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Lipopolysaccharide and the Glycoside Ring of Staurosporine Induce CD14 Expression on Bone Marrow Granulocytes by Different Mechanisms

THIERRY PEDRON, ROBERT GIRARD, KENGO INOUE, DANIEL CHARON, and RICHARD CHABY

Molecular Immunophysiology Unit, URA-1961, National Center for Scientific Research, Pasteur Institute, Paris, France (T.P., R.G.), Pharmaceutical Research and Development Group, Kyowa Hakko Kogyo, Ltd., Chiyoda-ku, Tokyo, Japan (K.I.), and Endotoxin Group, URA-1116, National Center for Scientific Research, University of Paris-Sud, Centre d'Etudes Pharmaceutiques, 92290 Châtenay-Malabry (D.C.) and 91405 Orsay (R.C.), France

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SUMMARY

We established previously that lipopolysaccharide (LPS) can induce the expression of LPS-binding sites on bone marrow cells (BMC). We now report that staurosporine (STP), a glycosylated indolocarbazole alkaloid with potent inhibitory activity for various protein kinases, can induce the same effect. With both agents, the newly expressed LPS receptor was found to be CD14. The STP-induced effect was independent of its protein kinase inhibitory activity because several other protein kinase inhibitors, such as the indolocarbazole K-252a, the bisindolylmaleimide RO-31–8220, the perylenequinone calphostin C, and the isoquinolinesulfonamide H7, did not induce CD14 expression. The observation that the STP analog K-252a with an identical polyaromatic aglycon moiety was inactive yet the analog UCN-01 with an identical glycoside ring was active suggests that the induction of CD14 expression is triggered by the sugar moiety of STP. Three lines of evidence show that the mechanism of CD14 expression induced by STP differs from that induced by LPS: (i) unlike LPS, STP can stimulate BMC from LPS-unresponsive C3H/HeJ mice, (ii) LPS and STP effects are additive at a saturating dose of LPS, and (iii) the protein kinase inhibitor K-252a inhibits the LPS-induced but not STP-induced stimulation. Therefore, our findings show that both a protein kinase-dependent (LPS-induced) and a protein kinase-independent (STP-induced) mechanism can lead to the expression of the LPS receptor CD14 on BMC. We also found that the STP-induced stimulation of BMC is modulated by cyclosporin A, vinblastine, and verapamil. This observation may suggest that the inducible effect of STP could be initiated by its interaction with P-glycoprotein, a membrane pump with drug efflux function that plays a critical role in the multidrug resistance of cancer cells.

LPS, a major cell wall component of all Gram-negative bacteria, has marked pathophysiological effects in mammals (1). Because monocytes/macrophages play a determinant role in the initiation of these deleterious effects, a number of studies have focused on the mechanisms of LPS-induced production of cytokines and other mediators in this cell type. However, little is known about other LPS-induced effects, particularly about modifications in the expression of various cell surface constituents. Some surface molecules are down-modulated, whereas others are newly expressed or up-modulated after exposure of the cells to LPS. In this second group, LPS has been shown to elicit the expression of sIg on pre-B cells, transferrin receptors on B cells, Ia antigens and glucocorticoid receptors on macrophages, and formyl-methionyl-leucyl-phenylalanine receptors and CD11b/CD18 molecules on neutrophils.

In previous studies (2, 3), we reported that LPS receptors, constitutively undetectable on the neutrophilic granulocytes of BMC, are expressed on these cells after *in vivo* or *in vitro* exposure to nanomolar concentrations of LPS. In human BMC, we identified the inducible LPS receptors as the GPI-anchored differentiation antigen CD14. The inducible LPS receptor of mouse BMC exhibited similar characteristics, but its definite identification awaited the availability of antibodies specific for mouse CD14.

The initial purpose of this study was to gain some insight into the mechanism of expression of the inducible LPS receptor on mouse BMC. Because activation of protein kinases is involved in several other LPS-induced effects (4), we decided to examine whether it is also involved in the expression of the LPS receptor of mouse BMC. We analyzed the influence of

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ABBREVIATIONS: LPS, lipopolysaccharide; BMC, bone marrow cells; CM, culture medium; FCS, fetal calf serum; FITC-LPS, lipopolysaccharides from *Salmonella choleraesuis* labeled with fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; PMA, phorbol-12-myristate-13-acetate; PBS, phosphate-buffered saline; PS, polysaccharide; STP, staurosporine; VitD3; 1,25-dihydroxyvitamin D_3.

STP, and of structurally related analogs, on the expression of the receptor. STP is a glycosylated indolo-2,3-α-carbazole alkaloid produced by *Streptomyces staurosporeus*. This antifungal agent is one of the most potent and well known inhibitors of protein kinases described in the literature, and it is often used to study the involvement of protein kinases in signal transduction pathways. It has an inhibitory effect on a variety of serine/threonine kinases (5), including protein kinase C, cAMP- and cGMP-dependent protein kinases, myosin light-chain kinase, and calcium/calmodulin-dependent protein kinase. It also inhibits the tyrosine kinase activity of p60 (6), insulin receptor (7), epidermal growth factor receptor (8), and platelet-derived growth factor receptor (8).

This broad spectrum of inhibition activity suggested that STP might inhibit the differentiation of bone marrow granulocytes and the concomitent expression of the LPS receptor. Surprisingly, this study showed that STP is another inducer of the expression of this receptor. We compared several characteristics of the LPS- and STP-induced stimulation of BMC.

Experimental Procedures

Mice. We used 8–10-week-old female C3H/HeOU and C3H/HeJ mice from the Breeding Center of the Pasteur Institute (Paris, France).

Materials and cell lines. CM was RPMI-1640 (GIBCO, Grand Island, NY) containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2-mercaptoethanol (5×10^{-5} M), and 10% heat-inactivated (56° for 30 min) FCS (ATGC Biotechnologie, Noisy le Grand, France). Verapamil, PMA, cytochrome *c* (from horse heart), and FITC were purchased from Sigma Chemical (St. Louis, MO). VitD3 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Cyclosporin A was kindly provided by Dr. Paolo Truffa-Bachi (Pasteur Institute). The rat anti-mouse CD14 monoclonal antibody (rmC5–3 mAb) was from PharMingen (San Diego, CA). The FITC-labeled goat anti-rat Ig was from Southern Biotechnology Associates (Birmingham, AL). The monocytic cell line Mono Mac 6 was from a human clone originally isolated by Dr. Ziegler-Heitbrock.

