

controls (MyD88^{+/+}, *n* = 5) were injected i.p. with 30 µg flagellin in 0.5 cc of saline. Blood was sampled at 0, 1, 2, 4 and 8 h after injection, and IL-6 levels were determined by ELISA (DuoSet, R&D Systems).

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Correspondence and requests for materials should be addressed to A.A. (e-mail: aderem@systemsbiology.org).

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TREM-1 amplifies inflammation and is a crucial mediator of septic shock

Axel Bouchon*[†], Fabio Facchetti*[‡], Markus A. Weigand*[‡] & Marco Colonna*

* *Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland*

[†] *Institute of Pathology, University of Brescia, Spedali Civili 1, Brescia, Italy*

[‡] *Clinic of Anaesthesiology, University of Heidelberg and Tumor Immunology Program, German Cancer Research Institute, (DKFZ), INF 110,280, 69120 Heidelberg, Germany*

[‡] *These authors contributed equally to this work*

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Host innate responses to bacterial infections are primarily mediated by neutrophils and monocytes/macrophages^{1,2}. These cells express pattern recognition receptors (PRRs) that bind conserved molecular structures shared by groups of microorganisms^{3,4}. Stimulation of PRR signalling pathways initiates secretion of proinflammatory mediators^{3,4}, which promote the elimination of infectious agents and the induction of tissue repair. Excessive inflammation owing to bacterial infections can lead to tissue damage and septic shock^{5–9}. Here we show that inflammatory responses to microbial products are amplified by a pathway mediated by triggering receptor expressed on myeloid cells (TREM)-1. TREM-1 is an activating receptor expressed at high levels on neutrophils and monocytes that infiltrate human tissues infected with bacteria. Furthermore, it is upregulated on peritoneal neutrophils of patients with microbial sepsis and mice with experimental lipopolysaccharide (LPS)-induced shock. Notably, blockade of TREM-1 protects mice against LPS-induced shock, as well as microbial sepsis caused by live *Escherichia coli* or caecal ligation and puncture. These results demonstrate a critical function of TREM-1 in acute inflammatory responses to bacteria and implicate TREM-1 as a potential therapeutic target for septic shock.

We have identified recently a new receptor of the immunoglobulin superfamily expressed on human neutrophils and monocytes, called TREM-1 (ref. 10), which promotes cell activation through an associated signal transduction molecule DAP12 (ref. 11). The ability of TREM-1 to trigger secretion of proinflammatory mediators¹⁰ prompted us to investigate its role in inflammation caused by bacteria. We determined TREM-1 expression by flow cytometry on neutrophils and monocytes incubated *in vitro* with heat-inactivated Gram-positive bacteria, Gram-negative bacteria, mycobacteria, or with bacterial cell-wall components. TREM-1 expression was strongly upregulated by extracellular bacteria, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, as well as lipoteichoic acid (LTA) and LPS (Fig. 1a–b). In contrast, intracellular bacteria, such as *Bacillus Calmette-Guerin* (BCG), and mycolic acid had no effect. These results indicate that TREM-1 is upregulated in the presence of extracellular bacteria. On ligation, TREM-1 synergized with LPS to induce secretion of proinflammatory cytokines (tumour necrosis factor (TNF)-α and interleukin (IL)-1β) in monocytes (Fig. 1c). Thus, TREM-1 signalling amplifies responses to microbial products.

In vivo TREM-1 expression was determined in tissue specimens derived from acute or granulomatous inflammatory lesions caused by bacterial, fungal or non-microbial agents. TREM-1 was highly expressed in neutrophils associated with skin lesions caused by *S. aureus*, such as folliculitis and impetigo (Fig. 2a–d). In addition, increased TREM-1 expression was observed in neutrophils associated with granulomatous lymphadenitis caused by *Bartonella henselae* and *Aspergillus fumigatus* (Fig. 2e–h). In the latter, TREM-1 was also expressed in epithelioid and multinucleated giant cells

surrounding the granulomas (Fig. 2g). In contrast, TREM-1 was hardly detectable in non-microbial inflammations, such as psoriasis, ulcerative colitis and vasculitis caused by immune complexes, despite a considerable infiltration of neutrophils and monocytes (Fig. 3). These results are consistent with a predominant role of TREM-1 in acute and granulomatous inflammations caused by extracellular bacteria and fungi.

