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Glycogen synthase kinase-3 regulates inflammatory tolerance in astrocytes

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Abstract

Inflammatory tolerance is the down-regulation of inflammation upon repeated stimuli, which is well-established to occur in peripheral immune cells. However, less is known about inflammatory tolerance in the brain although it may provide an important protective mechanism from detrimental consequences of prolonged inflammation, which appears to occur in many psychiatric and neurodegenerative conditions. Array analysis of 308 inflammatory molecules produced by mouse primary astrocytes after two sequential stimulations with lipopolysaccharide (LPS) distinguished three classes, tolerant, sensitized and unaltered groups. For many of these inflammatory molecules, inhibition of glycogen synthase kinase-3 (GSK3) increased tolerance and reduced sensitization. Focusing on LPS-tolerance in interleukin-6 (IL-6) production, we found that microglia exhibited a strong tolerance response that matched that of macrophages, whereas astrocytes exhibited only partial tolerance. The astrocyte semi-tolerance was found to be regulated by GSK3. GSK3 inhibitors or knocking down GSK3 levels promoted LPS-tolerance and astrocytes expressing constitutively active GSK3 did not develop LPS-tolerance. These findings identify the critical role of GSK3 in counteracting IL-6 inflammatory tolerance in cells of the CNS, supporting the therapeutic potential of GSK3 inhibitors to reduce neuroinflammation by promoting tolerance.

Keywords

neuroinflammation; GSK-3; astrocytes; LPS

(i) Introduction

Tolerance to inflammatory signals, which down-regulates inflammatory molecule production in spite of repeated stimuli, may be a particularly crucial protective mechanism in the brain. Although initial inflammatory reactions, particularly cytokine production by astrocytes and microglia, are crucial for responses to injury, infection, or stress, prolonged neuroinflammation, which can even be induced by psychological stress, damages neuronal function and contributes to many psychiatric and neurodegenerative conditions (Dantzer et

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al., 2008, Miller et al., 2009). However, little information is available concerning the possibility that tolerance may contribute to regulating the extent and duration of cytokine production in the central nervous system (CNS) (Craft et al., 2005). Lipopolysaccharide (LPS)-preconditioning provides neuroprotection against ischemic brain injury in mouse models of stroke (reviewed in (Kariko et al., 2004)). Furthermore, repeated exposures to LPS are associated with tolerance to mechanical hyperalgesia (Guo and Schluesener, 2006) and with a reduction of tumor necrosis factor (TNF) but not prostaglandin E2 (PGE2) production by primary glia (Shemi and Kaplanski, 2005). This contrasts with the peripheral immune system, where tolerance is well-characterized. For example, it is well-established that pre-conditioning macrophages with LPS desensitizes the cytokine producing machinery to subsequent exposure to LPS (Freudenberg and Galanos, 1990, Ziegler-Heitbrock, 1995, Medvedev et al., 2000, Cavaillon and Adib-Conquy, 2006, Schneider and Ayres, 2008). Opposite to tolerance, some inflammatory molecules become sensitized to multiple exposures to LPS, an outcome that also could contribute to the deleterious effects of long-term or repeated inflammatory stimuli.

Inflammatory cytokine production is promoted by glycogen synthase kinase-3 (GSK3) (Martin et al., 2005, Beurel and Jope, 2009b). GSK3 is a highly conserved Ser/Thr protein kinase that is constitutively active (Cohen and Frame, 2001, Doble and Woodgett, 2003, Jope and Johnson, 2004). GSK3 is predominantly regulated by inhibitory phosphorylation of its two isoforms, serine-9 in GSK3 β and serine-21 in GSK3 α , which is abrogated in GSK3 knockin mice that express serine-to-alanine mutations in both isoforms (McManus et al., 2005). In this study, we tested if LPS-tolerance or sensitization occurs in astrocytes, and focusing on IL-6 production in response to LPS stimulation, we tested if tolerance is regulated by GSK3.

