

TREM-1 Activation Alters the Dynamics of Pulmonary IRAK-M Expression In Vivo and Improves Host Defense during Pneumococcal Pneumonia¹

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Triggering receptor expressed on myeloid cells-1 (TREM-1) is an amplifier of TLR-mediated inflammation during bacterial infections. Thus far, TREM-1 is primarily associated with unwanted signs of overwhelming inflammation, rendering it an attractive target for conditions such as sepsis. Respiratory tract infections are the leading cause of sepsis, but the biological role of TREM-1 therein is poorly understood. To determine the function of TREM-1 in pneumococcal pneumonia, we first established TREM-1 up-regulation in infected lungs and human plasma together with augmented alveolar macrophage responsiveness toward *Streptococcus pneumoniae*. Mice treated with an agonistic TREM-1 Ab and infected with *S. pneumoniae* exhibited an enhanced early induction of the inflammatory response that was indirectly associated with lower levels of negative regulators of TLR signaling in lung tissue in vivo. Later in infection, TREM-1 engagement altered *S. pneumoniae*-induced IRAK-M (IL-1R-associated kinase-M) kinetics so as to promote the resolution of pneumonia and remarkably led to an accelerated elimination of bacteria and consequently improved survival. These data show that TREM-1 exerts a protective role in the innate immune response to a common bacterial infection and suggest that caution should be exerted in modulating TREM-1 activity during certain clinically relevant bacterial infections. *The Journal of Immunology*, 2009, 183: 2027–2036.

S *treptococcus pneumoniae* is the most frequently isolated pathogen in community-acquired pneumonia and is responsible for an estimated 10 million deaths annually, making pneumococcal pneumonia a major health threat worldwide (1). Adding to the clinical significance of this disease, respiratory tract infections are the major sources of systemic inflammation and sepsis (2). Furthermore, the continuous rise in antibiotic resistance stresses the need for better insight into the host response mechanisms involved in pneumococcal pneumonia (3).

Triggering receptor expressed on myeloid cells-1 (TREM-1)⁴ is a surface receptor expressed on myeloid cells such as neutrophils,

monocytes, and macrophages that belongs to the Ig superfamily. The ligand for TREM-1 is unknown, and many studies on TREM-1 have made use of an agonistic Ab that induces receptor cross-linking. This causes the transmembrane domain of TREM-1 to associate with the ITAM of the adaptor protein DAP-12 (DNAX activation protein-12), resulting in downstream signal transduction events that lead to the release of proinflammatory mediators such as IL-8, TNF- α , and IL-1 β (4–6). Furthermore, the simultaneous activation of TREM-1 and pattern recognition receptors (PRRs) such as members of the TLR family results in an enhanced inflammatory response compared with that of with either stimulus alone (5, 7). Studies using agents that either interfere with or activate TREM-1 signaling revealed its importance in bacterial infection and sepsis in vivo. Blocking experiments demonstrate down-regulated inflammation, resulting in improved survival in models of murine endotoxemia, septic peritonitis, and *Pseudomonas aeruginosa* pneumonia (5, 8). In models of LPS-induced shock, activation of TREM-1 signaling with an agonistic Ab has been shown to double the mortality rate (7). TREM-1 is therefore considered a potent amplifier of the inflammatory response to invading microbes, and TREM-1 blocking agents have been discussed as novel therapeutic options in sepsis (9). These have been particularly discussed for pneumonia, because the presence of soluble TREM-1 (sTREM-1) within the bronchoalveolar lavage (BAL) fluid (BALF) of humans is an independent predictor of bacterial or fungal pneumonia (10), and blockage of TREM-1 has previously been shown to be beneficial in *P. aeruginosa* pneumonia (8).

Contrary to systemic and septic inflammation, the host defense to *S. pneumoniae* pneumonia is primarily localized to the lungs and depends on the immediate recognition of bacteria via TLRs to mount a proper inflammatory response. The early

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⁴ Abbreviations used in this paper: TREM-1, triggering receptor expressed on myeloid cells-1; AM, alveolar macrophage; BAL, bronchoalveolar lavage; BALF, BAL fluid; IRAK-M, IL-1R-associated kinase-M; KC, keratinocyte-derived chemokine; MPO, myeloperoxidase; PRR, pattern recognition receptor; shRNAi, short hairpin RNA interference; sTREM-1, soluble TREM-1; Tollip, Toll-interacting protein.

production of the proinflammatory cytokines TNF- α and IL-1, which are released upon recognition of *S. pneumoniae*, is crucial for bacterial clearance from the pulmonary compartment and, consequently, survival (11, 12). Contrastingly, the same proinflammatory cytokines that are so important in *S. pneumoniae* pneumonia have been associated with impaired bacterial clearance in *P. aeruginosa*-mediated pneumonia (13, 14). TLR2, TLR4, and TLR9 all play important and partially redundant roles in recognizing *S. pneumoniae* in vivo (15–18). Given that TREM-1 signaling amplifies TLR signals to cause enhanced proinflammatory cytokine synthesis, we considered it feasible that unlike during *P. aeruginosa*-induced pneumonia, when inflammation is excessive and thereby harms the host, TREM-1 might actually contribute to an improved host defense during *S. pneumoniae*-induced pneumonia. In this report we show a vital role for TREM-1 in vivo as its activation accelerated the induction of the early pulmonary host response to *S. pneumoniae*, resulting in augmented bacterial elimination together with accelerated resolution of inflammation and ultimately improved survival. These data indicate that TREM-1 is indispensable in the innate immune response to *S. pneumoniae* pneumonia in vivo. This is the first report illustrating a beneficial role for TREM-1 during a clinically relevant model of bacterial infection.

Materials and Methods

Human plasma samples

Plasma samples were collected from patients with community-acquired pneumonia admitted to the General Hospital of Vienna, Austria after informed consent had been obtained. Blood TREM-1 concentrations were measured in samples from patients diagnosed with pneumococcal pneumonia (microbiologic diagnosis of *S. pneumoniae* in blood samples, further referred to as “bacteremia” samples, or pneumococcal Ag detection in urine samples, further referred to as “pneumonia” samples). The Ethical Review Board of the Medical University of Vienna, Austria approved these procedures.

Mouse pneumonia model

Age- and sex-matched, pathogen-free, 8- to 10-wk-old C57BL/6 mice were used in all experiments. The Animal Care and Use Committee of the Medical University of Vienna approved all experiments. Pneumonia was induced as described previously (15, 19, 20). Briefly, *S. pneumoniae* serotype 3 obtained from American Type Culture Collection (ATCC 6303) were grown for 6 h to mid-logarithmic phase at 37°C using Todd-Hewitt broth (Difco), harvested by centrifugation at 1500 \times g for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of 10⁴ CFU per 50 μ l as determined by plating serial 10-fold dilutions on sheep blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Baxter) and 50 μ l of the bacterial suspension was inoculated intranasally. Agonistic TREM-1 mAbs or the respective isotype control Abs (R&D Systems) were i.p. administered at a dose of 250 μ g/kg in 200 μ l of PBS immediately after bacterial inoculation. For experiments lasting longer than 24 h, this treatment was repeated at *t* = 24 h. Control mice received the carrier (200 μ l of PBS i.p.) at the indicated time points.

