Enhancement of Macrophage Stimulation by Lipoteichoic Acid and the Costimulant Hemoglobin Is Dependent on Toll-Like Receptors 2 and 4\textsuperscript{7}

Kathleen H. Cox\textsuperscript{1}, Itzhak Ofek\textsuperscript{2}, and David L. Hasty\textsuperscript{1,3}\textasteriskcentered*

\textit{Department of Anatomy and Neurobiology, University of Tennessee Health Science Center, 800 Madison Avenue, Memphis, Tennessee 38163\textsuperscript{3}; Department of Human Microbiology, Tel Aviv University, Tel Aviv 69978, Israel\textsuperscript{2}; and Research Service (151), Department of Veterans Affairs Medical Center, 1030 Jefferson Avenue, Memphis, Tennessee 38104\textsuperscript{4}}

Received 16 August 2006/Returned for modification 14 November 2006/Accepted 2 February 2007

Macrophage stimulation by lipoteichoic acid (LTA) and hemoglobin (Hb) requires Toll-like receptors 2 and 4 (TLR2 and -4). There are two distinct temporal phases of interleukin-6 (IL-6) production. The first results in a slight enhancement of IL-6 secretion in response to LTA plus Hb compared to that with LTA alone and is TLR4 independent. The second requires TLR4 and accounts for most of the additional stimulation seen with LTA plus Hb.

It has been argued that lipoteichoic acid (LTA) is the gram-positive counterpart of lipopolysaccharide (LPS) in inducing host responses to infection, including septic shock (6). This concept is based in part on the fact that both molecules stimulate macrophages to secrete proinflammatory cytokines, albeit via different macrophage receptors and signaling pathways (1). It is generally accepted that most kinds of LPS act exclusively through Toll-like receptor 4 (TLR4) (3, 13) and that LTAs activate macrophages through TLR2 (11, 14, 15, 21). Recently, we showed that stimulation of human monocytic and murine macrophages by LTA is markedly enhanced by a costimulatory molecule, namely, hemoglobin (Hb) (7). Hb appeared to form complexes with LTA, which may be responsible for the enhanced activity. In the present study, we found that the enhanced cytokine secretion obtained when macrophages are incubated with LTA-Hb mixtures can be divided into two temporal phases which are dependent upon not only TLR2 but also TLR4.

Wild-type TLR4 (HeNC2) and TLR4 mutant (GG2EE) macrophage cell lines, derived from C3H/HeN and C3H/HeJ mice, respectively (4, 8), were used in most of the studies presented here. For incubation with test substances, 2.5 \times 10^5 cells were seeded into 24-well plates in macrophage SFM medium (Invitrogen Life Technologies), and after 2 h, the medium was replaced with fresh medium containing test substances at the concentrations indicated. After incubation at 37°C in a humidified 5% CO\textsubscript{2} incubator for the times indicated in the figures, medium samples were removed from triplicate wells and centrifuged to remove detached cells or cellular debris, and supernatants were stored at -80°C until assay by enzyme-linked immunosorbent assay (ELISA) (for interleukin-6 [IL-6] and tumor necrosis factor alpha [TNF-\alpha]; eBioscience). In some experiments, resident peritoneal macrophages from TLR4 mutant (C3H/HeJ) and TLR2\textsuperscript{-/-} (B6.129-\textsuperscript{Thr2m1Kir/J}; Jackson Laboratory) mice, along with their appropriate wild-type controls (cells from C3H/HeOuJ and C57BL/6J mice, respectively), were used as described previously (7). LTA was purified as described previously (7), and Hb was purchased from Sigma Chemical Co. (St. Louis, MO). The level of endotoxin was determined using the OCL-1000 quantitative chromogenic Limulus amoebocyte lysate assay according to the manufacturer’s directions (Bio-Whittaker). In our experiments with LTA, we ruled out the possibility that trace contamination with endotoxin accounted for the responses observed in several different ways. First, the concentrations of LPS detected in our LTA preparations were <4 pg/ml in the test solutions. LPS at such concentrations did not cause secretion of IL-6, with or without the addition of Hb (data not shown). Secondly, polymyxin B (5 \mu g/ml), a known inhibitor of LPS activity, was added to all stimulation assays, except for the wells testing LPS. All experiments contained unstimulated and Hb-only controls, but since IL-6 was never produced at detectable levels (>10 pg/ml) under these conditions in any of our experiments, these data are not included in the figures.