Protein kinase inhibitors. STP and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (compound H7) were from Sigma Chemical. Compounds K-252a, K-252b, KT-5823, GF-109203X, and RO-31–8220 were from Calbiochem (La Jolla, CA). 7-Hydroxy STP (compound UCN-01) was from Kyowa Hakko (Tokyo, Japan). The structures of these compounds are given in Fig. 1. Stock solutions of H7 (1 mg/ml in water), vinblastine sulfate (20 mg/ml in methanol), and all other compounds (in dimethylsulfoxide) were stored at −20°. Further dilutions were made into CM just before use.

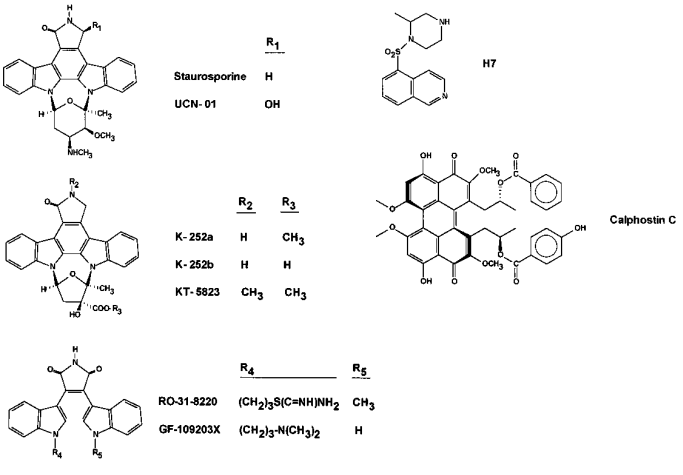

Fig. 1. Structures of study compounds.

LPS and FITC-LPS. The LPS from *Salmonella choleraesuis* (serotypes 6_2, 7, 14) was prepared by the phenol-water extraction procedure (9). Its PS fragment was isolated after mild acid hydrolysis (10). FITC-LPS was prepared as described previously (2). Briefly, FITC (250 μl; 1 mg/ml in dimethylsulfoxide) was incubated (150 min, 20°) with a suspension of lysine-LPS (0.9 ml in 0.1 M $NaHCO_3$; pH 9) obtained through incubation of CNBr-activated LPS (5.2 mg; 700 μl; pH 10) with lysine chloride (200 μl, 5 mg/ml in 1 M $NaHCO_3$). After dialysis against PBS, FITC-LPS (3.7 mg/ml) was stored in the dark (4°) until used.

Analysis of BMC for LPS-binding capacity and membrane CD14. BMC collected from mouse femurs (5×10^5 cells in 200 μl of CM) were incubated (18–24 hr, 37°) with the inducer (usually 20 ng/ml LPS or 20 nM STP in 200 μl of CM). The cultures were then maintained for 1 hr at 4°. For analysis of LPS-binding capacity, the cells were incubated (18 hr, 4°) with FITC-LPS (1 μg/ml in 100 μl of CM). For detection of membrane CD14, the cells were incubated first (30 min, 0°) with the rat anti-mouse CD14 monoclonal antibody (rmC5–3) and stained by reincubation (30 min, 0°) with an FITC-labeled goat-anti-rat antibody (Southern Biotechnologies, Birmingham, AL). In each case, stained cells were layered onto a 50% FCS solution and centrifuged, and the cell pellet was resuspended in 0.5 ml of staining buffer (PBS, 5% FCS, and 0.02% sodium azide) containing propidium iodide (0.2 μg/ml) to stain dead cells. Fluorescent cells were detected by analysis (5000 cells/sample) with a FACS flowcytometer (FACScan; Becton-Dickinson Electronic Laboratories, Mountain View, CA) using Cell Quest software. Dead cells, which incorporated propidium iodide, were gated out of analysis. Cells with a fluorescence intensity higher than the maximal level of autofluorescence were scored as fluorescent cells.

Determination of superoxide production by BMC. BMC were preincubated (18 hr, 37°) in CM with or without STP (5 nM) in the absence of serum. The cells were washed and suspended in PBSG (PBS containing 7.5 mM D-glucose, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$). Suspensions (10 μl) of LPS (0–2.5 μg/ml in PBSG) were added to the suspensions of BMC (2.5×10^5 cells/well in 210 μl of PBMC) in 96-well plates. After incubation for 30 min at 37°, PMA-inducible secretion of superoxide was estimated by a kinetic microplate assay based on the ability of O_2^- to reduce ferricytochrome *c* to its ferrous form. Briefly, cytochrome *c* (20 μl, 0.94 mM in PBSG) and PMA (10 μl, 5 μg/ml in PBSG) were sequentially added to the cells. Absorbance at 550 nm was determined at different time points with a Dynatech MR 5000 microplate spectrophotometer (Marnes-la-Coquette, France). The rate of variation of the absorbance (ΔA_{550nm}/min) and its standard deviation were determined by linear regression analysis of the data collected during the first 20 min.

Results

STP induces expression of LPS-binding sites on BMC. In previous studies (2, 3), we established that exposure of mouse and human BMC to small amounts of LPS induces the expression of cell surface LPS-binding sites. To determine whether a protein kinase can be involved in this mechanism, the most straightforward approach was to analyze the influence of a potent and membrane-permeant protein kinase inhibitor on LPS-induced expression of LPS-binding sites on mouse BMC. As shown previously (2, 3), LPS-binding sites are easily detectable with the fluorescent ligand FITC-LPS. We first used the microbial alkaloid STP, one of the most potent but nonselective inhibitors of a wide range of serine/threonine-specific protein kinases, which has been widely used to investigate the role of these kinases in cellular responses. Unexpectedly, we observed in a preliminary exper-

iment that STP alone behaves as an inducer rather than an inhibitor: it enhanced the LPS-binding capacity of the cells. To ensure that this effect was not due to a contamination of STP with endotoxin, we compared the responses of BMC from LPS-responsive C3H/HeOU mice with those from LPS-unresponsive C3H/HeJ mice. Responses were analyzed as a function of the time of incubation with STP and of STP concentration. Fig. 2 shows that BMC from C3H/HeJ mice do respond to STP. This demonstrates that the observed activity is due to STP and not to contamination with endotoxin. The kinetics of STP-induced stimulation of BMC (Fig. 2A) were rather slow; no significant effect was detectable after 6 hr. Optimal stimulation (90% of cells expressing LPS-binding sites) was obtained after exposure of the cells for 24 hr at 37° to a 10 nM concentration of STP (Fig. 2B).

