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Glycogen synthase kinase-3 promotes the synergistic action of interferon- γ on lipopolysaccharide-induced IL-6 production in RAW264.7 cells¹

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Abstract

Macrophages are the major effector cells of the innate immune system. Their function requires the integration of signals from pathogens, such as those induced by lipopolysaccharide (LPS), and from the host, such as those induced by interferon- γ (IFN γ). The priming by IFN γ of Toll-like receptor-induced macrophage activation has long been recognized, but the mechanisms underlying this priming action remain unclear. We report in this study that the priming of macrophage-derived RAW264.7 cells by IFN γ is highly dependent on glycogen synthase kinase-3 (GSK3). Cooperative interactions of GSK3 and signal transducer and activator of transcription-3 (STAT3) were revealed by the findings that GSK3 inhibitors, or knockdown of the GSK3 β isoform, strongly reduced the activation of STAT3, but not STAT1, induced by IFN γ without affecting upstream signaling events, and GSK3 was associated with STAT3. Direct inhibition of STAT3 activation abolished the synergistic action of IL-6 production by IFN γ administered with LPS. Similarly, inhibition of GSK3 abolished the synergistic stimulation of IFN γ on IL-6 production, and GSK3 was recruited to the IFN γ receptor by co-treatment with IFN γ and LPS. These results demonstrate the dependency of macrophage priming by IFN γ on STAT3 and GSK3, providing novel targets for intervention.

Keywords

Cytokines; macrophage; signal transduction; lipopolysaccharide

1. Introduction

Macrophages initiate the innate immune response by recognizing pathogens, phagocytosing them, and secreting inflammatory mediators such as the proinflammatory cytokine interleukin-6 (IL-6)². These host inflammatory responses are mediated by a family of Toll-like receptors (TLR), each of which is activated by specific components of microorganisms [1]. In the context of bacterial pathogen recognition, macrophages detect bacterial products, such as lipopolysaccharide (LPS), and proinflammatory cytokines, such as interferon- γ (IFN γ). LPS activates TLR4, which can induce cytokine production by two pathways, one involving the recruitment of the adapter molecule MyD88 and the activation of NF- κ B [2], and the other

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independent of MyD88 involving Toll-IL-1R domain-containing adaptor-inducing IFN β leading to IFN β production [3]. Co-stimulation with IFN γ synergizes with LPS to greatly enhance cytokine production. IFN γ signals through the IFN γ transmembrane receptor that activates receptor-associated kinases in the Janus kinase (JAK) family that phosphorylate signal transducer and activator of transcription (STAT) transcription factors [4,5]. Mice with targeted deficits in IFN γ signaling display compromised innate and acquired immunity [6] and are highly resistant to LPS-induced endotoxic shock [7]. This highlights the physiological significance of IFN γ priming in vivo, as it suggests that IFN γ is normally produced during the response to LPS and functions to amplify the TLR-induced cellular responses. Many mechanisms have been proposed for IFN γ priming of macrophage responses to LPS involving the reciprocal cross-regulation of signaling molecules between the IFN γ and LPS signaling pathways [reviewed in [8]]. Proposed cross-talk mechanisms include induction of TLR4 and inhibition of LPS-induced TLR4 down-regulation, induction of MyD88, potentiation of NF- κ B activity, and transcription factor synergy at the promoters of target genes [9–12].

Recently, glycogen synthase kinase-3 (GSK3), a constitutively active Ser/Thr kinase with two isoforms, α and β , was identified as a crucial regulator of innate inflammatory processes [[13], reviewed in [14]]. GSK3 promotes TLR-induced production of pro-inflammatory IL-6, tumor necrosis factor- α (TNF α), IL-12p40 and IFN γ , at least in part by promoting NF- κ B activity in monocytes [13]. In addition, GSK3 has been shown to be a key regulator of IFN γ -induced IL-10 production and TLR2 responses in innate immunity and inflammation [15]. However, regulation by GSK3 of components of the IFN γ signaling pathway in macrophage has not been examined. In this study, we identified a new critical cooperative interaction between GSK3 and STAT3 that supports the potentiation by IFN γ of LPS-induced IL-6 production in macrophages.

