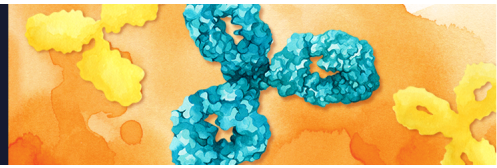


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Cutting Edge: Expression Patterns of Surface and Soluble Triggering Receptor Expressed on Myeloid Cells-1 in Human Endotoxemia

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CUTTING EDGE

Cutting Edge: Expression Patterns of Surface and Soluble Triggering Receptor Expressed on Myeloid Cells-1 in Human Endotoxemia¹Sylvia Knapp,^{2*} Sébastien Gibot,[‡] Alex de Vos,^{*} Henri H. Versteeg,^{*} Marco Colonna,[§] and Tom van der Poll^{*†}

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a recently identified molecule involved in the amplification of inflammation. To determine the regulation of TREM-1, we studied TREM-1 expression and soluble TREM-1 plasma levels upon i.v. LPS challenge in healthy humans in vivo and in vitro. Granulocyte TREM-1 expression was high at baseline and immediately down-regulated upon LPS exposure along with an increase in soluble TREM-1. Monocytes displayed a gradual up-regulation of TREM-1 upon LPS in vivo and in vitro. In vitro studies extended these findings to highly purified lipoteichoic acid and Streptococcus pneumoniae. Non-bacterial TLR ligands such as polyinosine-polycytidylic acid and imidazoquinoline, as well as the TLR9 ligand CpG, did not impact TREM-1 expression. The LPS-induced alterations in TREM-1 surface expression were not a result of increased TNF- α or IL-10. Inhibitor studies disclosed a PI3K-dependent pathway in LPS-induced up-regulation of TREM-1 on monocytes, whereas MAPK played a limited role. The Journal of Immunology, 2004, 173: 7131–7134.

Sepsis is a leading cause of mortality and morbidity worldwide and characterized by a dysregulated host response to pathogens. The excessive release of inflammatory mediators such as TNF- α contributes to the multiorgan failure and high lethality of septic patients (1, 2). Granulocytes and monocytes are key effector cells in sepsis by virtue of their capacity to recognize pathogen-associated molecular patterns, which in turn leads to the induction of the inflammatory response following infection.

Triggering receptor expressed on myeloid cells-1 (TREM-1)³ is a recently discovered cell surface molecule expressed on granulocytes, monocytes, and a subset of macrophages (3, 4). It belongs to the Ig superfamily and interacts with the adaptor protein DAP12 for signaling and function (3). Engagement of

TREM-1 results in the production of chemokines such as IL-8 and the proinflammatory cytokines TNF- α and IL-1 β (3). Although the endogenous ligand is unknown, costimulation with TLR ligands LPS (TLR4 ligand), LTA, or *Mycobacterium tuberculosis* 19-kDa lipopeptide (TLR2 ligands) synergistically enhances the inflammatory response (3, 5–7). The functional significance of TREM-1 has been underlined in murine models of LPS-induced shock and peritonitis, because blocking TREM-1 down-regulated inflammation and protected mice from lethality (5). Therefore, TREM-1 is considered an amplifier of inflammation in response to LPS and bacteria, making it an attractive therapeutic target in hyperinflammatory states like sepsis.

However, before potential therapeutic implications for TREM-1 in humans can be seriously considered, knowledge about the in vivo regulation and expression of TREM-1 has to be expanded. Investigations on TREM-1 expression are thus far limited to infected tissues obtained in later stages of infection. The in vivo expression of TREM-1 at the onset of inflammation is unknown. Therefore, we investigated the dynamics of surface and soluble TREM-1 during endotoxemia in humans.

Materials and Methods

Human endotoxemia

Endotoxemia was induced as described previously (8). Briefly, eight healthy males received an i.v. injection with *Escherichia coli* LPS, lot G (U.S. Pharmacopeial Convention, Rockville, MD), at a dose of 4 ng/kg. For FACS analysis, EDTA-anticoagulated blood was obtained before and 2, 4, 6, and 24 h after LPS challenge.

The institutional scientific and ethics committees of the Academic Medical Center (Amsterdam, The Netherlands) approved the study, and written informed consent was obtained from all study subjects.

