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Toll-like receptor—mediated cytokine production is differentially regulated by glycogen synthase kinase 3

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Abstract

The cellular mechanisms that directly regulate the inflammatory response after Toll-like receptor (TLR) stimulation are unresolved at present. Here we report that glycogen synthase kinase 3 (GSK3) differentially regulates TLR-mediated production of pro- and anti-inflammatory cytokines. Stimulation of monocytes or peripheral blood mononuclear cells with TLR2, TLR4, TLR5 or TLR9 agonists induced substantial increases in interleukin 10 production while suppressing the release of proinflammatory cytokines after GSK3 inhibition. GSK3 regulated the inflammatory response by differentially affecting the nuclear amounts of transcription factors NF- κ B subunit p65 and CREB interacting with the coactivator CBP. Administration of a GSK3 inhibitor potently suppressed the proinflammatory response in mice receiving lipopolysaccharide and mediated protection from endotoxin shock. These findings demonstrate a regulatory function for GSK3 in modulating the inflammatory response.

The ability of the innate immune system to recognize and respond to microbial components has been largely attributed to the family of type I transmembrane receptors called Toll-like receptors (TLRs)^{1,2}. TLRs are expressed mainly on antigen-presenting cells such as monocytes-macrophages and dendritic cells and show an ability to discriminate among distinct molecular patterns associated with microbial components. Recognition of microbial products by TLRs leads to a variety of signal transduction pathways that regulate the nature, magnitude and duration of the inflammatory response³. Although the production of proinflammatory cytokines is important for mediating the initial host defense against invading pathogens⁴, an inability to regulate the nature or duration of the host's inflammatory response can be detrimental, as in chronic inflammatory diseases. The underlying cellular mechanisms that directly control pro- versus anti-inflammatory cytokine production after TLR stimulation are unresolved, but studies have shown the TLR signaling pathway can activate phosphatidylinositol 3-OH kinase (PI(3)K)⁵ to limit production of tumor necrosis factor (TNF) and interleukin 12 (IL-12)^{6–8}. Moreover, TLR2-induced activation of the PI(3)K pathway enhances IL-10 production, whereas IL-12 production is reduced⁹. Those studies suggested critical involvement of the PI(3)K pathway in differentially controlling pro- and anti-inflammatory cytokine production, but it is unclear at present if this pathway is limited to TLR2 signaling and how production of other inflammatory mediators induced by other TLR ligands is affected.

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Here we have characterized how intracellular TLR signaling affected ‘downstream’ effector molecules of the PI(3)K pathway to determine if a central effector molecule is responsible for mediating the ability of this pathway to differentially dictate the host’s inflammatory response. Inhibition of glycogen synthase kinase 3 (GSK3), the constitutively active downstream kinase of the PI(3)K pathway, mediated the ability of this pathway to selectively augment anti-inflammatory cytokine production while concurrently suppressing proinflammatory cytokine production in response to TLR stimulation.

RESULTS

TLR4-mediated phosphorylation of GSK3- β

PI(3)K is a heterodimeric enzyme that consists of a regulatory subunit (p85) and a catalytic subunit (p110)¹⁰. PI(3)K activation occurs through binding of its regulatory subunit to adaptor proteins bound to phosphotyrosine residues present in activated cellular receptors located on the plasma membrane, including TLRs^{10–12}. Activation of PI(3)K can mediate the recruitment and subsequent activation of signaling proteins with pleckstrin homology domains, including the serine-threonine kinase Akt^{13–15}. After recruitment, Akt is activated by phosphorylation at Thr308 and Ser473 (refs. 13–15). Akt is a key physiologic mediator of the PI(3)K pathway. Activated Akt phosphorylates several downstream targets of the PI(3)K pathway, including the constitutively active serine-threonine kinase GSK3- β (Ser9). Phosphorylation of GSK3- β (Ser9) results in its inhibition¹⁶. GSK3 can both positively and negatively affect a variety of transcription factors that are critical in regulating pro- and anti-inflammatory cytokine production^{17–22}. Therefore, we initially determined if LPS stimulation of human monocytes mediated Ser9 phosphorylation of GSK3 in a PI(3)K-Akt-dependent way. LPS stimulation of monocytes mediated the phosphorylation of Akt (Ser473; Fig. 1a). Use of the PI(3)K inhibitor LY294002 abolished the ability of *Escherichia coli* LPS to induce Ser473 phosphorylation of Akt ($P < 0.05$; Fig. 1a). We obtained similar results for the phosphorylation of Akt at Thr308 (data not shown). We next investigated if LPS stimulation of monocytes induced Ser9 phosphorylation of the constitutively active kinase GSK3- β in a PI(3)K-Akt-dependent way. Human monocytes stimulated with *E. coli* LPS demonstrated increased Ser9 phosphorylation of GSK3- β at multiple time points (Fig. 1b). In contrast, LY294002 significantly reduced the ability of *E. coli* LPS to phosphorylate GSK3- β on Ser9 ($P < 0.05$; Fig. 1b). We next used a selective Akt inhibitor to determine if this kinase was responsible for GSK3- β phosphorylation by *E. coli* LPS (Fig. 1c). The ability of *E. coli* LPS to induce Ser9 phosphorylation of GSK3- β was Akt dependent, because the Akt inhibitor abrogated LPS-induced phosphorylation of GSK3- β on Ser9 to nearly unstimulated amounts ($P < 0.05$; Fig. 1c). Phosphorylation of GSK3- β on Ser9 results in GSK3- β inactivation; thus, we next assessed how LPS-mediated Ser9 phosphorylation of GSK3- β affected its downstream target, glycogen synthase¹⁶. Glycogen synthase was phosphorylated on Ser641 in unstimulated human monocytes, but this was substantially reduced after LPS stimulation. Moreover, treatment of monocytes with the GSK3 inhibitor SB216763 alone completely abrogated Ser641 phosphorylation of glycogen synthase (Fig. 1d). Thus, whereas LPS-mediated Ser9 phosphorylation of GSK3 did reduce the constitutive ability of GSK3 to phosphorylate Ser641 of glycogen synthase, it did not completely abolish the ability of GSK3 to phosphorylate glycogen synthase, as noted with the GSK3 inhibitor SB216763 (Fig. 1d).

