Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors

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Abstract: Triggering receptor expressed on myeloid cells-1 (TREM-1) is a member of a new family of myeloid receptors, encoded by a gene cluster linked to the MHC. Engagement of TREM-1 stimulates intracellular signals, resulting in activation of phagocytosis, neutrophil degranulation, and amplification of cytokine production induced by TLRs. In the present study, a novel property following engagement of TREM-1 is described, namely the amplification of cytokine production induced by the second major class of pattern recognition receptors, the NAIP, CIITA, HET-E, TP-1-leucine-rich repeat (NACHT-LRR; NLR) receptors, which recognize intracellular microorganisms through sensing their muropeptide components of peptidoglycan. The TREM-1/NLR synergism was observed for the production of TNF-α, IL-1β, and IL-6, leading to an increase in cytokine production up to tenfold greater than the additive value of TREM-1 or muropeptide stimulation alone. Several putative mechanisms are proposed to be involved in the synergism between NLRs and TREM-1, including the increase in TREM-1 expression by NLR ligands, and of the expression of nucleotide oligomerization domain-2 receptor by TREM-1 engagement. In contrast, although caspase-1 modulates IL-1β and IL-6 production after stimulation with anti-TREM-1 antibodies or NLR ligands, it does not appear to be responsible for the synergism between these two pathways. These findings demonstrate that TREM-1 acts on both major recognition pathways of bacterial structures: the extracellular TLR receptors, and the intracellular NLR molecules. This latter finding supports the concept that TREM-1 provides optimal amplification of cytokine-induced inflammation during the initiation of host defense. *J. Leukoc. Biol.* 80: 1454–1461; 2006.

Key Words: *NOD2 · cytokines · caspase-1*

INTRODUCTION

During the initial phase of the host defense, the innate immune system relies mainly on the coordinated action of phagocytes and NK cells. These cells recognize microorganisms through pattern recognition receptors (PRRs), resulting in the release of proinflammatory cytokines and phagocyte activation [1]. Microbial recognition is accomplished by two main classes of PRRs: the TLRs, which are expressed primarily on the surface of the cell membrane, are responsible for recognition of extracellular microbial components [2], whereas the cytoplasmic NAIP, CIITA, HET-E, TP-1-leucine-rich repeat (NACHT-LRR; NLR) receptors are the main recognition system of intracellular microorganisms and their ligands [3]. However, efficient activation of host defense mechanisms by these receptors requires amplification signals provided by additional routes such as lectin-like molecules (e.g., dectin-1, mannose receptors) [4, 5], high-mobility group box protein-1 [6], and a newly described class of molecules on the surface of leukocytes, called triggering receptors expressed on myeloid cells (TREMs) [7].

TREM-1 is a 30-kDa glycoprotein of the Ig family, consisting of a single extracellular Ig-like domain of the V-type, a transmembrane region, and a short intracytoplasmic tail [8]. TREM-1 is expressed on neutrophil and monocytes, whereas TREM-2 is a family member which is expressed on macrophages and dendritic cells [7]. Both receptors associate with DNAX-activating protein 12 (DAP12) for signaling, and TREM-1 engagement triggers TNF, IL-8, and MCP-1 production, as well as neutrophil degranulation and release of NO [9, 10]. It has been also proposed that TREM-1 provides a vital amplification loop for the proinflammatory responses induced by TLR ligands [10, 11]. This concept has been validated in animal models characterized by overwhelming inflammation (e.g., lethal endotoxaemia, *Escherichia coli* peritonitis, caecal ligation, and puncture), in which neutralization of TREM-1 prevented hyperinflammatory responses and death [12].

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We hypothesized that engagement of TREM-1 amplifies not only the TLR-dependent signals but also the activation of NLR-dependent pathways. Engagement of NLRs by the muropeptide components of bacterial peptidoglycans activates the NF-κB pathway and proinflammatory cytokine production [3, 13]. In this respect, the NLR family members nucleotide oligomerization domain-2 (NOD2) and NALP3 recognize the muramyl dipeptide (MDP) of Gram-positive bacteria [14–16], and NOD1 recognizes the meso-diaminopimelic acid-containing muropeptides (Mur-Tri-DAP) of Gram-negative bacteria [17]. In the present study, we demonstrate that TREM-1 activation amplifies the induction of the proinflammatory cytokine production by bacterial muropeptides, through mechanisms involving increased NOD2 gene transcription and activation of caspase-1-dependent pathways.

MATERIALS AND METHODS

Isolation of PBMC and stimulation of cytokine production by TREM-1 engagement

After informed consent, venous blood was drawn from the antecubital vein of healthy volunteers into heparin tubes. The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Histopaque (Sigma Chemical Co., St. Louis, MO). Cells were washed twice in saline and suspended in culture medium (RPMI 1640) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml).

To activate PBMC through TREM-1, 96-well flat-bottom plates were precoated with 10 μg/ml (unless otherwise indicated) of an agonist anti-TREM-1 mAb (Clone 193015, R&D Systems, Minneapolis, MN) or an isotype-matched control antibody (mouse IgG1, R&D Systems), as previously used also by others [10]. To study the effects of human plasma, 5% human pooled plasma was added into some of the wells. After 4 h incubation at 37°C, the wells were washed with sterile PBS, and 5×10^5 PBMC in a 200-μl vol were added. Proinflammatory cytokine production was measured in the supernatants after an additional 24-h incubation. The role of the endogenous TNF, IL-1, or IL-18 in the induction of cytokines by TREM-1 was investigated by preincubating the PBMC for 30 min with the respective natural cytokine antagonists: TNF-binding proteins (TNFbp; 10 μg/ml, R&D Systems), IL-1 receptor antagonist (IL-1Ra; 10 μg/ml, R&D Systems), or IL-18 binding protein (IL-18BP; 10 μg/ml; ref. [18]).

