



# STAT6 induces expression of Gas6 in macrophages to clear apoptotic neutrophils and resolve inflammation

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**Efferocytosis of apoptotic neutrophils (PMNs) by alveolar macrophages (AMΦs) is vital for resolution of inflammation and tissue injury. Here, we investigated the role of AMΦ polarization and expression of the efferocytic ligand Gas6 in restoring homeostasis. In the murine model of lipopolysaccharide (LPS)-induced acute lung injury (ALI), we observed augmented temporal generation of cytokines IL-4 and TSG6 in bronchoalveolar fluid (BALF). Interestingly, we also observed increased expression of antiinflammatory markers consistent with a phenotype shift in AMΦs. In particular, AMΦs expressed the efferocytic ligand Gas6. In vitro priming of bone marrow-derived macrophages (BMMΦs) with IL-4 or TSG6 also induced MΦ transition and expression of Gas6. TSG6- or IL-4-primed BMMΦs induced efferocytosis of apoptotic PMNs compared with control BMMΦs. Adoptive transfer of TSG6- or IL-4-primed BMMΦs i.t. into LPS-challenged mice more rapidly and effectively cleared PMNs in lungs compared with control BMMΦs. We demonstrated that expression of Gas6 during AMΦ transition was due to activation of the transcription factor signal transducer and activator of transcription-6 (STAT6) downstream of IL-4 or TSG6 signaling. Adoptive transfer of Gas6-depleted BMMΦs failed to clear PMNs in lungs following LPS challenge and mice showed severely defective resolution of lung injury. Thus, activation of STAT6-mediated Gas6 expression during macrophage phenotype transition resulting in efferocytosis of PMNs plays a crucial role in the resolution of inflammatory lung injury.**

macrophage | STAT6 | Gas6

**A**lveolar macrophages (AMΦs) are essential for recognition and clearance of pathogens from the airways, initiation of host defense, and tissue repair (1). During resolution of lung injury, AMΦs clear apoptotic neutrophils (PMNs), tissue debris, and bacteria from the alveolar space through a tightly coordinated process known as efferocytosis (2, 3). Efferocytosis is critical for resolution of lung injury since impaired clearance of apoptotic PMNs by AMΦs leads to continued inflammation and injury (1, 4). Efferocytosis is a well-conserved process as evident from the observation that IL-13 secreted by Treg cells resolves lung inflammation through activating MΦ-mediated efferocytosis (5).

A distinct family of tyrosine kinase receptors known as TAM receptors, which comprise Tyro3, Axl, and Mer proteins, are essential for efferocytosis mediated by MΦs (6, 7). MΦs also generate the ligand growth arrest specific-6 (Gas6), which bridges MΦ-expressed TAM receptors to the phosphatidylserine on the surface of apoptotic cells to trigger efferocytosis (8). The inflammatory response is associated with inflammatory MΦ phenotype M1, which secretes proinflammatory cytokines, whereas resolution of inflammation is associated with MΦ M2 phenotype shift, with MΦs expressing antiinflammatory M2 markers (e.g., arginase-1 and CD206) (9, 10). The signaling events mediating up-regulation of efferocytotic components during MΦ phenotype shift are not well understood. IL-4 and IL-13 generated by MΦs promote MΦ phenotype transition via activation of the transcription factor STAT6

(11–13). We showed that MΦ secretes the protein TNF- $\alpha$ -stimulated gene-6 (TSG6), which is also essential for MΦ phenotype transition; further, it promotes the resolution of sepsis-induced acute lung injury (14). To elucidate mechanisms of efferocytosis activated during MΦ phenotype transition, we focused on the role of IL-4 and TSG6 signaling. We observed that both TSG6 and IL-4 induced increased STAT6-dependent expression of the efferocytosis ligand Gas6 in MΦs and that Gas6 expression was required for the clearance of apoptotic PMNs. Furthermore, our results showed that adoptive transfer of TSG6- or IL-4-primed MΦs i.t. into lipopolysaccharide (LPS)-challenged mice rapidly and effectively clears PMNs, raising the prospect of cell-based therapy in resolving inflammatory lung injury.

## Results

**MΦ Phenotype Transition Promotes Efferocytosis in Alveoli.** To determine whether AMΦ phenotype shift occurs during resolution of endotoxin-induced inflammatory lung injury, we injected LPS (10 mg/kg i.p.) into wild-type (WT) mice. At different time points, we analyzed the concentrations of IL-4 and TSG6 in bronchoalveolar lavage fluid (BALF). We found significantly increased concentrations of IL-4 and TSG6 in BALF at 48 h after LPS challenge compared with controls (Fig. 1A). We also observed that AMΦs obtained from BALF using anti-CD11b antibody at this time exhibited augmented expression of antiinflammatory M2 markers

## Significance

**Clearance of apoptotic neutrophils by alveolar macrophages (AMΦs) is critical for the resolution of acute lung injury (ALI). Here, we demonstrated that induction of the M1 phenotype in MΦs activates the transcription factor STAT6 and thereby promotes resolution of lung injury by the increased expression of Gas6, the ligand for efferocytosis. Therefore, targeting the STAT6 activation pathway in AMΦs and Gas6 may be a potential therapeutic strategy for resolution of inflammatory lung injury.**

