

# IFN- $\gamma$ Suppresses IL-10 Production and Synergizes with TLR2 by Regulating GSK3 and CREB/AP-1 Proteins

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## Summary

The control of IL-10 production and mechanisms that mediate synergy between IFN- $\gamma$  and TLR ligands are not well understood. We report that IFN- $\gamma$  augments induction of TNF $\alpha$  by TLR ligands, immune complexes, and zymosan by suppressing IL-10 production and thereby interrupting Stat3-mediated feedback inhibition. IFN- $\gamma$  altered TLR2-induced signal transduction by increasing GSK3 activity and suppressing MAPK activation, leading to diminished IL-10 production. Inhibition of GSK3 or ablation of the GSK3 $\beta$  gene ameliorated TLR2-induced peritonitis and arthritis. IFN- $\gamma$  suppressed the activity of CREB and AP-1, transcription factors that induce IL-10 expression and are regulated in part by MAPKs and GSK3. These results yield insight into mechanisms by which IFN- $\gamma$  regulates IL-10 production and TLR2-mediated inflammatory responses and identify inhibition of CREB and AP-1 as part of the macrophage response to IFN- $\gamma$ . GSK3 and CREB/AP-1 are key players in integrating IFN- $\gamma$  and TLR2 responses in innate immunity and inflammation.

## Introduction

A key effector function of activated macrophages is the production of inflammatory cytokines and chemokines. Potent activators of inflammatory cytokine production include microbial products, immune complexes, and TNF. Macrophages respond to microbial products with pattern recognition receptors (PRRs) that recognize conserved molecules, termed pathogen-associated molecular patterns or PAMPs, expressed by microbes. The Toll-like receptor (TLR) family of PRRs is comprised of at least 11 receptors that play a key role in mediating inflammatory responses to microbial infections (Akira and Takeda, 2004). Examples include TLR2 and TLR4 that mediate recognition of, respectively, bacterial lipo-

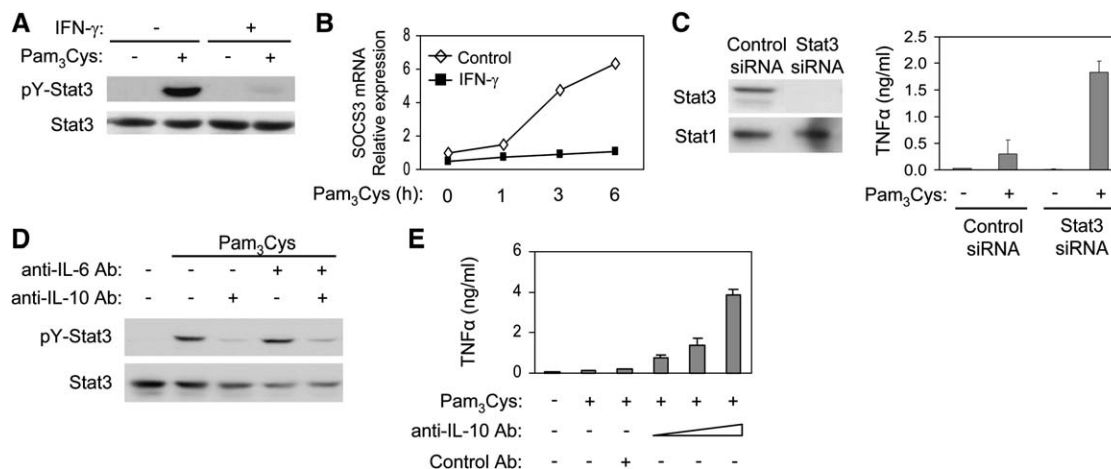
peptides and lipopolysaccharide (LPS). TLRs have also been implicated in regulating the transition from innate to acquired immunity (Hoebe et al., 2004; Iwasaki and Medzhitov, 2004). Although the strong inflammatory activation of dendritic cells (DCs) and macrophages by TLRs has been linked to the development of Th1 responses (Schnare et al., 2001), there is accumulating evidence that activation of specific TLRs, such as TLR2, can be less inflammatory and favor the development of Th2 responses (Agrawal et al., 2003; Dillon et al., 2004; Pulendran et al., 2001; Re and Strominger, 2001; Redecke et al., 2004).

Excessive inflammatory cytokine production results in tissue damage and toxicity that is harmful to the host. Thus, strong inducers of inflammatory cytokines also activate homeostatic mechanisms that serve to limit cell activation, cytokine production, and tissue damage. One key homeostatic mechanism is the induction of IL-10, a potent anti-inflammatory cytokine that mediates a feedback inhibition loop that limits inflammatory cytokine production. IL-10 inhibits multiple macrophage and DC effector functions and plays a critical role in limiting tissue injury during infections and in preventing autoimmunity by limiting the duration and intensity of immune and inflammatory reactions (Moore et al., 2001). Immune complexes and TLR2 and TLR4 ligands are potent inducers of IL-10. IL-10 induced by these factors limits endotoxin toxicity and mediates the paradoxical suppressive effects of immune complexes (Berg et al., 1995; Gerber and Mosser, 2001; Miles et al., 2005). Induction of IL-10 by TLR2 is particularly biologically significant, as TLR2-induced IL-10 promotes Th2 responses in vivo (Agrawal et al., 2003; Dillon et al., 2004; Pulendran et al., 2001; Redecke et al., 2004) and represents an important pathogenic mechanism by which microbes that activate TLR2, such as *Yersinia pestis*, suppress host immune responses (Netea et al., 2004; Sing et al., 2002a, 2002b). The suppressive effects of IL-10 on myeloid cells are dependent upon the signal transducer and activator of transcription Stat3 (Takeda et al., 1999).

Glycogen synthase kinases 3 (GSK3)  $\alpha$  and  $\beta$  are homologous serine/threonine kinases involved in signal transduction downstream of Wnt, receptor tyrosine kinases, and G protein-coupled receptors (Frame and Cohen, 2001). GSK3 phosphorylates multiple substrates, including Wnt signaling components and transcription factors NFATc, Jun, CREB, Myc, and HSF-1 (Doble and Woodgett, 2003). Phosphorylation by GSK3 results in inhibition of many of its substrates. For example, phosphorylation of Jun by GSK3 negatively regulates Jun's DNA binding capacity (Boyle et al., 1991). GSK3 regulates many cellular functions, including glycogen metabolism, cell-cycle control, and proliferation, has been implicated in the pathogenesis of diabetes and Alzheimer's disease, and is under active investigation as a therapeutic target in these diseases (Cohen and Goedert, 2004). In vivo, homozygous deletion of the GSK3 $\beta$  gene led to embryonic lethality characterized by extensive liver degeneration (Hoeftlich et al., 2000). GSK3 activity is regulated by serine phosphorylation,

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**Figure 1. IFN- $\gamma$  Abrogates a TLR2-Induced, IL-10-Stat3-Dependent Feedback Inhibition Loop**

(A) Control human primary macrophages or macrophages activated with 100 U/ml of IFN- $\gamma$  were stimulated with 10 ng/ml of Pam<sub>3</sub>Cys for 6 hr. Whole cell extracts were analyzed by immunoblotting with antibodies against tyrosine-phosphorylated Stat3 (pY-Stat3), followed by probing the same filter with antibodies against Stat3. One representative experiment out of eight performed is shown.

(B) Control or IFN- $\gamma$ -activated human macrophages were treated with 10 ng/ml of Pam<sub>3</sub>Cys for the indicated periods. mRNA expression levels of SOCS3 were assessed by quantitative real-time PCR and normalized relative to levels of GAPDH.

(C) THP-1 monocytic cells were transduced with lentiviral particles encoding control small interfering RNA (siRNA) or Stat3 siRNA. Left: whole-cell extracts were analyzed for Stat3 and Stat1 protein levels by immunoblotting. Right: virally transduced cells were stimulated with 10 ng/ml of Pam<sub>3</sub>Cys for 18 hr and TNF $\alpha$  protein levels in the culture supernatants were quantitated by ELISA (mean  $\pm$  SD of three independent experiments). (D) Control human macrophages were pretreated with anti-IL-6 or anti-IL-10 neutralizing antibody for 30 min prior to a 6 hr stimulation with Pam<sub>3</sub>Cys. Cell extracts were assayed for both pY-Stat3 and total Stat3 levels by immunoblotting.

(E) Human primary macrophages were stimulated with 10 ng/ml of Pam<sub>3</sub>Cys for 18 hr in the presence of increasing concentrations of anti-IL-10 neutralizing antibody. The concentration of isotype-matched control antibody corresponded to the highest dose of anti-IL-10 antibody. Levels of TNF $\alpha$  in culture supernatants were determined by ELISA.

which suppresses kinase activity. GSK3 is phosphorylated and inactivated by serine kinases including Akt and thus is regulated by the PI3K-Akt pathway that is activated by multiple immune receptors.

