RESEARCH ARTICLE

TREM-1 interaction with the LPS/TLR4 receptor complex

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ABSTRACT. Triggering receptor expressed on myeloid cells 1 (TREM-1) is an activating receptor expressed on neutrophils and monocytes that amplifies inflammation induced by stimulation of pattern-recognition receptors. In this study, several lines of evidence are presented that TREM-1 interacts with the toll-like receptor 4 (TLR4) receptor complex, or is a component of this complex. Blocking anti-TREM-1 antibodies specifically inhibited LPS-induced TNF-α production, while the alternative approach of blocking TLR4 by a specific inhibitor led to a down-regulation of the effects of TREM-1 cross-linking. These data are in line with the TLR4-TREM1 co-localization in human neutrophils and suggests that, at least some of the biological effects of TREM-1 may be due to its interaction with the TLR4/LPS-receptor complex.

Key words: triggering receptor expressed on myeloid cells 1, lipopolysaccharide, toll-like receptor 4

Triggering receptor expressed on myeloid cells 1 (TREM-1) is an activating receptor expressed on neutrophils and monocytes that can be upregulated by stimulation with pathogenic micro-organisms and their ligands such as lipopolysaccharide (LPS) [1]. Structurally, TREM-1 is a 30-kDa glycoprotein receptor of the immunoglobulin (Ig) super family that contains a short, intracytoplasmic domain without signaling motives, a transmembrane part, and an ectodomain of the Ig-like V-type [1, 2]. TREM-1 is considered an amplifier of the inflammatory response through its associated molecule DAP12, a transmembrane protein with an immune receptor tyrosine-based activation motif (ITAM) [3]. On the one hand, engagement of TREM-1 can exert beneficial effects through the activation of antibacterial host defense, as shown in an experimental model of pneumonia [4]. On the other hand, during sepsis TREM-1 is up-regulated and appears to be a crucial mediator of the overwhelming systemic inflammation that leads to septic shock [5, 6].

It is therefore clear that knowledge of the biology of TREM-1 is important, and it has been proposed that immunotherapeutic strategies based on the modulation of TREM-1 effects may have beneficial effects in severe infections. This approach is hampered by the poor knowledge of the natural ligands of TREM-1. Several lines of evidence have suggested that TREM-1 (and other members of this family) may contribute to recognition of microbial ligands. Firstly, synergistic effects between TREM-1 and pathogen-associated molecular patterns (PAMPs) binding Toll-like receptors (TLRs) or NOD2 has been demonstrated in *in vitro* studies [7-11]. Secondly, pattern recognition of anionic ligands from pathogenic micro-organisms has been reported for the related receptor TREM-2 [12]. Finally, and most importantly, TREM-1 has been reported to co-localize with the LPS receptor TLR4 in human neutrophils, and LPS primes the effects induced by TREM-1 engagement in these cells [10].

These data suggest that TREM-1 contributes to the pattern recognition of bacterial PAMPs (especially LPS), and this may represent an important biological role of TREM-1. We addressed this hypothesis by assessing the cross-modulation between the LPS-recognition pathway and the TREM-1 stimulation pathway in primary human mononuclear cells. The data presented here suggest a contribution of TREM-1 to the receptor complex contributing to the recognition of LPS.

DONORS AND METHODS

Isolation of PBMCs: peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood collected from the antecubital vein of healthy volunteers, after obtaining informed consent. The PBMC fraction was separated by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Histopaque (Sigma Chemical Co., St. Louis, MO, USA). Afterwards, cells were washed twice in saline and subsequently suspended in RPMI 1640 culture medium, supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL).

Cytokine stimulation assay: PBMCs were preincubated in polypropylene tubes for two hours at 37°C with

