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Serum-independent binding of lipopolysaccharide to human monocytes is trypsin sensitive and does not involve CD14

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SUMMARY

The nature of the binding sites for lipopolysaccharide (LPS) on human monocytes was investigated using fluorescein isothiocyanate (FITC)-labelled LPS from *Salmonella minnesota* R595 (ReLPS). In the absence of serum, ReLPS bound to monocytes and this interaction was trypsin sensitive. A concentration of 0·1 mg/ml resulted in a 90% loss of LPS binding, while low concentrations increased this binding. Trypsin-treated monocytes recovered FITC–ReLPS binding after 20 hr culture, which was abrogated in the presence of cycloheximide and actinomycin D. This showed that *de novo* protein and mRNA synthesis were essential. A number of different proteins have been implicated in cellular binding of LPS to monocytes. In this paper we show that CD14 is not involved in direct binding of FITC–ReLPS to monocytes, since anti-CD14 monoclonal antibody (mAb) (3C10) and removal of most of cell-surface CD14 by phosphatidylinositol-specific phospholipase C did not prevent FITC–ReLPS binding. Furthermore, LPS also bound to CD14-deficient cells from a patient with paroxysmal nocturnal haemoglobinuria (PNH). FITC–ReLPS binding was not mediated by the CD11/CD18 complex since mAb to the α and β chains of the CD11/CD18 complex did not alter the binding of FITC–ReLPS to cells. These observations indicate that ReLPS may interact with monocyte membrane protein(s) in the absence of serum. This binding site(s) for LPS might be different from those previously described by others.

INTRODUCTION

The lipopolysaccharide (LPS, endotoxin) from Gram-negative bacteria has been implicated as the major contributing factor in the pathogenesis of septic shock.[1] The LPS molecule interacts with and stimulates a variety of host cells which subsequently produce the potentially lethal mediators of septic shock.[2] Monocytes and macrophages have been shown to play a pivotal role in the elaboration of mediators after interaction with LPS, such as synthesis and release of interleukin-1 (IL-1),[3] tumour necrosis factor (TNF),[4] IL-6,[5] neutrophil-activating factor (IL-8),[6] interferon-α/β,[7] prostaglandins,[8] and the third complement component.[9]

Although the lipid A moiety of LPS is the principal bioactive part of the molecule, the earliest steps by which LPS causes cellular activation remain poorly understood. There is experimental evidence to support the concept that cells recognize LPS specifically, suggesting the existence of a receptor(s) for LPS. Several candidates for cellular binding sites and/or receptors for LPS on monocytes have been described: the leucocyte integrins;[10,11] a 73,000 MW LPS-binding protein expressed on mouse and human leucocytes;[12–14] a lectin-like monocyte membrane molecule interacting with the polysaccharide moiety of LPS;[15] and the glycolipid-anchored CD14 molecule recognizing LPS complexed to LPS binding proteins.[16–18]

In this paper we have investigated the binding of fluorescein isothiocyanate (FITC)-labelled LPS from *Salmonella minnesota* R595 (ReLPS) to human peripheral blood monocytes using flow cytometry. Our initial studies have shown that in the absence of serum proteins, FITC–ReLPS binds to mononuclear cells.[19] The results presented here indicate that LPS binding proteins exist on the surface of human peripheral blood monocytes which differ from CD14 and the CD11/CD18 complex.

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MATERIALS AND METHODS

Cell isolation

Leucocytes were isolated from buffy-coat obtained from healthy donors and from a PNH patient by Percoll centrifugation.[20] Peripheral blood mononuclear cells (PBMC) were collected at the interface of the Percoll gradient (Pharmacia, Uppsala, Sweden) and washed twice with RPMI-1640 (Flow Laboratories, Irvine, U.K.) containing 0·1% gelatin (RPMI–G). In some

experiments monocytes were further purified by adherence to plastic culture flasks (Costar, Cambridge, MA). Washed PBMC were suspended to a density of 10×10^6 in medium (RPMI containing 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with 10% heat-inactivated pyrogen-free fetal calf serum (FCS) and incubated for 2 hr at 37° in culture flasks precoated with fibronectin for 30 min at 37°. Non-adherent cells were removed by washing with warm medium and the adherent cells were incubated overnight in RPMI–10% FCS. The adherent cells were washed twice with warm phosphate-buffered saline (PBS) and detached by treatment with PBS supplemented with 3 mM EDTA–0·1% bovine serum albumin (BSA) for 10 min at room temperature, followed by gentle scraping with a rubber policeman in the presence of cold PBS. Collected monocytes were washed and resuspended in Hanks' balanced saline solution (HBSS)–0·1% gelatin (HBSS–G). The recovered cell suspension consisted of ⩾90% $CD14^+$ cells, as determined by flow cytometry using LeuM3 monoclonal antibody (mAb) (Becton Dickinson, Mountain View, CA).