GSK3 modulates IL-10 and IL-12 production

Although the involvement of GSK3 in regulating a variety of cellular functions, including the regulation of glycogen synthesis, has been described in considerable detail²³, the ability of GSK3 to regulate the production of inflammatory mediators has not been demonstrated. In unstimulated cells, GSK3 constitutively phosphorylates β -catenin and thus targets β -catenin for degradation²³. Therefore, to initially determine the ability of a panel of various GSK3

inhibitors to inhibit GSK3 activity in human monocytes, we assessed β -catenin phosphorylation in the presence or absence of the GSK3 inhibitors LiCl, SB216763, azakenpaulone and BIO^{24–27}. Unstimulated monocytes had detectable β -catenin phosphorylation (Fig. 1e). In contrast, monocytes treated with the GSK3 inhibitors showed no detectable phosphorylated β -catenin (Fig. 1e), thus demonstrating their ability to inhibit GSK3 activity in human monocytes.

Because inhibition of the PI(3)K pathway augments IL-12 production while concurrently suppressing IL-10 in response to a TLR2 ligand, we next investigated whether inactivation of GSK3 mediated a functional effect on pro- and anti-inflammatory cytokine production by human monocytes after TLR4 stimulation (Fig. 2). In the absence of LPS stimulation, inhibitors of Akt, PI(3)K and GSK3 had no discernible effect on pro- or anti-inflammatory cytokine production compared with that of untreated controls (data not shown). Production of the anti-inflammatory cytokine IL-10 was increased by three- to fivefold when human monocytes were stimulated with *E. coli* LPS in the presence of any of the GSK3 inhibitors tested compared with LPS-stimulated cultures (Fig. 2a, Fig. 2c). In contrast, inhibition of PI(3)K or Akt using LY294002 or the Akt inhibitor, respectively, both of which abrogated the ability of *E. coli* LPS to inactivate GSK3- β by phosphorylating Ser9 (Fig. 1b, Fig. 1c), resulted in a substantial reduction in IL-10 compared with that of monocytes treated with *E. coli* LPS (Fig. 2a). In sharp contrast to IL-10, IL-12p40 was increased by greater than 50% when we used LY294002 or the Akt inhibitor (Fig. 2b). We obtained similar results with the PI(3)K inhibitor wortmannin and cells transfected with small interfering RNA (siRNA) targeting Akt (data not shown). However, IL-12p40 production was reduced more than 70% when human monocytes were pretreated with a GSK3 inhibitor and stimulated with LPS, compared with LPS-treated controls (Fig. 2b, Fig. 2d) or LPS-treated controls containing 0.1% DMSO or 10 mM NaCl (Fig. 2d). In LPS-stimulated monocytes, GSK3 inhibition resulted in a 60–80% reduction in IL-6 and TNF production compared with that of LPS-stimulated control cells (Fig. 2e, Fig. 2f). Using GSK3- β -deficient mouse embryonic fibroblasts (MEFs)¹⁷, we also found that LPS stimulation resulted in a reduction in TNF and IL-6 production of more than 70%, whereas IL-10 production was significantly enhanced compared with that of wild-type MEFs ($P < 0.05$; Fig. 2g–Fig. 2i). These data demonstrate that the inhibition of GSK3 differentially affects the ability of the TLR4 signaling pathway to induce pro- and anti-inflammatory cytokines.

The two mammalian GSK3 isoforms, GSK3- α and GSK3- β , are encoded by separate genes²⁸. Although the GSK3 isoforms share 95% identity in their kinase domains, GSK3- α and GSK3- β share only 36% identity in the 76 amino residues at the C terminus²⁸. This divergence in homology has functional relevance, as deletion of the gene encoding GSK3- β is embryonically lethal and GSK3- α is unable to ‘rescue’ GSK3- β -null mice¹⁷. Probing for both isoforms of GSK3 using a phosphorylation-specific antibody to GSK3- α and GSK3- β (Ser21 and Ser9, respectively; Fig. 1b, Fig. 1c) demonstrated that only Ser9 phosphorylation of GSK3- β was evident in response to LPS stimulation; GSK3- α was not phosphorylated on Ser21. To definitively demonstrate that GSK3- β was responsible for differentially regulating IL-10 and IL-12 production after LPS stimulation, we next used siRNA specific for GSK3- β (Fig. 3). ‘Silencing’ of GSK3- β by RNA interference for 96 h reduced GSK3- β by more than 70% compared with that of untransfected cells or cells transfected with control siRNA (Fig. 3a). In the absence of LPS stimulation, cells pretreated with control siRNA or siRNA targeting GSK3- β showed no substantial increase in IL-10 or IL-12 production compared with that of untransfected control cells (data not shown). In contrast, monocytes pretreated for 96 h with siRNA specific for GSK3- β and subsequently stimulated with *E. coli* LPS showed an increase in IL-10 production of more than twofold compared with that of untransfected cells or cells transfected with control siRNA (Fig. 3b). Moreover, IL-12p40 production by LPS-stimulated cultures pretreated for 96 h with siRNA against GSK3- β was reduced by more than 60% compared with that of untransfected cells or cells transfected with control siRNA (Fig. 3c).

