We reported previously that bone marrow granulocytes respond to small amounts of enterobacterial lipopolysaccharide (LPS) via a CD14-independent and TLR4-mediated mechanism by de novo expression of an inducible receptor (CD14) and by down-modulation of a constitutive receptor (L-selectin). In this report we address another effect of LPS: the down-regulation of receptors for tumor necrosis factor-α. In mouse bone marrow cells (BMC), this down-regulation is detectable soon (20 min) after exposure of the cells to low levels (0.5 ng/ml) of LPS. This temperature-dependent effect is rather selective for LPS and requires the presence of a conventional lipid A structure in the LPS molecule and a functional TLR4 molecule in the cells. The down-modulation, due to a shedding of the receptors, is blocked by p38 MAPK inhibitors, by a furin inhibitor, and by three metalloproteinase inhibitors (BB-3103, TIMP-2, and TIMP-3). In contrast, inhibitors of MEK, protein kinase C, cAMP-dependent protein kinase, and kinases of the Src family do not block the shedding. Analysis of BMC from mice lacking tumor necrosis factor receptor-1 (CD120a⁺/−) or tumor necrosis factor receptor-2 (CD120b⁺/−) indicates that the LPS-induced shedding is specific for CD120b. Thus, exposure of BMC to LPS triggers a rapid shedding of CD120b via a protein kinase C- and Src-independent pathway mediated by p38 MAPK, furin, and metalloproteinase. The additive effects of furin and metalloproteinase inhibitors suggest that these enzymes are involved in parallel shedding pathways.

Two major cell types, the neutrophilic granulocyte and the monocyte/macrophage, are associated with susceptibility to infections (1). Both derive from granulocyte-type progenitor cells present in the bone marrow. Therefore, a better knowledge of the events following the interaction of bacterial lipopolysaccharide (LPS) with bone marrow granulocytes can help us to understand some of its in vivo effects. We have shown in a previous study (2) that granulocytes from the bone marrow of LPS-responsive mice express an inducible LPS-binding site on their cell surface after exposure in vivo or in vitro to nanomolar concentrations of LPS. This cell surface molecule was identified later as CD14 (3, 4). In contrast to this expression of a receptor, many surface markers are actually down-modulated after exposure of cells to LPS. This has been observed in macrophages with mannose receptors (5), scavenger receptors (6), interleukin 6 receptors (7), CSF-M receptors (8), and TNF-α receptors (TNF-R) (9). Concerning BMC, we found in a previous study (10) that LPS induced in these cells a marked down-regulation of TNF-R, in the absence of any direct binding of LPS to TNF-R.

Because TNF-α and its receptors play a prominent role in inflammation and in LPS-induced septic shock (11, 12), we considered that the mechanism by which LPS induces the down-regulation of TNF-R in BMC would deserve closer scrutiny. The two known TNF-α receptors CD120a (p55) and CD120b (p75) have gained wide interest because of their outstanding pattern of activities. On the level of individual cells, CD120a is involved in various aspects of innate immunity, for example by inducing death of pathogen-afflicted cells. At the multicellular level, CD120a contributes to lymphoid organ development and controls defense by coordinating the inflammatory process. At the level of the whole organism, it induces changes such as fever and elevation of acute-phase serum proteins. The extracellular domain of the TNF receptors contains membrane-proximal regions that render these molecules susceptible to shedding. The membrane form of the TNF-α cytokine has, like TNF receptors, an extracellular domain sensitive to the TNF-α convertase (TACE/ADAM-17), a transmembrane disintegrin metalloproteinase of the ADAM family of proteases (adamalysin). It has been shown that TACE can cleave a number of cell surface molecules, such as CD120b (the p75 TNF-R2), TGF-α, CD62-L (L-selectin) (13), IL-1 receptor II, CD120a (the p55 TNF-R1) (14), and the L1 adhesion molecule (15) and thus participate in inflammatory and pathological reactions.

However, other mechanisms of TNF-R down-regulation, which are not mediated by metalloproteinases, have been suggested, including shedding by serine proteases (16) or even internalization of the receptors (17).

To date, the nature and the specificity of the mechanism by which LPS down-regulates TNF-R in BMC have not been explored.

This paper is available on line at http://www.jbc.org
PMA, cholera toxin (CT), Carlson (University of Georgia, Athens, GA). biovar cells (BMC) were collected from mouse femurs. CM is RPMI 1640. C3H/HeJ mice were bred at the Pasteur Institute (Paris, France).