Endotoxin

Rough LPS was isolated from *Salmonella minnesota* R595 (ReLPS) by the phenol-chloroform petroleum ether method, as described elsewhere.[21,22] ReLPS was labelled with FITC using a modification of the procedure of Skelly *et al.*,[23] as described previously.[19]

Antibodies

The following monoclonal antibodies (mAb) were used: anti-LFA1α (Becton Dickinson) against CD11a; anti-Leu15 (Becton Dickinson) and 44A (a generous gift of R. Todd, University of Michigan, Ann Arbor, MI) detecting CD11b; IB4 directed against the common β chain of CR3, LFA-1 and p150,95 (CD18) (a generous gift of Dr S. D. Wright, Rockefeller University New York, NY); CD14-specific mAb anti-LeuM3 (Becton Dickinson) and 3C10 (a generous gift of Dr S. D. Wright). Anti-HLA-DR mAb, FITC- and phycoerythrin (PE)-conjugated goat anti-mouse Ig were purchased from Becton Dickinson.

Saturating amounts of mAb were added to 5×10^5 PBMC for 30 min at 4°. After two washes, cells were stained with FITC-conjugated anti-mouse Ig for an additional 30 min, washed twice and resuspended in HBSS–1% FCS, and fluorescence was measured by flow cytometry (FACStar; Becton Dickinson).

Binding studies of FITC–ReLPS

Binding of ReLPS to monocytes was studied by incubating 5×10^5 PBMC with 50 μl FITC–ReLPS (50 μg/ml) in HBSS–G for 1 hr at 4°. Cells were washed twice with ice-cold HBSS–G, resuspended in 400 μl HBSS–1% FCS and kept on ice until analysis or fixed with 1% paraformaldehyde in HBSS for prolonged storage before analysis.

Measurement of FITC–ReLPS binding to monocytes was performed by flow cytometry. To analyse monocytes separately, a dot plot display of forward and right angle scatter was used for gating, excluding lymphocytes from the analysis.[24] In key experiments the monocyte population was selected by mAb anti-LeuM3 and PE-labelled goat anti-mouse Ig staining.

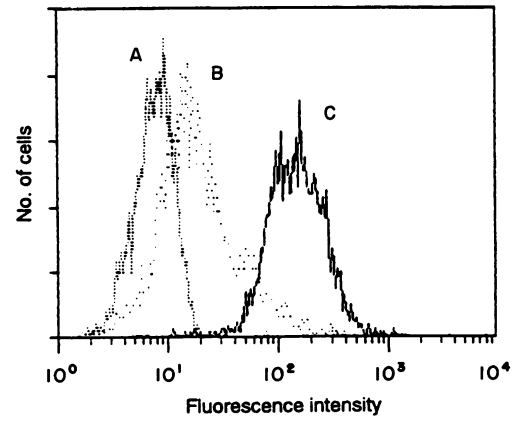

Figure 1. FITC–ReLPS binding to trypsinized and non-trypsinized monocytes. PBMC (1×10^6 cells) were incubated for 30 min at 37° with buffer or with 0·1 mg/ml of trypsin and subsequently incubated with FITC–ReLPS (2·5 μg) for 1 hr at 4°. The fluorescence of monocytes was analysed selecting the monocyte population on the basis of forward and right angle side scatter. The fluorescence intensity is shown on the abscissa (ordinary units) and the cell number on the ordinate. Background fluorescence of cells incubated with buffer only (A); fluorescence of trypsinized cells (B); and non-trypsinized cells (C) incubated with FITC–ReLPS.

Protease treatment

PBMC (1×10^6) were incubated in HBSS–G at pH 7·0 in the presence of freshly prepared solutions of trypsin for 30 min at 37°. Protease action was stopped by the addition of ice-cold HBSS–10% FCS or soybean trypsin inhibitor (SBTI, Type II-S; Sigma, St Louis, MO) using 10 times molar excess of trypsin. Control cells were treated with buffer only or with a combination of trypsin and SBTI added simultaneously. When phosphatidylinositol-specific phospholipase C (PI–PLC; kindly provided by Dr T. Schumacher, Cancer Institute, Amsterdam, The Netherlands) was used, PBMC (1×10^6 cells) were incubated for 60 min at 37° with 15 μg/ml PI–PLC in RPMI–5% FCS. The protease-treated cells were washed twice in HBSS–G and subsequently assayed for FITC–ReLPS binding and expression of surface antigens, as described above.