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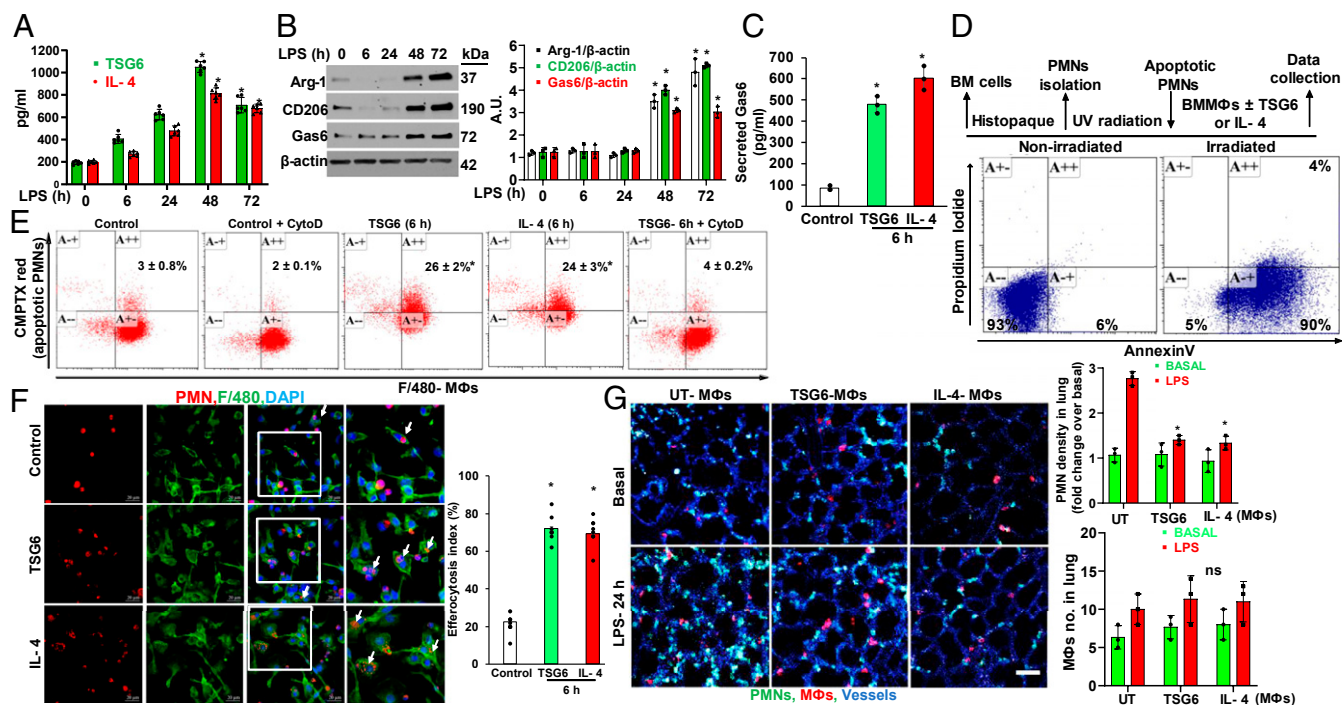
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**Fig. 1.** MΦ phenotype transition promotes efferocytosis. (A) BALF collected from mice challenged with LPS for the indicated time points was used for the measurement of TSG6 and IL-4 by ELISA.  $n = 6$  mice per time point.  $*P < 0.05$  vs. basal. (B) Immunoblots showing arginase-1, CD206, and Gas6 expression in AMΦs isolated from BALF from mice challenged with LPS for the indicated time points. Results shown are representative blots with  $\beta$ -actin as a loading control.  $n = 6$  mice per time point.  $*P < 0.05$  vs. basal. (C) Gas6 secreted in BMMΦ culture media was measured by ELISA. Results shown are mean  $\pm$  SD of three experiments.  $*P < 0.001$  vs. control. (D) Scheme of experimental protocol for PMN isolation and apoptosis induction. Apoptosis induction was analyzed by flow cytometry after staining with FITC Annexin V and Propidium iodide. More than 90% of PMNs were apoptotic (Lower- and Upper-right quadrants) in irradiated group. (E and F) Assessment of efferocytosis by BMMΦs in vitro. (E) BMMΦs treated with TSG6 (400 ng/mL) or IL-4 (100 ng/mL) for the indicated time points were overlaid for 2 h with apoptotic PMNs labeled with CellTracker CMPTX red dye in the presence or absence of cytochalasin D (2  $\mu$ M, 2 h). Then the cells were fixed in 1% PFA and analyzed by flow cytometry. BMMΦ populations positive for both F4/80 and CMPTX PMNs were considered to be the BMMΦs engulfing apoptotic PMNs (Upper-right quadrant). Results shown are from separate experiments.  $*P < 0.05$  vs. control. (F) same as E with confocal analysis to assess efferocytosis by BMMΦs. F4/80 antibody BMMΦs in green; apoptotic PMNs in red. Quantified data from three different experiments are shown in bar graph (Lower right);  $*P < 0.05$  vs. control. (G) In vivo imaging of PMNs in alveolar space and lung microvessels using high-resolution 2-photon excitation microscopy. BMMΦs were stimulated with TSG6 (400 ng/mL) or IL-4 (100 ng/mL) for 6 h and then i.t.-instilled into mice ( $2 \times 10^6$  cells/mouse). Animals simultaneously received LPS (10 mg/kg i.p.) and BMMΦs i.t. At 24 h after LPS challenge and BMMΦs i.t., 2-photon images were collected as described in *Materials and Methods*. (Scale bar, 50  $\mu$ m.) Quantitative analysis of PMN density in lung ( $n = 30$ –33 for each bar of wild type) and number of MΦs in alveolar space ( $n = 5$ , Right). Alveoli were outlined and fluorescent intensities of PMNs were quantified; value of the basal condition in wild-type mice with unstimulated BMMΦs was normalized as 1 (Right).  $*P < 0.001$  vs. basal level with unstimulated BMMΦs.