Assessment of the synergistsic activity between TREM-1 and TLR/NLR ligands

To investigate the effect of TREM-1 engagement on the TLR- and NLR-induced stimulation, PBMC were added in the wells preincubated with anti-TREM-1 or control antibodies. In addition, 100 μl culture medium (RPMI 1640) or the various TLR ligands were added to the culture: TLR2 was stimulated with synthetic lipoteichoic acid (LTA; kindly provided by Dr. Thomas Hartung, University of Konstanz, Germany, 5 μg/ml), TLR3 with polyinosinic:polycytidylic acid (poly I:C; 50 μg/ml), TLR4 stimulation with purified *E. coli* LPS (1 ng/ml), TLR5 with flagellin (10 ng/ml), and TLR9 with CpG (5 μg/ml). The synergism between IL-32 and the NLR ligands MDP (0.1–10 μg/ml) and MurNAc-L-Ala-D-Glu-meso-diaminopimelic acid (Mur-Tri-DAP; 1 μg/ml) was also investigated. All TLR and NLR ligands were checked for the contamination with LPS in the *Limulus* amoebocyte lysate assay and found to be negative. After 24 h incubation at 37°C, the supernatants were collected and stored at −70°C until cytokine assays were performed. Cytokine concentrations were determined by specific electrochemiluminescence assays, as described previously [19]. In separate experiments, PBMC were sequentially incubated for 4 h with anti-TREM-1 antibodies, before adding the NLR ligands to the cells. Alternatively, the sequence was changed, and the cells were preincubated with NLR ligands before stimulation with anti-TREM-1 antibodies.

Stimulation of TREM-1 expression by TLR and NLR ligands

To investigate the effects of the various TLR and NLR ligands on TREM-1 expression, 5×10^5 PBMC in a 200-μl vol were added to flat-bottom 96-wells plates and were stimulated for 24 h with the TLR and NLR ligands described above. After stimulation, 20 μl Triton-X 100 was added to the wells to induce cell-membrane disruption, and the samples were frozen-thawed twice. TREM-1 concentrations were measured with a commercial sandwich ELISA from R&D Systems, with a detection limit of 30 pg/ml.

RT-PCR for the NOD2 expression

Ten million freshly isolated PBMC were stimulated with 10 μg/ml control mouse IgG1 or the monoclonal anti-human TREM-1. After 4 h incubation at 37°C, total RNA was extracted in 1 ml TRIzol reagent, an improved single-step RNA isolation method based on the method described by Chomczynski and Sacchi [20]. Thereafter, RNA was precipitated with isopropanol, washed with 70% ethanol, and redissolved in water. Isolated RNA was treated with DNase before being reverse-transcribed into cDNA using oligo(dT) primers and Moloney murine leukemia virus RT. PCR was performed using a Peltier (Watertown, MA) Thermal Cycler-200. Primer sequences for the NOD2 were: 5-TGG-TTC-AGC-CTC-TCA-CGA-TG-3 (forward) and 5-TGC-TGA-AGA-GCT-CCT-CCA-GG-3 (reverse). GAPDH was used as a reference gene, for which the primers were: 5-GGC-AAA-TTC-AAC-GGC-ACA-3 (forward) and 5-GTT-AGT-GGG-GTC-TCG-CTC-TG-3 (reverse). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 30 cycles of PCR reaction at 94°C for 45 s, 70°C for 2 min, and 59°C for 1 min. The PCR products were run on 1% agarose gels stained with ethidium bromide.

The role of caspases for the synergism between TREM-1 and NLR ligands

To investigate the role of caspases for the synergism between TREM-1 and the muropeptides MDP and Mur-Tri-DAP, we added 20 μM Z-Val-Ala-Asp-fluoromethylketone, a pan-caspase inhibitor (Bachem, King of Prussia, PA) to the medium. In addition, specific inhibition of caspase-1 with 20 μM Ac-Tyr-Val-Ala-Asp-2,6-dimethylbezoyloxymethylketone (Alexis Biochemicals, San Diego, CA) or simultaneous inhibition of caspase-1 and caspase-5 with 20 μM Ac-Trp-Glu-His-Asp-aldehyde (Bachem) was performed. The role of the endogenous IL-1β and IL-18 for the synergistic effects of TREM-1 and muropeptides on IL-6 production was investigated by blocking their activity with IL-1Ra (10 μg/ml) or IL-18BP (10 μg/ml).

Statistical analysis

The human experiments were performed in triplicate with PBMC obtained from eight volunteers. The mouse experiments were performed in 10 mice per group on two different occasions, and the data are presented as cumulative results. The differences between groups were analyzed by unpaired *t*-test, and where appropriate by paired *t*-test. The data are given as means ± SEM.

RESULTS

TREM-1 engagement stimulates cytokine production in a plasma-dependent manner

When anti-TREM-1 antibodies were added in the liquid phase to the PBMC, no stimulation of cytokines was apparent. In contrast, as shown in **Figure 1**, when the anti-TREM-1 antibodies were fixed on the bottom of the wells, the TREM-1 engagement strongly induced the production of the proinflammatory cytokines TNF-α, IL-6, and IL-1β, probably as a result of the cross-linking of TREM-1 on the cell membrane. No stimulation was observed in the presence of the control antibody. Addition of human plasma greatly potentiated the cytokine production induced by the anti-TREM-1 antibody (Fig. 1).