For experiments measuring the recovery of ReLPS binding after trypsin treatment, cells were resuspended at a concentration of 1×10^6 cells/ml in RPMI supplemented with 10% human AB serum (HABS) and incubated in teflon bags (Flits B.V., Almere, The Netherlands)[25] at 37°. After incubation, the cells were analysed for their ability to bind FITC–ReLPS. The viability of cells was >90% as determined by trypan blue dye exclusion or propidium iodine (5 μg/ml) staining and flow cytometry.

RESULTS

Binding of FITC–ReLPS to human monocytes and the effect of trypsin

In a previous study from our laboratory the optimal conditions for binding of FITC–ReLPS to leucocytes were established.[19] Incubation of 10^6 PBMC with 2·5 μg LPS in 50 μl for 60 min at 4° yielded a fluorescence signal sufficient to detect LPS binding to monocytes, as shown in Fig. 1. Treatment of PBMC with 0·1 mg/ml trypsin reduced the subsequent binding of FITC–ReLPS to monocytes to nearly backgrounds level (Fig. 1).

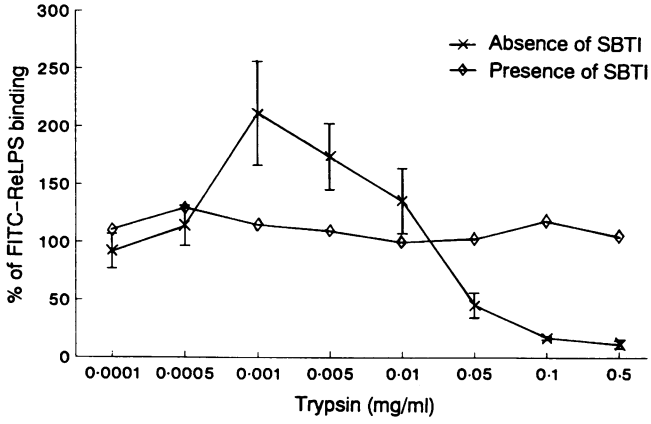

Figure 2. Dual effect of trypsin treatment on the binding of FITC-ReLPS to monocytes. PBMC (1×10^6 cells) were incubated in buffer with or without indicated concentrations of trypsin. After 30 min trypsin was inactivated by addition of HBSS-10% FCS, cells were washed twice in HBSS-G and the FITC-ReLPS binding to monocytes was determined as described in Materials and Methods. Binding of FITC-ReLPS to trypsinized cells is expressed as the percentage of binding relative to buffer-treated cells (mean ± SEM of six experiments).

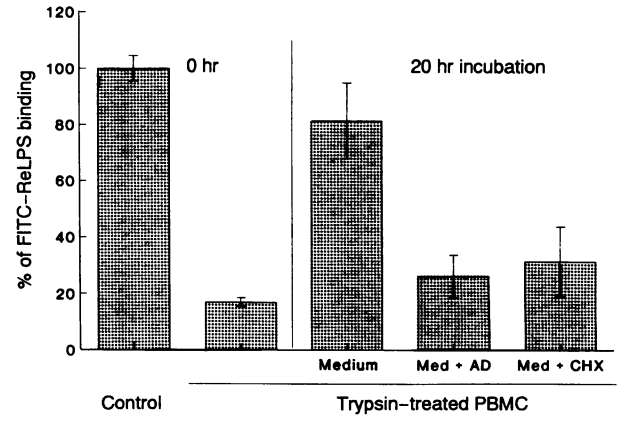

Figure 3. Effect of AD and CHX on the recovery of FITC-ReLPS binding to trypsin-treated cells. PBMC were incubated in buffer (control) or 0·1 mg/ml trypsin (trypsin-treated PBMC) and subsequently incubated with FITC-ReLPS (see Materials and Methods). Trypsin-treated cells were further incubated for 20 hr in RPMI with 10% HABS in the presence or absence of AD (0·05 μg/ml) or CHX (1 μg/ml) and washed; then FITC-ReLPS binding was determined. LPS binding is expressed as the percentage of binding relative to control cells at time-point 0 hr (mean ± SEM of four experiments).

