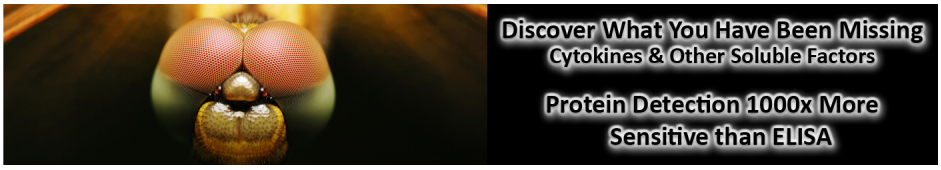

The Journal of Immunology

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Metalloproteinases Shed TREM-1 Ectodomain from Lipopolysaccharide-Stimulated Human Monocytes[1]

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Triggering receptors expressed on myeloid cell (TREM) proteins are a family of cell surface receptors that participate in diverse cellular processes such as inflammation, coagulation, and bone homeostasis. TREM-1, in particular, is expressed on neutrophils and monocytes and is a potent amplifier of inflammatory responses. LPS and other microbial products induce up-regulation of cell surface-localized TREM-1 and the release of its soluble form, sTREM-1. Two hypotheses have been advanced to explain the origin of sTREM-1: alternative splicing of TREM-1 mRNA and proteolytic cleavage(s) of mature, membrane-anchored TREM-1. In this report, we present conclusive evidence in favor of the proteolytic mechanism of sTREM-1 generation. No alternative splicing forms of TREM-1 were detected in monocytes/macrophages. Besides, metalloproteinase inhibitors increased the stability of TREM-1 at the cell surface while significantly reducing sTREM-1 release in cultures of LPS-challenged human monocytes and neutrophils. We conclude that metalloproteinases are responsible for shedding of the TREM-1 ectodomain through proteolytic cleavage of its long juxtamembrane linker. *The Journal of Immunology,* **2007, 179: 4065–4073.**

Triggering receptors expressed on myeloid cells (TREM)[3] are a family of cell surface receptors that possess an ectodomain homologous to Ig-like modules. The founding member of this family, TREM-1, is expressed mainly on monocytes and neutrophils, and has been identified as an amplifier of the immune response that strongly enhances leukocyte activation in the presence of microbial products (1, 2). Levels of TREM-1 at the cell surface are up-regulated upon challenge with LPS and other microbial stimuli (1, 3). However, and despite several investigations, the nature of TREM-1 ligand(s) remains elusive. Recently, it has been reported that human platelets express a coreceptor for TREM-1 (4). These authors indicate that interactions between platelets and polymorphonuclear neutrophils, in the presence of LPS, result in an amplification of inflammatory responses. In addition, another study shows the presence of a TREM-1 ligand in sera from septic patients (5). Finally, an exogenous ligand is present in filoviruses such as Marburg and Ebola virus (6).

In contrast, cellular signaling events downstream of TREM-1 engagement are only poorly understood. Receptor expression induced by LPS stimulation appears to be at least partly mediated by endogenous PGE_2 and triggers EP4 and cAMP/protein kinase A-dependent mechanisms, which are followed by p38 MAPK activation and PI3K-mediated signaling (7). Nevertheless, recently reported data suggest that receptor expression plays an important role in mounting an adequate inflammatory and cytotoxic response to polymicrobial sepsis, as demonstrated, e.g., by in vivo silencing of *TREM-1* (2, 8). In this regard, receptor levels are significantly higher in the course of infectious diseases.

In addition to the membrane-bound form, a soluble TREM-1 variant (sTREM-1) has been detected in mouse and human serum (9, 10). In particular, clinical studies have reported the presence of sTREM-1 in patients' serum and in bronchioalveolar lavage fluid (11). This soluble form has been found at high concentrations only in fluids from patients with microbial infections (2, 9, 12). Further, increased sTREM-1 levels have been observed in patients with ventilator-associated pneumonia (13). Other studies also suggest an up-regulation of soluble TREM-1 in plasma from patients who suffer from sepsis vs those undergoing a systemic inflammatory response syndrome (14). Of particular note, recently reported results indicate that sTREM-1 is a useful marker for the diagnosis of nosocomial sepsis (15). Finally, we note that in vivo modulation of TREM-1 activity protects endotoxinic mice from death (16, 17). Altogether, current evidence strongly suggests an important role for sTREM-1 in the evolution of infectious diseases, and indicates that the soluble receptor variant represents a reliable marker of infection, particularly during sepsis and pneumonia (18, 19).

The clinical significance of TREM-1 has been confirmed in several studies in which sTREM-1 is detected in patients with chronic obstructive pulmonary disease (20), peptic ulcer disease (21), or inflammatory bowel disease (22). The latter results underscore the involvement of TREM-1 also in noncritically ill patients and suggest that the receptor plays a general role in the innate inflammatory response (2). Interestingly, some authors suggest that a progressive decline of plasma sTREM-1 levels points to a favorable clinical evolution during the recovery phase of sepsis (9).

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[3] Abbreviations used in this paper: TREM, triggering receptors expressed on myeloid cells; sTREM, soluble form of TREM; MMP, matrix metalloproteinase; PI, propidium iodide; INH, standard proteinase inhibitor mixture.

Previous works suggest that in vitro stimulation of monocytes and neutrophils with microbial products results in the release of sTREM-1 into the culture supernatant (reviewed in Ref. 2). Despite several studies focused on sTREM-1, the actual source of this variant remains unknown. At present, there are two possible explanations for the origin of sTREM-1: translation of an alternative TREM-1 mRNA splice variant and proteolytic cleavage (shedding) of mature, cell surface-anchored TREM-1 (2). Some authors have detected an alternative TREM-1 splicing variant in monocytes and $CD34^+$ bone marrow cells (23, 24), but the predicted molecular mass of this variant would be lower than that of actual sTREM-1 found in supernatants of monocyte cultures (10). In contrast, a number of findings indicate that the soluble receptor form is generated via limited proteolysis of TREM-1. For instance, careful analysis of published data indicates that appearance of sTREM-1 coincides with a decrease of TREM-1 expression at the cell surface (3). In contrast, it is well-known that several metalloproteinases are overexpressed and/or activated in monocytes upon stimulation with microbial products such as LPS (25, 26). Protease activation proceeds partly through the PGE_2 pathway, which is also responsible for TREM-1 induction (7, 27).

On the basis of these observations, we hypothesized that sTREM-1 is generated via proteolytic cleavage(s) of membrane-bound TREM-1. We have now tested this hypothesis in human monocytes stimulated with LPS.

Materials and Methods

Reagents

All reagents were of the highest quality available and were obtained from Merck, Boehringer Mannheim, or Sigma-Aldrich. LPS (from *Salmonella abortus*) was provided by Dr. C. Galanos (Max-Planck-Institut für Immunobiologie, Freiburg, Germany). The anti-TREM-1 Ab was purchased from R&D Systems; anti-CD14-PE, anti-CD16b-FITC, and anti-CD1a-FITC were obtained from Serotec. The standard protease inhibitor mixture was obtained from Roche Diagnostics, and the general matrix metalloproteinase (MMP) inhibitor GM6001 (also known as Ilomastat) was purchased from Chemicon International.

Human monocytes isolation and culture

PBMC were isolated from blood of healthy donors by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences) as we previously reported (28, 29). Cells were initially cultured for 2 h to a density of 10^6 cells/ml in DMEM supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). After this period, the supernatant was removed and adherent cells (2×10^6/well) were cultured in the same medium supplemented with 10% heat-inactivated FBS. Purity of all cultures was verified by $CD14^+$ staining; on average, 90% of the cells presented this surface marker. Cells were then cultured in the presence or absence of 10 ng/ml LPS for different periods of time (range: from 1 to 48 h). For some experiments, a standard mixture of protease inhibitors or the general MMP and ADAM-family inhibitor, GM6001, was added 6 h after LPS challenge; the cultures were kept for a maximum of 48 h. Both the mixture of protease inhibitors and GM6001 were endotoxin-free, as assayed with a Limulus Amebocyte test (Cambrex).

Human neutrophils isolation and culture

Neutrophils were isolated from total blood in a dextran solution following a previously reported protocol (30). Purity of all cultures was verified by CD16b staining; on average, we obtained 88% of CD16b-positive cells. Cells were then cultured in the presence or absence of 10 ng/ml LPS for different periods of time. In some experiments, the general MMP inhibitor GM6001 was added 1 h after LPS challenge; the cultures were kept for a maximum of 10 h.