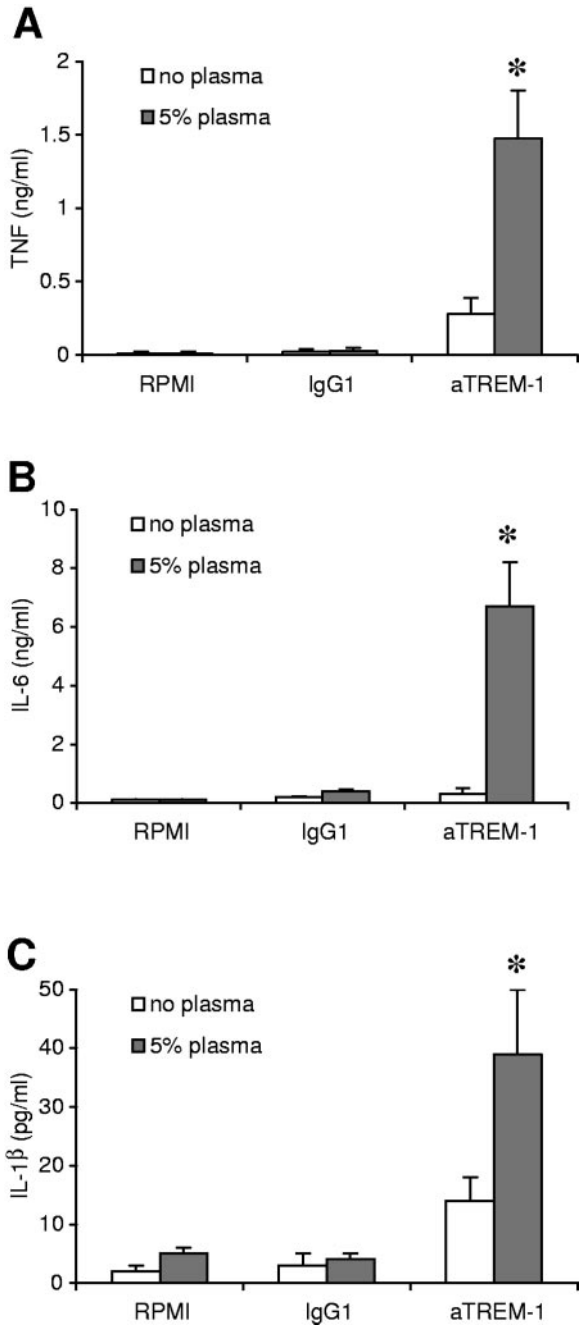

Fig. 1. TREM-1 engagement stimulates cytokine production. PBMC were added to antihuman TREM-1 (aTREM-1)-coated wells in the absence (open bars) or presence (hatched bars) of 5% frozen, pooled human plasma. After 24 incubation, the concentrations of TNF-α (A), IL-6 (B), and IL-1β (C) were measured by specific ECL. Data are presented as means ± SEM (n=8); *, $P < 0.01$.

In separate experiments, to investigate whether this stimulation was dependent on the intermediary production of TNF, IL-1, or IL-18, the cells were preincubated for 30 min with the natural inhibitors of these cytokines: TNFbp, IL-1Ra, or IL-18BP. No effects of these inhibitors on the TREM-1-induced cytokines were apparent, in the absence or in presence of human plasma, demonstrating that the stimulatory effects of TREM-1 were direct and not a result of intermediate TNF, IL-1, or IL-18 (**Fig. 2**). Positive control stimulation of IL-6 by TNF and IL-1β or IFN-γ by IL-18/IL-12 costimulation has been performed to assess the efficacy of the various cytokine inhibitors. TNFbp, IL-1Ra, and IL-18BP completely blocked the biological effects of the respective cytokines (not shown).

TREM-1 amplifies cytokine induction by TLR and NLR ligands

Optimal induction of proinflammatory cytokines after recognition of bacteria by the major PRRs needs amplification through additional pathways. The protective effects of anti-TREM-1 antibodes in models of endotoxic shock suggest that TREM-1 is one of these amplification mechanisms. To test the validity of this hypothesis, human PBMC were costimulated with anti-TREM-1 agonistic antibodies and TLR or NLR ligands. As shown in **Figure 3, A** and **B**, TREM-1 synergized with the signals induced by ligands of TLRs and the muropeptide ligands of NLRs: MDP (ligand of NOD2, NALP1, and NALP3) and Mur-Tri-DAP (NOD1). There was a dose-dependent effect of the anti-TREM-1 concentrations used for coating the plates (expressed in μg/ml) on the synergism with MDP-induced TNF-α (Fig. 3C) and IL-6 (Fig. 3D).

