Production of Soluble Triggering Receptor Expressed on Myeloid Cells by Lipopolysaccharide-Stimulated Human Neutrophils Involves De Novo Protein Synthesis

Amr M. Mahdy,* Damon A. Lowes, Helen F. Galley, Jane E. Bruce, and Nigel R. Webster

Academic Unit of Anaesthesia and Intensive Care, School of Medicine, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom

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The triggering receptor expressed on myeloid cells (TREM-1) is a recently identified receptor expressed on neutrophils and monocytes. Activation of the receptor induces neutrophils to release the enzyme myeloperoxidase and inflammatory cytokines such as interleukin-8. TREM-1 has an alternatively spliced variant that lacks the transmembrane region, resulting in the receptor being secreted in a soluble form (sTREM-1). Soluble TREM-1 has been detected in plasma during experimental and clinical sepsis and has been advocated as a diagnostic marker of infection for pneumonia and as a prognostic marker for patients with septic shock. We studied TREM-1 surface expression, using flow cytometry, and simultaneously measured sTREM-1 concentrations in culture supernatants of lipopolysaccharide (LPS)-stimulated neutrophils. TREM-1 surface expression was constitutive and was not upregulated upon LPS stimulation. However, sTREM-1 release from neutrophils was significantly upregulated by LPS stimulation (P < 0.0001), an effect that was abrogated by cycloheximide. Soluble TREM-1 is therefore secreted by human neutrophils in response to LPS challenge in a process involving de novo protein synthesis that is not accompanied by an upregulation of the TREM-1 receptor on the surfaces of the cells.

MATERIALS AND METHODS

Power calculation. A pilot study was conducted (n = 10), and sTREM-1 concentrations were found to be 85.1 ± 43.3 and 135.6 ± 57.8 pg/ml (mean ± standard deviation [SD]) in culture supernatants of unstimulated and LPS-stimulated neutrophils, respectively. A power calculation based on neutrophil sTREM-1 release indicated that 18 subjects were required to achieve a power of 80% and a significance level of <0.05.

* Corresponding author. Mailing address: Academic Unit of Anaesthesia & Intensive Care, Institute of Medical Sciences, Forresthill, Aberdeen AB25 2ZD, United Kingdom. Phone: (0)1224 553019. Fax: (0)1224 555766. E-mail: a.mahdy@abdn.ac.uk.
Neutrophil isolation. Following Local Research Ethics Committee approval and informed consent, 30 ml venous blood was collected into sodium heparin-coated tubes, using a sterile closed system, from 18 volunteers (male/female ratio, 10/8; age range, 22 to 52 years). Neutrophils were immediately separated by single-step density gradient centrifugation, using Polymorphprep (Axis-Shield, Oslo, Norway) as previously described (16). The harvested cells were resuspended in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum, 20 mmol/liter L-glutamine, and 1% penicillin-streptomycin. Cell counts were adjusted to 1 × 10⁷ cells/ml. Neutrophil viability was assessed using trypan blue exclusion.

The cell suspension was divided into three tubes: one tube was left unstimulated, whereas LPS was added to the two other tubes to a final concentration of 1 μg/ml in the presence or absence of 10 μg/ml cycloheximide. The cells were then incubated in a humidified incubator at 5% CO₂–95% air at 37°C for 16 h. Following incubation, tubes were centrifuged, and culture supernatants were collected and stored at −20°C until further use. Cells were then harvested from culture, washed twice, and resuspended in phosphate-buffered saline. Viability was reassessed, and cell suspensions were readjusted to 1 × 10⁷ cells/ml. Neutrophil viability was consistently above 90%.

Flow cytometry analysis. Before antibody staining, cells were incubated with 10% normal human serum in phosphate-buffered saline for 20 min at 4°C to block nonspecific Fc binding. Approximately 1 × 10⁶ cells were incubated with a monoclonal anti-human TREM-1 antibody (R&D Systems, Minneapolis, Minn.), followed by an isotype-specific secondary antibody (fluorescein isothiocyanate-conjugated rat anti-mouse IgG1; BD Pharmingen, CA). Cells were subsequently incubated with a phycoerythrin-conjugated mouse anti-human CD16b antibody (BD Pharmingen, CA). Isotype-matched control antibodies were used in order to exclude nonspecific staining. Flow cytometry was carried out on a BD LSR system (Becton Dickinson), and data were analyzed using CellQuest Pro software (Becton Dickinson). The purity of the neutrophil population was initially assessed by the mean fluorescence intensity (MFI) of phycoerythrin-conjugated CD16b antibody staining (Fig. 1a) and was consistently above 95%. Neutrophils were gated according to side scatter and CD16b antibody binding characteristics (Fig. 1b), and cells within this gate were subsequently analyzed for TREM-1 surface expression (Fig. 1c).

Enzyme immunoassay. Soluble TREM-1 was measured in culture supernatants of unstimulated and LPS-stimulated neutrophils by using the commercially available DuoSet enzyme-linked immunosorbent assay development system (R&D Systems). All assays were performed in a single session, with an intra-assay coefficient of variation of 8.7% in our hands (n = 8).

Statistical analysis. Soluble TREM-1 data were not normally (log normally) distributed and were thus reported as medians (with ranges) and statistically analyzed using the Wilcoxon signed-rank test. TREM-1 expression data obtained from flow cytometry analysis were normally distributed and thus were reported as means ± SD and statistically analyzed using Student’s paired t test. Statistical analysis was performed using the Analyze-it software package, and P values of <0.05 were considered significant.