IFN- $\gamma$  is a potent macrophage activating factor that promotes antigen presentation, microbial killing, and cytokine production (Schroder et al., 2004). Many of these activating functions can be explained on the basis of IFN- $\gamma$ -induced expression of immune/inflammatory genes. Less is understood about another key property of IFN- $\gamma$ , its capacity to dramatically enhance macrophage responses to other inflammatory factors such as TLR ligands, immune complexes, and TNF $\alpha$ . Investigation of the synergistic activation of macrophages by IFN- $\gamma$  and TLRs/TNF has focused on enhanced activation of proinflammatory pathways. For example, IFN- $\gamma$  enhances LPS- and TNF-induced NF- $\kappa$ B activation and increases TLR expression (Schroder et al., 2004). We reasoned that suppression of TLR-induced homeostatic responses and feedback inhibitory pathways would provide an additional mechanism by which IFN- $\gamma$  synergizes with TLRs and, in this study, analyzed the effects of IFN- $\gamma$  on TLR2-induced production of IL-10 and activation of Stat3. We found that IFN- $\gamma$  inhibits IL-10 production by regulating the activity of GSK3 and MAPKs downstream of TLR2, with a concomitant suppression of CREB and AP-1 transcription factors that are required for IL-10 production. Our results yield insight into mechanisms by which IFN- $\gamma$  regulates TLR-mediated inflammatory responses and identify GSK3 and AP-1 proteins as key players in the integration of IFN- $\gamma$  and TLR responses in innate immunity and inflammation.

## Results

### IFN- $\gamma$ Abrogates IL-10-Stat3-Mediated Feedback Inhibition by Suppressing IL-10 Expression

TLR4 ligation induces indirect activation of Stat3 that is mediated predominantly by autocrine IL-10 (Carl et al., 2004). Feedback inhibition of inflammatory cytokine production by autocrine IL-10 is well established (Moore et al., 2001), but the role of Stat3 that is activated downstream of TLR ligation has not been defined. We had previously shown that IFN- $\gamma$  partially suppresses Stat3 function in the absence of any effect on Stat3 tyrosine phosphorylation (Herrero et al., 2003; Hu et al., 2005b) and wished to investigate the effects of IFN- $\gamma$  on TLR-induced Stat3 and its potential homeostatic functions. We chose to study Stat3 activation downstream of TLR2 because of the reported suppressive effects of TLR2 that would be consistent with Stat3 activation (Netea et al., 2004; Sing et al., 2002b). Primary human macrophages were stimulated with Pam<sub>3</sub>CysSer(Lys)<sub>4</sub> (a triacylated peptide that stimulates via TLR2, hereafter termed Pam<sub>3</sub>Cys), and activation of Stat3 was measured. TLR2 stimulation of control macrophages resulted in phosphorylation of Stat3 that became apparent 3 hr after stimulation and was sustained for at least 6 hr (Figure 1A and data not shown). Expression of the Stat3 target gene suppressor of cytokine signaling 3 (SOCS3) was induced in parallel with Stat3 activation (Figure 1B), indicating that Stat3 was activated sufficiently to induce gene expression. To assess the functional role of Stat3 in limiting TLR2-induced inflammatory cytokine production, we used RNA interference

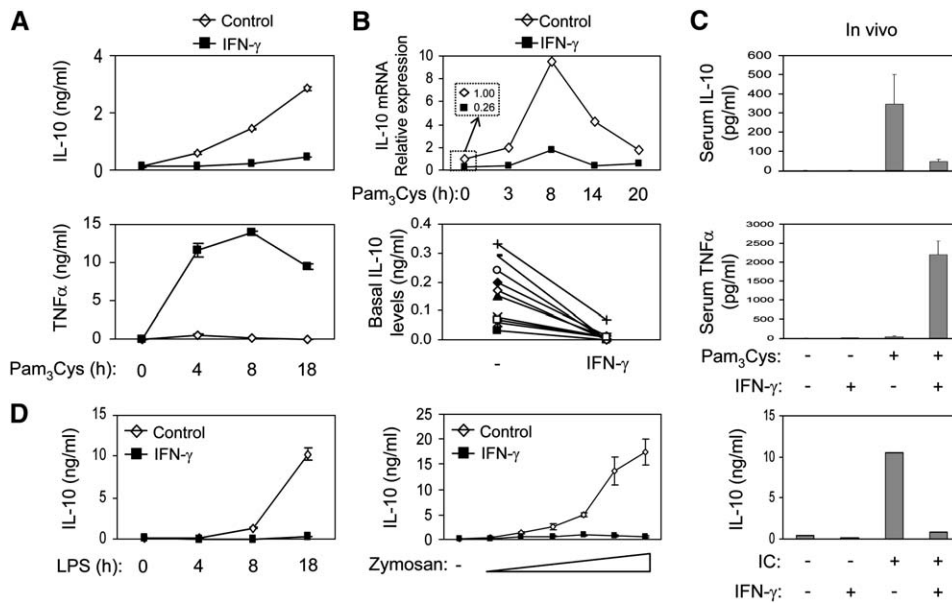


Figure 2. IFN- $\gamma$  Inhibits IL-10 Expression In Vitro and In Vivo

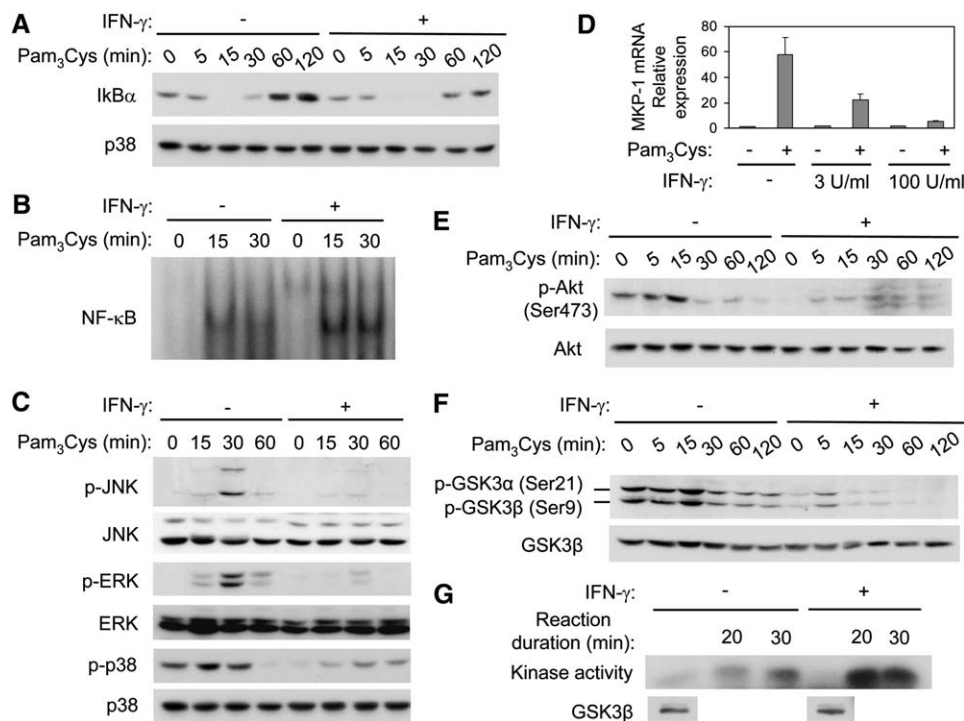
(A) Control or IFN- $\gamma$ -activated human macrophages were stimulated with 10 ng/ml of Pam<sub>3</sub>Cys for indicated periods of time, and levels of IL-10 and TNF $\alpha$  in culture supernatants were determined by ELISA.  
(B) Top: IL-10 mRNA levels were assessed by real-time PCR. Bottom: basal IL-10 protein levels in culture supernatants, prior to Pam<sub>3</sub>Cys addition, are shown. Each line represents an individual experiment performed with an independent blood donor.  
(C) 6 week old C57BL/6J mice were injected intraperitoneally with 4  $\mu$ g of IFN- $\gamma$  followed by injection of 100  $\mu$ g of Pam<sub>3</sub>Cys. 1.5 hr post Pam<sub>3</sub>Cys injection, serum levels of IL-10 and TNF $\alpha$  were determined by ELISA (mean  $\pm$  SD of four mice in each treatment group). Data represent two independent experiments.  
(D) Control or IFN- $\gamma$ -activated human macrophages were stimulated with 10 ng/ml of LPS for indicated periods of time (left), increasing doses of zymosan for 18 hr (middle), or 75  $\mu$ g/ml of immune complexes (IC) for 18 hr (right). IL-10 levels in culture supernatants were determined by ELISA.