Excessive inflammatory response to infectious agents can lead to septic shock⁵⁻⁹, which is characterized by massive release of pro-inflammatory mediators, such as TNF- α , IL-1 β , macrophage migration inhibitory factor (MIF) and high mobility group (HMG)-1 protein. This process leads not only to tissue damage, but also to haemodynamic changes, multiple organ failure, and ultimately death^{6,12-15}. Consistent with the function of TREM-1 in response to bacterial infections, TREM-1 surface expression was strongly increased on infiltrating neutrophils isolated from the peritoneal cavity of patients with septic shock due to bacterial peritonitis (Fig. 4a, right panel). In contrast, peritoneal lavage cells of patients with a systemic inflammatory response syndrome (SIRS)

caused by non-microbial peritoneal inflammation showed normal levels of TREM-1 (Fig. 4a, left panel).

To assess the direct involvement of TREM-1 in inflammatory responses, we tested whether a TREM-1 receptor decoy reduces inflammation and lethal shock in murine models of sepsis. We cloned the murine homologue of human TREM-1 and generated a murine TREM-1-specific monoclonal antibody (data not shown). Using this antibody, we observed that murine TREM-1 expression was upregulated in peritoneal neutrophils during experimental LPS-induced shock (Fig. 4b). A fusion protein containing murine TREM-1 extracellular domain and human immunoglobulin- γ (IgG1) Fc portion (mTREM-1/IgG1) was produced and injected in the peritoneal cavity 1 h before the induction of endotoxaemia. Lethality was monitored over time and compared with animals that had received control injections of human IgG1 (hIgG1), control IgG1 fusion protein (ILT-3/IgG1)¹⁶ or heat-inactivated mTREM-1/IgG1 before LPS administration. As shown in Fig. 5a, 76% of the mice treated with mTREM-1/IgG1 survived endotoxaemia as compared with 6% of control mice. To quantify the protection provided by mTREM-1/IgG1, groups of mice pre-treated with mTREM-1/IgG1 or hIgG1 were challenged with various doses of LPS. The median lethal dose (LD₅₀) of LPS in animals treated with mTREM-1/IgG1 (LD₅₀ = 621 μ g) was significantly higher than the LD₅₀ in control animals (LD₅₀ = 467 μ g) (Fig. 5b). We further monitored whether mTREM-1/IgG1 was still protective when administered 1,

