

TNF α -stimulated gene-6 (TSG6) activates macrophage phenotype transition to prevent inflammatory lung injury

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TNF α -stimulated gene-6 (TSG6), a 30-kDa protein generated by activated macrophages, modulates inflammation; however, its mechanism of action and role in the activation of macrophages are not fully understood. Here we observed markedly augmented LPS-induced inflammatory lung injury and mortality in TSG6^{-/-} mice compared with WT (TSG6^{+/+}) mice. Treatment of mice with intratracheal instillation of TSG6 prevented LPS-induced lung injury and neutrophil sequestration, and increased survival in mice. We found that TSG6 inhibited the association of TLR4 with MyD88, thereby suppressing NF- κ B activation. TSG6 also prevented the expression of proinflammatory proteins (iNOS, IL-6, TNF α , IL-1 β , and CXCL1) while increasing the expression of anti-inflammatory proteins (CD206, Chi3l3, IL-4, and IL-10) in macrophages. This shift was associated with suppressed activation of proinflammatory transcription factors STAT1 and STAT3. In addition, we observed that LPS itself up-regulated the expression of TSG6 in TSG6^{+/+} mice, suggesting an autocrine role for TSG6 in transitioning macrophages. Thus, TSG6 functions by converting macrophages from a proinflammatory to an anti-inflammatory phenotype secondary to suppression of TLR4/NF- κ B signaling and STAT1 and STAT3 activation.

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TNF α -stimulated gene-6 (TSG6) is a 30-kDa hyaluronan (HA)-binding protein produced by monocytes/macrophages, mesenchymal stem cells (MSCs), and fibroblasts in inflammatory diseases (1–4). However, there appears to be little constitutive expression of TSG6 (3). TSG6 is synthesized in response to proinflammatory mediators, including lipopolysaccharide (LPS), TNF α , and IL-1 β (3–5). TSG6 is composed of an HA-binding LINK domain and a CUB (complement protein subcomponents C1r/C1s, urchin embryonic growth factor, and bone morphogenetic protein) domain (3, 4). To date, only the HA-binding protein CD44 has been shown to function as a TSG6 receptor (6).

TSG6 has a potent but poorly understood function in suppressing inflammation through inhibition of neutrophil migration and production of proinflammatory cytokines (7, 8). This effect of TSG6 has been demonstrated in several models. TSG6 was shown to prevent inflammation and tissue injury in rodent models of arthritis (9), myocardial infarction (10), corneal injury (11), and peritonitis (12). Both IL-1 β and TNF α induced the synthesis of TSG6 in MSCs, monocytes/macrophages, and fibroblasts (2, 4, 5). Administration of human MSCs in mice prevented endotoxin (LPS)-induced acute lung injury (ALI), and, importantly, this effect was abrogated using cells pretreated with TSG6-siRNA to suppress TSG6 expression (13). TSG6 also delayed the onset of autoimmune diabetes by suppressing the activation of T cells and antigen-presenting cells (14), further supporting its role in dampening the adaptive immune response.

In the present study, we addressed the role of TSG6 in ALI, an acute inflammatory disease that injures the lung's vascular barrier, resulting in severe protein-rich pulmonary edema and hypoxemia, associated with ~50% mortality (15). There is a profound release of proinflammatory cytotoxic mediators from activated alveolar

macrophages and other inflammatory cells sequestered in lungs (15). Macrophages in ALI are activated through the sensing of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) via Toll-like receptors (TLRs). On binding of LPS to TLR4, macrophages produce proinflammatory cytokines, such as IFN γ , TNF α , and IL-1 β , which signal the recruitment of neutrophils and lymphocytes at the site of infection and aid in the clearance of pathogens (16). However, macrophages also display marked plasticity and functional heterogeneity depending on such factors as their niche, interaction with other cells (e.g., endothelial cells), and cytokines to which they are exposed (17, 18).

Macrophages are classified as classically activated macrophages, or M1 type, and alternatively activated macrophages, or M2 type (17, 18). Within this classification, macrophages are polarized to the M1 in response to LPS and other mediators, such as IFN γ , whereas M2 macrophages are generated in response to IL-4, IL-10, and glucocorticoids (18–20). Each type is characterized by distinct molecular signatures, such as iNOS, IL-12, IL-6, TNF α , and STAT1 for M1 and arginase-1, chitinase-3-like-3 (Chi3l3), IL-10, IL-4, and STAT6 for M2 (17–20). However, recent transcription data indicate that macrophages exhibit a far more complex range of phenotypes than are accounted for by the M1/M2 classification scheme (17–20). The basis for macrophage polarization in vivo is not clear, but NF- κ B activation downstream of TLR4 and up-regulation of specific cytokines and transcription factors, such as STAT6, appear to be important in mediating macrophage plasticity (20–24).

We examined the role of TSG6, a macrophage releasate, in mediating the phenotypic shift of macrophages and in counteracting LPS-induced ALI in mice. We found that TSG6 is crucial in mediating the transition of macrophages and thereby inhibiting the inflammatory feed-forward cascade activated by LPS. We show that the TSG6-mediated reprogramming of macrophages is essential

Significance

We found that TNF α -stimulated gene-6 (TSG6), a secreted 30-kDa immunomodulatory protein, resolved LPS-induced inflammatory lung injury by shifting macrophages from a proinflammatory to an anti-inflammatory phenotype. Macrophages from mice genetically deficient in TSG6 failed to transition, demonstrating the essential role of TSG6 in mediating macrophage plasticity. The finding that TSG6 induced the marked transition in anti-inflammatory macrophages lays the foundation for its therapeutic application.

