

Stat2 loss leads to cytokine-independent, cell-mediated lethality in LPS-induced sepsis

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Deregulated Toll-like receptor (TLR)-triggered inflammatory responses that depend on NF- κ B are detrimental to the host via excessive production of proinflammatory cytokines, including TNF- α . Stat2 is a critical component of type I IFN signaling, but it is not thought to participate in TLR signaling. Our study shows that LPS-induced lethality in *Stat2*^{-/-} mice is accelerated as a result of increased cellular transmigration. Blocking intercellular adhesion molecule-1 prevents cellular egress and confers survival of *Stat2*^{-/-} mice. The main determinant of cellular egress in *Stat2*^{-/-} mice is the genotype of the host and not the circulating leukocyte. Surprisingly, lethality and cellular egress observed on *Stat2*^{-/-} mice are not associated with excessive increases in classical sepsis cytokines or chemokines. Indeed, in the absence of Stat2, cytokine production in response to multiple TLR agonists is reduced. We find that Stat2 loss leads to reduced expression of NF- κ B target genes by affecting nuclear translocation of NF- κ B. Thus, our data reveal the existence of a different mechanism of LPS-induced lethality that is independent of NF- κ B triggered cytokine storm but dependent on cellular egress.

Sepsis is a complex clinical disorder that may culminate in multiorgan failure and death (1, 2). Many infectious agents induce a severe inflammatory response by triggering Toll-like receptor (TLR) signaling pathways. In Gram-negative sepsis, bacterial LPS binds TLR4 and relays signals through adapter molecules and transcription factors, including the NF- κ B family, to mount an inflammatory response (3). This includes production of cytokines (e.g., TNF- α , IL-6, and IFN- γ) and chemokines [e.g., regulated on activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1)] that recruit innate and adaptive components of inflammation to sites of injury.

Infusion of LPS into rodent models recapitulates cytokine surges that correlate with the pathophysiology of sepsis (4, 5). Mice deficient in genes encoding components of the TLR signaling pathway [*Tlr4* (6), *Md-2* (7), *Cd14* (7), and *MyD88* (8)] survive doses of LPS that are lethal to WT animals. In all these models, survival is associated with reduced cytokine production, appearing to confirm that a sepsis-induced cytokine storm is necessary and sufficient for inflammation associated mortality. This pathway and consequent cytokine production have been targeted for pharmacological intervention, but drugs that inhibit cytokines have limited clinical value (9).

Production of inflammatory mediators, e.g. TNF- α , is amplified by release of “priming cytokines” chiefly IFN- β , a type I IFN (10). The significance of type I IFN is demonstrated by survival of animals deficient in components of the type I IFN signaling cascade (Tyk2 and Ifn- β) when exposed to lethal doses of LPS (11). Stat2 mediates canonical signaling through the type I IFN receptor. IFN- α/β binds its receptor (Ifnar), which activates the intracellular members of the Janus-activated kinase (JAK) family of tyrosine kinases. Jak1 and Tyk2 phosphorylate and activate Stat1 and Stat2, which form the heterotrimeric IFN-stimulated gene factor 3 (ISGF3) transcriptional complex with IRF9 (12).

Other signaling complexes (phosphorylated Stat1 homodimers and Stat-independent pathways) have been identified (13). Stat2 is essential to nuclear-cytoplasmic shuttling of the ISGF3 complex and necessary for expression of type I ISGs (14). Stat2 is protective in viral infection (15–17), but its role in other human diseases has received little attention. However, reduced levels of Stat2 have been reported in alcoholic hepatitis (18) and inflammatory bowel disease (19).

The present study tests the hypothesis that loss of Stat2 protects against LPS-induced sepsis, as reported in mice deficient in other components of type I IFN signaling. Unexpectedly, we find that loss of Stat2 leads to accelerated LPS-induced mortality resulting from increased cellular extravasation and inflammation with enhanced peritonitis and hepatitis. Contrary to the established view, we find that accelerated death occurs without surges in proinflammatory cytokines. These data reveal a regulatory role for Stat2 in responses to TLR stimulation and introduce an alternative mechanism of LPS-induced death that occurs in the absence of excessive cytokine production.

Results

Effect of LPS on Survival in Mice Deficient in Stat2 and Other Components of Type I IFN Signaling Cascade. To examine effects of Stat2 loss on the inflammatory response, mice were treated with a lethal dose of i.p. LPS (70 mg/kg). *Stat2*^{-/-} mice showed increased mortality compared with WT (Fig. 1A). Conversely, mice deficient in other components of type I IFN signaling, *Ifnar1* ($n = 6$; $P = 0.0084$) and *Tyk2* ($n = 5$, $P = 0.049$), were protected against LPS-induced lethality. *Stat1*^{-/-} mice also showed reduced mortality, but this did not reach statistical significance ($n = 5$; $P = 0.07$).

Cardiovascular collapse is the hallmark of septic shock (20) with high mortality (21). No physiological changes that would account for the accelerated death in *Stat2*^{-/-} mice were detected. No significant differences in the rate of myocardial depression (reduction in heart rate, stroke volume, peak velocity, aortic velocity time integral) or core temperature at baseline or after 18 h LPS stimulation were observed (Fig. S1).