Animals and Cell Culture—C57BL/10 ScSn and C57BL/6 mice were purchased from Harlan (Gannat, France). C57BL/10 ScCr mice were a gift from Dr. Marina Freudenberg (Freiburg, Germany). Mice lacking TNF-α receptor 1 (CD120a+−) (18) and TNF-α receptor 2 (CD120b+−) (19) were backcrossed for 10 generations to C57BL/6 and kindly provided by Dr. H.R. Blautenthal (Hoffmann-La Roche). C3H/HeJ and C3H/HeJ mice were bred at the Pasteur Institute (Paris, France).

8–10-Week-old female mice were used in all experiments. Bone marrow cells (BMC) were collected from mouse femurs. CM is RPMI 1640 (Sigma) containing 20 mM Hepes, 1 mM sodium pyruvate, 2 mM l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. FCS was from Invitrogen.

Lipopoly saccharides—The LPSs from the Re-595 mutant of Salmonella enteria serovar minnesota (LPS-Sm) and from the JS mutant of Escherichia coli (LPS-Ec) were from Sigma. The LPSs from S. enterica serovar choleraesuis (LPS-Sc), from S. enterica serovar senftenberg (LPS-Ss), and from Bordetella pertussis (LPS-Bp), and the lipid A fraction of the latter two were prepared as described previously (20, 21). The LPSs from Rhizobium species Sin-1, Rhizobium leguminosarum biovar trifoli, 24AR were provided by Dr. R. W. Carlson (University of Georgia, Athens, GA).

Reagents—The synthetic furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (DVALCK) was purchased from Bachem (Bubendorf, Switzerland). Cytochalasin B, MG132, GF109203X, PP2, K252a, H89, and KT5720 were from Calbiochem. Phenanthroline, leupeptin, PMMA, cholora toxin (CT), N-tosyl-1-phenylalanine chloromethyl ketone (TPCK), N-tosyl-l-lysine chloromethyl ketone (TLCK), anti-goat IgG-peroxidase conjugate, and the goat anti-mouse soluble TNF receptor type II antisera were from Sigma. SB202190, SB203580, U0126, AG 126, sorbactin (CAMP), and non-fat milk in PBS). After extensive washings, sites with peroxidase activity were detected with the Supersignal West Pico chemiluminescent substrate (Pierce) according to the guidelines of the manufacturer. Molecular weight standards (ProServe color protein markers) were from BioWhittaker (Rockland, ME). All other electrophoresis reagents, including autoradiography Hyperfilm MP, were from Amersham Biosciences.

Statistics—Student’s t test was used for statistical analysis. A probability of p < 0.05 was considered significant.

RESULTS

Rapid Down-regulation of TNF-Rs Induced by LPS in BMC—We have shown in previous reports (2, 20) that very small amounts of LPS can induce the expression of CD14 within a few hours in BMC, in the absence of serum. We also reported that LPS induces in the same cells a down-modulation of the binding of TNF-α receptor (10). To investigate further the features of the latter effect and compare them to the former, we examined the influence of five parameters: LPS concentration, time, temperature, presence of serum, and presence of accessory adherent cells. In a first experiment, BMC were exposed for 1 h at 37 °C to relatively high concentrations of LPS (0.1–10 μg/ml), in the presence or absence of fetal calf serum. The cells were then washed, and the binding of 125I-TNF-α to the TNF receptors (TNF-R) was analyzed. We observed a similar down-modulation of TNF-R in the presence or absence of serum (Fig. 1A). The effect was already optimal at the lowest concentration of LPS used (0.1 μg/ml). A second experiment performed with a 1000-fold lower range of LPS concentration (0.1–10 ng/ml), and in the absence of serum, we found that a significant down-modulation of the binding of 125I-TNF-α occurs with as little as 0.5 ng/ml LPS (Fig. 1B). These results confirmed our previous finding of LPS-induced down-modulation of the binding of TNF-α and indicated that this LPS-induced effect does not require serum.

The kinetics of this LPS effect appeared markedly more rapid than that of the LPS-induced expression of CD14 mentioned above; several hours are required for LPS-induced CD14 expression (20), whereas only 20 min of exposure to LPS allows a significant reduction of the binding of 125I-TNF-α (Fig. 1C).

