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ORIGINAL ARTICLE

STAT1 plays a role in TLR signal transduction and inflammatory responses

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Activation of the Toll-like receptor (TLR) family of innate immune sensors stimulates multiple signal transduction pathways. Previous studies have suggested that TLR2, TLR4 and TLR9 induce serine phosphorylation of Signal Transducers and Activators of Transcription-1 (STAT1) at residue 727 (S727), although its role in TLR signaling was unclear. We report here that STAT1 rapidly undergoes phosphorylation following TLR4 challenge with lipopolysaccharide (LPS) in a model of LPS hypersensitivity *in vivo*. Importantly, genetic ablation of STAT1 protected against LPS-induced lethality suggesting that STAT1 may have a key role in TLR-induced inflammation. We have found that multiple TLRs induce Ser727 phosphorylation of STAT1, which is dependent on MyD88 and TRIF signaling, but independent of interferon (IFN) regulatory factor (IRF)-3, IRF7 and the IFN receptor complex, suggesting that activation is a direct TLR response rather than autocrine activation via IFN. Importantly, we found that STAT1 interacts with tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6), a key mediator of TLR signaling after TLR challenge and that following activation, STAT1 translocates to the nucleus. Critically, macrophages generated from mice in which the S727 residue was replaced with alanine (STAT1 S727A mice) display significantly reduced TNFα protein production, but not reduced interleukin-6 or RANTES protein in response to multiple TLR challenges, as compared with wild-type macrophages. These results clearly demonstrate cross-talk between the TLR and JAK/STAT signaling pathways with direct recruitment of STAT1 by TRAF6 and that the direct activation of STAT1 by TLR signaling suggests a crucial role for STAT1 in TLR-induced inflammation.
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Innate immune detection of pathogens is mediated by germ line-encoded pattern recognition receptors that recognize molecular patterns shared broadly by pathogens, known as pathogen-associated molecular patterns. Toll-like receptors (TLRs) are one family of pattern recognition receptors and are expressed widely across immune cells, acting as primary sentinels to detect pathogen challenge, in the form of pathogen-associated molecular patterns, and to initiate subsequent pro-inflammatory responses. Activation of TLRs involves an orchestrated signaling pathway, described below, leading to expression of cytokines that act through members of the extracellular hematopoietin receptor family, such as the interleukin (IL)-6 co-receptor gp130, which themselves amplify their actions through activation of the JAK/signal transducers and activators of transcription (STAT) signaling cascade.

Upon ligand-induced TLR dimerization, a well-characterized signal transduction pathway follows that involves one or more Toll/IL receptor domain-containing adapter molecules. There are five Toll/IL receptor domain-containing adaptors, among them MyD88. All TLRs, with the exception of TLR3, recruit MyD88 to the receptor complex, which subsequently recruits kinases including IL-1 receptor-associated kinase-1 (IRAK-1), IRAK-4 and tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6) to the complex. Activation of the multimeric signaling complex leads to activation of the prototypic inflammatory transcription factors, nuclear factor κB (NF-κB) and the antiviral interferon (IFN) regulatory factor (IRF)-3, which regulate the production of NF-κB-dependent pro-inflammatory cytokines (such as IL-6, TNFα, IL-1) and the IRF3-dependent IFN, IFNβ.[1,2]

TLR3 and TLR4 also use a MyD88-independent pathway, which is defined by the activation of IRF3, leading to the expression of IFNβ. TRIF is essential for MyD88-independent TLR3 and TLR4 signaling.[3,4] Although TRIF associates directly with TLR3, it associates indirectly with TLR4 via the bridging adapter Toll/IL-1R (TIR) domain-containing adapter-inducing IFN-β (TRIF)-related adapter molecule (TRAM),[5] which is critical for TLR4-mediated activation of IRF3.

TLR4 is the primary pathogen recognition receptor for LPS, a key component of Gram-negative bacteria, which is responsible for the often lethal systemic inflammatory response syndrome, septic or endotoxic shock.[6,7] Regulatory mechanisms for these TLR4-induced signaling cascades are still unclear, but we have recently shown that in the LPS-hypersensitive *gp130*$^{F/F}$ knock-in mouse strain, IL-6

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feeds back via STAT3 to modulate the magnitude and duration of TLR4-dependent responses via its interaction with the MyD88 signaling pathway, independently of type I IFN signaling.[8,9] Specifically, the *gp130*$^{F/F}$ mice are homozygous for a Y757F substitution in the IL-6 co-receptor gp130, which abolishes suppressor of cytokine signaling-3 binding to gp130 leading to IL-6-induced hyperactivation of the gp130/JAK/STAT3 signaling axis.[9,10] In addition to STAT3, the related STAT1 factor is also strongly activated by gp130-acting cytokines.[11] In contrast to STAT3, however, early work has shown that the potent pro-inflammatory actions of STAT1, including those involved in LPS-driven inflammatory events, are essential to transduce IFN-dependent inflammatory responses to microbial challenge[12–14] and are thus considered involved in autocrine responses.

We reveal here that STAT1 undergoes rapid phosphorylation of S727 in response to stimulation by multiple TLR ligands, an event that is independent of IRF3, IRF7 and the IFN receptor complex, but dependent on MyD88/TRIF signaling. Moreover, STAT1 interacts with key TLR signaling protein TRAF6. Importantly, when compared with wild-type (WT) bone marrow-derived macrophages (BMMs), BMMs generated from mice expressing a S727A STAT1 mutation display significantly reduced TNFα production, but comparable IL-6 and RANTES protein production, in response to stimulation by a number of different TLR ligands. Taken together, these results demonstrate that STAT1 is directly recruited into the TLR signal transduction pathway and mediates specific pro-inflammatory cytokine responses following TLR stimulation, providing a new paradigm of cross-talk between TLR and cytokine signaling pathways.

RESULTS

LPS-induced STAT1 hyperactivation in *gp130*$^{F/F}$ mice correlates with LPS hypersensitivity

Previous reports have demonstrated that TLR4 induces rapid STAT1 S727 phosphorylation in macrophages,[15] although the biological impact of this was not evident. We have previously reported that non-hematopoietic (that is, liver) gp130 signaling is a key regulator of systemic inflammatory responses during LPS-induced endotoxemia,[9] therefore we initially investigated whether STAT1 phosphorylation was hyperactivated in *gp130*$^{F/F}$ mice in response to LPS. As shown in Figure 1a, immunoblots of tissue lysates from LPS-treated *gp130*$^{F/F}$ and control WT mice over 180 min indicated that STAT1 S727 and STAT1 Y701 phosphorylation were enhanced within 90 min post LPS challenge as compared with WT mice. The observation of increased STAT1 S727 phosphorylation was further supported by densitometric analysis of multiple LPS-treated mice (Figure 1b).