Determination of bacterial outgrowth

Six, 24, or 48 h after infection, mice were anesthetized with ketamine (Pfizer) and sacrificed by bleeding out via heart puncture. Blood was collected in EDTA-containing tubes. Whole lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products). In some experiments BAL was performed 6 h after infection. For this purpose, the trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abott). Bilateral BAL was performed by instilling two 0.5-ml aliquots of sterile saline. Approximately 0.9 ml of BALF was retrieved per mouse. BALF supernatant was stored at –20°C for cytokine measurements. CFU were determined from serial dilutions of lung homogenates and BALF plated on blood agar plates and incubated at 37°C for 16 h before colonies were counted.

Preparation of lung tissue for protein measurements

For cytokine measurements, lung homogenates were diluted 1/2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100, and pepstatin A, leupeptin, and aprotinin (all 20 ng/ml; pH 7.4; Sigma-Aldrich) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 \times g at 4°C for 15 min, and supernatants were stored at –20°C until assays were performed.

Cytokines, chemokines, and myeloperoxidase activity ELISA

Cytokines and chemokines (TREM-1, TNF- α , IL-1 β , IL-6, keratinocyte-derived chemokine (KC), and MIP-2) were measured using specific ELISAs (R&D Systems) according to the manufacturer's instructions. The detection limits were 62.5 pg/ml for TREM-1, 31 pg/ml for TNF- α , 8 pg/ml for IL-1 β , 16 pg/ml for IL-6, 12 pg/ml for KC, and 94 pg/ml for MIP-2. Myeloperoxidase (MPO) activity was assayed using a commercially available ELISA (HyCult Biotechnology).

Western blotting of lung homogenates

Lung homogenate (100 μ g) was separated by electrophoresis on a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Abs specific for IL-1R-associated kinase-M (IRAK-M; Chemicon) and β -actin (Sigma-Aldrich) were used at dilutions of 1/1000 and 1/500, respectively. Immunoreactive proteins were detected by enhanced chemiluminescent protocol (GE Healthcare).

Histology

Lungs for histology were harvested at 24 or 48 h after infection, fixed in 10% formalin, and embedded in paraffin. Four-micrometer sections were stained with H&E and analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: interstitial inflammation, edema, endotheliitis, bronchitis, pleuritis, and thrombi formation. Each parameter was graded on a scale of 0 to 4 as follows: 0, absent; 1, mild; 2, moderate; 3, moderately severe; and 4, severe. The “inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 24. The presence of confluent infiltrates was termed “pneumonia” and scored for its presence (1, pneumonia present; 0, pneumonia absent) and quantified in relation to the total lung surface (0.5 points per 10% infiltrate). The “pneumonia score” was expressed as the sum of the scores for the latter parameters. The sum of both scores was called the “total lung inflammation score.”

For immunohistochemistry, tissue samples were deparaffinized in xylene and ethanol and subjected to Ag retrieval using 10 mM citrate buffer (pH 6.0). Thereafter, endogenous peroxidase activity was blocked with 1.6% H₂O₂ in TBS. Following washing and blocking steps using 10% goat serum (Vector Laboratories) in TBS, sections were incubated overnight at 4°C with IRAK-M Ab (Abcam) in TBS with 1% BSA. After washing, the sections were incubated with goat anti-rabbit biotinylated Ab (Sigma-Aldrich) in TBS containing 1% BSA, and binding was visualized using the Vectastain ABC kit (Vector Laboratories). Sections were counterstained with hematoxylin, dehydrated, and subjected to microscopy. Isotype controls were those sections treated with rabbit IgG (Jackson ImmunoResearch Laboratories). Immunohistochemistry was scored blinded and the entire lung surface was given a score of 0–10, where 0 represented a negative signal and 10 a highly positive signal. Immunohistochemistry scoring was conducted at the same time on at least triplicate slides, representing stainings on different days.

Evaluation of mRNA levels by RT-PCR

Total lung RNA was isolated using TRIzol (Invitrogen) followed by DNase digestion (Invitrogen) and converted to cDNA using the SuperScript III First-Strand synthesis system as described by the supplier (Invitrogen). RNA from cell culture was extracted using an RNeasy kit from Qiagen according to the manufacturer's instructions, which included a DNase step (Qiagen). Real-time PCR was conducted according to the LightCycler FastStart DNA Master PLUS SYBR Green I system using the Roche LightCycler II sequence detector (Roche Diagnostics). Cycling conditions were set at 1 cycle at 95°C for 10 min, 50 cycles at 95°C for 5 s, 68°C for 5 s, and 72°C for 10 s. The exception to these conditions was made for 5-aminolevulinic synthase (ALAS), where an annealing temperature of 72°C was used instead of 68°C. To confirm the specificity of the reaction products in each experiment, the melting profile of each sample was analyzed using LightCycler software 3.5 (Roche). An analysis of the melting curves demonstrated that each pair of primers amplified a single product; this was also verified using electrophoresis on 2% agarose gels. The mouse gene-specific primer sequences used are shown in Table I.

Table I. Sequences of primers used for RT-PCR

Gene	Sense (5'→3')	Antisense (5'→3')
TNF- α	GAACCTGGCAGAAGAGGCACT	GGTCTGGGCCATAGAAGTGA
IL-6	CCACGGCCTTCCCTACTTCA	TGCAAGTGCATCGTTGTTC
MIP-2	AGTGAACCTGCCTGTCAATG	C'TTGGTTC'CGTTGAGG
IRAK-M	GCCAGAAGAATACATCAGACAGG	GTCTAAGAAGGACAGGCAGGAGT
Tollip	ATTATGGCATGACTCGTATGGAC	CTATACCACTCG TCCTCCACTTG
TREM-1	ATGACCTAGTGGAGGGCCAG	GCACAACAGGGTCATTCCGGAG
ALAS ^a	CAGCCACATCATCCCCATCA	CTCCTCACCACGAGGCACAG
HPRT ^b	GTTAAGCAGTACAGCCCCAAAAG	AAATCCAACAAAGTCTGGCCTGTA

^a ALAS, 5-Aminolevulinatase synthase.

^b HPRT, Hypoxanthine phosphoribosyltransferase.

Cell culture and treatment

MH-S cells (ATCC) were cultured in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin, 10% FCS, and 50 μ M 2-ME at 37°C with 5% CO₂. Freshly isolated alveolar macrophages were cultured in supplemented RPMI 1640 lacking 2-ME. Adherent cells, seeded at a final concentration of 1 \times 10⁶ cells/ml, were preincubated with agonistic anti-TREM-1 mAb or isotype Ab at a concentration of 10 μ g/ml followed by heat-killed or live *S. pneumoniae* (2 \times 10⁶⁻⁷ CFU/ml; ATCC 6303), respectively, or RPMI 1640 for the indicated times.

MLE-12 cells were cultured in HITES medium, which is RPMI 1640 containing 2 mM L-glutamine, penicillin, streptomycin, 2% FCS, 5 μ g/ml insulin (Sigma-Aldrich), 10 nm of hydrocortisone (Sigma-Aldrich), 10 nM β -estradiol (Sigma-Aldrich), 100 μ g/ml transferrin (Sigma-Aldrich), 30 nM (5 pg/ml) sodium selenite (Sigma-Aldrich), at 37°C with 5% CO₂. Adherent MLE-12 cells seeded at 1 \times 10⁶ were stimulated with pooled BALF (cleared of cells and bacteria by cytospin) from each of the three groups of mice (PBS, isotype Ab, or TREM Ab) for 6 h.

