

Molecules from *Staphylococcus aureus* that Bind CD14 and Stimulate Innate Immune Responses

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Summary

Mammals mount a rapid inflammatory response to gram-negative bacteria by recognizing lipopolysaccharide (LPS, endotoxin). LPS binds to CD14, and the resulting LPS-CD14 complex induces synthesis of cytokines and up-regulation of adhesion molecules in a variety of cell types. Gram-positive bacteria provoke a very similar inflammatory response, but the molecules that provoke innate responses to these bacteria have not been defined. Here we show that protein-free, phenol extracts of *Staphylococcus aureus* contain a minor component that stimulates adhesion of neutrophils and cytokine production in monocytes and in the astrocytoma cell line, U373. Responses to this component do not absolutely require CD14, but addition of soluble CD14 enhances sensitivity of U373 cells by up to 100-fold, and blocking CD14 on monocytes decreases sensitivity nearly 1,000-fold. Deletion of residues 57–64 of CD14, which are required for responses to LPS, also eliminates CD14-dependent responses to *S. aureus* molecules. The stimulatory component of *S. aureus* binds CD14 and blocks binding of radioactive LPS. Unlike LPS, the activity of *S. aureus* molecules was neither enhanced by LPS binding protein nor inhibited by bactericidal/permeability increasing protein. The active factor in extracts of *S. aureus* is also structurally and functionally distinct from the abundant species known as lipoteichoic acid (LTA). Cell-stimulating activity fractionates differently from LTA on a reverse-phase column, pure LTA fails to stimulate cells, and LTA antagonizes the action of LPS in assays of IL-6 production. These studies suggest that mammals may use CD14 in innate responses to both gram-negative and gram-positive bacteria, and that gram-positive bacteria may contain an apparently unique, CD14-binding species that initiates cellular responses.

Gram-positive and gram-negative bacteria provoke similar inflammatory responses, yielding equivalent cardiovascular abnormalities (1), similar TNF and IL-1 production, similar tissue injury (2, 3) and death (1, 3). In baboons, an equivalent number of *Staphylococcus aureus* and *Escherichia coli* are required for an LD50 (3), and in vitro, equivalent numbers of *S. aureus* and *E. coli* are required to initiate TNF release from monocytes (4) or macrophages (5). The similarity of the responses of cells to gram-positive and gram-negative bacteria suggests a common intermediate.

The principal inflammatory component of gram-negative bacteria is LPS (endotoxin), the dominant lipid on the outer leaflet of the outer membrane. Recent studies have described an innate immune mechanism for responding to LPS that involves the proteins CD14 and LPS-binding protein (LBP)¹ (see reference 6 for review). LBP (7) is a lipid transfer protein that facilitates movement of LPS from mi-

celles to CD14 (8). CD14 expresses a binding site for one to two LPS molecules, and LPS-CD14 complexes potently stimulate cellular responses (8–10). CD14 is found both as a glycosylphosphatidylinositol-linked membrane protein on monocytes and PMN (mCD14) and as a soluble form (sCD14) in plasma, and both forms of CD14 mediate cellular responses to LPS (7, 8, 11, 12).

In contrast with gram-negative bacteria, our understanding of the mechanism by which gram-positive bacteria induce inflammatory changes is far less developed. Most work on inflammatory molecules of gram-positive bacteria has centered on the cell wall and membrane because these portions of the bacterium are exposed and because heat-killed organisms and purified walls actively induce inflammation in animal models (13). Gram-positive bacterial walls are composed of highly cross-linked peptidoglycan (PG) decorated

¹Abbreviations used in this paper: AF, active fraction; BPI, bactericidal/permeability increasing protein; HSA, human serum albumin; LBP, LPS-

binding protein; LTA, lipoteichoic acid; NHP, normal human plasma; PG, peptidoglycan; rsCD14, recombinant soluble CD14; SACE, *Staphylococcus aureus* crude extracts; sCD14, soluble CD14.

to a variable extent with teichoic acid polymers. Teichoic acid polymers are also linked to plasma membrane phospholipids, and these lipoteichoic acids (LTA) comprise another major surface component (14, 15). Gram-positive bacteria contain no LPS, and LTA is the major macroamphiphile of gram-positive bacteria. The physicochemical properties of LTA are similar to those of LPS, and it can be extracted with phenol by procedures similar to those used for purification of LPS (16).

Published work has ascribed inflammatory activity to each of the surface components described above. PG and PG fragments have been shown to stimulate production of TNF, IL-1, IL-6, and IL-8 in monocytes and macrophages (4, 17–20). Similarly, LTA has been shown to provoke secretion of TNF, IL-1, IL-8, and NO from monocytes or macrophages (2, 21–23). While these data suggest a role for the numerically dominant envelope molecules in provoking inflammation, in nearly all cases very large amounts of PG or LTA are required to stimulate responses of cells in vitro: 1–10 $\mu\text{g/ml}$ of LTA (2, 21–23) and 10–100 $\mu\text{g/ml}$ PG (4, 18, 20) are necessary to elicit cellular responses while LPS elicits responses in the picogram to nanogram per milliliter range. The low sensitivity to the purified gram-positive products raises the possibility that a trace contaminant, even if present as 1 part in 10,000, may be responsible for the activity observed.

Here we show that <10 ng/ml of a trace constituent of *S. aureus* strongly stimulates cytokine production in both monocytes and the astrocytoma cell line, U373. The response of monocytes to extracts of *S. aureus* was strongly blocked by anti-CD14. Moreover, the response of U373 cells, which do not express CD14, was strongly enhanced by addition of sCD14. Finally, we show that the active molecule from *S. aureus* binds sCD14 and blocks binding of LPS to sCD14. These data suggest the presence of a novel, proinflammatory, CD14-binding molecule in *S. aureus*.

Materials and Methods

***S. aureus* Crude Extracts (SACE).** Phenol extracts of *S. aureus* suspensions, which are commercially available as LTA from *S. aureus*, were purchased from Sigma Chemical Co. (St. Louis, MO). They were prepared by the method of Fischer et al. (16). Briefly, mechanically broken *S. aureus* were extracted with phenol at 65°C and the aqueous portion dialyzed. Nucleic acids were removed by digestion with nucleases followed by reextraction, dialysis, and lyophilization. This procedure, which is similar to the Westphal procedure for purification of LPS from gram-negative bacteria, yields not only LTA, but also some polysaccharides and other soluble components of the cell. The material had no optical density at either 280 or 214 nm, indicating the absence of protein. We designated this product SACE and used it as our starting material. It yielded clear or slightly opalescent solutions in physiological saline, and hydrolysis of 1 mg/ml of SACE (see below) yielded 4 mM of phosphate. An abbreviated protocol was used in one experiment (Fig. 4). Tryptic soy broth was inoculated and incubated overnight at 37°C with vigorous shaking with *S. aureus* strain DSM20233 (a gift of Dr. Seva Polotsky, Yale University).

Cultures were centrifuged, mechanically broken, and extracted with phenol at 65°C as described above. The aqueous portion was collected, dried down in a SpeedVac concentrator (AS160; Savant Instruments, Inc., Farmingdale, NY), and resuspended in H₂O.

Other Reagents. LPS from *Salmonella minnesota* strain R595 (Re) and ³H-labeled LPS from *E. coli* K12 strain LCD25 were purchased from List Biologicals (Campbell, CA). Recombinant human soluble CD14 (rsCD14) and LBP were expressed and purified as described (8). Mutant rsCD14 terminating at position 152 of the mature protein (rsCD14_{1–152}) and a second CD14 mutant lacking amino acids 57–64 (rsCD14_{Δ57–64}) were expressed and purified as described (24, 25). Bactericidal/permeability increasing protein 21 (BPI₂₁) (26) was a gift of Dr. J. Weiss, New York University. Anti-CD14 mAb 60b (27) and anti-CD18 mAb IB4 (28) were purified from ascites fluid by chromatography on protein G–Sepharose. Fresh frozen normal human plasma (NHP) was supplied by the New York Blood Center. *Limulus* amoebocyte assay reagents were from BioWhittaker, Inc. (Walkersville, MD), and human serum albumin was from Armour Pharmaceutical Co. (Kankakee, IL).