(RNAi) to “knock down” Stat3 expression. Pam<sub>3</sub>Cys induced higher amounts of TNF $\alpha$  in cells that expressed low levels of Stat3 (Figure 1C). These results demonstrate a role for Stat3 in feedback inhibition of TLR2-induced cytokine production.

The delayed activation of Stat3 suggested that TLR2 activated Stat3 indirectly, via autocrine action of cytokines induced by TLR2. Experiments using neutralizing antibodies showed that TLR2 activation of Stat3 in control cells was predominantly dependent upon IL-10, whereas endogenous IL-6 made a minimal contribution to Stat3 activation (Figure 1D). To confirm the role of endogenous IL-10 in suppressing TLR2-induced TNF $\alpha$  production, we used anti-IL-10 neutralizing antibodies. Blocking endogenous IL-10 resulted in a substantial enhancement of TNF $\alpha$  production (Figure 1E), consistent with previous reports of the role of autocrine IL-10 in regulating TLR function (Berg et al., 1995). Taken together, the results show that IL-10 and Stat3 mediate feedback inhibition of TLR2-induced TNF $\alpha$  production. Compared with TLR2, TLR4 induced higher levels of TNF $\alpha$ , with an incremental increase in TNF $\alpha$  production when IL-10 was neutralized (Figure S1).

We investigated the effects of IFN- $\gamma$  on TLR2-induced Stat3 activation. Surprisingly, TLR2-dependent induction of Stat3 tyrosine phosphorylation was abolished in IFN- $\gamma$ -activated macrophages (Figure 1A, lane 4), as was induction of SOCS3 expression (Figure 1B). These experiments suggested that IFN- $\gamma$  suppressed either the production of IL-10 or the ability of IL-10 to activate Stat3. We then used ELISA to measure IL-10 levels in cul-

ture supernatants of TLR2-stimulated control or IFN- $\gamma$ -activated macrophages. As expected, TLR2 stimulation induced the time-dependent production of substantial amounts of IL-10 in control cultures (Figure 2A, top). In striking contrast, TLR2 stimulation of IL-10 production was strongly suppressed in IFN- $\gamma$ -activated macrophages (Figure 2A, top). Concomitant with diminished IL-10 production, Pam<sub>3</sub>Cys induced dramatically higher levels of TNF $\alpha$  in IFN- $\gamma$ -activated macrophages (Figure 2A, bottom). These results were highly reproducible among 25 experiments using macrophages derived from independent blood donors. In parallel with suppression of IL-10 protein production, IFN- $\gamma$  strongly suppressed TLR2-induced expression of IL-10 mRNA (Figure 2B, top). Interestingly, IFN- $\gamma$  suppressed basal (pre-TLR2 stimulation) IL-10 mRNA expression (Figure 2B, top); basal IL-10 mRNA expression was suppressed by an average of 80% in six independent experiments ( $p < 0.0001$  by paired Student's  $t$  test). Basal levels of IL-10 protein in culture supernatants were also suppressed by IFN- $\gamma$  (Figure 2B, bottom). In addition, IFN- $\gamma$  inhibited induction of IL-10 by Pam<sub>3</sub>Cys in vivo, with a concomitant increase in TNF $\alpha$  production (Figure 2C), thus recapitulating the reciprocal regulation of TNF $\alpha$  and IL-10 by IFN- $\gamma$  that was observed in cultured cells (Figure 2A). We wished to test whether IFN- $\gamma$  suppressed induction of IL-10 production by receptors other than TLR2. IFN- $\gamma$  effectively inhibited induction of IL-10 by repurified LPS, zymosan, and immune complexes that activate, respectively, TLR4, TLR2 and Dectin-1, and Fc $\gamma$ Rs (Akira and Takeda, 2004; Ravetch and Bolland, 2001; Rogers



**Figure 3. IFN- $\gamma$  Inhibits MAPK and PI3K Pathways and Increases GSK3 Activity**

Control or IFN- $\gamma$ -activated human macrophages were stimulated with 100 ng/ml of Pam<sub>3</sub>Cys, and cell extracts and mRNA were prepared. (A–C) Extracts were analyzed by immunoblotting (A and C) and EMSA with an NF- $\kappa$ B binding radiolabeled oligonucleotide (B) P = phosphorylated. (D) mRNA levels of MKP-1 were determined by real-time PCR. (E) Cell extracts were analyzed for p-Akt (ser473) and Akt protein levels by immunoblotting. (F) Cell extracts were assayed for both p-GSK and total GSK3 $\beta$  levels by immunoblotting. (G) GSK3 kinase assays with cell extracts from control and IFN- $\gamma$ -activated human macrophages were performed as described in [Experimental Procedures](#). One experiment representative of three is shown.

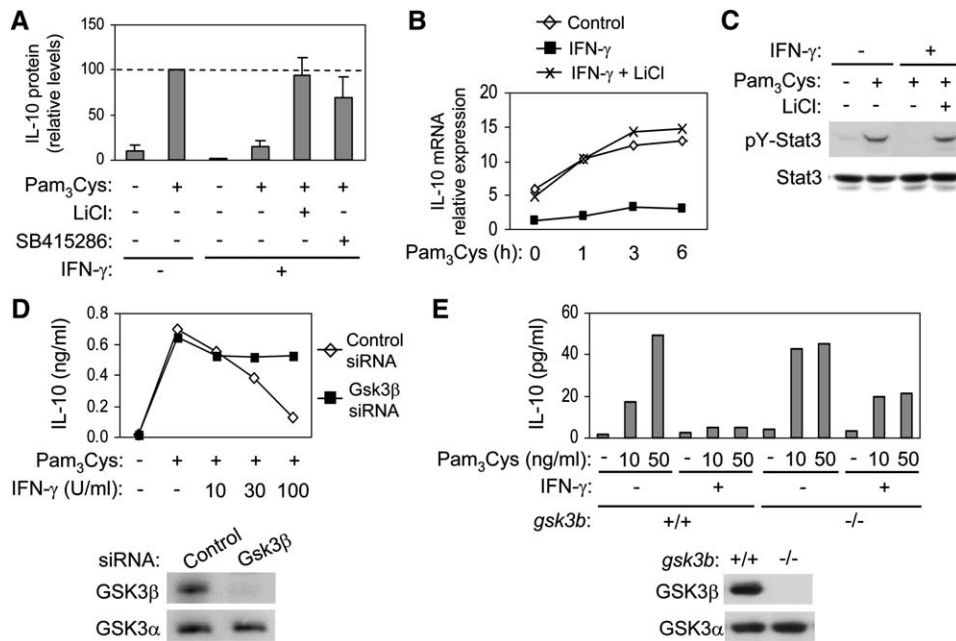
[et al., 2005](#)) (Figure 2D). Taken together, the results demonstrate that IFN- $\gamma$  abrogates IL-10-Stat3-mediated feedback inhibition by inhibiting induction of IL-10 by multiple receptors.

#### IFN- $\gamma$ Suppresses TLR2 Activation of MAPKs and Akt and Increases GSK3 Activity

We reasoned that IFN- $\gamma$  may disrupt a TLR2-induced signal that is required for IL-10 expression and thus investigated the effects of preactivating macrophages with IFN- $\gamma$  on TLR2 signaling. TLR2 signaling results in the downstream activation of NF- $\kappa$ B, MAPKs (ERKs, JNKs, p38), and PI3K (reviewed in [Akira and Takeda, 2004](#)). MAPK and PI3K activation is required for induction of IL-10 production by TLRs ([Foey et al., 1998](#); [Ma et al., 2001](#); [Martin et al., 2003](#)), and PI3K has been shown to suppress TNF $\alpha$  production by a mechanism that has not yet been clarified ([Fukao and Koyasu, 2003](#)). TLR2-induced activation of NF- $\kappa$ B, as assessed by degradation of I $\kappa$ B $\alpha$ , was comparable in control and IFN- $\gamma$ -activated macrophages, although resynthesis of I $\kappa$ B $\alpha$  was diminished in IFN- $\gamma$ -activated macrophages in some experiments (Figure 3A and data not shown). Activation of nuclear NF- $\kappa$ B DNA binding activity was comparable or slightly enhanced in IFN- $\gamma$ -activated relative to control macrophages (Figure 3B). These results are consistent with a mostly preserved activation of NF- $\kappa$ B and a dispensable role for NF- $\kappa$ B in driving IL-10 expression ([Moore et al., 2001](#)). Next, we investigated the

