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GSK3β and the control of infectious bacterial diseases

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Abstract

Glycogen synthesis kinase 3β (GSK3β) has been shown to be a critical mediator of the intensity and direction of the innate immune system responding to bacterial stimuli. This review will focus on: (i) the central role of GSK3β in the regulation of pathogen-induced inflammatory responses through the regulation of pro- and anti-inflammatory cytokine production. (ii) The extensive ongoing efforts to exploit GSK3β for its therapeutic potential in the control of infectious diseases. (iii) The increasing evidence that specific pathogens target GSK3β-related pathways for immune evasion. A better understanding of complex bacterial–GSK3β interactions is likely to lead to more effective anti-inflammatory interventions and novel targets to circumvent pathogen colonization and survival.

Keywords

GSK3β; cytokines; immune evasion; inflammation; septic shock; TLRs

Glycogen synthase kinase 3 action and isoforms

Glycogen synthase kinase 3 (GSK3) is a highly conserved, constitutively active, S/T protein kinase. GSK3 was originally named after its ability to phosphorylate the critical rate-limiting metabolic enzyme, glycogen synthase, which controls the final step of glycogen synthesis. However, this name does not adequately describe the diverse substrates and multiple functions now attributed to GSK3, including embryonic development, cell cycle control, cell differentiation, cell mobility, apoptosis, migration, and the focus of this review - a central regulator of the inflammatory response to bacterial infection and other insults [1].

In mammals, two isoforms, GSK3α and GSK3β, have been identified and each are widely expressed [2]. Sequence analysis has revealed that while these two isoforms are 98% homologous within their kinase domains and 85% homologous overall [2], the final 76 C-terminus amino acids exhibit only 36% homology [2]. This may explain functional

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differences between GSK isoforms, with GSK3β deficiency embryonic lethal in mice in a manner that cannot be compensated by GSK3α [3].

GSK3β appears to play important roles in the host response to viral [4], fungal [5] and parasitic infections, including malaria [6]. However, this review will focus specifically on the significance of GSK3β in the mammalian immune response, therapeutic targeting of GSK3β during bacterial infection, and exploitation of GSK3β by pathogens.

A distinct feature of GSK3 is the display of high activity under basal conditions. This activity is differentially regulated by tyrosine and S/T phosphorylation. Tyrosine phosphorylation (Tyr279 for GSK3α and Tyr216 for GSK3β) enhances activity, while N-terminal serine phosphorylation (Ser21 for GSK3α and Ser9 for GSK3β) is suppressive. Other GSK3β sites, such as ERK- and p38–MAPK-phosphorylated Thr43 and Ser389, are also reported to influence GSK3β activity in the brain and thymocytes [1]. The major GSK3β regulating event, however, seems to be Ser9 phosphorylation. Multiple extracellular signals induce a rapid Ser9 phosphorylation, resulting in a dramatic decrease in enzymatic activity, with the upstream activators phosphoinositide 3-kinase (PI3K)–Ak thymoma / protein kinase b (Akt / PKB) best studied (Figure 1). Early studies identified the insulin-mediated signaling pathway as an activator of PI3K and, subsequently, a phospho-inactivator of both GSK3α (Ser21) and GSK3β (Ser9) [7]. Other molecules, which include growth factors, phorbol esters, amino acids, interleukin receptors, Toll-like receptors (TLRs), T cell receptors, and CD28, as well as KIT/stem cell factor receptor activation have since been shown to be involved in the phosphorylation, and thus inactivation, of GSK3 [1].

In addition to the PI3K–Akt pathway, several other kinases, such as 90 kDa ribosomal protein S6 kinase 1 (p90RSK), serum and glucocorticoid-regulated kinase 1 (SGK1), and MAPK–p38 have also been shown to phospho-inactivate GSK3β [8–10]. As these kinases all belong to the protein kinase A, G, and C (AGC) family, it is possible that GSK3β could also be phospho-inactivated by other members of this large kinase group which control a multitude of physiological processes.