FACS analysis of TREM-1 expression

LPS-treated cells were washed in PBS and incubated with anti-TREM-1 Ab (R&D Systems) followed by an anti-goat FITC-conjugated secondary polyclonal Ab (Jackson ImmunoResearch Laboratories). To correct for unspecific binding, appropriate isotype control Abs were used. Samples were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) equipped with a 25 mW argon laser.

ELISA quantitation of sTREM-1

Concentrations of sTREM-1 in supernatants of human monocyte cultures were determined using a commercially available ELISA (DuoSet; R&D Systems), following the manufacturer's instructions.

Apoptosis assay

LPS-challenged cells were washed and resuspended in PBS. The apoptosis assay was performed using an Annexin $V^{FITC/PI}$ Apoptosis Detection kit (Oncogene) according to the manufacturer's recommendations. Stained cells were analyzed in a FACSCalibur (BD Biosciences) flow cytometer equipped with a 25 mW argon laser. The proportion of apoptotic cells was quantitated by plotting Annexin V^{FITC} against propidium iodide (PI) fluorescence.

Immunohistochemistry

Cells were cultured in glass wells and fixed for 15 min with 2% paraformaldehyde in PBS (pH 7.3). Then, samples were blocked using a blocking serum (Universal Quick kit; Vector Laboratories), rinsed, and finally labeled by incubating overnight at 4°C with anti-TREM-1 Ab (R&D Systems; dilution 1/1000 in PBS). The cells were washed twice in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% Tween 20 (TTBS) and incubated with a secondary FITC-conjugated Ab (1/4000) for 1 h. After rinsing with TTBS, cell nuclei were labeled by incubating with Hoechst 33342 (Molecular Probes), a cell-permeable fluorescent dye that can therefore stain nuclei of both living and fixed cells. Briefly, Hoechst stock solution (2 mM in saline) was diluted in PBS (1/8000), added to samples, and incubated for 5–10 min at room temperature. Cells were then rinsed three times with PBS; excess buffer was drained and samples finally mounted in anti-fade mounting medium. Cells were viewed and photographed on a Leica DMI6000 B (Leica Microsystems).

RNA isolation

Cells were washed once in PBS and their RNA was isolated using Tri-reagent (Imico). Purified RNA was treated with RNase-free DNase I (Amersham Biosciences) and cDNA was obtained by reverse transcription of 1 μg of RNA using a poly(dT) oligonucleotide primer (Roche). Protein fractions were isolated as recommended (Roche), and the final pellets were dissolved in a solution containing 1% SDS.

Southern blot analysis

Total cDNA was obtained from PBMCs, which were isolated from the blood of healthy donors by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences) as indicated above. Cells were initially cultured for 2 h to a density of 10^6 cells/ml in DMEM supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), and then stimulated with LPS for 1, 3, 6, 24, or 48 h. After treatment, cells were washed once with PBS and their RNA was isolated using Tri-reagent (Imico). The purified RNA was treated with RNase-free DNase I (Amersham Biosciences), and cDNA was obtained by reverse transcription of 1 μg of RNA using a poly(dT) oligonucleotide primer (Roche Diagnostics). The cDNA (40 μg/sample) was electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane (Amersham) by capillary blotting. After hybridization with a biotin-labeled probe (5′-AAATGACGCGCAGGCTTACC-3′; Bonsai), the membrane was blocked, incubated with streptavidin-peroxidase conjugate, and washed. Signals corresponding to hybridized cDNA were detected by chemiluminescence, using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham) following previously established protocols (31, 32).

Western blot analysis

Cell extracts were boiled in Laemmli buffer, resolved on 15% SDS-polyacrylamide gels in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS), and transferred to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad) at 300 mA for 1.5 h at 4°C. After blocking for 1 h in TTBS containing 5% nonfat milk, membranes were washed three times in TTBS alone and probed for 20 h with anti-TREM-1 Ab (R&D Systems) diluted in TTBS. Following washing in TTBS, membranes were incubated for 45 min with a secondary HRP-conjugated Ab (diluted 1/4000), and finally washed three times with TTBS. The bound Abs were detected using ECL Plus reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech).

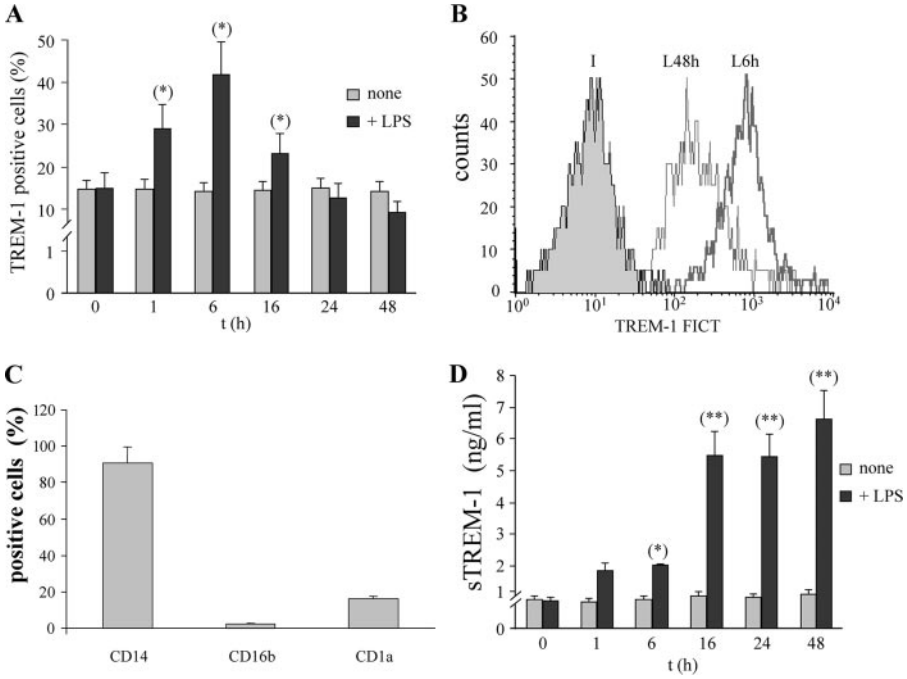

FIGURE 1. TREM-1 is expressed at the cell surface of human monocytes and released upon LPS stimulation. Cultures of human monocytes were stimulated (■) or not (▨) with 10 ng/ml LPS for indicated times. *A*, Cells were washed and harvested in PBS, stained with an anti-TREM-1 Ab as indicated in *Materials and Methods*, and finally analyzed by flow cytometry (FACS). The fraction of cells stained with anti-TREM-1 is given ($n = 4$). ∗, Value of $p < 0.05$ with respect to the control (no treatment). *B*, A typical histogram obtained from flow cytometer analysis of TREM-1 expression is shown (I: isotype; L6h and L48h: human monocytes treated with 10 ng/ml LPS for 6 and 48 h, respectively). *C*, Control cells were stained with anti-CD14-PE, anti-CD16b, and anti-CD1a Abs and then analyzed by flow cytometry; the fraction of cells stained with each Ab is given ($n = 4$). *D*, The concentration of sTREM-1 in the supernatants of the same cultures was analyzed with a commercial ELISA. The mean concentration of sTREM-1 in nanograms per milliliter is represented ($n = 4$). ∗, $p < 0.05$ and ∗∗, $p < 0.01$ with respect to the control.

