

# Activation of the Adhesive Capacity of CR3 on Neutrophils by Endotoxin: Dependence on Lipopolysaccharide Binding Protein and CD14

By Samuel D. Wright, Robert A. Ramos,  
Anne Hermanowski-Vosatka, Pamela Rockwell,  
and Patricia A. Detmers

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*From the Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021*

## Summary

Tumor necrosis factor  $\alpha$ , granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, and formyl peptide were each found to cause a twofold increase in expression of CD14 on the surface of polymorphonuclear leukocytes (PMN). Upregulation of CD14 was complete by 20 min and thus appeared to result from expression of preformed stores of protein. The CD14 on the surface of PMN was shown to serve two biological functions. It bound particles coated with complexes of lipopolysaccharide (LPS) and LPS binding protein (LBP). This binding activity was enhanced by agonists that upregulated CD14 expression and may serve in the clearance of Gram-negative bacteria opsonized with LBP. Interaction of CD14 with LPS in the presence of LBP or serum also caused a dramatic, transient increase in the adhesive activity of CR3 (CD11b/CD18) on PMN. Enhanced activity of CR3 and other members of the CD11/CD18 family underlies many of the known physiological responses of PMN to LPS and may be a central feature of the *in vivo* responses of PMN to endotoxin.

**B**acterial LPS (endotoxin) is known to have profound physiological effects on PMN both *in vivo* and *in vitro*. Animals respond to intravenous LPS with a rapid fall in the number of circulating PMN and a concomitant accumulation of PMN in the lungs (1). Isolated PMN respond to LPS with increased adhesion to protein-coated surfaces (2), enhanced ability to mount an oxidative burst (3), and enhanced microbicidal powers (4). Many of these responses to LPS appear to depend at least in part on enhanced function of the three members of the CD11/CD18 family of adhesion-promoting receptors (LFA-1, CR3, and p150,95, also known as the  $\beta_2$  or leukocyte integrins) on the surface of PMN. Adhesion of PMN to endothelium requires the participation of CD11/CD18 molecules (5–7), and blockade of CD18 with mAbs prevents accumulation of PMN in the lungs of endotoxin-treated animals (8). Adhesion of PMN to protein-coated glass (5), enhanced oxidative burst in response to soluble agonists (9), and microbicidal activity (5) are also dependent on increased CD11/CD18 function. Here, we directly measure the effect of LPS on the adhesive function of CR3 (CD11b/CD18) and describe a dramatic enhancement of its adhesive activity by complexes of LPS with proteins from the serum.

Recent studies have described a novel mechanism by which mononuclear cells may respond to LPS (10, 11). LPS is first

bound by the serum protein LPS binding protein (LBP)<sup>1</sup>, and the resulting LPS-LBP complex is then recognized by CD14, a 55-kD glycoprotein that is strongly expressed on monocytes and macrophages. LBP and CD14 serve two physiological roles. These proteins act as opsonin and opsonic receptor, respectively, to promote the phagocytic uptake of bacteria or LPS-coated particles by macrophages (12). They also dramatically enhance the ability of mononuclear cells to synthesize TNF in response to endotoxin. Addition of LBP speeds the synthesis of TNF and enables a response to doses of LPS 100-fold lower than are otherwise required (10). Blockade of CD14 with mAbs prevents the synthesis of TNF in response to ng/ml concentrations of LPS by monocytes in the presence of plasma (11).

We have asked whether CD14 and LBP also participate in the physiological responses of PMN to LPS. Though these cells express  $\sim 10$ -fold less CD14 than monocytes, we find that the CD14 on PMN is capable of mediating the binding of LPS-coated erythrocytes (ELPS) opsonized with LBP. We

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<sup>1</sup> *Abbreviations used in this paper:* ELPS, LPS-coated erythrocytes; fNLLP, formyl-norleucyl-leucyl-phenylalanine; G-CSF, granulocyte CSF; GM-CSF, granulocyte/macrophage CSF; LPB, LPS binding protein; NIM, neutrophil isolation medium.

also find that PMN contain an intracellular pool of CD14 that is rapidly upregulated upon stimulation with TNF- $\alpha$ , granulocyte CSF (G-CSF), granulocyte/macrophage CSF (GM-CSF), and formyl peptide, and that the newly expressed CD14 serves in recognition of particles opsonized with LPS-LBP complexes. In addition, LBP and CD14 appear to play a critical role in responses of PMN to LPS, since the enhancement of CR3 activity in response to LPS requires serum or the serum protein LBP, and is completely blocked by antibodies against CD14.