Soluble TREM-1 (sTREM-1) concentrations

sTREM-1 levels were measured with an immunoblot technique, as described previously (9, 10). One hundred microliters of each plasma sample was dotted onto nitrocellulose membrane, dried, and then coated in PBS supplemented with 3% BSA and incubated with monoclonal anti-human TREM-1 (21C7) (3) for 60 min. After thorough rinsing, the nitrocellulose sheet was incubated with goat anti-mouse Ig (DakoCytomation, Glostrup, Denmark) for another

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³ Abbreviations used in this paper: TREM-1, triggering receptor expressed on myeloid cells-1; s, soluble; LTA, lipoteichoic acid; poly dI-dC, polyinosine-polycytidylic acid; h, human; MF, mean fluorescence.

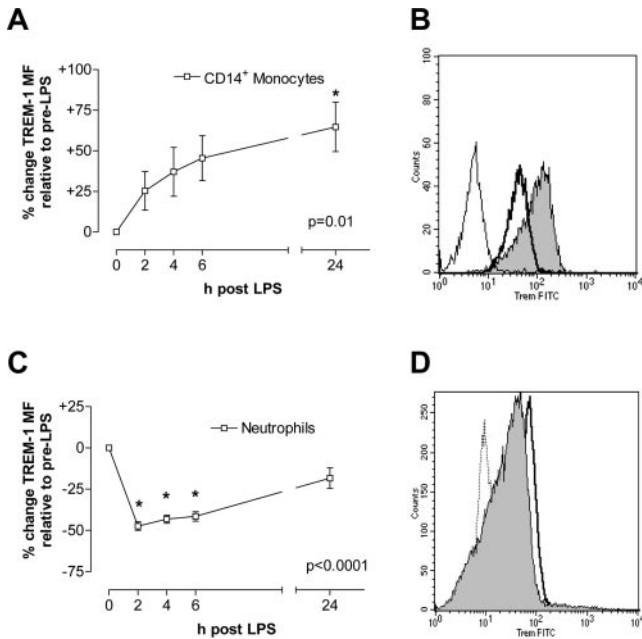


FIGURE 1. TREM-1 cell surface expression in vivo. Subjects received an i.v. injection of LPS (4 ng/kg), and the change in TREM-1 expression was monitored over 24 h. Monocytes were defined as CD14^{high} HLA-DR^{pos} cells (A and B), and granulocytes were gated according to their scatter pattern (C and D). Data shown represent results of two independent experiments. Representative histograms for monocytes (B) ($t = 0$, bold line; $t = 24$ h, filled gray; and isotype control, thin line) and granulocytes (D) ($t = 0$, bold line; $t = 2$ h, filled gray; and isotype control, thin line) are shown. Mean \pm SE of $n = 8$ per time point. *, $p < 0.05$ vs $t = 0$; p value of ANOVA is depicted in figure.

60 min. Following a washing step with PBS supplemented with 20% DMSO, the membrane was incubated with HRP-conjugated streptavidin (Bio-Rad, Marnes-la-Coquette, France) for 30 min before the enzyme substrate Opti-4CN (Bio-Rad) was added. Each sheet also contained calibration samples of a known concentration of sTREM (0–5,000 ng/ml). The staining intensity was colorimetrically compared with the standard curve with the help of a reflectance scanner and Quantity One Quantitation software (Bio-Rad). All measurements were performed in triplicate, and results are expressed as mean \pm SE. To ensure and control the specificity of the anti-TREM Ab and this technique, Western blots were performed on a routine basis (as depicted in Fig. 2B).

In vitro experiments

Whole-blood stimulations were performed as described (11), using aseptically collected blood from five to six volunteers. For some experiments, PBMC were isolated using Histopaque (Sigma-Aldrich, St. Louis, MO). The following stimuli were used: LPS, lot G (100, 1, or 0.01 ng/ml); purified lipoteichoic acid

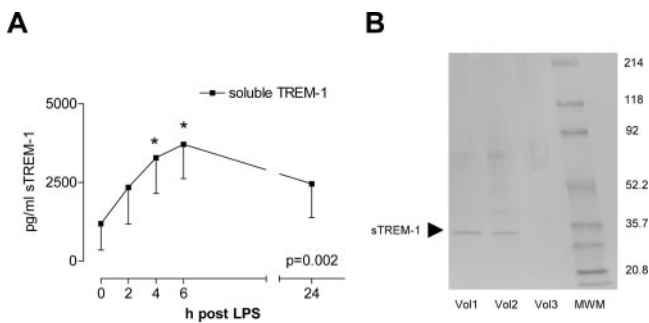


FIGURE 2. Early increase in sTREM-1 levels in vivo. A, At indicated time points, sTREM-1 levels were measured in plasma samples from volunteers that received an i.v. injection of LPS (4 ng/kg). Data are mean \pm SE of $n = 8$ per time point. *, $p < 0.05$ vs $t = 0$; p value of ANOVA is depicted in figure. B, A representative sTREM-1 blot of three volunteers (vol) at $t = 2$ h is shown.