(arginase-1 and CD206) (Fig. 1B). Importantly, LPS challenge elicited augmented expression of Gas6 in the AMΦs (Fig. 1B).

Since TSG6 and IL-4 are known to promote MΦ phenotype transition, we next investigated the possibility that TSG6- or IL-4-primed bone marrow-derived macrophages (BMMΦs) may functionally activate the efferocytosis of apoptotic PMNs. We observed that BMMΦs exposed to either TSG6 or IL-4 elicited marked release of Gas6 in the medium (Fig. 1C). To study efferocytosis, we isolated PMNs from mice bone marrow and exposed them to UV radiation for apoptosis induction (15) (Fig. 1D). These PMNs were labeled with CellTracker CMPTX red dye. We observed that BMMΦs primed with TSG6 or IL-4 effectively efferocytosed PMNs, as evident from the population of F4/80+ MΦs engulfing apoptotic PMNs (Fig. 1E). To confirm whether our experimental conditions could distinguish between ingested apoptotic PMNs and PMNs merely bound on the MΦ cell surface, we pretreated BMMΦs with cytochalasin D [which inhibits actin rearrangements (16)] and then measured efferocytosis. Here we observed complete blockade of PMN ingestion in cytochalasin D-treated BMMΦs (Fig. 1E), suggesting that the efferocytosis assay measured ingested and not cell surface-bound apoptotic PMNs. Confocal imaging analysis also showed enhanced

PMN uptake by TSG6- or IL-4-primed BMMΦs compared with untreated BMMΦs (Fig. 1F). Next, using high-resolution 2-photon excitation microscopy, we determined efferocytosis in vivo by the control BMMΦs and BMMΦs primed with TSG6 or IL-4. Here, mice were challenged with LPS i.p. and BMMΦs were instilled i.t. after having been either primed by TSG6 or IL-4 or not. In this experiment, blood vessels were labeled with anti-CD31 antibody (blue); PMNs, with anti-Ly6G antibody (green); and i.t. instilled BMMΦs, with CellTracker CMPTX red dye. We observed that i.t. instillation of BMMΦs primed with TSG6 or IL-4 effectively cleared the PMNs present in the air-space post-LPS compared with control BMMΦs (Fig. 1G). Interestingly, we did not observe any difference in the number of adoptively transferred BMMΦs present in lungs between the experimental groups (Fig. 1G, Right).

**STAT6 Expression in Response to TSG6 or IL-4 Induces Gas6 Expression in MΦs.** The efferocytotic receptors (Tyro3, Axl, and Mer) constitutively expressed in MΦs bind the secreted ligand Gas6, which bridges the MΦ-expressed TAM receptors to the phosphatidylerine on the surface of apoptotic cells to trigger efferocytosis (8). We observed that expression of Gas6 in AMΦs increased during the

inflammation resolution phase after LPS challenge (Fig. 1B) and that IL-4 or TSG6 priming of BMMΦs increased Gas6 expression (Figs. 1C and 2D and E). To gain insights into transcriptional mechanisms of Gas6 expression during MΦ phenotype transition, we analyzed the 5'-regulatory regions of both mouse (*m*) and human (*h*) genes encoding Gas6. We identified five putative binding sites for the transcription factor STAT6 upstream of the transcriptional start site (TSS) in the *mGas6* promoter (Fig. 2A) and four binding sites in the *hGas6* gene (Fig. 2A). We treated BMMΦs with STAT6-specific inhibitor and measured TSG6- or IL-4-induced STAT6 phosphorylation, which induces STAT6 nuclear translocation and transcriptional activity (12). The STAT6 selective inhibitor (AS1517499) prevented TSG6- and IL-4-induced STAT6 phosphorylation (Fig. 2B). We next used chromatin immunoprecipitation (ChIP) assay to assess binding of STAT6 to consensus sites of the Gas6 promoter in response to IL-4 or TSG6 in BMMΦs. We saw that either IL-4 or TSG6 challenge caused STAT6 binding to three sites (SB1, SB3, and SB4) in the proximal promoter region of the *mGas6* gene (Fig. 2C). Pharmacological inhibition of STAT6 prevented TSG6- and IL-4-induced Gas6 mRNA and protein expression in BMMΦs (Fig. 2D and E).