STP does not enhance the LPS-binding capacity of Mono Mac 6 cells. To determine whether the inducing activity of STP can be observed with another cell type, we decided to examine a cell line on which inducible LPS-binding sites have been reported, such as the human promonocytic cell line THP-1 (11) or the monocytic cell line Mono Mac 6 (12), in which the well-characterized LPS receptor CD14 is inducible. One of the best inducers of this expression is VitD3 (13); therefore, we compared the influences of STP and VitD3 on the LPS-binding capacity of Mono Mac 6 cells. Fig. 3 shows that VitD3 is a potent inducer of LPS-receptor expression, whereas STP is inactive on these cells. This suggests that the activation pathways induced by LPS and STP and leading to the expression of LPS receptors on BMC are not shared by monocytes, even though the latter cells are potentially able to express these receptors when exposed to an appropriate agent (VitD3).

Specificity and nature of the STP-induced LPS-binding sites. We reported previously (2) that the binding sites for FITC-LPS expressed on BMC after stimulation with LPS are LPS specific. To determine whether this is also the case after stimulation with STP, we analyzed the capacity of STP-stimulated cells to bind FITC-LPS in the presence or absence of unlabeled LPS or its PS fragment. We found (Table 1) that an excess (10 μg/ml) of unlabeled LPS completely blocked the binding of FITC-LPS to the newly expressed receptors, whereas the same concentration of PS was ineffective. This result suggests strongly that both LPS and STP induce the expression of receptors specific for the lipid A moiety of LPS.

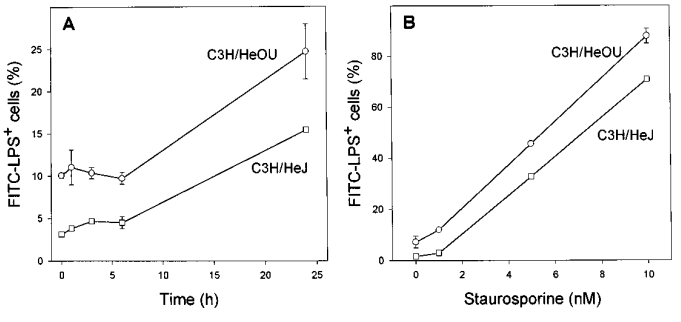

Fig. 2. STP-induced expression of LPS-binding sites on mouse BMC. BMC (5 × 10^5 cells) from C3H/HeOU (○) or C3H/HeJ mice (□) were incubated at 37° for different times with 10 nM STP (A) or for 24 hr with various concentrations of STP (B) in CM (400 μl) in the absence of serum. The stimulated cells were then incubated (18 hr, 4°) with FITC-LPS (0.2 μg/ml) in medium containing 10% FCS. The percentage of fluorescent cells was determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic mean ± standard deviation of duplicates.

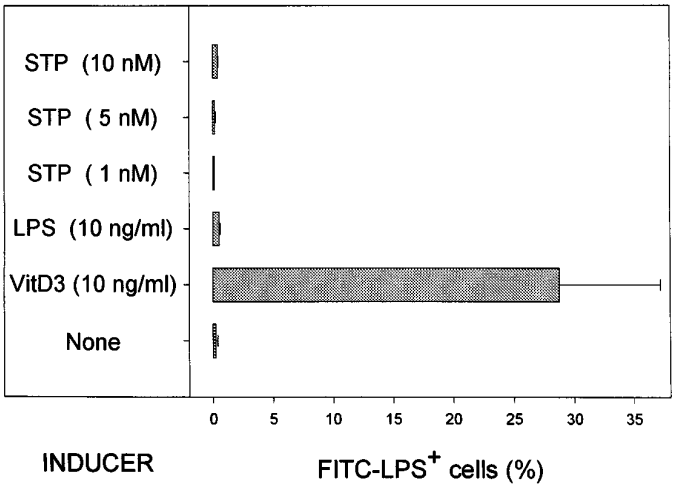

Fig. 3. Induced expression of LPS-binding sites on a human monocytic cell line. Mono Mac 6 cells (2 × 10^5 cells) were incubated for 48 hr at 37° with different inducers. After equilibration for 1 hr at 4°, the cells were incubated (18 hr, 4°) with FITC-LPS (0.2 μg/ml) in medium containing 10% FCS. The percentage of fluorescent cells was determined by FACS analysis. Values represent the mean ± standard deviation of two determinations.

TABLE 1

Inhibition of FITC-LPS binding to LPS-binding sites expressed on stimulated BMC

BMC from C3H/HeOU and C3H/HeJ mice were first exposed (24 hr, 37°C) to LPS (10 ng/ml) or STP (5 nM) in CM (400 μl) in the absence of serum. The LPS- and STP-stimulated cells were then equilibrated for 1 hr at 4°C in medium containing 10% FCS, incubated (2 hr, 4°C) with the inhibitor alone, and reincubated (18 hr, 4°C) with FITC-LPS (0.2 μg/ml) in the presence of the inhibitor (10 μg/ml). Data represent the percentage of fluorescent cells determined by FACS analysis. Values represent the mean ± Standard deviation of duplicate determinations.

Inhibitor	C3H/HeOU cells stimulated with		C3H/HeJ cells stimulated with	
	LPS	STP	LPS	STP
None	62.8 ± 0.8	69.0 ± 2.6	1.9 ± 0.5	61.4 ± 1.6
LPS (10 μg/ml)	1.8 ± 0.3	1.5 ± 0.0	0.1 ± 0.0	0.4 ± 0.1
PS (10 μg/ml)	63.3 ± 0.5	70.5 ± 0.2	2.0 ± 0.3	57.6 ± 5.4

We have also shown (3) that in both mouse and human BMC, the inducible LPS-binding sites expressed after exposure to LPS are susceptible to cleavage by a phosphatidylinositol-specific phospholipase C. The human inducible receptor was then identified as CD14 with anti-human CD14 antibodies (3). The recent availability of a rat anti-mouse CD14 (14) allowed us to examine whether the LPS receptors induced by LPS and STP on mouse BMC can also be identified as CD14. FACS analysis of LPS- and STP-stimulated BMC from C3H/HeOU and C3H/HeJ mice showed complete parallelism between the stainings with FITC-LPS and anti-mouse CD14 (Fig. 4). The binding of both reagents was enhanced with STP-treated C3H/HeOU and C3H/HeJ cells and with LPS-treated C3H/HeOU cells (Fig. 4, C, F, and B, respectively) but was unaffected with LPS-treated C3H/HeJ cells (Fig. 4E). These results show that STP induces CD14 expression in both C3H/HeOU and C3H/HeJ BMC, whereas LPS induces this effect only in the former.