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Conflict of interest statement: D.J.P. is chair of the Scientific Advisory Committee and has a small equity stake (<5%) in Temple Therapeutics, LLC, with an interest in TSG6.

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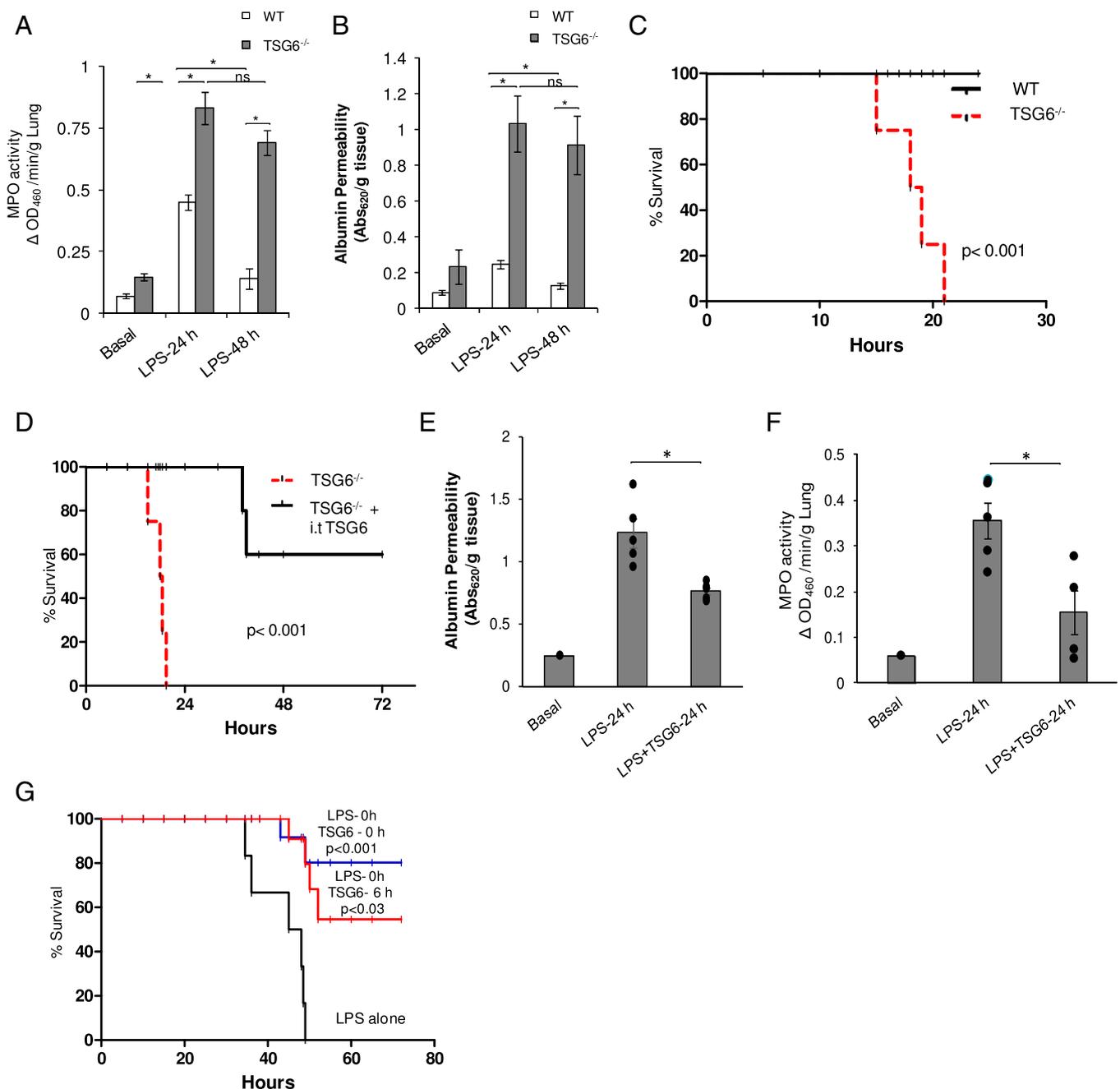


Fig. 1. TSG6 deletion in mice (*TSG6*^{-/-}) augments LPS-induced inflammatory lung injury. (A and B) WT (*TSG6*^{+/+}) and *TSG6*^{-/-} mice were challenged with LPS (10 mg/kg i.p.). (A) Lungs obtained at 0, 24 h, and 48 h after LPS challenge were used to measure MPO activity in lungs. (B) After LPS challenge, lung vascular permeability (uptake of EBA) was assessed. *n* = 4 mice per time point. **P* < 0.05, WT vs. *TSG6*^{-/-}. (C) Survival of age- and weight-matched WT and *TSG6*^{-/-} mice monitored after challenge with a lethal dose of LPS (20 mg/kg i.p.). *TSG6*^{-/-} mice showed the highest mortality within 20 h after LPS injection compared with WT mice. *n* = 10 per group. **P* < 0.001, WT vs. *TSG6*^{-/-}. (D) Survival of *TSG6*^{-/-} mice challenged with LPS (20 mg/kg, i.p.) with and without simultaneous i.t. instillation of TSG6 (5 μg/mouse). TSG6 instillation rescued the high mortality seen in the *TSG6*^{-/-} mice, increasing survival by 60% compared with untreated controls. *n* = 8 mice per group. **P* < 0.001. (E and F) WT mice were simultaneously challenged with LPS (10 mg/kg, i.p.) and saline (i.t) or TSG6 (5 μg/mouse, i.t.). (E) At 0 and 24 h after LPS/saline or LPS/TSG6 administration, mice were used for lung EBA permeability measurements as above. Mice treated with TSG6 showed significantly reduced lung vascular permeability compared with untreated control mice. *n* = 5 mice per group; **P* < 0.05, LPS vs. LPS + TSG6. (F) At 0 and 24 h after LPS/saline or LPS/TSG6 administration, lungs were used to measure MPO activity, expressed as change in absorbance (ΔA). MPO activity was significantly reduced in lungs of TSG6-treated mice compared with saline-treated control mice. *n* = 4 mice per group. **P* < 0.05, LPS vs. LPS + TSG6. (G) Survival in WT mice, monitored for 72 h after a lethal dose of LPS (20 mg/kg i.p.) alone or in combination with TSG6 (5 μg/mouse) given i.t. either concomitantly with or 6 h after LPS challenge. *n* = 10 per group. **P* < 0.001, LPS vs. LPS + TSG6; **P* < 0.03, LPS vs. LPS + TSG6 post-LPS.