Stimulation and Assay of IL-6 Production. The human astrocytoma cell line, U373, was obtained from the American Type Culture Collection (HTB 17; Rockville, MD) and maintained in RPMI 1640 medium (BioWhittaker, Inc.) supplemented with 10% FCS. U373 cells were plated in 72-well terasaki plates (Robbins Scientific Corp., Sunnyvale, CA) at a density of 10,000 cells/well 24 h before stimulation. In some experiments, PBMC were purified from the blood of healthy volunteers as described (29), and plated in the same way 2 h before stimulation. The cells were washed extensively with AIM-V serum-free medium (GIBCO BRL, Grand Island, NY) and then incubated with various stimuli in AIM-V medium containing 0.5 mg/ml of human serum albumin (AIM-V-HSA). After 24 h, the supernatants were collected, diluted five times in PBS containing 1 mg/ml of HSA, and IL-6 levels were quantitated using a human IL-6 ELISA (Biosource International, Camarillo, CA) with slight modification. Briefly, terasaki plates were coated with 5 $\mu\text{l/well}$ of anti-IL-6 mAb B-E8, 40 $\mu\text{g/ml}$, overnight at 4°C. After blocking with 0.1% gelatin, 5 μl of sample and 5 μl of biotin-labeled anti-human IL-6 were added per well and incubated for 2 h at 37°C. After washing, bound antibodies were detected by adding 10 $\mu\text{l/well}$ of streptavidin-conjugated alkaline phosphatase (1:1,000; Calbiochem Corp., La Jolla, CA), and incubating for 1 h at room temperature. A fluorescent signal was generated using the fluorogenic substrate Attophos (JBL Scientific, San Luis Obispo, CA) and was measured using a Cytofluor 2300 (Millipore Corp., Bedford, MA).

In some experiments, heparinized whole peripheral blood from healthy volunteers was used for the assay. 90 μl of the whole blood was added to plastic tubes (Sarstedt, Inc., Newton, NC), 10 μl of various stimuli was added, and the tubes were incubated for 5 h at 37°C. After centrifugation, the supernatant was collected, diluted five times in PBS containing 1 mg/ml of HSA, and IL-6 levels were quantitated as described above.

Assay of PMN Adhesion to Fibrinogen. Adhesion of human PMN to fibrinogen-coated surfaces in response to LPS or SACE fractions was measured as described (8, 30). In this assay, adhesion of PMN in response to LPS is mediated by the leukocyte integrin CD11b/CD18 (CR3, Mac1) (30) and requires either the presence of LBP to hasten interaction of LPS with mCD14 on the PMN or preformed LPS–sCD14 complexes made by prolonged incubation of LPS with sCD14 (8). Briefly, mixtures containing LPS or SACE fractions, prepared as described in the legend to Fig. 12, were diluted to 125% of their final concentrations in 40 μl

HAP (Dulbecco's PBS with 0.5 U/ml aprotinin, 0.05% HSA, 3 mM D-glucose). 10 μ l of freshly isolated PMN (2×10^7 cells/ml in HAP) fluorescently labeled with 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester as described (30) were added and incubated for 10 min at 37°C to stimulate the cells. PMN were then washed into HAP and added to a 72-well terasaki plate pre-coated with fibrinogen. After 15 min at 37°C, adherence of PMN to the plate was quantitated: The fluorescence in each well was determined before and after washing using a Cytofluor 2300, and the percent adhesion was calculated from triplicate wells as (fluorescence after washing/fluorescence before washing) \times 100. Donor-to-donor variation in maximal responses prohibited averaging results of separate experiments, but the pattern of responses was highly reproducible.

Phosphate Assay. The absolute amount of phosphate in samples was measured by the method of Broekman et al. (31). Briefly, 25 μ l of sample was mixed with 100 μ l perchloric acid and digested at 180–190°C for 30 min. After cooling, 1.45 ml phosphate reagent (6 N H₂SO₄, 2.5% ammonium molybdate, 2.5% ascorbic acid, and water mixed in a 1:1:4:4 ratio) was added, samples were held at 50°C for 1 h, and 830 nm was read. The amount of phosphate in each sample was calculated from a standard curve prepared with KH₂PO₄ solutions.

Column Chromatography. A C8-HPLC column (Nova-Pak C8, 3.9 mm \times 150 mm; Waters Associates, Millipore Corp., Marlborough, MA) was equilibrated in 0.05 M sodium acetate (pH 4.7) containing 5% propanol. Sample (100 μ l) was applied at a flow rate of 0.5 ml/min, and the column was subsequently eluted with 5–100% propanol gradient in the same buffer. 20 fractions (1.5 ml each) were collected, dried down in a SpeedVac concentrator, and resuspended in 100 μ l H₂O.

Gel Electrophoresis. Tris-glycine polyacrylamide step-gradient gels (Novex, San Diego, CA) were used for native PAGE. Sample buffers and running buffers were made according to the manufacturer's suggested recipes. Samples were electrophoresed at 100–150 V for 2 h and stained with ammoniacal silver according to published procedures (32). ³H-LPS was visualized by fluorography using ENHANCE (DuPont Co., Boston, MA).

Results

Extracts of *S. aureus* Stimulate IL-6 Production in Monocytes. Addition of SACE to whole blood caused a strong, dose-dependent production of IL-6 (Fig. 1). This response was dependent on CD14 since anti-CD14 mAb 60b strongly blocked IL-6 production, while a control antibody, anti-CD18 mAb IB4, was without effect. IL-6 production with the same magnitude and CD14 requirement was observed in parallel experiments using LPS as stimulant (not shown), in keeping with previous work (33). This finding suggests that crude extracts of *S. aureus* contain a factor that stimulates monocytes in a CD14-dependent fashion.

SACE Stimulates IL-6 Secretion by U373 Astrocytoma Cell Line in a CD14-dependent and in a CD14-independent Manner. The role of CD14 in responses to SACE was further explored in the U373 astrocytoma cell line which expresses no mCD14. These cells secrete IL-6 in response to LPS, but only in the presence of exogenously added sCD14 (12). Monolayers of U373 cells were incubated with dilutions of SACE for 24 h in serum-free medium in the presence or

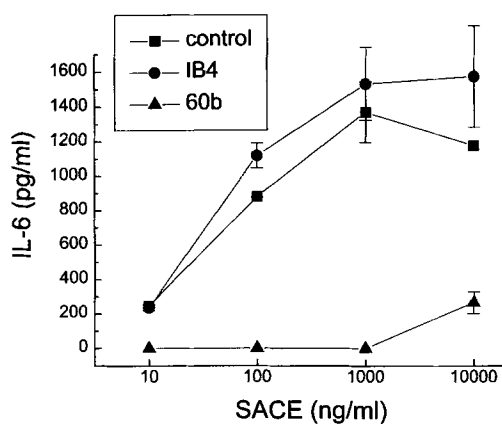


Figure 1. SACE induces IL-6 production in whole blood in a CD14-dependent manner. Whole peripheral blood was incubated for 5 h with different concentrations of SACE in the presence or absence of 50 μ g/ml of either control anti-CD18 mAb (IB4) or anti-CD14 mAb (60b), and the supernatants were assayed for IL-6 by ELISA. Results are means \pm SD of triplicate determinations of a representative experiment repeated two times.

absence of rsCD14, and the supernatant was assayed for IL-6. High concentrations of SACE (1–10 μ g/ml) alone caused production of IL-6 (Fig. 2 A). The amount of IL-6 produced in response to optimal concentrations of SACE was similar to that produced in response to LPS and sCD14 (Fig. 2 B). Addition of rsCD14 enhanced the sensitivity of cells to SACE, with a strong response observed with as little as 10 ng/ml SACE. The response to SACE was dependent on the concentration of rsCD14 added, with strong potentiation of both SACE and LPS responses observed with 100 ng/ml rsCD14 (Fig. 3). The CD14-dependent response of U373 cells to SACE was completely abolished by the addition of blocking anti-CD14 mAbs (not shown). These results indicate that SACE can stimulate cytokine production in U373 cells and that CD14 can strongly potentiate this response.