2. Materials and methods

2.1. Reagents

Protein-free *E. coli* (K235) LPS was prepared as described [16]. IFN γ was obtained from R&D Systems, SB216763 and SB415286 from Tocris, kenpaullone, indirubin-3'-monoxime, 6-bromoindirubin-3'-oxime (BIO), GSK3 inhibitor II, and AG490 from Calbiochem, LiCl from Sigma, PP2 from Alexis, and JSI-124 (cucurbitacin) from the National Cancer Institute Developmental Therapeutic Program (NIH).

2.2. Cell culture

RAW264.7 cells were cultured in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin. IFN γ (1 ng/mL), LPS (100 ng/mL), or both, were added to cell culture medium without supplements for 15 min to 6 h before cells were harvested. Cell viability was assessed by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A multiwell scanner was used to measure the absorbance at 570–630 nm dual wavelengths.

2.3. Immunoprecipitation and immunoblotting

Samples were incubated with the indicated primary antibody overnight at 4°C with gentle agitation, followed by incubation with protein A or G sepharose beads (Amersham) for 2 h at 4°C, and the immune complexes were washed three times.

For immunoblotting, cells were lysed with a triton lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol) supplemented with protease and phosphatase inhibitors (1 μ g/mL leupeptin, aprotinin, pepstatin A, 1 mM orthovanadate, 50 mM NaF, 0.1 μ M okadaic acid, 1 mM PMSF). Membrane and soluble fractions were separated

using a membrane extraction kit (Calbiochem) and nuclear and cytosolic fractions were fractionated using a nuclear extraction kit (Active Motif). Proteins were resolved with SDS-PAGE and nitrocellulose membranes were immunoblotted with antibodies to phospho-Tyr705-STAT3, phospho-Ser727-STAT3, phospho-Tyr701-STAT1, phospho-Tyr1022/Tyr1023-JAK1, phospho-Tyr1007/Tyr1008-JAK2, phospho-Thr202/Tyr204-ERK, STAT3, STAT1, CREB (Cell Signaling Technology), TLR4 (eBioscience), JAK1, CD119 (BD Pharmingen), β -actin (Sigma), GSK3 α/β (Millipore), and JAK2 (Biosource).

2.4. STAT3 DNA binding

Nuclei from RAW264.7 cells were extracted with a nuclear extraction kit (Active Motif). Nuclear proteins (100 μ g) from RAW264.7 cells were immunoprecipitated with a STAT3 consensus oligonucleotide agarose conjugate (Santa Cruz Biotechnology) according to the manufacturer's instructions.

2.5. Measurement of cytokines

Mouse IL-6 and TNF α in cell culture supernatants was measured with an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience) according to the manufacturer's instructions.

2.6. siRNA transfection

The prevalidated siRNA oligonucleotide sequence for GSK3 α and GSK3 β (Smart pool) were purchased from Dharmacon Research, Inc and Silencer negative control were obtained from Ambion. Cells were transfected using liposome-mediated transfection reagent LipofectAMINE RNAiMAX (Invitrogen, Carlsbad, CA) with 50 nM siRNA according to the manufacturer's instructions.

2.7. Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical significance between groups was evaluated by Mann Whitney or Wilcoxon non parametric tests. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. GSK3 promotes IFN γ signaling to STAT3

IFN γ signals through a transmembrane receptor and receptor-associated JAK kinases that activate STAT transcription factors, primarily STAT1 and STAT3, by phosphorylation on regulatory Tyr701 and Tyr705, respectively [4]. Examination of the kinetics in RAW264.7 cells showed that after stimulation with a relatively low concentration of IFN γ (1 ng/mL) the tyrosine phosphorylation of STAT1 was increased throughout the 15 min to 4 h treatment time, and co-stimulation with 100 ng/mL LPS did not affect STAT1 tyrosine phosphorylation (Figure 1). The total level of STAT1 remained unaffected following each treatment. Stimulation with IFN γ also caused a rapid but biphasic increase in Tyr705 phosphorylation of STAT3 with increases evident at 30 min to 1 h (Figure 1), while the total level of STAT3 remained unaltered. In contrast to STAT1, treatment with LPS alone increased the tyrosine-phosphorylation of STAT3, but this occurred only after 3–4 h, suggesting that it occurred at the later times in response to a cytokine produced in response to LPS, likely IL-6, which is well-known to strongly activate STAT3 [4]. At the early 30 min to 1 h treatment times, co-stimulation with IFN γ plus LPS increased phospho-Tyr705-STAT3 to a similar degree as attained following treatment with IFN γ alone. In contrast, at the later 3 to 4 h treatment times there was a synergistic effect of LPS plus IFN γ on phospho-Tyr705-STAT3, likely due to co-stimulation of STAT3 activation by a cytokine, such as IL-6, that was synergistically produced in response to LPS plus IFN γ treatment.