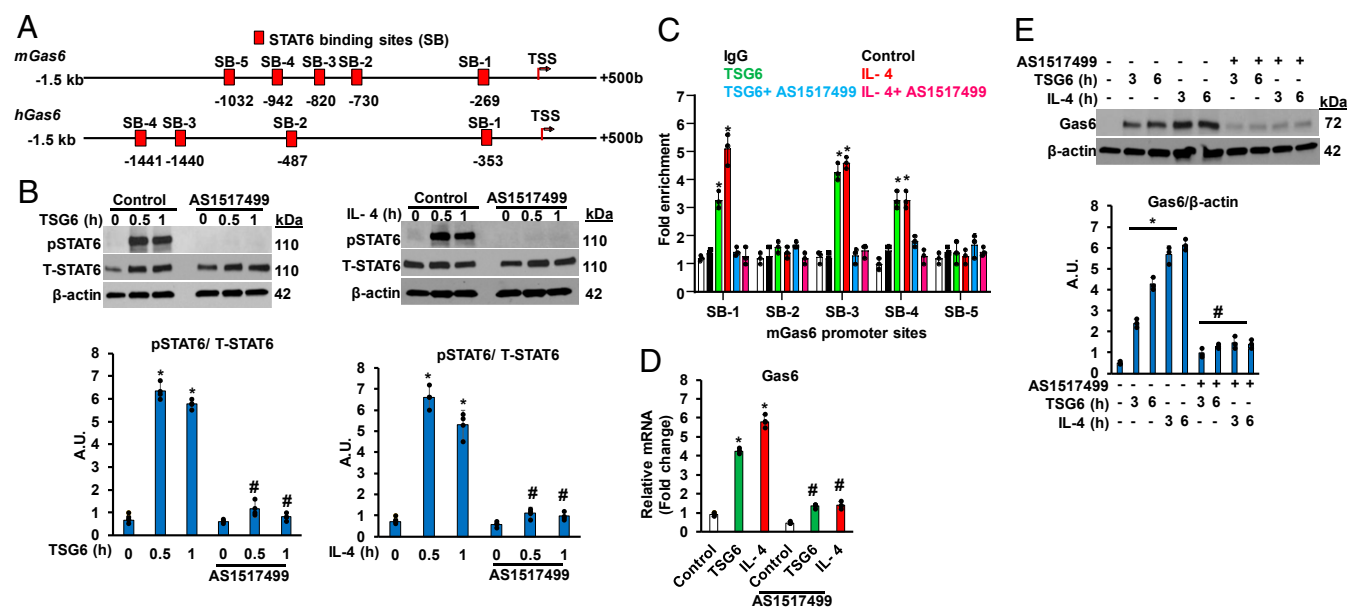
**Gas6 Expression in MΦs Is Required for Efferocytosis.** To address the role of Gas6 in mediating efferocytosis, we suppressed Gas6 expression by transfecting BMMΦs with Gas6 small interfering RNA (siRNA). We observed markedly reduced Gas6 protein expression compared with control siRNA-transfected cells (Fig. 3A). Next, we determined the effects of Gas6 knockdown on MΦ efferocytosis in vitro and observed significantly reduced engulfment of PMNs compared with control TSG6-primed BMMΦs (Fig. 3B). We also suppressed Gas6 expression using siRNA in

MΦs, and observed decreased MΦ efferocytosis in vitro (SI Appendix, Fig. S1A and B), further supporting the requisite role of Gas6 in the mechanism of efferocytosis in phenotype-shifted MΦs. In addition, we determined the effects of Gas6 depletion in BMMΦs in clearing lung airspace PMNs in vivo. In contrast to the active efferocytosis of airspace PMNs induced by instillation of TSG6-primed BMMΦs (Fig. 1G), we found that instillation of Gas6-depleted BMMΦs failed to clear PMNs (Fig. 3C). Based on these results, we propose a model for the mechanism of resolution of lung injury by MΦs through activation of PMN efferocytosis following phenotype transition of MΦs (Fig. 3D).