Results

LPS stimulation induces cell surface overexpression of TREM-1 in human monocytes

As part of our efforts to understand the molecular basis of monocyte reactions to pathogen challenge, we first set out to establish conditions under which TREM-1 expression pattern can be reliably reproduced in vitro. As Fig. 1, *A* and *B*, shows, stimulation with 10 ng/ml LPS up-regulated basal levels of cell surface-anchored TREM-1 in cultured human monocytes. A time-course analysis of TREM-1 expression by FACS revealed that levels of

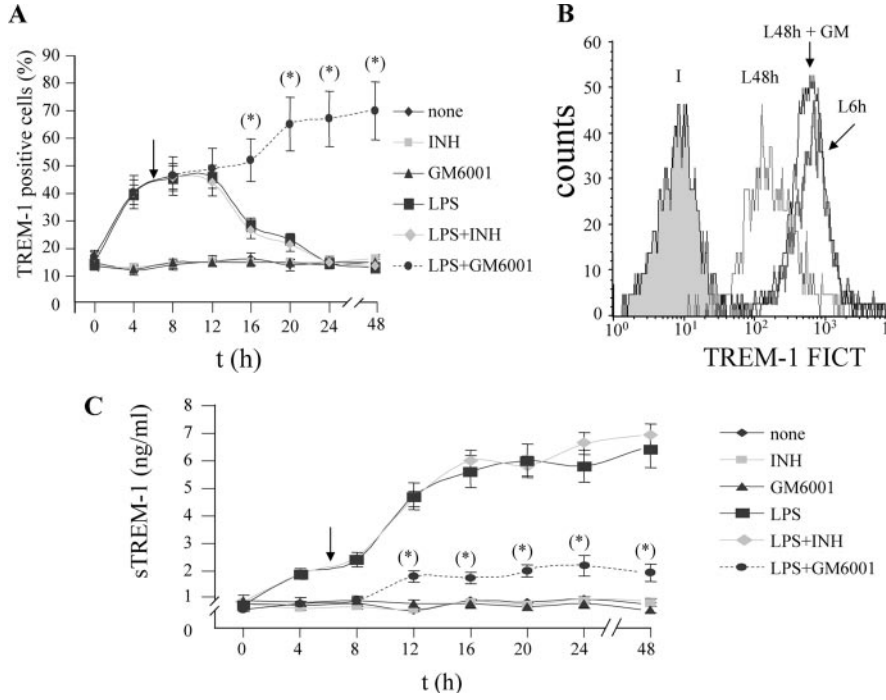

FIGURE 2. A general matrix metalloproteinase inhibitor blocks TREM-1 removal from the cell surface and reduces production of sTREM-1 in human monocyte cultures. Human monocytes were cultured in the presence or absence of 10 ng/ml LPS for indicated periods of time. In some cultures, a standard proteinase inhibitor mixture (INH) or a pan-MMP inhibitor (GM6001) were added 6 h after LPS stimulation (indicated with an arrow). *A*, TREM-1 expression at the cell surface was analyzed by flow cytometry as described in the legend to Fig. 1 and in *Materials and Methods*. The fraction of stained cells is given ($n = 3$). ∗, Value of $p < 0.01$ with respect to LPS treatment alone. *B*, A typical histogram obtained from flow cytometer analysis of TREM-1 expression is shown (I: isotype, L6h: human monocytes stimulated with 10 ng/ml LPS for 6 h, L48h: human monocytes treated with 10 ng/ml LPS for 48 h, and L48h + GM: the same condition in the presence of 10 μM GM6001). *C*, sTREM-1 concentration in the supernatants from the same cultures as in *A* was quantitated using a commercial ELISA. The results shown are the mean of three independent experiments. ∗, Value of $p < 0.01$ with respect to LPS treatment alone.

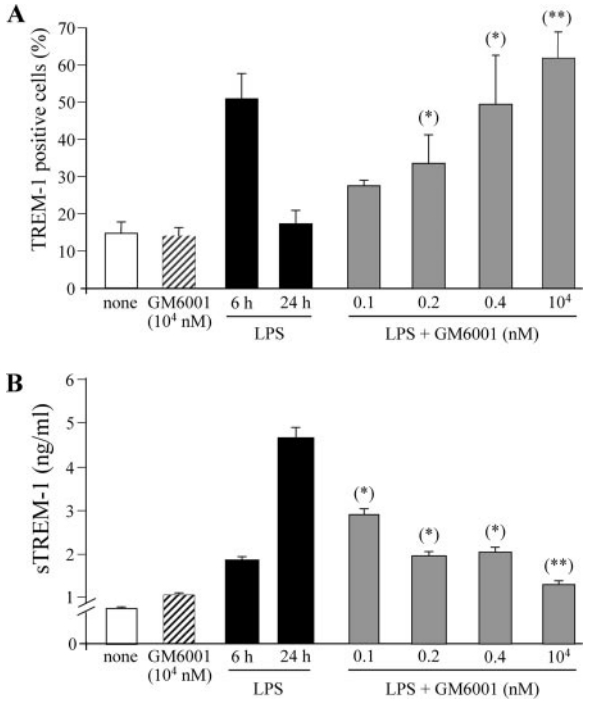

FIGURE 3. GM6001 impairs LPS-induced removal of surface-anchored TREM-1 in a dose-dependent manner. *A*, Cultures of human monocytes were stimulated with 10 ng/ml LPS alone for indicated times (■), or treated in addition with different doses of GM6001 6 h after LPS stimulation, and kept for 24 h (▨). Controls include nontreated monocyte cultures (□) and monocytes treated with the highest inhibitor dose used in these experiments (▨). Cell surface expression of TREM-1 was analyzed by flow cytometry ($n = 3$) and the fraction of stained cells is given. *B*, sTREM-1 concentration in the supernatants from the same cultures was determined using a commercial ELISA. The results shown are the mean of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ with respect to the treatment with LPS alone for 24 h.

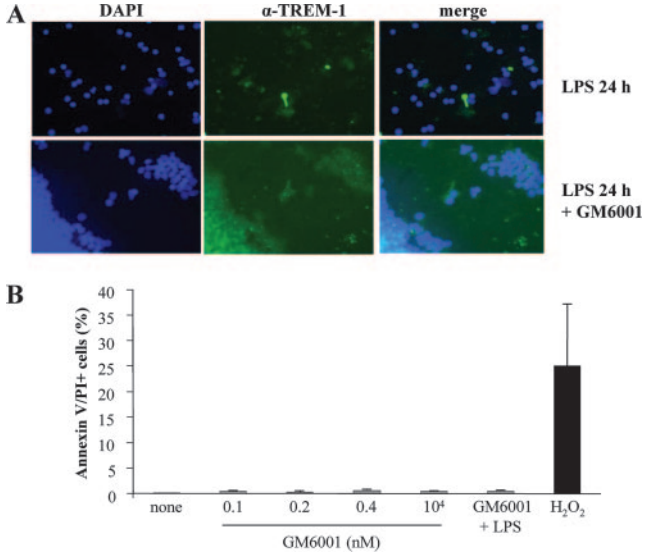

FIGURE 4. The metalloproteinase inhibitor, GM6001, blocks removal of membrane-bound TREM-1 without affecting cell viability. *A*, Cultures of human monocytes were treated with 10 ng/ml LPS for 24 h. In some cultures, GM6001 was added 6 h after LPS stimulation (*lower panel*). Then, cells were stained with an anti-TREM-1 Ab (green) and nuclei were localized via standard staining with Hoechst 33342 (blue). Cells treated for 24 h with LPS alone are shown in the *upper panel.* A typical result of three independent experiments is shown. *B*, Monocytes treated with different doses of GM6001 for 24 h (▨) were stained for annexin V and PI and analyzed by flow cytometry. The fraction of double-stained, apoptotic cells is given; cells treated with 1 mM H_2O_2 are presented as positive control of apoptosis induction.

this receptor increase in a time-dependent manner, reaching a maximum (3-fold increase over background) about 6 h after LPS challenge. These findings are in line with previous reports of significant TREM-1 overexpression in the presence of microbial products such as LPS (2, 3). However, we also observed that levels of membrane-anchored TREM-1 decreased thereupon, and returned to background values 24 h after LPS stimulation. We note that monocyte cultures were essentially pure, as illustrated by the presence of ≈90% CD14-positive cells on average, with negligible levels of CD16b staining (Fig. 1*C*).

Production of sTREM-1 by human monocytes coincides with TREM-1 removal from the cell surface

Having observed a decrease in the amount of cell surface-bound TREM-1 in human monocytes stimulated with LPS 16 to 24 h after challenge (Fig. 1*A*), and hypothesizing that limited proteolysis was involved, we next wished to determine the concentration of the soluble variant in supernatants from these cultures. In our model, sTREM-1 is not detected in supernatants of untreated cultures of human monocytes isolated from healthy volunteers, which implies levels below 15 pg/ml (data not shown). However, and in striking contrast, concentration of this soluble form was notably enhanced in the supernatants of LPS-treated cultures after 16 h, and its levels remained high for at least 48 h (Fig. 1*D*). In four independent experiments, reduced cell surface expression of TREM-1 was always accompanied by a concomitant increase in sTREM-1 levels in culture supernatants.