The kinetics of this LPS effect appeared markedly more rapid than that of the LPS-induced expression of CD14 mentioned above; several hours are required for LPS-induced CD14 expression (20), whereas only 20 min of exposure to LPS allows a significant reduction of the binding of 125I-TNF-α (Fig. 1C). On the other hand, when the cells were exposed to LPS at 4 °C, there was no detectable down-modulation of TNF-R (Fig. 1D), thus indicating that rapid temperature-dependent reactions are involved. In all experiments, the LPS-induced decrease of
indicated temperatures (C). The cells were then washed, and binding of 
C and of LPS-Sc (A) in the absence of fetal calf serum (A) was performed with total BMC, and the cells have been exposed to 
C at 37 °C, in the absence of 
R. galegae, and 
R. leguminosarum biovar trifolii indicate that the LPS-induced down-modulation of TNF-R in 
BMC requires the presence of a conventional lipid A structure.

We also observed that the LPS-induced down-modulation of TNF-R still occurs after removal of B lymphocytes from the BMC population with magnetic beads coated with an anti-B220 antibody, or after removal of adherent cells by incubation for 3 h at 37 °C in plastic dishes (data not shown). In the following sections of this paper, all experiments have been performed with total BMC, and the cells have been exposed to LPS, or to other agents, for 1 h at 37 °C, in the absence of serum.

Specificity and TLR4 Dependence of the LPS-induced Down-modulation of TNF-R—Information on the specificity of the triggering agent for TNF-R down-modulation was obtained by comparison of the effects induced by the proper TNF-R ligand (TNF-α), and by several agents selected either because they represent major components exposed on the surface of different microorganisms (LPSs in Gram-negative bacteria, peptidoglycan in Gram-positive bacteria, and lipophosphoglycans in parasites) or because they can induce another response (CD14 expression) in BMC (cAMP derivatives and cholera toxin) (23). BMC were exposed to the agents and analyzed for their capacity to bind 125I-TNF-α as mentioned above. The results (Table I) show that TNF-α, the natural ligand of TNF-R, reduced the capacity of the cells to bind 125I-TNF-α. We will show further (end of the next paragraph) that this effect is not due to binding inhibition by the unlabeled ligand, but rather to an efficient down-regulation of the receptor. On the other hand, among various other agents tested, only LPSs or a lipid A fragment thereof exhibited a down-modulation effect. The polysaccharide fragments isolated from two Salmonella LPSs were inactive. It should be noted that cAMP derivatives and cholera toxin, which induce CD14 expression in BMC as efficiently as LPS (23), were both unable to down-regulate TNF-R in these cells (Table I).

Toll-like receptor-4 (TLR4) is involved in several effects induced by LPSs of conventional structures (24). However, some LPS effects independent of TLR4 have been reported, such as induction of matrix metalloproteinase-9 (MMP-9, gelatinase) (25), production of Mn-superoxide dismutase (26), and induction of bacterial clearance (27). Therefore, it appeared important to determine whether the LPS-induced down-modulation of TNF-R was mediated or not by TLR4. In addition to normal C3H/HeOU mice, we used two mouse strains that have mutations in the Tlr4 gene as follows: the C3H/HeJ strain, which expresses non-functional TLR4 molecules, and the C57BL/10 ScCr strain, in which TLR4 is completely missing (28, 29). The results in Fig. 2 show that an LPS with a conventional lipid A structure of Sin-1, R. galegae, and R. leguminosarum biovar trifolii 24AR, reported previously (30) to induce CD14 expression in BMC by a TLR4-independent pathway, were unable to down-regulate TNF-R (data not shown). Taken together, these results clearly indicate that the LPS-induced down-modulation of TNF-R in BMC requires the presence of a conventional lipid A structure in the molecule and the presence of a functional TLR4 molecule in the cells.

Absence of LPS-induced Internalization of TNF-R—Cell surface molecules can be down-modulated by internalization or by shedding. To determine whether LPS-induced TNF-R down-modulation could be due to internalization, we used an inhibitor of cytoskeleton-dependent internalization, cytochalasin B. In a preliminary experiment, internalization of Fc receptors...
LPS-induced Shedding of CD120b in Granulocytes

(FcR) was used as a model to evaluate the efficiency of cytochalasin B as an inhibitor of internalization in BMC. The cellular density of FcR was estimated by determining the specific binding of $^{125}$I-labeled immunoglobulins at 4 °C (binding inhibitible with a large excess of unlabelled ligand). We found that in conditions where shedding is blocked (in the presence of 5 mM phenanthroline), incubation of the cells at 37 °C for 1 h induced an internalization of 93% of FcR, and 48.4% of that internalization was inhibited in the presence of 10 µg/ml cytochalasin B. Because this compound is not cytotoxic for BMC at the concentration used (as assessed by the absence of propidium iodide incorporation), it can be concluded that, as already observed in other cell types (31), cytochalasin B efficiently inhibits internalization processes in BMC. We then examined the influence of cytochalasin B on LPS-induced TNF-R down-modulation. We found that the down-modulation of TNF-R induced by 50 ng/ml LPS was 54 ± 1% in the absence of cytochalasin B and 46 ± 1% in the presence of cytochalasin B. This moderate effect of the inhibitor indicates that the large majority (85%) of the LPS-induced down-modulation of TNF-R is not attributable to internalization.