To determine whether the aberrant LPS-induced phosphorylation of STAT1 in *gp130*$^{F/F}$ mice contributed to their heightened sensitivity to endotoxic shock, we investigated whether genetic ablation of STAT1 in *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice would protect against LPS-driven endotoxic shock. Consistent with our previous findings,[9] intraperitoneal injection of a sub-lethal (for WT mice) dose of 4 mg kg^{-1} LPS, resulted in 100% of *gp130*$^{F/F}$ mice failing to survive during a 72-h experiment (Figure 1c). By contrast, *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice were resistant to the low dose of LPS and demonstrated 100% survival (Figure 1c), commensurate with that observed of WT mice. Together, these results suggest that *gp130*$^{F/F}$ mice display increased STAT1 S727 phosphorylation and that genetic ablation of STAT1 in mice protects against detrimental hyper-phosphorylation of STAT1 that is associated with the hyper-sensitivity of *gp130*$^{F/F}$ mice to LPS-induced endotoxemia. Given previous studies suggesting TLR4 induces rapid STAT1 S727 phosphorylation, we next investigated whether S727 phosphorylation was a direct or indirect consequence of TLR activation.

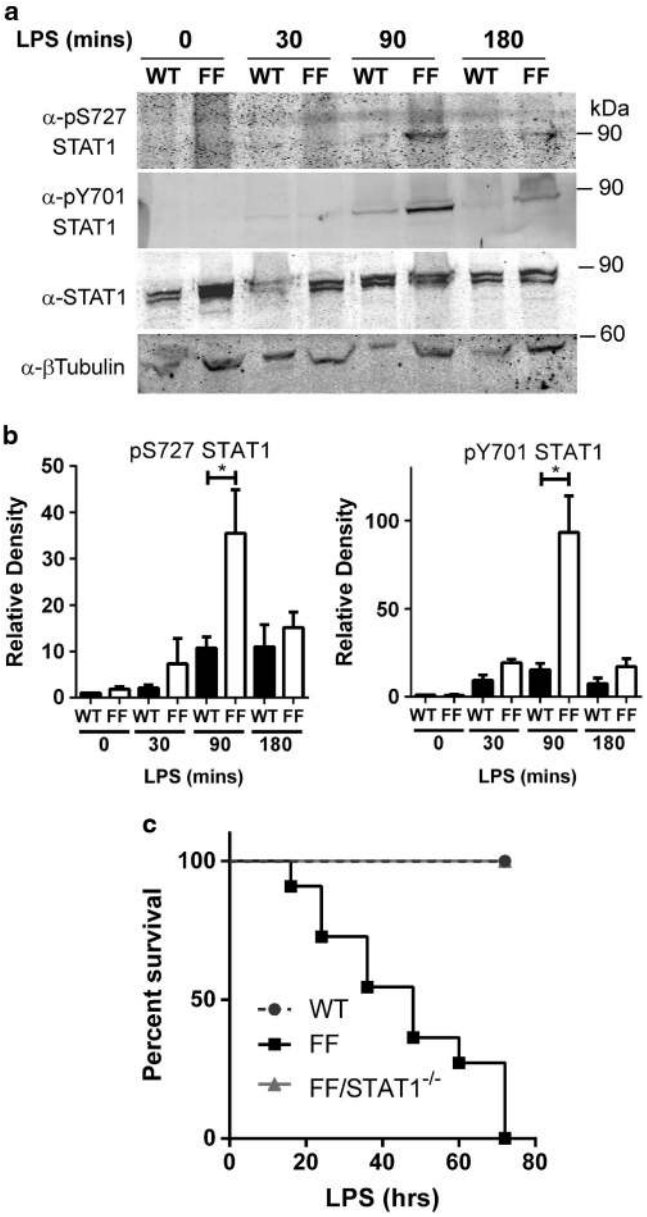


Figure 1 Genetic reduction of STAT1 in *gp130*$^{F/F}$ mice protects against LPS endotoxic shock. (**a**) Wild-type (WT) and *gp130*$^{F/F}$ (F/F) mice were intraperitoneal (i.p.) administered with LPS (4 mg/kg), and at defined intervals the levels of STAT1 S727 phosphorylation (pS727 STAT1), total STAT1 and β-tubulin were measured by immunoblotting liver lysates. Results shown are representative of three mice per genotype per time point. (**b**) Densitometric analysis of pS727 STAT1 as compared with total cellular STAT1 of three mice per time point per genotype (*$P<0.05$). (**c**) Survival of WT (8 mice, circle symbol, dashed line), *gp130*$^{F/F}$ (F/F, 11 mice, square symbol, solid line) and *gp130*$^{F/F}$:*Stat1*$^{-/-}$ (F/F:STAT1$^{-/-}$, 8 mice, triangle symbol, solid line) mice over 72 h following i.p. administration of 4 mg kg^{-1} LPS.

Multiple TLRs induce rapid S727 phosphorylation of STAT1, but not Tyr701

As it has been reported previously that ligand-induced activation of TLR2, TLR4 and TLR9 induce STAT1 S727 phosphorylation in macrophages,[15,16] we examined whether this observation also

applied to a broader range of ligands, including those used to stimulate TLR3 and TLR7 specifically.

Consistent with the earlier studies, we found that stimulation with Pam_3Cys-targeting TLR2 (Figure 2a), and LPS-targeting TLR4 (Figure 2c), induced rapid STAT1 S727 phosphorylation within 10 min in murine RAW264.7 macrophages, comparable to that observed in cells stimulated with IFNα, which was used as a control for inducing STAT1 S727 and tyrosine (Y)701 phosphorylation (Figure 2f). Furthermore, although TLR2 challenge did not induce STAT1 tyrosine phosphorylation over the time course examined, TLR4 challenge did induce tyrosine phosphorylation, but only at the later time points, distinct from those observed for serine phosphorylation. In contrast to TLR2 and TLR4 challenge, challenge with the TLR3 ligand poly I:C demonstrated substantially delayed STAT1 serine phosphorylation, visible only at 60 and 120 min post stimulation, whereas tyrosine phosphorylation was also observed at 120 min post challenge (Figure 2b). Consistent with our observations for TLR2 and TLR4 ligands, both TLR7 (Figure 2d) and TLR9 ligands (Figure 2e) induced rapid serine phosphorylation 20–30 min post stimulation. Neither TLR7 nor TLR9 ligands induced STAT1 tyrosine phosphorylation, as measured for up to 120 min post challenge. Densitometry analysis of STAT1 S727 phosphorylation further supported our observation that multiple TLR ligands induced STAT1 S727 phosphorylation (Supplementary Data 1) in a time-dependent manner.