STP treatment enables BMC to be primed by LPS. It has been shown that exposure of phagocytic cells (neutrophils, granulocytes, monocytes/macrophages) to LPS primes

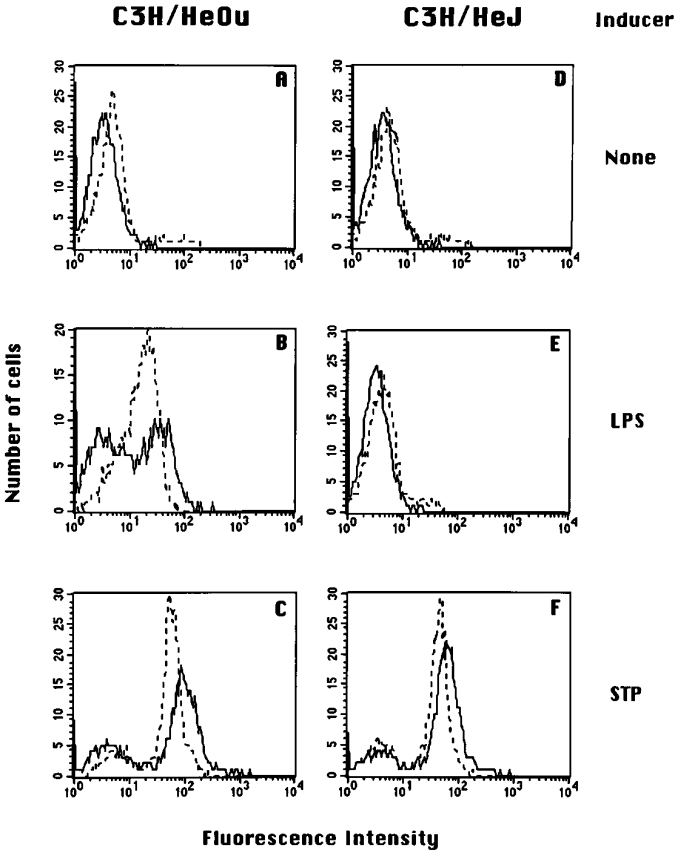

Fig. 4. Analysis of the induced LPS receptor with FITC-LPS and anti-CD14 antibody. BMC from C3H/HeOU (A–C) or C3H/HeJ mice (D–F) untreated (A and D) or cultured for 24 hr at 37° with 10 ng/ml LPS (B and E) or 10 nM STP (C and F) and then incubated with FITC-LPS (0.2 μg/ml, 18 hr, 4°) (*solid line*) or rmC5–3 (2.5 μg/ml, 30 min, 0°) (*dashed line*). The binding of rmC5–3 was detected by further incubation (30 min, 0°) with an FITC-labeled goat anti-rat Ig mAb. Fluorescence was analyzed by FACS on the gated granulocyte population.

the cells for enhanced superoxide production in response to PMA, chemotactic peptides, and many other stimuli (15). This priming effect of LPS has been reported to occur after interaction of LPS with CD14 (15). Therefore, to evaluate the biological significance of the expression of CD14 induced by STP, we examined the priming effect of LPS for PMA-induced generation of superoxide. BMC pretreated with STP were compared with untreated cells. The results in Fig. 5 show that untreated BMC produce superoxide in response to PMA, but LPS cannot enhance this production. On the other hand, in cells pretreated with STP, the direct response to PMA was reduced, but this response was significantly enhanced by LPS. This result shows that after STP treatment, BMC acquire both a new differentiation antigen (CD14) and a new functional potentiality (sensitivity to LPS priming for enhanced superoxide production).

Inhibition of protein kinases does not account for the inducing activity of STP. One obvious interpretation of the STP-induced expression of CD14 could be that a protein kinase is involved in a retroactive control that down-regulates the level of membrane CD14. If this hypothesis is correct, other inhibitors of protein kinases should also enhance the LPS-binding capacity of BMC. Therefore, we compared the influence of STP with that of another structurally

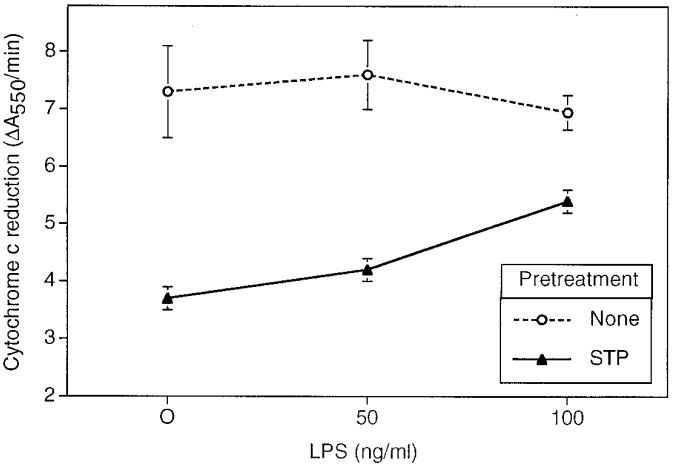

Fig. 5. Enhanced superoxide production induced by LPS in STP-treated BMC. BMC were preincubated (18 hr, 37°) with (▲) or without (○) STP (5 nM). After washings, the cells (2.5×10^5 cells/well) were incubated (30 min, 37°) with various concentrations of LPS. Cytochrome *c* (75 μM) and PMA (0.2 μg/ml) were then added, and absorbance at 550 nm (A_{550}) was measured (four replicates) at different times. The rates of superoxide generation are represented by the mean ± standard deviation of the rates of variation of A_{550} (ΔA_{550}/min) and were calculated by linear regression analysis of absorbances collected during the first 20 min.

unrelated and nonselective inhibitor of serine/threonine protein kinases: the synthetic compound H7. We found that exposure to compound H7 did not trigger the expression of the inducible LPS receptor detectable with FITC-LPS (Fig. 6B) or with the anti-CD14 mAb rmC5–3 (data not shown). Another protein kinase inhibitor, calphostin C, was also inactive in this test (Fig. 6C). Because compound H7 and calphostin C (isoquinolinesulfonamide and perylenequinone, respectively) are structurally unrelated to the alkaloid STP, it follows that the inducing activity of STP must be attributed to some structural characteristic that is unrelated to its protein kinase inhibitory activity. Therefore, in further analyses, only compounds with structures related closely to STP (indocarbazoles and bisindolylmaleimides) were examined for their capacity to stimulate BMC.