\[\text{Binding of } ^{125}\text{I-TNF-}\alpha \text{ (%)}\]

\[\text{LPS (ng/ml)}\]

| Mice          | C3H/HeOU | C3H/HeJ | C57BL/10ScCr |
|---------------|----------|---------|--------------|
| 0             | 100      | 100     | 100          |
| 1             | 90       | 90      | 90           |
| 10            | 80       | 80      | 80           |
| 100           | 70       | 70      | 70           |
| 1000          | 60       | 60      | 60           |

**Fig. 2.** Comparison of the effect of LPS on the binding of TNF-α in BMC from different mouse strains. BMC (1.5 × 10^6 in 0.5 ml CM) from normal (●) or TLR4-deficient (○ and △) mice were incubated for 1 h at 37 °C with different concentrations of LPS, in the absence of serum. The cells were then washed, and their capacity to bind $^{125}$I-TNF-α was determined as in Fig. 1. Values represent the arithmetic mean ± S.D. from the mean of duplicate experiments. Two independent experiments gave similar results.

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**Influence of Protease Inhibitors**—To evaluate the second possible hypothesis consisting of a shedding of the receptors, we analyzed the effects of several protease inhibitors. The concentration at which each inhibitor was used was selected on the basis of its cellular effect reported in the literature or previously obtained with BMC in our laboratory. The protease inhibitors were not cytotoxic at the concentration used, as assessed by the absence of propidium iodide incorporation, determined by FACS analysis. Among the four inhibitors tested, we found that two serine protease inhibitors (TLCK and TPCK) induced a marked reduction of the LPS effect (Table II). On the other hand, MG-132 and leupeptin (inhibitors of proteasome and serine/cysteine proteases, respectively) were ineffective.

The marked effect of the serine protease inhibitors, particularly TPCK, indicated that a proteolytic step is involved in TNF-R down-modulation. We examined whether TPCK can block in the same cells the down-modulation of another marker, CD16. By FACS analysis with a specific fluorescent antibody, we established in a preliminary experiment that a 27% down-modulation of CD16 occurs in BMC exposed to 100 ng/ml PMA for 1 h at 37 °C. When we examined the effects of TPCK, we found that at a concentration of 20 µM, it inhibited 90% of the LPS-induced down-modulation of TNF-R, and only 9% of the PMA-induced down-modulation of CD16. This suggests that in BMC the proteolytic step involved in LPS-induced down-modulation of TNF-R is not a common step involved in all down-modulation effects.

**Influence of Metalloproteinase Inhibitors**—Because proteolysis can lead to receptor shedding, and because shedding is often due to metalloproteinases, we examined the influence of metalloproteinase inhibitors. We first observed that 5 mM 1,10-phenanthroline, a metalloproteinase inhibitor of relatively broad specificity, markedly reduced (74 ± 9%) the LPS-induced down-modulation of TNF-R. The involvement of a metalloproteinase was confirmed by the use of a second metalloproteinase inhibitor, BB-3103. This compound is more selective than phe- nanthroline and inhibits only a restricted group of metalloproteinases, including TACE. We found (Fig. 3A) that BB-3103 inhibited a clear shedding (33%) of the LPS effect. Inhibition by BB-3103 was statistically significant at the three concentrations used (at 25 µM, p = 0.0009 in a Student’s t test).

Because TNF-α can also induce a down-modulation of TNF-R (Table I), we compared the effects of BB-3103 on TNF-α and LPS-induced down-modulations. We found that BB-3103 did not inhibit the TNF-α-induced effect (Fig. 3A). The same absence of inhibition was observed in cells from C3H/HeJ mice (data not shown). Therefore, the metalloproteinase involved in the TLR4-dependent down-modulation induced by LPS (sensitive to BB-3103) is different from the metalloproteinase involved in the TLR4-independent down-modulation induced by TNF-α, which is insensitive to BB-3103 but sensitive to phenanthroline (data not shown).