(ii) Experimental procedures

Cell culture

Primary glia were prepared from cerebral cortex of 1 day old C57Bl/6 mice as described (McCarthy and de Vellis, 1980), and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 0.3% glucose, 2 mM L-glutamine, 10 U/mL penicillin and 10 μ g/mL streptomycin. For separation of astrocytes and microglia, after 10 days of culture the cells were shaken (30 h; 250 rpm), resulting in >99% pure astrocytes as determined by immunostaining with the astrocyte marker glial fibrillary acidic protein (GFAP). After the first hour of shaking, the medium containing microglia cells was collected and microglia were cultured in the same medium as astrocytes. RAW264.7 cells were cultivated as described previously (Beurel and Jope, 2009a). Protein-free *E. coli* (K235) LPS was prepared as described (Hirschfeld et al., 2000). Cells were left untreated (naive, 0) or stimulated with 100 ng/mL LPS for 24 h (to establish LPS-tolerance) in medium supplemented with 10% FBS, washed twice with warm medium and given fresh media (0/0 or LPS/0) or 10 ng/mL LPS (0/LPS, LPS/LPS) for 1 h or 24 h. Where indicated, cells were treated with 10 μ M kenpaullone, 10 μ M TDZD-8 (Calbiochem), 10 μ M Chiron99021 (University of Dundee, UK), or 20 mM LiCl (Sigma).

Biochemical methods

The levels of 308 inflammatory proteins were measured with antibody arrays according to the manufacturer's instructions (Raybiotech). IL-6 levels were measured by ELISA according to the manufacturer's instructions (eBioscience). For knocking down GSK3 levels, cells were transfected using liposome-mediated transfection reagent LipofectAMINE RNAiMAX (Invitrogen) with 50 nM siRNA according to the manufacturer's instructions with silencer negative control (Ambion), or GSK3 α and GSK3 β siRNA (Smart pool;

Dharmacon). For overexpressing GSK3, cells were rinsed twice with DMEM/F12 medium without supplements, infected with 50 moi of the designated adenovirus for 30 min, supplemented medium was added for incubation for 36–48 h, and infected cultures were examined for adequate infection efficiency (80%) as assessed by GFP fluorescence after infection with GFP adenovirus. Western blots were carried out as described previously (Beurel and Jope, 2008) using antibodies to phospho-Ser21GSK3 α , phospho-Ser9GSK3 β , (Cell Signaling Technology), total GSK3 α/β (Millipore), and β -actin (Sigma). Statistical significance between groups was evaluated by ANOVA.

(iii) Results

Inflammatory tolerance and sensitization in astrocytes

Array analysis of inflammatory molecules produced by mouse primary astrocytes was used to identify those that display adaptive responses to repeated LPS exposure and to determine which are regulated by GSK3. Cells were treated according to the paradigm of Foster *et al.* (2007) that was developed to examine LPS-tolerance in macrophages. This involved comparing the production of inflammatory molecules stimulated by 10 ng/mL LPS for 24 hr in astrocytes that either had been preincubated for 24 hr in media with no addition (0/LPS group) or with 100 ng/mL LPS (LPS/LPS group) (Figure 1A). With both conditions, to test if GSK3 regulated adaptive responses to repeated stimulation with LPS, during the first incubation, additional cells were treated with 20 mM lithium to fully inhibit GSK3 (Klein and Melton, 1996, Stambolic *et al.*, 1996), followed by thoroughly washing out the lithium before the second exposure to LPS. Of the 308 inflammatory molecules assessed on the array, 125 were reliably increased in media from LPS-treated astrocytes (Figure 1B). Of these, 13% displayed LPS-tolerance, defined as molecules that were produced by <50% during the second LPS stimulation (LPS/LPS) compared to the amounts produced by a single LPS stimulation (0/LPS) (Figure 2; white area in center circle). Opposite to LPS-tolerance, 34% of the inflammatory molecules displayed sensitization after repeated LPS administration, defined as >50% increased production by the second LPS stimulation compared with a single LPS stimulation (Figure 2; shaded area in center circle), and 53% were unaffected by pretreatment with LPS.