These results demonstrate that GSK3- β inhibition is responsible for enhancing IL-10 while suppressing IL-12 by LPS-stimulated monocytes.

GSK3 regulates TLR-induced inflammatory response

Next we determined if the ability of GSK3 to regulate ‘classical’ proand anti-inflammatory cytokine production was more global by assessing the production of a variety of inflammatory cytokines. We also determined whether GSK3 was able to differentially modulate proand anti-inflammatory cytokine production mediated by multiple TLR signaling pathways. Therefore, we used selective agonists for TLR2 (lipoteichoic acid from *Streptococcus pneumoniae*), TLR4 (synthetic lipid A; compound 506), TLR5 (flagellin protein FljB from *Salmonella typhimurium*) and TLR9 (human CpG) and assessed how inhibition of GSK3 in conjunction with a specific TLR agonist affected the inflammatory response. Human peripheral blood mononuclear cells (PBMCs) stimulated with a TLR2, TLR4, TLR5 or TLR9 agonist in the presence of the specific GSK3 inhibitor SB216763 showed a selective reduction of 50–90% in the production of the proinflammatory cytokines IL-1 β , interferon- γ (IFN- γ), IL-12p40 and IL-6 (Fig. 4a–Fig. 4d). In contrast, production of the anti-inflammatory cytokine IL-10 was increased by three- to eightfold compared with that of controls regardless of which TLR agonist we used (Fig. 4e). These data demonstrate that GSK3 potently suppresses the production of proinflammatory cytokines while concurrently augmenting production of anti-inflammatory IL-10 in response to multiple TLR signaling pathways.

GSK3 differentially regulates transcription factor activity

To investigate the underlying cellular mechanism responsible for the ability of GSK3 to suppress proinflammatory cytokine production while augmenting anti-inflammatory cytokine production, we assessed how GSK3 influenced the activation of downstream transcription factors involved in the inflammatory response. GSK3- β has been linked to regulation of the main eukaryotic transcription factor NF- κ B, which regulates many diverse cellular processes, including proinflammatory cytokine responses^{17–21}. Because NF- κ B regulation can be mediated at multiple steps, including degradation of I κ B inhibitory molecules, processing of the NF- κ B p105 and p100 molecules and phosphorylation-dependent association with cellular coactivators, including cAMP response element—binding protein (CREB)—binding protein (CBP)²⁹, we sought to elucidate what step(s) of the NF- κ B pathway could be affected by GSK3 inhibition. I κ B α degradation induced by *E. coli* LPS was evident at 30 min after exposure (Fig. 5a). Presence of the GSK3 inhibitor SB216763 failed to alter the rate or extent of degradation or resynthesis of I κ B α (Fig. 5a). We obtained similar results for the degradation and resynthesis of I κ B α (data not shown). Because GSK3 can mediate p65 phosphorylation²¹, we determined if GSK3 inhibition was exerting an effect on the phosphorylation status of the p65 subunit. Neither the amount nor the duration of p65 phosphorylation (Ser276 or Ser536) was affected after stimulation of human monocytes with *E. coli* LPS in the presence of the GSK3 inhibitor SB216763 compared with that of cultures stimulated with *E. coli* LPS alone (Fig. 5b). Therefore, we measured the nuclear NF- κ B subunits p50 and p65 in the presence and absence of the GSK3 inhibitor SB216763 (Fig. 5c, Fig. 5d). We found no alterations in the amount of nuclear p50 or p65 in unstimulated cells treated with SB216763 alone compared with that of untreated control cells (data not shown). Additionally, the DNA binding of nuclear p50 or p65 in LPS-stimulated cultures was not influenced by the presence of SB216763 compared with that in monocytes stimulated with LPS alone (Fig. 5c, Fig. 5d).

Optimal transcriptional activity of the NF- κ B p65 subunit is mediated by its association with the nuclear coactivator CBP^{30,31}. Additionally, nuclear amounts of CBP are limiting, and phosphorylated CREB (Ser133) and NF- κ B p65 (Ser276) compete for CBP binding. Increased association of CREB and CBP suppress NF- κ B activity^{30–33}. Because GSK3 can negatively regulate the activation and DNA-binding activity of CREB²², we investigated how GSK3

inhibition affected nuclear CREB (Ser133) binding (Fig. 5e). In the absence of LPS stimulation, the GSK3 inhibitor SB216763 did not substantially affect nuclear phosphorylated CREB (Ser133) compared with that of untreated control cells (data not shown). In contrast, the DNA-binding properties of CREB (Ser133) were significantly 'upregulated' when LPS-stimulated monocytes were treated with the GSK3 inhibitor SB216763 or with siRNA targeting GSK3- β (Fig. 5e). Because the CREB DNA-binding activity was augmented by GSK3 inhibition, we also sought to determine if GSK3 inhibition affected the ability of CREB (Ser133) and NF- κ B p65 (Ser276) to associate with CBP (Fig. 5f, Fig. 5g). LPS-stimulated monocytes had more association of p65 with CBP than did unstimulated control cells (Fig. 5f, Fig. 5g). However, LPS-stimulated cultures that were pretreated with SB216763 showed a considerable decrease in association of p65 with CBP (Fig. 5f), whereas the binding of CREB to CBP was potently augmented (Fig. 5g). These results demonstrate that GSK3- β inhibition augments the binding of CREB (Ser133) and suppresses the binding of NF- κ B p65 (Ser276) to the nuclear coactivator CBP.