effects of IFN- $\gamma$  on Pam<sub>3</sub>Cys/TLR2 activation of MAPK pathways. As expected, TLR2 activated ERKs, JNKs, and p38 rapidly and transiently (10–60 min) in control macrophages (Figure 3C). In contrast, activation of ERKs, JNKs, and p38 was suppressed in IFN- $\gamma$ -primed cells (Figure 3C). We were surprised by this result, but diminished TLR2-induced MAPK activation has been consistently detected in over 15 independent experiments and could not be explained by increased degradation of TRAF6 in IFN- $\gamma$ -activated cells, as was previously described in RANK signaling ([Takayanagi et al., 2000](#)) (data not shown). We obtained the following additional evidence to support the validity of this result: (1) Pam<sub>3</sub>Cys induction of a MAPK-dependent gene, MAP kinase phosphatase 1 (MKP-1) ([Sun et al., 1993](#)), was diminished in IFN- $\gamma$ -activated macrophages (Figure 3D), consistent with diminished MAPK activation in IFN- $\gamma$ -activated cells; (2) MAPK activation by LPS was readily detected in IFN- $\gamma$ -activated cells ([Hu et al., 2005a](#)), confirming that we were able to detect MAPK signals and demonstrating differences in MAPK activation by LPS (which activates Trif-dependent in addition to MyD88-dependent signaling pathways) and Pam<sub>3</sub>Cys in IFN- $\gamma$ -activated macrophages. The residual p38 and JNK activity in IFN- $\gamma$ -activated, Pam<sub>3</sub>Cys-stimulated macrophages (Figure 3C, lanes 5–8) was sufficient for the TNF $\alpha$  production that was observed in Figure 2A as this TNF $\alpha$  production was reduced by the p38 inhibitor SB203580 and the JNK inhibitor SP600215 (Figure S2).





**Figure 4. IFN- $\gamma$  Inhibition of TLR2-Induced IL-10 Production Is Dependent on GSK3**

(A) Human macrophages treated with or without 100 U/ml of IFN- $\gamma$  and the GSK3 inhibitors LiCl (20 mM) and SB415286 (10  $\mu$ M) were stimulated with 10 ng/ml of Pam<sub>3</sub>Cys for 18 hr. Results are means  $\pm$  SD of five independent experiments.

(B) Human macrophages were pretreated with IFN- $\gamma$  and LiCl as in (A) prior to 10 ng/ml of Pam<sub>3</sub>Cys stimulation. IL-10 mRNA expression was measured by real-time PCR.

(C) Human macrophages were pretreated with IFN- $\gamma$  and LiCl as in (A), stimulated with 100 ng/ml of Pam<sub>3</sub>Cys for 6 hr, and cell extracts were subjected to immunoblotting.

(D) Primary human macrophages transfected with control or GSK3 $\beta$ -specific short interfering RNAs were treated with IFN- $\gamma$  and Pam<sub>3</sub>Cys as in (A) and IL-10 was measured by ELISA.

(E) Bone marrow-derived murine macrophages were generated from chimeric C57BL/6 mice that had been reconstituted with wild-type or gsk3b<sup>-/-</sup> fetal liver cells. Cells were treated with IFN- $\gamma$  (100 U/ml) overnight, stimulated for 18 hr with Pam<sub>3</sub>Cys, and IL-10 protein concentrations in culture supernatants were determined by ELISA. A representative experiment is shown; mean inhibition was 5-fold in control cells and 2-fold in gsk3b<sup>-/-</sup> cells (n = 4).

Overall, the results show that IFN- $\gamma$  specifically suppressed the rapid and transient activation of MAPKs by Pam<sub>3</sub>Cys/TLR2.

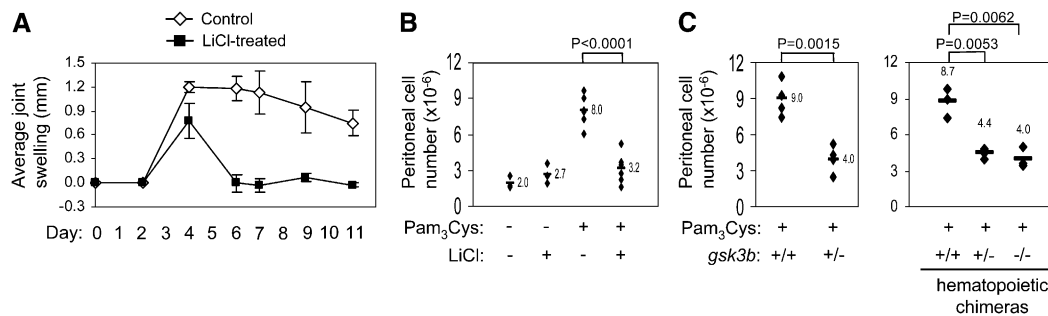
Next, we assessed activation of the PI3K-Akt pathway by analyzing the phosphorylation of Akt downstream of PI3K. In control macrophages, Akt was phosphorylated at baseline, and phosphorylation increased, as predicted (Arbibe et al., 2000), after stimulation with Pam<sub>3</sub>Cys (Figure 3E). In contrast, basal phosphorylation of Akt was diminished in IFN- $\gamma$ -activated macrophages, and the induction of phosphorylation by TLR2 was suppressed (Figure 3E). We then determined the role of the PI3K-Akt pathway in regulation of IL-10 production in our system. Inhibition of Akt suppressed IL-10 production (Figure 3A), as did deficiency of PI3K subunit p85 $\alpha$  (Figure S3B). Thus, macrophage IL-10 production was dependent on the PI3K-Akt pathway.

Akt regulates the activity of GSK3, which, in turn, regulates the function of the AP-1 and CREB transcription factors important for IL-10 production (Boyle et al., 1991; Bullock and Habener, 1998; Cross et al., 1995; Fiol et al., 1994; Grimes and Jope, 2001). Therefore, we investigated the regulation of GSK3 by IFN- $\gamma$  and TLR2. Consistent with the basal activity of Akt (Figure 3E), GSK3 $\alpha$  and GSK3 $\beta$  were phosphorylated at baseline on the inactivating residues, serine-21 and serine-9, respectively, in control macrophages, and phos-

phorylation increased transiently after treatment with Pam<sub>3</sub>Cys (Figure 3F). IFN- $\gamma$  strongly suppressed basal GSK3 serine phosphorylation and inhibited TLR2-induced phosphorylation (Figure 3F). Since phosphorylation of serine-21 and serine-9 is inhibitory for GSK3 activity, these results suggested that IFN- $\gamma$  treatment increased GSK3 activity. Indeed, GSK3 $\beta$  kinase activity was substantially higher in IFN- $\gamma$ -activated than in control macrophages (Figure 3G). Overall, the results indicate that IFN- $\gamma$  treatment of macrophages suppressed MAPK and PI3K-Akt signaling pathways downstream of TLR2, with a concomitant increase in GSK3 activity.

#### IFN- $\gamma$ Inhibition of TLR2-Induced IL-10 Production Is Partially Mediated by GSK3

We considered GSK3 as a good candidate regulator of IL-10 production because GSK3 regulates the function of the AP-1 and CREB transcription factors that are important for IL-10 production (Boyle et al., 1991; Bullock and Habener, 1998; Cross et al., 1995; Fiol et al., 1994; Grimes and Jope, 2001). Therefore, we investigated the role of GSK3 in mediating the regulation of IL-10 production by IFN- $\gamma$  and TLR2. Inhibition of GSK3 activity with two different GSK3 inhibitors that work by different mechanisms, LiCl (Stambolic et al., 1996) and SB415286 (Smith et al., 2001), reversed IFN- $\gamma$ -mediated suppression of IL-10 protein and mRNA production (Figures 4A



**Figure 5. Inhibition or Deficiency of GSK3 In Vivo Attenuates TLR2-Dependent Arthritis and Peritonitis**

(A) Collagen-antibody-induced arthritis was induced in 6 week old C57BL/6J mice as described in [Experimental Procedures](#). 3 mM of LiCl was present in the drinking water of the LiCl-treated group from day 2. In addition, LiCl was injected i.p. at the dose of 50  $\mu$ mol/mouse daily beginning on day 2. The data represent mean  $\pm$  SD of four mice in each of two independent experiments.

(B) 6 week old C57BL/6J mice were i.p. injected with 100  $\mu$ g of Pam<sub>3</sub>Cys. Starting from the day of Pam<sub>3</sub>Cys injection, half of the injected mice were fed LiCl-containing drinking water (3 mM), and LiCl was injected i.p. at the dose of 50  $\mu$ mol/mouse daily. 2 days after Pam<sub>3</sub>Cys administration, peritoneal cells were harvested and counted. The results are derived from three to six mice in each group and are representative of two independent experiments. P value was calculated by unpaired Student's t test.