To determine what pathways may be responsible for inducing STAT1 serine phosphorylation, we next examined whether a variety of TLRs could induce serine phosphorylation of STAT1 in immortalized WT and gene-deficient BMMs.[17] Consistent with our previous results, all TLR ligands, except poly I:C (TLR3) and our positive control, IFNα (lane 7), induced serine phosphorylation at 60 min post-stimulation in WT BMMs (Figure 3a). We next examined if MyD88 and TRIF, as central signaling mediators of TLR signaling, were required to induce phosphorylation. MyD88/TRIF double-deficient immortalized BMMs demonstrated an absence of STAT1 serine phosphorylation at 60 min post TLR challenge; however, IFNα-induced phosphorylation was unaffected in the absence of MyD88 and TRIF (Figure 3b). To examine if STAT1 serine phosphorylation may be induced via autocrine production of type I IFN following TLR stimulation, we next treated IRF3/IRF7-deficient immortalized BMMs with multiple TLR ligands for 60 min. As observed in Figure 3c, TLR-induced serine phosphorylation was unaffected in the absence of IRF3/IRF7, suggesting that TLR-mediated STAT1 S727 phosphorylation was independent of TLR-induced type I IFN expression. Finally, we stimulated primary BMMs derived from IFNAR1 gene-deficient mice with TLR ligands and IFNα. As can be observed in Figure 3e, TLR2-, TLR4-, TLR7- and TLR9-induced STAT1 S727 phosphorylation was unaffected in the absence of the IFNAR1 receptor complex; however, we saw no evidence of STAT S727 phosphorylation induced by TLR3 and IFNα.

Together, these results clearly establish that TLR-induced STAT1 serine phosphorylation is mediated by direct activation of the TLR signaling pathway and independent of TLR-induced autocrine type I IFN activation of the JAK/STAT pathway. Interestingly, it appears TLR3-induced pS727 STAT1, however, may be due to indirect activation via IFN production and the IFNAR receptor.

STAT1 is integrated into the TLR signaling pathway via its interaction with TRAF6

To identify how STAT1 may interact with the TLR signaling pathway, we tested if STAT1 could interact with TRAF6. TRAF6 is a signaling mediator common to both MyD88- and TRIF-dependent signaling pathways following TLR activation and is also known to interact

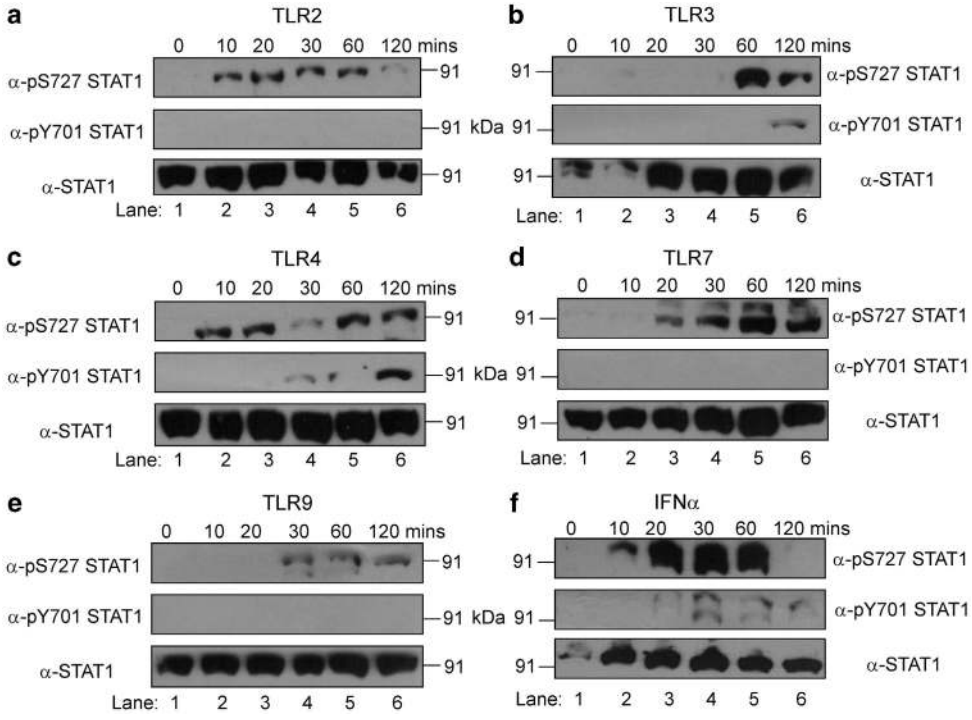


Figure 2 TLR activation directly induces rapid phosphorylation of STAT1 S727 but not Y701. RAW 264.7 cells were stimulated with (**a**) TLR2 ligand Pam_3Cys (100 ng ml^{-1}), (**b**) TLR4 ligand LPS (100 ng ml^{-1}), (**c**) TLR3 ligand poly I:C (10 μg ml^{-1}), (**d**) TLR7 ligand loxoribine (500 μM), (**e**) TLR9 ligand CpG-DNA (500 nM) and (**f**) IFNα (1000 IU ml^{-1}) over a time course of 120 min. Immunoblots were performed with antibodies against pSTAT1 (S727), pSTAT1 (Y701) and total STAT1 where indicated. Results are representative of three individual experiments.