GSK3 regulates inflammatory tolerance and sensitization in astrocytes

GSK3 was found to regulate many of the adaptive responses to repeated LPS exposure. Of the tolerant molecules, lithium pretreatment increased the tolerance of 19% (Figure 2, white area in upper right circle), lithium pretreatment blocked tolerance for half of the tolerant molecules (shaded area in upper right circle), and 31% were unaffected by lithium (dark area in upper right circle). Sensitization may be a critical response involved in heightened dysfunction in the brain associated with unabated neuroinflammation, and remarkably, inhibition of GSK3 with lithium during the first LPS exposure dampened the production of 63% of the sensitized inflammatory molecules (Figure 2; shaded area in upper left circle). Lithium also promoted tolerance of eight inflammatory molecules that otherwise demonstrated a sensitization response to repeated LPS treatment (Figure 2, white area in upper left circle). The group of inflammatory molecules least affected by lithium were those that were unaffected by pretreatment with LPS (the "No Change" group in the center circle). These results demonstrate that GSK3 contributes to the tolerance and sensitization processes of several inflammatory molecules in astrocytes exposed to repeated LPS exposure, indicating that GSK3 can influence the repertoire of inflammatory molecules that are produced after repeated exposure to LPS. Considering these differential responses to LPS with inhibition of GSK3, it is evident that GSK3 does not act only at the level of the initial LPS-induced activation of the toll-like receptor 4 (TLR4) signaling pathway, but must act

after dispersal of the signal to differentially modulate the expression of inflammatory molecules.

IL-6 production displays LPS-tolerance in astrocytes, macrophages, and microglia

Since IL-6 is involved in many neuroinflammatory conditions and promotion of tolerance to LPS would provide a mechanism to reduce neuroinflammation (Cerciat et al., Lee et al., Sironi et al., 1993, Bull et al., 2009), we examined in greater detail the regulation by GSK3 of LPS-tolerance in IL-6 production. As indicated by array analysis (Figure 1), astrocytes preincubated with LPS produced significantly less IL-6 upon restimulation with LPS than did astrocytes not pre-exposed to LPS (Figure 3A), demonstrating a phenotype of semi-tolerance. Preconditioning of astrocytes with LPS for up to 96 h induced semi-tolerance (Figure 3B), and astrocyte semi-tolerance to LPS persisted for at least 96 h after the initial 24 h stimulation (data not shown). However, more complete tolerance was achieved by two or more sequential 24 h treatments with 100 ng/mL LPS (Figure 3C). In contrast to astrocytes, complete LPS-tolerance occurred after a single LPS stimulus in macrophage-derived RAW264.7 cells, as very little IL-6 was produced by a second application of 10 ng/mL LPS following a first tolerance-inducing exposure to 100 ng/mL LPS (Figure 3D), consistent with previous studies (Foster et al., 2007). Microglia, which in common with macrophages are bone marrow derived haematopoietic cells, also developed complete LPS-tolerance in IL-6 production after an initial LPS stimulation (Figure 3E). The LPS semi-tolerance characteristic of astrocytes provides a model system to study the bi-directional modulation of LPS-tolerance.

GSK3 regulates LPS-tolerance of IL-6 production in astrocytes

To examine the involvement of GSK3 in LPS-tolerance of IL-6 production, astrocytes were pretreated with or without LPS in the absence or presence of GSK3 inhibitors, the inhibitors were then washed out, and the response to LPS was measured. Pretreatment with GSK3 inhibitors kenpaullone (Leost et al., 2000), Chiron99021 (Wagman et al., 2004), or TDZD-8 (Martinez et al., 2002) in the absence of LPS had no effect on the subsequent LPS-induced production of IL-6 (Figure 4A). However, all three GSK3 inhibitors greatly increased the development of tolerance when present with LPS in the initial incubation (Figure 4B). Incubation with the three GSK-3 inhibitors for the last one hour of the initial LPS treatment had no effect on subsequent LPS-induced IL-6 production (Figure 4C), demonstrating that more than 1 hr exposure to GSK3 inhibitors in the presence of LPS was required for the inhibitors to promote tolerance. Lithium is the most widely used GSK3 inhibitor (Klein and Melton, 1996, Stambolic et al., 1996), and like the other GSK3 inhibitors, lithium pretreatment with LPS during the initial treatment also promoted LPS-tolerance in IL-6 production (Figure 5A) and this did not occur if lithium was only present for the last one hour of the pretreatment (Figure 5B). Unlike the other GSK3 inhibitors, pretreatment with lithium alone, in the absence of LPS (0/LPS condition), tended to reduce the subsequent LPS-induced production of IL-6 (Figure 5A). Besides directly inhibiting GSK3, lithium treatment also indirectly inhibits GSK3 through an increase in the inhibitory serine-phosphorylation of GSK3 (Jope 2003), as shown in Figure 5C (right lane), but there was no residual increased serine-phosphorylation of GSK3 in the samples pretreated with lithium followed by a second incubation (Figure 5C lanes 4–6 compared with lanes 1–3). Nonetheless, the time necessary for inhibitory serine-phosphorylation of GSK3 to dissipate during the second incubation may have contributed to the lower production of IL-6 in samples pretreated with lithium followed by stimulation with LPS. Since LPS-tolerance was 100% in microglia and RAW264.7, the lack of IL-6 produced in response to a second LPS stimulus was not modulated by GSK3 inhibitors (data not shown). These results demonstrate that inhibition of GSK3 promotes the induction of LPS-tolerance on IL-6 production, indicating that active GSK3 counteracts LPS-tolerance. Comparing GSK3 levels in