When stimulation of cells was performed sequentially (4 h prestimulation by anti-TREM-1, followed by NOD2 stimulation), a similar amplification of NOD2 signals was observed: a

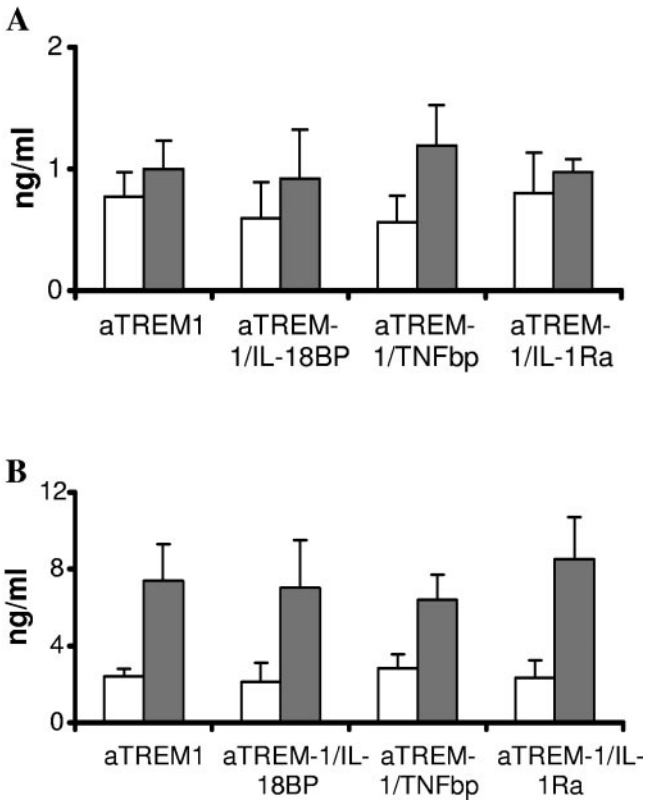

Fig. 2. TREM-1 stimulates cytokine synthesis independently of endogenous TNF, IL-1, or IL-18. Human PBMC were incubated in wells coated with a monoclonal anti-TREM-1 (aTREM-1) antibody, in the absence or presence of specific anticytokine inhibitors: IL-18BP, TNFbp, and IL-1Ra (each at 10 μg/ml). TNF (open bars) and IL-6 (hatched bars) were measured after stimulation in the absence (A) or in presence (B) of 5% human plasma. Data are presented as means ± SEM (n=8).