for the resolution of ALI. The expression of anti-inflammatory macrophage signatures (arginase-1 and Chi3l3) was abrogated in *TSG6*^{-/-} mice, whereas the expression of proinflammatory

transcription factors STAT1 and STAT3 was enhanced. TSG6 expression in macrophages was itself increased in response to LPS, resulting in a cell population displaying a distinct anti-inflammatory

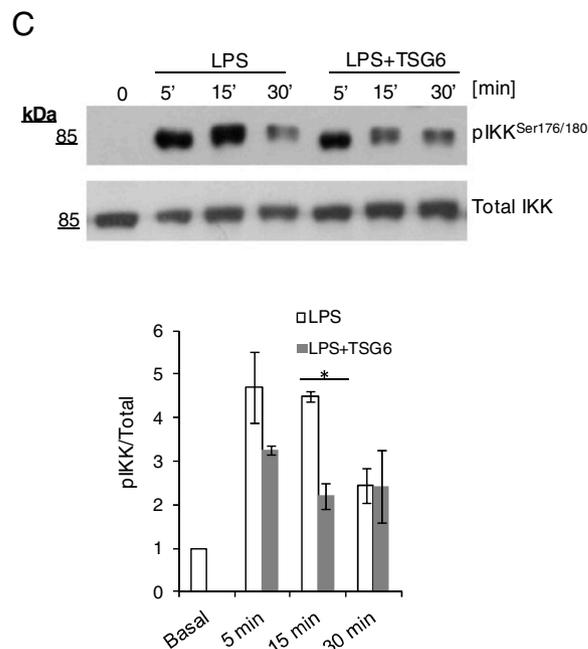
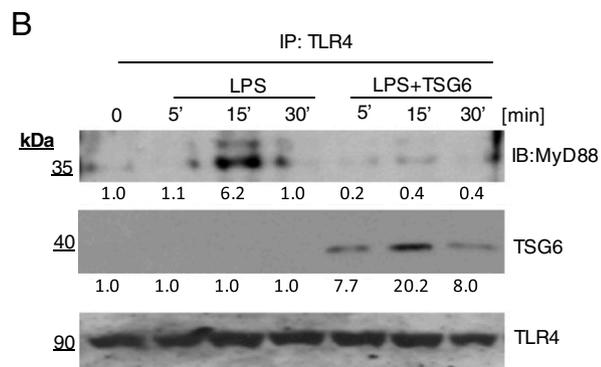
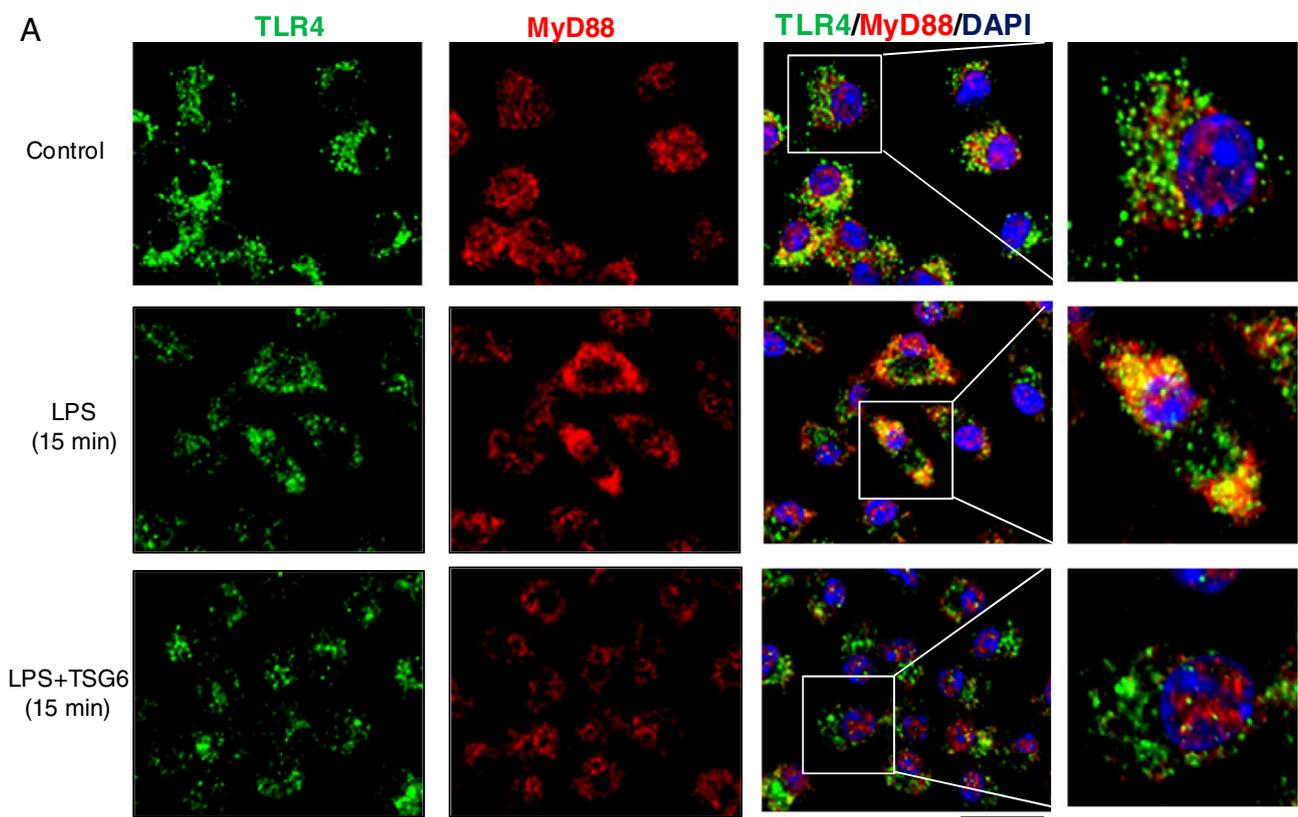