In addition to synthetic inhibitors, it appeared important to examine the effect of physiologic inhibitors of metalloproteinase, belonging to the group of tissue inhibitors of matrix metalloproteinases (TIMPs). Among the different TIMPs, it was particularly important to test TIMP-2 because, unlike TIMP-1 and TIMP-3, no inhibition of ADAMs by TIMP-2 has been reported so far. The results in Fig. 3B show that TIMP-2 inhibited a significant but quite minor (20%) inhibition of the LPS-mediated down-regulation of TNF-R. This supports the notion that matrix metalloproteinases (MMPs) play only a marginal role in this LPS effect. We also examined the effect of TIMP-3, which is known to inhibit ADAM17 (TACE) quite well. We found again that the inhibition of the LPS effect was rather low (20%) and similar to that obtained with TIMP-2 (Fig. 3B).

**TNF-R Down-regulation Is Due to the Shedding of Receptor Fragments**—The absence of internalization, and the blocking effect of protease and metalloproteinase inhibitors, suggested that TNF-R down-modulation was due to an enzymatic cleavage. Therefore, we looked for the presence of a soluble form of

### Table II

| Name       | Specificity         | Concentration (%) | Inhibition of LPS effect (%) |
|------------|---------------------|-------------------|------------------------------|
| MG 132     | Proteasome          | 10                | 12 ± 10                      |
| Leupeptin  | Serine/cysteine protease | 100           | 70 ± 7                      |
| TLCK       | Serine protease     | 100               | 90 ± 11                      |
| TPCK       | Serine protease     | 20                | 90 ± 11                      |

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TNF-R in the cell culture supernatant of LPS-treated cells. BMC from C3H/HeOU mice were exposed to 50 ng/ml LPS for 1 h at 37 °C. The capacity of the culture supernatant to inhibit the binding of \(^{125}\text{I}\)-TNF-α at 4 °C was then examined. For comparative purposes, we used the culture supernatant of an untreated cell, in which LPS was added at the same concentration (50 ng/ml). In an experiment carried out on five replicates of undiluted culture supernatants, we observed with culture supernatants of LPS-treated cells an inhibition of \(^{125}\text{I}\)-TNF-α binding (52 ± 4%) significantly higher (\(p = 0.002\) in a Student’s \(t\) test) than the effect of supernatants from untreated cells (37 ± 4%). This inhibition was observed in the cold (4 °C), suggesting that it was independent of cellular metabolism.

We also analyzed more directly the shedded components that were able to interact with TNF-α. Culture supernatants of BMC from C3H/HeOU mice, exposed to LPS or to medium alone, were analyzed by SDS-PAGE after removal of membrane blebs by centrifugation at 145,000 \(\times\) g for 1 h. TNF-R or derived fragments with TNF binding capacity were detected with \(^{125}\text{I}\)-TNF-α. Three components (59, 52, and 41 kDa) with TNF binding capacity were detected in the supernatant of BMC exposed to LPS and were not detectable in LPS-untreated cells (Fig. 4B). These components were clearly distinct from the two major intact TNF receptors (bands at 55 and 78 kDa) detected in a 1% Triton X-100 extract of LPS-untreated BMC (Fig. 4A).

Taken together, this result and that mentioned above show that three fragments of TNF-R are shed from the cell surface upon exposure to LPS and retain the capacity to bind \(^{125}\text{I}\)-TNF-α and to block the binding of this cytokine to its cellular receptors. In addition, the shedding of a 59-kDa fragment, which cannot occur from the 55-kDa membrane TNF-R1, strongly suggests that TNF-R2 is at least partially cleaved upon exposure to LPS, which cannot occur from the 55-kDa membrane TNF-R1, strongly suggests that TNF-R2 is at least partially cleaved upon exposure to LPS.

Influence of a Furin Inhibitor—The above results suggest that exposure to LPS activates a metalloproteinase involved in the shedding of TNF-R. Most metalloproteinases are regulated through a cysteine-switch mechanism that requires pro-domain removal for the protease to become active. This processing is essentially mediated by serine endoproteinases among which furin is the best characterized. This calcium-dependent transmembrane enzyme of the subtilisin family can process proteins containing the recognition motif RXKR. In an attempt to clarify the putative implication of furin in the LPS-induced shedding of TNF-R, we treated BMC with increasing concentrations of the synthetic furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (DAVLACK), and we examined the ability of these cells to shed TNF-R after exposure to LPS.
life so that shedding would require continuous processing by furin to generate an active form of the “sheddase.” A second possibility could be that a yet unidentified serine protease sensitive to the furin inhibitor (a furin-like serine protease) was activated by LPS and played a direct role in cleaving TNF-R.