RESULTS

A preliminary dose-response experiment was performed using neutrophils from six volunteers and different concentrations of LPS. All samples were incubated for 16 h. There was a gradual increase in sTREM-1 levels in response to increasing concentrations of LPS up to 1 μg/ml; however, increasing the LPS concentration to 2 μg/ml was not accompanied by further increases in the sTREM-1 concentration (Table 1). In contrast, we did not observe any upregulation in TREM-1 surface expression in response to LPS concentrations ranging from 1 ng to 2 μg (Table 2). Based on the results of this dose-finding experiment, an LPS concentration of 1 μg/ml was used for the current study.

In our study, following 16 h of incubation, TREM-1 surface expression was similar in both LPS-stimulated and unstimulated neutrophils (P = 0.093), as judged by flow cytometry, and was not affected by coinoculation with cycloheximide (Fig. 2). Soluble TREM-1 concentrations were, however, significantly higher (P < 0.0001) in culture supernatants from LPS-stimulated neutrophils (147 [48 to 331] pg/ml) than in those from unstimulated neutrophils (89 [25 to 265] pg/ml). Coincubation of LPS-stimulated neutrophils with cycloheximide abrogated the increase in sTREM-1 (69 [25 to 161] pg/ml), and sTREM-1 release in the presence of cycloheximide was significantly lower (P < 0.001) than that from unstimulated neutrophils (Fig. 3).

DISCUSSION

TREM-1 is a transmembrane glycoprotein with a single extracellular Ig-like domain, a transmembrane region, and a short intracellular region. When TREM-1 is engaged by a
ligand, it associates with DAP12, triggering several intracellular pathways that lead to intracellular calcium mobilization, actin cytoskeleton rearrangement, and the activation of transmembrane domains of toll-like receptors (TREM)-1 ligands. In murine models, ligation of TREM-1 with monoclonal agonistic antibodies stimulates the production of inflammatory cytokines such as IL-8 and leads to rapid neutrophil degranulation and oxidative burst (3, 17). In addition, the activation of TREM-1 in the presence of a Toll-like receptor ligand amplifies the production of proinflammatory cytokines and inhibits the release of IL-10 (2).

TREM-1 mRNA has an alternatively spliced variant which encodes a 30-kDa protein that is specifically recognized by monoclonal antibodies directed against the extracellular domain of TREM-1. The variant lacks the transmembrane region, resulting in the receptor being secreted (sTREM-1). Both membrane-bound and soluble TREM-1 have the same extracellular domain, and they can compete for the same ligand(s). However, the secreted form will be incapable of transmitting a signal, suggesting a way for cells to downregulate the TREM-1 pathway (10, 12). Plasma sTREM-1 levels increased in response to LPS challenge in volunteers (13) and were shown to be higher for patients admitted to an intensive care unit with sepsis than for nonseptic patients (6). In addition, sTREM-1 levels upon admission to the intensive care unit were higher in septic patients with established septic shock, at any time point during the 14-day study period, compared with patients with shock of noninfectious origin or healthy controls (11).

Although there is evidence that sTREM-1 is produced through alternative splicing of the TREM-1 pre-mRNA (12), (3) and Knapp and colleagues reporting a downregulation of expression after in vivo administration of LPS in a human experimental endotoxemia model (13). The authors of the latter study explained the unintuitive apparent downregulation of neutrophil TREM-1 expression, which was accompanied by increased monocyte expression, as an artifact of endotoxin-mediated neutrophil sequestration (13). There are experimental differences which may explain the discrepancy between our results and the upregulation previously reported by Bouchon and colleagues (3). Firstly, they used dextran sedimentation followed by single-step density gradient centrifugation designed to optimally separate lymphocytes. Polymorphonuclear leukocytes were then retrieved, along with contaminating erythrocytes, which were removed by hypotonic shock. In contrast, we used a single-step separation system designed and validated solely for the isolation of polymorphonuclear leukocytes, which negates the need for removal of red cells and reduces in vitro activation and, thus, the potential for cell damage. Perhaps more importantly, we measured TREM-1 expression only after selection of CD16b neutrophils, whereas Bouchon and coworkers included all eosinophils and basophils in their analysis (3). Moreover, in support of our findings, a more recent study by Gibot and colleagues found no difference in neutrophil TREM-1 expression in patients with established septic shock, at any time point during the 14-day study period, compared with patients with shock of noninfectious origin or healthy controls (11).

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FIG. 3. Soluble TREM-1 concentrations in culture supernatants from neutrophils isolated from 18 subjects and incubated ex vivo for 16 h. No LPS, unstimulated cells; LPS, cells stimulated with 1 μg/ml LPS; LPS/CHX, cells stimulated with 1 μg/ml LPS in the presence of 10 μg/ml cycloheximide. Box and whisker plots show medians, 25th and 75th percentiles, and full ranges. **, P < 0.001; ***, P < 0.0001 compared to unstimulated cells.

In summary, we have demonstrated for the first time that sTREM-1 is produced as a de novo protein by LPS-activated neutrophils. It is also possible, however, that sTREM-1 might have been prestored intracellularly and required the synthesis of another protein in order to be released. We therefore concluded that sTREM-1 was released through a process involving de novo protein synthesis. This finding is in line with the finding of Gingras and colleagues, who reported sTREM-1 as an alternatively spliced variant of TREM-1, not a shed extracellular domain of the cell surface receptor (12).

In summary, we have demonstrated for the first time that sTREM-1 is produced by human neutrophils in response to LPS challenge in a process involving de novo synthesis that is not accompanied by TREM-1 receptor upregulation. This could be seen as a protective mechanism through which neutrophils produce their own TREM-1 decoy receptor as a down-regulatory response to limit the effects of persistent activation of the TREM-1/DAP12 pathway. Manipulation of the ratio of expression of sTREM-1 to TREM-1 in a favorable direction may represent a future novel therapeutic approach for the treatment of sepsis.

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