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Prognostic value and therapeutic potential of TREM-1 in *Streptococcus pyogenes* induced sepsis

Running title: TREM-1 in *S. pyogenes* severe sepsis

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ABSTRACT

The triggering receptor expressed on myeloid cells (TREM-1) is a surface molecule expressed on neutrophils and macrophages, which has been implicated in the amplification of the inflammatory responses triggered during infection. In the present study we have investigated the clinical significance of TREM-1 in *Streptococcus pyogenes* severe sepsis in both experimentally infected mice as well as in patients with streptococcal toxic shock. We found that *S. pyogenes* induced a dose-dependent up-regulation of TREM-1 in *in vitro*-cultured phagocytic cells and in the organs of *S. pyogenes*-infected mice. Furthermore, we reported a positive correlation between serum levels of soluble TREM-1 (sTREM-1) and disease severity in infected patients as well as in experimentally infected mice. Hence, sTREM-1 may represent a useful surrogate marker for streptococcal sepsis. We found that modulation of TREM-1 by administration of the TREM-1 decoy receptor rTREM-1/Fc, substantially attenuated the synthesis of inflammatory cytokines. More importantly, treatment of *S. pyogenes*-infected septic mice with rTREM-1/Fc or the synthetically produced conserved extracellular domain LP17 significantly improved the disease outcome. In summary, our data suggests that TREM-1 may not only represent a valuable marker for *S. pyogenes* infection severity but it may also be an attractive target for the treatment of streptococcal sepsis.

INTRODUCTION

Sepsis is a serious medical condition caused by the body's response to an infection [1], [2]. Sepsis can be presented with a spectrum of severity with septic shock being the most severe form of this syndrome. Early intervention and diagnosis have been shown to have a significant impact in reducing the sepsis-associated morbidity and mortality [3,4]. In this regard, several potential biomarkers have been evaluated for diagnosis and for identification of patients at risk for a severe outcome. Among the evaluated candidates, the triggering receptor expressed on myeloid cells-1 (TREM-1) has recently attracted attention as a diagnostic/prognostic biomarker for sepsis [5], [6]. TREM-1 belongs to the immunoglobulin superfamily and is expressed by monocytes and neutrophils [7-9]. Expression of TREM-1 can be induced by bacterial products such as LPS and LTA [7-9] and has been reported to be highly up-regulated in neutrophils and peritoneal macrophages of mice subjected to cecal ligation and puncture (CLP) [7-9]. TREM-1 is believed to be a potent amplifier of the inflammatory response to invading pathogens since activation of this receptor during infection results in enhanced production of pro-inflammatory cytokines [7-9]. Interestingly, modulation of TREM-1 has been shown to down-regulate the inflammatory response and improve the disease outcome in murine models of sepsis [8,10] as well as in a rat model of overwhelming pseudomonas pneumonia [11]. These observations suggest that anti-TREM-1 interventions may be an efficient therapeutic strategy for the treatment of sepsis. On the other hand, Lagler *et al*. [12] reported that TREM-1 confers protection and is required for the control of pneumococcal pneumonia infection. Therefore, the beneficial effect of TREM-1 modulation seems to be highly dependent of the type of infection.

A soluble form of TREM-1 (sTREM-1) is shed from activated phagocytic cells and can be quantified in human plasma and body fluids [13]. Plasma sTREM-1 level appears to be a reliable parameter in differentiating patients with sepsis from those with systemic inflammatory response syndrome [14]. Furthermore, Gibot and co-workers [15] reported that

the concentrations of sTREM-1 remained stable or even higher in non-surviving septic patients whereas they decreased in survivors suggesting that the followed-up of plasma levels of sTREM-1 may have prognostic value during sepsis. Other studies have, however, questioned the prognostic value of sTREM-1 in sepsis [16-18], particularly since the causative microorganism can influence the pattern of the elicited immune response [19]. The prognostic and therapeutic value of TREM-1 may therefore require careful validation for each particular situation.