Cell Stimulating Activity of SACE Is Distinct from That of LPS. *Limulus* amoebocyte lysate assays showed that a stimulatory dose of SACE (10 ng/ml) contained <3 pg/ml LPS. This level is 300-fold lower than the amount of LPS required for a comparable response. Several additional observations confirm that LPS is not responsible for cytokine induction by SACE. (a) Phenol extracts of culture medium incubated overnight without a bacterial inoculum yielded no cell-stimulating activity (Fig. 4). A parallel flask inoculated with a single colony of *S. aureus* yielded strong, dose-dependent, CD14-enhanced activity. The activity must, therefore, derive from *S. aureus*. (b) LPS induced strong IL-6 production by U373 cells that was absolutely dependent on the presence of rsCD14 (12, and Fig. 2 B). SACE, on the other hand, induced IL-6 production in a CD14-independent way (Fig. 2 A), thus distinguishing it from LPS. (c) We observed an opposite effect of NHP on cell stimulation by SACE and LPS. LPS activity was markedly enhanced by adding 1% NHP (Fig. 2 D), again consistent with previous

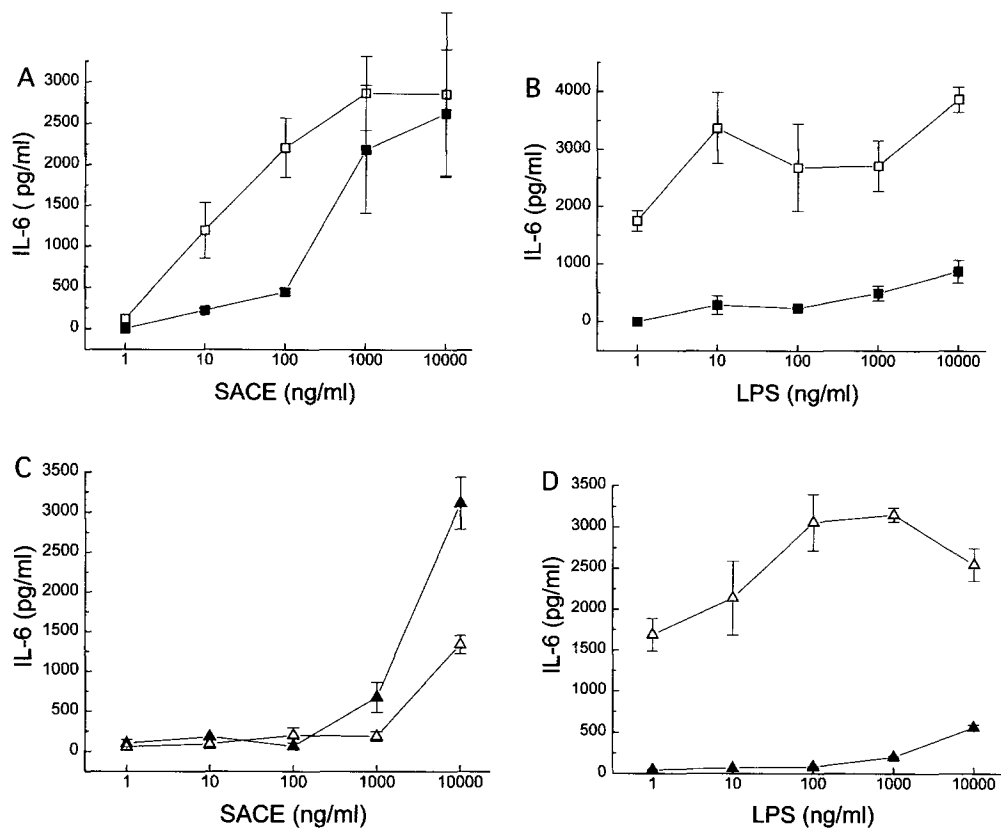


Figure 2. IL-6 production by U373 cells in response to SACE or LPS. Monolayers of U373 cells were incubated for 24 h with different concentrations of (A and C) SACE or (B and D) LPS in the presence (open symbols) or absence (filled symbols) of either (A and B) rsCD14 (1 μ g/ml) or (C and D) 1% NHP. The supernatant was assayed for IL-6 by ELISA. Results are means \pm SD of triplicate determinations of a representative experiment repeated three times.

results (12, 34). In contrast, NHP did not enhance the activity of SACE (Fig. 2 C) but caused a reduction of IL-6 production at high concentrations of SACE. (d) LPS-stimulated production of IL-6 by purified PBMC in serum-free medium was strongly enhanced by LBP (7, 35, and Fig. 5 B). Concentrations of SACE of 100 ng/ml or greater also stimulated IL-6 production by monocytes (Fig. 5 A). However, LBP had no effect on this response, suggesting that LBP does not recognize the active factor in SACE. This

finding is consistent with the observation of Mathison et al. (35) that LBP did not enhance the response of murine macrophage to whole heat-killed *S. aureus*. (e) BPI specifically binds LPS and blocks its ability to stimulate cells (26, and

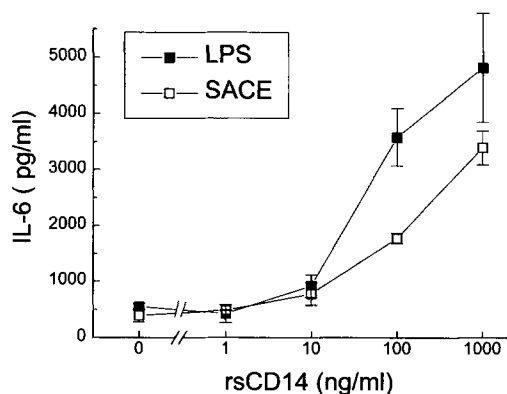


Figure 3. CD14 enhances production of IL-6 in response to SACE. Monolayers of U373 cells were incubated for 24 h with either SACE (100 ng/ml, 400 nM phosphate) or LPS (100 ng/ml) in the presence or absence of different concentrations of rsCD14. The supernatants were assayed for IL-6 by ELISA. Results are means \pm SD of triplicate determinations of a representative experiment repeated three times.

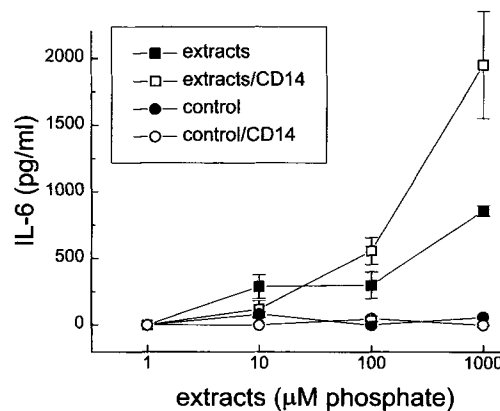


Figure 4. The cell-stimulating activity of phenol extracts derived from *S. aureus*. Culture medium was incubated overnight at 37°C with vigorous shaking in the presence (squares) or absence (circles) of an inoculum of *S. aureus* strain DSM20233. Both cultures were centrifuged and extracted in an identical way with phenol as described in Materials and Methods. The extracts were diluted to the indicated concentration of phosphate and were applied to monolayers of U373 cells with or without sCD14 (1 μ g/ml), and incubated for 24 h. Control extracts had no phosphate, but were diluted exactly the same way for comparison. The supernatants were assayed for IL-6 by ELISA. Results are means \pm SD of triplicate determinations of a representative experiment repeated two times.