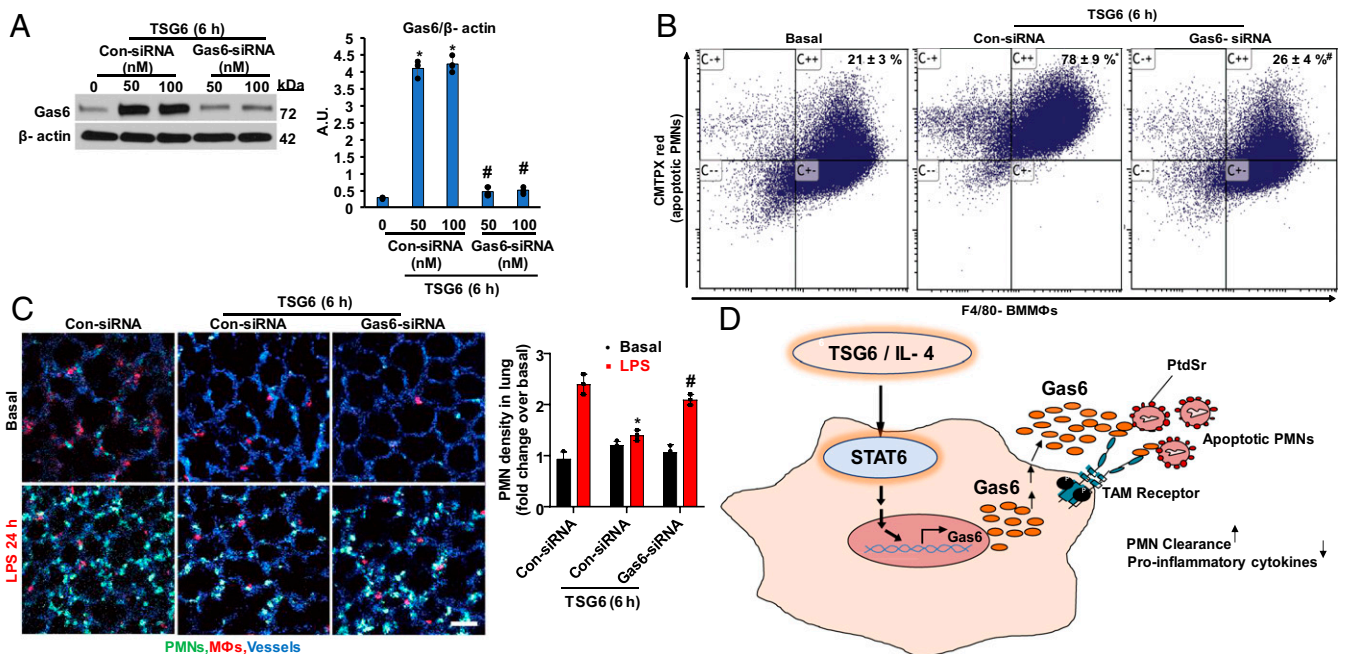
**TSG6-Primed BMMΦs Resolve Inflammatory Lung Injury in Mice.** We next determined whether MΦ phenotype transition is sufficient to resolve LPS-induced inflammatory lung injury. Mice were challenged with a lethal dose of LPS (20 mg/kg i.p.) and simultaneously i.t.-instilled with BMMΦs treated and not treated with TSG6. We observed 100% mortality in mice challenged with LPS plus i.t.-instilled with control BMMΦs (Fig. 4A), whereas LPS-challenged mice receiving i.t. instillation of BMMΦs primed with TSG6 or IL-4 showed markedly reduced mortality (Fig. 4A). In other experiments, we determined the effects of TSG6-primed BMMΦs on resolution of LPS-induced lung injury. We observed that i.t. instillation of TSG6-primed BMMΦs significantly reduced lung vascular permeability (Fig. 4B), PMN accumulation in BALF (Fig. 4C), and generation of inflammatory cytokines in BALF (Fig. 4D).

## Discussion

Dead PMNs prolong inflammation and fail to restore tissue homeostasis through release of chemokines, chemoattractants, and tissue debris (17). Therefore, effective clearance of apoptotic



**Fig. 2.** Transcription factor STAT6 activation promotes Gas6 expression in BMMΦs. (A) Schematics of STAT6 binding sites in the 5'-regulatory regions of mouse (*m*) and human (*h*) genes encoding Gas6. (B) BMMΦs were pretreated with STAT6 inhibitor AS1517499 (1 μM) for 1 h, followed by TSG6 (400 ng/mL; *Left*) or IL-4 (100 ng/mL; *Right*) stimulation for the indicated time points. Cell lysates were used for immunoblot analysis to determine STAT6 phosphorylation at Y641 and total STAT6 expression. Representative blots from three independent experiments are shown. \**P* < 0.05 vs. control. (C) BMMΦs pretreated with AS1517499 (1 μM, 1 h) followed by either TSG6 (400 ng/mL) or IL-4 (100 ng/mL) stimulation for 1 h were used for ChIP. TSG6 or IL-4 induced STAT6 binding to three sites (SB-1, SB-3, and SB-4) in the *mGas6* promoter. Results are mean of three experiments normalized to those of input DNA and presented relative to control IgG values. \**P* < 0.05 vs. control. (D) BMMΦs pretreated with AS1517499 were stimulated with either TSG6 (400 ng/mL) or IL-4 (100 ng/mL) for 1 h, and Gas6 mRNA expression was determined by RT-qPCR. \**P* < 0.05 vs. control; #*P* < 0.05 vs. TSG6- or IL-4-treated BMMΦs without AS1517499 pretreatment. (E) Immunoblot analysis of Gas6 protein expression in BMMΦs stimulated with TSG6 or IL-4 for the indicated time points with or without AS1517499 pretreatment. Results are representative of three independent experiments. \**P* < 0.05 vs. control; #*P* < 0.05 vs. TSG6- or IL-4-treated BMMΦs without AS1517499 pretreatment.