Streptococcus pyogenes is a common human pathogen responsible for mild infections such as pharyngitis, impetigo or scarlet fever [20] but it can also cause severe invasive infections such as bacteraemia, sepsis and septic shock associated with high mortality rates [21]. In recent years, there has been an increase in the variety and severity of *S. pyogenes* infections, including those that are invasive. The rapid progression and death in patients with severe *S. pyogenes* infections underscores the importance of early diagnosis and prompt initiation of antimicrobial and supportive therapy [22]. In this regard, the aim of the present study was to investigate the relevance of TREM-1 for the progression of streptococcal sepsis. We found a significant correlation between the levels of sTREM-1 in the plasma of *S. pyogenes*-infected septic patients and disease severity. This correlation was confirmed using an experimental murine model of *S. pyogenes* sepsis. We further demonstrated that by modulating TREM-1 signalling using the fusion protein rTREM-1/Fc or the synthetically produced conserved extracellular domain LP17, the disease outcome in septic mice was significantly improved. Our results underscore the potential prognostic as well as therapeutic value of TREM-1 in patients affected by streptococcal sepsis.

MATERIALS AND METHODS

Ethical issues. The study involving human subjects was approved by the regional ethical committee and drug agency authority in each country [23]. Written informed consent was obtained from all subjects, or their legal guardian. The study was performed in accordance with the Declaration of Helsinki. All experiments involving animals were performed in strict accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. All experiments were approved by the ethical board Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany.

Patient material. Plasma samples collected from patients with streptococcal toxic shock syndrome (STSS) (n=18) were available from a placebo-controlled trial of intravenous immunoglobulin G (IVIG) (8). STSS was defined according to established criteria [24]. Infections were caused by *S. pyogenes* of varying serotypes with T1 strains dominating. Only samples collected at baseline (prior to study drug-administration) were used to assess the correlation between plasma concentration of sTREM-1 and disease severity. Only patients that had received the placebo (n=10) were included in the study of sTREM over the first 3 days of observation.

Bacteria. *S. pyogenes* strain A20 is a clinical isolate obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (DSM 2071). Stocks were cultured in Todd-Hewitt broth (Oxoid), supplemented with 1% yeast extract and collected in mid-log-phase for experimental infections. When required, *S. pyogenes* was labelled with carboxyfluorescein (Molecular Probes, Göttingen, Germany) as previously described [25].

Experimental model of S. pyogenes sepsis. C3H/HeN female mice (Harlan-Winkelmann, Borchen, Germany) were intravenously inoculated with 10^5 CFU of *S. pyogenes* via a lateral tail vein. Infected mice were sacrificed by CO_2 asphyxiation at progressive times of infection and bacteria were enumerated in the blood, liver and spleen by preparing homogenates of these organs in PBS and plating 10-fold serial dilutions on blood agar.

To modulate TREM-1 *in vivo,* mice were intravenously injected with 5 µg (0.2mg / kg body weight) of rTREM-1/Fc, a fusion protein consisting of the extracellular domain of mouse TREM-1 and the Fc portion of human IgG1 (R&D systems, Minneapolis, USA), 2 h prior and 2 h after bacterial inoculation. Alternatively, mice were injected every 24 h intravenously with 200 µl of a 300 µM solution of the synthetically produced conserved extracellular domain LP17 (117 µg) [11] or with the inactive LP17 scrambled peptide (LP17-CTR) starting at 4 h after the onset of infection. Peptide sequence of LP17: LQVTDSGLYRCVIYHPP and LP17-CTR: TDSRCVIGLYHPPLQVY.

Measurement of sTREM-1 in serum. sTREM in plasma samples were measured by Quantikine human or murine TREM-1 ELISA (R&D systems, Minneapolis, USA) according to the manufacturer's instructions.

Cytokines ELISA. Cytokine levels were determined in serum by ELISA according to the recommendations of the manufacturer (BD Pharmingen, San Diego, CA, USA) using matched antibody pairs and recombinant cytokines as standards.

RT-PCR. Total RNA was prepared using the RNeasy RNA extraction Kit (Qiagen, Hilden, Germany). The PCR primer sequences were for the murine *trem-1*: 5'-CGG AAT TCG AGC TTG AAG GAT GAG GAA GGC-3' and 5'-GGA TCA ATC CAG AGT CTG TCA CTT GAA

GGT CAG TC-3', for the murine housekeeping gene *β-actin*: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' and 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. The resultant PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualised under UV. Quantitative real-time PCR was carried out using the SensiFAST SYBER NO-ROX-kit (Bioline, Luckenwalde, Germany) and RotorGene Q (Quiagen, Hilden, Germany).