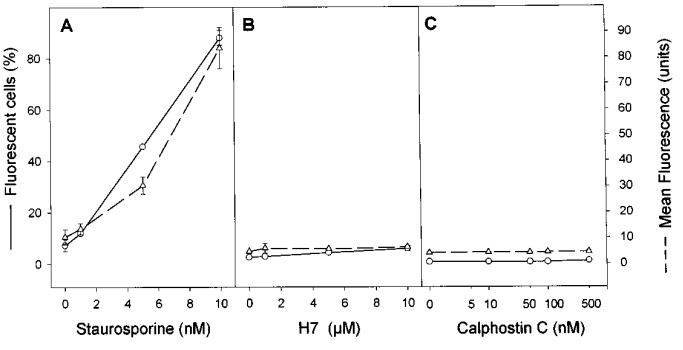

Fig. 6. Influences of STP, H7, and calphostin C on LPS receptor expression. BMC (5×10^5 cells) from C3H/HeOU mice were incubated at 37° for 24 hr with various concentrations of STP (A), H7 (B), or calphostin C (C) in CM (400 μl) in the absence of serum. The stimulated cells were then incubated (18 hr, 4°) with FITC-LPS (0.2 μg/ml) in medium containing 10% FCS. The percentage of fluorescent cells (○) and the mean fluorescence intensity (Δ) were determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic mean ± standard deviation of duplicates.

The inducing activity of STP is due to its glycoside moiety. The STP structure consists in a pyranose-like ring (glycoside moiety) linked to two nitrogen atoms belonging to an indolo-2,3-α-carbazole skeleton (aglycon moiety). To determine which of these two regions is involved in the inducing activity of STP, we used two STP analogs, K-252a and UCN-01. The former has an aglycon region identical to that of STP and differs in its glycoside moiety, which is a five-atom ring, whereas the latter has a glycoside region identical to that of STP but differs in its aglycon moiety, which is hydroxylated in position 7 of the indolocarbazole skeleton (Fig. 1). The ability of these compounds to enhance the LPS-binding capacity of BMC was examined. We found that compound UCN-01 was active, whereas compound K-252a was not (Fig. 7). This result suggests strongly that the inducing activity of STP and UCN-01 is due to the only region absent from the K-252a structure: the pyranose-like ring.

Comparison of several STP analogs for CD14 induction in BMC from C3H/HeJ mice. We showed above that a change in the glycoside region of STP, leading to compound K-252a, abolished the activity; however, to confirm that the particular glycoside ring of STP is required, we wanted to demonstrate that small modifications of the aglycon skeleton cannot also give active analogs. Therefore, we compared the activities of the three compounds used above (STP, UCN-01, and K-252a) with those of four other analogs exhibiting small structural differences in the aglycon region: compounds: K-252b, KT-5823, RO-31–8220, and GF-109203X (Fig. 1). To be quite certain that differences in possible endotoxin contamination would not lead to misinterpretations of the results, a comparison of the various compounds was performed with BMC from endotoxin-unresponsive C3H/HeJ mice. The expression of CD14 was analyzed with both the specific antibody rmC5–3 (Fig. 8A) and the FITC-LPS ligand (Fig. 8B). The results were identical with both reagents and showed clearly that only STP and UCN-01, which bear the same six-atom glycoside-like ring, were active.

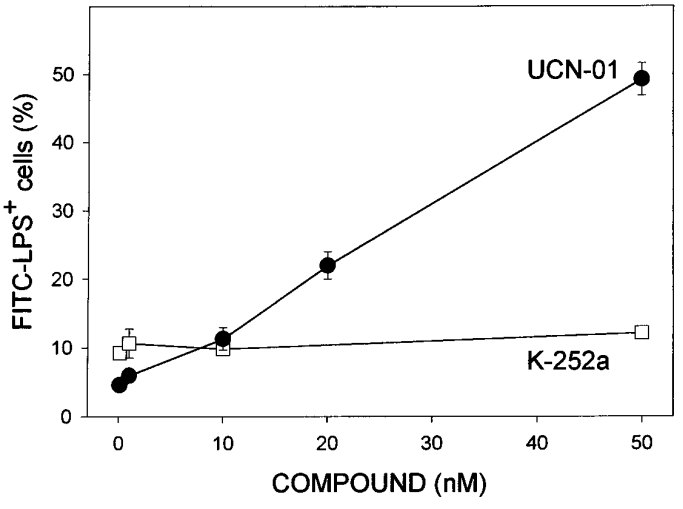

Fig. 7. Influence of K-252a and UCN-01 on LPS receptor expression. BMC (5×10^5 cells) from C3H/HeOU mice were incubated at 37° for 24 hr with various concentrations of K-252a (□) or UCN-01 (●) in CM (400 μl) in the absence of serum. The stimulated cells were then incubated (18 hr, 4°) with FITC-LPS (0.2 μg/ml) in medium containing 10% FCS. The percentage of fluorescent cells was determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic mean ± standard deviation of duplicates.