GSK3 was found to promote STAT3 activation in RAW264.7 cells, as inhibition of GSK3 with lithium at a concentration reported to fully inhibit GSK3 [17] particularly reduced the tyrosine-705 phosphorylation of STAT3 stimulated by LPS plus IFN γ (Figure 2A). Examination of the effects of inhibition of GSK3 with lithium on the kinetics of STAT activation showed inhibition of both the early and late phases of tyrosine-705 phosphorylation of STAT3 but no effect on tyrosine-701 phosphorylation of STAT1 (Figure 2B). Treatment with two other selective GSK3 inhibitors, 6-bromoindirubin-3'-oxime [18] and SB415286 [19] (Figure 2C) and the knockdown by siRNA of GSK3 β , but not of GSK3 α (Figure 2D), confirmed the requirement for GSK3 β in the activating tyrosine phosphorylation of STAT3 and the lack of effect on phospho-Tyr701-STAT1. Altogether, these results raised the possibility that GSK3 may contribute to the synergistic action of IFN γ on the response to LPS by promoting IFN γ -induced STAT3 activation.

Since GSK3 is required for the full activation of STAT3 induced by IFN γ , we examined if GSK3 is involved in the initial signaling events associated with STAT3 tyrosine phosphorylation, JAK and Src activation. The tyrosine phosphorylation of STAT3 induced by IFN γ and LPS was abolished by the JAK2 inhibitor AG490, but not by the Src-family inhibitor PP2, indicating that a JAK-family kinase likely mediates GSK3-dependent STAT3 tyrosine phosphorylation stimulated by IFN γ in RAW264.7 cells (Figure 3A). However, inhibition of GSK3 by several structurally diverse selective inhibitors did not reduce tyrosine phosphorylation of JAK2 or JAK1, or phosphorylation of ERK1/2 induced by IFN γ and LPS (Figure 3B), and the GSK3 inhibitors also did not affect the kinetics of activation of these kinases (Figure 3C). These results demonstrate that GSK3 does not function upstream of JAK, but instead regulates the signaling of JAK to STAT3.

To test if GSK3 may regulate STAT3 at the plasma membrane receptor after stimulation with IFN γ , membrane fractions were prepared and STAT3 was immunoprecipitated to determine its association with GSK3. Quantitative analyses of three independent experiments revealed that stimulation with IFN γ rapidly increased the association of both GSK3 isoforms with STAT3 in the membrane fraction (Figure 4A). Similarly, co-immunoprecipitation demonstrated GSK3 β was associated with STAT3 in the nucleus and this association was increased by stimulation with IFN γ (Figure 4B), suggesting that GSK3 by activating STAT3 also modulated its transcriptional activity. Examination of the DNA binding activity of STAT3 using SIE-oligo-agarose beads (Figure 4C) showed that treatment with the GSK3 inhibitor lithium reduced IFN γ -induced STAT3 DNA binding, consistent with the conclusion that depressed STAT3 DNA binding activity is the consequence of reduced tyrosine phosphorylation of STAT3 caused by inhibition of GSK3.