Substrate recognition by GSK3β is well defined. The kinase has a 100–1000-fold predilection for phosphorylation of substrates that are pre-primed (pre-phosphorylated) on a serine or threonine located around a five amino acid consensus sequence, Ser/Thr–X–X–X–Ser/Thr-P with the first serine or threonine of this motif the residue phosphorylated by GSK3β [11]. Frame *et al.* demonstrated that mutation of Arg96 to Ala96 has been identified as a critical residue in the phosphorylation of pre-primed (pre-phosphorylated), but not non-primed substrates [12]. The GSK3β crystal structure has revealed that Tyr276/216 is also important conformationally, with phosphorylation of this residue enhancing substrate binding [13].

GSK3β is a central mediator of the immune response to bacterial infection

The immune system recognizes and responds to bacterial challenge primarily through pattern recognition receptors (PRRs), such as TLRs, retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide binding oligomerization domain-like receptors (NLRs). Recognition of microbe-associated molecular patterns (MAMPs) by PRRs results in

the initial activation of innate immune cells with an increase in the expression of inflammatory cytokines, co-stimulatory molecules, and major histocompatibility complex (MHC) I and II molecules, all of which work together to promote the subsequent activation of the adaptive immune response. Activation of PRRs by various microbial components results in the recruitment of different downstream signaling adaptors, which confer selectivity on the repertoire of cytokines induced. Broadly speaking, signaling pathways in innate cells can be classified as myeloid differentiation primary response 88 (MyD88)-dependent, which initiate the expression of pro- (e.g. IL-1β, IL-6, TNF, IL-12) and anti-inflammatory (IL-10) cytokines, or MyD88-independent, which control the production of type I interferon through TRIF-related adaptor molecule (TRAM)–TIR-domain-containing adapter-inducing interferon-β (TRIF) [14–15]. While GSK3β is a central regulator of adaptive (including B cell expansion and T cell polarization and cytokine production) and innate immunity, we will focus on the role of the innate arm of defense and the increasing evidence that GSK3β inactivation has potent therapeutic potential in the control of bacterial-driven inflammatory diseases [1, 14, 16] (Figure 2).

It has been known for many years that phospho-inactivativation of GSK3β suppresses MyD88-dependent cytokine production. The consequence is a downregulation of pro-inflammatory cytokines, but upregulation of IL-10 production, in innate cells exposed to bacterial stimuli [14–15, 17]. Nuclear factor-κB (NF-κB) and cyclic AMP response element-binding protein (CREB) represent prototypical transcription factors for pro-inflammatory cytokines and anti-inflammatory IL-10, respectively. Phospho-inactivation of GSK3β enhances interactions of CREB with the co-transcriptor, CBP, attenuating CBP binding with NF-κB [17]. Moreover, active GSK3β is able to directly phosphorylate NF-κB p65 and B-cell lymphoma 3-encoded protein (BCL-3), a transcriptional co-activator of the NF-κB p50 homodimer [18–19]. Phosphorylation of BCL-3 leads to its proteosomal degradation [18]. Thus, GSK3β facilitates NF-κB activity by targeting phospho-activation of NF-κB p65 and limits NF-κB activation in unidentified BCL-3 dependent pathways. GSK3β has also been shown to phosphorylate p105 at residues Ser903 and Ser907, stabilizing p105 by preventing its degradation in unstimulated cells [20–21]. However, in TNF stimulated cells, GSK3β has been found to phosphorylate p105 and prime it for subsequent degradation [20]. Therefore, GSK3β exerts dual effects on p105 either suppressing or augmenting NF-κB activity, depending on the context of the signal. In addition, a recent publication has reported that deficiency of MyD88 does not influence GSK3β phosphorylation levels and that inhibition of GSK3β results in a robust increase of type I interferon (IFNβ) production *in vitro* and *in vivo* [22]. Augmentation of IFNβ occurs via phosphorylation of the transcription factor c-Jun and the enhancement of nuclear AP-1–ATF-2 complex [22].

Apart from the classic cytokines discussed above, other inflammatory mediators, including IL-1Ra, CCL3, CCL4 and nitric oxide (NO) have been reported to be regulated by GSK3β in different contexts. For example, Rehani *et al*. have shown that GSK3β activity negatively regulates the level of the anti-inflammatory cytokine IL-1Ra, while concurrently increasing the levels of IL-1β, in TLR4-stimulated human monocytes. GSK3β inhibition abrogates Rac1 inhibition at residue Ser71, increasing the activities of Rac-1 and its downstream