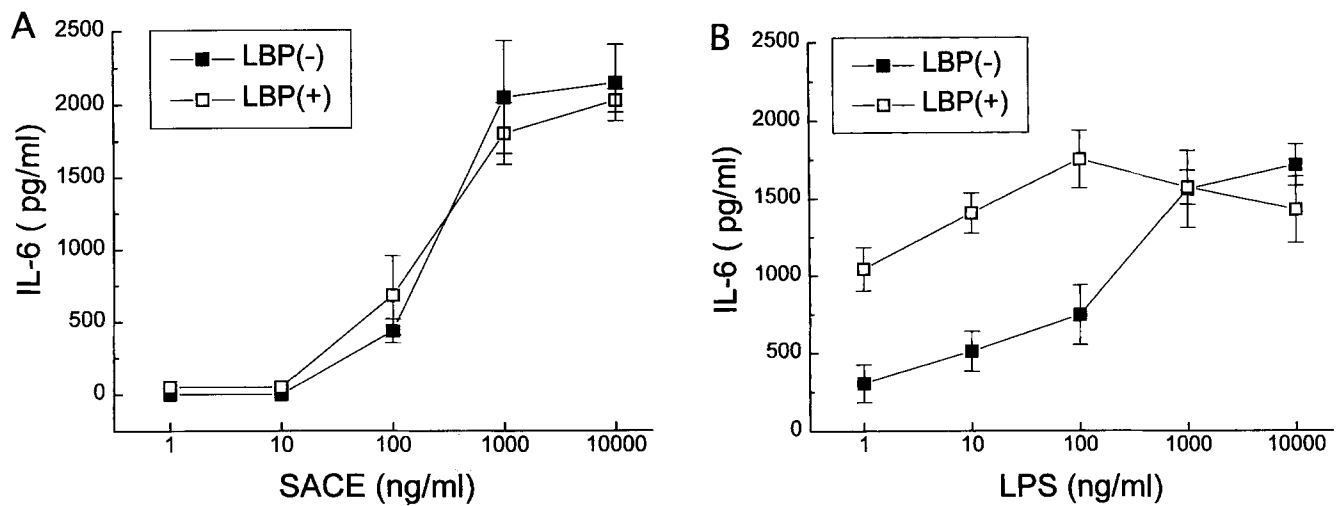


Figure 5. Effect of SACE and LPS on IL-6 production by PBMC with or without LBP. Monolayers of PBMC were incubated for 24 h with different concentrations of (A) SACE or (B) LPS in the presence or absence of LBP (1 μ g/ml), and the supernatants were assayed for IL-6 by ELISA. Results are means \pm SD of triplicate determinations of a representative experiment repeated two times.

Fig. 6 B). In contrast, BPI did not significantly block CD14-dependent stimulation of U373 cells by SACE (Fig. 6 A). The above experiments indicate the existence of stimulatory molecules in SACE that are functionally distinct from LPS but which may nevertheless use a CD14-dependent mechanism of cell stimulation.

SACE Components Bind rsCD14. The above studies indicate that SACE contains a strong activator of IL-6 production and that responses of both monocytes and U373 cells to this activator are enhanced by CD14. By analogy with LPS, we hypothesized that the active component of SACE may function by binding to rsCD14. We have previously shown that binding of LPS to rsCD14 causes a characteristic increase in the mobility of rsCD14 on native

gels (8) (and see Fig. 7 A). Since LPS does not cause a change in the conformation of rsCD14 (36), we believe that the shift in migration is caused by the strong negative charge of LPS. Addition of SACE also caused an increase in electrophoretic mobility of rsCD14 (Fig. 7 A), suggesting that SACE binds directly to sCD14. The mobility shift observed upon binding of SACE to rsCD14 was greater in magnitude than that seen with any LPS, smooth or rough, previously assayed (Fig. 7 A, and not shown). This finding suggests that SACE components bind rsCD14 and are distinct from LPS. In contrast with LPS, binding of SACE to rsCD14 was not accelerated by the addition of LBP (not shown), further confirming that SACE is distinct from LPS.

We also measured the ability of SACE to compete the

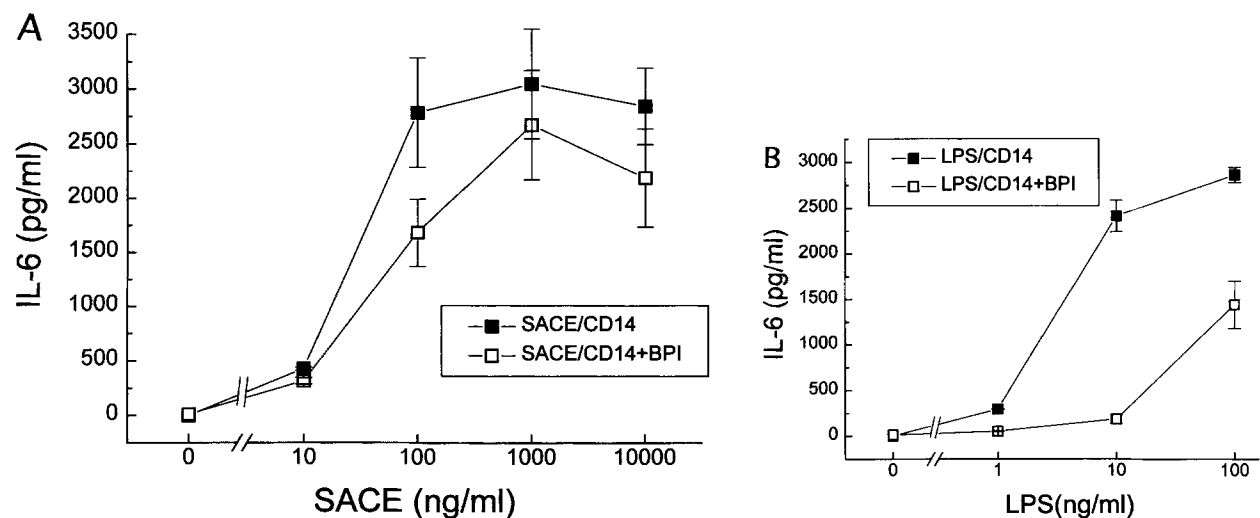


Figure 6. BPI₂₁ does not block the IL-6-inducing activity of SACE. Monolayers of U373 cells were incubated for 24 h with CD14 (1 μ g/ml) and different concentrations of either (A) LPS or (B) SACE in the presence or absence of BPI₂₁ (200 nM). The supernatants were assayed for IL-6 by ELISA. Results are means \pm SD or triplicate determinations of a representative experiment repeated two times.