3.2. IFN γ potentiation of LPS-induced IL-6 production requires STAT3

The synergistic action of IFN γ on LPS signaling was measured by examining the production of IL-6 in RAW264.7 cells. The kinetics showed significantly greater IL-6 production after 6 h of treatment with 100 ng/mL LPS and 1 ng/mL IFN γ compared to LPS alone (Figure 5A). Treatment with IFN γ alone for 6 h did not induce IL-6 production and a 6 h treatment with LPS alone only slightly increased IL-6 production. Therefore, the synergistic stimulation of IL-6 production by IFN γ and LPS is particularly evident after 6 h treatment with these low concentrations of stimulants. To test the hypothesis that STAT3 may be involved in the synergistic action of IFN γ , RAW264.7 cells were treated with agents that inhibit STAT3 activation and IL-6 production was measured. A low concentration of the specific JAK2 inhibitor AG490 (30 μ M) [20] blocked the synergistic action of IFN γ on LPS-induced IL-6 production (Figure 5B). Although a higher concentration of AG490 still efficiently blocked this synergy without affecting the cell viability (Figure 5D), it also reduced the production of IL-6 induced by LPS, raising the possibility that JAK2 or STAT3 activation is necessary for

IL-6 production induced by LPS. To further examine the necessity for active STAT3 in the IFN γ -induced synergistic stimulation of IL-6 production caused by LPS treatment, cells were pretreated with the selective STAT3 inhibitor JSI-124 [21]. Treatment with JSI-124 effectively blocked IL-6 produced in response to IFN γ plus LPS, as well as to LPS alone (Figure 5C), but did not affect cell viability (Figure 5D). Co-stimulation with LPS plus IFN γ recruited STAT3 to the IFN γ receptor, and this was blocked by treatment with the GSK3 inhibitor lithium (Figure 5E) or by knocking down GSK3 β , but not GSK3 α (Figure 5F). Altogether, these results show that GSK3 β is required for IFN γ -induced STAT3 activation and STAT3 is required for the synergistic action of IFN γ on LPS-induced IL-6 production.

3.3. GSK3 is associated with the IFN γ receptor and is required for IL-6 production

Since GSK3 regulates STAT3 activation in response to IFN γ , we examined if GSK3 is involved in the priming by IFN γ of RAW264.7 cells. Co-immunoprecipitation experiments revealed that both GSK3 α and GSK3 β associated with the IFN γ CD119 receptor (Figure 6A). GSK3 β was rapidly (5 min) recruited to the IFN γ receptor and its increased association was maintained through 30 min of treatment, while the association of GSK3 α or GSK3 β to TLR4 was not changed by the same stimuli (data not shown). In order to examine if GSK3 is involved in the synergistic effect of IFN γ and LPS, RAW264.7 cells were treated with a selective inhibitor of GSK3, lithium [17], to evaluate if IFN γ was still able to augment the LPS-dependent induction of IL-6. Lithium greatly attenuated the production of IL-6 induced by LPS and IFN γ (Figure 6B), suggesting that GSK3 plays an important role in the synergy between IFN γ and LPS. To confirm the involvement of GSK3, RAW264.7 cells were treated with four other structurally diverse specific GSK3 inhibitors and each completely abolished the production of IL-6 induced by LPS and IFN γ (Figure 6C), which was not due to loss of cell viability (Figure 6D). In contrast to IL-6 production, IFN γ did not potentiate LPS-induced production of TNF, and TNF production was minimally affected by GSK3 inhibitors, which also verified the lack of toxicity of these agents (Figure 6E). Knocking down each isoform of GSK3 individually revealed that GSK3 β but not GSK3 α was required for IL-6 production induced by LPS plus IFN γ (Figure 6F). These results show that the IFN γ potentiation of LPS-induced IL-6 production requires active GSK3 β .

4. Discussion

A novel mechanism for the synergistic actions of IFN γ and LPS, also known as IFN γ priming of macrophage responses to LPS, was found to involve the recruitment of active GSK3 and STAT3 into the inflammatory signaling pathway. Thus, each of several structurally diverse selective GSK3 inhibitors or inhibition of STAT3 activation almost completely blocked IFN γ potentiation of LPS-induced IL-6 production by RAW264.7 cells. This identification of GSK3 and STAT3 as necessary for the enhanced production of IL-6 induced by LPS in the presence of IFN γ raises the possibility that administration of GSK3 inhibitors may be beneficial for limiting macrophage inflammatory signaling in vivo, particularly since lithium, which effectively inhibits GSK3 [17,22], has been used therapeutically in humans for other purposes for many years [23].