Figure 2 shows that the effect of trypsin on LPS binding was concentration dependent. The binding of FITC-ReLPS to PBMC treated with 0·5–0·1 mg/ml trypsin was reduced to 17% of buffer-treated cells. However, treatment of PBMC with low concentrations of trypsin (0·01–0·001 mg/ml) resulted in a 1·5–2-fold increase in binding of FITC-ReLPS compared with buffer-treated cells. In order to exclude the possibility that the enhancement observed with low concentrations of trypsin was due to contaminating substances, trypsin was directly inactivated by SBTI before addition to the cells. Incubation of PBMC with this mixture for 30 min had no effect on FITC-ReLPS binding, showing efficient trypsin inactivation (Fig. 2).

The effect of trypsin treatment of cells on LPS binding was not due to cell death as there was no significant increase in the percentage of cells staining by trypan blue or propidium iodide after trypsin treatment.

To study whether the loss of ReLPS binding to trypsinized cells was transient, trypsin-treated PBMC were allowed to recover for different periods of time at 37° before analysis of FITC-ReLPS binding. Restoration of ReLPS binding was 25 ± 2·8% of control cells after 4 hr of incubation at 37°. Maximal recovery of ReLPS binding was to 76 ± 11% of control cells and was reached after 20 hr (Fig. 3). This suggests that protein synthesis might be required. In order to test whether active protein synthesis was required, trypsin-treated cells were cultured for 20 hr in medium containing actinomycin D (AD), an inhibitor of mRNA synthesis, or cycloheximide (CHX), an inhibitor of protein synthesis (Fig. 3). The addition of 0·05 μg/ml of AD or 1 μg/ml of CHX prevented the restoration of ReLPS binding to trypsinized cells. These data indicate that *de novo* protein and mRNA synthesis are required for the recovery of ReLPS binding.

FITC-ReLPS binding does not involve the CD11/CD18 complex or CD14

To determine whether the CD11/CD18 complex or CD14 played a role in the LPS binding, cells were preincubated with various mAb before measuring FITC-ReLPS binding. PBMC were preincubated for 30 min at room temperature with saturating concentrations of anti-CR3, anti-CD14 or isotype control mAb anti-HLA-DR and assayed for subsequent FITC-ReLPS binding. Monoclonal antibodies directed against the α-chain of CR3 (anti-Leu 15 and 44A) and the common β-chain (IB4) did not inhibit the binding of FITC-ReLPS to monocytes. CR3-dependent rosette formation of monocytes with C3bi-coated red blood cells was diminished by mAb 44A and IB4 (data not shown). Preincubation of PBMC with anti-CD14 mAb anti-LeuM3 and 3C10, which recognize different epitopes on the CD14 molecule, had no effect on ReLPS binding to monocytes in the absence of serum (data not shown), but 3C10 antibody completely inhibited the LPS-mediated priming of granulocytes in whole blood for enhanced oxidative burst response (manuscript in preparation) and inhibited LPS-induced TNF production by PBMC (data not shown).

To address the possible involvement of CD14 further, isolated monocytes were treated with PI-PLC. This resulted in the removal of 60% of cell-surface CD14 (Fig. 4), while FITC-ReLPS binding was not altered by PI-PLC treatment. Furthermore, monocytes from a patient with PNH showed measurable FITC-ReLPS binding within the range seen in normal monocytes (Fig. 5B).[19] CD14 was completely absent on PNH monocytes, as determined with anti-LeuM3 mAb staining (Fig. 5A).

Although CD14 is anchored to the membrane by a phosphatidylinositol linkage, treatment of isolated monocytes with high concentrations of trypsin (≥0·05 mg/ml) also resulted in reduction of CD14 expression, as determined with mAb LeuM3 (Fig. 6). This reduction of CD14 expression did not correlate with that observed for ReLPS binding. On the other hand, concentrations of trypsin below 0·05 mg/ml did not alter the CD14 expression but increased ReLPS binding. The expression of CR3, as measured with mAb Leu-15, was not altered at any trypsin concentration used.