microglia and astrocytes revealed that astrocytes contain $362 \pm 40\%$ ($n=3$) greater levels of GSK3 β , the predominant isoform, than microglia (Figure 5D), raising the possibility that elevated GSK3 in astrocytes provides a stronger signal to counteract LPS tolerance compared with microglia. This was further indicated by the increased inhibitory serine-phosphorylation of GSK3 β in the tolerant condition in microglia, but not in astrocytes (Figure 5D). Molecular means were also used to define the role of GSK3 in LPS-tolerance in IL-6 production by astrocytes. Knocking down GSK3 β levels with siRNA promoted LPS-tolerance, as indicated by greater reduction of IL-6 production following pretreatment with LPS than in control siRNA cells (Figure 6A). Conversely, overexpression of active mutants of GSK3 that cannot be inhibited by serine-phosphorylation, S21AGSK3 α and S9AGSK3 β , blocked the induction of LPS-tolerance for IL-6 production in astrocytes (Figure 6B). This was further examined by measuring the production of IL-6 in astrocytes isolated from GSK3 knockin mice (McManus et al., 2005). These mice express GSK3 α and GSK3 β at normal levels but with serine-to-alanine mutations at serine-21 in GSK3 α and serine-9 in GSK3 β to abrogate inhibitory serine-phosphorylation of GSK3 (Figure 4C), the major target for inhibition of GSK3, so GSK3 is constitutively maximally active, but within its normal physiological range. In astrocytes from GSK3 knockin mice there was a complete loss of LPS-tolerance, as IL-6 production was the same with or without a preconditioning LPS treatment (Figure 6C). LPS-tolerance was restored in GSK3 knockin astrocytes by treatment with GSK3 inhibitors (Figure 6D). Altogether, these results demonstrate that GSK3 β counteracts LPS-tolerance in the production of IL-6 by mouse primary astrocytes.

(iv) Discussion

The brain appears to be particularly sensitive to inflammation, as neuroinflammation has been implicated in deleterious outcomes in many neurological and psychiatric diseases (Miller et al., 2009). This may be due to the long lifetimes of neurons and likely partially underlies the barriers that evolved to isolate the brain from the life-sustaining peripheral inflammatory responses that are required for pathogen resistance (Rivest, 2009). However, a further protective mechanism could develop to minimize the deleterious effects of repeated inflammation by down-regulating inflammatory responses, i.e., the development of inflammatory tolerance. Here we found a wide range of responses to repeated LPS stimulation of astrocytes, including tolerance, sensitization, and no changes in response, indicating that the inflammatory environment of the brain following repeated episodes of inflammation is much different than following a single episode, and that much remains to be learned about inflammatory responses in the brain following repeated stimulation. Our survey of inflammatory molecules regulated by lithium treatment demonstrated broad effects, showing that inhibition of GSK3 can not be simply classified as producing an anti-inflammatory outcome but inflammatory molecules must be examined on an individual basis.