**Fig. 3.** Gas6 expression in MΦs is required for efferocytosis. (A) BMMΦs transfected with control or Gas6 siRNA (sc-35451) were used to determine Gas6 protein expression by immunoblot. \* $P < 0.05$  vs. control siRNA group. (B) BMMΦs transfected with control or Gas6 siRNA were primed with TSG6 and then incubated with CMTPIX-labeled PMNs for 2 h. In vitro efferocytosis was analyzed by flow cytometry as in Fig. 1E. \* $P < 0.05$  vs. basal; # $P < 0.05$  vs. TSG6-treated control siRNA group. (C) BMMΦs transfected with control or Gas6 siRNA were primed with TSG6 and adoptively transferred i.t. into mice challenged with LPS, followed by in vivo imaging of mouse lungs using high-resolution 2-photon excitation microscopy as in Fig. 1G. \* $P < 0.05$  compared with control siRNA group without TSG6 stimulation. # $P < 0.05$  vs. control siRNA BMMΦs primed with TSG6. (D) Proposed signaling mechanisms of inflammation resolution induced by TSG6 or IL-4 reprogramming of MΦs. TSG6- or IL-4-induced STAT6 activation promotes Gas6 expression in MΦs. Once secreted, Gas6 links phosphatidylserine (PtdSer) on apoptotic PMNs to TAM receptor kinases expressed on MΦs. This results in PMN clearance and suppression of proinflammatory cytokine production, thereby resolving lung injury.

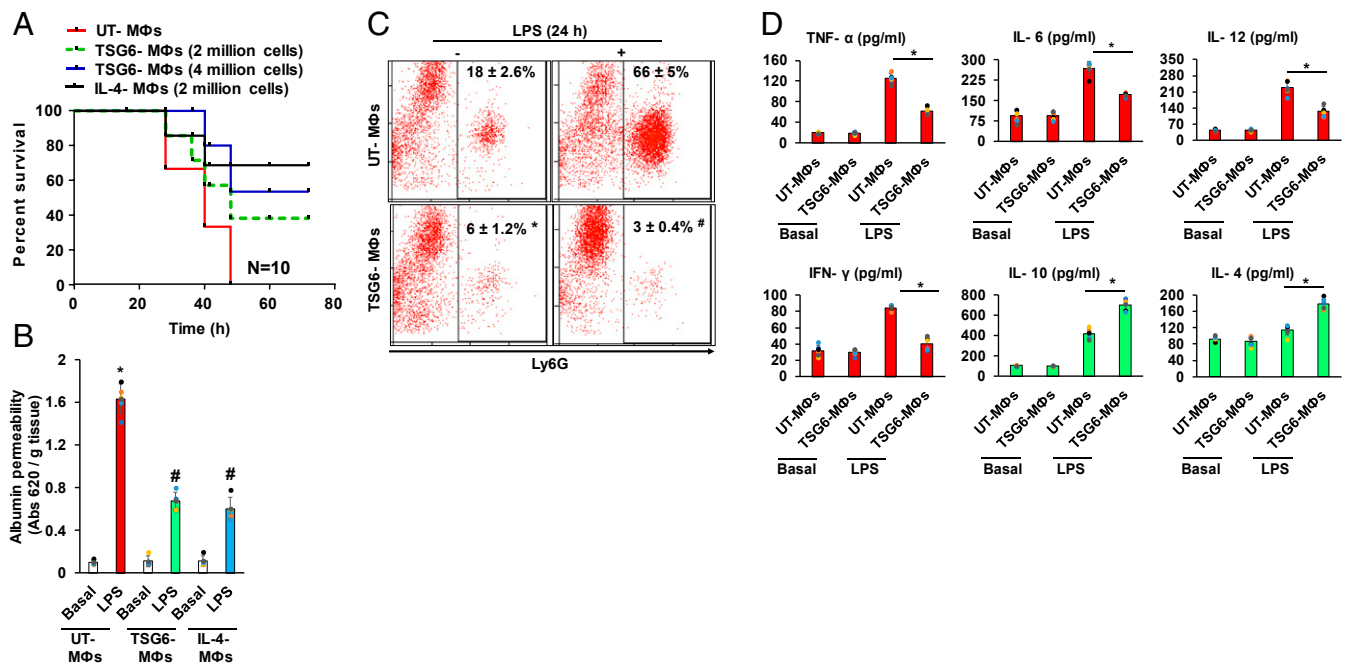
PMNs via efferocytosis by AMΦs, an immunologically silent process, is a prerequisite for resolution of inflammation and tissue injury (18, 19). Studies showed that MΦ phenotype transition from inflammatory to antiinflammatory cells terminates inflammatory signals in MΦs (9, 10, 20). Efferocytosis is mediated by TAM receptors in phagocytes such as AMΦs and the highly conserved recognition “eat me” signaling molecule phosphatidylserine present on target cells (2, 11). The recognition of dead cells removed by efferocytosis is distinct from the recognition of foreign materials by innate and adaptive immune systems (2, 21). Although clearing of dead cells, cell fragments, and other effete material through phagocytosis is essential for resolution of inflammation (1, 3, 11), the mechanistic links between MΦ phenotype transition and activation of efferocytosis are unclear.

