Induction of a Novel Mechanism of Accelerated Bacterial Clearance by Lipopolysaccharide in CD14-Deficient and Toll-Like Receptor 4-Deficient Mice

Alain Haziot, Naoki Hijiya, Sophie C. Gangloff, Jack Silver and Sanna M. Goyert

*J Immunol* 2001; 166:1075-1078; doi: 10.4049/jimmunol.166.2.1075
http://www.jimmunol.org/content/166/2/1075

**References**
This article cites 28 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/166/2/1075.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
Induction of a Novel Mechanism of Accelerated Bacterial Clearance by Lipopolysaccharide in CD14-Deficient and Toll-Like Receptor 4-Deficient Mice

Alain Haziot, Naoki Hijiya, Sophie C. Gangloff, Jack Silver, and Sanna M. Goyert

Despite the lack of a proinflammatory response to LPS, CD14-deficient mice clear Gram-negative bacteria (Escherichia coli 0111) at least 10 times more efficiently than normal mice. In this study, we show that this is due to an early and intense recruitment of neutrophils following the injection of Gram-negative bacteria or LPS in CD14-deficient mice; in contrast, neutrophil infiltration is delayed by 24 h in normal mice. Similar results of early LPS-induced PMN infiltration and enhanced clearance of E. coli were seen in Toll-like receptor (TLR) 4-deficient mice. Furthermore, the lipid A moiety of LPS induced early neutrophil infiltration not only in CD14-deficient and TLR-4-deficient mice, but also in normal mice. In conclusion, the lipid A component of LPS stimulates a unique and critical pathway of innate immune responses that is independent of CD14 and TLR4 and results in early neutrophil infiltration and enhanced bacterial clearance. The Journal of Immunology, 2001, 166: 1075–1078.

I

n Gram-negative infection, the release of LPS, a component of the outer membrane of the bacteria (1), induces a wide range of inflammatory responses including the production of cytokines such as TNF-α and IL-1 (2, 3). The production of large amounts of these, as well as other proinflammatory mediators, is responsible for the development of endotoxic shock, a leading cause of mortality in septic patients (4). Most of these responses result from the interaction of LPS with CD14 (5, 6), a glycoprotein that is expressed as a glycosyl phosphatidylinositol surface-anchored molecule on monocytes, macrophages, and granulocytes (7, 8); CD14-deficient mice injected with a dose of LPS 10-fold higher than that required to kill control mice produce little or no cytokines (TNF, IL-1), display little or no symptoms of endotoxic shock (ruffled fur, etc.), and show 100% survival (6). Similarly, administration of a lethal dose of live Gram-negative bacteria (Escherichia coli 0111:B4) to CD14-deficient mice results in little or no production of proinflammatory cytokines and 100% survival. Surprisingly, despite this inability to respond to LPS and does not require the expression of Toll-like receptor (TLR)3 4, a signaling molecule that is required for most other responses to LPS (10–13) and that this response can be induced in normal CD14-expressing or TLR4-expressing mice using a derivative of LPS, monophosphoryl lipid A (MPLA).

Materials and Methods

Animals

Mouse strains used in these studies include CD14-deficient (6) of C57BL/6J or BALB/c genetic background (sixth backcross), age- and weight-matched control C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) or BALB/c (Harlan Sprague Dawley, Indianapolis, IN), 12- wk-old C57BL/10ScN (Harlan Sprague Dawley), and C57BL/10SnJ (The Jackson Laboratory). Hamsters (Chinese, obtained from Cytogen Research and Development, Roxbury, MA). All animals were maintained and studied in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences) and the North Shore University Hospital Institutional Animal Care and Use Committee.