The pro-inflammatory action of GSK3 appears to be mediated by targeting critical transcription factors in the signaling pathways activated by LPS and IFN γ . These include STAT3 in the synergistic signaling induced by LPS plus IFN γ in RAW264.7 cells in addition to the previously identified modulatory effects of GSK3 on the activation of NF- κ B and CREB in monocytes that regulate the production of IL-6 and other cytokines by monocytes following TLR stimulation [13,15]. Particularly intriguing was the selective requirement for GSK3 in the IFN γ -induced activation of STAT3, while STAT1 activation was completely independent of GSK3. This demonstrates that although GSK3 was associated with the IFN γ receptor, GSK3

did not regulate all signals emanating from the receptor. This conclusion was further supported by the lack of effects of GSK3 inhibitors on the IFN γ -induced activation of JAKs or ERKs. The selective action of GSK3 on STAT3 may be linked with the capacity of GSK3 to associate with STAT3, but not STAT1 (data not shown). GSK3 was found associated with STAT3 both in the membrane fraction and in the nucleus, suggesting that GSK3 may both promote STAT3 activation at the membrane-localized IFN γ receptor and have an additional role in the nucleus. Although the precise mechanism remains to be identified, it is evident that GSK3 has a strong, and selective, regulatory influence on the activation of STAT3 induced by IFN γ . These findings also indicate that the synergistic action of IFN γ on LPS-induced IL-6 production is independent of STAT1 signaling since STAT1 activation was unaffected by inhibitors of GSK3 that blocked IFN γ -potentiated IL-6 production, whereas STAT3 appears to play a crucial role.

This investigation also revealed a strong regulatory influence of STAT3 on IL-6 production in addition to identifying the regulatory effect of GSK3 on STAT3 in RAW264.7 cells. The established STAT3 inhibitor JSI-124 [21,24,25] abolished IL-6 production stimulated by LPS plus IFN γ . This was also supported by the finding that inhibition of JAK signaling by AG490 inhibited IL-6 production. This regulation of IL-6 production by STAT3 is unlikely to be a direct action on the IL-6 promoter since it does not contain a STAT3 consensus sequence. Instead the effect of STAT3 may be indirect due to its regulatory effects on other proteins associated with the promoter, notably the recent finding that STAT3 associates with NF- κ B on the IL-6 promoter [26–28]. Whether or not this underlies the regulatory effect of STAT3, it is notable that GSK3 promotes the activation of both NF- κ B and STAT3, which together act to induce IL-6 production. These dual actions reinforce the possibility that GSK3 may be a viable target for modulating cytokine production.

Although it is known that STAT3 mediates the anti-inflammatory effects of the cytokine IL-10 (reviewed in [29]), other evidence indicates that STAT3 can exert proinflammatory actions besides anti-inflammatory functions depending on the cell lineage [30], and the findings reported here show that STAT3 exerts a proinflammatory action by promoting IL-6 production in macrophage-derived RAW264.7 cells. An interesting picture has emerged for the plasticity of macrophage function based on two distinct polarization states of macrophages that have been described [31]. A proinflammatory macrophages phenotype characterized by the release of inflammatory cytokines such as TNF, IL-1 β , and IL-6, and an immunosuppressive phenotype characterized by the enhanced release of anti-inflammatory cytokines such as IL-10. Therefore, it may be speculated that GSK3 is involved in the macrophage polarization switch mechanism by promoting macrophage responses to IFN γ , and thus in consequence changing the cytokine environment. Different inflammatory pathologies involve distinct patterns of cytokines and growth factors, and improved therapies should be individually tailored toward each particular type of pathologic inflammation. Therefore, modulating GSK3 activity may provide a new strategy to control the function of macrophages and to target their dysfunction in pathology.

5. Conclusions

The function of macrophages requires the integration of signals from pathogens, such as those induced by lipopolysaccharide (LPS), and from the host, such as those induced by interferon- γ (IFN γ). The mechanisms underlying the priming by IFN γ of Toll-like receptor-induced macrophage activation remain unclear. We found that the priming of macrophage-derived RAW264.7 cells by IFN γ is highly dependent on GSK3. Cooperative interactions of GSK3 and STAT3 were revealed by the findings that inhibiting GSK3 strongly reduced the activation of STAT3 induced by IFN γ without affecting upstream signaling events, and GSK3 was associated with STAT3. Inhibition of STAT3 activation abolished the synergistic action of IL-6 production by IFN γ administered with LPS. Similarly, inhibition of GSK3 abolished the

synergistic stimulation of IFN γ on IL-6 production, and GSK3 was recruited to the IFN γ receptor by co-treatment with IFN γ and LPS. These results demonstrate the dependency of macrophage priming by IFN γ on STAT3 and GSK3, providing novel targets for intervention.