Here, utilizing the murine model of PMN-dependent ALI induced by LPS (22, 23), we observed (i) the temporal transition of AMΦs to an antiinflammatory phenotype coupled with generation of the cytokines IL-4 and TSG6, (ii) increases in AMΦs expressing antiinflammatory markers indicative of phenotype shift in AMΦs, and (iii) enhanced expression of the efferocytotic ligand Gas6 inducing the activation of efferocytosis machinery in AMΦs. These findings are consistent with the MΦ phenotype shift occurring in association with activation of efferocytosis. We focused on Gas6, a central component of efferocytosis on the MΦ surface. The binding of Gas6 to TAM receptors expressed on the MΦ membrane bridges with the phosphatidylserine signal expressed on apoptotic PMN membrane (6, 8). We identified the requisite role of STAT6-mediated expression of Gas6 in response to IL-4 and TSG6 signaling as a central mechanism of activation of efferocytosis in AMΦs.

MΦs play a key role in resolving inflammation through their ability to engulf bacteria and dead cells (1, 21). MΦs are also

plastic in that they adopt a proinflammatory or antiinflammatory property (9, 10). This shift depends on the microenvironment (or niche) and release of specific cytokines such as IL-4 and TSG6 (3, 14), which we used in the present study to address the role of MΦ shift in efferocytosis. Studies showed that efferocytosis can itself lead to MΦ antiinflammatory phenotype transition, as evidenced by the release of antiinflammatory and proresolving mediators IL-10 and TGF- $\beta$  (2, 24–26). In this context, IL-13 (a MΦ antiinflammatory transition cytokine) generated by regulatory T cells stimulated IL-10 production in MΦs, which in turn induced efferocytosis and resolution of inflammation. Studies also showed that engagement of the efferocytosis machinery is only one feature of a broader antiinflammatory phenotype shift in MΦs (11, 27).

The transcription factor STAT6 induces MΦ phenotype transition in response to IL-4 and IL-13 signaling (28, 29). IL-4 binding to IL-4 receptor- $\alpha$  phosphorylates STAT6, resulting in its dimerization and translocation to the nucleus and triggering expression of target genes (12, 29). Activation of STAT6 was shown to repress transcription of inflammatory genes and resolve inflammation (13, 30). This was evident in studies in which IL-4 administration via i.p. injection accelerated resolution of lung inflammation in a STAT6-dependent manner (3). In the present study, we found that STAT6 binding to the Gas6 promoter induced the expression of Gas6 in AMΦs. Release of cytokines is a likely cause of STAT6 activation (28, 31). We observed the generation of IL-4 and TSG6 in BALF of LPS-challenged mice, both TSG6- and IL-4-induced STAT6 activation, and transcription of Gas6. Suppression of STAT6 abrogated both Gas6 expression and efferocytosis of dead PMNs, demonstrating that the antiinflammatory MΦ phenotype inducing STAT6 plays a vital role in mediating efferocytosis through Gas6 expression.



**Fig. 4.** Adoptive transfer of TSG6-primed MΦs via i.t. instillation suppresses LPS-induced lung injury. (A) Age- and weight-matched mice were challenged with a lethal dose of LPS (20 mg/kg i.p.) and i.t.-instilled with indicated number of TSG6- or IL-4-stimulated or unstimulated BMMΦs. Mortality was monitored for 72 h. The mice instilled with TSG6- or IL-4-stimulated BMMΦs (TSG6 MΦs and IL-4 MΦs) showed markedly increased survival compared with the unstimulated BMMΦs (UT-MΦs) group. \* $P < 0.05$  vs. UT MΦs group;  $n = 10$  per group. (B) Lung vascular permeability (uptake of EBA) was assessed in LPS-challenged (10 mg/kg i.p.) mice concurrently instilled with UT or TSG6- or IL-4-stimulated MΦs. The mice instilled with TSG6- or IL-4-treated MΦs showed significantly reduced lung vascular permeability;  $n = 6$  mice per group. \* $P < 0.05$  vs. UT MΦs group. (C) Ly6G population was measured by flow cytometric analysis in BALF of mice i.t.-instilled with either UT- or TSG6-treated BMMΦs, and challenged with i.p. LPS (10 mg/kg) for 24 h. (D) BALF concentrations of proinflammatory (TNF- $\alpha$ , IL-6, IL-12, and IFN- $\gamma$ ) and antiinflammatory (IL-10 and IL-4) cytokines measured with ELISA. \* $P < 0.05$  vs. UT MΦs group.

We investigated whether efferocytosis in the endotoxemia model is essential for resolution of inflammatory lung injury. We observed that adoptive transfer of BMMΦs primed with STAT6-activating mediators in which Gas6 was induced effectively cleared apoptotic airspace PMNs, suppressed production of inflammatory cytokines and lung vascular leaking in endotoxemic mice, and significantly reduced mortality. The protective effects of phenotype-shifted BMMΦs required the expression of Gas6, indicating the necessity of priming the MΦs and intact efferocytotic machinery for resolution of inflammation. We cannot, however, rule out the possibility that the adoptively transferred BMMΦs may have in some way affected resident AMΦs, which could account for the protection. Nevertheless, our findings raise the intriguing possibility of MΦ-based therapy through instillation of the shifted MΦs in restoring homeostasis in ALI patients through activation of efferocytosis of PMNs in the airspace.