## Materials and Methods

**Reagents.** The recombinant human cytokines TNF- $\alpha$  (Genentech, So. San Francisco, CA), GM-CSF (Amgen, Thousand Oaks, CA), and G-CSF (Amgen) were gifts from the manufacturers. Formyl-norleucyl-leucyl-phenylalanine (fNLLP) and aprotinin were purchased from Sigma Chemical Co. (St. Louis, MO), and pyrogen-free human serum albumin was from Armour Pharmaceuticals (Kankakee, IL). LPS (Re 595) was from List Biochemicals (Campbell, CA).

Human LBP was purified from fresh frozen normal human plasma by published methods (13), and rabbit LBP was a gift from Dr. R. Ulevitch (La Jolla, CA). mAbs 3C10 (14) and 26ic (15) directed against CD14, and W6/32 (16) directed against HLA, were purified to homogeneity by chromatography on protein G.

**Cells.** PMN for rosetting experiments were isolated from normal human blood on Ficoll gradients (17). PMN for flow cytometry were isolated using neutrophil isolation medium (NIM; Los Alamos Diagnostics, Los Alamos, NM). 8 ml of whole heparinized blood was layered on 4 ml of NIM and centrifuged 30 min at 400 g at ambient temperature. The layer containing PMN was removed to a separate tube and contaminating erythrocytes were removed by hypotonic lysis. PMN isolated by either method were suspended to  $2 \times 10^6$ /ml in Dulbecco's PBS containing 0.5 mg/ml HSA, 0.3 U/ml aprotinin, and 3 mM glucose (HAP buffer) and were used within 1 h of isolation.

Sheep erythrocytes were coated with LPS or C3bi to yield ELPS or EC3bi by methods previously described (12, 18). The dose of LPS used to prepare the ELPS was  $1 \mu\text{g}/4 \times 10^7$  erythrocytes.

**Assays.** To measure the binding of erythrocytes coated with LPS-LBP complexes to human PMN, monolayers of cells were formed by adding  $10^4$  PMN to Terasaki culture wells and incubating 30 min at 37°C. Cells were washed with PBS containing 1 mM EDTA and incubated with  $5 \times 10^5$  ELPS and 1  $\mu\text{g}/\text{ml}$  LBP for 15 min at 0°C, then warmed to 21°C for 15 min. Removal of divalent cations eliminates contributions of CD18 molecules to the binding of ELPS (18) but does not affect binding of LPS-LBP complexes to macrophages (12). Cultures were inverted for 20 min to allow gravity to gently remove nonadherent erythrocytes from the PMN. Attachment of erythrocytes was scored by phase contrast microscopy and data are presented as attachment index, the number of erythrocytes bound per 100 PMN.

The adhesive activity of CR3 was measured by adding  $5 \times 10^5$  EC3bi to monolayers of PMN and incubating for 30 min at 37°C. Nonadherent EC3bi were removed by vigorous washing, and the attachment index was enumerated as above.

**Flow Cytometry.** Isolated cells were exposed to mAb (5  $\mu\text{g}/\text{ml}$ ) for 15 min at 0°C, washed, and incubated with fluoresceinated affinity-purified goat anti-mouse IgG F(ab')<sub>2</sub> (7  $\mu\text{g}/\text{ml}$ ; Tago Inc., Burlingame, CA) for 45 min at 0°C. Fluorescence was measured on a FACScan® cell analyzer (Becton Dickinson & Co., Mountain