Fig. 2. TSG6 inhibits the interaction of TLR4 with MyD88 and prevents NF- κ B activation. (A) Macrophages cultured from bone marrow of WT mice were challenged with LPS (1 μ g/mL) alone or TSG6 (0.1 μ g/mL) plus LPS (1 μ g/mL) together for 0 and 15 min. Cells were fixed, permeabilized, and stained, and images were acquired by confocal microscopy. Green, TLR4; red, MyD88; blue, DAPI. Strong colocalization of TLR4 with MyD88 was seen after LPS challenge, and this response was prevented by rTSG6. (Scale bar: 20 μ m.) (B) WT macrophages cultured from bone marrow were treated with LPS (1 μ g/mL) alone or with rTSG6 (0.1 μ g/mL) plus LPS (1 μ g/mL) for different times. They were then lysed and immunoprecipitated with anti-TLR4 polyclonal antibody (pAb). Immunoprecipitated samples were blotted with anti-MyD88 or anti-TSG6 pAb, and blots were reprobed with anti-TLR4 pAb. A strong interaction of MyD88 with TLR4 was observed at 15 min after LPS treatment, which was abolished by rTSG6. (C) The cell lysates were immunoblotted with anti-phos-Ser177/181-IKK β pAb. Quantified results are shown as ratio of phosphorylated to total protein (Bottom). rTSG6 treatment decreased LPS-induced phosphorylation of IKK β . * P < 0.05, LPS vs. LPS + TSG6.

phenotype. This indicates that TSG6 plays a vital role in the resolution of inflammatory lung injury *in vivo* by converting macrophages to the anti-inflammatory phenotype, and furthermore, that TSG6 expression is a strong indicator of the innate immune function of macrophages.

Results

TSG6 Deletion ($TSG6^{-/-}$) in Mice Augments Lung Vascular Injury, Neutrophil Sequestration, and Mortality Induced by LPS. We challenged $TSG6^{-/-}$ mice with 20 mg/kg LPS *i.p.* and measured lung vascular permeability (an index of vascular injury) using Evans blue dye conjugated with albumin (EBA) tracer and neutrophil sequestration by assaying lung tissue myeloperoxidase (MPO) content. $TSG6^{-/-}$ mice showed a higher basal MPO level compared with WT mice, although the basal vascular permeability value was not significantly greater than that in WT mice (Fig. 1*A* and *B*). However, at 24 h after LPS challenge, $TSG6^{-/-}$ mice demonstrated significantly greater lung vascular injury and neutrophil accumulation compared with WT mice (Fig. 1*A* and *B*). In contrast, at 48 h after LPS challenge, lung vascular injury and neutrophil sequestration both recovered to basal values in WT mice, but persisted in $TSG6^{-/-}$ mice (Fig. 1*A* and *B*). We also observed 100% mortality in $TSG6^{-/-}$ mice (generated on a BALB/c background) within 20 h of the LPS challenge, whereas all WT mice of the same background survived (Fig. 1*C*), consistent with the survival results reported in other studies in mice of the BALB/c strain given LPS (25).

Next, to determine whether exogenous administration of TSG6 was itself protective, we evaluated the effects of intratracheal (*i.t.*) instillation of recombinant (*r*) TSG6. We observed that $TSG6^{-/-}$ mice treated with *r*TSG6 before LPS challenge showed 60% survival, compared with no survival of $TSG6^{-/-}$ mice (Fig. 1*D*). Thus, TSG6 administration significantly reduced the mortality seen in $TSG6^{-/-}$ mice.

TSG6 Treatment Prevents LPS-Induced Lung Vascular Injury, Neutrophil Infiltration, and Mortality. We challenged WT mice with *i.p.* LPS and simultaneously instilled *r*TSG6 *i.t.*, and then at 24 h post-LPS challenge assessed lung vascular injury and neutrophil uptake as above. *r*TSG6 significantly reduced LPS-induced lung vascular injury and neutrophil sequestration (Fig. 1*E* and *F*). To investigate the prophylactic vs. therapeutic effects of *r*TSG6, we divided the mice into three groups. Group 1 was challenged with *i.p.* LPS alone, group 2 received concomitant *i.p.* LPS and *i.t.* *r*TSG6, and group 3 received *i.t.* *r*TSG6 at 6 h post-LPS challenge, when the mice had a high probability of dying. In group 1 (LPS alone), all mice died within 48 h of the LPS challenge (Fig. 1*G*). Group 2 (concomitant *r*TSG6 and LPS) showed 80% survival, and, surprisingly, group 3 mice (*r*TSG6 at 6 h post-LPS) showed 50% survival (Fig. 1*G*).