A general MMP inhibitor but not standard proteinase inhibitors prevents generation of sTREM-1

To prove our hypothesis, we challenged cultured human monocytes with LPS, but in contrast to the previous experiment (Fig. 1), we added either a mixture of proteinase inhibitors or the general inhibitor of MMP and ADAM-family proteases, GM6001, 6 h after LPS stimulation. At this time, TREM-1 is clearly detected on the surface of LPS-stimulated monocytes (Fig. 1, *A* and *B*). No significant changes in TREM-1 expression levels were observed when we added a standard mixture of proteinase inhibitors that block activity of a broad range of serine and cysteine proteases but not metalloproteinases (standard proteinase inhibitor mixture (INH); see *Materials and Methods* and Refs. 33 and 34). However, in the presence of GM6001, TREM-1 expression at the cell surface was clearly maintained for at least 48 h, in striking contrast to the previously observed decline (Fig. 2, *A* and *B*). In line with these findings, we observed a notable decrease in the sTREM-1 concentration in supernatants of LPS-stimulated monocyte cultures that were additionally incubated in the presence of GM6001, but not in those treated with the standard inhibitor mixture (Fig. 2*C*).

Inhibition of TREM-1 cleavage by a metalloproteinase inhibitor is dose dependent

GM6001 potently inhibits MMPs 1, 2, 3, 8, and 9 in a concentration-dependent manner, with K_i values of 0.4, 0.5, 27.0, 0.1, and 0.2 nM, respectively (35, 36). To verify that the observed effect of GM6001 was indeed due to inhibition of a metalloproteinase and to narrow the range of possible MMPs actually responsible for TREM-1 cleavage, we conducted additional experiments using different doses of the general MMP inhibitor. Human monocytes cultured in the presence of LPS for 24 h exhibited basal levels of

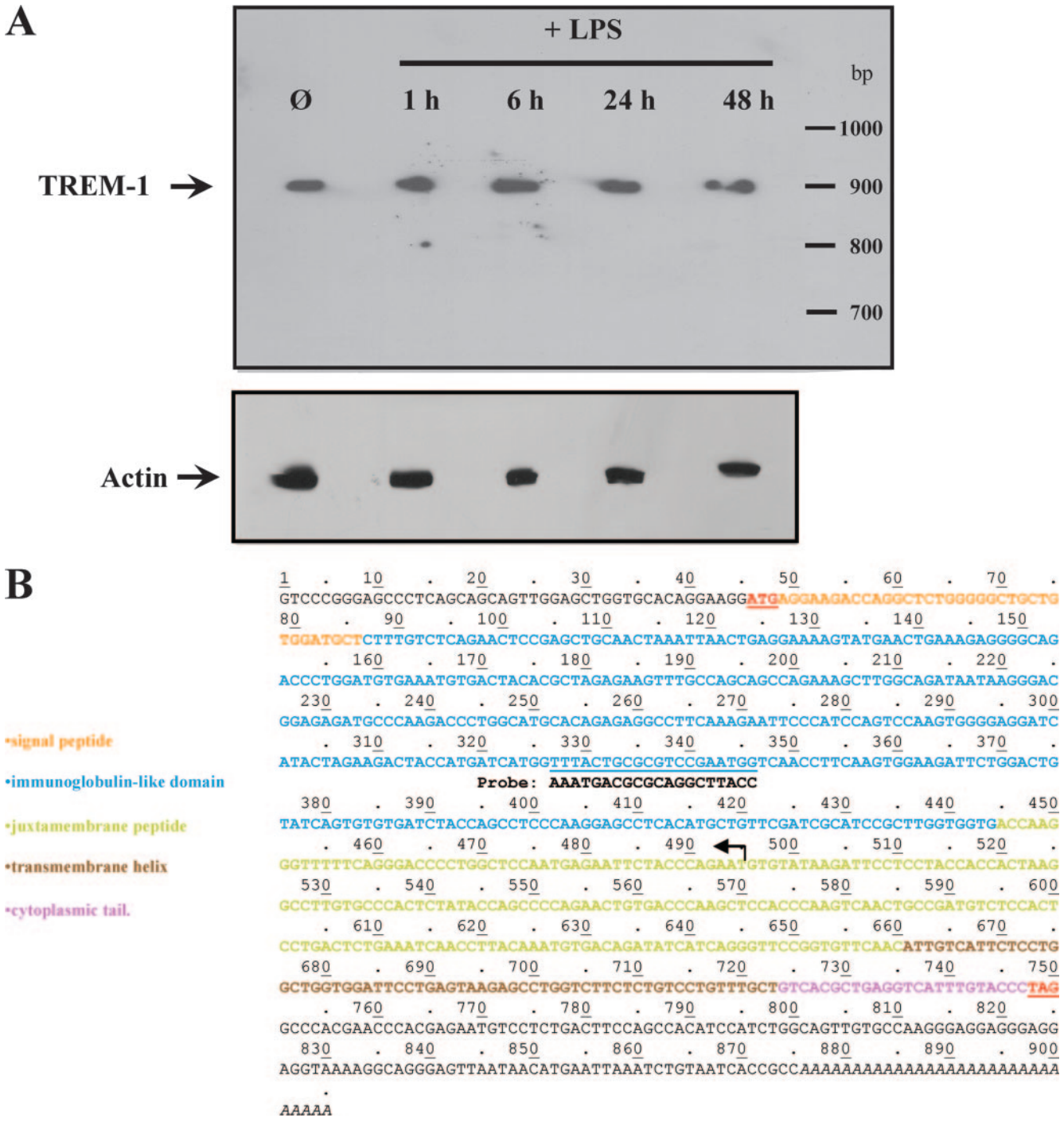

FIGURE 5. A single TREM-1 mRNA form corresponding to the full-length receptor is detected by Southern blot analysis. *A*, Human monocytes were challenged with 10 ng/ml LPS for indicated periods of time. After treatment, cells were washed with PBS and their RNA was isolated. The purified RNA was treated with RNase-free DNase I and cDNA was obtained by reverse transcription of 1 μg RNA using a poly(dT) oligonucleotide primer (Roche Diagnostics). The cDNA was electrophoresed on an agarose gel and transferred into a nitrocellulose membrane by capillary blotting. The membrane was hybridized with two biotin-labeled probes (TREM-1, *upper panel* and β-actin, *lower panel*), blocked, incubated with streptavidin/peroxidase conjugate, and washed. Signal generation was performed by chemiluminescence. A typical result is shown ($n = 4$). *B*, cDNA sequence of *TREM-1*. The regions coding for different structural elements are color coded, as indicated. The poly(A) tail is in italics and the 3′ end of the putative splice variant is indicated with an arrow. The probe used for Southern blot analysis is shown below the sequence to which it hybridizes; notice that it would bind to both full-length TREM-1 and to the reported splicing variant.

TREM-1 expression on their cell surface (Fig. 3*A*). However, the addition of GM6001 at a commonly used concentration (10 μM) not only reinstated TREM-1 expression but slightly raised the maximum level observed in the absence of the pan-metalloproteinase inhibitor (6 h after LPS stimulation). In addition, the TREM-1 decline was inhibited at much lower concentrations of GM6001 (from 0.1 to 0.4 nM). Concomitantly, sTREM-1 levels were clearly decreased in supernatants from monocyte cultures in a concentration-dependent manner (Fig. 3*B*).

Complementary results were obtained by immunofluorescence analysis, as shown in Fig. 4. Significant TREM-1 expression is observed in cultures of human monocytes treated with LPS for

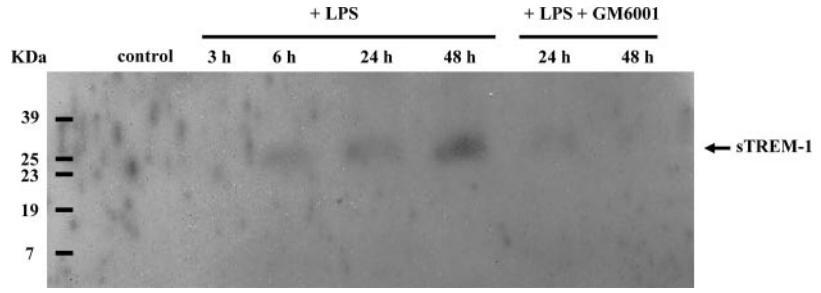

FIGURE 6. Detection of sTREM-1 in supernatants of human monocyte cultures by Western blot. Human monocytes were treated with 10 ng/ml LPS for 48 h (+LPS). In some cultures, GM6001 (10 μM) was added 6 h after LPS stimulation (+LPS + GM6001). Then, cell supernatants were centrifuged at 400 $\times$ *g* to remove detached cells and possible cellular debris. A Western blot analysis of supernatants was performed using an anti-TREM-1 commercial Ab that recognizes extracellular domain of TREM-1. Notice detection of a band of ~27 kDa 6 h after LPS challenge (marked with an arrow), whose intensity increased up to the end of the experiment (48 h). A typical experiment from three is shown.