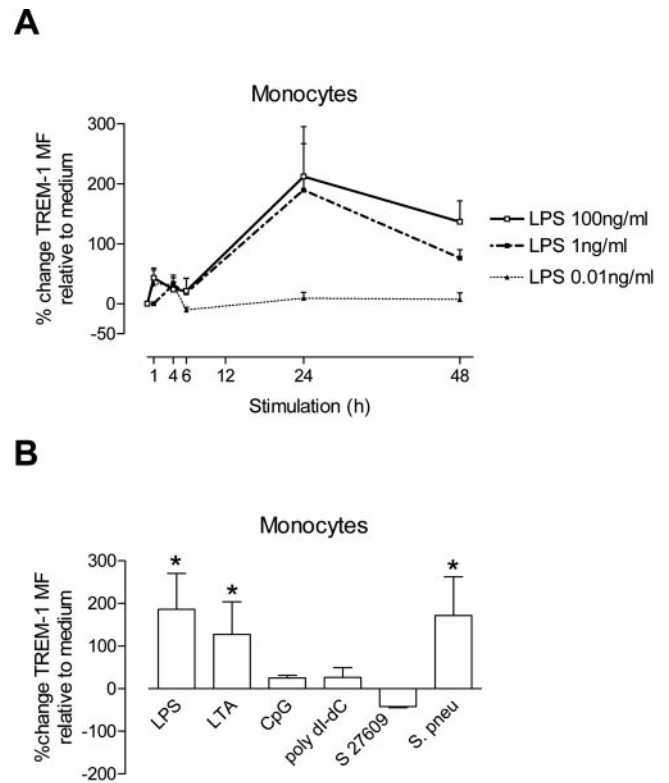


FIGURE 3. Dynamic of TREM-1 surface expression in vitro. Whole-blood stimulation was performed for indicated time points. A, Time- and dose-dependent TREM-1 up-regulation on monocytes in response to 100, 1, or 0.01 ng/ml LPS. B, Dynamic of TREM-1 surface expression on monocytes incubated with indicated TLR ligands for 24 h: LPS (100 ng/ml), LTA (10 μ g/ml), *S. pneumoniae* (10^8 CFU/ml), CpG (10 μ M), poly dI-dC (50 μ g/ml), imidazoquinoline S27609 (0.5 μ g/ml). Data are mean \pm SE of $n = 6$; differences were calculated with ANOVA; *, $p < 0.05$ vs medium; #, $p < 0.05$ vs LPS.

(LTA; 10 μ g/ml; *Staphylococcus aureus*; kind gift of T. Hartung (University of Konstanz, Konstanz, Germany)) (12); CpG oligodeoxynucleotide (10 μ M; Eurogentec, Seraing, Belgium; TLR9 ligand); polyinosine-polycytidylic acid (poly dI-dC; 50 μ g/ml, Sigma-Aldrich; TLR3 ligand); imidazoquinoline S27609 (0.5 μ g/ml; 3M Pharmaceuticals, St. Paul, MN; TLR7 ligand); recombinant human (rh)TNF- α (20 ng/ml) and rhIL-10 (10 ng/ml) (both Strathmann Biotech, Hannover, Germany); and heat-killed *Streptococcus pneumoniae* (10^8 CFU/ml; 6303; American Type Culture Collection, Manassas, VA). All stimuli were independently tested for their bioactivity in our laboratory (our unpublished data, and Refs. 13 and 14). In some experiments, cells were preincubated for 30 min with the following inhibitors: p38 MAPK inhibitor SB203580 (10 μ M), MEK kinase inhibitor PD98059 (20 μ M), PI3K inhibitor LY294002 (10 μ M) (all Alexis, Leiden, The Netherlands), or anti-TNF- α (25 μ g/ml; etanercept; Wyeth, Hoofddorp, The Netherlands). The dose of the respective kinase inhibitors was chosen after ruling out additional cytotoxicity by the compounds themselves (data not shown). LY294002 was chosen as a PI3K inhibitor due to its longer half-life as compared with other PI3K inhibitors such as wortmannin. Stimulations were performed for the indicated time points at 37°C and repeated independently at least two times.