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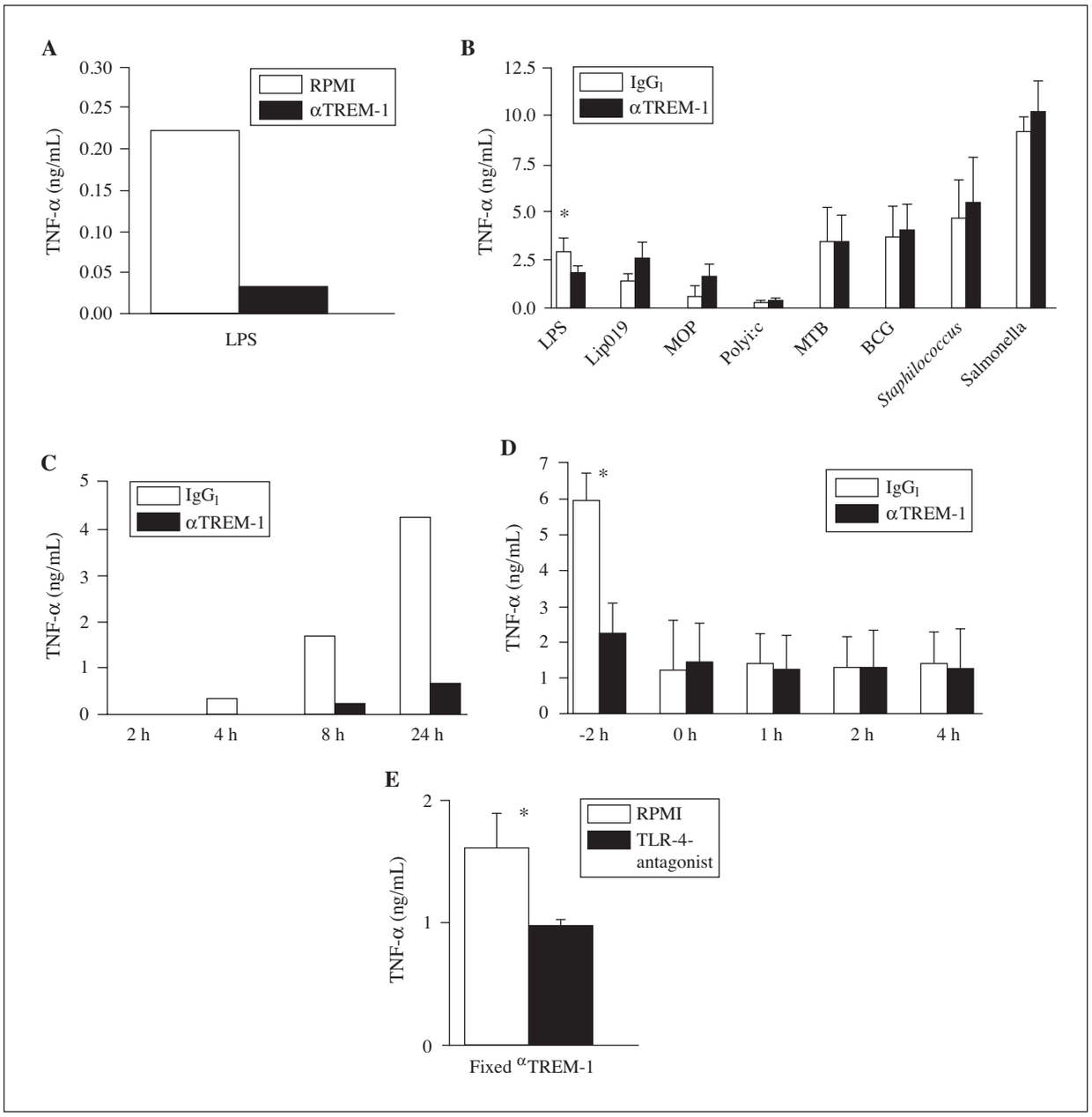

Figure 1

TREM-1 interacts with the LPS receptor complex. **A)** Preincubation of human PBMCs for 2h with blocking anti-TREM-1 antibodies (αTREM-1) significantly inhibited LPS-induced cytokine production. **B)** Except for LPS, no effect of anti-TREM-1 antibodies was apparent on TNF induction by other TLR ligands or whole micro-organisms. **C)** The effect of anti-TREM-1 antibodies on LPS-induced TNF production is already apparent after a 4 h stimulation period. **D)** PBMCs were incubated with anti-TREM-1 antibodies before (-2 h), at the same time (0 h) or after stimulation with LPS. Only preincubation of cells with anti-TREM-1 inhibited LPS-induced TNF production. **E)** Stimulation of cytokine production, by cross-linking of TREM-1 receptors by fixed anti-TREM-1 antibodies, was inhibited by an antagonist of the LPS receptor TLR4. (Data are give as means+SD, n = 4 experiments).

20 μg/mL of either an antagonistic anti-TREM-1 monoclonal antibody (Clone 193015, R&D systems, Minneapolis, MN, USA) or an isotype-matched control antibody (mouse IgG_1, R&D Systems). After preincubation, PBMCs were washed with sterile PBS, then 5×10^5 PBMCs were added to 96-well, flat-bottom plates. Subsequently, cells were stimulated with 1ng/mL LPS (*E. coli* O55:B5, highly purified as previously described [13], or other PRR ligands: lipoprotein 19 kDa (5 μg/mL; TLR2 ligand); polyinosinic:polycytidylic acid (poly I:C; 5 μg/mL; TLR3 ligand); muramyl dipeptide (MDP; 0.5 μg/mL; NOD2 ligand); sonicated *Mycobacterium tuberculosis* and *Bacillus Calmette-Guerin* (MTB and BCG; 10 μg/mL); heat-killed *Staphylococcus aureus* (1×10^7 micro-organisms/mL); and heat-killed *Salmonella typhimurium* (1×10^6 micro-organisms/mL). After a 24-hour incubation period, supernatant was collected and TNF-α was measured.

In a separate set of experiments, 96-well, flat-bottom plates were precoated for four hours with 10 μg/mL of either anti-TREM-1 or IgG_1. In the meantime, PBMCs were preincubated for two hours with RPMI or 10 μg/mL of a

specific TLR4 antagonist (*Bartonella quintana* LPS) [14]. After preincubation with the TLR4 antagonist, PBMCs were added to the wells with fixed anti-TREM-1 of IgG_1 and stimulated for 24 hours. Supernatants were thereafter collected to determine the concentration of TNF-α stimulated upon engagement of TREM-1.