LTA induced secretion of IL-6 from the wild-type, i.e., HeNC2, macrophage cell line, and this response was potentiated >4-fold by Hb (Fig. 1). As expected, the TLR4 mutant macrophage cell line GG2EE, in which all other TLRs are functional, secreted similar levels of IL-6 when induced by LTA. Surprisingly, this macrophage cell line lacking functional TLR4 could not respond to stimulation by LTA-Hb to the same level as that in wild-type cells (i.e., enhancement was <1.5-fold). The level of alanylation of LTA does not play a role here, since macrophages responded similarly to D-Ala-rich (LTA) and D-Ala-poor (dLTA) LTAs from wild-type and \textit{dtA} mutant \textit{Streptococcus pyogenes}, respectively (7). Furthermore, Hb increased the response to LPS in wild-type macrophages (<2-fold), as shown previously. However, neither LPS nor LPS-Hb mixtures could induce macrophages with mutant TLR4.

To confirm the results obtained with the macrophage cell lines, we tested resident peritoneal macrophages from C3H/
HeOuJ (i.e., wild-type TLR4) and C3H/HeJ (i.e., TLR4 mutant) mice. The results show that there was little enhancement of stimulation by LTA-Hb mixtures in TLR4 mutant macrophages. This is consistent with the data in Fig. 1 and demonstrates that the dependence upon TLR4 for potentiation of cytokine induction by LTA and Hb is not restricted to the macrophage cell line (Fig. 2A). While the response trends were the same between cell lines and resident peritoneal macrophages, there was a notable difference in the level of responses to the various stimuli. At this time, the reason for this quantitative variation is unknown.

To determine the role of TLR2 in responding to stimulation by LTA and Hb, resident peritoneal macrophages from TLR2-deficient mice and control mice were stimulated by LTA, with or without Hb. Stimulation by LTA alone was TLR2 dependent (Fig. 2B), consistent with data from other laboratories (3, 15). Stimulation with LTA-Hb mixtures demonstrated that while this response is largely dependent on TLR2, a low level (≤10%) is TLR2 independent. The requirement for TLR2 in this signaling pathway was further confirmed in experiments employing an inhibitory antibody against TLR2 (TLR2.5; Bio- science). Figure 3 shows that this antibody inhibited stimulation by LTA completely and stimulation by LTA-Hb mixtures by 85% compared to an isotype-matched control (IgG control) for 1 h prior to adding LTA (2 µg/ml), with or without Hb (50 µg/ml). Antibodies were added to a final concentration of 6.7 µg/ml. Results are means ± SD (n = 3).

TLR2 and TLR4 have never been linked in a response to LTA induction. To begin to define how these receptors are stimulated, we examined the temporal responses to LTA and LTA-Hb in macrophage cell lines expressing either wild-type or mutant TLR4. The data in Fig. 4 show that the response to LTA-Hb can be divided into two phases. Detectable IL-6 secretion was first observed 2 hours after exposure of macrophages to LTA or LTA-Hb (data not shown). Until 6 h post-stimulation, the response to LTA and Hb is TLR4 independent (Fig. 4A) and that results in a sevenfold enhancement of IL-6 secretion when Hb is added as a costimulant with LTA at the 20-h time point (Fig. 4A). Secretion of TNF-α follows a similar temporal pattern (Fig. 4B), although the difference between wild-type cells and TLR4 mutant cells was not as great at the 20-h time point.

FIG. 1. Effects of Hb on LTA- and LPS-induced production of IL-6 from wild-type (WT) and TLR4 mutant macrophage cell lines. Cell lines were exposed to LTA (2 µg/ml), dLTA (2 µg/ml), or LPS (10 ng/ml) overnight in the presence and absence of Hb (50 µg/ml), and IL-6 levels secreted into the culture medium were measured by ELISA. The data are means ± standard deviations (SD) (n = 3).