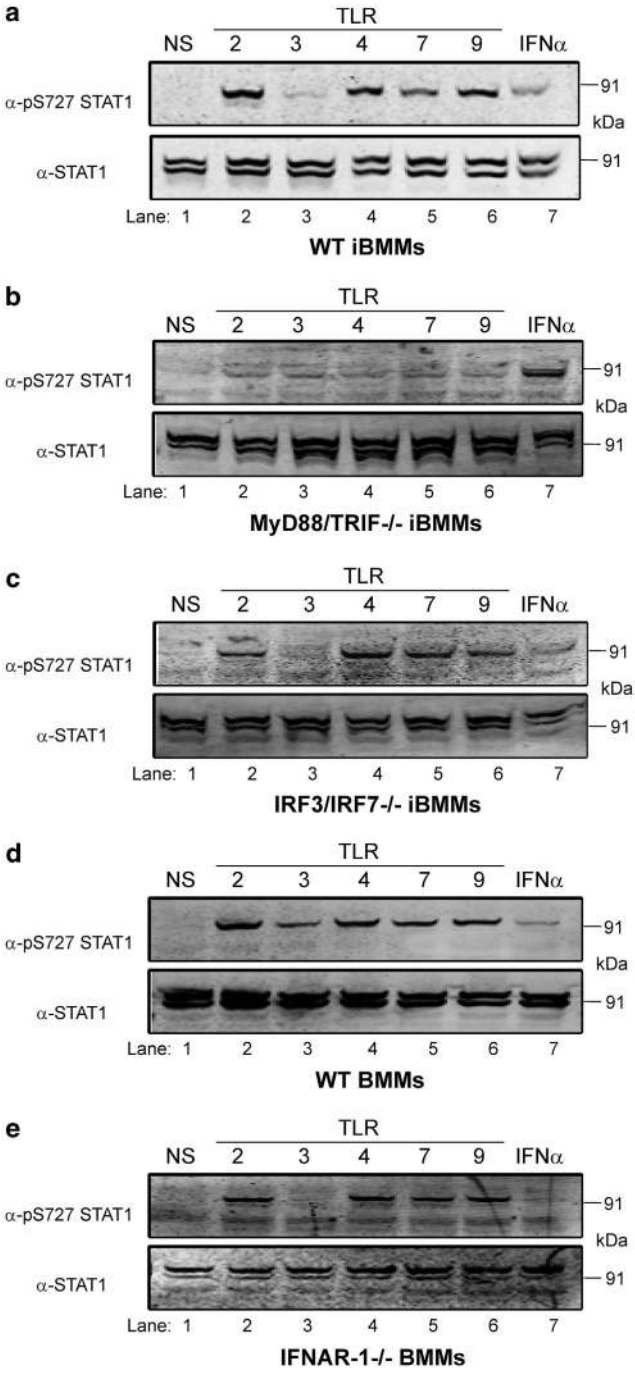


Figure 3 TLR-induced STAT1 S727 phosphorylation is MyD88/TRIF-dependent, but IRF3/IRF7- and IFNAR1-independent. Immortalized bone marrow-derived macrophages (iBMMs) derived from (**a**) WT, (**b**) IRF3/IRF7-deficient and (**c**) MyD88/TRIF-deficient mice were stimulated with Pam_3Cys (lane 2), poly I:C (lane 3), LPS (lane 4), loxoribine (lane 5), CpG-DNA (lane 6) and IFNα (lane 7) for 60 min. Primary BMMs generated from WT (**d**) and IFNAR1-deficient mice (**e**) were also stimulated with TLR agonists and IFNα for 60 min. Immunoblots were performed with antibodies against either total STAT1 or pSTAT1 (S727) as indicated. Results are representative of three individual experiments. NS, non-stimulated.

with another known STAT1 kinase, IRAK1.[18] We performed immunoprecipitation experiments in HEK293T cells transiently transfected with Myc-tagged STAT1 protein and probed cell lysates with recombinant glutathione S-transferase (GST) fused to TRAF6.

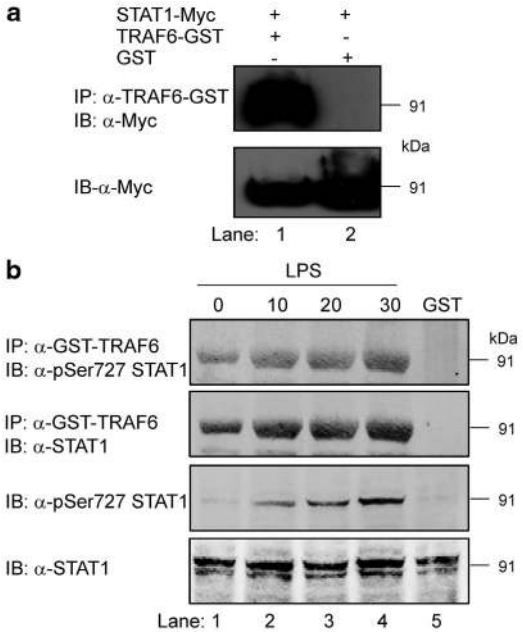


Figure 4 STAT1 immunoprecipitates (IP) with TRAF6 following TLR4 stimulation. (**a**) HEK293T cells were co-transfected with STAT1-Myc 24 h before probing lysates with GST-TRAF6 fusion protein or GST alone for 3 h to IP STAT1. Samples were then subjected to immunoblotting (IB) with the indicated antibodies. These results are a representation of three individual experiments. RAW 264.7 cells were stimulated with (**b**) 100 ng ml^{-1} of LPS for indicated times (0–30 min). Cell lysates were then probed with GST-TRAF6 beads (IP), and samples were subjected to IB with the indicated antibodies. Results are representative of three individual experiments.

As seen in Figure 4a, TRAF6 was able to specifically recognize ectopically expressed STAT1, but GST alone did not.

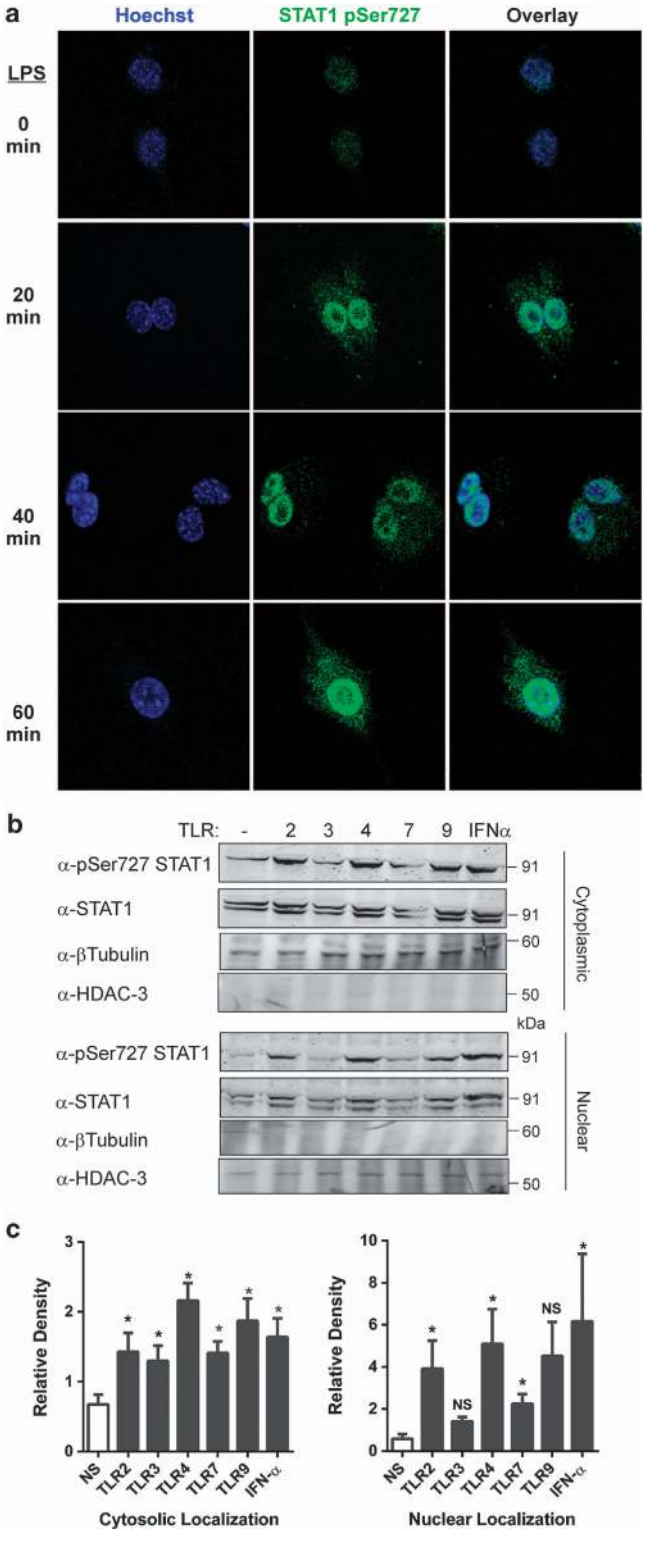
To further establish if TRAF6 could interact with STAT1 following TLR activation, we next probed TLR4-activated RAW264.7 macrophage cell lysates with recombinant GST-TRAF6. As can be seen in Figure 4b (top panel), there is an increase in TRAF6 recognition of serine-phosphorylated STAT1 within 10 min of TLR4 stimulation with LPS, which is commensurate with the observed increase in total cellular STAT1 serine phosphorylation observed earlier. A similar result was observed for TLR2-induced TRAF6 interaction with STAT1 in human monocytic THP-1 cells (Supplementary Data 2).