target, ERK1/2, enhancing IL-1Ra secretion [23]. Jing *et al.* demonstrated that prostaglandin E (PGE)-induced phospho-inactivation of GSK3β leads to a robust decrease in production of CCL3/4, as well as of several pro-inflammatory cytokines, in lipopolysaccharide (LPS) stimulated dendritic cells, suggesting that GSK3β may also regulate the inflammatory response to Gram-negative bacteria by modulating eukaryotic chemotaxis [24]. Expression of inducible nitric oxide synthase (iNOS) has been demonstrated to play an important role in the clearance of intracellular pathogens. Several findings indicate that GSK3β inhibition is also capable of attenuating production of NO by suppressing the expression of iNOS in TLR-stimulated cells [25]. GSK3β thus differentially regulates TLR-mediated production of pro- and anti-inflammatory cytokines, and such studies enhance the robustness of the original proposal by Martin *et al*. that GSK3β in its active form acts as a positive regulator of inflammation and that manipulation of GSK3β could be employed to up- or downregulate the inflammatory response, depending on the clinical necessity [17].

Our understanding of the control of inflammation by GSK3β grows ever more complex and is likely context specific. For example, Hu *et al.* have demonstrated synergy between IFNγ and TLR2, increasing GSK3β activity and augmenting TNF production, while suppressing IL-10, in macrophages. GSK3β inhibition reversed this phenomenon [26]. Furthermore, it appears that there is crosstalk between the PI3K–Akt–GSK3β pathway, the transcription factor STAT3, and other endogenous signaling networks that control inflammation, i.e., the α7 nicotinic acetylcholine receptor (α7nAChR)-initiated cholinergic anti-inflammatory pathway and wingless-type mouse mammary tumor virus integration site (Wnt)-related cascades [1, 27]. Phosphorylation of STAT3 at Tyr705 is important for the production of IL-1β and IL-6 production in LPS-stimulated innate cells [28]. The inhibition of GSK3β in various cell types upon stimulation IFNγ suppresses STAT3 phosphorylation at Tyr705 [29]. On the other hand, GSK3β inhibition enhances the production of IL-10, a prototypic inducer of STAT3 phosphorylation [30], and leads to the later enhancement of phosphorylation of STAT3. Thus, it will be interesting to determine how these opposing effects of GSK3 inhibition on STAT3 phosphorylation, both by directly suppressing phosphorylation of STAT3 and enhancing STAT3 phosphorylation by augmented IL-10, affects the overall anti-inflammatory property of GSK3 inhibition.

The PI3K–Akt–GSK3β pathway has been demonstrated as an important signaling pathway responsible for the acetylcholine-mediated anti-inflammatory response [27]. The cholinergic anti-inflammatory pathway is activated by the interaction of neuronal- or locally-derived non-neuronal acetylcholine with α7-nicotinic acetylcholine receptors (α7nAChRs) on the surface of immune cells [27, 31]. Classic studies by Tracey's group, provided evidence that stimulation of the vagus nerve, and α7nAChRs agonists (acetylcholine and nicotine) prevented cytokine release and pathological sequelae in experimental sepsis, endotoxemia, ischemia/reperfusion injury, hemorrhagic shock, arthritis, and other inflammatory disorders [32]. Activation of α7nAChR by cotinine, a major metabolite of nicotine, leads to activation of PI3K which then phospho-activates Akt, which subsequently phospho-inactivates GSK3β. Cotinine-induced inactivation of GSK3β does not affect the absolute or phosphorylated levels of NF-κB but does increase the levels of the CREB transcription factor and by which augments the production of anti-inflammatory IL-10 in monocytes

stimulated with LPS or other surface-exposed TLR-agonists [27, 33]. Such innate suppression by tobacco alkaloids may be an important clue to the increased susceptibility of smokers to multiple bacterial diseases, including those caused by *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Neisseria meningitidis*, *Chlamydia trachomatis*, *Porphyromonas gingivalis*, and *Helicobacter pylori* as well as post-surgical and nosocomial infections [34]. Indeed, cotinine, a stable molecule that circulates at approximately ten times the levels of nicotine, suppresses the innate response to bacterial stimuli at concentrations similar to those exposed to second-hand smoke [27].