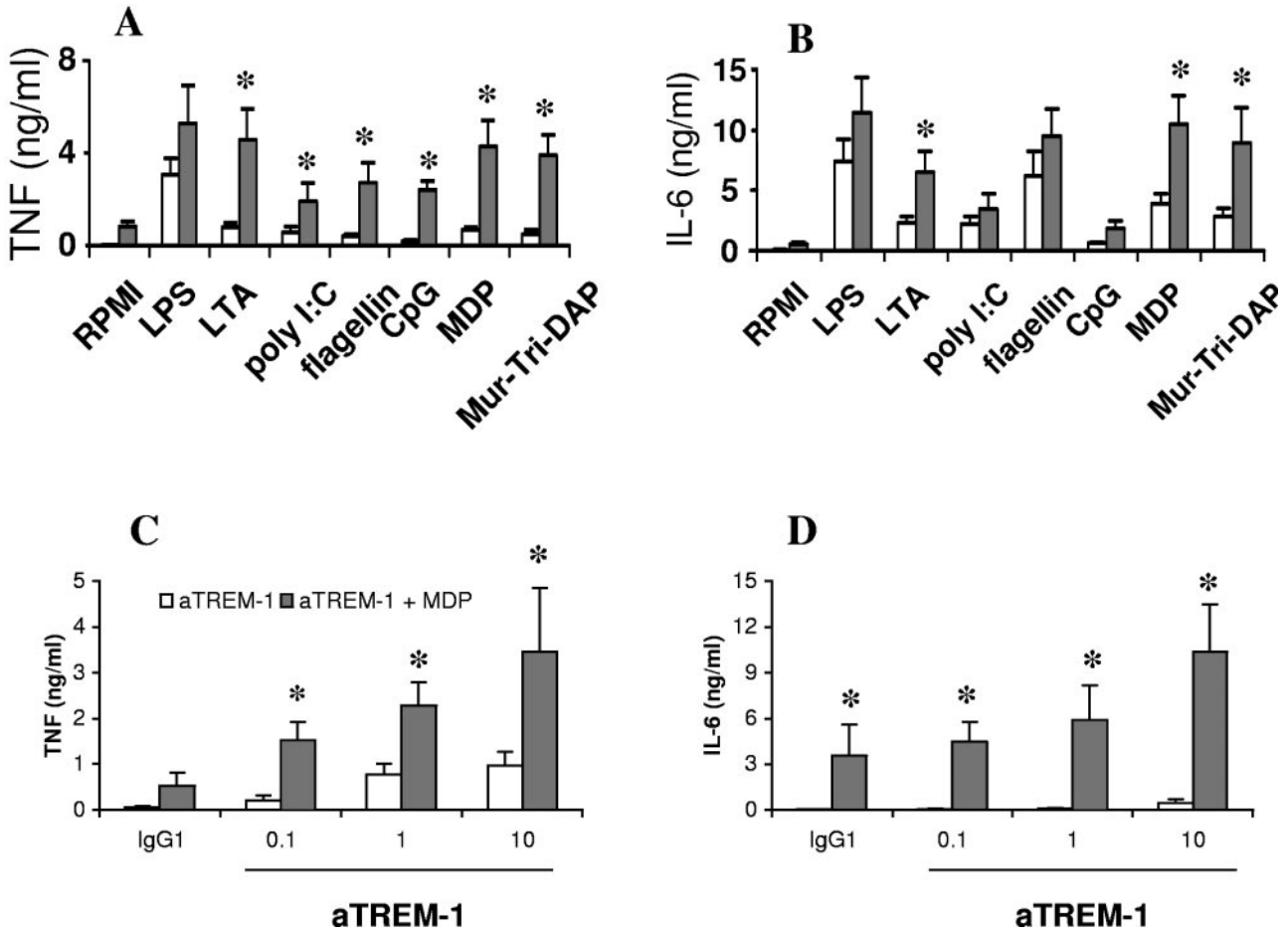

Fig. 3. TREM-1 synergizes with TLR and NLR ligands. Human PBMC were incubated in wells coated with anti-TREM-1 antibodies in the presence of ligands of TLRs: LPS (10 ng/ml), LTA (5 μg/ml), poly I:C (5 μg/ml), flagellin (100 ng/ml), CpG DNA (10 μg/ml), and the muropeptide ligands of NLRs: MDP (10 μg/ml) and Mur-Tri-DAP (10 μg/ml). TNF-α (A, C) and IL-6 (B, D) concentrations were measured by ECL after 24 stimulation. Hatched bars represent the stimulation induced by the combination of the anti-TREM-1 antibodies with the TLR/NLR ligands, and open bars are stimulations with TLR/NLR ligands alone. Data are presented as means ± SEM (n=6); *, $P < 0.05$.

threefold higher TNF production compared with NOD2 stimulation alone ($P<0.05$) and a fivefold enhancement of the production of IL-1β ($P<0.02$). In contrast, no synergism was observed when cells were first preincubated 4 h with MDP followed by TREM-1 ligation (TNF production 356±127 pg/ml after MDP stimulation and 524 ± 201 pg/ml after MDP, followed by anti-TREM-1 antibodies; P=not significant).

TREM-1 signals induce the expression of NOD2

Several possible mechanisms to explain the synergism between TREM-1 and NOD2 were investigated. First, the effects of NLR and TLR ligands on the expression of TREM-1 were investigated. Human PBMC were stimulated with a panel of TLR and NLR stimuli. After 24 h, the levels of TREM-1 protein in the cell lysates were measured by a specific ELISA. Whereas LPS increased twofold the amount of TREM-1, the other TLR and NLR ligands displayed only a marginal influence on TREM-1 expression, with 50–70% increase after stimulation by MDP or Mur-Tri-DAP (**Fig. 4A**). In contrast, after stimulation for 4 h with an anti-TREM-1 agonistic antibody, the level of steady-state NOD2 mRNA was significantly higher compared with that of IgG1-stimulated cells, in each four PBMC preparations tested (Fig. 4B). GAPDH levels were similar in each sample (not shown). Consistent with the increase of NOD2 expression, PBMC treated with the anti-TREM-1 antibody exhibited an increased response to low concentrations of MDP (Fig. 4C).

The role of caspase-1 in the synergistic effects of TREM-1 on NLR stimulation

Proinflammatory caspases, such as caspase-1 and caspase-5, interact with the caspase-associated recruitment domain (CARD) of NLRs, resulting in the processing of the proinflammatory cytokines IL-1β and IL-18 [21]. As IL-1β often controls IL-6 production, proinflammatory caspases also influence the production of the latter cytokine. To investigate whether caspase activation contributes to the synergistic effects of TREM-1 and muropeptides, the effect of a pan-caspase inhibitor, a specific caspase-1 inhibitor, and of a caspase1/5 inhibitor on the synergistic effects between TREM-1 and MDP (**Fig. 5 A–C**) or Mur-Tri-DAP (Fig. 5, D–F) was examined. Whereas the synergy between TREM-1 and the muropeptides for the stimulation of TNF was not significantly influenced by the caspase inhibitors (Fig. 5, A and D), the amplification of

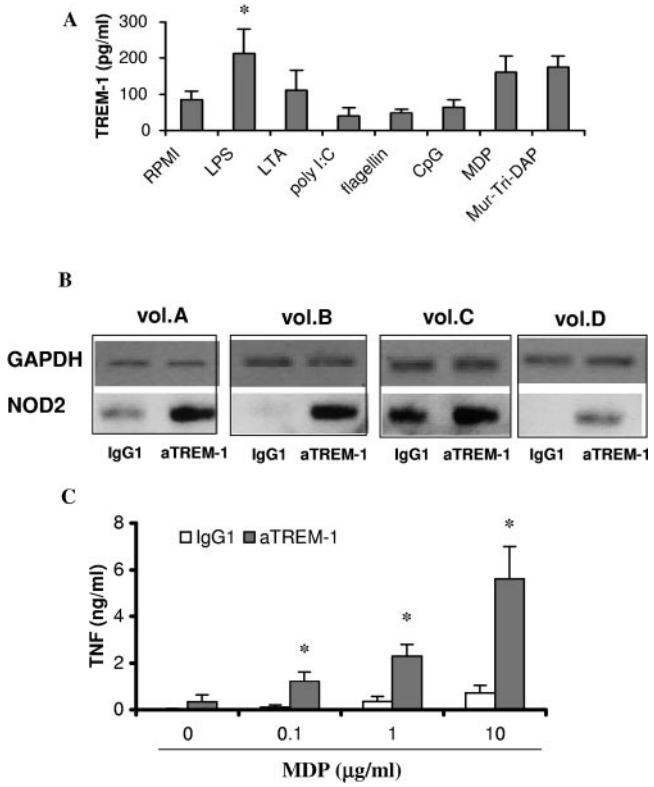

Fig. 4. TREM-1 activation induces the expression of NOD2. (A) Human PBMC were stimulated with a panel of TLR and NLR stimuli. After 24 h, the levels of TREM-1 protein in the cell lysates were measured by a specific ELISA (n=6, means±SEM). (B) After stimulation for 4 h with an anti-TREM-1 agonistic antibody, steady-state levels of NOD2 mRNA were determined by RT-PCR. Incubation of PBMC from four separate individuals is shown in the presence of a control IgG1 antibody (Lanes 1, 3, 5, and 7) or the anti-TREM-1 antibody (Lanes 2, 4, 6, and 8). (C) To investigate the effect of TREM-1 engagement on low concentrations of MDP stimulation, PBMC were added to wells coated with the anti-TREM-1 antibody and then stimulated with MDP in concentrations ranging from 0.1 to 10 μg/ml. Data are presented as means ± SEM (n=6); *, $P < 0.05$.