The ability of STAT1 to interact with TRAF6 following TLR activation would suggest that STAT1 is recruited to the TLR signaling pathway via association with TRAF6.

Serine phosphorylation of STAT1 promotes its translocation to the nucleus following TLR activation

A key step in the activation of STAT1 is its translocation to the nucleus following tyrosine phosphorylation-induced dimerization,[19] whereas serine phosphorylation is thought to act as a potentiating factor to boost gene transcription.[20] To further understand the consequence of TLR-induced STAT1 serine phosphorylation on its activation status, we next examined STAT1 cellular localization by fluorescence microscopy. We initially observed STAT1 (green) diffuse throughout the cytosol of RAW264.7 macrophages before LPS stimulation (Supplementary Data 3). However, we noted steady accumulation of STAT1 in the nucleus as visualized by Hoechst staining (blue) within 20 min of LPS stimulation, which became highly enriched in the nucleus by 60 min post stimulation. Consistent with our earlier results, immunostaining for phosphorylated S727

STAT1 (Figure 5a) was observed in both the cytosol and nucleus within 20 min of LPS stimulation, substantially increasing in the nucleus by 60 min. In addition, STAT1 Y701 was again delayed compared with STAT1 S727, however, weak STAT1 Y701 staining was observed at 40 min of LPS challenge, although not clearly observed until 60 min post-stimulation; this was localized to the nucleus (Supplementary Data 4).



To further support TLR-mediated nuclear localization of serine-phosphorylated STAT1, we fractionated cytosolic and nuclear fractions of RAW264.7 following LPS treatment for 60 min. As can be seen in Figure 5b, we found STAT1 S727 phosphorylation in both the cytosolic and nuclear fractions of TLR-stimulated cells, which is supported by densitometry analysis of pS727 STAT1 localization (Figure 5c), consistent with our earlier results.

S727 phosphorylation of STAT1 modulates TLR-induced NF-κB activity

Having established that TLRs mediate STAT1 S727 phosphorylation and subsequent translocation to the nucleus, we wished to establish if this phosphorylation event may affect TLR-induced NF-κB activity. For this purpose, we used the STAT1 S727A mutant, unable to undergo serine phosphorylation. As can be seen in Figure 6a, although HEK293 cells expressing TLR2 demonstrate a Pam_3Cys dose-dependent increase in NF-κB-luciferase reporter activity in the presence of WT STAT1, cells expressing STAT1 S727A (Supplementary Data 5A) demonstrate significantly decreased NF-κB reporter activity, suggesting STAT1 S727A can act as a dominant negative in TLR2-mediated NF-κB activity.

To further explore the effect of TLR-induced STAT1 activation on the inflammatory responsiveness of primary cells to TLR ligand stimulation, we compared the ability of murine embryonic fibroblasts (MEFs) derived from *gp130*$^{F/F}$ mice (displaying STAT1 hyper-activation) and STAT1-deficient *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice to respond to multiple TLR ligands. Interestingly, we found that although *gp130*$^{F/F}$ MEFs displayed robust TNFα expression (Figures 6b–d) in response to TLR2, TLR4 and TLR9 ligands, *gp130*$^{F/F}$:*Stat1*$^{-/-}$ MEFs showed a significant reduction in total TNFα protein secreted in response to TLR2 and TLR4 ligands, but not TLR9 stimulation. By contrast, the production of IL-6 in response to these TLR ligands was comparable among MEFs from both genotypes (Supplementary Data 5B-D). Taken together, these results suggest that S727 phosphorylation of STAT1 has a critical role in augmenting TLR-induced NF-κB activation, and STAT1 can differentially modulate pro-inflammatory cytokines in response to TLR ligands.

STAT1 phosphorylated at S727 regulates inflammation in macrophages induced by multiple TLRs

To further elucidate the role of STAT1 S727 phosphorylation in TLR-induced inflammatory responses, we next profiled the production of pro-inflammatory mediators following TLR challenge in BMMs derived from STAT1 S727A mice.[14] BMMs from these mice display a selective deficiency in TNFα protein expression in response to a

Figure 5 Serine phosphorylated STAT1 rapidly translocates to the nucleus following TLR4 challenge. (**a**) RAW 264.7 cells were stimulated with LPS (100 ng ml^{-1}) for indicated times, following which they were stained with Hoechst, incubated with α-pSTAT1 (S727) antibody and counterstained with Alex Fluor 488 antibody. All images are single confocal sections taken with a ×60 oil objective lens. Results are a representation of three individual experiments, where >300 cells were examined per condition. (**b**) RAW 264.7 cells were stimulated using Pam_3Cys (100 ng ml^{-1}: '2'), poly (I:C) (10 μg ml^{-1}: '3'), LPS (100 ng ml^{-1}: '4'), loxoribine (500 μM: '7'), mCpG DNA (500 nM: '9') and IFNα (1000 IU ml^{-1}: lane 8) for 60 min. Cell lysates were immunoblotted (IB) with α-STAT1 p-S727 or α-STAT1. Membranes were also stained with α-β-tubulin or α-HDAC3 to identify cytosolic or nuclear fractions, respectively. Results are representative of three individual experiments. (**c**) Densitometry analysis of three independent experiments of pS727 STAT1 as compared with β-tubulin (cytosolic) and HDAC-3 (nuclear) staining of untreated samples, respectively. NS, non-stimulated. *$P<0.05$.