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Abbreviations

GSK3	glycogen synthase kinase-3
IL-6	interleukin-6
IFN	interferon
JAK	Janus kinase
LiCl	lithium chloride
LPS	lipopolysaccharide
NF-κB	nuclear factor kappa B
STAT	signal transducer and activator of transcription
TLR	Toll-like receptor
TNFα	tumor necrosis factor- α

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FIGURE 1. IFN γ activates STAT1 and STAT3

RAW264.7 cells were treated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, for 15 min to 4 h and cell lysates were immunoblotted for phospho-Tyr701-STAT1, phospho-Tyr705-STAT3, and total STAT1 and STAT3 (n=3).

FIGURE 2. GSK3 regulates STAT3 tyrosine-705 phosphorylation

(A) RAW264.7 cells were treated with 100 ng/mL LPS, 1 ng/mL IFN γ , or both, in the absence or presence of 20 mM lithium (LiCl) for 30 min, followed by analysis of cell lysates by immunoblotting with phosphorylation-specific antibodies to assess the phosphorylation of STAT3 (Tyr705, Ser727) and STAT1 (Tyr701) (n=4). (B) Kinetics of inhibition of phospho-Tyr705-STAT3 by 20 mM lithium in RAW264.7 cells (n=3). (C) RAW264.7 cells were treated with 100 ng/mL LPS and 1 ng/mL IFN γ , without or with the GSK3 inhibitors 10 μ M bromindirubin-3'-oxime (BIO) or 10 μ M SB415286, for 30 min and cell lysates were analyzed by immunoblotting (n=3). (D) The level and phosphorylation of STAT3 (Tyr705) and STAT1

(Tyr701) were assessed after 48 h siRNA-mediated knockdown of GSK3 α or GSK3 β and stimulation with IFN γ (1 ng/mL) for 30 min (n=3).

A **B** **C** **FIGURE 3. Modulation of signaling induced by stimulation with LPS and IFN γ**

RAW264.7 cells were stimulated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both. (A) JAK was inhibited by treatment with 100 μ M AG490 for 6 h (*left panels*) and Src was inhibited with 10 μ M PP2 for 30 min (*right panels*) and cell lysates were immunoblotted for phospho-Tyr705-STAT3 and total STAT3 (n=3). (B) GSK3 was inhibited by treatment with the indicated selective GSK3 inhibitors (10 μ M) and cell lysates were immunoblotted for phospho-Tyr1007/1008-JAK2, total JAK2, phospho-Tyr1022/1023-JAK1, total JAK1, phospho-ERK1/2 (p44 and p42), and total ERK1/2 (n=4). Immunoblots were reblotted with β -actin to ensure equal protein loading. (C) RAW264.7 cells were treated for 15, 30 min or 1 h without

or with the indicated selective GSK3 inhibitors 10 μ M bromindirubin-3'-oxime (BIO) or 10 μ M SB216763 and cell lysates were immunoblotted as in (**B**) (n=2).

FIGURE 4. STAT3 is regulated by GSK3

(A) RAW264.7 cells were treated with 100 ng/mL LPS, 1 ng/mL IFN γ , or both, for 5 min, membrane fractions were prepared for immunoprecipitation of STAT3, and immunoprecipitants were immunoblotted for GSK3 α/β and STAT3. To ensure the specificity of the immunoprecipitation, an immunoprecipitation with a non-specific isotypic IgG was included as a control. Quantified levels of GSK3 α and GSK3 β are shown on the right and values represent the mean $\pm$ SEM.; n=3 *p<0.05 compared to controls (Mann Whitney test). (B) RAW264.7 cells were treated with 100 ng/mL LPS, 1 ng/mL IFN γ , or both, for 5, 30, or 60 min and GSK3 β was immunoprecipitated from nuclear fractions, and immunoprecipitants were immunoblotted for GSK3 β and STAT3. (C) STAT3 DNA binding activity was measured in RAW264.7 cells after stimulation with 100 ng/mL LPS, 1 ng/mL IFN γ , or both for 30 min, with or without a 30 min pretreatment with 20 mM lithium. STAT3 immunoprecipitated with SIE-oligoSTAT3 beads was visualized by immunoblotting (n=3).