TSG6 Prevents the Interaction of TLR4 with MyD88 and NF- κ B Activation. We next determined whether *r*TSG6 interfered with TLR4 signaling, which induces NF- κ B activation via MyD88-dependent and MyD88-independent pathways (26). In control bone marrow-derived macrophages, LPS induced I κ B kinase β (IKK β) phosphorylation within 5 min; however, a strong TLR4–MyD88 association was observed only within 15 min (Fig. 2*A–C*). *r*TSG6 suppressed IKK β activation and inhibited the association of MyD88 with TLR4 (Fig. 2*A–C*); thus, TSG6 functions by preventing MyD88-dependent NF- κ B activation.

TSG6 Reprograms Macrophage Phenotype *In Vivo*. To investigate mechanisms of TSG6 inhibition of LPS-induced ALI, we injected mice with *i.p.* LPS (10 mg/kg) alone or concomitantly with *i.t.* *r*TSG6 (5 μ g/mouse). At 6 h and 24 h after LPS, we measured concentrations of proinflammatory cytokines IL-6, TNF α , IFN γ , and IL-12p70 and anti-inflammatory cytokines IL-10 and IL-4 in bronchoalveolar lavage (BAL) fluid. TSG6 treatment significantly

reduced the generation of IL-6, IFN γ , TNF α , and IL-12p70 while increasing the generation of IL-10 and IL-4 (Fig. 3*A* and *B*). In other experiments, we obtained CD11b⁺ cells (alveolar macrophages) from BAL fluid of mice at 24 h post-LPS. TSG6 treatment of these mice suppressed the LPS-mediated induction of proinflammatory transcripts iNOS, IL-6, TNF α , IL-1 β , and CXCL1 while augmenting anti-inflammatory markers CD206 and Chi3l3 in the alveolar macrophages (Fig. 3*C*).

We also isolated CD11b⁺ macrophages from digested lungs at 24 h after challenging mice with either *i.p.* LPS or *i.p.* LPS plus TSG6, and assessed the expression of proinflammatory and anti-inflammatory proteins by Western blot analysis and ELISA. *r*TSG6 suppressed the phosphorylation of the proinflammatory transcription factors STAT1 and STAT3, as well as the expression of IL-1 β in these cells (Fig. 4*A*). Interestingly, the CD11b⁺ cells obtained from the *r*TSG6-treated mice showed increased expression of arginase-1 and Chi3l3, indicative of the anti-inflammatory phenotype shift (Fig. 4*A*).

We next placed the CD11b⁺ cells in culture for 3 d, and then used the culture supernatants for cytokine measurements. We observed that CD11b⁺ macrophages obtained from the mice treated with TSG6 exhibited reduced production of TNF α , IL-6, IFN γ , and IL-12p70 and increased production of IL-10 and IL-4 (Fig. 4*B*).

In these studies, we also determined whether LPS itself could up-regulate TSG6 expression. We observed that LPS given *i.p.* induced the TSG6 expression in the lungs of WT mice, but not of $TSG6^{-/-}$ mice (Fig. 4*C*). LPS also induced phosphorylation of the proinflammatory transcription factors STAT1 and STAT3 at 6 h of the LPS challenge in $TSG6^{+/+}$ mice, followed by a return to baseline at 24 h (Fig. 4*C*). In contrast, LPS induced sustained phosphorylation of STAT1 and STAT3 in $TSG6^{-/-}$ mice (Fig. 4*C*), indicating that TSG6 regulates STAT1 and STAT3 activities. Importantly, LPS induced a sustained expression of IL-1 β in $TSG6^{-/-}$ mice, but not in $TSG6^{+/+}$ mice (Fig. 4*C*), suggesting the crucial role of TSG6 in dampening inflammation. We also observed that expression of anti-inflammatory proteins arginase-1 and Chi3l3 was increased in $TSG6^{+/+}$ mice post-LPS, whereas their expression was defective in $TSG6^{-/-}$ mice (Fig. 4*C*).

Discussion

TSG6 was first described as an anti-inflammatory factor released by TNF α -treated fibroblasts (27, 28). IL-1 β and TNF α have been shown to induce TSG6 expression in multiple cell types (2–5, 29). Human and murine TSG6 proteins are ~94% identical (4). Transgenic mice overexpressing TSG6 were resistant to collagen-induced arthritis (CIA) (9), whereas inactivation of the TSG6 gene enhanced inflammation and increased the susceptibility to arthritis (30). Intravenous administration of MSCs in mice also prevented endotoxin-mediated ALI, apparently by reducing the expression of proinflammatory cytokines and neutrophil sequestration in lungs (13); however, siRNA-mediated suppression of TSG6 expression in MSCs prevented these protective effects, indicating the involvement of TSG6 in this protection. In another study, TSG6 prevented zymosan-induced peritonitis secondary to reduced TLR2-mediated nuclear translocation of NF- κ B (12). TSG6 also has been shown to reduce inflammation by inhibiting NF- κ B signaling in a model of sterile injury of the cornea (11, 31).