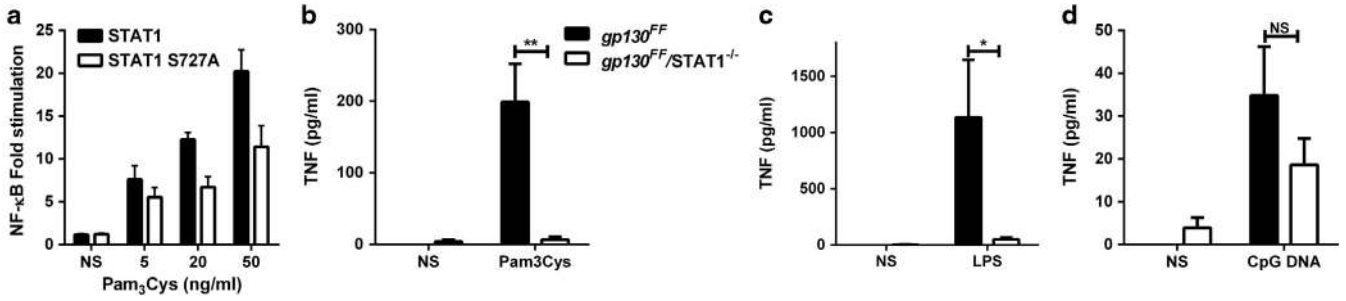


Figure 6 STAT1 S727A acts as a dominant negative to inhibit TLR responses. (**a**) HEK293 cells stably expressing TLR2 were co-transfected with NF-κB-luciferase promoter and thymidine kinase (TK)-renilla for transfection normalization in conjunction with either STAT1 WT or STAT1 S727A mutant. After 24 h, cells were stimulated with Pam_3Cys (5–50 ng ml^{-1}) for a further 6 h and assessed for luciferase activity. Results are normalized to each individual cell line and exhibited as pooled data presented as mean ± s.e.m. of four independent experiments performed in triplicate. *gp130*$^{F/F}$ or *gp130*$^{F/F}$:*Stat1*$^{-/-}$ MEFs were stimulated with (**b**) Pam_3Cys (10 ng ml^{-1}), (**c**) LPS (10 ng ml^{-1}) or (**d**) CpG DNA (100 nM) for 16 h. Supernatants were collected and TNFα protein expression measured by ELISA. Data are pooled results from triplicate samples of three independent experiments and presented as the mean ± s.e.m. (*$P<0.05$, **$P<0.01$).

dose range of ligands for TLR2 (Figure 7a), TLR3 (Figure 7b), TLR4 (Figure 7c), TLR7 (Figure 7d) and TLR9 (Figure 7e). Interestingly, inflammatory cytokine IL-6 production was not significantly altered between WT and STAT1 S727A BMMs for any TLR except for TLR4. Curiously, we noted no significant increase in IL-6 production in response to TLR3 stimulation at the doses used, in contrast to TLR3-induced TNFα and RANTES. Nor was STAT1 pS727 required for production of the chemokine RANTES, as it requires both NF-κB and IRF3 activation for gene expression.[21] These data clearly support our earlier observation that STAT1 S727 phosphorylation selectively regulates TNFα expression, but not IL-6 nor RANTES, in response to multiple TLR stimulation.

DISCUSSION

Activation of the optimal pro-inflammatory response by TLRs requires the coordinated recruitment of a series of signaling mediators. TNFα is a key pro-inflammatory cytokine produced by myeloid cells in response to pathogen challenge but is also a key chronicity factor in the pathology of many auto-inflammatory diseases and etiologies. Our finding shows that activation of STAT1 via its recruitment into the TLR signaling pathway provides a decisive insight into the specific production of TNFα in response to TLR activation and evidence of cross-talk between the TLR and JAK/STAT signaling pathways.

The activity profiles of STAT1 S727 phosphorylation suggest the recruitment of STAT1 into the TLR pathway is both rapid and direct. Previous studies have demonstrated that TLR2, TLR4[15] and TLR9[16] induce STAT1 S727 phosphorylation and our study now extends these observations to demonstrate that STAT1 S727 phosphorylation is a common event across TLRs in both the plasma membrane and endosome. Indeed, we noted that plasma membrane-associated TLRs such as TLR2 and TLR4 induce rapid S727 phosphorylation within 10 min post stimulation, whereas the induction of S727 phosphorylation by endosomal-localized TLRs was delayed until 20–30 min in the case of TLR7 and TLR9, which may reflect the requirement of endosomal trafficking and acidification for signaling. Interestingly, TLR3 activation, which signals exclusively via TRIF,[4,22] appears to induce STAT1 S727 phosphorylation indirectly, presumably via IFNβ production, as compared with other TLRs, which are MyD88-dependent. Importantly, our evidence for TLR-induced activation of STAT1 shows that it occurs consistently before STAT1 Y701 phosphorylation, suggesting that activation is independent of type I IFN production and the subsequent traditional activation of the JAK/STAT pathway. This is further supported by our observation of STAT1 S727 phosphorylation in IRF3/IRF7 double-deficient macrophages, which are unable to produce type I IFN and importantly, supported by the continued phosphorylation of STAT1 S727 as a consequence of TLR stimulation in IFNAR1-deficient macrophages. Interestingly, previous studies by Fenton and co-workers[15] found that MyD88 signaling was required for TLR4-induced S727 phosphorylation, but not STAT1 phosphorylation downstream of TLR2 stimulation. However, we found that macrophages deficient in both MyD88 and TRIF were refractive to TLR-induced S727 phosphorylation. One possibility, however, is that TLR-induced IL-6 production, which can also induce STAT1 S727[20] and Y701 phosphorylation, could be responsible for TLR-induced phosphorylation. However, the absence or delay of STAT1 Y701 phosphorylation, combined with the rapidity of STAT1 S727 phosphorylation in response to TLR stimulation (that is, within 10–20 min) would appear to negate the possibility of IL-6 gene transcription being responsible for the observations described.

TRAF6 appears to have a critical role in not only transducing the canonical MyD88 signal transduction pathway leading to NF-κB nuclear translocation, but also through interactions with ancillary signaling components, which allow 'fine-tuning' of the pro-inflammatory response. TRAF6 has now been demonstrated to interact with Mal[23,24] and TRIF[25] to modulate peripheral signaling pathways such as NF-κB transactivation and to provide another level of complexity to TLR responses. In addition, TRAF6 acts as branch point in TLR signaling to promote activation of the MAP kinase pathway allowing regulatory control over pro-inflammatory gene transcription. Our novel observation shows that TRAF6 can also interact with components of the JAK/STAT pathway, such as STAT1, further highlights the central role of TRAF6 in modulating and influencing TLR signal transduction, and suggests that TRAF6 interaction with alternative signaling components adds layers of specificity to TLR-mediated inflammation.