(C) Peritonitis was induced as in (B) by i.p. injection of 100  $\mu$ g of Pam<sub>3</sub>Cys per mouse. Left: peritonitis in 6 week old *gsk3b*<sup>+/-</sup> and wild-type littermate control mice (n = 4 in each group). Right: peritonitis in hematopoietic chimeras reconstituted with *gsk3b*<sup>+/+</sup>, *gsk3b*<sup>+/-</sup>, or *gsk3b*<sup>-/-</sup> fetal liver cells (n = 3 in each group). P value was calculated by unpaired Student's t test.

and 4B), resulting in the recovery of TLR2-induced Stat3 activation ([Figure 4C](#)). Consistent with relatively low basal GSK3 activity in human macrophages in our system ([Figures 3F and 3G](#)), inhibition of GSK3 in control macrophages resulted in modest superinduction of IL-10 production that was not observed in all blood donors (data not shown). To obtain additional evidence supporting a role for GSK3 in negative regulation of IL-10 production in primary human macrophages, we used RNA interference to knock down GSK3 $\beta$  expression ([Figure 4D](#)). Inhibition of TLR2-induced IL-10 production by IFN- $\gamma$  was strongly attenuated in macrophages expressing low levels of GSK3 $\beta$  ([Figure 4D](#)). In addition, we examined the regulation of IL-10 production in murine macrophages deficient in GSK3 $\beta$ . Consistent with basal GSK3 activity and its transient inactivation by TLR2 ([Martin et al., 2005](#)), higher levels of IL-10 were produced by GSK3 $\beta$ -deficient cells when subsaturating concentrations of Pam<sub>3</sub>Cys were used ([Figure 4E](#), bars 2 and 8). Strong suppression of IL-10 production by IFN- $\gamma$  was observed in control cells and was attenuated in GSK3 $\beta$ -deficient cells (mean 5-fold suppression in control cells versus 2-fold suppression in GSK3 $\beta$ -deficient cells [n = 4]), further supporting a role for GSK3 in mediating IFN- $\gamma$  suppression of IL-10 ([Figure 4E](#)). The partial effect of GSK3 $\beta$ -deficiency suggests that IFN- $\gamma$  also activates a GSK3 $\beta$ -independent mechanism of suppression of IL-10 production, and this will be further addressed below. These results establish GSK3 $\beta$  as a negative regulator of IL-10 and demonstrate that GSK3 $\beta$  mediates at least in part the inhibitory effects of IFN- $\gamma$  on IL-10 production. The results suggest that levels of IL-10 production are determined by the reciprocal regulation of GSK3 by IFN- $\gamma$  and TLR2 that was observed in [Figure 3F](#).

#### Role of GSK3 in TLR2-Induced Peritonitis and Arthritis

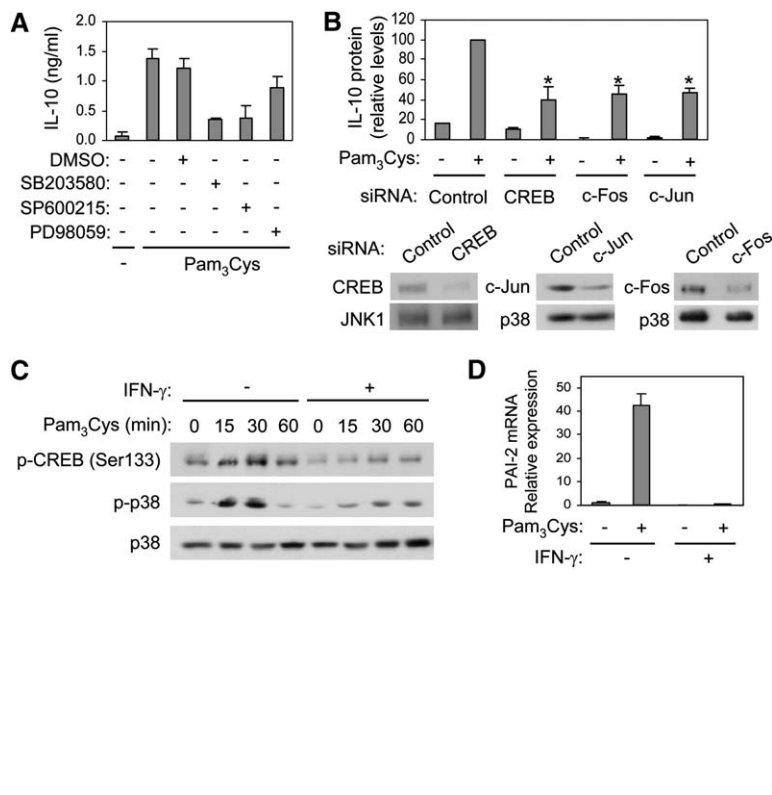
The key role of GSK3 in suppressing IL-10 production and thereby modulating the balance of pro- and anti-in-

flammatory cytokines led us to test the effects of inhibiting GSK3 on TLR2-induced inflammation in vivo in murine models of arthritis and peritonitis. Collagen antibody-induced arthritis (CAIA) is an established model of the effector phase of inflammatory arthritis ([Terato et al., 1992](#)). In this model, TLR-dependent activation of inflammatory cytokine production is required to induce arthritis in mice that also have been injected with anti-collagen antibodies. Inhibition of GSK3 strongly suppressed TLR2-dependent arthritis, as assessed by joint swelling ([Figure 5A](#)).

Inhibition of GSK3 activity in vivo significantly ameliorated Pam<sub>3</sub>Cys-induced peritonitis as evidenced by reduced inflammatory exudates ([Figure 5B](#)). We wished to test the effects of GSK3 deficiency on inflammatory responses in vivo. GSK3 $\alpha$ -deficient mice are not available, and GSK3 $\beta$  deficiency is embryonal lethal, but an in vivo phenotype was observed in heterozygous *gsk3b*<sup>+/-</sup> mice ([Hoefflich et al., 2000](#)). Therefore, we examined the effects of GSK3 $\beta$  haploinsufficiency on TLR2-dependent peritonitis. Pam<sub>3</sub>Cys-induced inflammatory peritoneal exudates were significantly reduced in *gsk3b*<sup>+/-</sup> mice ([Figure 5C](#)). We then used an adoptive transfer approach to determine whether the role of GSK3 $\beta$  in peritonitis was dependent on GSK3 $\beta$  expression in bone marrow-derived cells. Irradiated control C57BL/6 mice were reconstituted with fetal liver cells derived from *gsk3b*<sup>+/+</sup>, *gsk3b*<sup>+/-</sup>, and *gsk3b*<sup>-/-</sup> embryos. Pam<sub>3</sub>Cys-induced peritonitis was significantly attenuated in chimeric mice reconstituted with *gsk3b*<sup>+/-</sup> or *gsk3b*<sup>-/-</sup> hematopoietic cells ([Figure 5C](#)). These results indicate that GSK3 $\beta$  is an important regulator of inflammation in vivo and suggest that GSK3 is a potential therapeutic target for inflammatory disorders.

#### IFN- $\gamma$ Suppresses Activation of CREB

CREB and AP-1 transcription factors are important mediators of IL-10 production ([Agrawal et al., 2003; Dillon et al., 2004; Martin et al., 2005; Platzer et al., 1999; Wang et al., 2005](#)) that are activated by MAPKs and



**Figure 6.** Role of MAPKs and CREB/AP-1 in IL-10 Production and Suppression of CREB by IFN- $\gamma$

(A) Control human macrophages were stimulated with Pam<sub>3</sub>Cys for 6 hr in the presence or absence of the p38 inhibitor SB203580 (10  $\mu$ M), JNK inhibitor SP600215 (10  $\mu$ M), and the MEK-ERK inhibitor PD98059 (20  $\mu$ M). IL-10 levels in culture supernatants were determined by ELISA.

(B) Primary human macrophages were transfected with control or CREB-, c-Fos-, and c-Jun-specific short interfering RNAs, stimulated with Pam<sub>3</sub>Cys (10 ng/ml) for 18 hr, and IL-10 in culture supernatants was measured by ELISA. Results are presented as mean  $\pm$  SD of three independent experiments. Results obtained with two different control siRNAs were comparable. siRNA-mediated protein knockdown from one representative experiment is shown. Asterisk,  $p < 0.05$  by paired Student's  $t$  test.

(C) Primary human macrophages were treated overnight with IFN- $\gamma$  (100 U/ml), stimulated with Pam<sub>3</sub>Cys, and phosphorylation of p38 and CREB (serine-133) was measured by immunoblotting.

(D) RNA was extracted from human macrophages that had been treated with IFN- $\gamma$  overnight and stimulated with 10 ng/ml of Pam<sub>3</sub>Cys for 6 hr, and mRNA levels of the CREB-dependent gene PAI-2 were measured by qPCR and normalized relative to GAPDH.