GSK3 regulates inflammatory response through CREB

Studies characterizing the transcription factors important for IL-10 production in human monocytes have identified CREB as a critical component³⁴. Moreover, because we had demonstrated enhanced association of CREB with CBP, we investigated whether the ability of GSK3 inhibition to enhance CREB activity was responsible for the differential regulation of pro- and anti-inflammatory cytokine production. Treatment of monocytes with siRNA targeting CREB for 96 h reduced CREB by more than 80% compared with that of untransfected cells or cells transfected with control siRNA (Fig. 5h). To determine what function CREB was mediating in the ability of GSK3 inhibition to suppress the inflammatory response, we compared IL-10 production in LPS-stimulated monocytes pretreated for 96 h with siRNA specific for CREB and in the presence or absence of the GSK3 inhibitor SB216763 (Fig. 5i). LPS-stimulated cultures pretreated with siRNA targeting CREB had a decrease of more than 30% in IL-10 compared with that of cultures stimulated with LPS alone or with LPS in conjunction with control siRNA (Fig. 5i). LPS stimulation of cells pretreated with siRNA targeting CREB and in the presence of SB216763 did not produce any discernible increase in IL-10 release compared with that of LPS-stimulated control cells (Fig. 5i). In contrast, stimulation of cells pretreated with siRNA against CREB resulted in an increase in IL-12p40 production of approximately 20% compared with that of LPS-stimulated cells or LPS-stimulated cells transfected with control siRNA (Fig. 5j). Moreover, the production of IL-12p40 by LPS-stimulated cultures pretreated for 96 h with siRNA against CREB did not seem to be altered by the presence of SB216763 (Fig. 5j). These data demonstrate that the ability of GSK3 to regulate the production of pro- and anti-inflammatory cytokines by LPS-stimulated monocytes is dependent on regulation of CREB activity.

GSK3 inhibition protects mice from endotoxin shock

Next we defined the function of GSK3 inhibition in mediating protection against endotoxin shock and its effects on modulating the inflammatory response *in vivo*. Mice given the GSK3 inhibitor SB216763 2 h before receiving a 100% lethal dose (LD₁₀₀) of LPS showed significantly improved survival of more than 70%, compared with 0% for the LPS-only control group ($P < 0.001$; Fig. 6a). Next we determined if a 'delayed' dose of SB216763 could still mediate protective effects against LPS-induced lethality. SB216763 given 2 h after LPS challenge conferred a survival rate of more than a 50%, compared with 0% for the control mice given only LPS containing 0.1% DMSO. (Fig. 6b). Monitoring the survival rates of mice challenged with a LD₁₀₀ of LPS over a 10-day period further demonstrated that mice given SB216763 did not have any 'late' deaths (Fig. 6a, Fig. 6b). Thus, GSK3 inhibition did not simply prolong LPS-induced lethality. We next assessed how GSK3 inhibition regulated the inflammatory response in mice given an LD₁₀₀ of LPS. Assessment of proinflammatory

cytokine profiles in mice given SB216763 and challenged with LPS showed there was a significant reduction in IL-12p40, IFN- γ and IL-6 ($P < 0.001$; Fig. 6c–Fig. 6e). In contrast, production of the anti-inflammatory cytokine IL-10 was significantly enhanced by more than twofold in LPS-challenged mice given SB216763 compared with that of control mice receiving only LPS ($P < 0.001$; Fig. 6f). Thus, GSK3 inhibition conferred significant protection against LPS-induced lethality when given before or after LPS insult ($P < 0.001$). Moreover, these findings further demonstrate targeting GSK3 *in vivo* can potently suppress the production of proinflammatory cytokines, whereas production of the anti-inflammatory cytokine IL-10 is significantly augmented.

DISCUSSION

Studies directly comparing the biological activity of some TLR agonists have indicated a substantial divergence in their ability to influence the nature (pro- versus anti-inflammatory) and magnitude (absolute amounts) of the inflammatory response^{35–41}. Indeed, the ability of the TLR2 and TLR4 signaling pathways to mediate the relative production of the immunoregulatory cytokines IL-10 and IL-12, known to be key in the development of cell-mediated and humoral immunity as well as in controlling the amount of inflammatory mediators, differs widely^{36,38,42–44}. Whereas the TLR4 signaling pathway is typically a potent mediator of IL-12p70, TLR2 agonists induce mainly IL-12p40 with little to no detectable IL-12p70 (refs. 38,43). The ability of a TLR2 agonist to induce IL-12p70 from human monocytes is dependent on inhibition of the PI(3)K pathway, but this reduces IL-10 production⁹. Our findings now demonstrate the ability of the PI(3)K-Akt pathway to differentially regulate IL-10 and IL-12 production is through inhibition of GSK3- β . Furthermore, the ability of GSK3 inhibition to reduce IL-1 β , IL-6, TNF- α , IL-12 and IFN- γ substantially while augmenting IL-10 in response to a variety of TLR-agonists demonstrates the broad ability of GSK3 to attenuate the inflammatory response after TLR stimulation.