Isolation and infection of bone marrow-derived macrophages (BMDM). Macrophages were derived from bone marrow extruded from the femur and tibia of C3H/HeN mice and *in vitro* differentiated as previously described [26]. Differentiated BMDM were infected with *S. pyogenes* at a multiplicity of infection (MOI) ranging from 1:1 to 100:1 bacteria per macrophage for the indicated period of time at 37°C. Cells were then either fixed with 3.7 % paraformaldehyde and used for microscopy analysis or lysed with RLT buffer and used for RNA extraction, or further incubated in medium containing 100 µg/ml of gentamicin to kill all extracellular bacteria.

In some experiments, BMDM were treated with 50 µg/ml of anti TREM-1 antibodies (RD Systems) or rTREM-1/Fc (RD Systems) 1 h prior to infection. Control BMDM received the same amount of human IgG1.

Immunofluorescence microscopy. BMDM were seeded on glass coverslips and infected with carboxyfluorescein-labelled *S. pyogenes* for 1 h at 37°C and fixed with 3.7% formaldehyde overnight at 4°C. BMDM were then washed, permeabilised with 200 µl of 0.1% Triton X-100 for 5 min at room temperature, blocked with PBS + 10% FCS and incubated for 45 min at room temperature with FITC-conjugated rabbit anti-TREM-1 antibodies (BD Pharmingen). Coverslips were washed with PBS, mounted on glass slides with Moviol containing DAPI (Prolong Gold, Promega) and analysed by fluorescence microscopy using an AxioVision

microscope (Zeiss).

Histopathology. Liver tissue was removed and fixed in 10% formalin for 24 h and subsequently embedded in paraffin wax. Three-μm sections were stained with hematoxylin and eosin and examined by light microscopy using an Olympus BX51 microscope.

Statistical analysis. Statistical calculations were performed using GraphPad PRISM software (San Diego, CA, USA). Comparisons between two groups were made by use of two-tailed *t*-tests. Comparison between more than two groups was made by using the analysis of variance (ANOVA) followed by a Tukey's HSD test. Comparisons of survival time curves were performed by use of the Logrank test. The non-parametric Spearman test was used to determine correlations between groups. All data is presented as the mean ± SD; $p < 0.05$ was considered significant.

RESULTS

S. pyogenes induces expression of TREM-1.

First, we determined if *S. pyogenes* could induce up-regulation of TREM-1 on phagocytic cells. For this purpose, the expression of *trem-1* mRNA was assessed in *S. pyogenes*-infected BMDM at progressive times of infection. The semi quantitative PCR analysis displayed in fig. 1a and the quantitative RT-PCR data displayed in fig. 1b show that *trem-1* mRNA was induced on BMDM after exposure to *S. pyogenes* and that the degree of *trem-1* up-regulation was highly dependent on the infectious dose. The dose-dependency of TREM-1 induction was further evidenced at the protein level using fluorescence microscopy (fig. 1c). TREM-1 induction was also increased on bone marrow-derived murine neutrophils after exposure to *S. pyogenes* (Supplementary fig. 1).

The expression of TREM-1 during *S. pyogenes in vivo* infection was then investigated using a previously described murine infection model [27]. In this model, intravenous inoculation of C3H/HeN mice with 10^5 CFU of *S. pyogenes* strain A20 resulted in severe sepsis characterized by progressive bacterial growth in the blood and liver (fig. 2a), high levels of serum IL-6 (fig. 2b), which is an important mediator of septic shock [28,29], and 100% mortality by day 4 of infection (fig. 2c). The quantitative PCR analysis shown in fig. 2d demonstrated a progressive increase in the up-regulation of TREM-1 in the liver of mice during the course of *S. pyogenes* infection. TREM-1 was also highly up-regulated on peritoneal cells (macrophages and neutrophils) isolated from the peritoneal cavity of C3H/HeN mice after i.p. injection of *S. pyogenes* (Supplementary fig. 2).

Plasma levels of sTREM-1 correlate with severity of disease in *S. pyogenes*-infected mice.