NLR-induced IL-1β (Fig. 5, B and E) and IL-6 (Fig. 5, C and F) was reduced by a pan-caspase inhibitor and a caspase-1 inhibitor (80–90% reduction). An inihibitor blocking caspase-5 and caspase-1 had no additional effects on the synergism on top of the specific caspase-1 inhibitor.

The effects of caspase-1 inhibition on IL-6 production is a result of endogenous IL-1β

As caspase-1 does not directly influence IL-6 production, we sought to investigate whether this effect could be mediated by endogenous IL-1β or IL-18. The amplification of the IL-6 production induced by NLR ligands MDP and Mur-Tri-DAP by engagement of TREM-1 was investigated under IL-1R blockade with saturating concentrations of IL-1Ra or neutralizing concentrations of IL-18BP. As shown in **Figure 6**, IL-1Ra inhibited the synergism between TREM-1 and the muropeptides, demonstrating that the effects of caspase-1 on IL-6 release depend on neutralization of endogenous IL-1 activity. In contrast, IL-18BP had no effect on the synergism between TREM-1 and muropeptides (Fig. 6).

DISCUSSION

The novel finding of the present study is that engagement of TREM-1 amplifies not only TLR signals but also the NLR stimulation induced by the muropeptide components of peptidoglycan. This observation has a considerable biological relevance, as peptidoglycans are structures common to all clinically important bacteria, including mycobacteria. The amplification was observed for the production of TNF-α, IL-1β, and IL-6, leading to an increase in cytokine production up to tenfold greater than the additive value of TREM-1 or muropeptide stimulation alone. Several putative mechanisms appear to account for these effects: on the one hand, NLR ligands moderately stimulate TREM-1 expression, whereas an increase in NOD2 gene expression has been induced by TREM-1 stimulation. In addition, TREM-1/NOD2 amplification of IL-1β and IL-6 was strongly inhibited by a caspase-1 inhibitor. Thus, these findings support the concept that TREM-1 is an amplification route for both major responses to bacterial recognition: the extracellular TLRs and the intracellular NLR molecules.

TREM-1 engagement by agonist antibodies induces production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 as well as production of chemokines, as described previously [9–11]. This stimulation was dependent on the presence of serum, and we demonstrate here that the serum effect is independent of the intermediary production of endogenous TNF-α, IL-1, or IL-18, as their specific inhibitors did not influence the effects of TREM-1 stimulation. It is presently unclear why stimulation of cytokines by anti-TREM-1 antibodies is dependent on serum, although others [10] have also observed this. Cross-linking between a serum component and anti-TREM-1 antibodies on the one hand and membrane TREM-1 on the other may represent a possible explanation. Thus, a direct, proinflammatory action accounts for the cytokines induced by TREM-1 engagement. Experimental models of endotoxic shock support an important role of TREM-1 in systemic inflammation, in which blockade of TREM-1 results in improved survival [12, 22]. In addition, studies in a model of endotoxemia in human volunteers revealed that the expression of TREM-1 was up-regulated after challenge with LPS [23]. Increased levels of soluble TREM-1 (sTREM-1) have been measured in patients with septic shock [24, 25], and sTREM-1 has been proposed as an additional parameter of inflammation during severe infections [26].

The NLR family members NOD1, NOD2, and NALP3 are PRRs for the muropeptide components of bacterial peptidoglycans. They recognize different motifs of bacterial peptidoglycan: NOD1 recognizes the Mur-Tri-DAP of Gram-negative bacteria, which contains a meso-diaminopimelic acid in the third position of the peptidoglycan stem peptide [17], whereas NOD2 and NALP3 interact with MDP of Gram-positive bacteria, containing a lysine residue in the same position [14, 15]. By recognizing these specific motifs, NLRs are able to discriminate between Gram-negative and Gram-positive bacteria. This important feature of NLR, together with the ubiquitous presence of peptidoglycans in the structure of Gram-positive and Gram-negative bacteria and with the interaction of TLR and NOD pathways [27–30], results in a vital role for NLRs in the process of bacterial recognition and host defense. Studies in