TREM-1 small interfering RNA (siRNA) silencing

TREM-1 gene silencing was conducted by designing short hairpins using the siRNA Target Designer program (Promega) on the mouse TREM-1 transcript (accession no: NM_021406). Nucleotides 592–610 sense (5'-ACCGTCTCCACATCCAGTGTATTCAAGAGATAAACTGGATGTGGAGACTTTTTC-3') and antisense (5'-TGCAGAAAAGTCTCCACA TCCAGTGTATTCTCTTGAATAAACTGGATGTGGAGA-3') were chosen for annealing before ligation into the *Pst*I site of the psiSTRIKE vector, which is under the control of the U6 promoter and contains puromycin resistance, according to the manufacturer's instructions (Promega). As a control, nucleotides 592–610 were scrambled and blasted into the GenBank database for improper interaction with other mouse transcripts before cloning into the psiSTRIKE vector. The scrambled nucleotide sequences were sense (5'-ACCGCATCTAGATCCGTTTCCATTCAAGAGATGGAAACGGATCTAGATGCTTTTTC-3') and antisense (5'-TGCAGAAAAGCATCTAGATCCGTTTCCATCTCTTG AATGGAAACGGATCTAGATG-3'). Both plasmids were transformed into competent DH5- α cells and recombinant DNA was purified using a Maxiprep kit from Promega. Purified DNA (1 μ g) was used to transfect 2 \times 10⁶ MH-S cells using the Amaxa Cell Line Nucleofector kit V according to the supplier's instructions. Twenty-four hours later the cell medium was replaced by medium supplemented with 4 μ g/ml puromycin, and 48 h after that the concentration of puromycin was decreased to 2 μ g/ml and stable cell lines were generated.

Statistical analysis

Differences between groups were analyzed by Mann-Whitney *U* test or one-way ANOVA where appropriate using GraphPad software. For survival analyses, Kaplan-Meier analysis followed by log rank test was performed. Values are expressed as mean \pm SEM. A value of *p* \leq 0.05 was considered statistically significant.

Results

TREM-1 expression is up-regulated during pneumococcal pneumonia

In a first attempt, we intended to evaluate the expression and release of sTREM-1 during pneumococcal pneumonia in humans and measured sTREM-1 concentrations in the plasma of patients

diagnosed with this disease. Whereas sTREM-1 levels were below the detection limit in healthy individuals, elevated sTREM-1 concentrations were detectable in the plasma of patients with proven pneumococcal pneumonia, and the highest levels were found in those with systemic spread and microbiologic detection of *S. pneumoniae* in blood samples (so-called "bacteremia" patients) (Fig. 1A). To exploit the constitutive presence of TREM-1 within the pulmonary compartment and to elucidate its infection-induced expression during pneumococcal pneumonia in vivo, lungs collected from naive mice and following infection with *S. pneumoniae* were assayed for TREM-1 after 24 and 48 h, respectively. As depicted in Fig. 1B, TREM-1 was constitutively expressed in healthy murine lung tissue and markedly enhanced following pulmonary infection with pneumococci.

TREM-1 enhances responsiveness of alveolar macrophages to *S. pneumoniae* in vitro

To obtain insights into the function of TREM-1 in the pulmonary host response to *S. pneumoniae*, we determined the responsiveness of alveolar macrophages (AM) to heat-killed pneumococci. Having established TREM-1 up-regulation in AM in response to *S. pneumoniae* (Fig. 1C), primary mouse AM were stimulated with *S. pneumoniae* in the presence or absence of agonistic TREM-1 mAb and TNF- α release was assessed. As depicted in Fig. 1D, TNF- α was secreted after stimulation with heat-killed *S. pneumoniae*, whereas additional TREM-1 activation synergistically enhanced the inflammatory response to bacteria. Furthermore, we could confirm these results using MH-S cells (AM cell line) stimulated with either heat-killed bacteria (data not shown) or viable *S. pneumoniae* (Fig. 1E). To finally ensure specificity of the agonistic TREM-1 mAb, we generated stable MH-S cell lines where TREM-1 expression was knocked down using short hairpin RNA interference (shRNAi) directed against the C terminus of the mouse TREM-1 receptor. As shown in Fig. 1F, TREM-1 transcript levels were significantly decreased (80%) compared with the scrambled control cell line as determined by real-time PCR. Following treatment with the agonistic TREM-1 mAb and heat-killed *S. pneumoniae*, a synergistic up-regulation of TNF- α and MIP-2 secretion was observed in scrambled control cells (Fig. 1G, open bars), whereas no up-regulation was found in MH-S cells that had been silenced for TREM-1 (Fig. 1G, filled bars). These results strongly suggest that the augmented cytokine secretion following treatment of AM with agonistic TREM-1 mAb and *S. pneumoniae* compared with those cells treated with *S. pneumoniae* alone is dependent upon signaling pathways that travel solely through the TREM-1 receptor. These results provided us with the confidence to use the agonistic TREM-1 mAb in vivo to specifically activate TREM-1 signaling and to study the role of TREM-1 activation in the context of pneumococcal pneumonia.