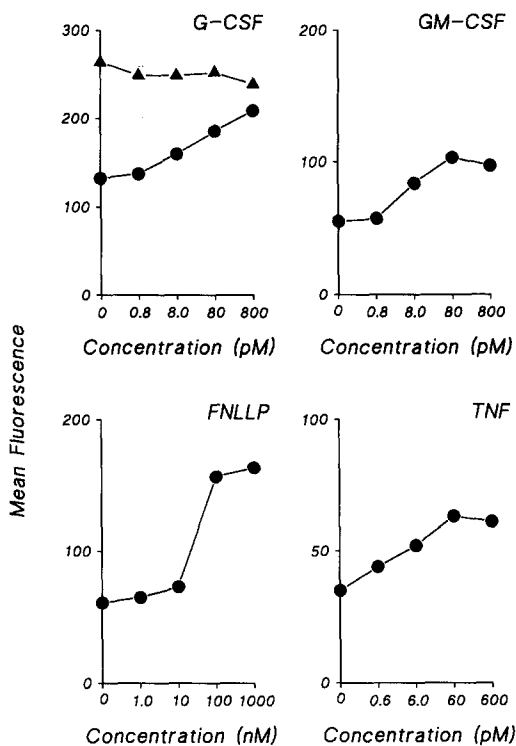
View, CA). mAb OKT3, directed against CD3, was used as a negative control, and the mean fluorescence channel of cells stained with OKT3 was subtracted from the experimental values.

In some experiments, washed cells from whole blood were stained as described above. Erythrocytes were then removed with FACS® lysing buffer (Becton Dickinson & Co.), and PMN and monocytes were gated by their light-scattering properties. Experiments with fluorescent standards indicated that our measurements of mean fluorescence were linearly related to the number of fluorescein molecules per cell.

## Results

**PMN Contain an Intracellular Pool of CD14.** Flow cytometry on blood leukocytes indicated that the mean fluorescence obtained after staining PMN with anti-CD14 was  $\sim 10\%$  ( $9.78 \pm 6.8\%$ ,  $n = 8$ ) of that observed on monocytes in the same blood sample. Approximately 50,000 CD14 molecules are expressed per monocyte (14), and PMN thus express  $\sim 5,000$  CD14 per cell.

Incubation of isolated PMN with the cytokines TNF- $\alpha$ , G-CSF, or GM-CSF, or with the chemotactic peptide fNLLP caused a dose-dependent 1.6–2.6-fold increase in the expression of CD14 on PMN (Fig. 1, Table 1). The dose of each



**Figure 1.** Cytokines increase expression of CD14 on the surface of PMN. PMN were incubated at 37°C for either 30 min with increasing concentrations of fNLLP or TNF, or 60 min with increasing concentrations of G-CSF or GM-CSF before staining with the anti-CD14 mAb 3C10. Parallel samples were stained with mAb W6/32 and one example is shown in the G-CSF panel (filled triangles). In all experiments the expression of HLA either remained the same or declined upon treatment with agonists. The panels shown are from separate experiments and are representative of at least three individual experiments.

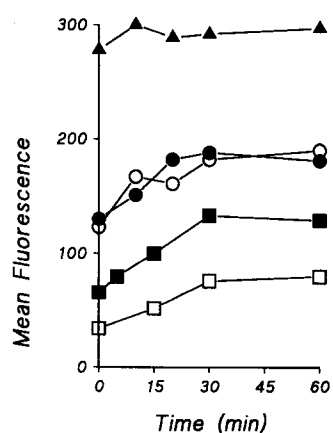
**Table 1.** Increase in Expression of CD14 in Response to Agonists

Agonist	Fold increase in expression
G-CSF (800 pM)	1.6 ± 0.1 (n = 3)
GM-CSF (800 pM)	1.7 ± 0.3 (n = 3)
fNLLP (1 μM)	2.6 ± 0.2 (n = 3)
TNF (600 pM)	2.0 ± 0.1 (n = 5)

PMN were incubated with agonists and stained as in Fig. 1. The fold increase in expression of CD14 is presented as mean ± SEM.

stimulant required for half-maximal upregulation of CD14 (Fig. 1) was consistent with the known potencies of these agonists in other assays of PMN function. Upregulation of CD14 was rapid and complete within 20 min of adding the stimulus (Fig. 2), suggesting that new synthesis of CD14 is not required for the increased expression. Parallel studies on the expression of HLA showed no increase, suggesting that CD14 is selectively externalized from an intracellular pool. Quantitatively similar upregulation of CD14 expression was measured with 3C10, an anti-CD14 mAb that blocks the ligand-binding site, or with 26ic (data not shown), a mAb that does not block the ligand-binding site (11). It is therefore unlikely that the apparent levels of CD14 expression were altered by the presence of ligand on the CD14 glycoprotein.