An inability to regulate the nature, magnitude or duration of the host's inflammatory response can be detrimental to the host. Our study has shown how GSK3 inhibition can modulate both the nature and magnitude of pro- versus anti-inflammatory cytokine production after TLR stimulation by differentially regulating the association of NF- κ B p65 and CREB with the nuclear coactivator CBP. These findings are consistent with published studies demonstrating that the relative amounts of active nuclear CREB and NF- κ B p65 determine subsequent association with CBP^{30–33}. A notable property of GSK3 after TLR stimulation was that GSK3 inhibition selectively influenced the amount of nuclear CREB (Ser133) DNA-binding activity without any discernible effects on the amount of nuclear NF- κ B p65. This is in agreement with published work showing CREB activity is potently suppressed by active GSK3 (ref. 22). Moreover, the finding that GSK3 inhibition augmented nuclear CREB activity and enhanced its association with CBP while reducing interactions between NF- κ B p65 and CBP provides molecular understanding of how TLR-mediated GSK3 inhibition suppresses the production of proinflammatory cytokines while concurrently augmenting IL-10.

The onset of sepsis has been associated with predominant production of proinflammatory cytokines that are believed to contribute to the pathology. Exogenous IL-10 can substantially reduce the toxicity associated with endotoxin shock³⁵. Moreover, various strategies targeting proinflammatory cytokines, including IL-1 β , TNF, IFN- γ and IL-12, in animal models have indicated these cytokines are key mediators of endotoxin lethality⁴⁵. Our study has demonstrated that GSK3 inhibition substantially reduced proinflammatory cytokine production induced after challenge of mice with an LD₁₀₀ of LPS while augmenting IL-10 release. Moreover, the GSK3 inhibitor SB216763 mediated significant protection against endotoxin lethality when given before or after LPS challenge. Mice fed a diet comprising 0.4% of the GSK3 inhibitor LiCl for 10 d (ref. 46) had substantially reduced proinflammatory

cytokines and approached survival rates of 60% when challenged with an LD₁₀₀ of *E. coli* LPS, compared with mice fed normal mouse chow, which had survival rates of 0% (M.M. and R.J., unpublished observations). Thus, because of the general ability of GSK3 to modulate the inflammatory response after TLR activation, GSK3 could potentially serve as a therapeutic target against sepsis or other inflammatory diseases.

In summary, we have identified and characterized a central mechanism by which the inhibition of GSK3 differentially affects the nature and magnitude of the inflammatory response. Inhibition of GSK3 resulted in a profound increase in IL-10 production after TLR stimulation, whereas the concurrent production of proinflammatory cytokines, including IL-1 β , IL-6, TNF, IL-12 and IFN- γ , by human monocytes and PBMCs was substantially reduced. Our findings identify a critical function for GSK3 in modulating pro- versus anti-inflammatory cytokines *in vivo* and provide a rationale for regulating the nature and severity of inflammation.

METHODS

Reagents

Protein-free *E. coli* (K235) LPS was prepared as described^{47,48}. Lipoteichoic acid from *S. pneumoniae* was obtained from M. Nahm (University of Alabama at Birmingham). Bacterial flagellin (*S. typhimurium* FljB) was obtained from A. Gewirtz (Emory University, Atlanta, Georgia). *E. coli* synthetic lipid A (compound 506) was obtained from T. Ogawa (Asahi University, Gifu, Japan). The CpG oligodeoxynucleotides 2216 (5'-GGGGGACGATCGTCGGGGG-3') and 2216 control (5'-GGGGGAGCATGCTGCGGGG-3') were purchased from InvivoGen. LiCl was purchased from Sigma. 'Prevalidated' siRNA kits targeting CREB and GSK3- β were purchased from Upstate Biotechnology, and assays were done according to the manufacturer's protocol. The GSK3 inhibitors SB216763, azakenpaullone and BIO were obtained from Sigma. Wild-type and GSK3- β -deficient MEFs were obtained from J. Woodgett (Ontario Cancer Institute, Division of Experimental Therapeutics, Toronto, Ontario, Canada)¹⁷. The Akt inhibitor II Akt-i was purchased from Calbiochem. Antibodies to CBP, total p38 and total GSK3- β and phosphorylation-specific antibodies to Akt (Ser473), Akt (Thr308), GSK3- α and GSK3 β (Ser21 and Ser9, respectively), β -catenin (Ser33, Ser37, Thr41), glycogen synthase (Ser641), NF- κ B p65 (Ser276 or Ser536) and CREB (Ser133) were purchased from Cell Signaling Technology, Biosource International and Santa Cruz Biotechnology.

Endotoxin shock model

Male C57BL/6 mice (8–12 weeks of age; 18–23 g body weight) were injected intraperitoneally with an LD₁₀₀ (10 μ g/g) of *E. coli* K235 LPS in 200 μ l of PBS containing 0.1% DMSO. Mice were monitored over a 10-day period for survival. All studies were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Measurement of cytokines

Mouse and human IL-1 β , IL-6, IL-10, TNF, IL-12p40 and IFN- γ in culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems or eBioscience according to manufacturer's instructions.

Immunoprecipitation analysis

Human monocytes (2×10^6 /ml) in 24-well plates were pretreated with medium, 0.01% DMSO, the Akt inhibitor, LiCl, SB216763, azakenpaullone and BIO before the addition of medium or LPS. At various time points, cells were washed with PBS and analyzed by immunoblot as described⁹. The AlphaImager 2000 documentation and analysis system (Alpha Innotech) was

used for densitometer scans of the blots. For immunoprecipitation of CBP (Santa Cruz Biotechnology) and subsequent probing for associated NF- κ B p65 (Ser276) or CREB (Ser133; obtained from Cell Signaling Technologies), the Catch and Release Immunoprecipitation System (Upstate Biotechnology) was used according to the manufacturer's instructions.