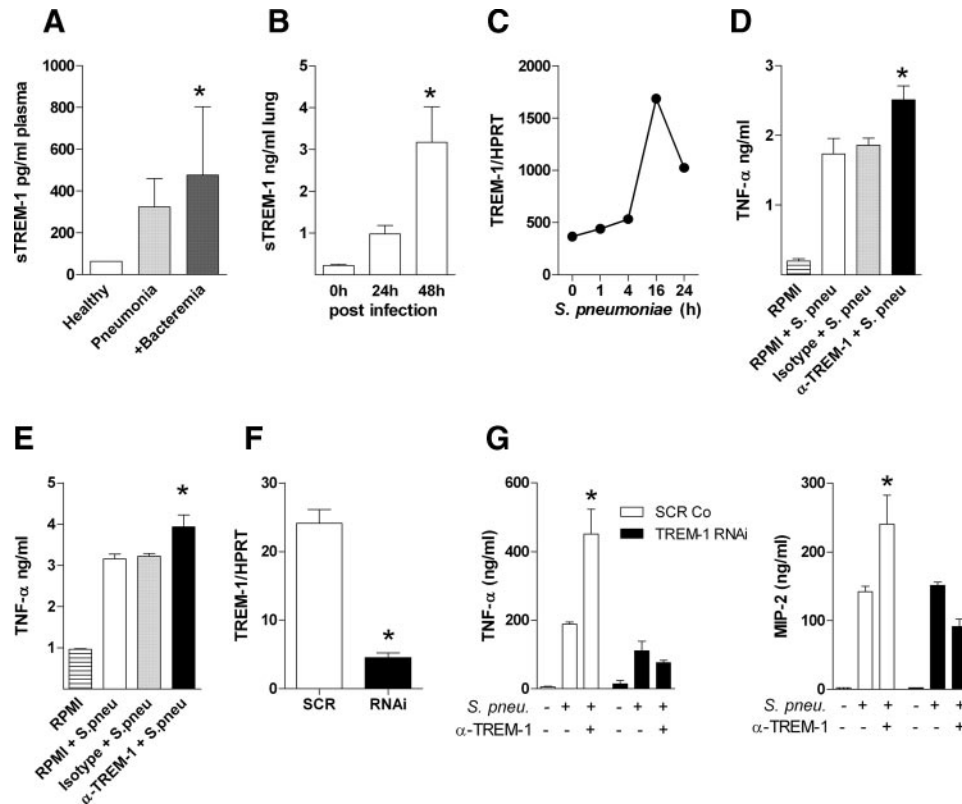


FIGURE 1. TREM-1 is expressed upon infection in vivo and specifically amplifies the responsiveness of alveolar macrophages to *S. pneumoniae* in vitro. **A**, TREM-1 plasma concentrations were measured in samples from patients newly diagnosed with pneumococcal pneumonia with ($n = 6$) or without ($n = 9$) concomitant bacteremia and in age-matched healthy controls ($n = 6$). *, $p < 0.05$ vs healthy controls. **B**, TREM-1 expression was determined in lung samples obtained from healthy mice (0 h) and 24 or 48 h after an intranasal challenge with 10^4 CFU *S. pneumoniae* ($n = 8$ per group). *, $p < 0.05$ vs 0 and 24 h. **C**, MH-S cells seeded at 1×10^6 /ml in RPMI 1640 were stimulated with 2×10^7 CFU/ml *S. pneumoniae* for the indicated times. RNA was isolated and TREM-1 expression quantified by real-time RT-PCR. Data show expression change normalized to hypoxanthine phosphoribosyl-transferase (HPRT). **D**, Primary AM were seeded in quadruplicate at 10^6 cells/ml, pretreated with $10 \mu\text{g/ml}$ isotype Ab or agonistic TREM-1 mAb (α -TREM-1), and stimulated for 6 h with or without 10^6 CFU/ml *S. pneumoniae* (*S. pneu*). **E**, MH-S cells were seeded at 10^6 cells/ml in quadruplicate, pretreated as in **D**, and stimulated with 10^6 CFU/ml live *S. pneumoniae* for 6 h. **D** and **E**, Supernatants were assayed for TNF- α . *, $p < 0.05$ vs cells stimulated with *S. pneumoniae* alone. **F**, MH-S cell were transfected with a plasmid encoding TREM-1 shRNAi or scrambled (SCR) control to generate stable cell lines. Both cell lines were seeded at 1×10^6 and RNA was isolated to quantify TREM-1 expression by real-time RT-PCR. TREM-1 levels normalized to HPRT are shown; *, $p < 0.05$ vs SCR. **G**, Both cell lines were seeded at 5×10^5 cells/ml, pretreated with $10 \mu\text{g/ml}$ agonistic TREM-1 mAb (α -TREM-1), and stimulated for 16 h with or without 2×10^7 CFU/ml *S. pneumoniae*. Supernatants were assayed for TNF- α (left) and MIP-2 (right). *, $p < 0.05$ vs cells stimulated with *S. pneumoniae* alone. **D–G**, Experiments were conducted in triplicate and a representative experiment is shown. Data presented are mean \pm SEM.

TREM-1 augments the early pulmonary inflammatory response

Having established that agonistic TREM-1 mAb amplifies *S. pneumoniae*-induced TNF- α and MIP-2 release by AM in vitro through the TREM-1 receptor and knowing that both cytokines contribute

importantly to host defense against this pathogen (11, 21), we next investigated whether this finding would translate into improved antibacterial defense mechanisms during pneumococcal pneumonia in vivo. For this purpose, mice were treated with agonistic

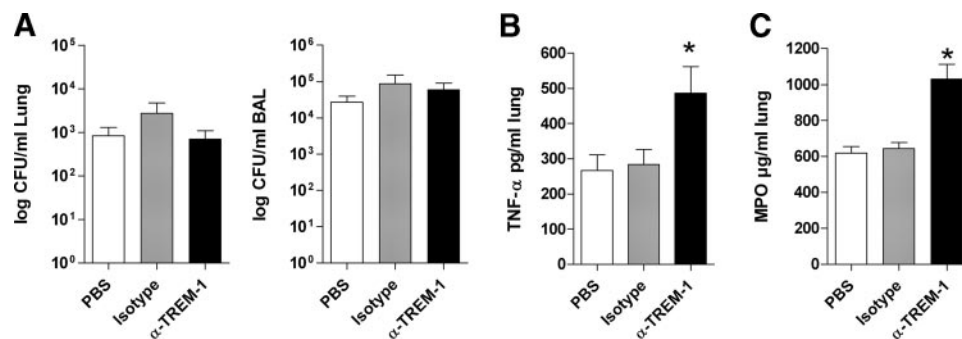


FIGURE 2. TREM-1 engagement accelerates the early immune response in vivo. **A**, Mice were inoculated with 1×10^4 CFU *S. pneumoniae* and treated with PBS, isotype Ab, or agonistic TREM-1 mAb (α -TREM-1) and bacterial outgrowth in lungs and BALF was assessed 6 h after infection. **B** and **C**, Lung TNF- α (**B**) and lung MPO (**C**) were measured using ELISA 6 h after infection. Data represent mean \pm SEM of eight mice per group; *, $p < 0.05$ vs both control groups, i.e., PBS and isotype control.

Table II. Increased pulmonary mRNA concentrations of TNF- α , IL-6, and MIP-2 in agonistic TREM-1 mAb (α -TREM-1)-treated mice 6 h after infection with *S. pneumoniae*^a

Fold Induction	PBS	Isotype	α -TREM-1
TNF- α	414 \pm 85	340 \pm 93	656 \pm 174*
IL-1 β	177 \pm 19	175 \pm 30	233 \pm 36
IL-6	3099 \pm 214	2375 \pm 544	5203 \pm 1106*
KC	2105 \pm 171	2102 \pm 514	2662 \pm 260
MIP-2	133 \pm 27	138 \pm 31	368 \pm 120*

^a Cytokine and chemokine mRNA levels quantified from lungs 6 h after infection with 10^4 CFU of *S. pneumoniae*. Data are means \pm SEM, $n = 8$ per group.

*, $p < 0.05$ versus PBS and isotype control group.

TREM-1 mAb at a concentration previously used in vivo (7), infected with 10^4 CFU *S. pneumoniae*, and sacrificed 6 h later. No significant difference in bacterial loads in lungs or BALF of agonistic TREM-1 mAb-treated animals and control mice was observed at this early time point, and blood cultures were all negative (Fig. 2A). Although cytokine protein levels were barely detectable at $t = 6$ h, pulmonary TNF- α was expressed and found significantly elevated in the agonistic TREM-1 mAb-treated group as compared with control mice (Fig. 2B). In addition, lung mRNA levels revealed significantly higher induction of TNF- α , IL-6, and MIP-2 in agonistic TREM-1 mAb-treated mice as compared with control mice (Table II). KC and IL-1 β mRNA concentrations did not differ between the three groups (Table II). The increased TNF- α and MIP-2 levels were associated with significantly ele-

vated numbers of lung neutrophils, as assessed by MPO measurements of whole lungs (Fig. 2C). Hence, activation of TREM-1 was associated with an earlier induction of the inflammatory response during pneumococcal pneumonia in vivo.

TREM-1 prevents the early induction of negative regulators of TLR signaling

Knowing that TREM-1 amplifies proinflammatory signaling pathways initiated via PPRs such as TLRs, we were curious as to whether the involvement of TREM-1 affects negative regulators of TLR signaling such as IRAK-M, Toll-interacting protein (Tollip), or A20 in vivo. For this purpose we quantified IRAK-M, Tollip, and A20 expression levels in lung homogenates obtained 6 h after the induction of pneumonia. Although A20 was not expressed in the lung at significant levels (data not shown), both IRAK-M and Tollip mRNA levels were significantly reduced in mice treated with agonistic TREM-1 mAb as compared with both control groups (Fig. 3A). Correspondingly, IRAK-M protein levels were diminished in the lungs of mice treated with agonistic TREM-1 mAb and infected with *S. pneumoniae* (Fig. 3B).