TREM-1 can be cleaved off the membrane of activated neutrophils and monocytes to release a soluble form of TREM-1 (sTREM-1) [13]. High levels of sTREM-1 have been

detected in the peritoneal lavage fluid of CLP septic mice [13] as well as in the plasma of patients at risk of sepsis [6,14,15]. Therefore, we investigated if sTREM-1 was released in the serum of *S. pyogenes*-infected C3H/HeN mice during the course of the infection. Serum sTREM-1 was readily detectable at 24 h after bacterial inoculation and increased significantly at 48 h of infection, the point of time when the mice undergo overwhelming sepsis and begin to die (fig. 3a). We next plotted the levels of sTREM-1 in the serum of infected mice against the amount of bacteria in the liver and blood. A strong positive correlation was found between the serum levels of sTREM-1 and the bacterial loads in the liver (fig. 3b; r = 0 .951, $p < 0.001$) and blood (fig. 3c; r = 0.9423, $p < 0.001$). Therefore, serum levels of sTREM-1 appear to be a suitable marker of the severity of *S. pyogenes* infection.

Plasma levels of sTREM-1 correlate with severity of disease in patients with streptococcal toxic shock.

Having demonstrated the potential prognostic value of serum sTREM-1 in experimental streptococcal sepsis, we next sought to validate these results in patients with streptococcal toxic shock. For this purpose, the levels of sTREM-1 were measured in plasma collected from patients with streptococcal toxic shock during the acute phase of infection. At baseline, all patients had elevated sTREM-1 levels with concentrations ranging from 257-3075 pg/ml (mean = 1011 pg/ml) (fig. 4a). Importantly, there was a significant correlation (r = 0.73, $p < 0.0009$) between the plasma concentration of sTREM-1 and the severity of disease as defined by the patient's baseline Simplified Acute Physiology Score (SAPS II) (fig. 4a). Longitudinally, plasma sTREM-1 concentrations declined over time in most cases (fig. 4b, continuous line) with the exception of 3 patients, which exhibited a completely different profile (fig. 4b, dashed lines). One of these patients had extremely high values of sTREM-1 (fig. 4b, white triangles) and the other two patients demonstrated a sharp increase of plasma sTREM-1 during days 1 to 3 (fig. 4b, white diamond and white squares). These patients were

the only ones with a fatal outcome in this cohort. These observations indicate that serum sTREM-1 levels can be used as predictor of outcome in patients with streptococcal sepsis. According to the specification of the sTREM kit used, levels in plasma of healthy controls showed a mean of sTREM-1 serum levels of 134 pg/ml.

TREM-1 modulates the inflammatory response of macrophages to *S. pyogenes in vitro*.

It has been previously shown that TREM-1 synergises with LPS and amplifies the synthesis of pro-inflammatory cytokines by monocytes/macrophages [8,9] and it has been also shown that rTREM-1/Fc is able to modulate and attenuate the inflammatory response towards bacterial infections *in vivo*, by trapping bacterial ligands and the therefore avoiding TREM-1 signalling on the cellular surface of monocytes/macrophages [11]. Hence, we investigated the potential contribution of TREM-1 to the production of inflammatory cytokines by macrophages after exposure to *S. pyogenes*. For this purpose, the levels of IL-6 and TNF-α were determined in the supernatant of macrophages infected with *S. pyogenes* in the presence or absence of rTREM-1/Fc, a fusion protein containing the extracellular domain of murine TREM-1 and the Fc portion of human IgG1 that acts as decoy receptor to compete with cell associated TREM-1 [8]. We found a significant attenuation of IL-6 (fig. 5a) and TNF-α (fig. 5b) production by macrophages treated with rTREM-1/Fc. Treatment with rTREM-1/Fc did not influence the capacity of BMDM to phagocyte or kill *S. pyogenes* (data not shown).

Modulation of TREM-1 *in vivo* has a beneficial effect during *S. pyogenes*-induced sepsis.