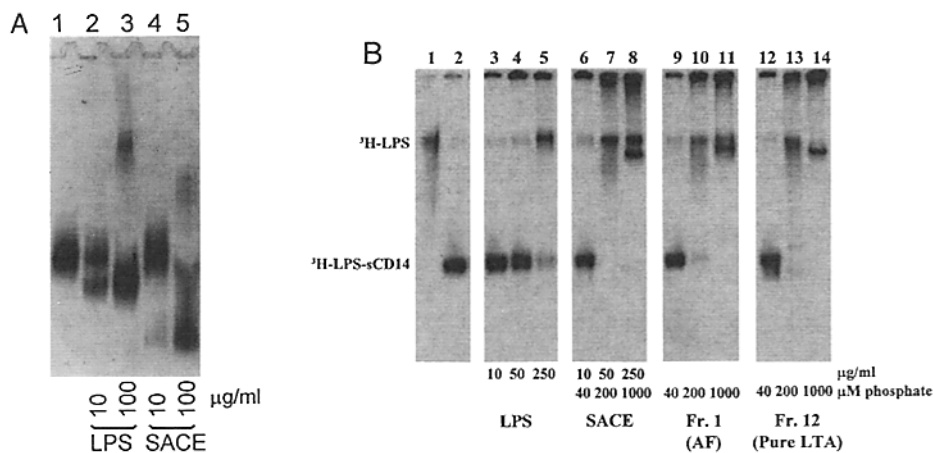


Figure 7. (A) SACE causes a shift in the electrophoretic mobility of rsCD14 in native PAGE. rsCD14 (30 µg/ml) was incubated alone (lane 1) or with 10 or 100 µg/ml of LPS (lanes 2 and 3) or SACE (lanes 4 and 5) overnight at 37°C in PD-EDTA (Dulbecco's PBS lacking divalent cations, with 1 mM EDTA). The samples were run on a 4–12% native polyacrylamide gel and stained with silver. A shift in mobility of rsCD14 by both LPS and SACE was confirmed in parallel experiments in which the location of rsCD14 was determined by Western blotting (not shown). (B) SACE competes for binding of ³H-LPS to sCD14. ³H-LPS (2 µg/ml) and sCD14 (50 µg/ml) were incubated overnight at 37°C in PD-EDTA alone

(lane 2) or with the indicated concentrations of Re LPS (lanes 3–5), SACE (lanes 6–8), AF (lanes 9–11) or pure LTA (lanes 12–14). ³H-LPS was incubated without sCD14 to show the electrophoretic pattern of free (aggregated) LPS (lane 1). The samples were run on an 8–16% native polyacrylamide gel and ³H-LPS was visualized by fluorography.

binding of ³H-LPS to rsCD14. ³H-LPS runs on native PAGE as a high molecular weight micelle and these micelles are well separated from rsCD14. Complexes of ³H-LPS with rsCD14 migrate at a low apparent molecular weight, near the position of rsCD14 (Fig. 7 B, lanes 1 and 2). Addition of unlabeled LPS blocked the binding of ³H-LPS to rsCD14 as evidenced by the lack of radioactivity at the position of ³H-LPS-rsCD14 as previously reported (Fig. 7 B, lanes 3–5) (8). Parallel studies showed that SACE also strongly blocked binding of ³H-LPS to rsCD14 (Fig. 7 B, lanes 6–8). The amount of SACE needed to inhibit binding of ³H-LPS to rsCD14 was lower than that for unlabeled LPS by weight, suggesting an avid interaction of an SACE component with the LPS-binding site of rsCD14.

LTA Binds sCD14 but Does Not Stimulate Cellular Responses. The dominant component in SACE is LTA, a lipid-linked glycerol-phosphate polymer with a well-defined structure (15). Reverse-phase HPLC of SACE yielded a dominant peak (70% of phosphate applied to column) at a position expected for LTA (Fig. 8, fractions 11 and 12).

We confirmed that this peak is LTA by measuring equimolar glycerol and phosphate in base hydrolyzates of the fractions (Kusunoki, T., and S.D. Wright, unpublished observations). Purified LTA competitively displaced ³H-LPS from sCD14 (Fig. 7 B) and caused a shift in mobility of sCD14 (not shown), indicating that it binds to sCD14. The purified LTA, however, did not stimulate U373 cells either in the absence (Figs. 8 and 9) or the presence (Fig. 9) of sCD14. Similarly, purified LTA failed to stimulate IL-6 production in monocytes (not shown) or adhesion in PMN (see below). The inability of LTA to stimulate cytokine production is consistent with the data of Keller et al. (23) who showed that highly purified LTA from *S. aureus* was incapable of stimulating NO secretion from macrophages.

The biosynthetic precursor of LPS known as lipid IVa also binds to sCD14 (8) but does not stimulate cytokine synthesis in human cells. Rather, it acts as an LPS antagonist, inhibiting responses of cells to complete LPS (37). In a similar fashion, we observed that LTA caused a dose-dependent inhibition of LPS-stimulated, CD14-dependent IL-6 re-

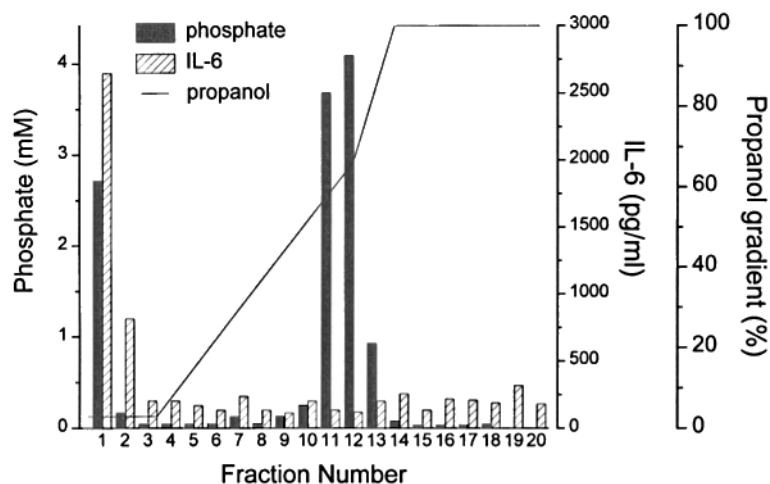


Figure 8. Purification of the stimulatory components of SACE with C8-HPLC. SACE (14 mM phosphate × 100 µl) was applied to a reverse-phase HPLC C8 column and eluted with a 5–100% propanol gradient. 20 fractions were collected and assayed as described in Materials and Methods. For the IL-6 assay, each fraction was further diluted 100-fold with AIM-V-HSA medium and applied to U373 monolayers. Results are means of triplicate determinations of a representative experiment repeated three times.

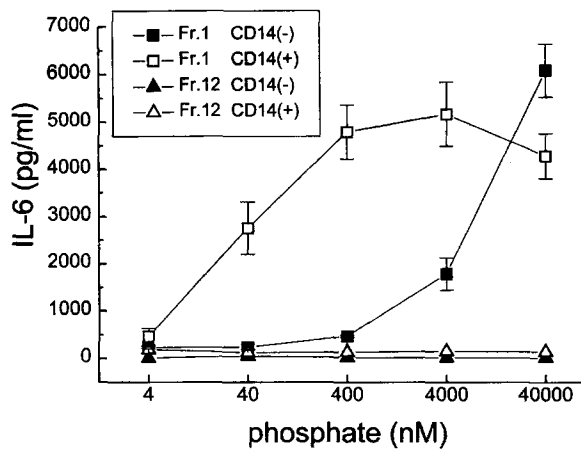


Figure 9. CD14-dependent and -independent activities of fractions 1 and 12 from C8 HPLC. Fractions 1 and 12 from C8 HPLC were diluted to the indicated concentrations of phosphate in AIM-V-HSA medium and applied to U373 monolayers with or without rsCD14 (1 $\mu\text{g}/\text{ml}$). After a 24-h incubation, IL-6 in the supernatant was assayed by ELISA. Results are means \pm SD of triplicate determinations of a representative experiment repeated three times.

lease from U373 cells (Fig. 10). This observation confirms that LTA does not stimulate cells and suggests that, by binding CD14, LTA may blunt responses to LPS. More importantly, it indicates that the active factor in SACE must be distinct from LTA.