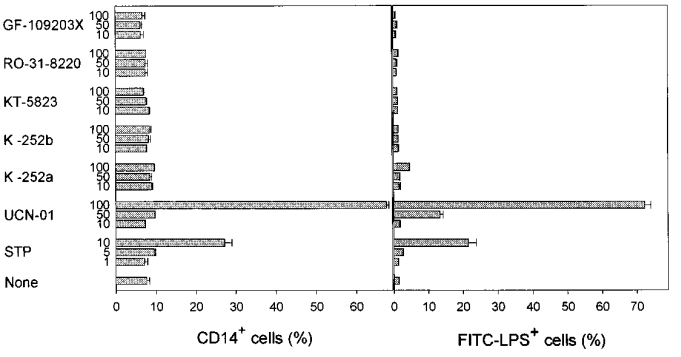

Fig. 8. Influence of various STP analogs on LPS receptor expression in C3H/HeJ cells. BMC (5×10^5 cells) from C3H/HeJ mice were incubated at 37° for 24 hr with different concentrations of STP (1 to 10 nM) or structural analogs (UCN-01, K-252a, K-252b, KT-5823, RO-31–2820, GF-109203X) (10–100 nM) in CM (400 μl) in the absence of serum. The stimulated cells were then incubated with rmC5–3 (2.5 μg/ml, 30 min, 0°) (A) or with FITC-LPS (0.2 μg/ml, 18 hr, 4°) (B) in medium containing 10% FCS. The binding of rmC5–3 was detected by further incubation (30 min, 0°) with an FITC-labeled goat anti-rat Ig mAb. The percentage of fluorescent cells was determined by FACS analysis of the gated granulocyte population. Values are mean ± standard deviation of duplicates.

Additive effects of LPS and STP. Because both LPS and STP can induce the LPS receptor CD14 in BMC, we analyzed the influence of simultaneous exposure of the cells to the two agents to determine whether these inducers will act synergistically, additively, or competitively. We examined the enhancement of the LPS-binding capacity of BMC from C3H/HeOU and C3H/HeJ mice. For this type of experiment, it was more appropriate to analyze the increase of the mean fluorescence of the cells than the increase of the percentage of fluorescent cells because cells with different levels of fluorescence are scored together in the latter type of analysis. We examined the influence of increasing concentrations of one of the agents in the presence or absence of a constant concentration of the second agent (10 ng/ml LPS or 5 nM STP). In the LPS-unresponsive C3H/HeJ cells, we observed, as expected, that the addition of LPS did not modify their response to STP (Fig. 9C) and that the addition of STP induced an increase of the response that was independent of the concentration of LPS (Fig. 9D). With the LPS-responsive C3H/HeOU cells, we observed that the dose-response curve to one of the agents (LPS or STP) was shifted to an upper parallel curve when a constant concentration of the second agent was added to the incubation medium (Fig. 9, A and B). It is noteworthy that this increase in cell response was also observed when STP was added to a saturating dose of LPS (10 ng/ml) (Fig. 9B). The observation that the influence of one agent was independent of the dose of the other agent with mixtures of LPS and STP demonstrates that the combined effects of the two agents are neither synergistic nor competitive; rather, they are purely additive.

Differential effects of K-252a on LPS- and STP-induced stimulation. The additivity of the effects of LPS and STP suggests strongly that the mechanisms leading to CD14 expression induced by these two agents are different. Because STP induces CD14 expression and simultaneously inhibits a wide panel of protein kinases, we can assume that protein kinases are not involved in the mechanism of STP-induced expression of CD14. To confirm this assumption and

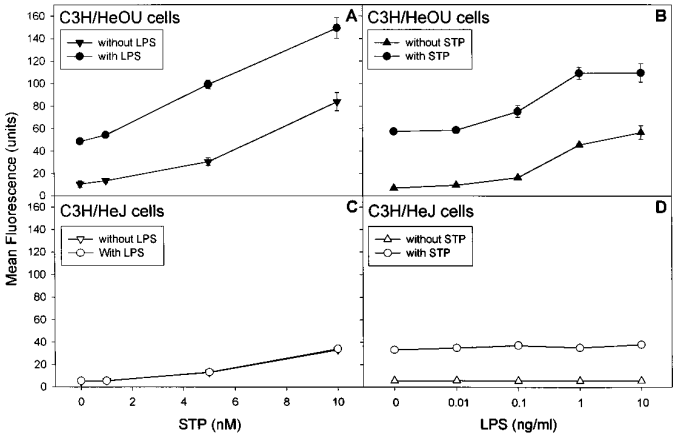

Fig. 9. Additive effects of LPS and STP. BMC from C3H/HeOU (A and B) or C3H/HeJ mice (C and D) were incubated (24 hr, 37°) with various concentrations of STP in the presence or absence of 10 ng/ml LPS (A and C) or various concentrations of LPS in the presence or absence of 5 nM STP (B and D). Incubations were carried out in CM (400 μl) in the absence of serum, and STP was always added 2 hr before LPS. The stimulated cells were then incubated (18 hr, 4°) with FITC-LPS (0.2 μg/ml) in medium containing 10% FCS. The percentage of fluorescent cells was determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic mean ± standard deviation of duplicates.

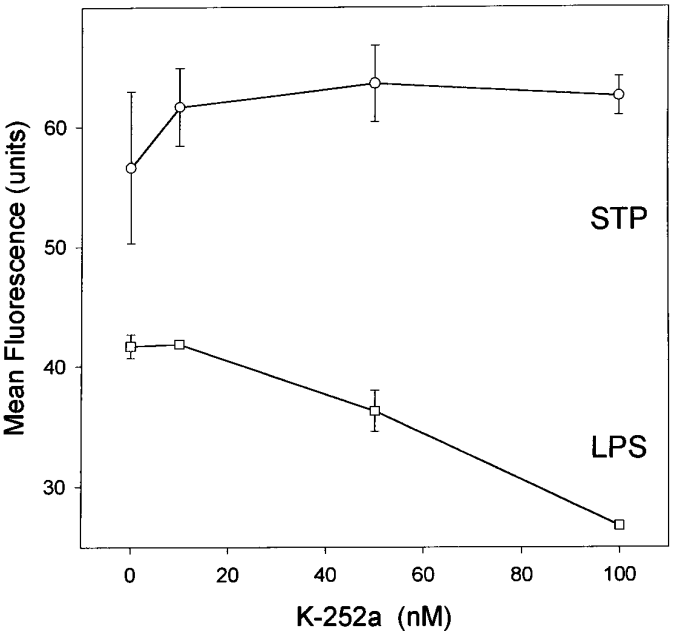

Fig. 10. Influence of K-252a on LPS receptor expression induced by LPS and STP. BMC (5×10^5 cells) from C3H/HeOU mice were preincubated for 2 hr at 37° with various concentrations of the protein kinase inhibitor K-252a in CM (400 μl) in the absence of serum. The cells were then stimulated for 24 hr at 37° with 10 ng/ml LPS (□) or 5 nM STP (○) in the presence of the inhibitor. The stimulated cells were then incubated (18 hr, 4°) with FITC-LPS (0.2 μg/ml) in medium containing 10% FCS. The mean fluorescence intensity of the cells was determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic mean ± standard deviation of duplicates.