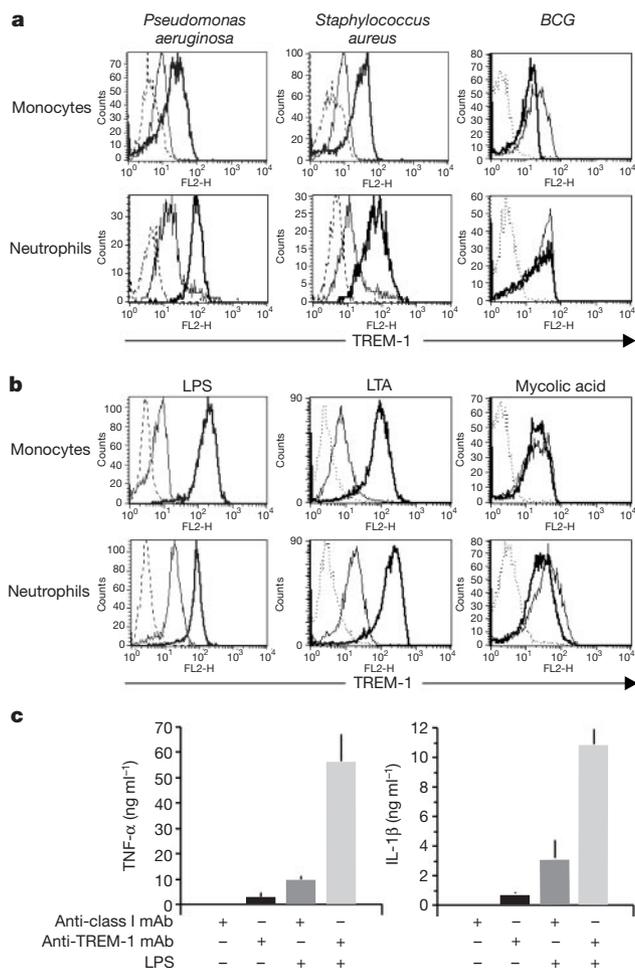


Figure 1 Regulation of human TREM-1 surface expression and function *in vitro*. **a, b**, TREM-1 is strongly upregulated after incubation of neutrophils and monocytes with heat-inactivated *S. aureus*, *P. aeruginosa*, LTA and LPS (left and middle panels) but not by BCG and mycolic acid (right panels). Stimulated (solid bold line) and non-stimulated (solid line) cells were analysed for cell-surface expression of TREM-1 (FL2-H). Dashed lines indicate background staining of stimulated cells with a control IgG1 monoclonal antibody (mAb). **c**, Ligand of TREM-1 potentiates LPS-mediated cytokine release. Monocytes were challenged for 16 h, as indicated. Supernatants were analysed for TNF- α (left panel) and IL-1 β (right panel). All data points correspond to the mean and the s.d. of four independent experiments.

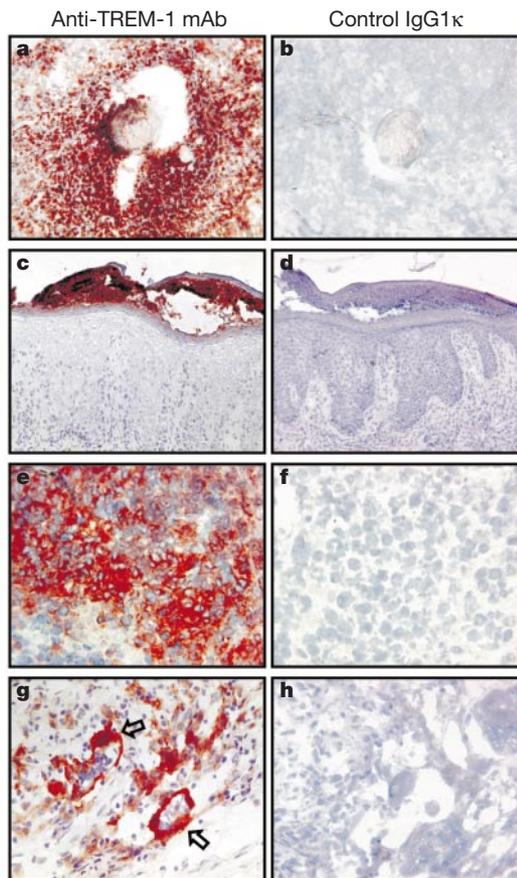


Figure 2 Human TREM-1 is strongly expressed in acute inflammatory lesions caused by bacteria and fungi. Staining with control mAb (**b, d, f, h**) and expression of TREM-1 (**a, c, e, g**). TREM-1 expression was detected using mAb 21C7 in acute cutaneous folliculitis (**a**) and impetigo (**c**) owing to *S. aureus*; in cat scratch granuloma induced by *B. henselae* (**e**); and in granuloma owing to *A. fumigatus* (**g**). Within the last (**g**), TREM-1 is expressed not only on infiltrating neutrophils but also on the multinucleated giant cells (arrows) surrounding the granuloma.

2, 4 and 6 h after LPS injection. Notably, mTREM-1/IgG1 conferred 80% protection against endotoxic shock when applied 1 h after LPS injection. Partial protection was also observed after 2 and 4 h (Fig. 5c). Thus, soluble TREM-1 is effective even when injected after the outbreak of endotoxaemia.

Analysis of blood samples taken from mice pre-treated with mTREM-1/IgG1 and control animals at different time points after LPS administration showed a significant reduction of the plasma concentrations of both TNF- α and IL-1 β (Fig. 5d, e). We also observed a significant reduction in the total cell number of neutrophils and monocytes/macrophages infiltrating the peritoneum 6–8 h after LPS injection in mTREM-1/IgG1-pre-treated animals as compared with controls (Fig. 5f, g). Injection of mTREM-1/IgG1 in normal mice did not affect the levels of circulating leukocytes. Furthermore, mTREM-1/IgG1 was effective against endotoxaemia even when the IgG1–Fc portion of the fusion protein was mutated to inhibit Fc receptor binding and complement fixation (data not shown). Thus, inhibition of TREM-1-mediated responses is sufficient to lower systemic levels of TNF- α and IL-1 β , and to reduce cellular infiltrates at the site of inflammation below levels that are lethal for the host, without causing leukopenia.