We examined tissues from organs from *Stat2*^{-/-} and WT animals at different times following LPS challenge. Loss of Stat2 led to increased neutrophilic hepatitis with microabscess formation

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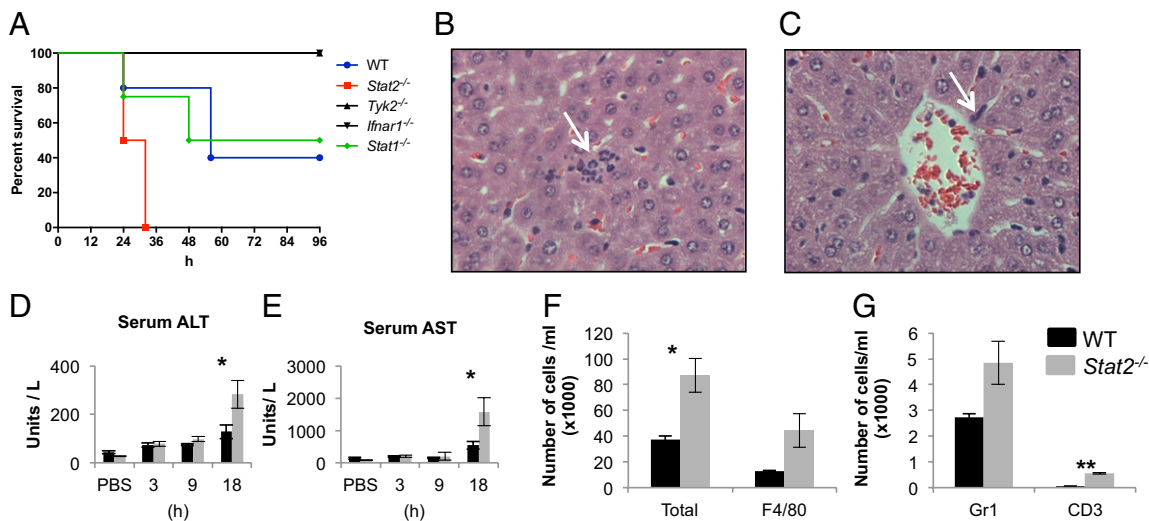


Fig. 1. LPS-induced mortality in mice lacking IFN signaling mediators and sepsis-induced tissue inflammation in *Stat2*^{-/-} mice. (A) Survival curves in WT, *Tyk2*^{-/-}, *Ifnar1*^{-/-}, *Stat1*^{-/-}, and *Stat2*^{-/-} mice following LPS challenge at a dose of 70 mg/kg. (B and C) Immune cells rapidly accumulate in the extravascular space of livers in *Stat2*^{-/-} mice compared with WT following LPS challenge. H&E-stained sections of *Stat2*^{-/-} mouse liver after 9 h LPS challenge (magnification of 600×) show signs of inflammation, microabscesses in liver parenchyma, and leukocytes within hepatic sinusoids (white arrows). (D and E) Serum concentrations (mean ± SEM) of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) from three to five animals following i.p. injection with PBS solution or LPS at time points indicated (**P* < 0.05). (F and G) Number of cells in peritoneal lavage fluid of mice after 6 h of LPS (20 mg/kg). Total number (**P* = 0.02), F4/80⁺, GR1⁺, and CD3⁺ (***P* < 0.001).

at 9 h and 18 h compared with WT mice (Fig. 1 *B* and *C*). Serum alanine aminotransferase and aspartate aminotransferase (markers of liver injury) were significantly higher in *Stat2*^{-/-} animals than WT at 18 h (*P* = 0.026; Fig. 1 *D* and *E*). We examined the possibility that loss of *Stat2* may directly affect hepatocytes but did not find any increases in hepatocyte proliferation [minichromosome maintenance 4 (MCM4) positivity] or apoptosis (cleaved caspase 3 positivity), after LPS, suggesting that the intrahepatic effects of *Stat2* loss are indirect (Fig. *S2A* and *B*). Instead, we observed a reduction in hepatocyte apoptosis in *Stat2*^{-/-} animals following LPS that probably reflects abrogated IFN signaling.

We found no other differences between WT and *Stat2*^{-/-} mice to account for animal death in brain, heart, lung, thymus, kidney, adrenal gland, pancreas, or skin at any time (*t* = 3, 9, and 18 h) during LPS or PBS challenge (Fig. *S2C–J*). Examination of the lungs revealed a similar increase in neutrophil infiltrates in WT and *Stat2*^{-/-} mice following LPS (Fig. *S2K*), with no other signs of lung injury. At 18 h after LPS, more apoptosis was noted in the colonic epithelium of *Stat2*^{-/-} mice, but this was not sufficient to account for death and culture of blood samples from mice showed no significant growth after aerobic and anaerobic culture (*n* = 5).