Purification of the Active Factor in SACE. The chromatogram of SACE showed that all cell-stimulating capacity appeared in a single peak (Fig. 8, fraction 1) that was well separated from LTA. This peak contained 20% of the total applied phosphate and caused both CD14-dependent and CD14-independent stimulation of U373 cells in a dose-

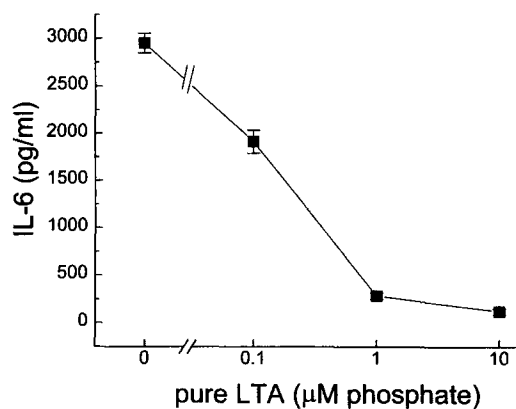


Figure 10. Pure LTA antagonizes the activity LPS. LPS (1 $\mu\text{g}/\text{ml}$), sCD14 (10 $\mu\text{g}/\text{ml}$), LBP (1 $\mu\text{g}/\text{ml}$), and different concentrations of pure LTA were mixed and incubated at 37°C overnight. The mixtures were then diluted 100-fold and applied to U373 monolayers and further incubated for 24 h. The x-axis depicts the final concentration of pure LTA present with cells. The supernatants were assayed for IL-6 by ELISA. Results are means \pm SD of triplicate determinations of a representative experiment repeated two times. The inhibitory effect of LTA is likely to be due to competition with LPS binding for sCD14 since the potency of LTA as an inhibitor was reduced \sim 100-fold if the preincubation step with sCD14 was omitted (data not shown).

dependent fashion (Fig. 9). Fraction 1 also bound sCD14 as shown by its ability to shift the mobility of sCD14 in native PAGE (not shown) and by its ability to block binding of ^3H -LPS to sCD14 (Fig. 7, lanes 9–11). These results suggest that fraction 1 contains the biologically active species in SACE. Since this active fraction (AF) is distinct from LTA, we suggest that it may contain a novel gram-positive compound.

Approximately 20% of the original IL-6-inducing activity was recovered in fraction 1 as judged by dose-dependence data (not shown). We noted, however, that LTA effectively antagonized the action of AF (not shown), and thus any additional activity that may have eluted with LTA would not have been detected because of this antagonist activity.

AF Interacts with a Site in the Amino Terminus of CD14.

Previous work has suggested that only the amino-terminal 152 residues of sCD14 are necessary for biological activity since residues 153–346 of rsCD14 (the carboxy-terminal two-thirds of the molecule) can be deleted without affecting its ability to mediate cellular responses to LPS (24). In a parallel fashion, we found that rsCD14_{1–152} enabled responses of U373 cells to AF with concentration dependence similar to that of full-length rsCD14 (Fig. 11). These studies indicate that residues in the carboxy-terminal two-thirds of rsCD14 are not necessary for the interaction of AF with rsCD14. Additional recent studies have identified residues 57–64 as crucial to the function of sCD14 (25). Deletion of these residues leads to a mutant (CD14 Δ _{57–64}) that cannot mediate cellular responses to LPS, and in a parallel fashion, we found that rsCD14 Δ _{57–64} did not enable responses of U373 cells to AF (Fig. 11). This finding indicates that AF requires residues 57–64 of CD14 to stimulate cells.

AF-rsCD14 Complexes Stimulate Neutrophils. LPS causes a rapid, CD14-dependent enhancement of integrin-mediated PMN adhesion (8, 11). Because adhesion is measured after exposure of PMN to LPS for only 10–20 min, LPS

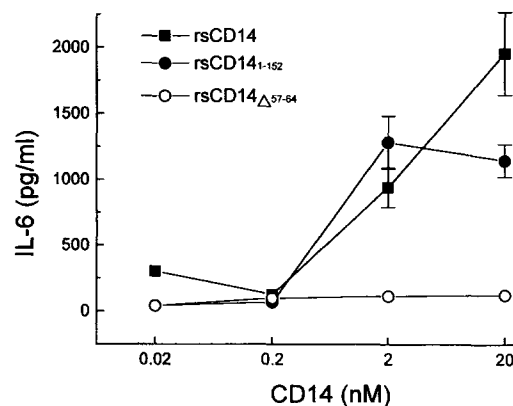


Figure 11. rsCD14_{1–152}, but not rsCD14 Δ _{57–64}, induces a dose-dependent cell-stimulatory activity of AF. Monolayers of U373 cells were incubated for 24 h with AF (400 nM phosphate) in the presence or absence of different concentrations of either rsCD14, rsCD14_{1–152}, or rsCD14 Δ _{57–64}. The supernatant was assayed for IL-6 by ELISA. Results are means \pm SD of triplicate determinations of a representative experiment repeated two times.

alone is ineffective and a response is only observed if LBP is added to speed movement of LPS into CD14 or if preformed LPS-sCD14 complexes are used (8). In a parallel fashion, we observed that AF alone did not stimulate PMN, but incubation of AF with sCD14 overnight to form AF-sCD14 complexes lead to strong stimulation (Fig. 12). To our knowledge, this is the first demonstration that gram-positive molecules may directly stimulate leukocyte integrin function in PMN. To determine if formation of AF-rsCD14 complexes was required for stimulation of PMN, AF and rsCD14 were mixed and added immediately to PMN, without time for binding of AF to rsCD14. Under these conditions, AF caused no PMN adhesion (Fig. 12). This observation suggests that binding of AF to sCD14 is a prerequisite for efficient PMN stimulation. We have observed in addition that pure LTA alone (not shown) or preincubated with rsCD14 (Fig. 12) failed to stimulate PMN adhesion, confirming that AF, not LTA, comprises the biologically active species in SACE.

Discussion

A large number of reports have described cytokine synthesis in response to bacterial cells or extracts (1-5, 13, 17-23, and see reference 38 for review), and several recent reports have implicated CD14 in these responses. Lipoarabinomannan from *Mycobacterium tuberculosis* stimulates TNF and IL-1 production from monocytes, and this response is blocked by anti-CD14 mAbs (39). Similarly, manuronic acid polymers from *Pseudomonas* (40), soluble peptidoglycan fragments from *S. aureus* (41), rhamnose-glucose polymers from *Streptococcus mutans* (42), chitosans from arthropods (43), and insoluble cell walls from several gram-positive species (44) have been shown to induce a cytokine response in

monocytes, and all these responses can be blocked with anti-CD14 antibodies. The studies reported here confirm a role for CD14 in responses of monocytes, PMN, and U373 cells to *S. aureus*. Addition of sCD14 enhanced responses of U373 cells by up to 100-fold, and blockade of CD14 decreased sensitivity of monocytes by ~1,000-fold. These studies suggest that the role of CD14 is to enhance the sensitivity of responses that can occur in the absence of CD14.

Additional experiments have suggested that stimulatory microbial substances may bind to CD14. Manuronic acid polymers compete with anti-CD14 for binding to monocytes (40), and sCD14 binds to insoluble walls of *Streptococcus mitis* (44). Our studies offer unequivocal evidence that molecules from *S. aureus* bind sCD14. They further show that binding to CD14 is necessary for enhancement of sensitivity to these molecules, and that residues 57-64 of CD14, previously defined as a binding site for LPS, are necessary for CD14 to enhance responses to *S. aureus* molecules.

Our studies extend previous work in that we have specifically sought the bacterial molecule that binds CD14 and stimulates cells. While prior authors have been meticulous in excluding LPS from their preparations, the whole bacteria, cell walls, or cell wall fragments used in past studies are inherently heterogeneous, and it is not possible to exclude a highly active subspecies as the source of the biological activity. We have observed that a minor component in extracts of *S. aureus* bears all of the cell-stimulating activity in the extract. This component has no optical density or other characteristic by which its presence could be detected. Therefore, it is possible that such a molecule may have been the source of the stimulatory activities reported for other bacterial preparations. In keeping with this hypothesis, Tsutsui et al. (45) and Kotani et al. (46) recently fractionated LTA from *Enterococcus hirae* and found cytokine-inducing activity only in the most lipophilic fractions. However, synthetic LTA molecules with the structure predicted from their studies did not have biological activity (47). It is thus clear that a minor, uncharacterized component of gram-positive bacteria may express the inflammatory activity. The precise structure of the active molecule is thus of considerable interest and is currently under study.