**CD14 on PMN Mediates Binding of LPS-LBP Complexes.** ELPS did not bind appreciably to resting PMN, but addition of 1 μg/ml LBP enabled binding (Table 2). The experiment shown in Table 2 was done in the absence of divalent cations to eliminate a small contribution of CD18 molecules to the binding of ELPS, but qualitatively similar results were obtained in the presence of divalents (data not shown). Additional experiments showed that pretreatment of the PMN with LBP did not enable the leukocytes to bind ELPS, but pretreatment of ELPS and LBP resulted in strong binding to PMN (data not shown). Thus, as previously described for macrophages (12), PMN recognize the stable complexes of



**Figure 2.** Time course of the increase in expression of CD14 in response to agonists. PMN were incubated for a total of 60 min at 37°C with G-CSF (800 pM; filled circles), GM-CSF (800 pM; open circles), fNLLP (10 nM; filled squares), or TNF (600 pM; open squares) added for the indicated times. PMN were stained with the anti-CD14 mAb 3C10. Parallel samples treated with G-CSF were stained with mAb W6/32 (filled triangles). The data are from separate experiments and are representative of at least three individual experiments.

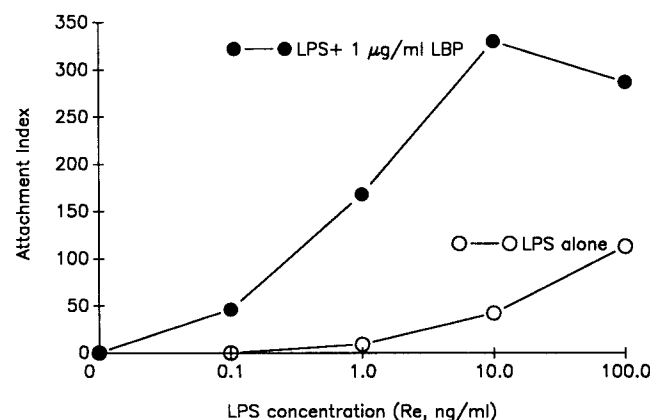
**Table 2.** CD14 on PMN Mediates Binding of Particles Coated with LPS-LBP Complexes

Cytokine	ELPS	ELPS + LBP	ELPS + LBP + 3C10
None	8.3	81	4.8
GM-CSF (70 pM)	27	152	6.3
G-CSF (70 pM)	2.5	138	2.8
TNF (300 pM)	58	161	9.5
fNLLP (10 <sup>-7</sup> M)	28	200	14

PMN were incubated in Terasaki wells for 30 min with the cytokines shown, and the monolayers were washed. 10 μg/ml of the anti-CD14 mAb 3C10 was added to some wells at this point. ELPS were then added in the presence or absence of 0.5 μg/ml human LBP. The preparations were incubated at 0°C for 15 min, then at 21°C for 15 min, and the attachment index was tabulated. This experiment is representative of more than five separate studies. Similar results were obtained with both human and rabbit LBP.

LBP with LPS formed on the surface of an erythrocyte, not soluble LBP.

Recognition of LPS-LBP complexes by PMN occurs via CD14, since the LBP-dependent binding of ELPS to PMN was completely blocked by a mAb directed against CD14 (Table 2). Furthermore, treatment of PMN with concentrations of fNLLP, TNF, G-CSF, or GM-CSF that caused an approximate doubling in the expression of cell surface CD14 also caused an approximate doubling in the LBP-dependent binding of ELPS (Table 2). Both the time course and dose dependence of cytokine-induced increases in binding activity were congruent with increases in expression of CD14 shown in Figs. 1 and 2 (data not shown), and the enhanced binding

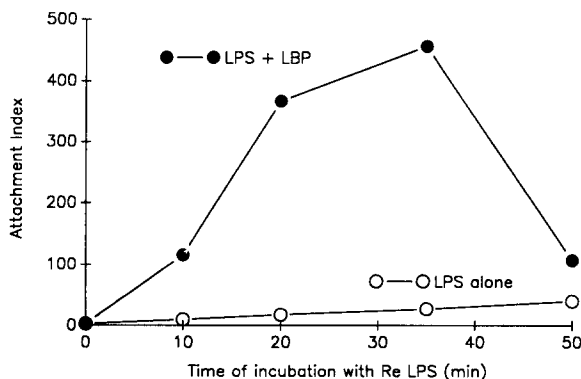


**Figure 3.** LPS-LBP complexes stimulate CR3 activity. PMN were incubated for 30 min with the indicated concentrations of LPS in either the absence (open circles) or presence (filled circles) of 1 μg/ml human LBP. The monolayers were then washed, EC3bi were added, and the attachment index was determined after a 30-min incubation at 37°C. Results are representative of three separate experiments. Identical results were obtained with rabbit LBP.