Neutrophil infiltration assay

Mice were injected i.p. with either E. coli O111:B4 (1 × 107 CFU) (6), protein-depleted LPS (500 ng/gbw) from E. coli K235, highly purified and free of contaminating protein (14, 15), Re-LPS (180 ng/gbw) from Salmonella minnesota R595 (Sigma, St. Louis, MO) dissolved in nonpyrogenic water (Allegiance, McGaw Park, IL) and added to 0.2 ml PBS (Life Technologies, Gaithersburg, MD), and MPLA (180 ng/gbw) from Salmonella minnesota R595 (List Biological Laboratories, Campbell, CA) dissolved in 0.5% triethylamine in nonpyrogenic water and added to 0.2 ml PBS (Life Technologies) or 0.2 ml PBS alone (<0.03 endotoxin units/ml). After 6 h, the mice were sacrificed by CO2 inhalation and the peritoneal cavity was washed with 10 ml RPMI 1640 (Life Technologies) supplemented with 10 mM HEPES and 1% FBS (Intergen, Purchase, NY). The total number of cells in the lavage fluid was counted and the percentage of neutrophils was determined by morphological analysis of Wright-Giemsa-stained cyto- spins. Hamsters were injected with PBS, MPLA, or LPS as described for mice and analyzed as described above.

Neutropenic mouse model

CD14-deficient and control BALB/c mice were injected s.c. with cyclophosphamide (Mead Johnson, Princeton, NJ) at the doses of 250 mg/kg on day 0 and 100 mg/kg on day 3 (16) or vehicle alone (mannitol; Abbott Laboratories, North Chicago, IL). This procedure produced neutropenia

Division of Molecular Medicine, North Shore University Hospital/New York University School of Medicine, Manhasset, NY 11030

Received for publication July 6, 2000. Accepted for publication October 16, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a grant from the National Institutes of Health (to S.M.G.) and by a grant-in-aid from the American Heart Association (to A.H.).

2 Address correspondence and reprint requests to Dr. Sanna M. Goyert, North Shore University Hospital/New York University School of Medicine, 350 Community Drive, Manhasset, NY 11030. E-mail address: sannag@aol.com

Copyright © 2001 by The American Association of Immunologists

0022-1767/01/$02.00

0222-1767/01/S02.00
Infiltration of PMN induced by Gram-negative bacteria and LPS in CD14-deficient mice

To identify the mechanism that might be responsible for the enhanced clearance of Gram-negative bacteria by CD14-deficient mice, the number and types of leukocytes in the peritoneal cavity was measured at various time points. CD14-deficient mice rapidly responded to LPS at early time points with a peak PMN response 6 h after injection, whereas control mice had no detectable neutrophils at this time point (Fig. 1). Microscopic analysis of the cells harvested from CD14-deficient mice showed bacteria attached to and/or phagocytosed by PMN (data not shown).

To determine whether LPS might be the bacterial component that elicits this early PMN influx in CD14-deficient mice, mice were injected i.p. with LPS (0.5 μg/gbw) (a gift from S. Vogel, Uniformed Services University of the Health Sciences, Bethesda, MD) that was highly purified and depleted of protein, as described previously (14, 15), and the number of neutrophils recruited to the peritoneal cavity was measured at various time points. CD14-deficient mice rapidly responded to LPS at early time points with a peak PMN response 6 h after injection, whereas control mice had no detectable neutrophils at this time point (Fig. 1b); indeed, the response of control mice did not become appreciable until more than 24 h after injection of LPS (Fig. 1b). The neutrophil response of CD14-deficient mice to LPS was extremely sensitive; neutrophil recruitment was strongly induced with a dose of LPS as low as 0.5 ng/gbw (Fig. 1c). Depletion of the LPS preparation of its LPS content on a polymyxin B agarose column as previously described (17) abrogated the ability of the preparation to induce neutrophil infiltration (data not shown), demonstrating that LPS is required for this response. In addition, early neutrophil infiltration could be induced with synthetic LPS-like molecules including a synthetic diphosphoryl lipid A (ICN Pharmaceuticals, Costa Mesa, CA) (data not shown), further confirming that LPS itself, and not a contaminant, is responsible for the response. Similar differences in the number of PMN in the blood of CD14-deficient and control mice could not be reliably detected (data not shown). These experiments demonstrate that the infiltration of neutrophils is specifically elicited by very low doses of LPS via a CD14-independent pathway. Moreover, the presence of CD14, as found in normal mice, strongly delays LPS-induced neutrophil influx.