## Materials and Methods

The detailed methods are discussed in the *SI Appendix*.

**Mouse Experiments.** Male and female C57BL/6 mice aged 8–10 wk (Jackson Laboratory) were used for experiments. Mice were bred and maintained under specific pathogen-free conditions at the University of Illinois at Chicago animal facility, and all protocols were approved by the Animal Care Committee administered through the Office of Animal Care and Institutional Biosafety.

**Collection of BALF and Cytokine ELISAs.** Bronchoalveolar lavage fluid (BALF) was collected from mice. The supernatant was used for the measurement of cytokines with an ELISA kit (eBiosciences), and the pelleted cells were stained with Ly6G (127628; Biolegend) and subjected to flow cytometry analysis.

**Isolation of AMΦs.** AMΦs were isolated from BALF as described previously (32).

**Bone Marrow-Derived Macrophages (BMMΦ).** Bone marrow cells were isolated from mouse femur and tibial cavities and incubated at  $\sim 2 \times 10^6$  cells/mL in DMEM supplemented with 10% (vol/vol) FBS, 1% (vol/vol) streptomycin/penicillin, and 10% (vol/vol) L929-conditioned media for 6 d. Cells were used for experiments on day 6 of culture. MΦs were labeled with CellTracker CMTPX red dye for 15 min (C34552, Thermo Fisher Scientific) and used for experiments.

**Isolation of PMNs and Induction of Apoptosis.** PMNs were isolated from bone marrow cells by density-gradient centrifugation using Histopaque as described previously (33). Isolated PMNs were exposed to UV irradiation (254 nm, UVS-26, 6-W bulb, 0.02 J/s/cm<sup>2</sup>) for 15 min to induce apoptosis and then incubated at 37 °C for 4 h in an incubator containing 5% CO<sub>2</sub> (15).

**Adoptive Transfer of BMMΦs in Mice.** BMMΦs were stimulated with 400 ng/mL rTSG6 (2104-TS-050; R&D Systems) or 100 ng/mL rIL-4 (214-14; Peprotech) for 6 h in culture conditions in vitro, and adoptively transferred by noninvasive i.t. instillation into mice. For the induction of lung injury, mice received a single dose of LPS i.p. (10 mg/kg body weight; *Escherichia coli* strain 0111: B4; Sigma-Aldrich).

**Immunostaining.** BMMΦs were grown on coverslips and stimulated with TSG6 (400 ng/mL) for 6 h. After 4 h of TSG6 treatment, MΦs were overlaid with apoptotic PMNs (1:10 ratio) labeled with CellTracker CMTPX red dye for 15 min for 2 h. After vigorous washing with PBS and fixation with acetone-methanol, MΦs engulfing apoptotic PMNs were analyzed by confocal microscopy (LSM 880; Zeiss).

**In Vivo Imaging of Lungs.** The surgical methods for gaining access to lungs were based on Looney et al. (34). BMMΦs stimulated with rTSG6 (TSG6 MΦs) or rIL-4 (IL-4 MΦs) were labeled with CellTracker CMTPX red dye for 15 min as described previously and adoptively transferred into mice. An Ultima resonant-scanning 2-photon microscope (Bruker) with an Olympus XLUMPlanFL N 20 $\times$  (NA 1.00) was used to collect dual-color images (emission filter: 460/50 nm for Brilliant Violet 421, 525/50 nm for Alexa 488, and 595/50 for CMTPX) with 820 nm excitation.

**Promoter Analysis and ChIP Assay.** Consensus binding sites for transcription factor STAT6 in the 5'-regulatory region of the Gas6 gene were analyzed with the Eukaryotic Promoter Database (SIB). ChIP assays were done as described previously (35).

**RNA Extraction and qRT-PCR.** Total RNA isolated from BMMΦs were reverse-transcribed for the synthesis of cDNA according to manufacturer's instructions (K1612; Thermo Fisher Scientific). The cDNA obtained was mixed with SYBR Green PCR Master Mix (Applied Biosystems), and gene-specific primers were used for PCR. An ABI Prism 7000 system was used for quantitative PCR analysis.

**Immunoblotting.** MΦs were lysed in RIPA buffer containing protease and phosphatase inhibitor mixture. Cell lysates were centrifuged at 20,000 × g for 15 min at 4 °C, and cleared supernatant was used for immunoblotting.

**Transfection with siRNA.** BMMΦs were transfected with control (sc-37007) or two independent Gas6 siRNAs (sc-35451; Santa Cruz Biotechnology; s66469; Life Technologies) for 36 h using Lipofectamine 3000 reagent (L3000015; Thermo Fisher Scientific) in 2 mL growth medium according to the manufacturer's instructions. For the assessment of transfection efficiency, immunoblotting was done as described earlier.

**Measurement of Lung Vascular Permeability.** Lung vascular permeability was measured as described previously (14).

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