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*J Immunol* 2001; 166:1075-1078; doi: 10.4049/jimmunol.166.2.1075

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

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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.

In 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 glycolipid phosphatidylinositol 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-α, 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 E. coli 0111:B4, CD14-deficient mice display a markedly accelerated clearance of the bacteria from the blood and tissues (6). In these mice, the bacterial load is dramatically reduced (>25-fold) as early as 6 h after the infection. A chronic model of abscess formation following infection with Bacteroides fragilis also shows enhanced clearance of bacteria from the blood of CD14-deficient mice as compared with control mice (9).

The studies described show that this accelerated clearance of Gram-negative bacteria in CD14-deficient mice is accompanied by a rapid infiltration of neutrophils that is normally delayed in CD14-expressing mice. In addition, we show that this response to LPS does not require the expression of Toll-like receptor (TLR) 3, 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 (Integen, 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 cytospins. 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

3 Abbreviations used in this paper: TLR, Toll-like receptor; MPLA, monophosphoryl lipid A; gbw, gram body weight.
The peritoneal cavity, 2 h after infection (3.2). Although mice normally have few, if any, neutrophils in the peritoneal cavity at early time points (2.5–5.5 h) than control mice (ANOVA) higher numbers of neutrophils (PMN) 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 $3 \times 10^7$ 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 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,
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 (\( 4 \times 10^7 \) CFU) and bacterial counts in the blood were determined 7 h later. As shown in Fig. 3b, TLR4-deficient B10ScN mice had a 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 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).

![FIGURE 2. Clearance of \( E. coli \) in neutrophil-depleted mice. Mice were treated with vehicle (pyrogen-free mannitol in saline) (CD14-deficient, \( n = 6 \); control BALB/c, \( n = 5 \)) or cyclophosphamide (CD14-deficient, \( n = 4 \); control BALB/c, \( n = 6 \)) as described (16), followed by i.p. injection with \( 3 \times 10^7 \) CFU \( E. coli \) O111:B4 (6). Eight hours later, bacterial counts in the blood were determined (6). * \( p < 0.0411 \) (Mann-Whitney U test). Results are presented as mean ± SEM and are representative of two independent experiments. When error bars are not seen, they fall within the symbol.

![FIGURE 3. Infiltration of neutrophils in the peritoneal cavity of TLR4-deficient mice after injection of LPS and resistance to infection with \( E. coli \). a, C57Bl10ScN (TLR4 deficient, \( n = 3 \)) and control C57Bl10SnJ mice (\( n = 3 \)) were injected i.p. with 0.5 \( \mu g/gbw \) of protein-free LPS from \( E. coli \) K235 and the cellularity in the peritoneal cavity was analyzed after a 6-h incubation. b, C57Bl10ScN (TLR4 deficient, \( n = 5 \)) and control C57Bl10SnJ mice (\( n = 3 \)) were injected i.p. with \( 1.3 \times 10^7 \) CFU/gbw \( E. coli \) O111:B4 and the bacterial counts in the blood were determined 7 h later. * \( p < 0.0357 \) (Mann-Whitney U test). Results are presented as mean ± SEM and are representative of two independent experiments. When error bars are not seen, they fall within the symbol.

![FIGURE 4. Infiltration of neutrophils induced by lipid A. CD14-deficient and control C57Bl10Jd mice (\( n = 3 \)) were injected i.p. with smooth LPS (\( E. coli \) ) (500 ng/gbw), rough Re-LPS (\( Salmonella \) minnesota R595) (180 ng/gbw), or MPLA (180 ng/gbw), a hydrolysate product of LPS isolated from \( Salmonella \) minnesota R595, in 0.2 ml of nonpyrogenic saline. The cellularity in the peritoneal cavity was analyzed after 6 h. Results are presented as mean ± SEM and are representative of two independent experiments. When error bars are not seen, they fall within the symbol.
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/TLR4 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 stimulator 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, at least 2 days before infection, doses of MPLA that are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the LPS product.

**FIGURE 5.** PMN infiltration induced by MPLA or LPS in Chinese hamsters carrying a nonfunctional Tlr2 gene. Chinese hamsters were injected i.p. with MPLA (0.18 μg/gbw; n = 3), LPS (n = 3), or PBS (n = 3). The peritoneal cavity was lavaged 2 h later and the number of PMN present in the lavage fluid was determined. Results are presented as mean ± SEM and are representative of two independent experiments.

References