24 h in the presence of GM6001, but not in the absence of the pan-MMP inhibitor (Fig. 4*A*). In addition, these experiments clearly demonstrate membrane localization of mature TREM-1.

We also verified that the observed effects were not due to the toxicity of GM6001 by staining human monocytes treated with different doses of the inhibitor and/or LPS using an Annexin^{PI} kit. As shown in Fig. 4*B*, no significant apoptosis was observed in the presence of these stimuli.

Alternative splicing of TREM-1 is not detected in LPS-stimulated human monocytes

To directly exclude alternative splicing as a source for sTREM-1, we performed a Southern blot analysis of *TREM-1* cDNA using a biotin-labeled probe that would hybridize both full-length TREM-1 and the reported spliced variant (Fig. 5). However, only a single band of ≈900 bases corresponding to the previously described TREM-1 mRNA (1, 7, 23, 24) was detected in our cultures of human monocytes treated with LPS for indicated times.

The apparent molecular mass of sTREM-1 suggests cleavage of glycosylated TREM-1 at a membrane-proximal site

TREM-1 ectodomain possesses a theoretical molecular mass (M_r) of 20.8 kDa. Unfortunately, we have not been able to accumulate enough sTREM-1 to accurately determine its experimental mass using mass spectrometry. However, the difference between the predicted value and the figure estimated from Western blot analysis after separation on SDS-polyacrylamide gels (≈27 kDa; Fig. 6) could be straightforwardly explained by asparagine *N*-glycosylation within three Asn-Xxx-Thr consensus sequences located in the juxtamembrane linker. To verify this possibility, we submitted the sequence corresponding to TREM-1 extracellular region to the

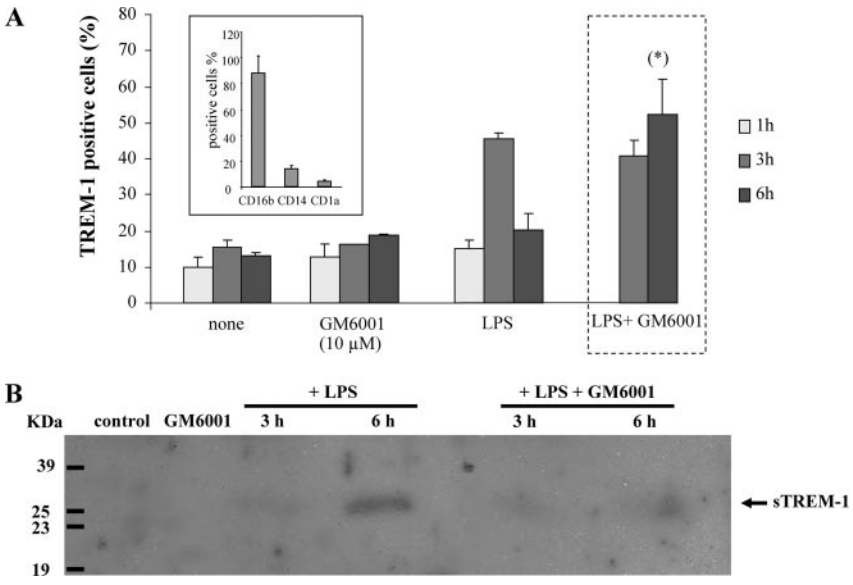

FIGURE 7. Human neutrophils challenged with LPS exhibit a similar TREM-1/sTREM-1 expression pattern as monocytes. *A*, Cultures of human neutrophils from healthy volunteers were treated with 10 ng/ml LPS for 6 h (LPS) or leaved untreated (none); some cultures were also treated with 10 μM GM6001 alone (GM6001), or the same concentration of the inhibitor was added 1 h after LPS challenge (LPS + GM6001). Next, cells were harvested and stained with anti-TREM-1-FITC and analyzed by flow cytometry. The fraction of positive cells is given ($n = 3$). *, $p < 0.01$ with respect to the treatment with LPS alone for 6 h. *Inset*, The results of flow cytometric analysis of control cells were stained with anti-CD16b, anti-CD14, and anti-CD1a Abs; the fraction of cells stained with each Ab is given ($n = 3$). *B*, Human neutrophils were treated with 10 ng/ml LPS for 3 and 6 h (+LPS). In some cultures, GM6001 was added 1 h after LPS stimulation (+LPS + GM6001). Supernatants were centrifuged at 400 $\times$ *g* to remove detached cells and possible cellular debris. A Western blot analysis of sTREM-1 was performed using a specific commercial Ab (see legend to Fig. 6), and a band of ~27 kDa was detected (see arrow). The results of a typical experiment are shown ($n = 3$).

GlycoMod server (www.expasy.org/tools/glycomod/), and multiple combinations of complex- or hybrid-type glycans were suggested as potential candidates to yield the experimental mass of 27,000 Da. In this regard, we notice that mass spectrometry analysis of several unrelated transmembrane and soluble human glycoproteins reveal frequent derivatization with complex-type oligosaccharide moieties, whose masses range between 1,800 and 3,200 Da (see e.g., Refs. 37–40). Thus, while experimental analysis of *N*-glycan composition in TREM-1 will have to await recombinant expression of this receptor, current evidence strongly suggests that two or all three potential *N*-glycosylation sites in its extracellular region are occupied by complex-type structures. sTREM-1, most probably generated via proteolytic cleavage within the membrane-proximal Val^{195}-Asn^{205} stretch would retain these oligosaccharide moieties, thus explaining migration as a species with a larger than expected molecular mass.

Human neutrophils exhibit similar patterns of TREM-1 expression and sTREM-1 generation as monocytes

Neutrophils have been reported as an important source of sTREM-1 in infectious processes (2, 6, 41). Once established that this soluble receptor form is shed from human monocytes upon metalloprotease cleavage, we tested whether a similar mechanism could be responsible for sTREM-1 generation in neutrophils. As Fig. 7 shows, human neutrophils isolated from healthy volunteers (purity by CD16b staining: ≈88%; see *inset* in Fig. 7*A*) also up-regulate expression of membrane-anchored TREM-1 after LPS challenge (Fig. 7*A*), as well as metalloprotease-mediated production of sTREM-1 (Fig. 7*B*). However, we note that proteolysis occurs markedly faster in neutrophils than in monocytes (compare Figs. 1*A* and 7*A*). In the latter cells, substantial amounts of membrane-anchored TREM-1 remain at the cell surface for at least 12 h after LPS treatment (Fig. 2*A*). By contrast, full-length, membrane-bound receptor reaches a maximum in human neutrophils ~3 h following LPS stimulation, and is down-regulated to basal levels around 6 h after this challenge (Fig. 7*A*). Despite these differences, and in line with our previous findings, presence of the metalloproteinase inhibitor GM6001 prevented both down-regulation of membrane-anchored TREM-1 (Fig. 7*A*) and sTREM-1 generation (Fig. 7*B*). Altogether, these observations suggest that the shedding mechanism described above for sTREM-1 production in human monocytes is also triggered in neutrophils in the presence of LPS. In this regard, it has been reported that neutrophils produce several metalloproteinases after LPS challenge (42).

Discussion

In previous reports, a soluble form of TREM-1, sTREM-1, has been detected in mouse and human fluids, and in vitro stimulation of monocytes and neutrophils with microbial products resulted in production of this truncated form (2). In particular, high levels of sTREM-1 were found in fluids from patients suffering from infectious diseases (9, 18, 43, 44). In our in vitro model, high concentrations of sTREM-1 are detected in supernatants of cultures of human monocytes after 16 h of LPS treatment, thus reproducing the in vivo situation. Interestingly, accumulation of sTREM-1 coincided with the disappearance of the cell surface-bound receptor (compare Fig. 1, *A* and *D*). Two alternative explanations could account for this observation: preferred translation of an alternative TREM-1 mRNA splice variant upon LPS challenge and proteolytic cleavage (shedding) of mature, cell surface-anchored TREM-1.