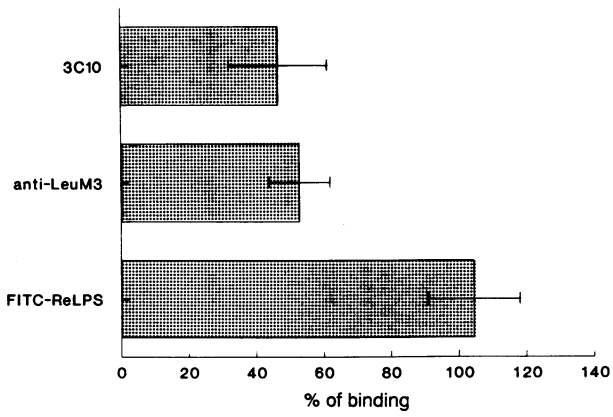

Figure 4. Effect of PI-PLC on the expression of CD14 and LPS binding. Isolated monocytes (1×10^6) were incubated with 15 μg/ml PI-PLC for 60 min at 37°, washed and further incubated with 2·5 μg FITC-LPS, anti-CD14 mAb 3C10 or LeuM3 for 60 min at 4°. The percentage of binding is expressed relative to the binding to buffer-treated cells (100%) (mean ± SEM of three experiments).

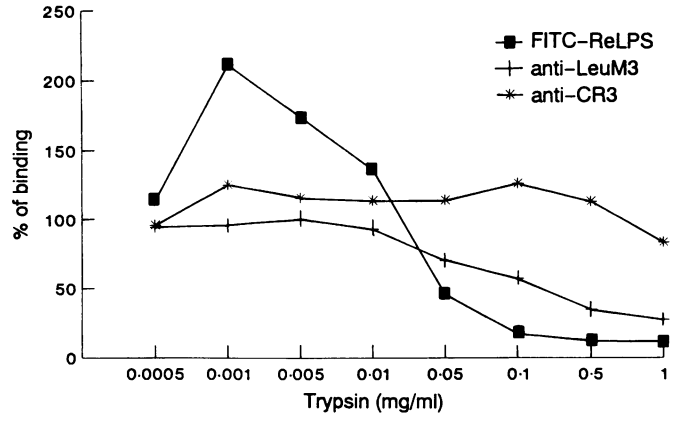

Figure 6. Effect of trypsin on the surface expression of CD14, CR3 and the binding of FITC-ReLPS. After incubation of isolated monocytes with increasing concentrations of trypsin, cells were washed and further incubated with LeuM3 (anti-CD14) or Leu-15 (anti-CD11b) followed by goat anti-mouse Ig-FITC. Binding of FITC-ReLPS was determined as described in the legend to Fig. 1. Binding is expressed relative to the binding of buffer-treated cells (mean of three experiments).

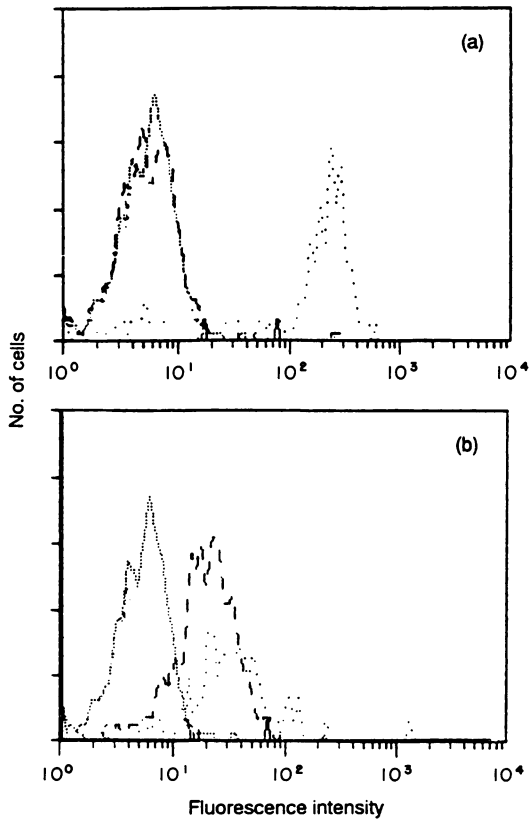

Figure 5. Binding of LPS and anti-CD14 mAb LeuM3 to monocytes of a PNH patient. Monocytes were incubated for 30 min with saturating concentrations of LeuM3 mAb or 60 min with 2·5 μg of FITC-ReLPS at 4°. LeuM3 was detected with goat anti-mouse Ig-FITC. (a) Binding of LeuM3 to normal monocytes (· · ·) and PNH monocytes (– – –). (b) FITC-ReLPS binding to normal monocytes (· · ·) and PNH monocytes (– – –). Background fluorescence of cells incubated in buffer only is shown by the dotted line (···) in both (a) and (b).