NF- κ B p50, NF- κ B p65 and CREB activity

Human monocytes in 24-well polystyrene tissue culture plates were pretreated for 1 h with SB216763 or 0.01% DMSO and then were incubated with medium alone or LPS for various times. Cells were collected and washed two times in PBS and then were assayed for activity with the TransAM kit specific for each transcription factor (Active Motif). The amount of nuclear NF- κ B p50, NF- κ B p65 or CREB was normalized by the absorbance at 450 nm from 2 mg NF- κ B p50 or NF- κ B p65 or 20 μ g of nuclear lysate (CREB).

Cell culture

Heparinized venous blood from healthy adult donors was used to obtain PBMCs by isolation of the buffy coat and elimination of red blood cell contamination using Histopaque (SG-1.077; Sigma) density gradients. Human monocytes were purified from PBMC samples by negative selection with a monocyte isolation kit purchased from Miltenyi Biotec. This procedure routinely resulted in samples that were more than 95% pure CD14⁺ cells, as shown by flow cytometry. Human monocytes or PBMCs were cultured in 24-well (2×10^6 cells/well) or 96-well (2×10^5 cells/well) plates containing RPMI 1640 medium supplemented with 10% FBS, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 50 U/ml of penicillin and 50 μ g/ml of streptomycin. Wild-type or GSK3- β -deficient MEFs were cultured as described¹⁷.

Statistical analysis

Data are expressed as mean $\pm$ s.d. Statistical significance between groups was evaluated by analysis of variance and the Tukey multiple-comparison test using the InStat program (GraphPad Software). Differences between groups were considered significant at $P < 0.05$.

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References

1. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997;388:394–397. [PubMed: 9237759]
2. Yang RB, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 1998;395:284–288. [PubMed: 9751057]
3. Dinarello CA. Proinflammatory cytokines. *Chest* 2000;118:503–508. [PubMed: 10936147]
4. O'Neill LA, Dinarello CA. The IL-1 receptor/Toll-like receptor superfamily: crucial receptors for inflammation and host defense. *Immunol. Today* 2000;21:206–209. [PubMed: 10782049]
5. Monick MM, et al. Lipopolysaccharide activates Akt in human alveolar macrophages resulting in nuclear accumulation and transcriptional activity of beta-catenin. *J. Immunol* 2001;166:4713–4720. [PubMed: 11254732]
6. Fukao T, et al. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat. Immunol* 2002;3:875–881. [PubMed: 12154357]
7. Fukao T, et al. Selective loss of gastrointestinal mast cells and impaired immunity in PI3K-deficient mice. *Nat. Immunol* 2002;3:295–304. [PubMed: 11850627]
8. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem* 2002;277:32124–32132. [PubMed: 12052830]

9. Martin M, et al. Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by *Porphyromonas gingivalis* lipopolysaccharide. *J. Immunol* 2003;171:717–725. [PubMed: 12847238]
10. Cantley LC. The phosphoinositide 3-kinase pathway. *Science* 2002;296:1655–1657. [PubMed: 12040186]
11. Arbibe L, et al. Toll-like receptor 2-mediated NF- κ B activation requires a Rac1 - dependent pathway. *Nat. Immunol* 2000;1:533–540. [PubMed: 11101877]
12. Toker A, Cantley LC. Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* 1997;387:673–676. [PubMed: 9192891]
13. Franke TF, Kaplan DR, Cantley LC, Toker A. Direct regulation of the Akt protooncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 1997;275:665–668. [PubMed: 9005852]
14. Lawlor MA, Alessi DR. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci* 2001;114:2903–2910. [PubMed: 11686294]
15. Stokoe DLR, et al. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* 1997;277:567–570. [PubMed: 9228007]
16. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995;378:785–789. [PubMed: 8524413]
17. Hoeflich KP, et al. Requirement for glycogen synthase kinase-3 β in cell survival and NF- κ B activation. *Nature* 2000;406:86–90. [PubMed: 10894547]
18. Demarchi F, Bertoli C, Sandy P, Schneider C. Glycogen synthase kinase-3 β regulates NF- κ B1/p105 stability. *J. Biol. Chem* 2003;278:39583–39590. [PubMed: 12871932]
19. Demarchi F, Verardo R, Varnum B, Brancolini C, Schneider C. Gas6 antiapoptotic signaling requires NF- κ B activation. *J. Biol. Chem* 2001;276:31738–31744. [PubMed: 11425860]
20. Nemeth ZH, et al. Lithium induces NF- κ B activation and interleukin-8 production in human intestinal epithelial cells. *J. Biol. Chem* 2002;277:7713–7719. [PubMed: 11756416]
21. Schwabe RF, Brenner DA. Role of glycogen synthase kinase-3 in TNF- α -induced NF- κ B activation and apoptosis in hepatocytes. *Am. J. Physiol. Gastrointest. Liver Physiol* 2002;283:G204–G211. [PubMed: 12065308]
22. Grimes CA, Jope RS. CREB DNA binding activity is inhibited by glycogen synthase kinase-3 β and facilitated by lithium. *J. Neurochem* 2001;78:1219–1232. [PubMed: 11579131]
23. Doble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell Sci* 2003;116:1175–1186. [PubMed: 12615961]
24. Kunick C, Lauenroth K, Leost M, Meijer L, Lemcke T. 1-Azakenpaullone is a selective inhibitor of glycogen synthase kinase-3 β . *Bioorg. Med. Chem. Lett* 2004;19:413–416. [PubMed: 14698171]
25. Cross DA, et al. Selective small-molecule inhibitors of glycogen synthase kinase-3 activity protect primary neurones from death. *J. Neurochem* 2001;77:94–102. [PubMed: 11279265]
26. Meijer L, et al. GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem. Biol* 2003;10:1255–1266. [PubMed: 14700633]
27. Stambolic V, Ruel L, Woodgett JR. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol* 1996;6:1664–1668. [PubMed: 8994831]
28. Woodgett JR. Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J* 1990;9:2431–2438. [PubMed: 2164470]
29. Ghosh S, May MJ, Kopp EB. NF- κ B and rel proteins: evolutionary conserved mediators of immune responses. *Annu. Rev. Immunol* 1998;16:225–260. [PubMed: 9597130]
30. Sheppard KA, et al. Transcriptional activation by NF- κ B requires multiple coactivators. *Mol. Cell. Biol* 1999;19:6367–6378. [PubMed: 10454583]
31. Zhong H, Voll RE, Ghosh S. Phosphorylation of NF- κ B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell* 1998;1:661–671. [PubMed: 9660950]
32. Parker D, et al. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Mol. Cell. Biol* 1996;16:694–703. [PubMed: 8552098]
33. Parry GC, Mackman N. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF- κ B-mediated transcription. *J. Immunol* 1997;159:5450–5456. [PubMed: 9548485]