FACS analysis

Erythrocytes were lysed, and cells were blocked for aspecific binding with 10% human Ig (Sigma-Aldrich) for 1 h on ice. Leukocytes were incubated with anti-TREM-1 Ab (21C7) (3), followed by goat anti-mouse secondary Abs (FITC-labeled for in vivo studies and allophycocyanin-labeled for in vitro studies) and finally anti-CD14 and anti-HLA-DR (all BD Pharmingen, San Diego, CA). Granulocytes were defined according to their scatter pattern and monocytes as CD14^{high} and HLA-DR^{pos}. To correct for aspecific staining, appropriate isotype control Abs were used. Samples were analyzed by flow cytometry using a FACScan (BD Biosciences, Mountain View, CA).

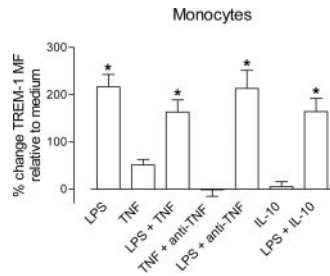


FIGURE 4. TREM-1 surface expression is independent of TNF- α or IL-10. Whole-blood stimulation was performed for 24 h, and TREM-1 up-regulation was assessed on monocytes in the presence of LPS (100 ng/ml) with or without TNF- α (20 ng/ml), anti-TNF- α (25 μ g/ml), or IL-10 (10 ng/ml). Monocytes were defined as CD14⁺ cells. Data are mean \pm SE of $n = 6$; differences were calculated with ANOVA; *, $p < 0.05$ vs medium.

Statistical analysis

Changes in time or between groups were analyzed by one-way ANOVA, and differences in response curves by repeated measurements. Data are presented as mean \pm SE; $p < 0.05$ was considered significant.

Results and Discussion

In light of the proinflammatory properties attributed to TREM-1, we studied the surface expression of TREM-1 on granulocytes and monocytes during the course of endotoxemia in humans. At baseline, TREM-1 was strongly expressed on blood granulocytes and monocytes. Endotoxemia induced a gradual up-regulation of TREM-1 on monocytes, peaking after 24 h (mean fluorescence (MF), 47.7 ± 4.3 at $t = 0$, 76.8 ± 7 at $t = 24$ h; Fig. 1A; $p = 0.008$). Granulocytes displayed a different TREM-1 pattern, reflected by an immediate decrease in receptor expression that returned to baseline values after 24 h (MF, 63 ± 4.9 at $t = 0$, 32.9 ± 2.8 at $t = 2$ h, 49.7 ± 2.7 at $t = 24$ h; Fig. 1B; $p < 0.0001$). In addition, we measured sTREM-1 concentrations and identified a rapid increase in sTREM-1 that reached peak levels 6 h after LPS administration (Fig. 2; $p = 0.002$). The latter finding adds to recent reports that demonstrated increased bronchoalveolar sTREM-1 levels in patients with pneumonia (9) and elevated sTREM-1 plasma concentrations in patients at risk for sepsis (10). Because administration of rTREM-1 consisting of the extracellular domain effectively blocked septic shock (5), sTREM-1 might represent a negative feedback regulator of the inflammatory response during endotoxemia. Although evidence exists that sTREM-1 is a splice variant of TREM-1, the precise cellular source of sTREM-1 has not been clarified (4, 15). Because endotoxemia leads to an immediate drop in the number of circulating granulocytes (8) due to sequestration to organs such as the lungs (16), the possibility exists that primarily TREM-1^{high} cells are sequestered, whereas TREM-1^{low} cells are released from the bone marrow, raising the impression of down-regulated TREM-1 expression on circulating granulocytes. To investigate this possibility, we stimulated whole blood with increasing concentrations of LPS in vitro and assessed changes in TREM-1 expression independent of potential sequestration. Indeed, irrespective of the LPS dose and incubation time, TREM-1 was not down-regulated in vitro (data not shown). This observation fits well with the finding of maturation stage-specific TREM-1 expression of myeloid cells (4).

TREM-1 surface expression on monocytes was also increased after stimulation with LPS in vitro. We extended these findings