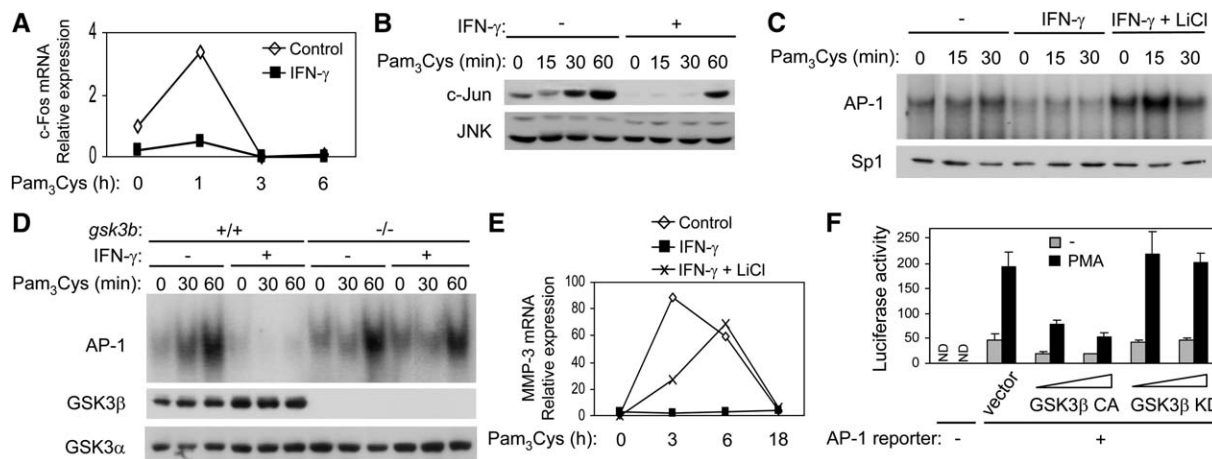
inhibited by GSK3 (Boyle et al., 1991; Bullock and Habener, 1998; Grimes and Jope, 2001; Shaulian and Karin, 2001). Since IFN- $\gamma$  inhibited MAPK activation and increased GSK3 activity, we investigated the effects of IFN- $\gamma$  on CREB and AP-1 proteins. First, we established the role of MAPKs, CREB, and AP-1 in IL-10 production in our system. Inhibition of p38, JNK, and to a lesser extent MEK-ERKs, diminished TLR2-induced IL-10 production (Figure 6A). RNAi-mediated knockdown of CREB, Fos, and Jun expression also resulted in diminished TLR2-induced IL-10 production (Figure 6B). These results confirm that MAPKs and CREB/AP-1 proteins regulate IL-10 production in our system, similar to their roles in IL-10 production in cells stimulated with different TLR ligands (Agrawal et al., 2003; Dillon et al., 2004; Foey et al., 1998; Lucas et al., 2005; Ma et al., 2001; Martin et al., 2005; Platzer et al., 1999; Wang et al., 2005; Yi et al., 2002).

Recent work from M. Karin's laboratory has demonstrated that TLR activation of CREB by phosphorylation of serine-133 is dependent on p38 (Park et al., 2005), which we confirmed in our system by using the p38 inhibitor SB203580 (data not shown). Since IFN- $\gamma$  inhibited TLR2 activation of p38, we investigated the effects of IFN- $\gamma$  on activation of CREB. IFN- $\gamma$  suppressed TLR2-induced phosphorylation of CREB on serine-133, concomitant with suppression of p38 activation (Figure 6C). Inhibition of CREB activation by IFN- $\gamma$  suppressed TLR2 activation of the CREB-dependent gene plasminogen activator 2 (PAI-2) (Park et al., 2005) (Figure 6D). Taken together, these results suggest that IFN- $\gamma$  suppresses TLR2-induced IL-10 production in part by suppressing p38-dependent activation of CREB.

### IFN- $\gamma$ and GSK3 Suppress AP-1 Expression and Function

ERKs and JNK phosphorylate, respectively, Fos and Jun, thereby activating their transcriptional function and also increasing their expression (Shaulian and Karin, 2001). We next investigated the effects of IFN- $\gamma$  on Fos and Jun. Interestingly, IFN- $\gamma$  strongly suppressed basal and TLR2-inducible expression of c-Fos mRNA ( $p < 0.005$  from three separate experiments by paired Student's  $t$  test) (Figure 7A) in a manner that correlated with suppression of IL-10 expression (Figures 2A and 2B). IFN- $\gamma$  also suppressed the expression and nuclear accumulation of Jun, AP-1 DNA binding activity, and TLR2-induced expression of the well-established AP-1 target genes MMP-3 (Hu et al., 1994) and MKP-1 (Figures 7B–7E and Figure 3D). Thus, IFN- $\gamma$  inhibited AP-1 expression and function in macrophages, and suppressed AP-1 activity explains the diminished expression of genes regulated directly or indirectly by AP-1, including IL-10, that was observed in IFN- $\gamma$ -activated macrophages.

GSK3 was previously reported to downregulate Jun DNA binding (Boyle et al., 1991), and we wished to address the role of GSK3 in mediating the inhibitory effects of IFN- $\gamma$  on AP-1 proteins. Inhibition of GSK3 reversed IFN- $\gamma$ -mediated suppression of AP-1 DNA binding, with concomitant re-expression of the AP-1 target genes MMP-3 (Figures 7C and 7E) and MKP-1 (data not shown). In addition, IFN- $\gamma$  no longer suppressed AP-1 DNA binding in GSK3 $\beta$ -deficient macrophages (Figure 7D). Consistent with this result and previous reports (Boyle et al., 1991; de Groot et al., 1993; Nikolakaki et al., 1993), GSK3 effectively suppressed expression of an AP-1-driven reporter gene (Figure 7F). Taken



**Figure 7. IFN- $\gamma$  and GSK3 Suppress AP-1 Expression and Function**

(A) Control and IFN- $\gamma$ -activated human primary macrophages were stimulated with 10 ng/ml of Pam<sub>3</sub>Cys. c-Fos mRNA expression was determined by real-time PCR.  
(B) c-Jun expression in nuclear extracts from human primary macrophages was assessed by immunoblotting.  
(C) Nuclear extracts from human primary macrophages were subjected to EMSA with a radiolabeled AP-1 binding oligonucleotide. The same extracts were analyzed by immunoblotting with Sp1 antibodies.  
(D) Bone marrow-derived murine macrophages were generated from chimeric C57BL/6 mice that had been reconstituted with *gsk3b*<sup>+/+</sup> or *gsk3b*<sup>-/-</sup> fetal liver cells. Cells were treated with IFN- $\gamma$  (100 U/ml) overnight, stimulated with Pam<sub>3</sub>Cys, and AP-1 DNA binding activity was measured by EMSA. The same extracts were analyzed by immunoblotting with GSK3 antibodies.  
(E) MMP-3 mRNA expression in human macrophages was determined by real-time PCR.  
(F) 293T cells were cotransfected in triplicate with an AP-1 reporter construct and GSK3 $\beta$  expression plasmids (CA, constitutive active; KD, kinase dead). 24 hr post transfection, cells from each transfection were split and stimulated with DMSO control or 100 ng/ml of PMA for 4 hr, and cell lysates were analyzed for luciferase activity. Results from a representative experiment out of three are shown.

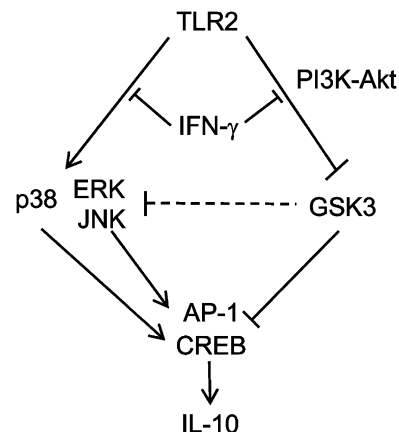
together, the results clearly establish a role for GSK3 $\beta$  in mediating the inhibitory effects of IFN- $\gamma$  on AP-1 DNA binding and downstream IL-10 expression.

## Discussion

In this study, we have discovered that IFN- $\gamma$  suppresses macrophage production of the anti-inflammatory cytokine IL-10 by regulating the function of GSK3 and of CREB/AP-1 transcription factors. IFN- $\gamma$  increased the activity of GSK3, which we established as a negative regulator of IL-10 production, and diminished the activity and expression of CREB and AP-1 proteins that are positive regulators of IL-10 production (Agrawal et al., 2003; Dillon et al., 2004; Martin et al., 2005; Platzer et al., 1999; Wang et al., 2005) (Figure 8). Our results provide the first link between IFN- $\gamma$  and GSK3 and CREB/AP-1, and yield insights into mechanisms of negative regulation of IL-10 production by IFN- $\gamma$ . The role of GSK3 in regulating cytokine production is physiologically significant, as inhibition or deletion of GSK3 attenuated inflammatory arthritis and peritonitis in vivo. The results also reveal mechanisms by which IFN- $\gamma$  inhibits gene expression and suggest that suppression of CREB- and AP-1-dependent genes is part of the IFN- $\gamma$  response of macrophages.