DISCUSSION

The use of FITC-labelled LPS and flow cytometry allowed reproducible measurements of the interaction of LPS with monocytes. The binding of LPS to monocytes in absence of serum was not saturable, which does not exclude the involvement of membrane proteins in the binding process. We provide evidence that trypsin-sensitive membrane proteins are involved in the binding of ReLPS to human monocytes. Due to the low labelling efficiency of ReLPS with FITC,[19] relatively high concentrations of FITC-ReLPS had to be used to ascertain a reliable positive fluorescence signal. Furthermore, the amphipatic structure of LPS favours the formation of micelles or aggregates, making it difficult to study classical ligand-receptor interactions. However, we have shown that the LPS binding was not due to merely sticking to cells. Double-labelling studies using specific mAb showed that natural killer (NK) cells did not bind LPS, B cells and monocytes showed a very high binding of LPS, whereas T cells were homogeneous in binding.[19] This interaction of LPS and leucocytes was inhibited in presence of two purified anti-lipid A IgM mAb preparations and polymyxin B.[19]

In this report it is shown that re-expression of FITC-ReLPS binding to monocytes after trypsin treatment required several hours and was impaired by cycloheximide and actinomycin D. This suggests *de novo* protein synthesis rather than receptor mobilization from intracellular stores or recycling after internalization, as shown for C5a and FMLP receptors with fluorescent probes.[26] Remarkably, treatment of monocytes with very low concentrations of trypsin increased the binding of FITC-ReLPS. This effect was attributed to the proteolytic activity of trypsin. Limited proteolysis may induce undirectional changes within cells and brings new physiological functions, as suggested for proteases, that act as a second signal following antigenic stimulation. The mechanism where trypsin enhances the FITC-ReLPS binding is not understood at present. It is possible that trypsin generates (new) LPS-binding sites or unmasks hidden sites by cleavage of adjacent cell-surface structures that interfere with LPS binding. Alternatively, low concentrations of trypsin

may increase the affinity of the putative LPS binding sites itself, as described for other surface receptors like FcRII,[27] while higher concentrations destroy the binding site.

A number of different proteins have been implicated in the binding of LPS to monocytes, using different experimental approaches. The CD11/CD18 complex on monocytes is known to be involved in binding of LPS-coated erythrocytes or intact bacteria.[10] Our and other studies have demonstrated that LPS binding to monocytes was not mediated by CD11b and CD18 because mAb to the α- and β-chain of the Leu CAM molecule did not interfere with the binding of vesicular FITC–ReLPS. On the other hand, it has been shown that, in the presence of plasma proteins such as LBP, complexes of LBP and LPS are recognized by the CD14 molecule.[18] However, under our experimental conditions, the CD14 molecule was not involved in direct binding of FITC–ReLPS to monocytes. The anti-CD14 mAb 3C10 and the removal of most cell-surface CD14 by PI-PLC did not prevent FITC–LPS binding. Furthermore, LPS bound to monocytes isolated from a PNH patient without detectable CD14 expression on leucocytes.

Although CD14 plays a major role when LPS is presented to monocytes in serum, a CD14-independent recognition of LPS is suggested by the fact that (1) LPS induces the production of IL-1 in CD14-deficient cells from patients with PNH;[28] (2) the blockade of the LPS response by anti-CD14 mAb can be overcome by higher concentrations of LPS;[29] and (3) the leucocyte response to LPS in the absence of serum and non-toxic deacylated LPS blocked the LPS response while the binding of radiolabelled LPS to CD14 was not affected.[30] In addition, these and our results indicate that alternative putative recognition sites for LPS are not GPI-anchored to the monocyte membrane. Using a photoactivable iodinated LPS, a 73,000 MW LPS-binding protein on human monocytes was identified.[31] Although some of the binding features for the 73,000 MW LPS binding protein are similar to those described in this paper, we currently do not have evidence for any resemblance. All these studies give evidence that LPS binding membrane proteins may be directed towards other membrane proteins in absence of serum. These pulsative other LPS-binding proteins co-operate with CD14, or they may recognize LPS with lower affinity.

There are multiple mechanisms for monocytes to recognize LPS. This study shows that trypsin-sensitive binding sites on human monocytes enable the binding of FITC–ReLPS under serum-free conditions that are not identical to CD11/CD18 or CD14. The identification and role of this protein(s) in cellular responses of monocytes to LPS remains to be established.

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