Cytokine assay: the concentration of TNF-α in the supernatant was measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Abbingdon, UK).

Statistical analysis: all experiments were performed at least four times. The differences between the two groups were analyzed by Wilcoxon rank sum test, and $p < 0.05$ was considered to be a significant difference. The data depicted in the figures are shown as means ± SD. All the statistical values were calculated using SPSS (Chicago, IL, USA).

RESULTS AND DISCUSSION

Previous experiments have suggested that receptors of the TREM family recognize bacterial ligands [12], and that TREM-1 co-localizes with the LPS receptor TLR4 in human neutrophils [10]. In order to assess whether TREM-1 contributes to LPS recognition in myeloid cells, TREM-1 receptor was blocked by a specific antagonistic antibody in human PBMCs before stimulation with LPS. TREM-1 blockade significantly inhibited the induction of TNF-α by LPS *(figure 1A)*, supporting the hypothesis that TREM-1 contributes to pattern recognition of LPS. This effect was specific for LPS, as no effect of TREM-1 blockade was apparent for cytokine stimulation by TLR2, TLR3 or NOD2 ligands, or the stimulation of cells with whole micro-organisms *(figure 1B)*.

Two different mechanisms could be envisaged to explain the effects of TREM-1 blockade on LPS-induced TNF-α. One possibility is that TREM-1 directly contributes to LPS recognition as a co-receptor, while another possible explanation involves induction of a putative TREM-1 ligand by LPS. In the latter case, the LPS signal transduction pathway would lead to synthesis and release of a TREM-1 ligand, which amplifies the pro-inflammatory response in an autocrine fashion, similar to IL-1 [15]. We tested this possibility by assessing the kinetics of TREM-1 blockade: in case of an autocrine TREM-1 ligand induction, blocking TREM-1 would inhibit the proinflammatory response only at late time points, while the rapid, direct induction of TNF-α by LPS is not affected. However, this does not seem to be the case, as TREM-1 blockade inhibited LPS-induced TNF-α as soon as TNF-α was detectable 4h after stimulation *(figure 1C)*. It is therefore most likely that TREM-1 blockade directly influenced LPS recognition and stimulation of TNF-α. This conclusion was supported by a complementary approach, in which TREM-1 blockade was performed at various times before or after LPS stimulation of PBMCs. Only early blocking of TREM-1 by preincubation of PBMCs with the monoclonal antibodies inhibited LPS-induced TNF-α (suggesting interference with LPS receptor recognition), while late addition of TREM-1 (which would putatively block interaction of a TREM-1 ligand with TREM-1) had no effect *(figure 1D)*.

The data presented above demonstrate that antibodies to TREM-1 decrease LPS-induced TNF-α production. This effect could have been exerted by the blockade of TREM-1, but also by a non-specific neutralizing effect on LPS. To exclude this possibility, we incubated LPS for 2h with either isotype-matched IgG_1 or anti-TREM-1 antibodies (both at 20 μg/mL), before the stimulation of PBMC. No effect of LPS preincubation with anti-TREM-1 antibodies on cytokine production was apparent (11.6 *versus* 11.8 ng/mL, $p = 0.33$).

TLR4 is the main component of the LPS-recognizing receptor [16], but other components of this receptor complex have also been described: MD2, CD14, CD11/18 and CXCR4 [17]. If TREM-1 interacts with the LPS-receptor complex, it would be expected that a blocker of this receptor such as *B. quintana* LPS would also sterically inhibit the engagement and cross-linking of TREM-1 by fixed antibodies, the only well-established method to induce TREM-1 activation [1, 18]. Indeed, cross-ligation of TREM-1 on PBMCs induced a significant induction of TNF-α, and this effect was significantly inhibited by preincubation of cells with a TLR4 blocker *(figure 1E)*.

In conclusion, this study presents several lines of evidence that TREM-1 interacts with the TLR4 receptor complex, or is a component of this complex. On the one hand, blocking anti-TREM-1 antibodies specifically inhibits LPS-induced TNF-α production, while stimulation of cytokine production by other PAMPs is not influenced. This effect is most likely direct, and not due to an indirect neutralization of a TREM-1 ligand induced by LPS. On the other hand, the alternative approach of blocking TLR4 by a specific blocker led to an inhibitory effect on TREM-1 cross-linking, strongly suggestive of a situation in which TREM-1 interacts with the LPS-receptor complex. These data confirm the putative cooperation of TLR4 and TREM-1 as a receptor complex [19], and are in line with the demonstration of TLR4-TREM1 co-localization in human neutrophils [10]. TREM-1 has been described as a receptor that strongly amplifies inflammation during infection. The attempt to interact with its effects has been greatly hampered by the lack of knowledge regarding its putative ligands. In this study, we present evidence that, at least part of its biological effects may be due to its interaction with the TLR4/LPS-receptor complex.

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None of the authors has any conflict of interest to disclose.

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