Experimental endotoxic shock reproduces human sepsis only in part, as it does not involve the replication and dissemination of bacteria. In these conditions a complete block of TREM-1 signalling could be deleterious by impairing the capacity of the immune system to fight infections, as observed for anti-TNF- α treatments^{17–23}. We therefore investigated whether mTREM-1/IgG1 protects against septic shock in two models of microbial peritonitis and sepsis caused by intraperitoneal (i.p.) administration of *E. coli* or by caecal ligation and puncture (CLP)^{17,24,25}. As shown in Fig. 6, injection of mTREM-1/IgG1 conferred significant protection

against lethal *E. coli* peritonitis and CLP-induced septic shock compared with control hIgG1, whereas treatment with TNF- α receptor I IgG1 (TNF-RI/IgG1) caused accelerated death of all animals (Fig. 6b). Thus, mTREM-1/IgG1 reduces inflammatory responses but allows sufficient control of the bacterial infection.

Our results demonstrate that TREM-1 is an amplifier of inflammatory responses that are triggered by bacterial and fungal infections. In the early phase of infection, neutrophils and monocytes initiate the inflammatory response owing to the engagement of PRRs by microbial products. At the same time, bacterial products induce upregulation of TREM-1. On recognition of an unknown ligand, TREM-1 activates DAP12-signalling pathways^{10,11}, which amplify inflammatory responses. We have shown that ligation of TREM-1 leads to sustained secretion of proinflammatory cytokines (TNF- α and IL-1 β) and chemokines (IL-8 and monocyte chemoattractant protein (MCP)-1)¹⁰ and may also result in prolonged survival of neutrophils and monocytes at the inflammatory site. Inhibition of functions mediated by TREM-1 in LPS-induced shock is sufficient to reduce serum TNF- α and IL-1 β to sublethal levels, preventing shock and death. Furthermore, mTREM-1/IgG1 protects against bacterial peritonitis, in contrast to treatment with TNF-RI/IgG1 (Fig. 6b) and anti-TNF- α antibodies¹⁷, which increase lethality. The residual levels of TNF- α and IL-1 β after mTREM-1/IgG1 treatment are probably sufficient for clearance of bacterial infections^{17–23}. Murine TREM-1-IgG1 was even protective after injection of LPS, an effect that was only reported for inhibition of MIF and HMG-1^{12,24}. Thus, administration of soluble TREM-1 after

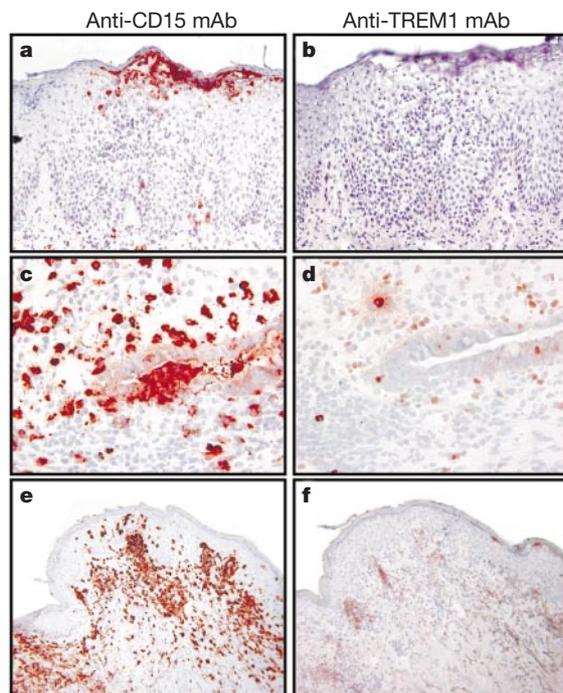


Figure 3 Human TREM-1 is only weakly expressed in neutrophils and monocytes accumulating in non-microbial inflammations. Psoriasis (**a, b**), ulcerative colitis (**c, d**), and vasculitis caused by immune complexes (**e, f**) are characterized by inflammatory infiltrates of neutrophils, as detected by anti-CD15 mAb (left panels). TREM-1 expression is weak or absent, however, in consecutive/serial sections (right panels).