34. Platzer C, et al. Cyclic adenosine monophosphate-responsive elements are involved in the transcriptional activation of the human IL-10 gene in monocytic cells. *Eur. J. Immunol* 1999;29:3098–3104. [PubMed: 10540320]
35. Berg DJ, et al. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Schwartzman reaction but not endotoxin tolerance. *J. Clin. Invest* 1995;96:2339–2347. [PubMed: 7593621]
36. Hirschfeld M, et al. Signaling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect. Immun* 2001;69:1477–1482. [PubMed: 11179315]
37. Jones BW, et al. Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leuk. Biol* 2001;69:1036–1044.
38. Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J. Biol. Chem* 2001;276:37692–37699. [PubMed: 11477091]
39. Rhee SH, Jones BW, Toshchakov V, Vogel SN, Fenton MJ. Toll-like receptors 2 and 4 activate STAT1 serine phosphorylation by distinct mechanisms in macrophages. *J. Biol. Chem* 2003;278:22506–22512. [PubMed: 12686553]
40. Schilling D, Thomas K, Nixdorff K, Vogel SN, Fenton MJ. Toll-like receptor 4 and Toll-IL-1 receptor domain-containing adapter protein (TIRAP)/myeloid differentiation protein 88 adapter-like (Mal) contribute to maximal IL-6 expression in macrophages. *J. Immunol* 2002;169:5874–5880. [PubMed: 12421970]
41. Toshchakov V, et al. TLR4, but not TLR2, mediates IFN- β -induced STAT1 α/β -dependent gene expression in macrophages. *Nat. Immunol* 2002;3:392–398. [PubMed: 11896392]
42. Dillon S, et al. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *J. Immunol* 2004;172:4733–4743. [PubMed: 15067049]
43. Re F, Strominger JL. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. *J. Immunol* 2004;173:7548–7555. [PubMed: 15585882]
44. Pulendran B, et al. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. *J. Immunol* 2001;167:5067–5076. [PubMed: 11673516]
45. Cohen J. The immunopathogenesis of sepsis. *Nature* 2002;420:885–891. [PubMed: 12490963]
46. O'Brien WT, et al. Glycogen synthase kinase-3 β haploinsufficiency mimics the behavioral and molecular effects of lithium. *J. Neurosci* 2004;24:6791–6798. [PubMed: 15282284]
47. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J. Immunol* 2000;165:18–22.
48. Tapping RI, Akashi S, Miyake K, Godowski PJ, Tobias PS. Toll-like receptor 4, but not Toll-like receptor 2, is a signaling receptor for *Escherichia* and *Salmonella* lipopolysaccharides. *J. Immunol* 2000;165:5780–5787. [PubMed: 11067937]

Figure 1.

E. coli LPS induces the phosphorylation of Akt (Ser473) and GSK3-β (Ser9) through the PI (3)K-Akt pathway in human peripheral blood monocytes. Human monocytes were preincubated with 20 μM LY294002 (LY), 1 μM Akt inhibitor (Akt-i), 10 mM SB216763 or 10 μM LiCl for 1 h before stimulation with 1 μg/ml of *E. coli* LPS. Unstimulated and LPS-stimulated cells not pretreated with an inhibitor were pretreated for 1 h with culture media containing 0.1% DMSO or 10 mM NaCl. Whole-cell lysates (10 μg) were analyzed by immunoblot with phosphorylation-specific antibodies (left margins) followed by enhanced chemiluminescence detection to assess phosphorylation of Akt (Ser473; **a**), GSK3-α and GSK3-β (Ser21 and Ser9, respectively; **b,c**), glycogen synthase (Ser641; **d**) and β-catenin (Ser33, Ser37 and Thr41; **e**). Data are representative of five separate experiments.

Figure 2.

