function of MTB 19-kDa lipoprotein is unknown, but it serves as an Ag to elicit Ab and T cell responses in humans and mice. It also induces innate immune responses by macrophages (34, 56), dendritic cells (57), and neutrophils (this report), but after prolonged exposure may inhibit macrophage MHC-II expression and Ag processing (36).

MTB 19-kDa lipoprotein is primarily associated with the cell wall of MTB (58, 59), but is also released into the medium during culture of MTB in the absence of host cells (58). After infection of host cells, MTB bacilli continue to release the 19-kDa lipoprotein, which then localizes to many intracellular compartments in macrophages (59). Thus, the 19-kDa lipoprotein is released by MTB both in the absence of host cells and during the course of infection and intracellular persistence.

Our results show that MTB 19-kDa lipoprotein is a potent agent for modulation of neutrophil activation, with activity at concentrations of ~200–1600 ng/ml. For example, 500–1600 ng/ml MTB 19-kDa lipoprotein decreased the expression of CD62L and increased the expression of CD11b/CD18, whereas CD35 was increased by 240–760 ng/ml MTB 19-kDa lipoprotein. Thus, the lipoprotein was active at concentrations as low as 10−8 M and at mass concentrations within 10-fold of the minimum concentration of LPS required for neutrophil activation in these assays. These observations suggest that MTB 19-kDa lipoprotein, like LPS, could be present in concentrations sufficient to activate neutrophils in vivo under pathophysiological conditions.

It is difficult to determine the actual concentration of MTB 19-kDa lipoprotein achieved in vivo under pathophysiological conditions, but it is likely to accumulate to levels of significant bioactivity. MTB 19-kDa lipoprotein is a major component of the cell wall, and it is also shed at significant levels into the surrounding medium (58). Our results indicate that MTB 19-kDa lipoprotein can accumulate to >70 ng/ml in the medium of MTB cultures (data not shown). In confined tissue spaces and intracellular compartments (e.g., phagosomes), the local concentration of MTB 19-kDa lipoprotein is likely to be much higher. Thus, MTB 19-kDa lipoprotein is a highly expressed component of MTB that is shed at substantial levels into the surrounding medium. Furthermore, it is likely that TLR2 may recognize MTB 19-kDa lipoprotein in phagosomes, where it may be highly concentrated.

Several lines of evidence support a role for neutrophils in the immune response to MTB. First, neutrophils are recruited early to the lung of BCG- or MTB-infected mice, and depletion of neutrophils results in increased bacterial growth in mouse models. Second, in vitro studies suggest that human neutrophils are capable of inhibiting the growth of MTB. Third, neutrophils are readily detected in sputum and bronchoalveolar lavage fluid from humans with active pulmonary tuberculosis. Finally, our data demonstrate that MTB 19-kDa lipoprotein can promote neutrophil activation. In the course of their responses to MTB, neutrophils may function as microbicidal effector cells and also have regulatory functions via secretion of proinflammatory chemokines or cytokines. Activation of neutrophils by MTB 19-kDa lipoprotein may initiate both functions and thus allow neutrophils to contribute significantly to host responses to MTB.

### Acknowledgments

We thank Erica Wetzler and Steve Potter for help with neutrophil protocols, Scott Fulton for helpful discussion, and our blood donors for their contributions. CpG ODN were generously provided by Arthur Krieg (University of Iowa, Iowa City, IA, and Coley Pharmaceuticals, Wellesley, MA). We appreciate the guidance of Prof. Ulf B. Göbel (Humboldt Universität, Berlin, Germany).

### References


### Table I. Responses of neutrophils from different donors to MTB 19-kDa lipoprotein

<table>
<thead>
<tr>
<th>Stimulating substance</th>
<th>Increased CD35</th>
<th>Increased CD11b/CD18</th>
<th>Decreased CD62L</th>
<th>Increased DHR</th>
<th>Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19 kDa LPS</td>
<td>19 kDa LPS</td>
<td>19 kDa LPS</td>
<td>19 kDa LPS</td>
<td>19 kDa LPS</td>
</tr>
<tr>
<td>Donor 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Donor 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Donor 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Donor 4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Donor 5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Donor 6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Donor 7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Neutrophils from different donors were incubated with MTB 19-kDa lipoprotein or LPS (Stimulating substance) and then analyzed for expression of activation markers (Response).*


Downloaded from http://www.jimmunol.org/ by guest on October 27, 2017