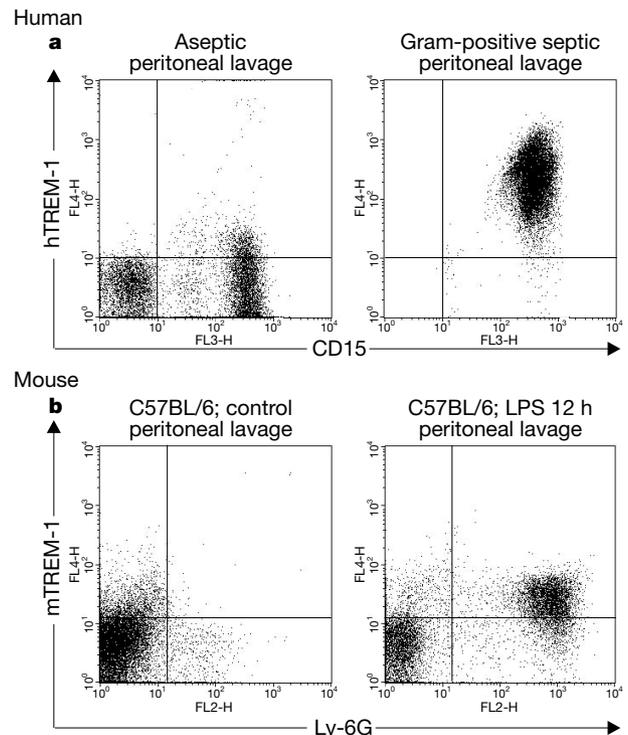


Figure 4 TREM-1 is strongly upregulated on peritoneal neutrophils during septic shock in humans and mice. **a**, Flow cytometric analysis of peritoneal lavage cells from patients with aseptic SIRS owing to aseptic cholecystitis (left) or polymicrobial Gram-positive sepsis caused by bowel perforation (right). CD15⁺ cells correspond to neutrophils. **b**, Four-colour analysis of peritoneal leucocytes from LPS-treated C57BL/6 mice (right) compared with control animals (left) Ly-6G⁺/TREM-1⁺ cells correspond to murine neutrophils. The Ly-6G⁺/TREM-1⁺ cells are CD11b/Mac-1⁺ (data not shown) and therefore correspond to peritoneal macrophages. Staining with isotype-matched control monoclonal antibodies were set to the indicated lower quadrants.