caused by cytokines was completely inhibited by anti-CD14 (Table 2). These data indicate that CD14 on PMN is capable of mediating the opsonic interaction of LPS-LBP complexes with PMN and that the intracellular pools of CD14 expressed in response to cytokines contribute to this activity.

**LPS-LBP Complexes Activate the Binding Activity of CR3.** We examined the ability of LPS to modulate CD18-dependent adhesivity by measuring the interaction of the numerically dominant CD18 molecule (CR3, CD11b/CD18) on PMN with its best-characterized ligand: C3bi attached to erythrocytes (EC3bi). As previously reported (19), unstimulated PMN have negligible ability to bind EC3bi. We found that extremely high concentrations of purified LPS (100 ng/ml) caused a slight activation of CR3, as evidenced by enhanced binding of EC3bi to PMN (Fig. 3). However, addition of LBP to the incubations enabled cells to respond to LPS with dramatically enhanced CR3 activity. LBP caused a >100-fold enhancement of sensitivity to LPS with half-maximal responses observed at <1 ng/ml LPS. Similar enhancement of CR3 binding activity in response to LPS was obtained when 1% human serum or plasma was substituted for purified LBP (data not shown). LBP or plasma in the absence of LPS had no effect on CR3 activity.

Activation of CR3 by LPS-LBP complexes was maximal after 35 min, but declined upon further incubation (Fig. 4). Such transient activation of CR3 has been previously noted for the agonists PMA (19), C5a (7), TNF (7), and fNLLP (S.D. Wright and P.A. Detmers, unpublished observations), and may underlie the transient binding of motile cells with the substrate over which they are migrating. Previous studies indicate that agonists such as PMA or fNLLP enhance the adhesive activity of CR3 by two mechanisms. Exocytosis of specific granules results in a two- to threefold rise in the expression of CR3, and simultaneous qualitative changes cause a 5–10-fold increase in the adhesive activity of existing CR3 (7, 19–22). LPS-LBP complexes caused only a modest rise (~40%) in cell surface CR3 (Table 3), and thus qualitative



**Figure 4.** LPS-LBP complexes cause transient activation of CR3. PMN were incubated at 37°C for 50 min. At intervals during this incubation, LPS (1 ng/ml) or LPS-LBP complexes (1 ng/ml LPS, 1 µg/ml LBP) were added so as to provide the indicated periods of stimulation with these agonists. The monolayers were washed, and attachment of EC3bi was then determined. Results are representative of three separate experiments.

**Table 3.** LPS-LBP Complexes Cause Little Increase Expression of CR3 on PMN

Stimulant	Mean fluorescence		
	CR3 (OKM10)	HLA (W6/32)	CD14 (26ic)
None	121	220	78
LPS	106	238	80
LBP	121	248	70
LPS-LBP	174	226	72

A suspension of  $2 \times 10^6$  PMN/ml in HAP buffer was incubated for 30 min at 37°C with LPS (1 ng/ml), LBP (1 µg/ml rabbit LBP), or both LPS and LBP. Cells were then washed, stained with the indicated mAbs, and the mean fluorescence was determined. Results represent averages of duplicate determinations, and the experiment shown is representative of two studies. The background fluorescence determined with mAb OKT3 (or without primary antibody) was 4.5 and was subtracted from the readings shown.

changes in existing CR3 may be principally responsible for the increased adhesive activity. Interestingly, LPS-LBP complexes did not cause upregulation of CD14 at the cell surface.

Addition of anti-CD14 mAb completely blocked activation of CR3 by LPS-LBP complexes, but control mAbs had no effect (Table 4). Similarly, anti-CD14 completely blocked the responses to LPS measured in the presence of whole serum (data not shown). These observations suggest that CD14 is required for activation of CR3 by LPS.