TREM-1 activation beneficially impacts pulmonary IRAK-M expression

IRAK-M is a key component of the feedback regulatory system of innate immunity as it down-regulates inflammatory responses, thereby promoting the resolution of inflammation (22). The functional role of IRAK-M in *S. pneumoniae* pneumonia is not known. Therefore, we wished to determine both the dynamics of IRAK-M

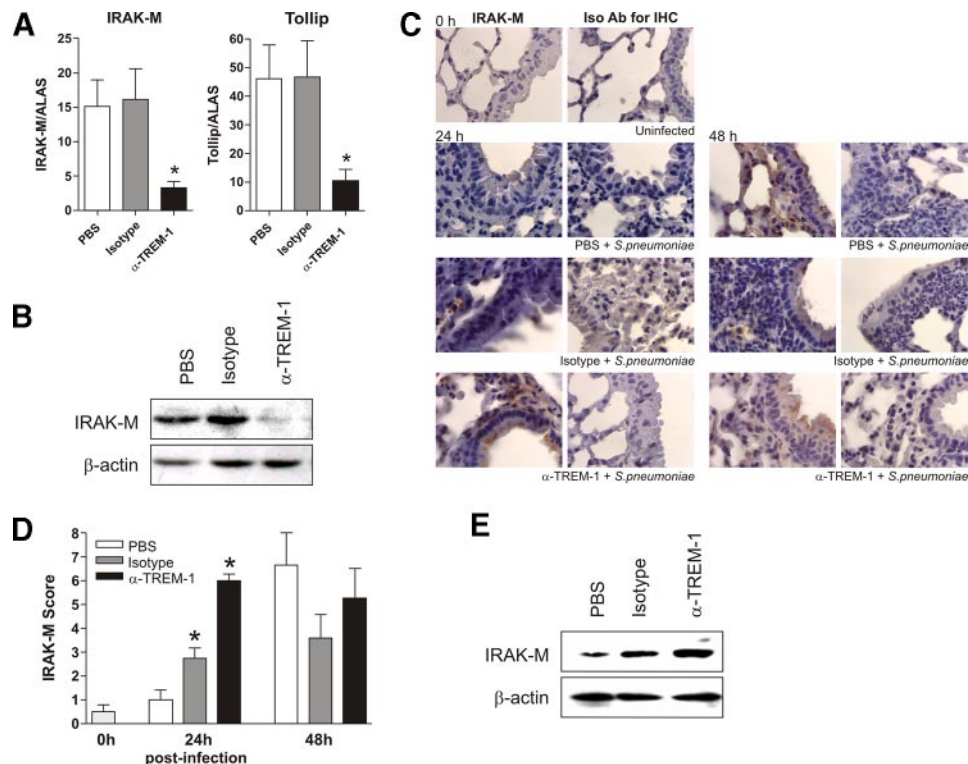
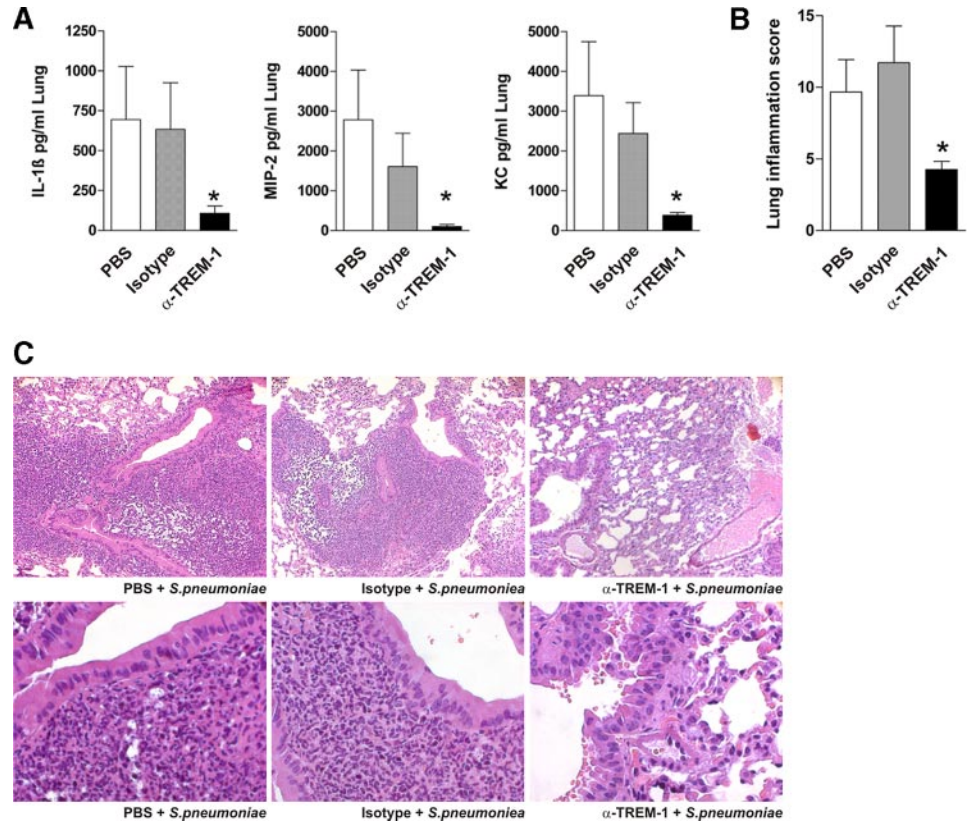


FIGURE 3. TREM-1 impacts induction of negative regulators of TLR signaling. *A*, Mice were inoculated with 1×10^4 CFU *S. pneumoniae* and treated with PBS, isotype Ab, or agonistic TREM-1 mAb (α -TREM-1) for 6 h and RNA was extracted and RT-PCR conducted for IRAK-M (left) and Tollip (right). Data are presented as the expression level normalized to 5-aminolevulinic synthase (ALAS) and represent mean \pm SEM of eight mice per group. *, $p < 0.05$ vs both control groups. *B*, Lung homogenates were prepared and blotted for IRAK-M as described in *Materials and Methods*; a representative blot is shown. *C*, Lungs from uninfected mice and mice infected and treated as described in *A* were harvested after 24 and 48 h and immunohistochemistry (IHC) for IRAK-M was conducted (Iso Ab, Isotype Ab). Representative pictures are depicted; original magnification was $\times 100$. *D*, Slides were scored blinded in triplicate as described in *Materials and Methods*. Data represent mean \pm SEM of $n = 8$ mice per group and time point. *E*, Lung homogenates 24 h after the induction of pneumonia were prepared and blotted for IRAK-M as described in *Materials and Methods*; a representative blot is shown.