Although the anti-inflammatory effects of TSG6 have been well described (1, 2, 7–14, 29, 32), how it modulates inflammation has not been addressed until now. Here, in studies performed in a model of LPS-induced inflammatory lung injury that simulates ALI, we have demonstrated that (*i*) $TSG6^{-/-}$ mice exhibit markedly augmented lung vascular injury and neutrophil sequestration and mortality, which is reversed by *i.t.* TSG6 instillation; (*ii*) *i.t.* TSG6 instillation even at 6 h after the onset of lung injury reduces mortality by 50% from the 100% mortality seen in untreated control mice; and (*iii*) TSG6 functions by interfering with TLR4–MyD88 interaction in macrophages to inhibit NF- κ B and STAT1 and STAT3 activation, and thereby promote the transition of

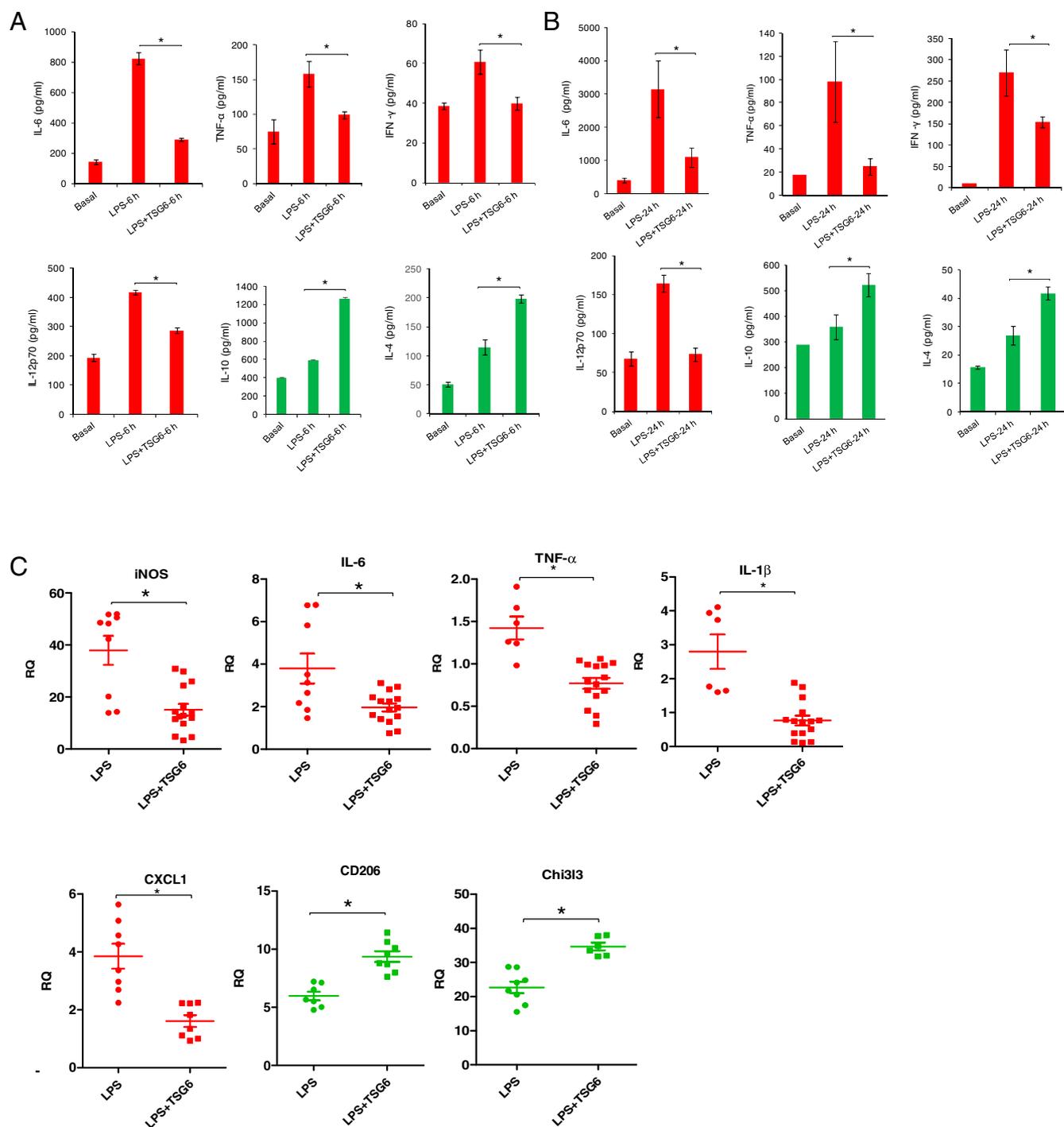


Fig. 3. TSG6 induces expression of anti-inflammatory factors while suppressing proinflammatory factors in alveolar macrophages. (A and B) Concentrations of proinflammatory cytokines (IL-6, TNF α , IFN γ , and IL12p70) and anti-inflammatory cytokines (IL-10 and IL-4) were measured in BAL fluid of mice challenged with either i.p. LPS (10 mg/kg) and i.t. saline or i.p. LPS (10 mg/kg) and i.t. TSG6 (5 μ g) for 6 h (A) or 24 h (B). TSG6 significantly decreased the production of IL-6, TNF α , IFN γ , and IL-12p70 while increasing the production of IL-10 and IL-4. $n = 4$ mice per group. * $P < 0.05$. (C) Quantification of transcript expression of iNOS, IL-6, IL-1 β , TNF α , CXCL1, Chi3l3, and CD206 by qPCR in CD11b $^{+}$ alveolar macrophages from BAL fluid of mice challenged with either i.p. LPS (10 mg/kg) for 24 h or i.p. LPS (10 mg/kg) and i.t. TSG6 (5 μ g) for 24 h. * $P < 0.05$, LPS vs. LPS + TSG6.

anti-inflammatory macrophages. We also observed that LPS itself up-regulated the expression of TSG6 in lung as reported previously (3–5, 29), consistent with its role as an autocrine mediator resolving inflammatory injury.