## Discussion

While CD14 is expressed on the surface of resting PMN, stimulation of cells with TNF-α, G-CSF, GM-CSF, or fNLLP caused a rapid twofold increase in its expression. These observations suggest that new CD14 is deployed on the cell

**Table 4.** Anti-CD14 Inhibits Activation of PMN by LPS-LBP complexes

Stimulant	Antibody	Attachment index (EC3bi)
-	-	9
LPS	-	12
LPS + LBP	-	485
LPS + LBP	W6/32 (10 µg/ml)	528
LPS + LBP	3C10 (1 µg/ml)	59
LPS + LBP	3C10 (10 µg/ml)	17

PMN were incubated for 15 min at 37°C with the antibodies indicated. LPS (1 ng/ml) or LPS/LBP complexes (1 ng/ml LPS, 1 µg/ml LBP) were added, and incubation was continued for 30 min at 37°C. EC3bi were then added and, after 30 min at 37°C, the attachment index was measured. This experiment is representative of four separate studies.

surface from a preexisting intracellular pool. The cytokines used here are known to cause fusion of at least three separate intracellular pools of membrane, a compartment marked by the presence of alkaline phosphatase (23, 24), a compartment marked by CR1 (25, 26), and specific granules marked by CR3 (25–30). Fusion of the compartment containing alkaline phosphatase occurs at concentrations of agonist lower than are required for fusion of CR1- or CR3-containing membranous compartments (26) and lower than are required for expression of CD14 (Fig. 1). In direct comparisons, the time of incubation and concentration of agonists required for upregulation of CD14 were identical with those required for upregulation of CR1 and CR3 (P.A. Detmers and A. Jakubowski, unpublished observations). Thus, CD14 is unlikely to reside in the easily mobilized alkaline phosphatase compartment and is more likely to reside in a compartment shared by CR1 or CR3. However, since these latter two compartments show similar responses to agonists, our data do not distinguish which contains the CD14.

We show here that CD14 on the surface of PMN is capable of binding particles opsonized with LPS-LBP complexes (Table 2). Exposure of PMN to stimulatory cytokines, with subsequent increases in the expression of CD14, enhances this ability to recognize LPS-LBP complexes. Thus, CD14 on PMN may play a role in the clearance of Gram-negative bacteria, and cytokines may augment this clearance mechanism. It should be noted, however, that binding of LPS-LBP complexes to CD14 represents only one of several mechanisms to clear LPS. Dispersions of LPS may be directly recognized by the scavenger receptor (also known as the acetyl-LDL receptor) (31), and LPS-bearing particles may be directly recognized by CD18

molecules (18, 32). PMN possess two of these mechanisms (CD18 and CD14), and each may serve in binding of bacteria to the leukocyte.

In addition to its function as an opsonic receptor, CD14 plays an important role in initiating the responses of leukocytes to LPS. LPS-LBP complexes stimulated a dramatic increase in the adhesive activity of CR3 (Figs. 3 and 4), and this response was completely blocked by anti-CD14 mAbs. Increased activity of CR3 (and the other leukocyte integrins) on PMN appears necessary for several well-known responses of PMN to LPS, such as binding of PMN to endothelium (5–7), migration to infected sites (5, 33), and enhanced microbicidal activity (5). Activation of leukocyte integrins by binding of LPS-LBP complexes to CD14 may thus represent a pivotal event in the responses of organisms to endotoxin.

The requirement for serum or LBP to support responses of PMN to LPS is consistent with several results in the literature. Cohn and Morse (4) reported rapid and dramatic enhancement of microbicidal activity of PMN by LPS, but only if serum was also present. A similar requirement for serum (34) or LBP (35) was observed in LPS-dependent priming of PMN for an oxidative burst, for LPS-dependent secretion of TNF by rabbit macrophages (10), and for the LPS-dependent production of cGMP in hepatocytes (36). In all of these studies, the responses of cells to LPS in the absence of LBP or serum were submaximal and occurred only at extremely high concentrations of LPS. Serum may serve as a source of LBP or other LPS-binding proteins (S.D. Wright, unpublished observations). These results suggest that soluble proteins such as LBP are a vital part of the normal process for responding to LPS.

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Address correspondence to Samuel D. Wright, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

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