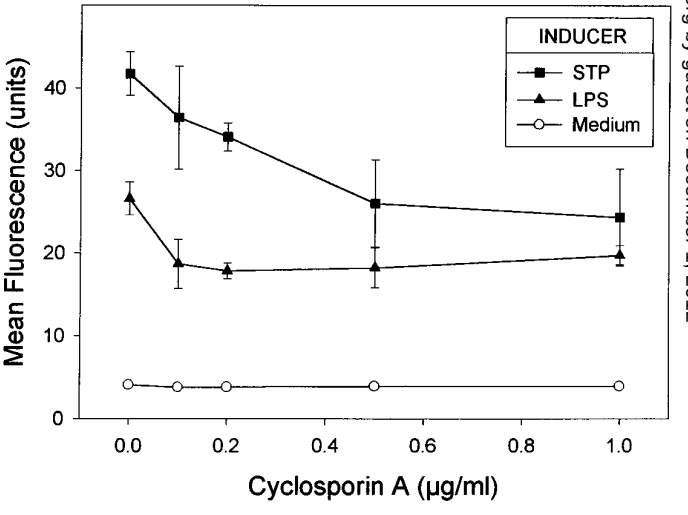

Fig. 11. Influence of cyclosporin A on LPS receptor expression induced by LPS and STP. BMC (5×10^5 cells) from C3H/HeOU mice were preincubated for 2 hr at 37° with various concentrations of cyclosporin A in CM (400 μl) in the absence of serum. The cells were then incubated for 24 hr at 37° in CM alone (○) or with 10 ng/ml LPS (▲) or 5 nM STP (■) in the continuous presence of cyclosporin A. The stimulated cells were then incubated (18 hr, 4°) with FITC-LPS (0.2 μg/ml) in medium containing 10% FCS. The mean fluorescence intensity of the cells was determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic mean ± standard deviation of duplicates.

determine whether the LPS-induced effect is also independent of protein kinases, we examined the influence of compound K-252a on LPS- and STP-induced stimulation. Like STP, K-252a is a potent and rather nonspecific protein kinase inhibitor. At low nanomolar concentrations, it inhibits protein kinase C (16) and cyclic nucleotide-dependent kinases (17). At micromolar concentrations, it also inhibits myosin light-chain kinase (18). We found that nanomolar concentrations of K-252a induced a partial but significant inhibition of LPS-induced stimulation (36% inhibition with 100 nM K-252a) (Fig. 10). On the other hand, the same concentrations of K-252a did not modify STP-induced stimulation. Furthermore, various other protein kinase inhibitors (H7, herbimycin A, KT-5823, and calphostin C) also seemed able to block the STP-induced expression of CD14 (data not shown). This means that at least one mechanism used by LPS to induce the LPS receptor CD14 requires protein kinase activity, whereas STP induces the same effect via a protein kinase-independent pathway.

Influence of P-glycoprotein ligands. The observation that the protein kinase inhibitory activity of STP does not account for its inducing activity (Fig. 6) is reminiscent of another activity of STP: its ability to inhibit the function of P-glycoprotein and reverse multidrug resistance in tumor cells (19). It is noteworthy that P-glycoprotein can be observed not only in cancer cells but also in several normal tissues. including BMC. It was therefore of interest to examine the influence of P-glycoprotein ligands on the LPS receptor expression induced by STP on this cell type. One of these agents is cyclosporin A. This immunosuppressive agent is a cyclic undecapeptide that has been shown to modulate multidrug resistance by interacting competitively with drug-binding sites of P-glycoprotein (20). We observed that cyclosporin A significantly inhibited the STP-induced stimulation of BMC but reduced only marginally the LPS-induced stimulation (Fig. 11). A similar inhibition of STP effect by cyclosporin A was observed in BMC from C3H/HeJ mice (data not shown). On the other hand, verapamil and vinblastine, two other P-glycoprotein ligands, induced the opposite effect;

both enhanced the STP-induced stimulation of BMC (Fig. 12). The observation that different agents known to interact with P-glycoprotein are able to modulate STP-induced stimulation could suggest that P-glycoprotein triggers BMC stimulation and CD14 expression after its interaction with STP.

Discussion

LPS is a potent stimulant of a wide variety of host cells. It induces phenotypic maturation of preb cells; proliferation of mature B cells; release of inflammatory cytokines and other mediators in monocytes/macrophages, endothelial cells, and astrocytes; and priming of polymorphonuclear leukocytes for degranulation and phagocytosis. Although the ability of LPS to interact with one of its receptors, the GPI-anchored differentiation antigen CD14, has been investigated extensively, little is known about the mechanisms of expression of this receptor, the presence of other LPS receptors on the different LPS-responsive cell types, and differences in downstream signaling mechanisms resulting from these LPS/receptor interactions.