JAK mediated signaling is also important in the cholinergic anti-inflammatory pathway. JAK2 has been reported to be recruited to α7nAChRs on the surface of macrophages upon cholinergic anti-inflammatory pathway engagement, leading to STAT3 activation and suppression of the innate response [35]. α7nAChR engagement promotes GSK3β phosphorylation and abrogates TLR-induced cytokine production [27]. De Jonge *et al.* found that activation of JAK2 controls the anti-inflammatory potential of nicotinic agonists in macrophages, and thus, JAK2 activation may be beneficial in infection control. JAK3 has now been established as a GSK3β-dependent negative regulator of inflammation with suggestions, from JAK3 knockout mice, that JAK3 is key in preventing bacterial-induced intestinal inflammation (neutrophil infiltration, IL-17 expression and epithelial damage) [36]. Thus, JAK2 and JAK3 may be differentially involved in the regulation of TLR-mediated inflammatory responses.

Wnt-related signaling events have also been shown to play an important role in the control of inflammation. The canonical Wnt (Wnt/β-catenin) pathway involves phospho-inactivation of GSK3β, which abrogates the degradation of β-catenin, promoting cytosolic accumulation and nuclear translocation of β-catenin [37]. β-catenin stabilization is known to suppress bacterial-induced inflammation, at least in part, due to physical interactions with NF-κB, in a similar manner to nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) [38]. Wnt3a has been proposed as the prototypic Wnt innate suppressor, partly due to its ability to suppress pro-inflammatory cytokine production in response to *M. tuberculosis* [39].

Furthermore, α7nAChR agonism has also been shown to augment Wnt signaling [40]. Thus, further interplay between endogenous pathways intended to minimize collateral damage consequential to infection is likely to emerge, increasing the possibility of more sophisticated and specific therapeutic manipulations of the innate response to microbial stimuli.

In summary, GSK3β is essential for the generation of MAMP-induced pro-inflammatory cytokines. Differential phosphorylation of GSK3β determines the inflammatory activity of this critical mediator. Ser9 phosphorylation, in particular, suppresses GSK3β and promotes CREB–CBP interactions, and the production of IL-10, over NFκB–CBP. α7nAChR agonists represent efficient endogenous or exogenous GSK3β inhibitors which may be exploited therapeutically to alter the innate response to bacterial infection, as required.

GSK3β is a promising therapeutic target for the control of bacterial-induced inflammation

While GSK3β is pleiotropic, it is a critical immune mediator with sufficient unique activities to have made it a much sought-after therapeutic target over the past decade or so. Indeed, GSK3β targeting has been proposed as a potential strategy for the treatment of multiple diseases that are primarily inflammatory or contain an important inflammatory component, including hypertension, diabetes, inflammatory bowel disease, pancreatitis, rhinosinusitis, psoriasis, cirrhosis, hyperoxia-induced lung injury, myocardial and brain ischemia complications, multiple sclerosis, Alzheimer's disease, neural inflammation, cystic fibrosis, and even traumatic spinal cord and brain injuries and inflammatory components underlying cancer progression.

Novel GSK3β inhibitors continue to emerge, such as SB216763, SB-415286, AR-A014418, thiadiazolidinones [e.g., 4-benzyl-2-methyl-1, 2, 4-thiadiazolidine-3, 5-dione (TDZD-8)], Ro3303544, CHIR98014 and GSK-silencing adenovirus. Several established plant-derived dietary components with reported anti-inflammatory properties, particularly the turmeric polyphenol, curcumin, are GSK3β inhibitors [41]. Of course, lithium has long been established as a potent GSK3β inhibitor. Unfortunately, current inhibitors are not as selective as hoped or are not effective *in vivo*, leading to ongoing efforts to generate new ones that are truly highly selective [42].

As GSK3β is a central regulator of the inflammatory response, it has been extensively studied as a therapeutic target for sepsis, the systemic response to bacteria and bacterial-derived toxins, and sepsis-induced multiple organ failure and death. Inhibition of GSK3β was established as a potent downregulator of TLR-mediated inflammatory responses in the seminal paper by Martin *et al.* [17]. This general phenomenon has been shown to hold true in various specific infections. In an elegant study in 2006, Dugo *et al.* examined the influence of several GSK3β inhibitors (TDZD-8, SB216763, and SB415286) on LPS- or LPS- plus peptidoglycan-induced mortality and organ-specific (kidney, liver, pancreas and neuromuscular) biomarkers of dysfunction. Each inhibitor effectively ameliorated shock-induced organ damage [43]. Recently, Noh *et al.* have shown that in LPS-induced toxemia, GSK3 inhibition by SB415286 induces protein kinase Cδ (PKCδ) and, subsequently, ERK1/2 activation, resulting in increased expression of anti-inflammatory IL-10 and abrogated septic shock [44].