The PI(3)K pathway differentially modulates pro- and anti-inflammatory cytokine production by inhibition of GSK3. (a–f) Cytokine production in monocytes preincubated for 1 h with medium only, 10 μ M SB216763, 10 mM LiCl, 200 nM azakenpaulone, BIO (50 nM), 1 μ M Akt inhibitor or 20 μ M LY294002 (keys) and then left unstimulated or stimulated with 1 μ g/ml of LPS for 20 h. Cell-free supernatants were analyzed by ELISA for production of IL-10 (a,c), IL-12p40 (b,d), TNF (e) or IL-6 (f). (g–i) Cytokine production by wild-type (WT) or GSK3- β -deficient MEFs left unstimulated or stimulated with LPS for 20 h; cell-free supernatants were analyzed by ELISA. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, compared with LPS (a–f) or wild-type MEFs (g–i). Results represent mean $\pm$ s.d. of five separate experiments.

Figure 3.

The siRNA targeting GSK3-β enhances IL-10 and suppresses IL-12p40 by LPS-stimulated monocytes. **(a)** Immunoblot of total GSK3-β and total p38 in monocytes pretreated for 96 h with medium (Untransfected), control siRNA (siRNA control) or siRNA targeting GSK3-β (siRNA (GSK3-β)) (left) and densitometer absorbance of GSK3-β/total p38 (right). **(b,c)** ELISA of IL-10 **(b)** and IL-12p40 **(c)** in cell-free supernatants of monocytes pretreated for 96 h with medium only (LPS), siRNA control (LPS + siRNA control) or siRNA targeting GSK3-β (LPS + (siRNA (GSK3-β))) and stimulated for 20 h with LPS. *, $P < 0.05$, compared with untransfected **(a)** or LPS-stimulated **(b,c)** cultures. Results represent mean $\pm$ s.d. of three separate experiments.

Figure 4.

Inhibition of GSK3 differentially regulates pro- and anti-inflammatory cytokine production after TLR2, TLR4, TLR5 and TLR9 stimulation. (a–d) Production of various cytokines in PBMCs pretreated for 1 h with SB216763 (concentration, horizontal axes) in culture medium containing 0.01% DMSO before the addition of TLR2 agonist (10 μg/ml of lipoteichoic acid; a), TLR4 agonist (1 μg/ml of *E. coli* synthetic lipid A; b), TLR5 agonist (5 μg/ml of FljB; c) or TLR9 agonist (5 μM human CpG; d) for 20 h. (e) Production of IL-10 after use of TLR agonists as in a–d. LTA, lipoteichoic acid. Concentrations of IL-1β, IFN-γ, IL-12p40, IL-6 and IL-10 were measured by ELISA. Stimulation of PBMCs with lipoteichoic acid from *S. pneumoniae* did not result in any detectable production of IFN-γ (data not shown). *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$, respectively, compared with TLR-treated controls containing 0.01% DMSO only without SB216763. Results represent mean ± s.d. of five separate experiments.

Figure 5.

GSK3 inhibition affects the association of NF-κB p65 and CREB with CBP that regulates the production of IL-10 and IL-12. (**a–g**) Monocytes were pretreated with medium only (Unstimulated) or with 10 μM SB216763 (SB) and were stimulated with 1 μg/ml of LPS (time, above lanes and below bars). (**a,b**) Immunoblots showing that the degradation and resynthesis of IκBα (**a**) and the phosphorylation of NF-κB p65 (Ser276 or Ser536; **b**) are not affected by SB216763. (**c–e**) Transcription factor binding assay of 2 μg (NF-κB p65 (**c**) or NF-κB p50 (**d**) or 20 μg (CREB (**e**)) of nuclear extracts obtained from human monocytes. GSK3 inhibition produces different effects on the DNA binding of NF-κB p65 (**c**), NF-κB p50 (**d**) and CREB (Ser133; **e**) after LPS stimulation. (**f,g**) Interaction of CBP with NF-κB p65 (**f**) and CREB (**g**), assessed by immunoprecipitation (IP) of CBP followed by immunoblot (IB) for NF-κB (**f**) or CREB (**g**). (**h–j**) The functional effect of GSK-3 in regulating CREB activity in LPS-stimulated monocytes, assessed by pretreatment of cells with siRNA targeting CREB or control siRNA for 96 h, followed by stimulation with LPS for 20 h. Cell-free supernatants were analyzed by immunoblot for total CREB and total p38 (**h**) and by ELISA for IL-10 (**i**) and IL-12p40 (**j**). *, $P < 0.05$. Results are representative of three separate experiments (**a,b,f–i**) or the mean $\pm$ s.d. of three separate experiments (**c–e**).

Figure 6.

The GSK3 inhibitor SB216763 protects mice from endotoxin shock. **(a,b)** Intravenous administration of the GSK3 inhibitor SB216763 (25 μ g/g) protects mice from an LD₁₀₀ (10 μ g/g) of *E. coli* K235 LPS given therapeutically (2 h before LPS insult; **a**) or prophylactically (2 h after LPS insult; **b**). Results represent the mean of three separate experiments ($n = 10$ per experiment). **(c-f)** *In vivo* IL-12p40 (**c**), IFN- γ (**d**), IL-6 (**e**) and IL-10 (**f**) in plasma, measured by ELISA 12 h after mice were given an LD₁₀₀ of LPS. Sham-immunized mice were given only PBS containing 0.1% DMSO. Results represent mean $\pm$ s.d. of three separate experiments ($n = 5$ per experiment). **, $P < 0.01$ and ***, $P < 0.001$, respectively, compared with control mice given only LPS containing 0.1% DMSO. All groups of mice were given PBS containing 0.1% DMSO with or without LPS or SB216763.