Role of PMN in the enhanced clearance of Gram-negative bacteria of CD14-deficient mice

Since neutrophils represent the first line of cellular defense in the elimination of bacteria, we sought to determine whether the early influx of neutrophils in CD14-deficient mice was responsible for the improved clearance of Gram-negative bacteria. Accordingly, the ability of neutropenic CD14-deficient animals to clear the bacteria was examined.

Cyclophosphamide-induced neutropenic mice (CD14-deficient and control BALB/c) were infected with 3 × 10^7 E. coli O111:B4 bacteria and 8 h later bacterial counts in the blood were measured. As shown in Fig. 2, whereas CD14-deficient mice pretreated with saline showed 10-fold fewer bacteria than similarly treated control mice (p < 0.0411, Mann-Whitney U test), the bacterial load in the blood of neutropenic mice was similar in normal and CD14-deficient mice. Thus, depletion of neutrophils abrogates the improved clearance observed in CD14-deficient mice. These studies indicate that PMN are required for the enhanced clearance in CD14-deficient mice. Thus, LPS stimulates a CD14-independent pathway leading to rapid neutrophil infiltration, and it is this rapid infiltration of PMN that is responsible for the enhanced bacterial clearance in CD14-deficient mice.

Role of TLR4 in the influx of PMN induced by LPS

Recently, it has been shown that the vertebrate homologue of a Drosophila Toll receptor protein, TLR4, is required for many responses to LPS, such as the production of inflammatory cytokines and the proliferation of B lymphocytes (10–13). To determine the role of TLR4 in the neutrophil infiltration induced by LPS, we tested the response of LPS-resistant C57BL/10ScN mice (B10ScN) that are deleted in Tlr4 (12, 13). As shown in Fig. 3a,
these TLR4-deficient mice display strong infiltration of neutrophils in the peritoneal cavity after injection of LPS in contrast to control C57BL/10SnJ mice (B10SnJ). Therefore, the expression of TLR4 is not required for the activation of the LPS pathway that leads to the early recruitment of neutrophils.

To determine whether the early neutrophil influx induced by LPS in the TLR4-deficient mice also enhances bacterial clearance, B10ScN and control mice were infected i.p. with E. coli O111:B4 (4 × 10^7 CFU) and bacterial counts in the blood were determined 7 h later. As shown in Fig. 3b, TLR4-deficient B10ScN mice had 14-fold fewer bacteria in the blood than control mice (p < 0.0357, Mann-Whitney U test). This indicates that, similar to CD14-deficient mice, the early neutrophil infiltration induced by LPS in TLR4-deficient mice is associated with an improved capacity to clear Gram-negative bacteria.

**Identification of substructures of LPS capable of inducing early neutrophil infiltration in normal mice**

To determine whether other forms of LPS can also induce early neutrophil infiltration, truncated forms of LPS were tested in both CD14-deficient and normal mice. Both Re-LPS and MPLA induced a rapid neutrophil influx in CD14-deficient mice (Fig. 4) and in TLR4-deficient B10ScN mice (data not shown). Surprisingly, MPLA, was also able to induce a strong rapid infiltration of neutrophils in normal mice 6 h after injection (Fig. 4); this response peaked as early as 2 h after injection (data not shown). Indeed, doses of MPLA as low as 1.8 ng/gbw were able to elicit this response (data not shown). However, Re-LPS, a truncated form of LPS lacking most of the polysaccharide chains, did not induce this response in normal mice (Fig. 4).

**Role of TLR2 in the influx of PMN induced by LPS or MPLA**

Studies of TLR2 suggested that it may also serve as a receptor for LPS (18, 19). To determine whether expression of TLR2 influences the early neutrophil infiltration induced by LPS and/or MPLA hamsters, previously shown to carry a null allele for TLR2 (20), were tested. As shown in Fig. 5, injection of MPLA induces a strong neutrophil infiltration to the peritoneal cavity of hamsters which lack functional TLR2 molecules 2 h after administration, whereas no infiltration is seen following administration of LPS. These studies show that TLR2 does not influence the PMN infiltration response to MPLA or LPS.