NF- κ B activation is a known driver of inflammatory signaling (33). Our results show that the rTSG6-mediated suppression of

MyD88-dependent NF- κ B activation is essential for reprogramming macrophages. Of note, the LPS-induced IKK β phosphorylation was observed in 5 min, but a strong association of TLR4 and MyD88 was not seen until 15 min. Although the reason for this discrepancy is not clear, the early IKK β phosphorylation could have been mediated by MyD88-independent pathways (26). In response to

rTSG6, the macrophages shifted from generating proinflammatory cytokines TNF α , IL-1 β , IL-6, and IL-12p70 to generating a distinct anti-inflammatory phenotype. NF- κ B is normally maintained in an inactive state in the cytoplasm by inhibitory proteins, such as I κ B (33). Macrophage polarization to the anti-inflammatory phenotype requires the formation of inactive dimers of the p50 subunit of NF- κ B, resulting in suppression of NF- κ B-driven macrophage polarization (22). We observed that rTSG6 prevents the interaction of TLR4 with MyD88, a requirement for the activation of NF- κ B signaling (33), and that this in turn induces the shift toward anti-inflammatory macrophages. Similar results were seen in a model of zymosan-induced peritonitis in which TSG6 prevented the interaction of TLR2 with MyD88 and thus inhibited the activation of NF- κ B (12).

Macrophage polarization is a central feature of inflammatory disease progression, such as in ALI (17, 18). In lungs, resident alveolar macrophages are the first line of defense against airway pathogens. They orchestrate the inflammatory response through activation of pattern recognition receptors that initiate both inflammatory and resolution pathways (17, 18). The phenotype of anti-inflammatory macrophages is characterized by the proteins arginase-1, Chi3l3, pSTAT6, IL-4, and IL-10, whereas proinflammatory macrophages express iNOS, pSTAT1, pSTAT3, TNF α , IL-6, IFN γ , IL-12p70, and IL-1 β (17–21, 23, 24). We have shown that TSG6 reprograms macrophages to the anti-inflammatory phenotype, which resolves ALI.

Mice genetically deficient in TSG6 (*TSG6*^{-/-}) exhibited far greater mortality than controls owing to the inability to reprogram macrophages, and thus to resolve ALI. Because TSG6 is released in the inflammatory milieu (3, 29), our finding that TSG6 induces the phenotype switch in macrophages suggests the therapeutic potential of TSG6 in sepsis-induced ALI. Of special note is that the effective dose of TSG6 administered i.t. was only 5 μ g, or one-tenth the dose required for efficacy with i.v. administration in several mouse models of inflammation (8–13, 32).

Materials and Methods

Mouse Experiments. All mice were housed at the University of Illinois Animal Care Facility in accordance with institutional guidelines and guidelines of the National Institutes of Health. Veterinary care of the mice and the related animal experiments was approved by the University of Illinois Animal Resources Center. The TSG6 knockout mice originally generated on a BALB/c background (30) were obtained from Jackson Laboratory. Age-matched *TSG6*^{+/+} and *TSG6*^{-/-} littermates were used for all experiments.

Endotoxin-induced ALI and i.t. rTSG6 Instillation. Age- and weight-matched *TSG6*^{+/+} and *TSG6*^{-/-} mice received a single i.p. dose of LPS (10 mg/kg body weight; *Escherichia coli* strain 0111:B4; Sigma-Aldrich). The mice were given a single 5- μ g dose of rTSG6 (2104-TS-050; R&D Systems) by noninvasive i.t. instillation. For i.t. instillation, the mice were anesthetized by ketamine/xylazine (45 mg/kg and 8 mg/kg, respectively) and were suspended on a flat board and placed in a semirecumbent position with the ventral surface and rostrum facing up. Using a curved blade forceps, the tongue was gently and partially retracted rostrally, and 50 μ L containing 5 μ g of rTSG6 was placed in the trachea. Saline (50 μ L) alone was similarly administered to the control mice. For survival studies, mice injected i.p. with 20 mg/kg LPS were monitored four times daily for up to 3 d.

MPO Assay. MPO activity in lung tissue was measured as described previously (34). The lungs were homogenized in 1 mL of PBS with 0.5% hexadecyltrimethylammonium bromide. The homogenates were sonicated, centrifuged at 40,000 \times g for 20 min, and run through two freeze-thaw cycles. The samples were homogenized and centrifuged a second time. The supernatant was then collected and mixed 1/30 (vol/vol) with assay buffer (0.2 mg/mL o-dianisidine hydrochloride and 0.0005% H₂O₂). The change in absorbance was measured at 460 nm for 3 min, and MPO activity was calculated as the change in absorbance over time.

Measurement of Lung Vascular Permeability. Lung vascular leak was measured using EBA as described previously (35). Mice anesthetized with 2.5% (vol/vol) isoflurane in room air were injected retro-orbitally with 150 μ L of EBA (30 mg/kg).

At 30 min after EBA injection, animals were killed, and lung tissue was harvested for EBA measurement.

Bone Marrow-Derived Macrophage Cultures. For the isolation of bone marrow cells, femur cavities from WT mice (C57Bl/6) were flushed, the cells were plated at approximately 2×10^6 cells/mL, and incubated in DMEM supplemented with 10% (vol/vol) FBS, 1% (vol/vol) streptomycin/penicillin, and 10% (vol/vol) L929-conditioned media for 1 wk. Cells were used for experiments at day 7 of culture.