FIGURE 5. STAT3 is required for the synergistic action of IFN γ

(A) IL-6 production was measured using RAW264.7 cells treated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, for 2 to 6 h. IL-6 in cell-free supernatants was analyzed by ELISA (n=3, *p < 0.05 compared to control, Wilcoxon test). RAW264.7 cells were treated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, for 6 h and IL-6 in cell-free supernatants was analyzed by ELISA after: (B) inhibition of JAK by treatment with 30 or 100 μ M AG490 for 6 h (n=5, *p < 0.05 compared to samples treated with LPS plus IFN γ , Mann Whitney test), (C) Inhibition of STAT3 activation by treatment with 10 μ M JSI-124 for 6 h (n=7, *p < 0.05 compared to samples treated with LPS plus IFN γ , Mann Whitney test). (D) Cell viability of RAW264.7 cells treated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, without or with 100 μ M AG490 or

10 μ M JSI-124 for 6 h (n=3). Values represent mean $\pm$ SEM. (E) CD119 was immunoprecipitated from membrane fractions prepared from RAW264.7 cells treated for 30 min with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, without or with treatment with 20 mM lithium, and immunoprecipitants were immunoblotted for GSK3 α/β and STAT3 (n=4). To confirm the efficiency of the immunoprecipitation, the supernatant (Sup) was immunoblotted after immunoprecipitation. (F) After 48 h siRNA-mediated knockdown of GSK3 α or GSK3 β , or incubation with a control siRNA (Ctl), RAW264.7 cells were treated for 30 min with 100 ng/mL LPS, 1 ng/mL IFN γ , or both. CD119 was immunoprecipitated, and immunoprecipitants were immunoblotted for GSK3 α/β and STAT3.

FIGURE 6. GSK3 controls the synergistic action of IFN γ on LPS-induced IL-6 production (A) The IFN γ receptor α -chain (CD119) was immunoprecipitated from membrane fractions prepared from RAW264.7 cells after 5 or 30 min treatment with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, and immunoprecipitants were immunoblotted for GSK3 α/β . (n=3). To ensure the specificity of the immunoprecipitation, an immunoprecipitation with a non-specific isotopic IgG was included as a control. Quantified levels of GSK3 α and GSK3 β are shown on the bottom and values represent the mean $\pm$ SEM.; n=3 *p<0.05 compared to controls (Mann Whitney test). (B) IL-6 production was measured using RAW264.7 cells treated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, without or with 20 mM lithium treatment for 4 or 6 h. (n=3; *p < 0.05 compared to samples treated with LPS plus IFN γ without lithium, Mann Whitney test). (C) IL-6 production was measured using RAW264.7 cells treated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, without or with 10 μ M GSK3 inhibitors (indirubin-3'-monoxime, kenpaullone, GSK3 inhibitor II and SB216763) for 6 h. (n=5; *p < 0.05 compared to samples treated with LPS plus IFN γ without GSK3 inhibitor, Mann Whitney test). (D) Cell viability of

RAW264.7 cells treated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, without or with 20 mM lithium or 10 μ M other GSK3 inhibitors (bromoindirubin-3'-monoxime (BIO), kenpaullone, GSK3 inhibitor II and SB216763). (E) TNF production was measured using RAW264.7 cells treated with 1 ng/mL IFN γ , 100 ng/mL LPS, or both, without or with 20 mM lithium or 10 μ M other GSK3 inhibitors (bromoindirubin-3'-monoxime, kenpaullone, GSK3 inhibitor II and SB216763) for 6 h. TNF in cell-free supernatants was analyzed by ELISA (n=3). (F) IL-6 production by RAW264.7 cells after 48 h siRNA-mediated knockdown of GSK3 α or GSK3 β and stimulation with LPS (100 ng/mL) alone or with IFN γ (1 ng/mL) for 6 h (n=3, *p<0.05 compared to corresponding control siRNA values, Mann Whitney test). Values represent mean $\pm$ SEM.