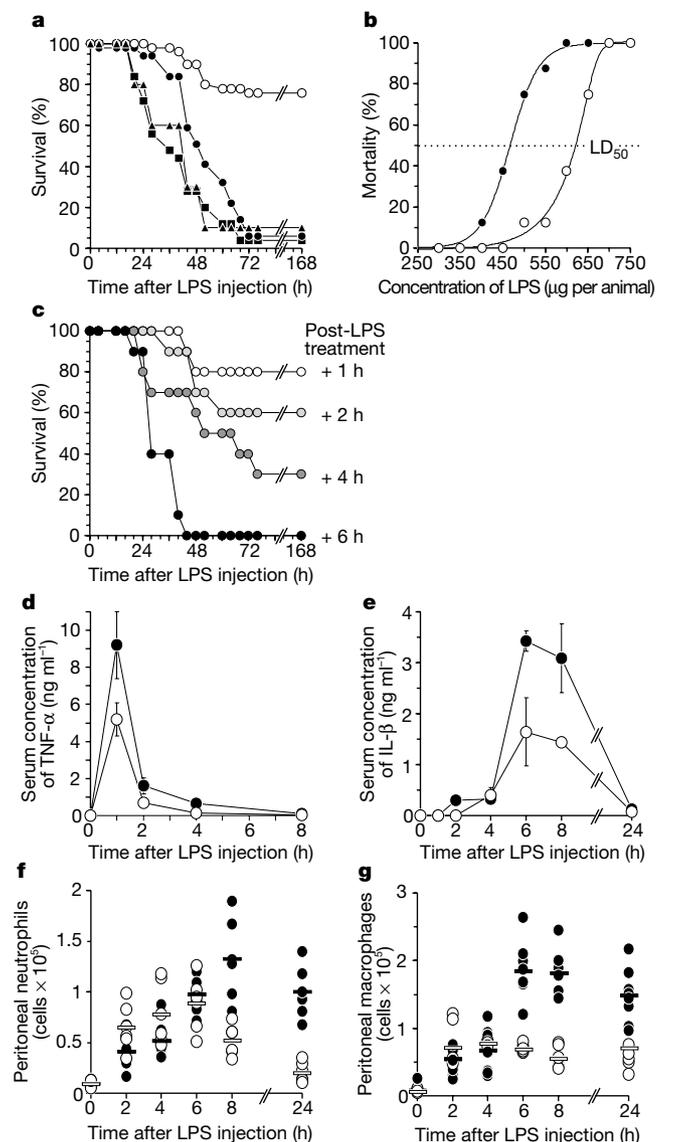


Figure 5 Inhibition of mTREM-1 signalling blocks endotoxic shock and inflammatory responses *in vivo*. **a**, C57BL/6 mice were treated with control hlgG1 (filled circles) or mTREM-1/IgG1 (open circles) 1 h before LPS administration. Data points are from seven independent experiments (5–10 animals per group). Survival was 76% (37 out of 49) in mice treated with mTREM-1/IgG1 and 6% (3 out of 49) in mice treated with hlgG1 ($P = 0.0002$, two-tailed Fisher's exact test). In additional controls, mice received injections with purified human ILT3-IgG1 (filled squares; $n = 25$) or heat-inactivated mTREM-1/IgG1 (filled triangles; $n = 10$) before induction of endotoxaemia. **b**, Estimation of LPS LD₅₀ in mice treated with mTREM-1/IgG1 or hlgG1. Mice were randomly assigned to 20 groups, each containing 10 animals. Ten groups received i.p. injections of mTREM-1/IgG1, and 10 groups were injected with hlgG1. One hour later, endotoxaemia was induced by application of LPS, as indicated. Calculation of LD₅₀ was accomplished as described (LD₅₀ for mTREM-1/IgG1 = 621 μg; LD₅₀ for hlgG1 = 467 μg; $P < 0.0001$)⁶. **c**, mTREM-1/IgG1 protects against LPS-induced lethal peritonitis when given after challenge. Mice were injected with LPS before administration of mTREM-1/IgG1. Data points are from two independent experiments (3–7 animals per group). Survival was 80%, 60% ($P = 0.0007$ and $P = 0.0108$, respectively; two-tailed Fisher's exact test), 40% and 0%, respectively. **d–g**, Analysis of inflammatory parameters during fatal endotoxaemia. Mice were treated as described in **a**. Serum levels of TNF-α (**d**) and IL-1β (**e**), and numbers of peritoneal neutrophils (**f**) and macrophages (**g**) were determined at the indicated time points. Data points correspond to the mean and the s.d. of two independent experiments (4–6 mice per treatment group).

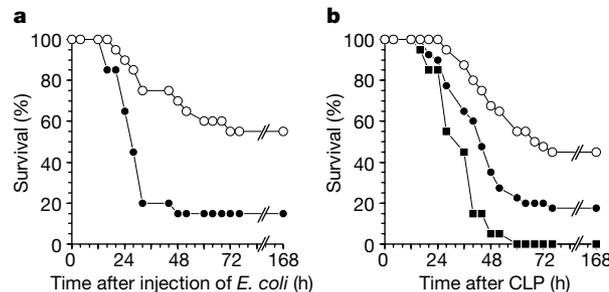


Figure 6 mTREM-1 is protective in bacterial peritonitis. **a**, C57BL/6 mice injected i.p. with mTREM-1/IgG1 (open circles) or hlgG1 (filled circles) 1 h before i.p. administration of *E. coli*. Data points are from two independent experiments (5–15 animals per group). Survival was 55% (11 out of 20) in mice treated with mTREM-1/IgG1 and 15% (3 out of 20) in mice treated with control hlgG1 ($P = 0.0187$, two-tailed Fisher's exact test). **b**, mTREM-1/IgG1 protects against fatal septic shock induced by CLP. Mice were injected i.p. with mTREM-1/IgG1 (open circles), hlgG1 (filled circles) or TNF-R1/IgG1 (filled squares) immediately after CLP. Data points are from four independent experiments (5–10 animals per group). Survival was 45% (18 out of 40) in mice treated with mTREM-1/IgG1, 17.5% (7 out of 40) in mice treated with control hlgG1 ($P = 0.015$, two-tailed Fisher's exact test) and 0% (0 out of 20) in mice treated with TNF-R1/IgG1.