IRAK-1, which interacts with TRAF6 in the canonical TLR signaling cascade, has been previously described as required for IL-1-induced STAT1 S727 phosphorylation independent of its kinase activity.[18] As IRAK-1 acts upstream of and interacts with TRAF6 in the MyD88-dependent canonical signaling cascade used by both IL-1 and TLRs, our findings suggest that TRAF6 and IRAK-1 may be responsible for recruiting STAT1 into the pathway, acting to recruit an as yet unknown IL-1/TLR-induced STAT1 serine kinase. Interestingly, Rhee *et al.*[15] proposed that p38 was the kinase responsible for

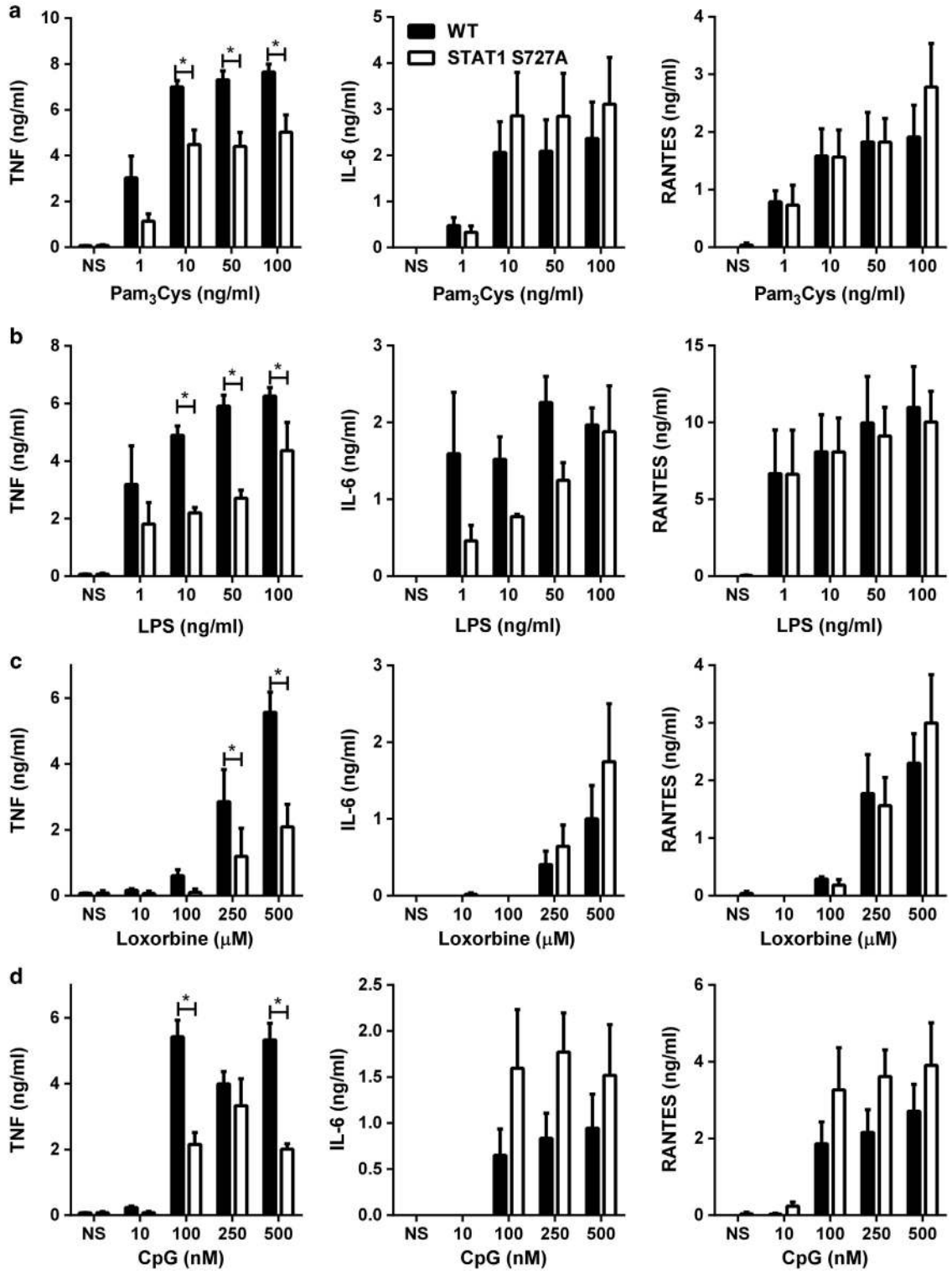


Figure 7 TNFα expression is suppressed in STAT1 S727A BMMs following multiple TLR ligand challenge. BMMs derived from WT and STAT1 S727A mice were stimulated with the indicated ranges of (**a**) Pam_3Cys, (**b**) poly I:C, (**c**) LPS, (**d**) loxoribine and (**e**) CpG-DNA for 16 h. Cellular supernatants were assayed for protein production of TNFα (left column), IL-6 (central column) and RANTES (right column) by ELISA. Results are presented as the mean ± s.e.m. of pooled results of three experiments performed in triplicate (*$P<0.05$). NS, non-stimulated.

TLR2- and TLR4-mediated STAT1 S727 phosphorylation; yet pharmacological inhibition of p38 did not affect IL-1-induced STAT1 S727 phosphorylation.[18] We also attempted to inhibit TLR-induced STAT1 serine phosphorylation in macrophages but found that chemical inhibitors of p38, MEK1/2, JNK, Akt and protein kinase R did not diminish TLR4-induced serine phosphorylation (data not shown). A potential explanation as to why these observations conflict one another may be due to the previous observations that STAT1 is a

poor substrate for all three MAP kinases.[18,26] Therefore, although TRAF6 has no intrinsic kinase activity itself, it would suggest that TRAF6 may act as the proximal 'bridge' for the recruitment of the STAT1 serine kinase into the MyD88-canonical pathway to NF-κB activation. Indeed, it is worth noting that it is the serine-phosphorylated form of STAT1 that TRAF6 immunoprecipitated following TLR2 and TLR4 challenge (Figure 4).

Interestingly, a previous study by Schroder *et al.* described that STAT1 S727 phosphorylation regulated TLR9 signaling within the context of differential induction of IFNβ by dendritic cells compared with macrophages.[16] Notably, the authors described exaggerated *Il-12p40* and *Cox-2* mRNA expression in BMMs generated from STAT1 S727A mice, whereas *Tlr4* and *Tlr9* mRNAs were repressed. Conversely, we found that STAT1 S727 phosphorylation had a positive regulatory function in multiple TLR inflammatory responses in STAT1 S727A BMMs. Importantly, however, it appears that STAT1 S727 phosphorylation differentially modulates the expression of specific subsets of inflammatory genes, an event that adds a further layer of complexity as to how TLRs induce and regulate the expression of inflammatory cytokines during pathogen challenge. Specifically, our studies clearly demonstrate that STAT1 S727 phosphorylation is required for TNFα expression for all TLRs tested, however, expression of IL-6 and RANTES were not similarly regulated. Indeed, in the case of TLR9, there appeared to be a substantial, but not significant, increase in both IL-6 and RANTES production (Figure 7), consistent with these previous observations. Thus, it would appear that S727 phosphorylation of STAT1 may act as a double-edged sword in TLR inflammation, negatively regulating a subset of genes such as *Cox-2* and *IL-12p40*,[16] but positively regulating inflammatory genes such as *Tnfa*, the context of which may contribute to the disparate toxicity observed among TLR ligands. For example, the apparent anti-inflammatory actions of activated STAT1 may be protective in response to TLR9 activation by CpG-DNA.[16] Conversely, S277 phosphorylation of STAT1 has a crucial role in LPS endotoxic shock as STAT1 S727A mice were protected from LPS lethality *in vivo*,[14] which is supported by our observations here that the absence of STAT1 in our $gp130^{F/F}$ hypersensitive mouse model protects against LPS lethality (Figure 1).