Our current results show that in the presence of GM6001, a general MMP and ADAM-family inhibitor with potential applications in cancer treatment (35), TREM-1 expression at the cell surface is clearly maintained for at least 48 h after LPS stimulation. Concomitantly, sTREM-1 concentrations were significantly reduced in supernatants of those cultures treated with the MMP inhibitor. These effects were not observed when a standard proteinase inhibitor was used instead (see Fig. 2, *A* and *C*). These findings strongly support our hypothesis that proteolytic cleavage(s) of membrane-anchored TREM-1 by one or several matrix metalloproteinases is responsible for sTREM-1 generation.

Translation of an alternative mRNA splice variant remains a possible source of soluble receptor, and a TREM-1-splicing form has been previously identified in human monocytes, $CD34^+$ bone marrow cells, as well as in fetal liver and spleen (23, 24). However, Southern blot analysis revealed a single band of ≈900 bases (Fig. 5*A*), which corresponds to the previously described TREM-1 mRNA coding for the full-length receptor (1, 7, 23, 24). This observation suggests that, at least in our system, alternative splicing is not a relevant source of sTREM-1.

In contrast, Western blot analysis of supernatants from our monocyte and neutrophil cultures indicates a molecular mass of ~27 kDa for the sTREM-1 isoform (Figs. 6 and 7*B*); this figure is similar to values reported by others for sTREM-1 detected in supernatants of LPS-treated monocytes (2, 10). The difference between experimental and theoretical mass (~6 kDa) is explained by attachment of complex-type glycans to two or three asparagine residues within Asn-Xxx-Thr *N*-glycosylation motifs found in the juxtamembrane region. We stress that the alternatively spliced mRNA (23, 24) codes for a receptor variant (isoform 2 in Swiss-Prot entry TREM1_HUMAN) whose theoretical M_r after removal of the signal peptide is much lower than the experimental value for sTREM-1 (≈15 kDa). In addition, this putative truncated protein lacks all three *N*-glycosylation motifs that are present in the full-length receptor, as residues 138–150 from the wild-type sequence (SGTPGSNENSTQN) are replaced by the unrelated peptide RC-STLSFSWLVDS, and residues C-terminally of 150 are missing. Even if the Asn^{146}-Ser-Thr^{148} *N*-glycosylation motif were conserved in the truncated variant, it is unlikely to be modified in vivo, because efficiently used glycosylation sites are usually located at least 60 residues away from the C terminus of the protein. In conclusion, it seems safe to exclude that sTREM-1 originates from posttranslational modification of a transcribed, alternatively spliced mRNA form.

Human monocytes produce an important number of metalloproteinases (45–47), several of which are induced upon stimulation with LPS or with a combination of GM-CSF and TNF-α (25, 48, 49). Metalloproteinases and, in particular, MMPs are being increasingly associated with the release of membrane-anchored signaling molecules in response to different stimuli. Perhaps the best-known example is TACE/ADAM17, which sheds the active form of TNF-α from its membrane-bound precursor (50, 51), but also liberates its receptor, TNFR (52), as well as several additional transmembrane proteins such as the GM-CSF receptor (53). Our current results indicate that MMPs also regulate inflammatory responses through shedding of TREM-1 ectodomain. Along these lines, it is noteworthy that uncontrolled expression of metalloproteinases accounts for loss of connective tissue associated with chronic inflammatory diseases (54, 55). In addition, our current data suggest that several metalloproteinases are involved in the enzymatic cleavage of TREM-1 (Fig. 3). However, the fact that notably low GM6001 concentrations (10 nM) sufficed to abolish LPS-mediated sTREM-1 generation seems to exclude an important role for MMP-3/stromelysin-1 in this process (K_i = 27 nM). This would be in line with the preferential expression of this MMP in skeletal muscle (www.genecards.org/cgi-bin/carddisp.pl?gene = MMP3), and its involvement in degradation of extracellular matrix

proteins (e.g., collagens, laminin, and fibronectin) rather than in signaling events.

Of particular relevance for the current investigation, all nine potential *N*-glycosylation sites are used in human ICAM-1/CD54, where mostly di- and trisialylated complex-type *N*-glycans were identified (56, 57). Interestingly, also in this case the ectodomain is shed in response to inflammatory stimuli (see e.g., Ref. 58), and this soluble form, sICAM-1, represents a marker of infection (59). TACE/ADAM17 has been recently identified as the metalloprotease that processes ICAM-1 in vivo (60). Furthermore, TACE is also responsible for stimulated shedding of VCAM-1/CD106 (61). Finally, a soluble form of PECAM-1/CD31 is also released during apoptosis (62) and in response to viral infections including HIV (63), but the responsible (metallo)protease has not been reported. We note that the extracellular regions of these important cell adhesion molecules contain from five (ICAM-1) to seven (VCAM-1) Ig-like modules, although they belong to a different subtype to those found in TREM proteins. Nevertheless, in the light of growing evidence that several members of the Ig subfamily of cell adhesion molecules are released via metalloprotease-mediated cleavage in response to inflammation/infection, we are tempted to speculate that TREM-1 cleavage is part of a broader response to diverse inflammatory clues (for a recent review on the roles of ectodomain shedding in inflammatory responses, see Ref. 64).

Finally, we also demonstrate that the soluble receptor form is produced by human neutrophils, in line with previous reports indicating that these cells contribute to sTREM-1 generation during infection (2, 6, 41). In our hands, neutrophils exhibited a different kinetics of TREM-1 expression as monocytes, and high receptor levels were observed as soon as 3 h after endotoxin challenge. Additionally, sTREM-1 was detected in supernatants of human neutrophils 6 h after LPS stimulation, also faster than in monocytes. Despite these differences between neutrophils and monocytes regarding the kinetics of TREM-1 expression and sTREM-1 shedding, presence of the metalloproteinase inhibitor GM6001 blocked in both cell types down-regulation of membrane-anchored TREM-1 and generation of the soluble receptor form. Taking together, these data strongly indicate that neutrophils also release sTREM-1 upon proteolysis of full-length TREM-1. The combined results of TREM-1 shedding from leukocytes would thus maintain high concentrations of circulating sTREM-1 from early infection times (neutrophils) and for at least 48 h (monocytes), in line with clinical observations.

In conclusion, our findings strongly suggest that limited proteolysis of membrane-anchored TREM-1 is a pathophysiologically relevant source of sTREM-1 in LPS-challenged human monocytes and neutrophils. Furthermore, our data indicate that MMPs are responsible for shedding TREM-1 ectodomain from the cell membrane, most likely through cleavage of a single peptide bond within its long juxtamembrane linker. These findings add TREM-1 to the list of membrane-bound proteins that are released by metalloproteinases to signal important inflammatory responses. We suggest that this fact should be taken into account for the development of pharmacological strategies to treat sepsis and other inflammatory diseases.