CD14 appears to serve in enhancing responses to an unexpectedly broad range of pathogens, and Pugin et al. (44) have suggested that CD14 may act as a "pattern recognition" receptor capable of binding and mediating responses to a broad range of bacterial molecules. Our results confirm a role for CD14 in binding and mediating responses to molecules other than LPS, but it is not clear that CD14 provides the specificity needed to discriminate LPS or AF from similar host molecules such as gangliosides or phospholipids. Indeed, CD14 binds inactive LPS analogues (8, 48), inactive LTA species (Fig. 5 B), and certain phospholipids (Hailman, E., and S. D. Wright, unpublished observations). Moreover, CD14 has been shown not to discriminate between LPS variants with opposite biological effects (49), and additional cellular molecules must contribute recognition specificity (48, 49). How could CD14 enhance responses to bacterial molecules without the recognition spec-

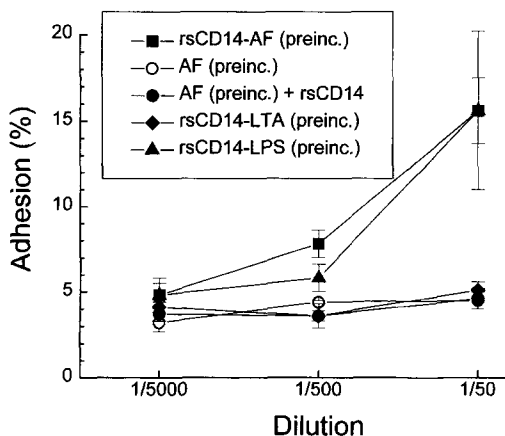


Figure 12. AF-rsCD14 complexes stimulate adhesion of PMN to fibrinogen. sCD14 (500 μ g/ml) was incubated overnight at 37°C in PBS with AF (1.5 mM phosphate) (■), pure LTA (1.5 mM phosphate) (◆), or LPS (5 μ g/ml) (▲). As control, AF was preincubated without rsCD14 (○). The samples were diluted 50-, 500-, or 5,000-fold, added to PMN, and adhesion of PMN to fibrinogen was measured as described in Materials and Methods. To determine if overnight incubation of AF with rsCD14 was necessary to observe cell stimulation, AF was mixed with sCD14 and added immediately to the PMN for assay (●).

ificity of IgG or the T cell receptor? We have recently shown that, by sequentially binding and releasing LPS, sCD14 serves to shuttle LPS from micelles into HDL particles (9) or lipid membranes (Wurfel, M.M., and S.D. Wright, manuscript in preparation). This type of shuttle action may thus concentrate LPS or AF in the membrane of a sensitive cell and facilitate recognition by additional cellular molecules. By concentrating a subset of bacterial molecules at the cell surface, CD14 may contribute both to the sensitivity and

selectivity of cellular responses even in the absence of decisive recognition specificity. We have thus proposed that CD14 acts in a combinatorial or sequential recognition scheme in which LBP and other molecules in plasma or at the cell surface each contribute to innate recognition of bacteria (6). We are currently seeking additional molecules that may contribute to specific recognition of the active molecules in *S. aureus*.

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References

- Natanson, C., R.L. Danner, R.J. Elin, J.M. Hosseini, K.W. Peart, S.M. Banks, T.J. MacVittie, R.I. Walker, and J.E. Parrillo. 1989. Role of endotoxemia in cardiovascular dysfunction and mortality. *Escherichia coli* and *Staphylococcus aureus*, challenges in a canine model, of human septic shock. *J. Clin. Invest.* 83:243–251.
- Wakabayashi, G., J.A. Gelfand, W.K. Jung, R.J. Connolly, J.F. Burke, and C.A. Dinarello. 1991. *Staphylococcus epidermidis* induces component activation, tumor necrosis factor and interleukin-1, a shock-like state and tissue injury in rabbits without endotoxemia. Comparison to *Escherichia coli*. *J. Clin. Invest.* 87:1925–1935.
- Hinshaw, L.B., T.E. Emerson, F.B. Taylor, A.C.K. Chang, M. Duerr, G.T. Peer, D.J. Fluornoy, G.L. White, S.D. Kosanke, C.K. Murray, et al. 1992. Lethal *Staphylococcus aureus*-induced shock in primates: prevention of death with anti-TNF antibody. *J. Trauma.* 33:568–573.
- Timmerman, C.P., E. Mattsson, L. Martinez-Martinez, L. De Graaf, J.A.G. Van Strijp, H.A. Verbrugh, J. Verhoef, and A. Fleer. 1993. Induction of release of tumor necrosis factor from human monocytes by *Staphylococci* and staphylococcal peptidoglycans. *Infect. Immun.* 61:4167–4172.
- Freudenberg, M.A., and C. Galanos. 1991. Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-galactosamine-treated mice. *Infect. Immun.* 59:2110–2115.
- Wright, S.D. 1995. CD14 and innate recognition of bacteria. *J. Immunol.* 155:6–8.
- Schumann, R.R., S.R. Leong, G.W. Flaggs, P.W. Gray, S.D. Wright, J.C. Mathison, P.S. Tobias, and R.J. Ulevitch. 1990. Structure and function of lipopolysaccharide binding protein. *Science (Wash. DC).* 249:1429–1431.
- Hailman, E., H.S. Lichenstein, M.M. Wurfel, D.S. Miller, D.A. Johnson, M. Kelley, L.A. Busse, M.M. Zukowski, and S.D. Wright. 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J. Exp. Med.* 179:269–277.
- Wurfel, M.M., E. Hailman, and S.D. Wright. 1995. Soluble CD14 acts as a shuttle in the neutralization of lipopolysaccharide by LPS-binding protein and reconstituted high density lipoprotein. *J. Exp. Med.* 181:1743–1754.
- Detmers, P.A., D. Zhou, and D.E. Powell. 1994. Different signalling pathways for CD18-mediated adhesion and Fc-mediated phagocytosis: response of neutrophils to LPS. *J. Immunol.* 153:2137–2145.
- Wright, S.D., R.A. Ramos, A. Hermanowski-Vosatka, P. Rockwell, and P.A. Detmers. 1991. Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: dependence on lipopolysaccharide binding protein and CD14. *J. Exp. Med.* 173:1281–1286.
- Frey, E.A., D.S. Miller, T.G. Jahr, A. Sundan, V. Bažil T. Espevik, B.B. Finlay, and S.D. Wright. 1992. Soluble CD14 participates in the response of cells to lipopolysaccharide. *J. Exp. Med.* 176:1665–1671.
- Tomasz, A., and K. Saukkonen. 1989. The nature of cell wall-derived inflammatory components of *Pneumococci*. *Pediatr. Infect. Dis. J.* 8:902–903.
- Lambert, P.A., I.C. Hancock, and J. Baddiley. 1977. Occurrence and function of membrane teichoic acids. *Biochim. Biophys. Acta.* 472:1–12.
- Fischer, W. 1989. Bacterial phosphoglycolipids and lipoteichoic acids. In *Handbook of Lipid Research*. D.J. Hannah, editor. Plenum, NY. 123–234.
- Fischer, W., H.U. Koch, and R. Haas. 1983. Improved preparation of lipoteichoic acids. *Eur. J. Biochem.* 133:523–530.
- Dinarello, C.A., R.J. Elin, L. Chedid, and S.M. Wolff. 1978. The pyrogenicity of the synthetic adjuvant muramyl dipeptide and two structural analogues. *J. Infect. Dis.* 138:760–767.
- Mattsson, E., L. Verhage, J. Rollof, A. Fleer, J. Verhoef, and H. van Dijk. 1993. Peptidoglycan and teichoic acid from *Staphylococcus epidermidis* stimulate human monocytes to release tumour necrosis factor- α , interleukin-1 β and interleukin-6. *FEMS Immunol. Med. Microbiol.* 7:281–288.
- Riesenfeld-Orn, I., S. Wolpe, J.F. Garcia-Bustos, M.K.