It has been reported that treatment with TREM-1 blocking agents has a beneficial effect in septic mice undergoing microbial sepsis caused by CLP [8,10] and in rats affected by severe pneumonia caused by *Pseudomonas aeruginosa* [11]. Therefore, our next step was to address the therapeutic effect of TREM-1 modulation in the course of *S. pyogenes*-induced

sepsis. For this purpose, C3H/HeN mice were intravenously infected with *S. pyogenes* and treated with the TREM-1 modulating agent rTREM-1/Fc or with human IgG1 as a control. The effect of rTREM-1/Fc in *S. pyogenes*-infected animals was evaluated by measuring survival times, bacterial growth and systemic inflammatory cytokines. As shown in fig. 6a, treatment with rTREM-1/Fc significantly extended the survival times of infected mice with respect to isotype IgG1-treated animals ($p < 0.01$). Bacterial loads were significantly lower in the blood, liver and spleens of rTREM-1/Fc–treated mice (fig. 6b) and the systemic levels of IL-6 (fig. 6c) and TNF-α (fig. 6d) were also significantly decreased when compared with the control group. Importantly, the modulation of TREM-1 significantly attenuated but did not abolish the production of IL-6 and TNF-α.

Similar results were obtained when *S. pyogenes*-infected mice were treated with the synthetically produced conserved extracellular domain LP17. This domain contains the highly conserved extracellular binding region of the surface TREM-1 protein based on the TREM-1 sequence in GenBank/EMBL/DDBJ (accession number AF241219). Thus, mice treated with LP17 survived longer than those treated with the inactive scrambled LP17 control peptide (Fig. 7). In addition, LP17-treated mice (Supplementary fig. 7a) exhibited lower levels of infection-associated pathology in the liver than scrambled LP17 control peptide treated animals (Supplementary fig. 7b) as shown by the reduced thrombus and less necrotic tissue formation.

DISCUSSION

One of the most severe clinical manifestations of *S. pyogenes* infections is streptococcal toxic shock syndrome [20]. Early diagnosis and timely therapeutic intervention is crucial for the successful outcome of this syndrome. In this study, we have investigated the role of TREM-1, a promising candidate for the diagnosis and prognosis of septic patients [5,6], in the pathogenesis of sepsis induced by *S. pyogenes*. We found that TREM-1 expression was dramatically up-regulated on macrophages and neutrophils after *in vitro* exposure to *S. pyogenes*. More importantly, we found a significant positive correlation between the serum levels of sTREM-1 (the soluble form of TREM-1) and the bacterial loads in the liver and blood of *S. pyogenes*-infected mice. We extended this investigation to patients with streptococcal toxic shock syndrome and found a similar positive correlation between the levels of plasma sTREM-1 and the severity of sepsis. Furthermore, sequential measurement of plasma concentrations of sTREM-1 in 10 of these septic patients showed that plasma sTREM-1 concentrations declined over time in survivors (7 patients) but sharply increased in two of the patients with a fatal outcome. Together, these observations highlight the value of plasma sTREM-1 as a surrogate biomarker in patients with streptococcal sepsis.

Regarding the physiological function of TREM-1, it has been proposed that TREM-1 is an amplifier of the inflammatory response to bacterial infection [7-9]. Thus, evidence has been provided which indicates that simultaneous triggering of TREM-1 and TLRs or Nod-like receptors results in a synergistic effect in the production of pro-inflammatory cytokines [7,8,30,31]. Therefore, TREM-1 seems to potentiate the signal transmitted by the pattern-recognition receptors to ensure a rapid host response upon pathogen recognition. In this study, we demonstrated that TREM-1 is involved in the immune response of phagocytic cells to *S. pyogenes* since attenuation of TREM-1 signalling using rTREM-1/Fc significantly decreased the production of the pro-inflammatory cytokines such as IL-6 and TNF-α. The potential

molecular mechanism underlining this protective effect might be the capturing of bacterial ligands by the rTREM-1/Fc protein reducing therefore TREM-1 signalling on the surface of immune cells and production of inflammatory cytokines by these cells. However, the nature of TREM-1 ligands is still a mystery. There is some evidence existing that besides bacterial molecules like LPS, also a ligand on human platelets might exists [32] which enhances the activation of neutrophils and monocytes/macrophages. It was shown that sTREM-1 binds to human platelets and that this interaction can be blocked by LP17 [32]. Recently it has been also demonstrated that high-mobility group box 1 (HMGB1) and heat shock protein 70 (Hsp70) could be potential ligands of TREM-1 [33]. Nevertheless there is still very little knowledge regarding the specific ligands involved in TREM-1 signalling of Gram-positive bacteria and also the ligands which might be involved in *S. pyogenes* infection, remains to be identified.