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Disclosures

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References

1. Bouchon, A., J. Dietrich, and M. Colonna. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* 164: 4991–4995.
2. Klesney-Tait, J., I. R. Turnbull, and M. Colonna. 2006. The TREM receptor family and signal integration. *Nat. Immunol.* 7: 1266–1273.
3. Knapp, S., S. Gibot, A. de Vos, H. H. Versteeg, M. Colonna, and T. van der Poll. 2004. Cutting edge: expression patterns of surface and soluble triggering receptor expressed on myeloid cells-1 in human endotoxemia. *J. Immunol.* 173: 7131–7714.
4. Haselmayer, P., L. Grosse-Hovest, P. V. Landenberg, H. Schild, and M. P. Radsak. 2007. TREM-1 ligand expression on platelets enhances neutrophil activation. *Blood* 110: 1029–1035.
5. Wong-Baeza, I., N. Gonzalez-Roldan, E. Ferat-Osorio, N. Esquivel-Callejas, R. Aduna-Vicente, L. Arriaga-Pizano, H. Astudillo-de la Vega, M. A. Villasis-Keever, R. Torres-Gonzalez, I. Estrada-Garcia, et al. 2006. Triggering receptor expressed on myeloid cells (TREM-1) is regulated post-transcriptionally and its ligand is present in the sera of some septic patients. *Clin. Exp. Immunol.* 145: 448–455.
6. Mohamadzadeh, M., S. S. Coberley, G. G. Olinger, W. V. Kalina, G. Ruthel, C. L. Fuller, D. L. Swenson, W. D. Pratt, D. B. Kuhns, and A. L. Schmaljohn. 2006. Activation of triggering receptor expressed on myeloid cells-1 on human neutrophils by Marburg and Ebola viruses. *J. Virol.* 80: 7235–7244.
7. Murakami, Y., H. Kohsaka, H. Kitasato, and T. Akahoshi. 2007. Lipopolysaccharide-induced up-regulation of triggering receptor expressed on myeloid cells-1 expression on macrophages is regulated by endogenous prostaglandin E_2. *J. Immunol.* 178: 1144–1150.
8. Gibot, S., F. Massin, M. Marcou, V. Taylor, R. Stidwill, P. Wilson, M. Singer, and G. Bellingan. 2007. TREM-1 promotes survival during septic shock in mice. *Eur. J. Immunol.* 37: 456–466.
9. Gibot, S., A. Cravoisy, M. N. Kolopp-Sarda, M. C. Bene, G. Faure, P. E. Bollaert, and B. Levy. 2005. Time-course of sTREM (soluble triggering receptor expressed on myeloid cells)-1, procalcitonin, and C-reactive protein plasma concentrations during sepsis. *Crit. Care Med.* 33: 792–796.
10. Gibot, S., M. N. Kolopp-Sarda, M. C. Bene, P. E. Bollaert, A. Lozniewski, F. Mory, B. Levy, and G. C. Faure. 2004. A soluble form of the triggering receptor expressed on myeloid cells-1 modulates the inflammatory response in murine sepsis. *J. Exp. Med.* 200: 1419–1426.
11. Determann, R. M., J. L. Millo, S. Gibot, J. C. Korevaar, M. B. Vroom, T. van der Poll, C. S. Garrard, and M. J. Schultz. 2005. Serial changes in soluble triggering receptor expressed on myeloid cells in the lung during development of ventilator-associated pneumonia. *Intensive Care Med.* 31: 1495–1500.
12. Gibot, S., and A. Cravoisy. 2004. Soluble form of the triggering receptor expressed on myeloid cells-1 as a marker of microbial infection. *Clin. Med. Res.* 2: 181–187.
13. Horonenko, G., J. C. Hoyt, R. A. Robbins, C. U. Singarajah, A. Umar, J. Pattengill, and J. M. Hayden. 2007. Soluble triggering receptor expressed on myeloid cell-1 (sTREM-1) is increased in patients with ventilator-associated pneumonia: a preliminary report. *Chest* 132: 58–63.
14. Gibot, S., and A. Cravoisy. 2004. Soluble form of the triggering receptor expressed on myeloid cells-1 as a marker of microbial infection. *Clin. Med. Res.* 2: 181–187.
15. Gibot, S., A. Cravoisy, R. Dupays, D. Barraud, L. Nace, B. Levy, and P. E. Bollaert. 2007. Combined measurement of procalcitonin and soluble TREM-1 in the diagnosis of nosocomial sepsis. *Scand. J. Infect. Dis.* 39: 604–608.
16. Gibot, S., C. Buonsanti, F. Massin, M. Romano, M. N. Kolopp-Sarda, F. Benigni, G. C. Faure, M. C. Bene, P. Panina-Bordignon, N. Passini, and B. Levy. 2006. Modulation of the triggering receptor expressed on the myeloid cell type 1 pathway in murine septic shock. *Infect. Immun.* 74: 2823–2830.
17. Gibot, S. 2006. The therapeutic potential of TREM-1 modulation in the treatment of sepsis and beyond. *Curr. Opin. Investig. Drugs* 7: 438–442.
18. Liu, C. L., W. Y. Hsieh, C. L. Wu, H. T. Kuo, and Y. T. Lu. 2006. Triggering receptor expressed on myeloid cells-1 in pleural effusions: a marker of inflammatory disease. *Respir. Med.* 101: 903–909.
19. How, C. K., C. H. Chern, M. F. Wu, L. M. Wang, C. I. Huang, C. H. Lee, and S. L. Hsieh. 2007. Expression of the triggering receptor expressed on myeloid cells-1 mRNA in a heterogeneous infected population. *Int. J. Clin. Pract.* In press.
20. Phua, J., E. S. Koay, D. Zhang, L. K. Tai, X. L. Boo, K. C. Lim, and T. K. Lim. 2007. Soluble triggering receptor expressed on myeloid cells-1 in acute respiratory infections. *Eur. Respir. J.* 28: 695–702.
21. Koussoulas, V., S. Vassiliou, M. Demonakou, G. Tassias, E. J. Giamarellos-Bourboulis, M. Mouktaroudi, H. Giamarellou, and C. Barbatzas. 2006. Soluble triggering receptor expressed on myeloid cells (sTREM-1): a new mediator involved in the pathogenesis of peptic ulcer disease. *Eur. J. Gastroenterol. Hepatol.* 18: 375–379.
22. Schenk, M., A. Bouchon, S. Birrer, M. Colonna, and C. Mueller. 2005. Macrophages expressing triggering receptor expressed on myeloid cells-1 are underrepresented in the human intestine. *J. Immunol.* 174: 517–524.
23. Gingras, M. C., H. Lapillonne, and J. F. Margolin. 2001. TREM-1, MDL-1, and DAP12 expression is associated with a mature stage of myeloid development. *Mol. Immunol.* 38: 817–824.
24. Begum, N. A., K. Ishii, M. Kurita-Taniguchi, M. Tanabe, M. Kobayashi, Y. Moriwaki, M. Matsumoto, Y. Fukumori, I. Azuma, K. Toyoshima, and