Our results support a model whereby IFN- $\gamma$  specifically inhibits TLR2-induced signaling via the MAPK and PI3K-Akt pathways (Figure 8). The direct targets that are inhibited by IFN- $\gamma$  remain to be identified, and we will investigate the effects of IFN- $\gamma$  on proximal TLR2-induced signaling events in future work. One significant consequence of the inhibition of PI3K-Akt signaling is the release of GSK3 from inhibition (Figure 8).

Active GSK3 then suppresses AP-1 DNA binding, which was previously shown to occur by GSK3-dependent phosphorylation of Jun on threonine 239, serine 243, and serine 249 in a region proximal to the DNA binding domain (Boyle et al., 1991). GSK3 can also contribute to inhibition of CREB (Bullock and Habener, 1998; Grimes and Jope, 2001). We have provided biochemical, pharmacological, and genetic evidence that GSK3 plays a key role in mediating the suppression of IL-10



**Figure 8. Model for Regulation of IL-10 Production by IFN- $\gamma$  and GSK3**

IFN- $\gamma$  inhibits TLR2-mediated activation of MAPK and PI3K-Akt signaling pathways, resulting in diminished p38, JNK, and ERK activity, and increased activity of GSK3. GSK3 suppresses AP-1 function by suppressing DNA binding. Diminished MAPK activity results in decreased activation of CREB (by p38) and decreased transcriptional activity and expression of Fos and Jun.



production by IFN- $\gamma$ . These results link the IFN- $\gamma$  regulation of TLR2 signaling and GSK3 activity with IL-10 production and provide mechanistic insight into the regulation of IL-10 expression. The regulation of IL-10 production by GSK3 downstream of PI3K and Akt provides a mechanism for the previously described negative regulation of macrophage inflammatory cytokine production by the PI3K pathway (Fang et al., 2004; Fukao et al., 2002; Guha and Mackman, 2002).

A second consequence of IFN- $\gamma$ -mediated alteration of TLR2 signaling is suppression of activation of the p38, JNK, and ERK MAPKs (Figure 8) that play a key role in induction of IL-10 expression (Foey et al., 1998; Lucas et al., 2005; Ma et al., 2001; Yi et al., 2002). Because p38 is a major activator of CREB downstream of TLRs (Park et al., 2005; X.H., unpublished data), suppression of p38 activation results in diminished CREB activity and IL-10 expression. Diminished p38 activity will also impact on other p38-dependent transcription factors important for IL-10 expression, such as Sp1 (Ma et al., 2001). JNK and ERK are major inducers of the transcriptional activity and expression of, respectively, Jun and Fos (Shaulian and Karin, 2001; Davis, 2000). Therefore, IFN- $\gamma$  suppression of JNK and ERK activation leads to decreased activity and expression of Jun and Fos and thus diminished production of IL-10 (Figure 8). MAPK-mediated phosphorylation of Jun that regulates its transcriptional activity occurs on different sites (serines 63 and 73) than GSK3-dependent phosphorylation of threonine 239 and serines 243 and 349 that regulates DNA binding. Thus, IFN- $\gamma$  suppressed AP-1 by two mechanisms—diminished activation by MAPKs and GSK3-dependent inhibition of DNA binding. Because Fos is a negative regulator of IL-12 production (Dillon et al., 2004, and references therein), it is possible that IFN- $\gamma$  inhibition of Fos regulates IL-10 expression indirectly, by increasing IL-12 production. Experiments with IL-12 neutralizing antibodies excluded a role for IL-12 in regulating IL-10 in our system, where relatively low levels of IL-12 were expressed (X.H., unpublished data). However, addition of high concentrations of exogenous IL-12 suppressed IL-10 production. The role of IL-12 in regulating IL-10 production under experimental conditions in which high levels of endogenous IL-12 are produced will be explored in future work.

An interesting question is whether there is crosstalk between GSK3 and MAPK pathways downstream of TLR2 and the extent to which increased GSK3 activity contributes to IFN- $\gamma$  suppression of MAPKs (Figure 8, dotted line). Experiments using GSK3 $\beta$ -deficient cells and cells in which GSK3 $\beta$  expression was knocked down by RNAi showed a role for GSK3 in attenuating JNK and ERK activation downstream of TLR2 (Figure S4A and data not shown). IFN- $\gamma$  was able to suppress MAPK activation independently of GSK3 $\beta$ , but residual JNK and ERK activation was higher in IFN- $\gamma$ -treated GSK3 $\beta$ -deficient cells than in IFN- $\gamma$ -treated control cells (Figure S4A, lanes 6 and 12) and was comparable to activation in non-IFN- $\gamma$ -treated control cells (lane 3). Consistent with this result, inhibition of GSK3 in human macrophages partially (but reproducibly) attenuated inhibition of MAPKs by IFN- $\gamma$  at the 60 min time point (Figure S4B). These results support a relatively minor role for GSK3 $\beta$  in IFN- $\gamma$  regulation of MAPKs and sug-

gest that IFN- $\gamma$  inhibits TLR2 signaling upstream of GSK3 and MAPKs. IFN- $\gamma$  regulation of MAPKs independently of GSK3 provides a plausible explanation for the incomplete reversal of IFN- $\gamma$ -mediated suppression of IL-10 production in GSK3 $\beta$ -deficient cells, although we have not excluded a role for GSK3 $\alpha$ . Taken together, our results support a model whereby IFN- $\gamma$  suppresses IL-10 production by two mechanisms: (1) IFN- $\gamma$ -dependent suppression of MAPKs (leading to diminished CREB phosphorylation and AP-1 transcriptional activity) and (2) IFN- $\gamma$  activation of GSK3 (leading to diminished AP-1 DNA binding). There is some crosstalk between these two mechanisms because GSK3 modulates MAPK activation, but IFN- $\gamma$  can inhibit MAPK activation independently of GSK3 $\beta$ .

Mechanisms by which IFN- $\gamma$  activates macrophages have been intensively studied (Schroder et al., 2004). The most straightforward and best understood involve IFN- $\gamma$  activation of the expression of multiple genes encoding inflammatory factors and immune effector molecules. In addition, IFN- $\gamma$  renders macrophages hyperresponsive to other inflammatory stimuli and microbial products, such as TNF $\alpha$  and TLR ligands (Adams and Hamilton, 1984; Schroder et al., 2004). Our results highlight that suppression of IL-10 production by IFN- $\gamma$  contributes to its proinflammatory properties. Suppression of IL-10 production interrupts an IL-10-mediated Stat3-dependent feedback inhibition loop that is induced by TLR ligands, with the resultant increased production of inflammatory cytokines. While this manuscript was under preparation, Michalek and colleagues reported that TLR4 transiently inactivates GSK3, leading to increased IL-10 production mediated by enhanced activity of CREB, and that pharmacological inhibition of GSK3 protects mice from endotoxin shock (Martin et al., 2005). We have substantially extended these results by using a genetic approach to definitively establish a role for GSK3 in inflammatory responses *in vivo* and by showing that GSK3 is also regulated by IFN- $\gamma$ . GSK3 is regulated by several signaling pathways (including PI3K-Akt), and GSK3 activity is regulated in opposing directions by TLRs (transient inactivation) and IFN- $\gamma$  (increase in kinase activity). Modulation of GSK3 activity by IFN- $\gamma$  provides a mechanism by which IFN- $\gamma$  alters cellular responses to other factors that regulate GSK3. GSK3 is thus located at a nodal point that integrates signals from IFN- $\gamma$  and PRRs to regulate the balance between macrophage pro- and anti-inflammatory cytokine production. The results provide a mechanism for synergy between IFN- $\gamma$  and TLR ligands in macrophage activation and identify GSK3 as an attractive therapeutic target for inflammatory diseases.

IFN- $\gamma$  induces gene expression by activating the transcription factor Stat1 that binds to gene promoters and initiates a cascade of gene activation. Multiple IFN- $\gamma$ -induced Stat1-dependent genes have been identified and their immune and inflammatory functions characterized (Schroder et al., 2004). In contrast, the identity and immune function of genes whose expression is suppressed by IFN- $\gamma$  are less well established. There is no clear evidence that Stat1 can act directly as a transcriptional repressor, and mechanisms by which IFN- $\gamma$  suppresses gene expression are not known. Our results provide a mechanism by which IFN- $\gamma$  downregulates

gene expression in macrophages by suppressing the activation of CREB and the activity and expression of Fos and Jun. This suppression results not only in down-regulation of IL-10, but also of the CREB-dependent PAI-2 gene and the AP-1-dependent MKP-1 and MMP-3 genes. It is likely that IFN- $\gamma$  regulation of CREB and AP-1-dependent genes contributes more broadly to the IFN- $\gamma$ -activated macrophage phenotype, and this notion will be tested in future work.