Increased peritonitis was detected in *Stat2*^{-/-} mice after LPS administration. There were significantly greater numbers of leukocytes in peritoneal lavage fluid from *Stat2*^{-/-} mice compared with WT (*P* = 0.02; 20 mg/kg; 6 h; Fig. 1*F*) that comprised mainly F4/80 macrophages (44,444 cells/mL vs. 12,963 cells/mL; Fig. 1*F*), Gr1⁺ granulocytes (5,016 cells/mL vs. 2,721 cells/mL; Fig. 1*G*), and CD3⁺ T cells (569 vs. 51 cells/mL; Fig. 1*G*), suggesting that *Stat2* loss is associated with cellular egress in peritoneum and liver.

Loss of *Stat2* Reduces LPS-Induced Production of Inflammatory Cytokines and Chemokines. Current models of sepsis associate adverse outcomes with elevated production of inflammatory cytokines, so we assessed changes in the serum levels of proinflammatory cytokines. Contrary to expectation, reduced levels of TNF-α (2 h; *P* = 0.0286), IL-6 (4 h; *P* = 0.0286), IL-12p70 (2 h; *P* = 0.0286), MCP-1 (4 h and 7 h; *P* = 0.0286), and IL-10 (2 h; *P* = 0.0294) were detected in *Stat2*^{-/-} mice compared with WT (Fig. 2 *A–E*). No differences in IFN-γ were found between the two strains (Fig. 2*F*). Increased mortality in *Stat2*^{-/-} mice was not a result of

susceptibility to TNF-α because WT and *Stat2*^{-/-} mice were similarly resistant to i.p. administration of TNF-α (2 μg per mouse). Even when high-dose TNF-α (20 mg per mouse) was used, *Stat2*^{-/-} mice showed no signs of sickness at any point. Conversely, WT mice showed piloerection and reduced movement at 10 h, indicating that *Stat2*^{-/-} mice are resistant to TNF-α.

To determine whether increased peritonitis in *Stat2*^{-/-} animals following LPS challenge was associated with overexpression of chemokine(s), peritoneal lavage fluid of *Stat2*^{-/-} and WT mice was evaluated for 32 chemokines and cytokines (Table *S1*). Of these, LPS stimulation led to an increase in the expression of 12 chemokines in WT mice (Fig. *S3A* and *B*); nine of these were reduced in *Stat2*^{-/-} mice. These results confirm that loss of *Stat2* was associated with down-regulation of inflammatory cytokines and chemokines.

Loss of *Stat2* in Endothelial/Stromal Compartment Modulates Cellular Migration.

Given that *Stat2* deficiency reduced LPS-induced cytokine production and increased leukocyte egress, we hypothesized that *Stat2* may be important in cell trafficking. Leukocyte–vessel wall interactions in *Stat2*^{-/-} mice were investigated by intravital microscopy using the cremaster muscle model (22). Locally injected LPS into WT mice induced a significant increase in leukocyte adhesion (*P* < 0.05) and transmigration (*P* < 0.01)

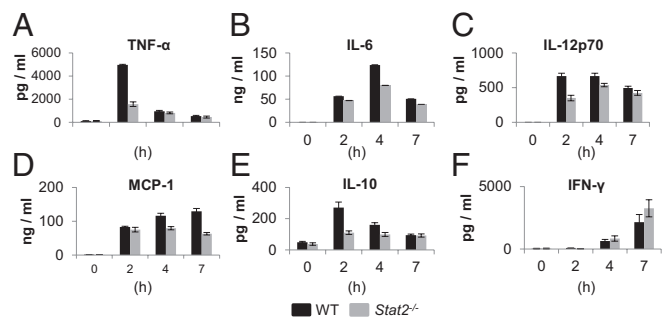


Fig. 2. Effects of LPS-induced sepsis on cytokine production in *Stat2*^{-/-} mice. Cytokine levels measured by ELISA in the serum of WT and *Stat2*^{-/-} mice following i.p. LPS challenge (50 mg/kg).

compared with saline solution-injected mice (Fig. 3A). *Stat2*^{-/-} mice showed an adherence response comparable to WT mice, but had a significantly elevated transmigration response ($P < 0.01$; Fig. 3A).

To determine in which cellular compartment (leukocyte vs. stroma/endothelium) *Stat2* affects leukocyte egress, equal number of bone marrow cells from WT and *Stat2*^{-/-} mice were labeled with Calcein-AM (AM-acetoxymethyl ester) or PKH-26 (no differential effect of dye labeling was detected) and injected i.v. into WT or *Stat2*^{-/-} animals before i.p. LPS injection. Peritoneal lavage fluid was collected 18 h later and analyzed by FACS to determine cell numbers, numbers of neutrophils, and number of labeled transferred cells. The main determinant of peritonitis was the genotype of the host animal, i.e., the stromal rather than the circulating leukocytes (Fig. 3B and C). Analysis of inflamed tissue in the intravital microscopy model demonstrated significantly greater endothelial expression of ICAM-1 protein in *Stat2*^{-/-} mice compared with WT following LPS stimulation ($P < 0.05$; Fig. 3G). There were no differences in platelet endothelial cell adhesion molecule-1 (PECAM-1) expression.