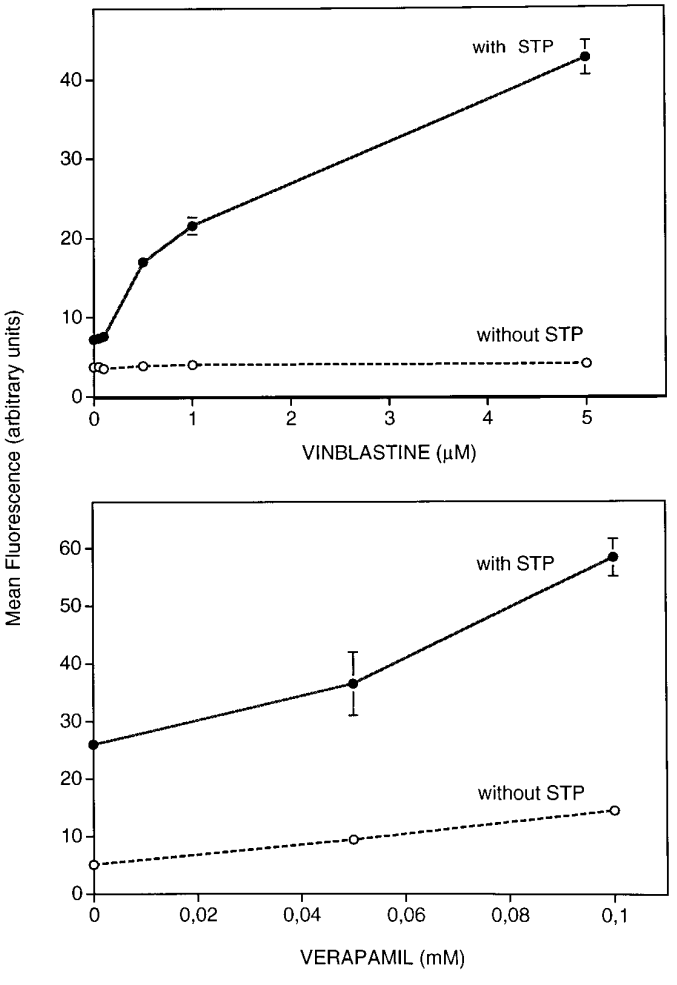

Fig. 12. Influence of vinblastine and verapamil on LPS receptor expression induced by STP. BMC (5×10^5 cells) from C3H/HeJ mice were preincubated for 2 hr at 37° with various concentrations of vinblastine (*top*) or verapamil (*bottom*) in CM (400 μl) in the absence of serum. The cells were then incubated for 24 hr at 37° in CM alone (○) or with 5 nM STP (●) in the continuous presence of vinblastine or verapamil. The stimulated cells were then incubated (18 hr, 4°) with FITC-LPS (0.2 μg/ml) in medium containing 10% FCS. The mean fluorescence intensity of the cells was determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic mean ± standard deviation of duplicates.

Such differences related to the cell type have been reported. For example, LPS-induced transcription of interleukin-1β is enhanced by cAMP in THP-1 cells ($CD14^+$ monocytes) but inhibited by the same agent in U-372GM cells ($CD14^-$ astrocytes) (21). On the other hand, it has been shown in several cell types that protein kinases inhibitable by STP play a central role in signal transduction in response to LPS. For instance, STP has been shown to inhibit LPS-induced production of nitric oxide in microglial cells (22) and suppress LPS-induced production of tumor necrosis factor-α- and LPS-induced priming for superoxide anion production (23) in alveolar macrophages. Recently, several groups documented that in monocytes/macrophages, LPS induced the activation of protein kinase C (4, 24, 25), of cAMP-dependent protein kinase (4) and of protein tyrosine kinase (4, 26, 27), all of which are inhibited by STP (6, 28). Therefore, we expected STP to also inhibit the expression of LPS receptors induced by LPS in BMC. However, the data we present show that STP does not inhibit but rather induces the expression of LPS receptors. This unexpected effect of STP is not unique. Although STP is a protein kinase C inhibitor in acellular systems, in intact cells, it may induce protein kinase C agonist effects (29, 30) and redistribution of protein kinase C isoforms (31). Cell activation by STP has thus been reported. For example, STP induces inducible nitric oxide synthase expression in rat vascular smooth muscle cells (32), release of secondary granules (vitamin B_{12}-binding protein) in neutrophils (33), increase of tumor necrosis factor-α-stimulated NO_2^- production in rat peritoneal macrophages (34), and tumor-promoting activity in normal (29) and neoplastic (30) mouse keratinocytes. In addition, STP can induce cell spreading of a human colon cancer cell line (35), morphological differentiation of human neuroblastoma cells (36), and neurotensin/neuromedin N gene expression in PC12 cells (37). Some of these effects have been interpreted as a consequence of the action of STP on protein kinases. In contrast, we showed that the STP-induced expression of LPS receptors in BMC is independent of this protein kinase inhibitory activity. This is reminiscent of another STP activity that has been reported to be independent of protein kinase inhibition: the ability to inhibit the function of P-glycoprotein (19), a 170-kDa plasma membrane transporter that works as an efflux pump for various drugs and confers multidrug resistance in tumor cells (38). The decrease of multidrug resistance induced by STP can be due to another characteristic of this agent: its ability to bind to P-glycoprotein (39). We observed that cyclosporin A, vinblastine, and verapamil, which interact with multidrug binding sites on P-glycoprotein (20), can modulate STP-induced stimulation (Figs. 11 and 12); an interesting hypothesis is that the interaction of STP with P-glycoprotein triggers a mechanism leading to the expression of LPS receptors.

This raises the question of the STP substructure that is recognized by the STP receptor. The data presented in the current study show that the induction of LPS receptor expression on BMC is triggered by the six-atom glycoside ring of STP and its analog UCN-01. Although six-atom glycoside rings are also part of the LPS structure, the possibility that a common receptor recognizes these two ligands and triggers

the same cellular effect is unlikely because the mechanisms of BMC stimulation elicited by LPS and STP seemed to be quite different. Three major differences were observed: (i) STP is active on C3H/HeJ cells, whereas LPS is inactive; (ii) at an optimal LPS concentration (1 ng/ml), the addition of more LPS does not enhance the cell response, whereas the addition of STP does; and (iii) the LPS effect is partially inhibited by compound K-252a, whereas the STP effect is not. The latter observation suggests that a protein kinase is involved in at least one of the mechanisms of LPS-induced stimulation of BMC but is not involved in STP-induced stimulation.

Another result of this study was that the LPS receptor expressed on mouse BMC after exposure to LPS or STP was identified as CD14. This myeloid differentiation antigen is a leucine-rich glycoprotein that lacks a transmembrane domain and is attached to the cell surface by a GPI anchor.Regarding other GPI-linked proteins, the anchor unit is preassembled in the endoplasmic reticulum. The addition of the anchor to the protein is an early post-translational process that requires the cleavage of a hydrophobic signal peptide at the COOH terminus of the protein, before the coupling of the GPI moiety (40). Our observation that CD14 expression can be induced by two different pathways in BMC but not with the same agents in monocytes is probably related to the complexity of the biosynthesis of GPI-anchored proteins, which depends not only on the biosynthesis of the protein moiety but also on mechanisms involved in GPI biosynthesis and attachment to the protein. The differential mechanisms elicited by LPS and STP in bone marrow granulocytes clearly deserve a better understanding, which would provide insight not only into the signaling pathways induced by endotoxins but probably also into important questions related to the biosynthesis of GPI-anchored proteins: the enzymatic processes involved in the synthesis of the anchor unit and their variations in different cell types.

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Send reprint requests to: Dr. Richard Chaby, Equipe "Endotoxines," Bâtiment 432, Université de Paris-Sud, 91405 Orsay, France. E-mail: rchaby@mailhost.pasteur.fr