Despite its important role in the initiation of a rapid immune response after pathogen recognition, activation of TREM-1 has been shown to contribute to the harmful amplification of the inflammatory reaction in situations of generalized inflammatory response to infection such as sepsis and septic shock [9,34]. Thus, interfering with TREM-1 signalling has been shown to attenuate systemic inflammation and promotes survival during experimental sepsis [8] as well as pulmonary inflammation during experimental *P. aeruginosa* pneumonia in rats [11]. Together, these studies suggested that modulating TREM-1 activity could be a viable treatment for sepsis. In contrast, Lagler *et al*. [12] reported that modulation of TREM-1 did not affect the inflammatory response or severity of infection in mice infected with *Streptococcus pneumoniae*. In our model of experimental *S. pyogenes*-induced sepsis, mitigation of TREM-1 signalling using rTREM-1/Fc as well as the synthetically produced conserved extracellular domain LP17 significantly improved the disease outcome in septic mice. This was demonstrated by the attenuated production of inflammatory cytokines, decreased bacterial loads and extended survival times observed in LP17-treated mice. Our

results are therefore in agreement with previous studies showing the detrimental effect of TREM-1 activation in the pathology of sepsis and imply that modulation of TREM-1 may help to optimize the efficacy of existing treatments in patients with streptococcal septic shock.

More importantly, we found that treatment of *S. pyogenes*-infected septic mice with rTREM-1/Fc signalling resulted in significant reduction of IL-6 and TNF-α but not in the complete inhibition of cytokine production. Inflammatory cytokines, in particular IL-6, are considered to be deleterious [28,29], yet they also have beneficial effects during severe *S. pyogenes* infections as shown by the more severe outcome of *S. pyogenes* invasive infection in mice deficient in the production of IL-6 [35].

In summary, the results of our study underscore the potential value of sTREM-1 as a surrogate marker for monitoring patients with streptococcal sepsis as well as a potential therapeutic agent for the treatment of this syndrome.

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AUTHOR CONTRIBUTIONS

SAH, AL, SL, CH, and AH carried out the experiments. SAH, AN-T, EM and OG conceived the experiments and analysed the data. The study was directed and interpreted by EM and OG.

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FIGURE LEGENDS

FIGURE 1. *S. pyogenes* induces expression of TREM-1 in BMDM. (a) Expression of *trem-1* mRNA on BMDM after infection with *S. pyogenes* at a MOI of 1:1, 10:1 and 100:1 bacteria per macrophage. BMDM were exposed to *S. pyogenes* for 1 h, washed and further incubated in the presence of gentamicin for 4 h. Total RNA was isolated followed by RT-PCR analysis of *trem-1* and *β-actin* gene expression. (b) Quantitative expression of *trem-1* mRNA on BMDM after infection with *S. pyogenes* at a MOI of 1:1, 10:1 and 100:1 bacteria per macrophage measured by real-time PCR. (c) Fluorescence microscope photographs of TREM-1 in BMDM infected with *S. pyogenes* at an MOI of 1:1 (Cii), 10:1 (Ciii) and 100:1 (Civ) bacteria per macrophage. TREM-1 expression on uninfected macrophages is shown in (Ci). TREM-1 appears in red and *S. pyogenes* in green. Macrophages nuclei are stained by DAPI (blue). Bar size, 25 μm.