**Discussion**

We have previously shown that mice deficient in CD14, a high-affinity receptor for LPS, can clear E. coli more efficiently than control mice (6). In this study, we show that in the absence of CD14, injection of LPS induces an early infiltration of neutrophils which peaks at 6 h. In contrast, neutrophil infiltration is delayed in control mice for 18–24 h. This early infiltration appears to be responsible for the enhanced bacterial clearance displayed by CD14-deficient mice as suggested by the experiments with cyclophosphamide-induced neutropenia. This pathway can be activated by LPS and other molecules of similar structure and does not require TLR4 as evidenced by the fact that mice deficient in TLR4 also display early PMN infiltration and enhanced bacterial clearance (Fig. 3, a and b). Indeed, the interaction of LPS with CD14 and subsequent transduction of a signal through TLR4 interferes with the rapid neutrophil infiltration and bacterial clearance induced by this pathway. This is consistent with previous studies demonstrating that pretreatment of rabbits with LPS delays the clearance of E. coli (21). However, it is not consistent with previous studies showing that C3H/HeJ mice, which have a defective Tlr4 gene (12, 13), are more susceptible to infection with E. coli than control mice (22). This discrepancy, however, can be readily explained by the fact that those studies used a strain of E. coli that was resistant to neutrophils. Under these circumstances, the activation of mechanisms that require other factors, such as inflammatory cytokines, may be necessary to eliminate neutrophil-resistant bacteria (23).
Although in normal mice the induction of this novel pathway of enhanced bacterial clearance is inhibited by the interaction of LPS with CD14, some truncated forms of LPS, such as MPLA, are able to trigger this pathway without triggering the inhibitory pathway. Previous studies have shown that the concentration of MPLA needed to elicit a response via the CD14/TLR-4 pathway is 2-3 logs higher than with LPS (24, 25). Thus, MPLA is an extremely weak stimulator of macrophages and PMN via the CD14 pathway; in contrast, it appears to be a potent stimulatory of the alternative pathway that elicits neutrophil infiltration in normal mice even in the presence of CD14 and TLR4 (Fig. 4), presumably acting through the same novel, non-CD14, non-TLR4, pathway as LPS. It should be noted that our model of MPLA treatment is distinct from that of MPLA-induced tolerance (26) where mice must be given, through the same novel, non-CD14, non-TLR4, pathway as LPS. It appears that models of MPLA-induced treatment are precluded a need to invoke a compensatory model for deleted or defective genes in CD14-deficient or TLR4-mutated mice and suggests that MPLA stimulates this pathway, which leads to enhanced bacterial clearance, without stimulating the inhibitory pathway which requires CD14 and TLR4.

We have also found that this pathway functions independently of TLR2, a signaling molecule proposed to account for some cellular responses to LPS (18, 19), since hamsters deficient in TLR2 (20) respond to MPLA in a manner similar to that of normal mice, i.e., administration of MPLA induces an early infiltration of PMN whereas LPS inhibits early infiltration. Furthermore, MPLA induced a normal influx of PMN in C5-deficient mice, in mice depleted of C3, and in mice treated with aprotinin, a protease inhibitor which blocks the intrinsic and extrinsic coagulation systems (27, 28) (data not shown), indicating that this pathway functions independently of both the complement and coagulation cascades.

In conclusion, these studies reveal a novel pathway for the induction of neutrophil infiltration that plays an important role in controlling dissemination and clearance of Gram-negative bacteria. The ability of MPLA and/or other LPS analogues to induce early neutrophil infiltration without activating the CD14/TLR4 inhibitory pathway may provide an important new method for controlling bacterial dissemination.

References

duced dissemination of Gram-negative bacteria in CD14-deficient mice. Immu-
nity 4:407.
cacies of amphotericin B, fluconazole, and itraconazole against a systemic Can-
dida albicans infection in normal and neutropenic mice. Antimicrob. Agents Che-
mother. 33:147.
ment with recombinant murine tumor necrosis factor α/cachectin and murine interleukin 1α protects mice from lethal bacterial infection. J. Exp. Med. 169:2021.
mediated host defense mechanism in mice against extraintestinally invasive Esch-
24. Kovach, N. L., E. Yee, R. S. Munford, C. R. Raetz, and J. M. Harlan. 1990. Lipid IVa inhibits synthesis and release of tumor necrosis factor induced by lipopoly-