Activation of TLR signaling pathways is dependent on a number of cytosolic Toll/IL receptor domain adapter proteins, such as Mal, TRIF, TRAM and MyD88. These adapter proteins orchestrate the dynamic pro-inflammatory response following TLR engagement: their alternate usage by different TLRs engenders an optimized inflammatory response and specific gene transcription. Specifically, TLR3 and TLR4 responses via TRIF include the induction of IFNβ expression through activation of the transcription factor IRF3, leading to STAT1 tyrosine phosphorylation via the IFNAR receptor complex and induction of STAT1-dependent genes. Several studies have also demonstrated that differential recruitment of signaling mediators to these pathways adds further complexity to the signaling pathways and modulates signals both positively and negatively. Indeed, we have previously demonstrated cross-talk between the TLR and JAK/STAT pathways via suppressor of cytokine signaling-1 negative regulation of Mal,[27] and recently, modulation of TLR4-dependent responses via the MyD88 signaling pathway, independent of type I IFN signaling.[8,9] Our current study has further extended the cross-talk between these pathways by demonstrating that STAT1 is also directly recruited into the TLR signaling pathway via interaction with TRAF6, leading to the activation of STAT1 S727 phosphorylation, which can sculpt the innate immune pro-inflammatory response via TLR signaling transduction. It also suggests that specific targeting of these peripheral signaling events may allow inflammatory signaling to be manipulated for therapeutic outcomes to repress specific subsets of genes (such as TNFα) in clinical situations such as septic shock. Indeed, previous studies have demonstrated that STAT1 S727A mice are protected from LPS-induced septic shock, although levels of inflammatory cytokines were not examined in this model.[14] Together, these studies further demonstrate the increasing incidence of cross-talk between the TLR/IL-1 and JAK/STAT signaling pathways, suggesting that differential activation of STAT family members may have a critical role in regulating the TLR pro-inflammatory response following pathogen challenge.

METHODS

Mice

The generation of $gp130^{F/F}$ and $gp130^{F/F}$:$Stat1^{-/-}$ mice has been previously described.[11,28] All experiments were performed following approval from the Monash University Animal Ethics Committees. Experiments used age-matched animals and included WT ($gp130^{+/+}$) littermate controls that were genetically matched to the experimental animals. All mice were maintained under specific pathogen-free conditions. Mice were subjected to intraperitoneal injection of highly purified *Escherichia coli* K-235 LPS[29] (Sigma-Aldrich, Castle Hill, NSW, Australia) at 4 mg kg^{-1} as described previously.[8] Mice were humanely euthanized by CO_2 asphyxiation at or before 3 days and their health was monitored during this period using a Clinical Signs Severity Score system[30] as approved by the Monash Medical Centre Animal Ethics 'A' Committee.

Cell culture

Immortalized BMMs (a kind gift of Professor E Latz, University of Bonn) were generated as previously described[17] and RAW264.7 murine macrophages were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine in 5% CO_2 at 37 °C. Primary BMMs generated from aged-matched WT and IFNAR1-deficient mice were generated as previously described.[31]

Protein extraction and immunoblot analysis

Lysates were prepared from snap-frozen liver tissue and subjected to immunoblot analyses as previously described.[9] Briefly, macrophages were seeded in six-well plates at 1×10^6 cells per well 24 h before stimulation. Cells were then stimulated with Pam_3Cys (100 ng ml^{-1}), poly (I:C) (10 μg ml^{-1}), LPS (100 ng ml^{-1}), loxoribine (500 μM), mCpG DNA (500 nM) and IFNα (1000 IU ml^{-1}) for 60 min. TLR ligands were sourced from Invivogen (San Diego, CA, USA). Following stimulation, cells were harvested at 4 °C in pre-chilled Kal B (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) solution supplemented with 10 mM sodium vanadate, 10 mM sodium fluoride, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail tablet (Roche, Castle Hill, NSW, Australia), then subjected to 30 min rotation and centrifuged to separate cellular debris (14 500 r.p.m., 4 °C, 5 min). Samples were boiled in Laemmeli reducing sample buffer and separated by SDS–polyacrylamide gel electrophoresis and immunoblotted with α-pSTAT1 (Ser-727) antibody, α-pSTAT1 (Tyr-701) and α-STAT1 antibody (Cell Signaling Technology, Danvers, MA, USA). Immunoblotting antibodies against pY-STAT1, pS-STAT1 and STAT1 were purchased from Cell Signaling Technology. Proteins were visualized using the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA) with the appropriate secondary antibodies.

Cytokine production

Cytokine concentrations in cultured supernatants were measured by ELISA kit for murine IL-6, TNFα (both BD Biosciences, North Ryde, NSW, Australia), and RANTES (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions.

Cellular fractionation

RAW264.7 cells were seeded and stimulated as above. Cells were pelleted in phosphate-buffered saline at 2000 r.p.m. for 5 min. The pellet was resuspended

in 80 µl plasma membrane lysis buffer (10 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Nonidet P-40) and incubated for 5 min. Nuclei were separated from the cytoplasmic fraction by centrifugation and washed three times in plasma membrane lysis buffer. The nuclear pellet was suspended in 30 µl nuclear lysis buffer (20 mM HEPES (pH 7.8), 420 mM NaCl, 20% glycerol, 0.2 mM EDTA, 1.5 mM $MgCl_2$) and incubated for 10 min. The nuclear fraction was extracted by centrifugation and samples were prepared as described above.

Microscopy

For all microscopy images, RAW 264.7 cells were grown on coverslips and stimulated for indicated times. After washing, cells were fixed using 10% formalin for 10 min at 37 °C, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 10 min, then nuclear staining was performed using Hoechst 33342 (Life Technologies Corporation, Melbourne, Victoria, Australia) for 10 min. Coverslips were blocked in 5% bovine serum albumin/phosphate-buffered saline for 120 min and stained with primary antibodies overnight at 4 °C. Cells were then stained in secondary antibodies for 60 min and mounted with Fluorescent Mounting Media (Dako, Noble Park, VIC, Australia). Cells were washed in phosphate-buffered saline between each step. Cells were imaged on a Nikon C1 inverted microscope with a $\times 60$ oil objective.

TLR stimulation of STAT1 S727A macrophages and cytokine analysis

STAT1 S727A BMMs and aged-matched WT controls were generated as previously described[31] and plated into 96-well plates at 4×10^4cells per well and were left untreated or stimulated with Pam_3Cys, LPS, loxoribine or CpG DNA for 16 h. Supernatants were collected and cytokines were measured for IL-6, TNF (both BD Biosciences), and RANTES (R&D Systems) by ELISA as per manufacturers' instructions.

Statistical analysis

Data are expressed as the means ± s.e.m., and statistical analyses of unpaired *t*-tests and one-way analysis of variance performed using the GraphPad PRISM software (GraphPad Software, La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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