In vivo and in vitro migration assays showed equal responses of WT and *Stat2*^{-/-} immortalized macrophages to a range of chemoattractants, further confirming that the migrating cell is unaffected by loss of *Stat2* (Fig. S3C and D).

Inhibition of Endothelial Cell Adhesion Reverses Inflammatory Extravasation and Prevents Mortality. To test the hypothesis that increased cellular egress caused the death of LPS-treated *Stat2*^{-/-} mice, animals received a single dose of anti-ICAM-1 blocking antibody [a global inhibitor of cellular egress (23)] 18 h before i.p. LPS injection. Blockade of cellular egress was confirmed by a reduction in total cell numbers in the peritoneal lavage (Fig. 3D). Pretreatment of mice with anti-ICAM-1 blocking antibody prolonged the survival of LPS-challenged mice (Fig. 3E and F).

Stat2 Is Needed for TLR-Mediated Cytokine Production. Paradoxically, our data describe reduced inflammatory cytokine production in a model of accelerated LPS-induced death. The response to LPS is mediated, at least in part, by an amplification loop involving IFN- β production (24). We excluded the possibility that the increase in mortality was attributable to enhanced type I IFN-induced responses as reported in macrophages from *Stat2*^{-/-} mice (25). We did not observe mortality or signs of distress in WT and *Stat2*^{-/-} mice injected with low- or high-dose IFN- β during 72 h. In addition, we confirmed that *Stat2*^{-/-} mice did not respond to type I IFN. Hepatocyte expression of key IFN-stimulated genes (*Ifit1*, *Ifit2*, and *Rsd2*) was impaired in *Stat2*^{-/-} mice injected with IFN- β (Fig. S4).

To confirm that *Stat2* is required for inflammatory cytokine production, immortalized WT and *Stat2*^{-/-} bone-marrow derived macrophage (BMDM) cell lines were established and tested for LPS responses. Expression of *Stat2*, TLR4, and macrophage markers (CD80 and F4/80) were confirmed (Fig. S5A and B), and we found no differences in TLR4 expression between WT and *Stat2*^{-/-} splenocytes (Fig. S5C).

Immortalized WT BMDMs produced large amounts of TNF- α and RANTES after LPS stimulation, whereas immortalized *Stat2*^{-/-} BMDM produced significantly less over 12 h ($P < 0.0001$; Fig. 4A and B). LPS stimulation for shorter periods did not reveal any early peaks in production of nitrite or TNF- α (Fig. S6A–C).

We next examined the effects of IL-1 and other TLR agonists: poly I:C, flagellin, and imiquimod. IL-1 induced TNF- α production in immortalized WT BMDMs, but not in *Stat2*^{-/-} BMDMs ($P = 0.0046$; Fig. S6D). Treatment with poly I:C led to the production of TNF- α and RANTES in immortalized WT BMDMs, with only minimal production in *Stat2*^{-/-} cells ($P < 0.0001$ and $P = 0.0046$, respectively; Fig. 4C and D). Neither TNF- α nor RANTES was produced by either cell type in response to flagellin or imiquimod.

The transcription of a set of genes triggered by TLR stimulation was analyzed by quantitative RT-PCR. In response to LPS