Additive Effects of Furin and Metalloproteinase Inhibitors—Because inhibitions by furin or metalloproteinase inhibitors were always incomplete, it appeared important to combine different inhibitors in order to determine whether different “sheddases” are activated by LPS and whether this activation occurs by sequential (dependent) or parallel (independent) pathways. We used the metalloproteinase inhibitor BB-3103, the two tissue inhibitors of MMP mentioned above (TIMP-2 and TIMP-3), and the furin inhibitor (DAVLACK), alone or combined by pairs, at the minimal concentration at which optimal inhibitions occur. The results (Fig. 6) show that inhibitions of the LPS effect by mixtures of BB-3103 and TIMP-2 (46%) or TIMP-3 (58%) were not significantly different from the inhibition obtained with BB-3103 alone (52%). In contrast, combinations of the furin inhibitor DAVLACK and one of the three metalloproteinase inhibitors produced nearly additive inhibitions, as compared with the effects of a single compound. This is particularly clear with DAVLACK and BB-3103, which produced 64 and 52% inhibition, respectively, when used separately and 94% inhibition when used together. This result suggests that two independent pathways, mediated by a metalloproteinase and a furin, may concur to the LPS-induced shedding of TNF-R.

Role of Protein Kinases—To analyze the signaling mechanisms involved in the shedding induced by LPS, we examined the influence of various protein kinase inhibitors. Again, the concentration at which each inhibitor was used was selected on the basis of literature data and of previous observations obtained with BMC in our laboratory (4, 34). For example, we found in preliminary experiments that 25 μM SB203580, SB202190, and U126 markedly reduced the LPS-induced expression of CD14 in BMC (69, 84, and 88% inhibition, respectively). We also checked that the protein kinase inhibitors were not cytotoxic at the concentration used (absence of propidium iodide incorporation determined by FACS). BMC were then incubated at 37 °C with the inhibitors, and LPS (50 ng/ml) was added 30 min later. After 90 min at 37 °C, the cells were washed and analyzed for their capacity to bind 125I-TNF-α. Values represent the arithmetic mean ± S.D. from the mean of triplicate experiments. Four independent experiments gave similar results.
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TABLE III
Influence of various kinase inhibitors on LPS-induced down-modulation of TNF-R

| Inhibitor | Name | Specificity | Concentration (μM) | Inhibition of LPS effect (%) |
|-----------|------|-------------|-------------------|-----------------------------|
| None      |      |             | 0.05              | 0 ± 0                       |
| K252a     | Serine/threonine protein kinase | 4                | 3 ± 1              |
| GF-109203X| PKC  |             | 0.1               | 4 ± 5                       |
| H89       | PKA  |             | 0.1               | 0 ± 3                       |
| KT5720    | PKA  |             | 80                | 2 ± 4                       |
| AG126     | PTK  |             | 25                | 2 ± 2                       |
| U126      | MEK  |             | 100               | 5 ± 1                       |
| PP2       | Src tyrosine kinase | 100              |                   |

Fig. 8. Comparison of TNF-R shedding in wild and TNF-R knockout mice. BMC (cells pooled from five mixed sex animals) from wild C57BL/6 mice and from CD120a<sup>-/-</sup> or CD120b<sup>-/-</sup> animals of the same genetic background (15 × 10<sup>6</sup> cells in 0.5 ml of CM) were incubated (1 h at 37 °C) with or without LPS-Sc (50 ng/ml) in the absence of serum. The cells were then washed, and their capacity to bind <sup>125</sup>I-TNF-α was determined as in Fig. 1. Without exposure to LPS, the binding of <sup>125</sup>I-TNF-α was of 11,700 ± 760, 13,600 ± 700, and 7300 ± 970 cpm, respectively. Down-modulation induced by LPS was the percentage of decrease of bound <sup>125</sup>I-TNF-α. Values represent the arithmetic mean ± S.D. of triplicate experiments in one representative experiment. Three independent experiments gave similar results.

DISCUSSION

Our efforts to elucidate the mechanisms of various responses of BMC to LPS led us to examine, in previous studies, the expression of CD14 and, in this study, the down-modulation of TNF-Rs. Expression of CD14 in BMC is a rather slow response (requiring several hours) that can be induced by low concentrations of LPS but also by various other agents such as TNF-α (10), staurosporine (3), cholera toxin, and cAMP analogs (23). A similar response to LPS and TNF-α can also be observed in macrophages (41). In contrast, the experiments described herein show that in BMC the shedding of TNF-R induced by LPS is a rather rapid and specific response. It requires less than 30 min and cannot be triggered by other activators of BMC, such as the CD14 inducers cholera toxin and cAMP analogs. Furthermore, unlike observations carried out in vivo and showing that C3H/HeJ mice respond to LPS by shedding...
TNF-R (42), we found that in vitro, there was no shedding of TNF-R in response to enterobacterial LPS, in BMC from TLR4-defective mice (C3H/HeJ and C57BL/10 ScCr).