- Hoffmann, and E. Tuomanen. 1989. Production of interleukin-1 but not tumor necrosis factor by human monocytes stimulated with pneumococcal cell surface components. *Infect. Immun.* 57:1890-1893.
20. Heumann, D., C. Barras, A. Severin, M.P. Glauser, and A. Tomasz. 1994. Gram-positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. *Infect. Immun.* 62:2715-2721.
 21. Bahkdi, S., T. Klonisch, P. Nuber, and W. Fischer. 1991. Stimulation of monokine production by lipoteichoic acids. *Infect. Immun.* 59:4614-4620.
 22. Standiford, T.J., D.A. Arenberg, J.M. Danforth, S.L. Kunkel, G.M. VanOtteren, and R.M. Strieter. 1994. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. *Infect. Immun.* 62:119-125.
 23. Keller, R., W. Fischer, R. Keist, and S. Bassetti. 1992. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. *Infect. Immun.* 60:3664-3672.
 24. Juan, T.S., M.J. Kelley, D.A. Johnson, L.A. Busse, E. Hailman, S.D. Wright, and H.S. Lichenstein. 1995. Soluble CD14 truncated at amino acid 152 binds lipopolysaccharide (LPS) and enables cellular response to LPS. *J. Biol. Chem.* 270:1382-1387.
 25. Juan, T.S., E. Hailman, M.J. Kelly, L.A. Busse, E. Davy, C.J. Empig, L.O. Narhi, S.D. Wright, and H.S. Lichenstein. 1995. Identification of a lipopolysaccharide-binding domain in CD14 between amino acids 57 and 64. *J. Biol. Chem.* 270:5219-5224.
 26. Elsbach, P., and J. Weiss. 1995. Prospects for use of recombinant BPI in the treatment of gram-negative bacterial infections. *Infectious Agents & Disease.* 4:102-109.
 27. Todd, R.F., III, A. Van Agthoven, S.F. Schlossman, and C. Terhorst. 1982. Structural analysis of differentiation antigens Mo1 and Mo2 on human monocytes. *Hybridoma.* 1:329-337.
 28. Wright, S.D., P.E. Rao, W.C. Van Voorhis, L.S. Craigmyle, K. Iida, M.A. Talle, E.F. Westberg, G. Goldstein, and S.C. Silverstein. 1983. Identification of the C3bi receptor on human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:5699-5703.
 29. Pawlowski, N.A., G. Kaplan, A.L. Hamill, Z.A. Cohn, and W.A. Scott. 1983. Arachidonic acid metabolism by human monocytes. Studies with platelet-depleted cultures. *J. Exp. Med.* 158:393-412.
 30. Van Kessel, K.P.M., C.T. Park, and S.D. Wright. 1994. A fluorescence microassay for the quantitation of integrin-mediated adhesion of neutrophil. *J. Immunol. Methods.* 172:25-31.
 31. Broekman, M.J. 1989. Endogenous phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol, and phosphatidic acid in stimulated human platelets. *Methods Enzymol.* 169:415-430.
 32. Harlow, E., and D. Lane. 1988. *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 651-652.
 33. Wright, S.D., R.A. Ramos, P.S. Tobias, R.J. Ulkevitch, and J.C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science (Wash. DC).* 249:1431-1433.
 34. Wright, S.D., R.A. Ramos, M. Patel, and D.S. Miller. 1992. Septin: a factor in plasma that opsonizes lipopolysaccharide-bearing particles for recognition by CD14 on phagocytes. *J. Exp. Med.* 176:719-727.
 35. Mathison, J.C., P.S. Tobias, E. Wolfson, and R.J. Ulevitch. 1992. Plasma lipopolysaccharide (LPS)-binding protein. A key component in macrophage recognition of gram-negative LPS. *J. Immunol.* 149:200-206.
 36. McGinley, M.D., L.O. Narhi, M.J. Kelley, E. Davy, J. Robinson, M.F. Rohde, S.D. Wright, and H.S. Lichenstein. 1995. CD14: physical properties and identification of an exposed site that is protected by lipopolysaccharide. *J. Biol. Chem.* 270:5213-5218.
 37. Lynn, W.A., and D.T. Golenbock. 1992. Lipopolysaccharide antagonists. *Immunol. Today.* 13:271-276.
 38. Bone, R.C. 1994. Gram-positive organisms and sepsis. *Arch. Intern. Med.* 154:26-34.
 39. Zhang, Y., M. Doerfler, T.C. Lee, B. Guillemin, and W.N. Rom. 1993. Mechanisms of stimulation of interleukin-1 β and tumor necrosis factor- α by *Mycobacterium tuberculosis* components. *J. Clin. Invest.* 91:2076-2083.
 40. Espevik, T., M. Otterlei, G. Skjak-Braek, L. Ryan, S.D. Wright, and A. Sundan. 1993. The involvement of CD14 in stimulation of cytokine production by uronic acid polymers. *Eur. J. Immunol.* 23:255-261.
 41. Weidemann, B., H. Brade, E.T. Rietschel, R. Dziarski, V. Bazil, S. Kusumoto, H. Flad, and A.J. Ulmer. 1994. Soluble peptidoglycan-induced monokine production can be blocked by anti-CD14 monoclonal antibodies and by lipid A partial structures. *Infect. Immun.* 62:4709-4715.
 42. Soell, M., E. Lett, F. Holveck, M. Scholler, D. Wachsmann, and J. Klein. 1995. Activation of human monocytes by streptococcal rhamnase glucose polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF- α release. *J. Immunol.* 154:851-860.
 43. Otterlei, M., K.M. Varum, L. Ryan, and T. Espevik. 1994. Characterization of binding and TNF- α -inducing ability of chitosans on monocytes: the involvement of CD14. *Vaccine.* 12:825-832.
 44. Pugin, J., A. Heumann, A. Tomasz, V. Kravchenko, Y. Akamatsu, M. Nishijima, P. Gluaser, P.S. Tobias, and R.J. Ulevitch. 1994. CD14 is a pattern recognition receptor. *Immunity.* 1:509-516.
 45. Tsutsui, O., S. Koikeguchi, T. Matsumura, and K. Kato. 1991. Relationship of the chemical structure and immunobiological activities of lipoteichoic acid from *Streptococcus faecalis* (*Enterococcus hirae*) ATCC 9790. *FEMS Microbiol. Immunol.* 76:211-218.
 46. Kotani, S. 1992. Bacterial cell surface biological response modifiers and their synthetic counterparts. *Adv. Exp. Med. Biol.* 319:145-164.
 47. Takada, H., Y. Kawabata, R. Arakai, S. Kusumoto, K. Fukase, Y. Suda, T. Yoshimura, S. Koikeguchi, K. Kato, T. Komuro, et al. 1995. Molecular and structural requirements of a lipoteichoic acid from *Enterococcus hirae* ATCC 9790 for cytokine-inducing, antitumor, and antigenic activities. *Infect. Immun.* 63:57-65.
 48. Kitchens, R.L., R.J. Ulevitch, and R.S. Munford. 1992. Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J. Exp. Med.* 176:485-494.
 49. Delude, R.L., R. Savedra, Jr., H. Zhao, R. Thieringer, S. Yamamoto, M.J. Fenton, and D.T. Golenbock. 1995. CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition. *Proc. Natl. Acad. Sci. USA.* 92:9288-9292.