infection might be a suitable therapeutic tool for the treatment of septic shock as well as other microbial-mediated diseases. □

Methods

Human cells and bacteria

Human peripheral blood mononuclear cells, neutrophils and CD14⁺ monocytes were purified from peripheral blood of healthy donors as described¹¹. Human peritoneal leukocytes were obtained from peritoneal lavage of patients diagnosed with aseptic systemic inflammatory response syndrome or polymicrobial sepsis, as defined in ref. 26. Cultures of *S. aureus*, *P. aeruginosa* and BCG were stopped in the logarithmic growth phase, washed twice in PBS and inactivated by incubating for 30 min at 80 °C.

Flow cytometric and functional analysis of human cells

Purified monocytes and neutrophils were cultured in the absence or presence of inactivated bacteria (monocytes/neutrophils: bacteria ratios were about 1/10–1/100), LPS (100 ng ml⁻¹), LTA (100 ng ml⁻¹) and mycolic acid (10 μg ml⁻¹). All cells were incubated with PBS or 20% human serum for 1 h on ice to block Fc receptors. After staining with either monoclonal antibody 21C7 (mouse IgG1, anti-TREM-1) or 1B7.11 (control mouse IgG1, anti-2,4,6 TNP; American Type Culture Collection), followed by human-adsorbed phycoerythrin (PE)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates), cells were analysed on a flow cytometer. Four-colour analysis of human peritoneal leukocytes was performed using anti-TREM-1, CD15 (Immunotech), CD14 (Immunotech) and CD16 (Immunotech) monoclonal antibodies conjugated with allophycocyanin (APC), CyChrome, PE and fluorescein isothiocyanate (FITC), respectively.

Immunohistochemistry

We took skin biopsies from *S. aureus* infections (with features of impetigo and folliculitis), psoriasis or leukocytoclastic vasculitis mediated by immunocomplexes. Colon biopsies were from patients with active ulcerative colitis. The infectious granulomas derived from lymph nodes or mediastinum were caused by *Mycobacterium tuberculosis*, *B. henselae* (cat scratch disease) or *A. fumigatus*; the latter derived from a patient affected by chronic granulomatous disease. We also analysed cases of sarcoidosis (lymph node) and a foreign-body giant-cell reaction associated with a vascular plastic prosthesis removed because of thrombosis. Staining with 21C7, anti-CD15 (Dako), or an isotype-matched (IgG1) antibody was performed on frozen sections. The primary antibody was detected by the streptavidine–biotin immunoperoxidase technique²⁷.

mTREM-1-fusion protein and antibodies

Complementary DNA of mTREM-1 (GenBank accession number NM 021406) was cloned by searching the GenBank expressed sequence database with the amino-acid sequence of human TREM-1. We produced mTREM-1/IgG1 as described¹⁰. Anti-mTREM-1-specific monoclonal antibody was produced by immunizing Lewis rats with soluble mTREM-1/IgG1 as described¹⁰.

LPS-induced endotoxaemia

Female C57BL/6 mice (8–10 weeks, 19–22 g) were randomly grouped (5–10 mice per group) and injected i.p. with different concentrations of LPS from *E. coli* 055:B5 (Sigma), in a blinded fashion. We administered 500 μg per mouse of purified hlgG1 (κ-light chain)

(Sigma), mTREM-1/IgG1, hILT-3/IgG1¹⁶, or heat-inactivated mTREM-1/IgG1 (30 min, 95 °C) by i.p. injection at 1, 2, 4 and 6 h after or 1 h before LPS administration. We monitored viability of treated mice 4–6 times a day for at least 10 days.