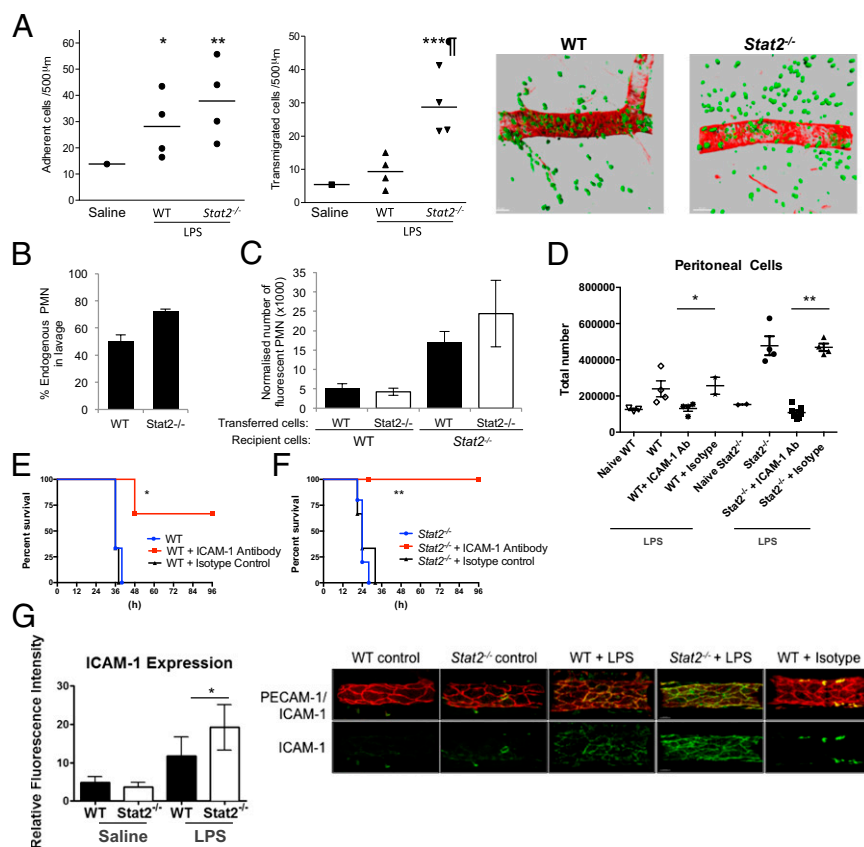


Fig. 3. LPS-induced death in *Stat2*^{-/-} mice is mediated by cellular extravasation. (A) WT or *Stat2*^{-/-} mice received intrascrotal injections of saline solution or LPS (300 ng). Leukocyte responses of adhesion (Left) and transmigration (Right) were quantified 4 h later in cremaster muscle by intravital microscopy (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, saline solution vs. LPS; † $P < 0.001$, WT vs. *Stat2*^{-/-} mice). Tissue immunofluorescence microscopy for markers of blood vessels (α -smooth muscle actin; red) and neutrophils [myeloid related protein-14 (MRP-14); green]. A representative example of LPS-stimulated WT (Left) and *Stat2*^{-/-} (Right) tissues. WT and *Stat2*^{-/-} recipient animals received an adoptive transfer of singly fluorescently labeled primary WT and *Stat2*^{-/-} bone marrow cells in equal amounts before i.p. administration of LPS (1 mg/kg). Peritoneal lavage was performed 18 h later, and the number and phenotype of WT and *Stat2*^{-/-} immune cells within the lavage was quantified by FACS analysis. (B) Percentage of endogenous polymorphonuclear neutrophils (PMN). (C) Total number of WT or *Stat2*^{-/-} fluorescent polymorphonuclear neutrophils retrieved from peritoneal cavities of WT or *Stat2*^{-/-} mice. (D) WT and *Stat2*^{-/-} mice received a single dose of ICAM-1 blocking antibody or isotype antibody control 18 h before i.p. LPS challenge (20 mg/kg). ICAM-1 inhibition reduced cellular egress into the peritoneum (* $P = 0.026$ and ** $P = 0.0001$) and (E and F) survival of mice (* $P = 0.02$ and ** $P = 0.007$). (G) WT and *Stat2*^{-/-} mice ($n = 6$; $n \sim 6$ vessels per animal) received LPS intrascrotally (300 ng) for 4 h and ICAM-1 expression was quantified (* $P < 0.05$). Examples of a maximum intensity projection depicting PECAM1 and ICAM-1 expression (and ICAM-1 isotype control) on WT and *Stat2*^{-/-} venules are also shown. (Scale bar: 30 μ m.)

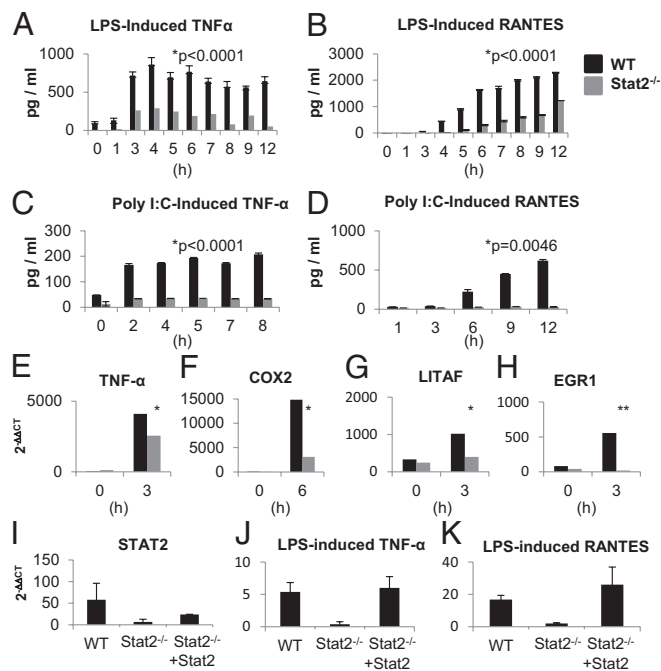


Fig. 4. TLR triggered cytokine production is diminished in the absence of Stat2. WT and Stat2^{-/-} immortalized BMDMs were stimulated with (A and B) LPS (1 μg/mL) or (C and D) Poly I:C (25 μg/mL). Secretion of TNF-α and RANTES was measured by ELISA [mean (±SEM) of three replicates; representative of at least two experiments]. (E–H) Quantitative PCR analysis of LPS-responsive genes is shown as mean fold change relative to unstimulated cells following normalization to β-actin (mean ± SEM of two to four experiments). Quantitative PCR analysis (I) of Stat2, (J) TNF-α, and (K) RANTES in Stat2^{-/-} and Stat2^{-/-} reconstituted with Stat2 immortalized BMDMs.

treatment, a reduction in mRNA expression of *TNF-α*, cyclooxygenase2 (*COX2*), LPS-induced *TNF-α* factor (*LITAF*), and early growth response 1 (*EGR1*) was detected in immortalized Stat2^{-/-} BMDM compared with WT BMDMs (Fig. 4 E–H). *MxA* (an IFN-induced gene) was included as a positive control of IFN response (Fig. S6E). In addition, TNF-related apoptosis-inducing ligand (*TRAIL*) mRNA levels were measured following stimulation with poly I:C and LPS. Regardless of the stimulus used, normal gene induction in immortalized WT cells was impaired in Stat2^{-/-} cells ($P = 0.0021$ and $P = 0.03$; Fig. S6 F and G).