Other agents, such as phorbol esters, are also known to induce the shedding of various cell surface molecules (40). For example, both LPS and PMA down-modulate TNF-R in macrophages (43, 44). However, the mechanisms by which LPS and PMA trigger shedding can depend considerably on the cell type. Indeed, it has been shown that in THP-1 cells, PMA activates a shedding of TNF-α, whereas LPS does not (45). In contrast, we observed here that in BMC, LPS induced a down-modulation of TNF-R, whereas PMA did not.

The sensitivity of the LPS-induced shedding of TNF-R to metalloproteinase inhibitors strongly suggests that like the majority of receptors in various cell types, the TNF-α receptors in BMC are probably cleaved by a metalloproteinase after activation of the cells with LPS. In most cases, shedding leading to the solubilization of the functional ectodomains of cell surface proteins is due to members of the ADAM family of metalloproteinases. These sheddases contain both metalloproteinase and disintegrin (integrin-binding) domains. Three members of this family have been particularly studied: ADAM-10, ADAM-17, and MDC9 (46–48). ADAM-17, also known as TNF-α-converting enzyme (TACE), appeared first as a good candidate for the LPS-induced shedding of TNF-R in BMC, because this effect was blocked by BB-3103 which is known to inhibit TACE with good selectivity (49), and because TACE is involved in the shedding of the two TNF-Rs (CD120a and CD120b) induced by PMA in other cell types (13, 14). However, two established features of TACE do not fit with our observations. First, TACE is PKC-regulated (50), and second, TACE-induced shedding of TNF-R is generally activated by PKC (14). Therefore, the failure of a PKC inhibitor to block LPS-induced shedding of TNF-R (Table III) and the failure of PMA to down-regulate TNF-R in BMC suggest that in this cell type TACE may not be involved in the LPS-induced shedding of TNF-R. This conclusion is also confirmed by the observation that TIMP-3, a good inhibitor of TACE, induced only a moderate inhibition of this LPS effect.

Shedding by other members of the metalloproteinase disintegrin family cannot be excluded. At least 23 ADAMs have been identified to date. ADAM-10 (the mammalian homolog of Kuzbanian) and ADAM-9 (MDC9, meltrin-γ) belong to this family. Involvement of ADAM-9 in the shedding of TNF-R is unlikely because of the specificity of ADAM-9 is very different from that of TACE (48). Concerning ADAM-10, it has been shown that processing of the transmembrane protein Notch by this disintegrin is neither augmented by PMA nor regulated by PKC (51). These features are very similar to those of the LPS-induced shedding of TNF-R. A second reported example of a shedding that is not up-regulated by phorbol esters is the cleavage of HER2, a tyrosine kinase receptor that is overexpressed in breast cancer (52). The metalloproteinase involved in this shedding is as yet unidentified.

Another important branch of the superfamily of metalloproteinases is represented by the zinc-dependent family of MMPs (53). TIMPs are sometimes used to determine whether a shedding process is ADAM- or MMP-dependent. However, TIMP-1 and TIMP-3 also inhibit ADAM-10 (54), and TIMP-3 inhibits ADAM-12 and ADAM-17 (TACE) (55). Our observation that TIMP-2, which has no reported activity against ADAMs, induces a partial inhibition of the LPS-mediated shedding of TNF-R2 suggests that a fraction of this shedding (about 20%) may be due to an MMP. The ability of this MMP to be regulated by LPS suggests that it might be a membrane-type MMP, such as MT1-MMP or MT4-MMP, which are both inhibited by TIMP-2 (56) and contain a furin-susceptible site (53). However, the contribution of this metalloproteinase in the total shedding is only partial, and cleavage of TNF-R likely depends also on another enzyme.

In this regard, we found that TLCK and TPCK, two inhibitors of serine proteases, inhibited very efficiently the LPS-induced shedding of TNF-R. This can probably be ascribed to an indirect maturation role of serine proteases on sheddases. Indeed, these metalloproteinases are themselves targets of proteolytic events that can either strip off their catalytic domain or allow their maturation to an active form. In this regard, it has been suggested that matrix metalloproteinases and ADAM proteases are synthesized with a prodomain that blocks their catalytic site. They become active only after proteolytic removal of this propeptide by a mechanism known as the “cysteine switch” (57), due to a furin-like convertase that cycles between the trans-Golgi network and the plasma membrane (58). Our observation of a blocking effect by a furin inhibitor (Fig. 5) supports the involvement of furin in the LPS-induced shedding of TNF-R. Because furin is a serine protease, the inhibitory effect of TLCK and TPCK (Table II) could be due in part to a nonspecific inhibition of furin.