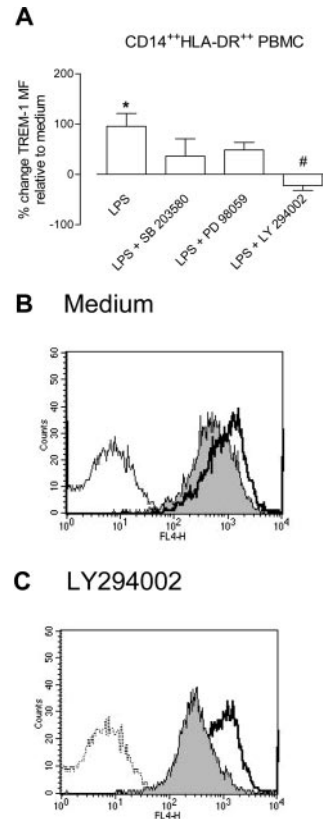


FIGURE 5. PI3K inhibition abrogates LPS-induced TREM-1 up-regulation. *A*, Influence of SB203580, PD98059, or LY294002 on LPS-induced TREM-1 up-regulation on PBMCs after 16 h. Monocytes were defined as CD14⁺HLA-DR⁺. Data shown represent results of two independent experiments. Mean \pm SE of $n = 5$; differences were calculated with ANOVA; *, $p < 0.05$ vs medium; #, $p < 0.05$ vs LPS in the absence of inhibitor. *B* and *C*, Representative histograms illustrate the influence of LPS stimulation (bold line) compared with medium (gray filled) (*B*) and the impact of LY294002 (gray filled) on LPS (bold line)-induced TREM-1 expression (*C*).

and demonstrate that this LPS-triggered up-regulation occurs in a dose- and time-dependent manner with a maximum expression after 24 h ($p < 0.001$ for 100 or 1 ng/ml LPS vs medium; NS for 0.01 ng/ml LPS; Fig. 3A) (MF, $t = 0$, 132.7 ± 20.4 ; $t = 48$ h, 614.4 ± 79.7 (medium), 1340.3 ± 108.4 (100 ng/ml LPS), 1048.2 ± 99.1 (1 ng/ml LPS), 720.5 ± 84.3 (0.01 ng/ml LPS)). Comparable results could be obtained after stimulation with the TLR2 ligand LTA ($p < 0.001$ vs medium) (MF, $t = 48$ h, 933.9 ± 98.6 (10 μ g/ml LTA)) and whole bacteria such as *S. pneumoniae* ($p = 0.001$ vs medium; Fig. 3B) (MF, $t = 48$ h, 939.4 ± 81.9) and flagellin (data not shown). As expected, the nonbacterial ligands poly dI-dC and imidazoquinoline as well as CpG oligodeoxynucleotide did not alter TREM-1 expression on monocytes (Fig. 3B). This can be explained by the fact that TLR3, -7, and -9 are predominantly expressed on dendritic cells and B cells (17, 18). The observation that TREM-1 is up-regulated on monocytes following stimulation with bacterial ligands fits well with the fact that monocytes are a major source of proinflammatory mediators like TNF- α that are released during endotoxemia. The gradual but transient increase in TREM-1 expression could in turn explain why neutralization of TREM-1 in mice proved still protective when administered 4 h after LPS administration (5).

Because the interaction with TREM-1 augments the release of proinflammatory mediators, we asked whether TREM-1 up-regulation following LPS challenge is mediated by TNF- α . However, stimulating whole blood in the presence of rhTNF- α did not significantly increase TREM-1 expression on monocytes, nor did the addition of anti-TNF- α alter the LPS-induced enhancement of receptor expression (Fig. 4). In addition, the anti-inflammatory mediator IL-10 did not affect TREM-1 expression levels (Fig. 4). This finding is somewhat in contrast to an earlier report by Bleharski et al. (6) who showed a modest influence of TNF- α and IL-10 that might be explained by the fact that we stimulated whole blood, whereas they used primary monocytes.

TLR signaling involves the activation of PI3K and MAPKs that influence LPS-induced inflammatory responses (19–21). Therefore, we investigated their role in LPS-mediated TREM-1 up-regulation with the use of synthetic inhibitors. Fig. 5 illustrates that PI3K inhibition (LY294002) completely abrogated the LPS-mediated TREM-1 up-regulation, whereas both MAPK inhibitors SB203580 (p38^{MAPK}) and PD98059 (ERK) had a modest effect on TREM-1 expression levels. Reports on the role of PI3K in LPS-induced inflammatory responses are conflicting; whereas some authors report that LPS induces cytokine production via the activation of PI3K in RAW macrophages (22), other groups have reported that LPS-induced cytokine production in THP-1 cells is negatively regulated by PI3K (20). However, we have found a positive role for PI3K in LPS-induced TREM-1 expression in isolated monocytes. It is currently not known why p38 MAPK and p42/p44 MAPK inhibitors only partially inhibit LPS-induced TREM-1 up-regulation, but a certain degree of redundancy may exist between these two kinases.

In conclusion, TREM-1 is up-regulated on monocytes during human endotoxemia together with an increase in sTREM-1. Receptor up-regulation is confined to bacterial molecular patterns but not a result of TNF- α production and involves a PI3K-dependent mechanism.

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