FIGURE 2. *S. pyogenes* induces expression of TREM-1 during *in vivo* infection. (a) Kinetics of bacterial growth in the blood (circles) and liver (squares) of C3H/HeN mice after intravenous inoculation with 10^5 CFU of *S. pyogenes*. Each symbol represents the mean value ± SD of 5 mice. One representative experiment out of three is shown. (b) Serum levels of IL-6 in C3H/HeN mice after intravenous inoculation with 10^5 CFU of *S. pyogenes*. Sera were collected at indicated time points and the levels of IL-6 in the serum were determined by ELISA. Each bar represents the mean value ± SD of 5 mice. One representative experiment out of three is shown. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$. (c) Survival time of C3H/HeN mice after intravenous infection with 10^5 CFU of *S. pyogenes*. (d) Expression of

trem-1 mRNA in the liver of C3H/HeN mice at progressive times after intravenous inoculation with 10^5 CFU of *S. pyogenes*. The induction of *trem-1* was determined by RT-PCR. *β-actin* expression serves as an internal control. (e) Quantification of *trem-1* mRNA in the liver of C3H/HeN mice at progressive times after intravenous inoculation with 10^5 CFU of *S. pyogenes* determined by real-time PCR.

FIGURE 3. sTREM-1 is shed in the plasma of *S. pyogenes*-infected mice. (a) Kinetics of sTREM-1 serum levels in *S. pyogenes*-infected C3H/HeN mice. (b) Correlation of sTREM-1 in serum samples with the amount of bacteria in the liver of *S. pyogenes*-infected mice. Spearman's correlation coefficient r = 0.951, $p < 0.001$. (c) Correlation of sTREM-1 in serum samples with the amount of bacteria in the blood of *S. pyogenes*-infected mice. Spearman's correlation coefficient r = 0.9423, $p < 0.001$. Each point represents one individual animal. **, $p < 0.01$ and ***, $p < 0.001$.

FIGURE 4. sTREM-1 in the plasma of patients affected by streptococcal toxic shock syndrome. (a) Spearman′s test correlation between sTREM-1 plasma levels and disease severity determined by the SAPS II score in patients with streptococcal septic shock. Each point represents the correlation value for one patient (n = 18). Spearman's correlation coefficient r = 0.73, $p < 0.0009$. (b) Line plot diagram showing plasma sTREM-1 concentration detected on 3 consecutive days. Each patient is shown by a specific symbol. Continuous lines indicate surviving patients and dashed lines those with a fatal outcome.

FIGURE 5. Effect of rTREM-1/Fc treatment in the production of inflammatory cytokines by BMDM. Production of IL-6 (a) and TNF-α (b) by uninfected or *S. pyogenes*-infected BMDM in the presence or absence of rTREM-1/Fc. BMDM were treated with 50 ng/ml of either rTREM-1/Fc or human IgG1 isotype control 1 h prior to infection with *S. pyogenes* at an MOI

of 10:1 bacteria per macrophage. After 1 h of incubation, the culture medium was replaced by medium supplemented with 100 μg/ml of gentamicin to kill all extracellular bacteria, 50 ng/ml of either rTREM-1/Fc or human IgG1 isotype control were retained in the medium and the levels of IL-6 and TNF-α measured in the supernatant 4 h thereafter. Each bar represents the mean ± SD of 10 different values obtained from two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

FIGURE 6. Effect of rTREM-1/Fc treatment in the course of *S. pyogenes* infection in mice. (a) Survival curves of *S. pyogenes*-infected mice treated with either isotype control IgG1 (black symbols) or rTrem-1/Fc (white symbols) 2 h prior and 2 h after intravenous inoculation with 10^5 CFU of *S. pyogenes.* Comparison of survival curves was performed by use of the Logrank test. (b) Bacterial loads in the blood, liver and spleen of *S. pyogenes*-infected mice treated with either isotype control IgG1 (black symbols) or rTrem-1/Fc (white symbols) at 48 h after intravenous inoculation with 10^5 CFU of *S. pyogenes*. Each symbol represents the bacterial load of one animal. One representative experiment out of three is shown. Levels of IL-6 (c) and TNF-α (d) in the serum of uninfected or *S. pyogenes*-infected mice treated with either isotype control IgG1 or rTrem-1/Fc at 48 h of infection. Each bar represents the mean ± SD of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$, and ***, $p < 0.001$.

FIGURE 7. Effect of LP17 treatment in the course of *S. pyogenes* infection in mice. Survival curves of *S. pyogenes*-infected mice treated with either LP17 peptide (white symbols) or scrambled LP17-control peptide (black symbols) after intravenous inoculation with 10^5 CFU of *S. pyogenes.* Comparison of survival curves was performed by use of the Logrank test. **, $p < 0.01$.