We focused on the regulation of tolerance in IL-6 production by GSK3 because IL-6 has been associated with several neurodegenerative and psychiatric diseases, including impairing higher functions such as cognition (Campbell et al., 1997, Marsland et al., 2008, Dugan et al., 2009). Microglia, like related macrophages, displayed a nearly complete tolerance in LPS-induced IL-6 production. This extends to LPS tolerance previous evidence of cross-tolerance that has been attributed to microglia in the brain between apparently unrelated noxious stimuli, such as LPS and ischemia (Shemi and Kaplanski, 2005, Guo and Schluesener, 2006). For IL-6 production astrocytes display a response of semi-tolerance, which allowed experimental manipulations to determine that active GSK3 counteracts tolerance and inhibition of GSK3 promoted more full tolerance. Interestingly, the relative magnitudes of tolerance in microglia and astrocytes inversely correlated with GSK3 levels in each cell type, as GSK3 expression levels were ~4-fold higher in astrocytes than

microglia. Although further mechanistic insight remains a goal for further studies, previously reported regulatory actions of GSK3 on transcription factors that control IL-6 production, such as NF- κ B and STAT3 (Hoeflich et al., 2000, Steinbrecher et al., 2005, Beurel and Jope, 2008) may contribute to the regulation of LPS tolerance by GSK3. Regardless of the mechanism, it is evident that inhibiting GSK3 not only reduces initial production of inflammatory molecules in the periphery and the brain (Martin et al., 2005, Beurel and Jope, 2009b, Yuskaitis and Jope, 2009), it also promotes IL-6 tolerance to repeated inflammatory stimuli.

The discovery that GSK3 inhibition promotes LPS-tolerance in IL-6 production besides participating in resistance to endotoxin shock (Martin et al., 2005) reveals new mechanisms of actions of GSK3 in the inflammatory process in the brain. Equally intriguing is the discovery that many inflammatory molecules are sensitized upon repeated LPS exposure, with greater production following an initial LPS stimulus. This characteristic raises the possibility that this sensitization process contributes to the difficulty in surmounting chronic neuroinflammation, although GSK3 appears to be a feasible target for controlling inflammatory sensitization as well as promoting tolerance to certain inflammatory molecules.

Abbreviations

The abbreviations used are:

GFAP	glial fibrillary acidic protein
GSK3	glycogen synthase kinase-3
IL-6	interleukin-6
LiCl	lithium chloride
LPS	lipopolysaccharide
TNF α	tumor necrosis factor- α .

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Figure 1.

Inflammatory molecule tolerance and sensitivity to repeated LPS exposures: Modulation by inhibition of GSK3 by lithium. **(A)** Scheme of tolerance paradigm in which cells were untreated (0), or stimulated with 100 ng/mL LPS for 24 h without or with GSK3 inhibitors, thoroughly washed with media, and reincubated for 24 h with or without 10 ng/mL LPS. **(B)** Mouse primary astrocytes were treated as described in **(A)** with or without the GSK3 inhibitor LiCl (20 mM). Supernatants from 2 experiments were pooled and analyzed by cytokine array detecting 308 molecules in duplicates. Four groups of six dark spots are positive control standards. The box indicates the changes observed with IL-6. Data are representative of 2 independent experiments.

Figure 2.

Classification of inflammatory molecule responses to repeated LPS stimulation. From the array data described in Figure 1, the ratio of LPS/LPS to 0/LPS was calculated for each inflammatory molecule. Classifications of inflammatory molecule production caused by repeated stimulation with LPS were defined as follows: "tolerance" was a ratio of <0.5 ($>50\%$ reduction), "sensitization" was a ratio of >1.5 ($>50\%$ increase), and "no change" indicates a ratio between 0.5 and 1.5. The center circle represents the distribution of molecules in the absence of lithium in the "tolerance", "sensitization" and "no change" groups. Each of these three groups was subclassified according to the effects of lithium exposure during the pretreatment phase and these are shown in the circles in the periphery.

For the original "no change" group, in the presence of lithium if the ratio was (a) <0.5 they were classified as "tolerance", (b) >1.5 they were classified as "sensitization", and (c) $0.5 - 1.5$ they were classified as "no change". For the original "tolerance" group, in the presence of lithium if the ratio was (a) still <0.5 and at least 0.1 lower than without lithium they were classified as "more tolerance", (b) still <0.5 and equal or greater than the ratio without lithium they were classified as "no change", and (c) >0.5 they were classified as "blocked tolerance". For the original "sensitization" group, in the presence of lithium if the ratio was (a) <0.5 they were classified as "tolerance", (b) $0.5 - 1.5$ they were classified as "blocked sensitization", (c) still >1.5 they were classified as "no change".

Figure 3.