Immunoprecipitation. Bone marrow-derived macrophages were stimulated with LPS (1 μ g/mL) alone or in combination with TSG6 (0.1 μ g/mL) for 0, 5, 15, and 30 min. After this treatment, cells were lysed in lysis buffer (final concentration: 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM sodium vanadate, 1% Triton X100, and 0.5% Nonidet P-40) supplemented with protease inhibitor mixture. The cell lysate was cleared of cellular debris by centrifuging at 12,000 \times g for 10 min. The clear supernatant collected was subjected to immunoprecipitation. Each sample was incubated overnight with TLR4 antibody (1 μ g/mL) at 4 $^{\circ}$ C on a rotating platform. Next day, protein A/G beads were added to the sample and incubated for 1 h at 4 $^{\circ}$ C. Immunoprecipitated samples were washed three times and used for immunoblotting.

Immunostaining. Bone marrow-derived macrophages from mice grown on coverslips were stimulated with LPS (1 μ g/mL) alone or in combination with TSG6 (0.1 μ g/mL) for 0 and 15 min. After this treatment, cells were fixed and used for immunostaining (36).

Collection of BAL Fluid and Cells. For collection of BAL fluid, the upper part of the trachea was cannulated and then lavaged three times with 0.8 mL of PBS supplemented with 0.4 mM EDTA and protease inhibitor mixture. The fluid recovery rate typically exceeds 90%. The lavage samples from each mouse were kept on ice. BAL fluid was centrifuged at 700 \times g for 5 min at 4 $^{\circ}$ C. The collected supernatant was then used for cytokine assays.

Immunoblotting. Cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture). Mouse lungs were homogenized in lysis buffer (35). Cell lysates or mouse lung homogenates were centrifuged at 14,000 \times g at 4 $^{\circ}$ C for 10 min, and cleared supernatant was used for immunoblot analysis. The protein lysates were resolved by SDS/PAGE on a 4–15% gradient separating gel under reducing conditions and transferred to PVDF membrane. The membrane was blocked with 3% (wt/vol) BSA in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature. The membranes were then probed with the indicated primary antibody (diluted in blocking buffer) overnight at 4 $^{\circ}$ C. Next, membranes were washed three times and then incubated with an appropriate HRP-conjugated secondary antibody. Protein bands were detected by enhanced chemiluminescence. The following antibodies were used for immunoblotting: TSG6 (MAB-2104; R&D Systems), TLR4 (sc-30002; Santa Cruz Biotechnology), MyD88 (sc-8196; Santa Cruz Biotechnology), phospho-STAT1^{Y701} (612233; BD Biosciences), pSTAT3^{Y705} (51-9002097; BD Biosciences), Chi3l3 (AF2446; R&D Systems), Arginase-1 (610708; BD Biosciences), IL-1 β (AF401; R&D Systems), phospho-IKK α/β (2697; Cell Signaling Technology), total IKK (2684; Cell Signaling Technology), and anti- β -actin (A5316, mouse monoclonal; Sigma-Aldrich). Band intensity was determined by densitometry with the aid of ImageJ software.

Cytokine ELISA. Cytokines were measured in BAL fluid and supernatants of cultured macrophages with commercially available ELISA kits (eBioscience) according to the manufacturer's instructions.

Isolation of CD11b⁺ Alveolar Macrophages. The CD11b⁺ cells were isolated from total BAL fluid by incubation with CD11b⁺ magnetic beads (Miltenyi Biotec) (37) according to the manufacturer's instructions. For isolation of CD11b⁺ cells from the lungs, the harvested lungs were minced into small pieces and then incubated in DMEM with 1% penicillin-streptomycin for 30 min at 37 $^{\circ}$ C and 5% (vol/vol) CO₂ in the presence of 1 mg/mL collagenase A (70499828; Roche). After incubation, the minced pieces of lungs were passed through 16G and 18G needles, and the resulting suspension was passed through a 70- μ m cell strainer and then washed with complete DMEM. The cells were centrifuged at 200 \times g for 3 min, incubated for 20 min at 4 $^{\circ}$ C with CD11b magnetic beads, and finally applied and eluted from MS columns (Miltenyi Biotec) for the positive selection of CD11b⁺ cells.

RNA Extraction and qRT-PCR. Total RNA isolated from CD11b⁺ cells was reverse-transcribed with random hexamers and MultiScribe reverse transcriptase (Applied Biosystems). The cDNA thus obtained was mixed with SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers for PCR. An ABI Prism 7000 was used for quantitative PCR analysis. Cyclophilin expression served as an internal control. The following mouse primers were used: iNOS (forward, 5'-AGTCTCAGACATGGCTTGCCCT-3'; reverse, 5'-GCTGCGGGAGCCATTTTGGT-3'), IL-6 (forward, 5'-TCCAGTTGCCTTCTGGGACTG-3'; reverse, 5'-AGCCTCCGACTGTGAAGTGGT-3'), TNF α (forward, 5'-GACCCTCACACTCAGATCATCT-3'; reverse, 5'-CCTCACTTGGTGGTTTGTCT-3'), IL-1 β (forward, 5'-GAATCTATACCTGCTCTGTG-3'; reverse, 5'-TTATGCTCTGACCCTGTTG-3'), CXCL1 (forward, 5'-CTGGGATCACCTCAAGAATC-3'; reverse, 5'-CAGGGTCAAGGCAAGCCTC-3'), Chi3l3 (forward, 5'-AGAAGGGATTTCAAC-

CTGGT-3'; reverse, 5'-GTCTTGCTCATGTGTGAAGTGA-3'), CD206 (forward, 5'-CCACGGATGACCTGTGCTCGAG-3'; reverse, 5'-ACACCAGACCCATCGTCCGA-3'), and CYP (forward, 5'-CTTGCCATGGCAAATGCTG-3'; reverse, 5'-GTGATCTTCTGCTGGTCTTG-3').

Statistical Analysis. Data were analyzed using the unpaired Student's *t* test and log-rank test. A difference in mean values was considered significant at a *P* value <0.05.

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