Because both furin and metalloproteinase are involved in TNF-R cleavage, we examined the influence of combinations of inhibitors of these two classes of enzymes (Fig. 6). We found that the effects of a combination of two metalloproteinase inhibitors were not additive, whereas almost additive effects were observed when the furin inhibitor was combined with one of the three metalloproteinase inhibitors. In particular, a combination of the furin inhibitor with the metalloproteinase inhibitor BB-3103 produced an almost complete inhibition of TNF-R shedding triggered by LPS. This result suggests the existence of two independent shedding pathways, in which a metalloproteinase and a furin are respectively involved.

Concerning the influence of protein kinase inhibitors, little is known about the signaling events involved in the regulation of the activity of sheddases. The cleavage of their substrates is regulated in different cell types by activation of PKC, calcium/camalmodulin kinases, or receptor tyrosine kinases. Unexpectedly, we found that among a number of kinases reported to mediate different cellular responses to LPS, many (PKC, cAMP-dependent protein kinase, protein tyrosine kinase, kinases of the Src family, MEK) are not involved in LPS-induced shedding of TNF-R, and only the MAP kinase p38 mediates this effect. It has been reported that the p38 MAPK can be activated by Src-dependent (59) and Src-independent (60) pathways. Our observation that PP2 did not inhibit LPS-induced shedding, whereas SB202190 did, indicates that an Src family independent activation of p38 mediates the shedding of CD120b. This observation is reminiscent to the study of Rizoli et al. (60) who...
found that shedding of L-selectin induced by hypertonicity is also mediated by Src-independent activation of p38.

The molecular mechanisms by which p38 is involved in the shedding of CD120b remain to be elucidated. We observed in previous studies on the same cell type an LPS-induced shedding of L-selectin (61) which occurred even when the cell nucleus was removed. This may suggest that in BMC, LPS-induced shedding of membrane-associated proteins is independent of the presence of the cell nucleus and, thus, that the p38-dependent shedding of TNF-α is probably not mediated by the activation of a transcription factor. Therefore, p38 could directly or indirectly trigger the activation of an involved enzyme (furin, sheddase) or make available the cleavage site of the substrate (TNF-R). We also observed a reproducible and significant difference in the ability of two p38 inhibitors to block the LPS-induced down-modulation of TNF-R. The concentration of SB203580 required for optimal inhibition of the LPS effect was lower than that of SB202190. This could be due to different sensitivities of p38 isomers to the inhibitors used.

Our study further addressed the question of the identification of the TNF-α receptor(s) actually released upon exposure to LPS. The observation that a decreased ability to bind TNF-α is found in LPS-treated CD120a knockout mice, but not in CD120b knockout mice, shows that the sheddase that is activated by LPS signaling is specific for CD120b. It should be remembered that the two TNF-α receptors differ significantly in their intracellular signaling domains (62). Most proinflammatory activities of LPS (63) and of soluble TNF-α (41, 62) are mediated through the CD120a receptor. However, it has been suggested that cell-associated TNF-α mediates some cellular responses through CD120b. The specific cleavage of CD120b that we observed in BMC is consistent with results obtained by others with monocytes and alveolar macrophages (44) showing that LPS induces mainly the release of CD120b in these cells.

The specificity of the cleavage of CD120b in BMC may have an important physiological relevance. The release of CD120b may desensitize BMC to cell-associated TNF-α of surrounding cells, and may also augment the clearance of soluble TNF-α produced in elevated amounts during inflammation. Another function of the cleavage of CD120b in the bone marrow environment could be related to the role ascribed to this receptor in hematopoiesis. CD120b is essential in TNF-mediated inhibition of primitive hematopoietic cells of the bone marrow that are involved in granulopoiesis (64). Therefore, LPS-induced shedding of CD120b from these granulocyte progenitors should enhance granulopoiesis. This can be of uttermost importance insofar as granulocytes are indispensable for efficient host defense against invading microorganisms. This may also explain the well known radioprotective effect of LPS.

In conclusion, the results show that in BMC exposed to low doses of LPS, CD120b is rapidly shed by a mechanism involving the p38 MAPK, furin, and a metalllopeptidase that may belong to the family of membrane-type MMPs. What remains unresolved is the identity of this sheddase and the exact mechanism by which it is activated via TLR4.

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2 T. Pedron, R. Girard, and R. Chaby, unpublished observations.
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