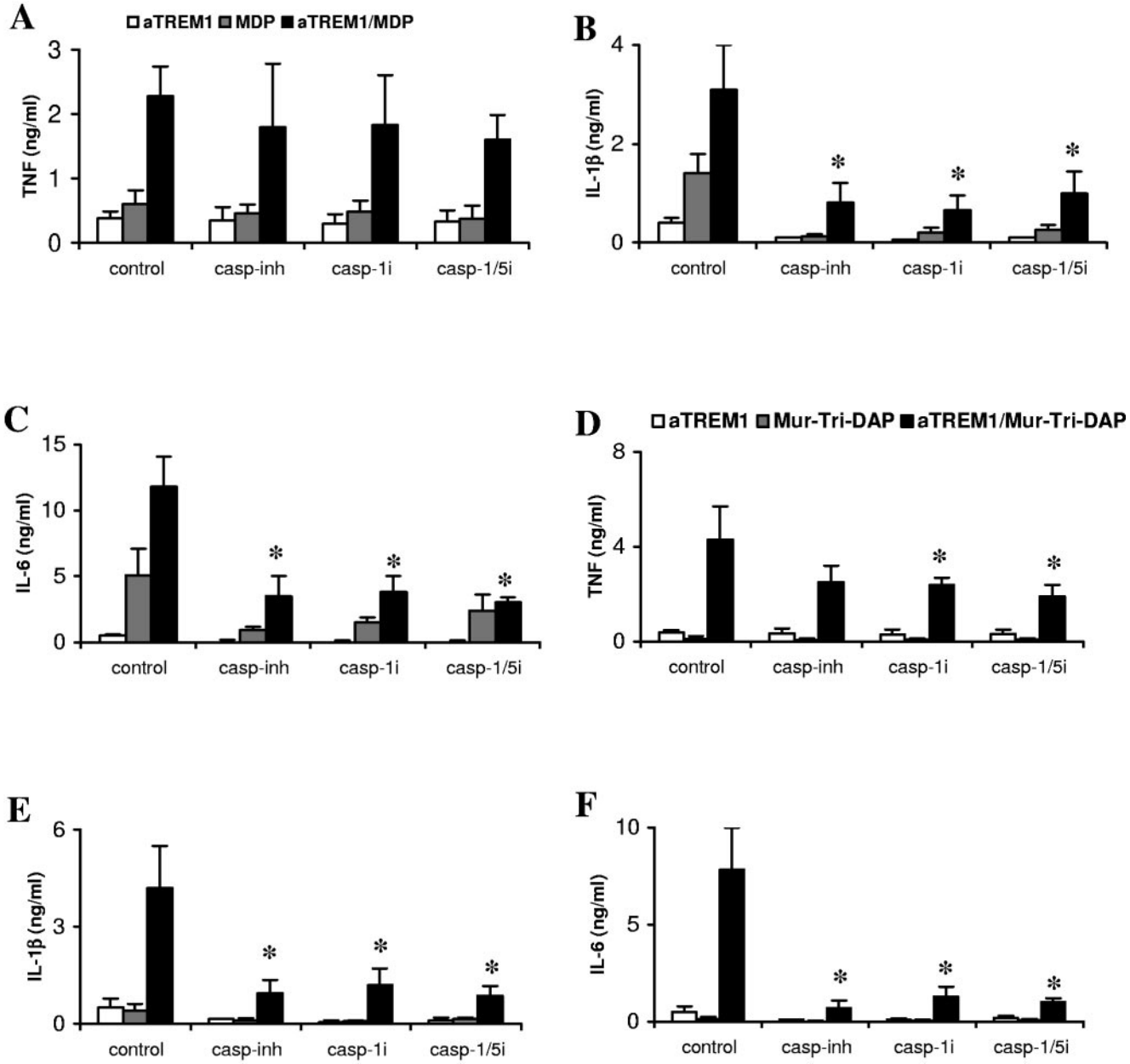

Fig. 5. The role of caspase-1 in the amplification of NLR-induced cytokines by TREM-1 engagement. PBMC were added to wells coated with an anti-TREM-1 antibody (aTREM-1; or mouse IgG1) and then stimulated with the NLR ligands MDP or Mur-Tri-DAP. The effects of a pan-caspase inhibitor (casp-inh), a caspase-1 inhibitor (casp-1i), and of a caspase1/5 inhibitor (casp1/5i; each at 20 mM) on the synergistic effects between TREM-1 and MDP (A–C) or Mur-Tri-DAP (D–F) were investigated. The stimulation of cells in the absence of caspase inhibitors is defined in the figure as "control" stimulation. TNF-α (A, D), IL-1β (B, E), and IL-6 (C, F) concentrations were measured after 24 h stimulation. Data are presented as means ± SEM (n=6); *, $P < 0.05$, compared with the stimulation without caspase inhibitors.

mice deficient in NOD2 reveal defective defensin production and increased susceptibility to intracellular pathogens [31, 32], whereas patients with Crohn's disease and homozygous for the 3020insC mutation in the LRR domain of NOD2 display defective MDP recognition, with absence of NF-κB activation and cytokine release [15, 27, 28, 30]. The present study is the first to demonstrate that TREM-1 synergizes with the muropeptide NLR ligands and implies that TREM-1 has a central role in inflammation, as it is an amplification signal for the two major classes of PRRs: TLRs and NLRs.

The synergism between TREM-1 and TLR/NLRs can have positive effects, as it would assure an efficient activation of host defense. However, a negative consequence is also possible as a result of induction of overwhelming inflammation. The protection of mice by the TREM-1 blockade in a model of lethal endotoxemia [12, 22] as well as experiments in mice overexpressing DAP12, who are highly susceptible to LPS-induced shock [33], are arguments in this latter respect. When considering the synergism between TREM-1 and NLR activation, it is important to remember the recent report of Schenk and colleagues [34], who demonstrated the low expression of TREM-1 in macrophages from intestine and colon compared with myeloid cells from the circulation, lymph nodes, or tonsils. As muropeptide recognition in the colon by the NLR family mem-