FIG. 2. Responses of resident peritoneal macrophages from inbred mouse strains to LTA and LTA-Hb mixtures. Macrophages were exposed to LTA (2 µg/ml) in the presence or absence of Hb (50 µg/ml) overnight, and IL-6 levels secreted into the culture medium were measured by ELISA. The data are means ± SD (n = 3). (A) C3H/HeOJ (wild type [WT]) and C3H/HeJ (TLR4–) cells. (B) C57BL/6J (wild type [WT]) and B6.129-Trtm1Kir/J (TLR2–/–) cells.

FIG. 3. Requirement of TLR2 and TLR4 for LTA and LTA-Hb activation. HeNC2 (TLR2+/+ TLR4+) and GG2EE (TLR2+/+ TLR4–) macrophages were incubated in the presence or absence of the TLR2 inhibitory antibody TLR2.5 (eBioscience) or with an isotype-matched control (IgG control) for 1 h prior to adding LTA (2 µg/ml), with or without Hb (50 µg/ml). Antibodies were added to a final concentration of 6.7 µg/ml. Results are means ± SD (n = 3).
The results in this paper show for the first time that there is a requirement for both TLR2 and TLR4 in the innate immune response to LTA when it is presented to macrophages along with the endogenous host protein Hb. The late requirement for TLR4, several hours after a TLR2-mediated response is initiated, suggests that this signaling pathway requires a second ligand. The nature of this second ligand is unknown, but the experiments in this study suggest that generation by, modified, or some type of interaction with a product(s) from the initial TLR4-independent reaction may be required, as suggested by Paul-Clark et al. (12). They showed that after TLR2 activation by gram-positive bacteria, expression of TNF-α was required for subsequent release of nitric oxide. An alternative explanation is that LTA-Hb mixtures contain or generate a ligand for a TLR2/TLR4 heterodimer. The potential for dimerization between these TLR family members was demonstrated by Lee et al. (10), but to date, no ligand for a TLR2/TLR4 dimer has been identified.

The experiments described here have examined LTA-macrophage interactions in vitro. For the response to occur in vivo, LTA would have to come into contact with free Hb (or methemoglobin) within the host. Free Hb and heme are normally found at low levels except during pathological conditions that result in hemolysis (5). Streptococcus pyogenes secretes two potent hemolysins, streptolysin O and streptolysin S (2). Although streptolysin O can function independent of streptolysin S requires a carrier to stabilize it in a functional form (19). One of the carriers identified by previous investigators is TLR4 (18). Such an LTA-streptolysin S complex may place LTA in a position to be exposed to microenvironments rich in Hb before other host components, such as haptoglobin, can sequester it.

It is not clear how LTA may be involved in gram-positive shock, but a number of studies accumulated over the last decade suggest that LTA is capable of inducing a strong cytokine response in combination with one or more host-derived or bacterial components. Indeed, peptidoglycan and LTA were shown to induce multiple organ failure and shock in experimental animals (9). Moreover, Hb was shown to markedly enhance the ability of LPS to cause mortality in rabbits (20) and mice (16, 17). The potentiation of the inflammatory activity of LTA by Hb or other macromolecules of either host or bacterial origin that are present as a result of host cell lysis, polymicrobial infections, or other pathogenic states could be the difference between the triggering of a protective innate immune response and the induction of tissue injury and shock. A thorough understanding of the interaction of LTA and co-stimulatory molecules may eventually help to explain how LTA contributes to shock or other serious sequelae of gram-positive infections.

We acknowledge the technical excellence and dedication of Susan Price. We thank Fabio Re for providing the macrophage-like cell lines and Elaine Tuomanen for providing TLR2-/- cells. We thank H. S. Courtney, J. B. Dale, F. Re, and B. K. English for carefully reading the manuscript and providing helpful suggestions.

This work was supported by research funds from the U.S. Department of Veterans Affairs.
binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J. Biol. Chem. 278:15587–15594.


Editor: V. J. DiRita