To confirm that this phenotype was a result of deficiency in Stat2, we transiently restored Stat2 expression in immortalized Stat2^{-/-} BMDMs (Fig. 4I). Rescue of Stat2 was effectively demonstrated by *MxA* mRNA expression in response to IFN-α stimulation (Fig. S6H). Stat2 reconstituted immortalized BMDM showed an increase in transcription of TNF-α and RANTES mRNAs following 6 h LPS stimulation (Fig. 4 J and K). These results demonstrate that loss of Stat2 impairs cytokine production at the transcriptional level.

Taken together, these data show that, although Stat2^{-/-} animals have increased mortality following LPS injection, TLR-mediated cytokine production in the absence of Stat2 is significantly impaired, and this is a global defect seen with several TLR agonists.

Effects of Stat2 Loss on Cytokine Production Are Independent of Type I IFN Signaling. To date, the only known role for Stat2 is as a transducer of IFN signaling. It is possible that the reduction in cytokine production in Stat2^{-/-} cells may have been a result of loss of a type I IFN-mediated amplification loop. However, this seems unlikely given the distinction between in vivo response to LPS seen in Stat2^{-/-} vs. Stat1^{-/-} and *Ifnar1*^{-/-} mice. To confirm that the phenotypes seen here were independent of type I IFN, primary BMDMs from Stat1^{-/-} and *Ifnar1*^{-/-} mice were stimulated

with LPS in vitro. No reduction in LPS-induced cytokine production was observed (Fig. S7 A and B).

To evaluate a role for type I IFNs in the Stat2-mediated LPS response, we blocked type I IFN signaling using a neutralizing antibody against the *Ifnar1* component of the type I IFN receptor (26). This led to impaired expression of the IFN target gene *MxA* (Fig. S8A). However, LPS induced TNF-α and RANTES production remained unaffected even when IFN signaling was blocked (Fig. S8 B and C).

Stat2 Is Needed for Timely Translocation of NF-κBp65 into the Nucleus. The widespread reduction in TLR response in the absence of Stat2 suggests that MyD88-dependent and MyD88-independent signaling pathways are affected. The NF-κB family of transcription factors represent a convergence point in all the signaling pathways noted to be defective in Stat2^{-/-} cells (27).

To determine the effect of Stat2 deletion on NF-κB-driven responses, we used an NF-κB luciferase reporter system. Following 6 h stimulation with TNF-α, immortalized Stat2^{-/-} BMDM showed a marked reduction in TNF-α-driven luciferase activity. This activity was recovered when Stat2 expression was restored (Fig. 5A). Similarly, immortalized Stat2^{-/-} BMDM reconstituted with a mutated Stat2 that lacked the classical Tyr-689 phosphorylation site (Y689F) showed restored TNF-α response. LPS-stimulation of WT cells in the presence of the anti-*Ifnar1* antibody and of *Ifnar1*^{-/-} and *Trlr4*^{-/-} splenocytes did not lead to phosphorylation of Stat2 Y689 (Fig. S8 D–F). These results indicate that defective cytokine production in the absence