FIGURE 4. Reduced lung inflammation in anti-TREM-1-treated mice 48 h after infection. *A* and *B*, Mice were inoculated with 1×10^4 CFU of *S. pneumoniae* and treated with PBS, isotype Ab, or agonistic TREM-1 mAb (α -TREM-1). Lung cytokine (IL-1 β) and chemokine (MIP-2, KC) concentrations were measured using ELISA 48 h after infection (*A*) and lung slides were scored for the presence of inflammation (*B*) as described in *Materials and Methods*. *C*, Representative lung histology slides of control, isotype Ab, or α -TREM-1-treated mice 48 h after infection with *S. pneumoniae* are depicted; data are representative of eight mice/group. Original magnification of H&E staining was $\times 10$ (*upper row*) and $\times 40$ (*lower row*). Data are presented as mean \pm SEM; *, $p < 0.05$ vs PBS and isotype control groups.



expression and inflammation during pneumococcal pneumonia at later time points and the effect of TREM-1 activation therein. Although IRAK-M expression was initially described as being myeloid cell specific (22, 23), it has been reported to be expressed in biliary epithelial cells (24). These studies complement our findings where we found it also expressed in bronchial and alveolar epithelial cells (Fig. 3C). IRAK-M expression was low in uninfected lungs and increased in response to infection, being maximal after 48 h in infected control mice (Fig. 3, C and D). The 48-h levels of IRAK-M were also high in mice that had been administered agonistic TREM-1 mAb. Importantly however, the highest IRAK-M expression was observed 24 h after the induction of pneumonia in the lungs of agonistic TREM-1 mAb-treated mice compared with control groups (Fig. 3, C and D). The increase in IRAK-M expression 24 h after the induction of pneumonia in mice that received the agonistic TREM-1 mAb was finally verified by Western blotting (Fig. 3E). Importantly, earlier induction of IRAK-M expression in mice treated with agonistic TREM-1 mAb was associated with a significant reduction of proinflammatory mediators such as IL-1 β , MIP-2, and KC within the pulmonary compartment 48 h postinfection (Fig. 4A). To further evaluate the role of TREM-1 in lung inflammation induced by *S. pneumoniae* in vivo, lung histology slides obtained 48 h after infection with 10^4 CFU *S. pneumoniae* were scored as described *Materials and Methods*. In line with reduced cytokine/chemokine levels, agonistic TREM-1 mAb-treated mice displayed significantly less inflammation, edema, and pleuritis when compared with both control groups (Fig. 4, B and C). Hence, activation of TREM-1 influenced the dynamics of IRAK-M and inflammation in vivo during pneumococcal pneumonia as it suppressed early (6 h) IRAK-M induction associated with enhanced inflammation 6 h after infection (Fig. 2), followed by a rapid increase in IRAK-M expression at 24 h and, consequently, accelerated resolution of inflammation 48 h after induction of pneumonia.

TREM-1 associated reduction in early IRAK-M is linked to epithelial cells

Considering our finding that IRAK-M is not restricted to myeloid cells but is strongly expressed in respiratory epithelial cells (Fig. 3C), we wondered whether the initial decrease in IRAK-M expression at 6 h (Fig. 3, A and B) was occurring in epithelial or myeloid cells, respectively. First, we stimulated alveolar macrophages (MH-S cells and primary alveolar macrophages) with agonistic TREM-1 mAb or controls followed by heat-killed or live *S. pneumoniae* to see whether IRAK-M expression is diminished in this cell type. As shown in Fig. 5, A and B, alveolar macrophage IRAK-M expression was not decreased upon TREM-1 engagement, rendering this direct pathway unlikely (stimulations with heat-killed or live bacteria resulted in identical results). Because respiratory epithelial cells do not express TREM-1 (data not shown) and *S. pneumoniae* stimulation results in IRAK-M up-regulation in epithelial cells (Fig. 5C), we then hypothesized that decreased pulmonary IRAK-M levels might occur through a soluble factor released by alveolar macrophages upon TREM-1 engagement. To test this hypothesis, we placed BALF from mice infected with *S. pneumoniae* and treated with agonistic TREM-1 mAb or controls (for 6 h) onto mouse lung epithelial (MLE-12) cells in vitro and conducted IRAK-M RT-PCR. As depicted in Fig. 5D, BALF from infected control mice (PBS treated) strongly induced IRAK-M expression by respiratory epithelial cells whereas BALF from agonistic TREM-1 mAb-treated mice induced significantly lower levels of IRAK-M transcription. However, stimulation of epithelial cells with BALF from isotype control mice also led to modestly reduced IRAK-M levels when compared with PBS-treated mice. Although our shRNAi experiments (Fig. 1G) proved that the agonistic TREM-1 mAb specifically amplified inflammation via the TREM-1 receptor, we wanted to be confident that the delay in IRAK-M expression was indeed occurring via a soluble