Analysis of blood and peritoneal lavage

Blood (250 µl) was collected and serum TNF-α and IL-1β were determined using cytokine-specific enzyme-linked immunosorbent assays as per the manufacturer's protocol (R&D Systems). Total cell numbers of peritoneal lavage cells collected from treated and control mice were determined on a coulter counter. We performed differential counts according to standard morphological criteria on cytospin preparations stained with Giemsa/May-Grünwald solution (Sigma). Four-colour analysis of peritoneal leukocytes was performed after blocking Fc receptors (FcR blocking agent; Pharmingen) for 30 min, using anti-mTREM-1, anti-Ly-6G (Pharmingen), anti-Mac-1 (Pharmingen) monoclonal antibodies conjugated with APC, PE and FITC, respectively. Dead cells were excluded by staining with propidium iodide.

Escherichia coli peritonitis model

E. coli peritonitis was induced in mice as described²⁵. Briefly, female C57BL/6 mice (8–10 weeks, 19–22 g) were weighed and randomly distributed into groups of 5–15 animals of equal body weight. Mice were injected i.p. with 500 µg of mTREM-1/IgG1 or control hIgG1 before i.p. administration of 500 µl of a suspension of *E. coli* O111:B4 (1.6–2.1 × 10⁶ colony forming units per mouse).

Caecal ligation and puncture

We performed CLP as described^{17,24}. Briefly, female C57BL/6 mice (8–10 weeks, 19–22 g) were anaesthetized by i.p. administration of 75 mg per kg Ketanest (Parke Davies) and 16 mg per kg Rompun (Bayer AG) in 0.2 ml sterile pyrogen-free saline (B. Braun Melsungen AG). The caecum was exposed through a 1.0–1.5-cm abdominal midline incision and subjected to a 50–80% ligation of the distal half followed by a single puncture with a G23 needle. A small amount of stool was expelled from the punctures to ensure patency. The caecum was replaced into the peritoneal cavity and the abdominal incision closed in layers with 5/0 Prolene thread (Ethicon). Sterile saline (500 µl) containing 500 µg mTREM-1/IgG1, 500 µg hIgG1κ (Sigma) or 100 µg TNF-RI/IgG1 (Pharmingen) (together with 400 µg hIgG1κ; Sigma) was administered by i.p. injection immediately after CLP. The CLP was performed blinded to the identity of the treatment group. We assessed survival after CLP 4–6 times a day for at least 7 days.

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Correspondence and requests for materials should be addressed to M.C. (e-mail: colonna@bii.ch).

IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity

Vijay Shankaran*, Hiroaki Ikeda*, Allen T. Bruce*, J. Michael White*, Paul E. Swanson*, Lloyd J. Old† & Robert D. Schreiber*

* Department of Pathology and Immunology, Center for Immunology, Washington University School of Medicine, 660 South Euclid Avenue, St Louis, Missouri 63110, USA

† Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA

Lymphocytes were originally thought to form the basis of a 'cancer immunosurveillance' process that protects immunocompetent hosts against primary tumour development^{1,2}, but this idea was largely abandoned when no differences in primary tumour development were found between athymic nude mice and syngeneic wild-type mice^{3–5}. However, subsequent observations that nude mice do not completely lack functional T cells^{6,7} and that two components of the immune system—IFNγ^{8,9} and perforin^{10–12}—help to prevent tumour formation in mice have led to renewed interest in a tumour-suppressor role for the immune response. Here we show that lymphocytes and IFNγ collaborate to protect against development of carcinogen-induced sarcomas and spontaneous epithelial carcinomas and also to select for tumour cells with reduced immunogenicity. The immune response thus functions as an effective extrinsic tumour-suppressor system. However, this process also leads to the immunoselection of tumour cells that are more capable of surviving in an immunocompetent host, which explains the apparent paradox of tumour formation in immunologically intact individuals.

Age-matched female wild-type mice and immunodeficient mice with a targeted disruption of the recombination-activating gene-2 (RAG2) that is expressed only in lymphocytes¹³, both on a pure 129/SvEv genetic background, were injected subcutaneously with 100 µg of the chemical carcinogen methylcholanthrene (MCA) and monitored for tumour development. RAG2^{-/-} mice developed tumours earlier than wild-type mice and with greater frequency (P < 0.01). After 160 days, 9/15 RAG2^{-/-} mice but only 2/15 wild-type mice formed MCA-induced tumours (Fig. 1a). Similar results