LPS-tolerance in IL-6 production differs in astrocytes, RAW264.7 cells and microglia. **(A)** Primary mouse astrocytes, **(D)** RAW264.7 cells or **(E)** primary mouse microglia were pretreated with 100 ng/mL LPS for 24 h, washed, and restimulated with 10 ng/mL LPS for 24 h. Media from the final 24 h were analyzed for IL-6 by ELISA. **(B)** Primary mouse astrocytes were pretreated with 100 ng/mL LPS for the indicated times, washed, and restimulated with 10 ng/mL LPS for 24 h. **(C)** Primary mouse astrocytes were pretreated with 100 ng/mL LPS for one (1×), two (2×) or three (3×) 24 h intervals, with washes between intervals, and restimulated with 10 ng/mL LPS for 24 h. Proliferation of astrocytes

resulted in greater production of IL-6 by 10 ng/mL LPS treatment without LPS-pretreatment in the 2× and 3× samples. Values represent mean $\pm$ SEM (n=3–6; *p<0.05, ANOVA).

Figure 4.

GSK3 inhibitors enhance LPS-tolerance. (A, B) Mouse primary astrocytes were pretreated without (0) or with 100 ng/mL LPS (LPS) with or without GSK3 inhibitors 10 μ M kenpaullone, 10 μ M Chiron99021, or 10 μ M TDZD-8 for 24 h, washed, and restimulated with 10 ng/mL LPS for 24 h and media from the second incubation were analyzed for IL-6 by ELISA. Values are shown as a percent of IL-6 production in the absence of GSK3 inhibitors. (C) Experiments were carried out as in A and B but GSK3 inhibitors were added only for the last 1 h of the first stimulation with LPS, and IL-6 was measured in the media after washing and restimulation with 10 ng/mL LPS for 24 h. (mean $\pm$ SEM; n=3–4; *p<0.05, ANOVA).

Figure 5.

Lithium enhances LPS-tolerance. (A) Mouse primary astrocytes were pretreated with 100 ng/mL LPS with or without the GSK3 inhibitor 20 mM LiCl for 24 h, or (B) LiCl was added for either 24 h or for the last 1 h of the first stimulation with LPS, cells were washed and restimulated with 10 ng/mL LPS for 24 h (mean $\pm$ SEM; $n=3$; $*p<0.05$, ANOVA). (C) Mouse primary astrocytes were treated as in (B) and were immunoblotted for phosphoSer21GSK3 α , phosphoSer9GSK3 β , and total GSK3 α/β (representative of 3 independent experiments). (D) After treatments as indicated, immunoblotting was used to measure the total level and serine-phosphorylation of GSK3 α and GSK3 β in mouse primary microglia (3.75 μ g protein) and mouse primary astrocytes (1.5 μ g protein). Results are

representative of three independent measurements. The level of GSK3 β in astrocytes was $362 \pm 40\%$ (n=3) the level in microglia in the 0/0 treated samples (taking into account the different amounts of protein loaded for each cell type, which was necessary to obtain accurate immunoblots of GSK3 β in each cell type measured on the same gel).

Figure 6.

GSK3 counteracts LPS-tolerance in astrocytes. (A) Mouse primary astrocytes were treated with control siRNA (SiCtl) or siRNA for GSK3 β for 48 h (values are shown as a percent of IL-6 production in the absence of GSK3 knockdown), or (B) control GFP or constitutively active S21A-GSK3 α and S9A-GSK3 β (α + β) were expressed in astrocytes for 48 h, followed by pretreatment with 100 ng/mL LPS for 24 h and restimulated with 10 ng/mL LPS for 24 h. Media were analyzed for IL-6 by ELISA (bar graph) or cell lysates were immunoblotted for GSK3 α and GSK3 β and β -actin or STAT3 loading controls (*top panels*) ($n=3-5$). (C, D) Mouse primary astrocytes isolated from GSK3 knockin mice (KI) or wild-type mice (WT) were pretreated with 100 ng/mL LPS (C) without or (D) with GSK3 inhibitors, 20 mM LiCl or 10 μ M TDZD-8, for 24 h and restimulated with 10 ng/mL LPS for 24 h. Media were analyzed for IL-6 by ELISA. Cell lysates were immunoblotted for phosphoSer21GSK3 α , phosphoSer9GSK3 β , and total GSK3 α / β (C, $n=3$, D, $n=2$). Values represent mean $\pm$ SEM (* $p<0.05$, ANOVA).