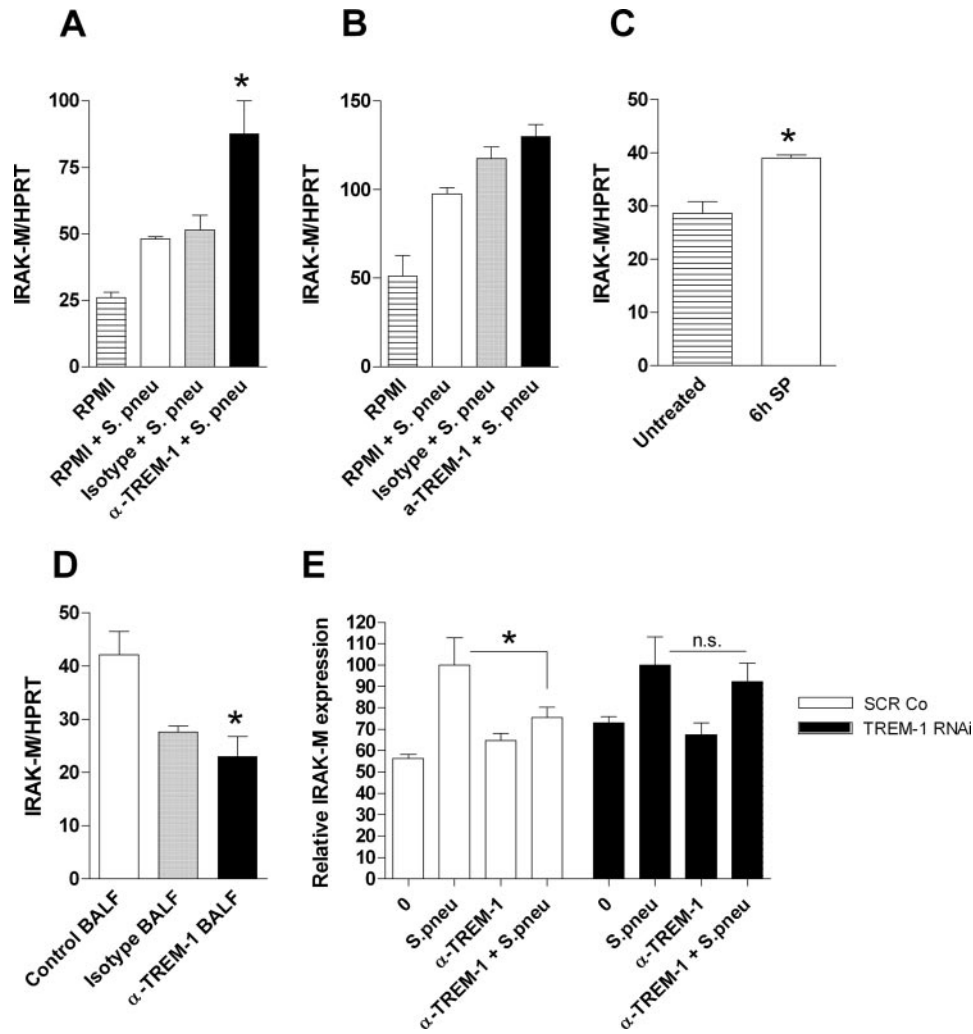


FIGURE 5. TREM-1-associated reduction in early IRAK-M is linked to epithelial cells. *A* and *B*, MH-S cells (*A*) or primary alveolar macrophages (*B*) were seeded at 10^6 cells/ml, preincubated with agonistic TREM-1 mAb (α -TREM-1) or isotype Ab (10 μ g/ml) followed by heat-killed *S. pneumoniae* (*S. pneu*) (2×10^7 CFU/ml in *A* or 10^6 CFU/ml in *B*) for 6 h. RNA was extracted and RT-PCR conducted on IRAK-M. *, $p < 0.05$ vs cells stimulated with *S. pneumoniae* alone. *C*, MLE-12 cells were stimulated with heat-killed *S. pneumoniae* for 6 h and IRAK-M expression was analyzed by RT-PCR. Data are representative (mean \pm SEM) of three independent experiments presented as expression levels normalized to hypoxanthine phosphoribosyltransferase (HPRT). *, $p < 0.05$ vs unstimulated cells. *D*, Mice ($n = 8$ per group) were inoculated with 10^4 CFU *S. pneumoniae*, treated with PBS, isotype Ab, or α -TREM-1 and BALF was performed after 6 h. Pooled BALF (cleared of cells and bacteria by centrifugation) from each group was used to stimulate triplicates of 1×10^6 /ml MLE-12 cells for 6 h, after which IRAK-M expression was analyzed. Data are mean \pm SEM; *, $p < 0.05$ vs PBS group. *E*, Scrambled control (open bars) or TREM-1 shRNAi (filled bars) MH-S cells were treated with either *S. pneumoniae* (2×10^7 CFU/ml), α -TREM-1 (10 μ g/ml), or a combination of both for 6 h and supernatant was removed, sterilized through a 0.2 μ m filter, and placed onto MLE-12 cells for 6 h. RNA was extracted and RT-PCR conducted on IRAK-M as described. Data are presented as mean \pm SEM of IRAK-M expression normalized to HPRT relative to *S. pneumoniae* induced levels; *, $p < 0.05$.

factor released by alveolar macrophages in a TREM-1-dependent manner. We therefore stimulated lung epithelial cells with supernatant from either scrambled control or TREM-1 shRNAi alveolar macrophages that had been treated with *S. pneumoniae* alone or in combination with agonistic TREM-1 mAb. As shown in Fig. 5E, lung epithelial cells treated with supernatant from scrambled control macrophages (open bars) that had been stimulated with *S. pneumoniae* and agonistic TREM-1 mAb for 6 h exhibited significantly lower levels of IRAK-M transcription compared with those cells treated with *S. pneumoniae* alone, and this did not occur in macrophages that had been silenced for TREM-1 (filled bars). These data indicate that diminished early expression of pulmonary IRAK-M in mice suffering from pneumococcal pneumonia occurs in lung epithelial cells through a yet to be identified factor(s) present in the lavage of TREM-1 treated mice that is produced in

an *S. pneumoniae*- and TREM-1-dependent manner via alveolar macrophages.

TREM-1 leads to enhanced bacterial clearance and survival during pneumococcal pneumonia

We next investigated whether agonistic TREM-1 mAb-treated mice would exhibit improved antibacterial defense mechanisms during pneumococcal pneumonia in vivo. For this purpose, we infected mice with 10^4 CFU of *S. pneumoniae* and enumerated lung bacterial counts after 24 and 48 h. Although no difference in lung CFU counts was observed 24 h following infection, agonistic TREM-1 mAb-treated animals disclosed significantly reduced lung CFU counts after 48 h when compared with isotype or PBS control animals ($p < 0.05$ vs PBS and isotype controls, respectively; Fig. 6A). These promising and exciting data opened the

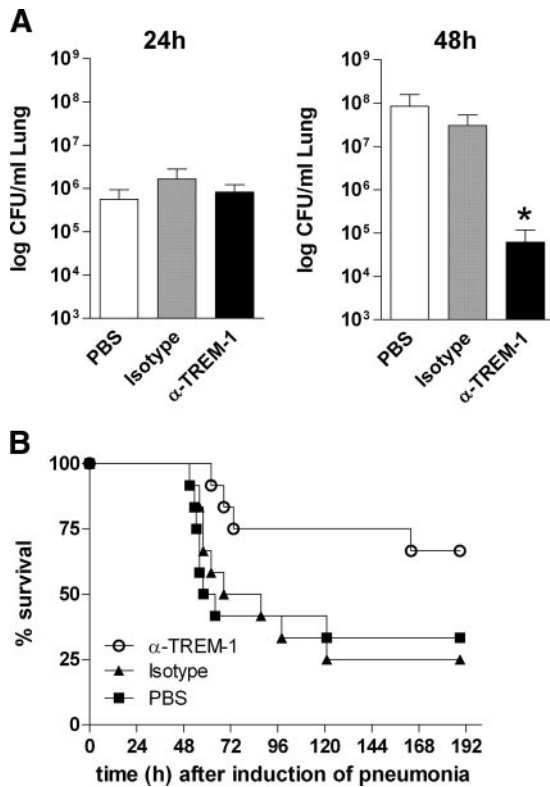


FIGURE 6. TREM-1 activation improves bacterial clearance and survival in vivo. *A*, Mice were inoculated with 1×10^4 CFU *S. pneumoniae* and treated with PBS, isotype Ab or agonistic TREM-1 mAb (α -TREM-1) and bacterial outgrowth in lungs was assessed at indicated time points. *B*, Survival was monitored over 8 days. Data represent mean \pm SEM of eight mice/group; $p < 0.05$ vs both control groups, i.e., PBS and isotype control.

possibility that agonistic TREM-1 mAb-treated mice might exhibit a survival advantage and we repeated the pneumonia experiments to observe potential differences in mortality. As depicted in Fig. 6B, activation of TREM-1 improved the survival of mice suffering from pneumococcal pneumonia.

Discussion

S. pneumoniae is the most frequently isolated pathogen in community-acquired pneumonia and is responsible for an estimated 10 million deaths annually, making pneumococcal pneumonia a major health threat worldwide (1, 25). Adding to the clinical significance of this disease, respiratory tract infections are the major sources of systemic inflammation and sepsis (2). TREM-1 is an amplifier of the inflammatory response to bacterial infections, which to date has been regarded harmful because TREM-1 involvement has been demonstrated to contribute to lethality in models of Gram-negative infection and sepsis (5, 7). In line with these findings, TREM-1 blocking agents have been discussed as novel therapeutic options in sepsis (9). Given the crucial importance of respiratory tract infections as the major source of sepsis and the significance of pneumococcal pneumonia herein, we deemed it mandatory to exploit the role of TREM-1 during pneumonia in vivo. We thereby demonstrated up-regulated TREM-1 in human plasma and murine lungs during pneumococcal pneumonia in vivo and used primary AM to illustrate that TREM-1 amplifies the responsiveness to *S. pneumoniae*. Follow-up studies revealed a vital role for TREM-1 in vivo, as its involvement augmented bacterial elimination together with accelerated resolution of inflammation and improved survival. We then could disclose that TREM-1 activation acceler-

ated the induction of the early pulmonary host response while concomitantly and indirectly inhibiting the early induction of negative regulators such as IRAK-M. Finally, we identified IRAK-M expression in respiratory epithelial cells and show that soluble factors present in the BALF of anti-TREM-1-treated mice are able to delay epithelial cell-associated IRAK-M expression ex vivo. Together, these data indicate that TREM-1 is indispensable in the innate immune response to *S. pneumoniae* pneumonia in vivo. This is the first report illustrating a beneficial role for TREM-1 during a clinically relevant model of bacterial infection.