## Experimental Procedures

### Cell Culture and Mice

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood from disease-free volunteers by density gradient centrifugation with Ficoll (Invitrogen, Carlsbad, CA). CD14<sup>+</sup> monocytes were purified from fresh PBMCs with anti-CD14 magnetic beads (Miltenyi Biotec, Auburn, CA), as recommended by the manufacturer. Purity of monocytes was greater than 97% as verified by FACS. Macrophages were derived by culturing monocytes in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Hyclone, Logan, UT) in the presence of 10 ng/ml of human macrophage colony stimulating factor (M-CSF) (Peprotech, Rocky Hill, NJ). IFN- $\gamma$ -activated macrophages were obtained by culturing monocytes with M-CSF and 100 U/ml of IFN- $\gamma$  (Roche, Indianapolis, IN) for 24 hr, unless otherwise noted. THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS. Murine bone marrow-derived macrophages were obtained as described (Hu et al., 2002) and maintained in DMEM supplemented with 20% FBS and 10 ng/ml of murine M-CSF (Peprotech). To generate hematopoietic chimeras, donor fetal livers were harvested from embryonic day 13.5 embryos of *gsk3b*<sup>+/-</sup>  $\times$  *gsk3b*<sup>+/-</sup> matings, and  $2 \times 10^6$  fetal liver cells were injected intravenously into each of the irradiated recipients. Recipient mice (6 week old C57/BL6 mice) were obtained from Jackson Laboratory (Bar Harbor, ME) and were lethally irradiated with 1100 rads (split dose of  $2 \times 550$  rads) on the day of transplantation. Chimeric mice were sacrificed 7 weeks post transplantation. The establishment of *gsk3b*<sup>-/-</sup> chimeras was confirmed by the absence of GSK3 $\beta$  protein expression in bone marrow-derived macrophages, alveolar macrophages, and peritoneal cells (Figure 4E and data not shown). The experiments with human and murine cells were approved by, respectively, the Hospital for Special Surgery Institutional Review Board and Institutional Animal Care and Use Committee.

### Reagents

Pam<sub>3</sub>Cys was purchased from EMC microcollections (Tuebingen, Germany) and was used at 10 ng/ml unless otherwise noted. Zymosan particles were from Molecular Probes (Eugene, OR). LPS, LiCl, SB415286, and the MAPK inhibitors SB203580, SP600215, and PD98059 were from Sigma-Aldrich (Milwaukee, WI). The p38 inhibitor SB203580 and the MEK-ERK inhibitor PD98059 were used as previously described (Ahmed and Ivashkiv, 2000; Sengupta et al., 1998) in which inhibition of kinase activity was monitored. Cell viability was comparable in control cells and cells treated with kinase inhibitors, as assessed by cell counts and annexin V and propidium iodide staining. Immune complexes were made as described with modifications (Nagarajan et al., 1995). Briefly, human IgG was mixed with goat anti-human IgG F(ab')<sub>2</sub> fragments at a molar ratio of 1:2. The mixture was incubated at 4°C overnight, and insoluble components were removed by centrifugation. The soluble materials were used as immune complexes.

### Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISAs) were performed with paired antibody sets, as recommended by the manufacturer (BD Pharmingen, San Diego, CA).

### Analysis of mRNA Levels

For real-time, quantitative PCR, DNA-free RNA was extracted with an RNeasy Mini kit (Qiagen, Valencia, CA), and 1  $\mu$ g of total RNA was reverse transcribed with a First Strand cDNA Synthesis kit (Fer-

mentas, Hanover, MD). Real-time PCR was performed in triplicate with the iCycler iQ thermal cycler and detection system (Biorad, Hercules, CA) and the PCR Core Reagents kit (Applied Biosystems, Foster City, CA). Relative expression was normalized for levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). When reverse transcriptase was omitted, threshold cycle number increased by at least ten, signifying lack of genomic DNA contamination or nonspecific amplification, and the generation of only the correct size amplification products was confirmed by agarose gel electrophoresis.

### Immunoblotting and Electrophoretic Mobility Shift Assay

Whole cell extracts and nuclear extracts were obtained, and protein levels quantitated by the Bradford assay (Biorad, Hercules, CA), as previously described (Hu et al., 2002). For immunoblotting, 10  $\mu$ g of cell lysates were fractionated on 7.5% or 10% polyacrylamide gels by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), incubated with specific antibodies, and enhanced chemiluminescence was used for detection. For AP-1 and NF- $\kappa$ B DNA binding assays, 5  $\mu$ g of nuclear extracts were incubated for 15 min at room temperature with 0.5 ng of <sup>32</sup>P-labeled double stranded AP-1 or NF- $\kappa$ B binding oligonucleotide (Ivashkiv et al., 1990) in the presence of 2  $\mu$ g of poly-dI-dC (Pharmacia), and complexes were resolved on nondenaturing 4.5% polyacrylamide gels.

### RNA Interference

Prevalidated siRNAs and nontargeting control siRNAs were purchased from Dharmacon (Lafayette, CO). siRNAs were transfected into primary human macrophages with the Amaxa Nucleofector device set to program Y-001 with the Human Monocyte Nucleofector kit (Amaxa, Cologne, Germany). For Stat3 RNA interference (RNAi) in THP-1 cells, lentiviral particles containing the sequence 5'-AGT CAGGTTGCTGGTCAAA-3' that targeted Stat3 were generated as described (Hu et al., 2005b). Lentiviral particles encoding interfering RNA against red fluorescence protein DSR2 were used as control for Stat3 RNAi experiments. THP-1 cells were incubated overnight with recombinant lentiviral particles at a ratio of 1:50 in the presence of 4  $\mu$ g/ml polybrene. The efficiency of transduction was evaluated by flow cytometry and fluorescence microscopy to monitor eGFP expression and was typically >90%.

### Immunoprecipitation and GSK3 Kinase Assay

$10^7$  human macrophages were lysed in the lysis buffer as described (Hu et al., 2002). Cell lysates were incubated with polyclonal anti-GSK3 $\beta$  antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 hr and then incubated with protein A/G agarose beads (Santa Cruz Biotechnology) for 1 hr at 4°C. The immunoprecipitates were pelleted and washed three times with cold lysis buffer and twice with cold kinase buffer (25 mM HEPES, 10 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol). The kinase reactions were performed at 30°C for periods as noted in figure legends in the presence of a GSK3 peptide substrate (Upstate Biotechnology, Charlottesville, VA), 10  $\mu$ M of ATP, and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. The reactions were terminated by adding an equal volume of tricine sample buffer (Biorad). The mixture was boiled and loaded onto 16.5% Tris-Tricine gels (Biorad).

### Collagen-Antibody-Induced Arthritis

Arthritis was induced in 6 week old C57BL/6 mice by intraperitoneal (i.p.) administration of 4 mg of Arthrogen-CIA arthritis inducing monoclonal antibody cocktail (Chemicon International, Temecula, CA) at day 0 followed by injection of 120  $\mu$ g of Pam<sub>3</sub>Cys on day 2. The severity of arthritis was monitored by measuring the thickness of both wrist and ankle joints. Joint swelling was measured with a dial type caliper (Bel-Art Products, Pequannock, NJ).

### Transient Transfection and Luciferase Assay

293T cells were transfected in triplicate in 24-well plates with an AP-1 reporter construct and an expression plasmid encoding GSK3 $\beta$  or a control empty vector and an internal control plasmid encoding Renilla luciferase by using Superfect reagents from Qiagen. The plasmid encoding constitutive active Gsk3 $\beta$  contained a Ser9  $\rightarrow$  Ala mutation, and the plasmid encoding kinase dead GSK3 $\beta$  contained a Lys85  $\rightarrow$  Ala mutation (Stambolic and Woodgett, 1994). Three hours posttransfection, cells were replenished with fresh medium and cultured overnight. On the next day, cells from the same

transfected well were divided, stimulated with phorbol myristate acetate (PMA) or a DMSO control for 4 hr, and cell lysates were prepared and analyzed for firefly and Renilla luciferase activity with the Luciferase Reporter Assay System from Promega (Madison, WI). Because PMA induced Renilla luciferase activity, results are shown normalized relative to amounts of cell lysate. Comparable results were obtained when results were normalized relative to Renilla luciferase activity in cells that did not receive PMA, which reflects transfection efficiency prior to splitting cells and subsequent treatment with DMSO or PMA. Three independent experiments were performed with triplicate transfections in each experiment.

#### Supplemental Data

Supplemental Data include four figures and are available at <http://www.immunity.com/cgi/content/full/24/5/563/DC1/>.

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