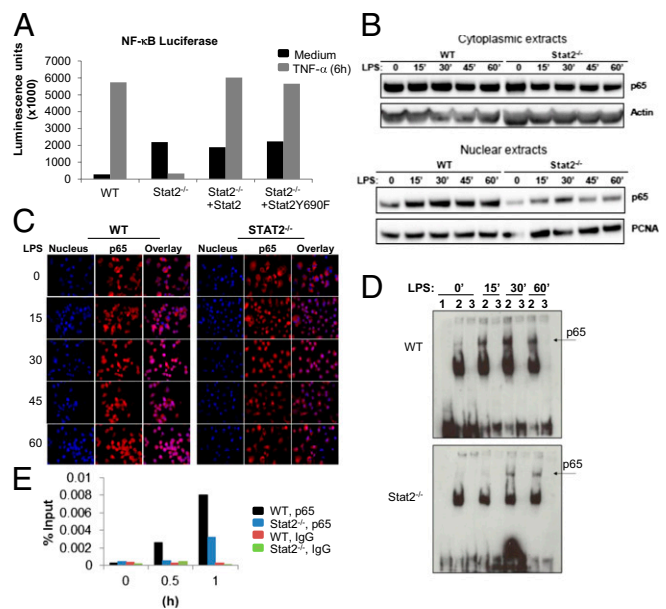


Fig. 5. Stat2 deficiency impairs nuclear localization of NF-κB p65. (A) Measurement of NF-κB luciferase reporter activity in WT and Stat2^{-/-} immortalized BMDMs reconstituted with Stat2 or mutant Stat2 (Y689F) and stimulated with TNF-α (10 ng/mL). Data are shown as mean luminescence units (magnification of 1,000×) from two experiments. (B) Immunoblot analysis of NF-κB p65 using nuclear and cytoplasmic extracts from immortalized BMDMs stimulated with LPS (1 μg/mL). (C) LPS induced nuclear translocation of NF-κB p65 (red) assessed by immunofluorescence microscopy. Nucleus is shown in blue. Images are representative of three independent experiments. (D) EMSA of nuclear extracts prepared from LPS-stimulated immortalized BMDMs. Lane 1 is the free probe, lane 2 the labeled probe, and lane 3 the labeled probe competed with 200× molar excess of unlabeled probe. Images shown are representative experiments using at least three independent extracts. (E) Quantitative ChIP assay performed at the indicated time points with antibody against p65 or irrelevant IgG as indicated. Specific quantitative PCR primers were used to amplify the proximal TNF-α promoter region.

of Stat2 is caused, at least in part, by impaired NF- κ B-mediated transcription, and Y689 phosphorylation of Stat2 is not involved.

Given the defective NF- κ B transcriptional activity in the absence of Stat2, mediators of LPS signaling upstream of the NF- κ B activation complex were evaluated. The protein level of MyD88 was comparable between immortalized WT and *Stat2*^{-/-} BMDMs following stimulation with LPS, and TNF receptor-associated factor 6 (TRAF6)-dependent reduction in IRAK1 did not require Stat2 (Fig. S9A). A key step in NF- κ B activation is its translocation to the nucleus, dependent on the degradation of I κ B α in the cytoplasm. Cytoplasmic levels of phosphorylated I κ B α increased in WT, but not in *Stat2*^{-/-} cells (Fig. S9B) following LPS stimulation; however, there was no difference in the abundance of total I κ B α protein or I κ B kinase B (IKKB) that is largely responsible for I κ B α phosphorylation (28). The basal increased expression of the I κ B kinase A (IKKA) in immortalized *Stat2*^{-/-} BMDMs was higher compared with immortalized WT BMDMs. These data suggest that loss of Stat2 is associated with aberrations in inducing or maintaining phosphorylated I κ B α .

To determine the effect of this defect on NF- κ B function, we assessed its localization to the nucleus and its binding to chromatin. A reduction in the amount of LPS-induced NF- κ Bp65 in the nucleus was confirmed by immunoblotting of nuclear and cytoplasmic extracts from immortalized BMDMs (Fig. 5B). Before stimulation, p65 was found predominantly in the cytoplasm; however, after 15 min LPS exposure, much less p65 was detected in the nuclei of *Stat2*^{-/-} than in WT BMDMs. Nuclear accumulation of p65 in immortalized *Stat2*^{-/-} BMDM was not increased later. Immunofluorescence microscopy confirmed that, in the absence of Stat2, p65 is not maintained in the nucleus (Fig. 5C). In immortalized WT BMDM, p65 persisted in the nucleus at 45 min and 60 min, but this was not the case in *Stat2*^{-/-} BMDMs. EMSA and ChIP analyses confirmed these results, as they also showed a reduction in protein complexes binding to an NF- κ B target sequence (Fig. 5D and E) in immortalized *Stat2*^{-/-} BMDM. These data indicate that Stat2 is needed for the timely nuclear accumulation of NF- κ Bp65.

Discussion

Here we show that Stat2 is a key regulator of LPS-induced sepsis, as loss of Stat2 leads to increased mortality caused by cellular extravasation, but paradoxically, without the expected surge in inflammatory cytokines classically associated with septic death. Our study reports the existence of an alternative mechanism of LPS-induced sepsis independent of an excessive inflammatory response that is limited by Stat2.

The pathophysiology of sepsis is poorly understood. Although cytokine surges accompany the septic phenotype, there is poor correlation between cytokine levels and clinical outcome. The *Stat2*^{-/-} mouse model shows accelerated mortality and a reduction in cytokine production following a single dose of LPS. A similar response was seen in early studies of LPS tolerance; monocyte hyporesponsiveness is accompanied by increased lethality in mice pretreated and rechallenged with sublethal doses of *Salmonella abortus-equi* LPS (29).

In this i.p. toxin model (30, 31), death in *Stat2*^{-/-} mice is accompanied by increased leukocyte migration; the most prominent pathological findings are hepatitis (histological and biochemical) and peritonitis. Significant bacteremia following LPS injection in *Stat2*^{-/-} mice was not detected; however, we have not excluded the possibility that bacterial fragments (including endotoxin) might be present in the blood to promote an immune response. Reduced levels of Stat2 have been reported in inflammatory liver (18) and bowel (19) diseases, indicating that the inverse association of Stat2 and cellular migration is likely to be a global effect. We show that transmigration is increased in *Stat2*^{-/-} mice and this is caused by a stromal defect rather than a leukocyte deficiency. It will be important to identify the role for Stat2 in the numerous cell types that constitute this microenvironment. Use of the anti-ICAM-1 blocking antibody as a global inhibitor of cellular egress (23) that reversed the mortality seen in *Stat2*^{-/-} mice

confirms our observation that, in the absence of a cytokine storm, cellular egress mediates death in this model of sepsis.