The beneficial effects of TREM-1 activation during pneumococcal pneumonia were associated with an early augmentation of the pulmonary inflammatory response. This early innate immune response during pneumococcal pneumonia is crucial for the host to survive and relies on the release of proinflammatory cytokines, such as TNF- α , produced upon recognition of bacteria. TLR2, TLR4, and TLR9 all play important and partially redundant roles in recognizing *S. pneumoniae* in vivo (15–18). All TLR signaling activates the transcription factor NF- κ B, which regulates the expression of many immune response genes, including TNF- α , IL-1 β , IL-6, and MIP-2. Knowing that TREM-1 enhances the early expression of all of the above genes in response to *S. pneumoniae* and that TREM-1 activation itself can induce NF- κ B DNA binding in alveolar macrophages in vitro (data not shown), we considered it possible that TREM-1 might augment TLR-associated inflammation by impacting the expression of negative regulators of TLR signaling. An array of negative regulators, including Tollip, IRAK-M, A20 or ST2, has been identified over the past years (22, 26–28). The main function attributed to these negative regulators is to ensure that production of proinflammatory cytokines is kept in check, as this can be detrimental to the host. However, at the onset of infection, low expression levels of negative regulators are required to ensure a proper immune response. We specifically decided to study Tollip and IRAK-M expression, because both have been shown to down-regulate TLR2-, TLR4-, and TLR9-mediated responses, all of which are important in *S. pneumoniae* infection (22, 26, 29). We indeed observed diminished early IRAK-M and Tollip expression in lungs of agonistic TREM-1 mAb-treated mice 6 h after infection with *S. pneumoniae*. It is tempting to speculate that TREM-1-associated augmentation of the early inflammatory response during pneumonia was additionally enhanced by the delayed induction of negative regulators in vivo.

From in vitro studies it is known that IRAK-M expression can be induced by the exposure of macrophages and monocytes to the TLR2 and TLR4 ligands peptidoglycan and LPS, respectively, the glycosaminoglycan hyaluronan, or NO (22, 29–31). Importantly, we thereby demonstrated that *S. pneumoniae* induces IRAK-M expression both in alveolar macrophages in vitro and in the lung in vivo. Although, IRAK-M expression was initially regarded as myeloid cell restrictive (22, 23), we found that IRAK-M is not myeloid cell specific but highly expressed in respiratory epithelial cells in vitro and in primary lung epithelial cells in vivo. These observations complement recent data showing IRAK-M expression in human biliary epithelial cells (24). Our finding of respiratory epithelial IRAK-M expression led us to consider the possibility that delayed pulmonary IRAK-M expression upon TREM-1 activation might arise from this cell type rather than from alveolar macrophages. In fact, TREM-1 engagement amplified IRAK-M expression in alveolar macrophages that had been stimulated with *S. pneumoniae* in vitro. Although *S. pneumoniae* itself can induce IRAK-M in epithelial cells in vitro, respiratory epithelial cells,

which do not express TREM-1, exhibited delayed IRAK-M expression when incubated with BALF of mice treated with the agonistic TREM-1 mAb. This finding points toward a soluble factor in the BALF of these mice that acts to delay levels of *S. pneumoniae*-mediated IRAK-M expression in alveolar epithelial cells. This hypothesis is supported by our experiments wherein we treated lung epithelial cells with supernatant from TREM-1-silenced alveolar macrophages and found no decrease in epithelial IRAK-M transcription. The identity of the soluble factor responsible for the IRAK-M decrease in agonistic TREM-1 mAb-treated mice remains a mystery. Interestingly, monocyte IRAK-M transcription was induced rapidly following exposure to fixed tumor cells or medium supplemented with cancer cell supernatants, but more slowly when monocytes were cocultured with tumor cells (30). Similar to our experiments, these data point to the existence of a soluble factor that delays the signaling cascade responsible for IRAK-M expression. The important consequence of this delay is the immediate influx of polymorphonuclear cells and rapid elimination of the causative pathogen. Our data are consistent with studies showing that septic IRAK-M^{-/-} mice challenged with *P. aeruginosa* have increased pulmonary MIP-2 production and polymorphonuclear cell influx 6 h postinfection, resulting in increased survival compared with control mice (32). Significantly, although there is a delay in IRAK-M expression at 6 h in agonistic TREM-1 mAb-treated mice, we found that IRAK-M expression peaked in these mice at 24 h relative to control mice. This suggests that at later time points TREM-1 influences IRAK-M expression in a manner that promotes the resolution of inflammation *in vivo*. In agreement with this hypothesis, pulmonary levels of proinflammatory cytokines are lower at 48 h, which correlates with a lower inflammation score in agonistic TREM-1 mAb-treated mice compared with controls.

What could be the functional relevance of this delayed IRAK-M induction *in vivo*? Although alveolar macrophages are thought to be the main producers of proinflammatory cytokines in response to *S. pneumoniae*, the contribution of epithelial cells to pulmonary inflammation cannot be underestimated (33, 34). Respiratory epithelial cells have been shown to express PPRs such as TLR2, TLR4, and TLR9 (33, 35–37). However, there is some controversy surrounding the role of bacteria such as *S. pneumoniae* in directly inducing NF- κ B activation and the corresponding cytokine/chemokine synthesis in lung epithelial cells (38–40). Interestingly, Quinton et al. reported that pneumococci did not cause NF- κ B activation in murine type II alveolar epithelial cells whereas BALF from infected mice induced the nuclear translocation of RelA, and this activation could be reversed by blocking TNF- α and IL-1 β (40). These data suggest an indirect activation of epithelial cells by cytokines such as IL- β and TNF- α (40). Therefore, the delayed epithelial IRAK-M expression we observed in this study might enhance cytokine transcription either directly by the capacity of *S. pneumoniae* to activate epithelial cells via TLRs or indirectly via cytokines released by macrophages in response to bacteria.

We propose the following model for TREM-1 function *in vivo* in response to *S. pneumoniae* infection. Early in infection, TREM-1 engagement directly augments the TLR-mediated macrophage response and indirectly decreases levels of epithelial IRAK-M expression *in vivo*. This dual mechanism results in an enhanced early inflammatory response that in turn induces IRAK-M expression, indicating a negative feedback loop, and stabilizes the homeostasis of the innate immune system. The up-regulation of IRAK-M expression occurs at 48 h in the control mice, which have high levels of bacteria and ongoing inflammation in their lungs at this time point. However, in ago-

nistic TREM-1 mAb-treated mice this up-regulation occurs earlier, which promotes the resolution of the inflammatory response in these mice. The net result of this dual action of TREM-1 on IRAK-M expression *in vivo* is improved survival. These data show that TREM-1 is beneficial in *S. pneumoniae* pneumonia but are contradictory to a previous study that used a sTREM-1 mimetic peptide (LP17) to block TREM-1 in a rat model of *Pseudomonas aeruginosa* pneumonia. Administration of LP17 led to reduced lung damage and improved survival in this model of overwhelming Gram-negative pneumonia in rats (8). We also made use of LP17 when studying pneumococcal pneumonia but were unable to discern any effect in terms of altered inflammatory response or bacterial clearance (data not shown). This could be because *P. aeruginosa* relies on different host defense mechanisms than those for *S. pneumoniae* (13, 14). Nonetheless, considering speculations about the potential therapeutic impacts of TREM-1 blocking agents during sepsis, our data are important as they suggest that blockade of TREM-1 may not necessarily be beneficial in all clinical infections.

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Disclosures

The authors have no financial conflict of interest.

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