T. Seya. 2004. Mycobacterium bovis BCG cell wall-specific differentially expressed genes identified by differential display and cDNA subtraction in human macrophages. *Infect. Immun.* 72: 937–948.
25. Lu, Y., and L. M. Wahl. 2005. Production of matrix metalloproteinase-9 by activated human monocytes involves a phosphatidylinositol-3 kinase/Akt/IKKα/NF-κB pathway. *J. Leukocyte Biol.* 78: 259–265.
26. Shapiro, S. D., D. K. Kobayashi, and T. J. Ley. 1993. Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. *J. Biol. Chem.* 268: 23824–22389.
27. Wahl, L. M., and L. L. Lampel. 1987. Regulation of human peripheral blood monocyte collagenase by prostaglandins and anti-inflammatory drugs. *Cell. Immunol.* 105: 411–422.
28. Escoll, P., C. del Fresno, L. Garcia, G. Valles, M. J. Lendinez, F. Arnalich, and E. Lopez-Collazo. 2003. Rapid up-regulation of IRAK-M expression following a second endotoxin challenge in human monocytes and in monocytes isolated from septic patients. *Biochem. Biophys. Res. Commun.* 311: 465–472.
29. del Fresno, C., K. Otero, L. Gomez-Garcia, M. C. Gonzalez-Leon, L. Soler-Ranger, P. Fuentes-Prior, P. Escoll, R. Baos, L. Caveda, F. Garcia, et al. 2005. Tumor cells deactivate human monocytes by up-regulating IL-1 receptor associated kinase-M expression via CD44 and TLR4. *J. Immunol.* 174: 3032–3040.
30. Kwak, J. Y., S. W. Park, K. H. Kim, Y. J. Na, and K. S. Lee. 2002. Modulation of neutrophil apoptosis by plasma and peritoneal fluid from patients with advanced endometriosis. *Hum. Reprod.* 17: 595–600.
31. Morgan, J. G., M. Dolganov, S. E. Robbins, L. M. Hinton, and M. Lovett. 1992. The selective isolation of novel cDNAs encoded by the regions surrounding the human interleukin 4 and 5 gene. *Nucleic Acids Res.* 20: 5173–5179.
32. Simmons, A. D., and M. Lovett. 1999. Direct cDNA selection using large genomic DNA targets. *Methods Enzymol.* 303: 111–126.
33. Chung, J., D. J. Haile, and M. Wessling-Resnick. 2004. Copper-induced ferroportin-1 expression in J774 macrophages is associated with increased iron efflux. *Proc. Natl. Acad. Sci. USA* 101: 2700–2705.
34. Betelli, E., M. Dastrange, and M. Oukka. 2005. Foxp3 interacts with nuclear factor of activated T cells and NF-κB to repress cytokine gene expression and effector functions of T helper cells. *Proc. Natl. Acad. Sci. USA* 102: 5138–5143.
35. Gupta, G. P., D. X. Nguyen, A. C. Chiang, P. D. Bos, J. Y. Kim, C. Nadal, R. R. Gomis, K. Manova-Todorova, and J. Massague. 2007. Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature* 446: 765–770.
36. Galardy, R. E., D. Grobelny, H. G. Foellmer, and L. A. Fernandez. 1994. Inhibition of angiogenesis by the matrix metalloprotease inhibitor *N*-[2R-2-(hydroxamidocarbonymethyl)-4-methylpentanoyl)]-L-tryptophan methylamide. *Cancer Res.* 54: 4715–4718.
37. Kapron, J. T., G. M. Hilliard, J. N. Lakins, M. P. Tenniswood, K. A. West, S. A. Carr, and J. W. Crabb. 1997. Identification and characterization of glycosylation sites in human serum clusterin. *Protein Sci.* 6: 2120–2133.
38. Forno, G., M. Bollati Fogolin, M. Oggero, R. Kratje, M. Etcheverrigaray, H. S. Conradt, and M. Nimtz. 2004. *N*- and *O*-linked carbohydrates and glycosylation site occupancy in recombinant human granulocyte-macrophage colony-stimulating factor secreted by a Chinese hamster ovary cell line. *Eur. J. Biochem.* 271: 907–919.
39. Kleinova, M., A. Buchacher, A. Heger, K. Pock, A. Rizzi, and G. Allmaier. 2004. Exact molecular mass determination of various forms of native and de-*N*-glycosylated human plasma-derived antithrombin by means of electrospray ionization ion trap mass spectrometry. *J. Mass Spectrom.* 39: 1429–1436.
40. Khoshnoodi, J., S. Hill, K. Tryggvason, B. Hudson, and D. B. Friedman. 2007. Identification of *N*-linked glycosylation sites in human nephrin using mass spectrometry. *J. Mass Spectrom.* 42: 370–379.
41. Gibot, S. 2005. Clinical review: role of triggering receptor expressed on myeloid cells-1 during sepsis. *Crit. Care* 9: 485–489.
42. Shimizu, T., K. Kanai, Y. Kyo, K. Asano, T. Hisamitsu, and H. Suzaki. 2006. Effect of tranilast on matrix metalloproteinase production from neutrophils in-vitro. *J. Pharm. Pharmacol.* 58: 91–99.
43. Kofoed, K., O. Andersen, G. Kronborg, M. Tvede, J. Petersen, J. Eugen-Olsen, and K. Larsen. 2007. C-reactive protein, procalcitonin, neutrophils, macrophage migration inhibitory factor, soluble urokinase-type plasminogen activator receptor, and soluble triggering receptor expressed on myeloid cells-1 plasma levels used in combination for diagnosing infections: a prospective study. *Crit. Care* 11: R38.
44. Gibot, S., A. Cravoisy, B. Levy, M. C. Bene, G. Faure, and P. E. Bollaert. 2004. Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. *N. Engl. J. Med.* 350: 451–458.
45. Speidl, W. S., W. G. Toller, C. Kaun, T. W. Weiss, S. Pfaffenberger, S. P. Kastl, A. Furnkranz, G. Maurer, K. Huber, H. Metzler, and J. Wojta. 2004. Catecholamines potentiate LPS-induced expression of MMP-1 and MMP-9 in human monocytes and in the human monocytic cell line U937: possible implications for peri-operative plaque instability. *FASEB J.* 18: 603–605.
46. Lai, W. C., M. Zhou, U. Shankavaram, G. Peng, and L. M. Wahl. 2003. Differential regulation of lipopolysaccharide-induced monocyte matrix metalloproteinase (MMP)-1 and MMP-9 by p38 and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases. *J. Immunol.* 170: 6244–6249.
47. Min, D., A. G. Moore, M. A. Bain, S. N. Breit, and J. G. Lyons. 2002. Activation of macrophage promatrix metalloproteinase-9 by lipopolysaccharide-associated proteinases. *J. Immunol.* 168: 2449–2455.
48. Campbel, E. J., J. D. Cury, S. D. Shapiro, G. I. Goldberg, and H. G. Welgus. 1991. Neutral proteinases of human mononuclear phagocytes: cellular differentiation markedly alters cell phenotype for serine proteinases, metalloproteinases, and tissue inhibitor of metalloproteinases. *J. Immunol.* 146: 1286–1293.
49. Woo, C. H., J. H. Lim, and J. H. Kim. 2004. Lipopolysaccharide induces matrix metalloproteinase-9 expression via a mitochondrial reactive oxygen species-p38 kinase-activator protein-1 pathway in Raw 264.7 cells. *J. Immunol.* 173: 6973–6980.
50. Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J Castner, K. L. Stocking, P. Reddy, S. Srinivasan, et al. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature* 385: 729–733.
51. Moss, M. L., S. L. C. Jin, M. E. Milla, W. Burkhart, H. L. Carter, W. J. Chen, W. C. Clay, J. R., Didsbury, D. Hassler, C. R. Hoffman, et al. 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. *Nature* 385: 733–736.
52. Peschon, J. J., J. L. Slack, P. Reddy, K. L. Stocking, S. W. Sunnarborg, D. C. Lee, W. E. Russell, B. J. Castner, R. S. Johnson, J. N. Fitzner, et al. 1998. An essential role for ectodomain shedding in mammalian development. *Science* 282: 1281–1284.
53. Rovida, E., A. Paccagnini, M. Del Rosso, J. Peschon, and P. Dello Sbarba. 2001. TNF-α-converting enzyme cleaves the macrophage colony-stimulating factor receptor in macrophages undergoing activation. *J. Immunol.* 166: 1583–1589.
54. Greenlee, K. J., Z. Werb, and F. Kheradmand. 2007. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. *Physiol. Rev.* 87: 69–98.
55. Gunia, S., K. Albrecht, M. May, and P. Stosiek. 2006. Imbalance of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinases: a novel approach for explaining the parenchymal liquefaction of the septic spleen? *Pathobiology* 73: 198–204.
56. Otto, V. I., T. Schurpf, G. Folkers, and R. D. Cummings. 2004. Sialylated complex-type *N*-glycans enhance the signaling activity of soluble intercellular adhesion molecule-1 in mouse astrocytes. *J. Biol. Chem.* 279: 35201–35209.
57. Otto, V. I., E. Damoc, L. N. Cueni, T. Schürpf, R. Frei, S. Ali, N. Callewaert, A. Moise, J. A. Leary, G. Folkers, and M. Przybylski. 2006. *N*-glycan structures and *N*-glycosylation sites of mouse soluble intercellular adhesion molecule-1 revealed by MALDI-TOF and FTICR mass spectrometry. *Glycobiology* 16: 1033–1044.
58. Rothlein, R., E. A. Mainolfi, M. Czajkowski, and S. D. Marlin. 1991. A form of circulating ICAM-1 in human serum. *J. Immunol.* 147: 3788–3793.
59. Figueras-Aloy, J., L. Gomez-Lopez, J. M. Rodriguez-Miguelez, M. D. Salvia-Roiges, I. Jordan-Garcia, I. Ferrer-Codina, X. Carbonell-Estrany, and R. Jimenez-Gonzalez. 2007. Serum soluble ICAM-1, VCAM-1, L-selectin, and P-selectin levels as markers of infection and their relation to clinical severity in neonatal sepsis. *Am. J. Perinatol.* 24: 331–338.
60. Tsakadze, N. L., S. D. Sithu, U. Sen, W. R. English, G. Murphy, and S. E. D'Souza. 2006. Tumor necrosis factor-α-converting enzyme (TACE/ADAM-17) mediates the ectodomain cleavage of intercellular adhesion molecule-1 (ICAM-1). *J. Biol. Chem.* 281: 3157–3164.
61. Garton, K. J., P. J. Gough, J. Philalay, P. T. Wille, C. P. Blobel, R. H. Whitehead, P. J. Dempsey, and E. W. Raines. 2003. Stimulated shedding of vascular cell adhesion molecule 1 (VCAM-1) is mediated by tumor necrosis factor-α-converting enzyme (ADAM 17). *J. Biol. Chem.* 278: 37459–37464.
62. Ilan, N., A. Mohsenin, L. Cheung, and J. A. Madri. 2001. PECAM-1 shedding during apoptosis generates a membrane-anchored truncated molecule with unique signaling characteristics. *FASEB J.* 15: 362–372.
63. Eugenin, E. A., R. Gamss, C. Buckner, D. Buono, R. S. Klein, E. E. Schoenbaum, T. M. Calderon, and J. W. Berman. 2006. Shedding of PECAM-1 during HIV infection: a potential role for soluble PECAM-1 in the pathogenesis of NeuroAIDS. *J. Leukocyte Biol.* 79: 444–452.
64. Garton, K. J., P. J. Gough, and E. W. Raines. 2006. Emerging roles for ectodomain shedding in the regulation of inflammatory responses. *J. Leukocyte Biol.* 79: 1105–1116.