Decreased cytokine production in *Stat2*^{-/-} cells correlates with reduced NF- κ B promoter activity, and our results show that Stat2 regulates NF- κ B signaling. Consistent differences between WT and *Stat2*^{-/-} BMDMs lead us to conclude that this is an important functional difference. Stat2 was found to mediate release of p65 from I κ B inhibitors and promote nuclear translocation of activated p65, subsequent retention of the activated complex in the nucleus, and binding to chromatin. Some of these traits have been reported to occur in virus infection. Measles virus V protein prevents NF- κ B gene expression by retaining NF- κ B in the cytoplasm (32), and, because V protein has been shown to bind Stat2 and inhibit IFN signaling (17), we speculate that inhibition of Stat2 may explain the effect on NF- κ B-mediated gene expression. The precise nature of the role of Stat2 in NF- κ B activation remains to be determined. Phosphorylation of I κ B α is impaired in immortalized *Stat2*^{-/-} BMDMs, suggesting that this may be the site of action of Stat2.

Loss of Stat2 did not completely abrogate cytokine production. We observe that, in the absence of Stat2, p65 nuclear entry is only reduced, not inhibited. Furthermore, in the absence of Stat2, p65 is not maintained in the nucleus. Timely nuclear entry of p65 is critical to normal function of NF- κ B (33–35), however, release of inhibition from I κ B α and nuclear translocation is not sufficient for p65 target gene expression (36). Active retention of p65 in the nucleus and the availability of specific associated proteins are necessary for transcriptional activity (37), and it is plausible that Stat2 [known to interact with p300/CBP (38)] may interact with p65 to maintain it in the nucleus.

This study shows that the effects of Stat2 on NF- κ B-mediated cytokine production are independent of type I IFN signaling. The increase in TNF- α observed in *Ifnar1*^{-/-} BMDMs is likely a result of loss of suppressor of cytokine signaling-1 (SOCS1)-mediated inhibition of TLR signaling pathways (39). Similarly, *Tyk2*^{-/-} mice have defective type I IFN signaling and yet LPS-induced production of cytokines (including TNF- α) is also unaffected (11). We also show that Stat2 does not appear to require Tyr689 phosphorylation as the Y689F mutation restored NF- κ B activity. Interest in noncanonical Stat signaling has increased; an ISGF3 complex containing nonphosphorylated Stat2 was identified as a significant mediator of IFN- γ antiviral effects (40) and alternative Stat2 posttranslational modifications may be required for normal NF- κ B function.

In summary, our results show a role for Stat2 in determining outcome from LPS sepsis independent of excessive immune inflammatory response. Loss of Stat2 is associated with accelerated death and marked hepatitis and peritonitis. Increased inflammatory cell migration is a property of increased permissiveness to extravasation in the endothelial/stromal compartment. Paradoxically, a surge in inflammatory cytokines is not seen as a result of a disruption in the nuclear localization of NF- κ B p65 and activity at its target promoter. These unexpected findings identify a role for Stat2 as a key mediator in the acute inflammatory response that is independent of its known functions as a transducer of IFN signaling.

Methods

Mice. *Stat2*^{-/-} mice in 129SvJ background were a gift from Christian Schindler (Columbia University, New York, NY). 129SvJ mice were purchased from National Cancer Institute (Frederick, MD) and used as WT controls. *Stat1*^{-/-} mice were purchased from Taconic Farms. *Tyk2*^{-/-} mice were a gift of Kazuya Shimoda (Miyasaki University, Miyasaki, Japan). These mice were then backcrossed seven times to 129SvE. *Ifnar1*^{-/-} mice (41) were obtained from Brian Kelsall (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Mice were housed in a conventional facility in individually ventilated cages and used at 6 to 10 wk of age. All animal studies were performed in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and approved by the Animal Care and Use Committee at Temple University. The UK Home Office and Local Ethics Committee approved the work done at Queen Mary, University of London.

Intravital microscopy was used to directly monitor leukocyte responses within mouse cremasteric venules as previously detailed (26). To investigate the contribution of leukocyte and endothelial Stat2 in leukocyte transmigration, a cell-transfer technique-modified version of bone marrow transfer from published methods was used (42).

Age- and sex-matched mice were injected i.p. with 200 μ L of the indicated doses of toxins or agonists. Mice were monitored for survival for as long as 7 d. In some experiments, antibodies were injected i.v. 18 h before LPS challenge. In other experiments, mouse organs were harvested at different time points and formalin-fixed before paraffin embedding for histological assessment.

Mice were bled by cardiac puncture at the indicated times after i.p. LPS injection, and serum samples were kept at -80°C . Sera were analyzed for cytokine levels and nitrite production.

In other experiments, mice were euthanized 6 h following i.p. injection with LPS or PBS solution. Five milliliters of cold PBS solution was injected into the peritoneal cavity. Peritoneal lavage fluid was collected for complete analysis of chemokines/cytokines by Milliplex magnetic bead assay according to the manufacturer's instructions (Millipore).

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