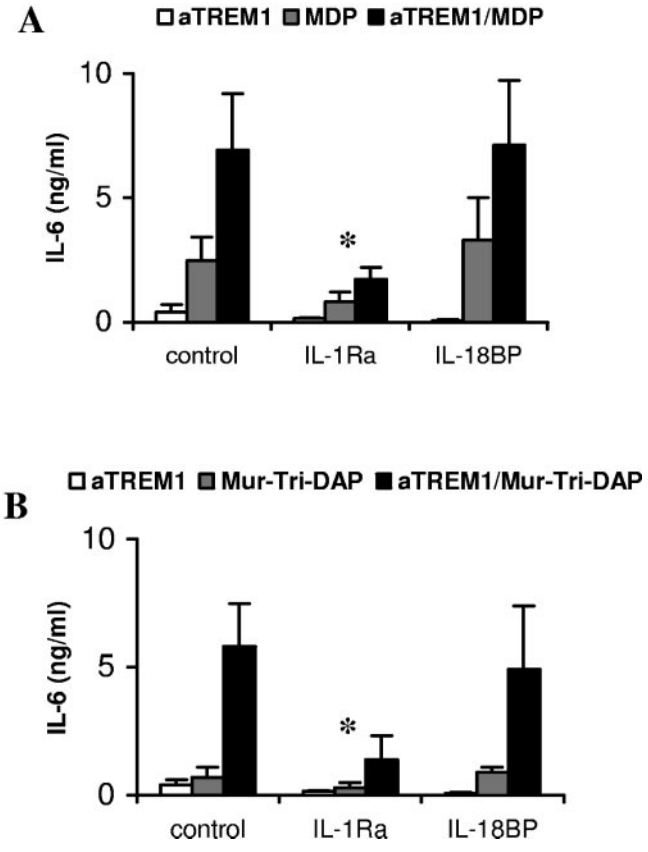

Fig. 6. The effects of caspase-1 on IL-6 production are a result of endogenous IL-1β. PBMC were added to wells coated with an anti-TREM-1 antibody (aTREM-1) or the control mouse IgG1 and then stimulated with MDP or Mur-Tri-DAP. The synergistic effects of the anti-TREM-1 agonistic antibody and the NLR ligands MDP (A) and Mur-Tri-DAP (B) on the level of IL-6 after 24 h were measured in the presence of the cytokine inhibitors IL-1Ra or IL-18BP (each at 10 μg/ml). Data are presented as means ± SEM (n=6); *, $P < 0.05$.

ber NOD2 appears to be a crucial event in the pathogenesis of Crohn's disease [35, 36], it is tempting to speculate that the low expression of TREM-1 in the intestine represents a protective mechanism against an overwhelming inflammation, which could be induced by the interaction of NOD2 and TREM-1 pathways.

It seems unlikely that an increase in TREM-1 expression by muropeptides (or TLR ligands) could play the main role in these effects: only 50% increase of the TREM-1 expression has been observed after stimulation of PBMC with TLR or NLR stimuli. However, engagement of TREM-1 increased the expression of NOD2 mRNA in each of the four volunteers. The enhancement of NOD2 expression by TREM-1 ligation was accompanied by increased sensitivity to low concentrations of MDP, and this may suggest a role of increased NOD2 expression in this synergism. In addition, the TREM-1/NLR synergism on IL-1β and IL-6 production was partially down-regulated by inhibitors of caspase-1. The CARD domain of NLR proteins interacts with caspase-1 and caspase-5, and this results in the processing of cytokine precursors such as pro-IL-1β and pro-IL-18 [21, 37]. A pan-caspase inhibitor and a specific caspase-1 inhibitor significantly (65–80%) reduced MDP-induced cytokines and the synergism between TREM-1 and muropeptides. Additional inhibition of caspase-5 by a dual inhibitor of caspase-1 and -5 did not have an additional effect on the TREM-1/NLR stimulation. These observations are consistent with a report showing that NOD1 interacts with caspase-1, enhancing the processing of pro-IL-1β [37]. Although this could suggest a possible role of caspase-1 in the TREM-1/NLR synergism, the effects of caspase-1 inhibition on NLR stimulation alone also may signify an effect of caspase-1 on NLR activation of the inflammasome and not on the synergism with TREM-1. In addition, using specific inhibitors of IL-1β and IL-18 activity, we demonstrate that although caspase-1 activation is responsible for the synergistic effects of TREM-1 and MDP on IL-1β production, it is IL-1β and not IL-18 that accounts for the secondary effect on IL-6 synthesis. Conversely, NOD2 activates the serine/threonine kinase receptor-interacting protein 2/RICK/CARDIAK, and this leads to NF-κB activation and transcription of proinflammatory cytokine genes such as TNF-α, IL-1β, and IL-6 [38]. The activation of this pathway may be potentiated by the increased NOD2 expression following TREM-1 engagement.

It is known that the short intracellular domain of TREM-1 associates with the adaptor molecule DAP12. Therefore, it is reasonable to hypothesize that the amplification signals induced by TREM-1 follow a DAP12-dependent route. This hypothesis is strengthened by a study showing increased inflammation in mice engineered to overexpress DAP12 [33]. This is in contrast to a report suggesting enhanced TLR responses in mice deficient in DAP12, which suggests that DAP12 is a negative regulator of TLR signaling [39], but is consistent with a study showing that DAP12 amplifies inflammation in models of endotoxemia and septic peritonitis [40]. Whether TREM-1 amplifies TLR and NLR signaling through a DAP12-independent mechanism or whether other DAP12-independent signals are responsible for the effects of TREM-1 remains to be elucidated. In addition, the nature of the natural agonist(s) of TREM-1 is yet to be found. Bacterial components have been reported to be ligands of TREM-2 [41], but no such reports have been published for TREM-1. Nevertheless, the Ig-like structure of TREM-1 is reminiscent of cytokine receptors rather than PRRs. However, the true nature of the TREM-1 ligand(s) remains to be elucidated.

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