Pulmonary and cardiac aspects of septic shock have received particular attention. In a murine cecal ligation and puncture sepsis model, glucan phosphate, which attenuates cardiac dysfunction in this system, suppresses sepsis-associated macrophage migration inhibitory factor (MIF) expression and cardiomyocyte apoptosis concomitant with activation of the Akt/GSK3β pathway [45]. The manipulation of GSK3β in cardiomyocytes, through the use of SB216763, dominant negative mutants or adenoviral overexpression, has also been shown to control LPS-induced TNF expression [46].

Acute respiratory distress syndrome (ARDS), associated with reduced lung surfactants, is the respiratory component of multiple organ failure. Acyl-CoA:lysophosphatidylcholine

acyltransferase 1 (LPCAT1) is a key enzyme in the synthesis of dipalmitoylphosphatidylcholine, an important surfactant. One potential mechanism of ARDS development is that, upon LPS-stimulation, LPCAT1 is phosphorylated by GSK3β, then ubiquinated and targeted for degradation [47]. Inhaled aerosolized insulin, known to inhibit GSK3β, has been proposed as a treatment for sepsis-induced ARDS [48]. In a cecal ligation and puncture sepsis model, LiCl reduced systemic cytokine (IL-1β, IL-6, and TNF) levels. LiCl treatment also reduced pulmonary 8-iso-prostaglandin F2α (8-ISO), increased lung SOD activity, and resulted in a dose-related reduction in pulmonary inflammation. Therefore, although GSK3β activity itself was not monitored, LiCl–the classic GSK3 antagonist–is a potential treatment for sepsis-induced lung injury [49].

Muscle loss is a well-established consequence of prolonged sepsis with LiCl or the thiadiazolidinone derivative TDZD-8 shown to efficiently suppress sepsis-induced protein breakdown [50].

The role of GSK3β in bacterial-induced bone pathologies has also been addressed. CD40 is a co-stimulatory molecule and interaction of CD40 with CD154 on T helper (T_h) cells is required for the activation of antigen-presenting cells, such as dendritic cells and macrophages. It is now established that bacterial infection with several osteopathogens – *Porphyromonas gingivalis*, *Staphylococcus aureus* and *Salmonella enterica* - promotes surface expression of CD40 on osteoblasts which may then contribute to osteoinflammation by interaction with T cells and the generation of pro-inflammatory mediators [51]. This enhanced immunological function may well correlate with reduced osteoblastic capacity to lay down new bone matrix. Die *et al.* have shown that SB216763 suppresses IkBα phosphorylation and decreases the expression of CD40 as well as the production of IL-6, TNF and IL-1β in *P. gingivalis* LPS-stimulated murine osteoblast-like cells [51]. Thus, GSK3β was identified as a potential therapeutic target for infection-induced bone loss. To this end, Adamowicz *et al.* have recently employed the GSK3β-inhibitor, SB216763, to completely abrogate *P. gingivalis*-elicited alveolar bone loss in a murine model of periodontitis [16] (Figure 3). *P. gingivalis* has also been associated with multiple chronic, systemic conditions. In a recent study examining *P. gingivalis* and diabetes, it has been shown that this pathogen translocates from the oral cavity to the liver, infects hepatic cells, and decreases glycogen synthesis in a manner likely to be driven by an insulin receptor substrate-1(IRS-1)–Akt–GSK3β signaling cascade [52].

Inhibition of GSK3β has been shown to reduce inflammation and mortality in other infectious disease models [53–55]. In meningitis studies, GSK3β inhibition (by SB216763 or LiCl) has been shown to suppress LPS-induced pro-inflammatory cytokines and promote IL-10 secretion in rat mixed glial-enriched cortical cultures [56]. Moreover, the anesthetic agent, propofol, suppresses LPS-induced pro-inflammatory cytokine production in LPS-stimulated BV2 microglia cells in a manner that could involve both TLR4 downregulation and the inactivation of GSK3β [54]. Microglia are producers of large concentrations of shock-inducing cytokines and others have confirmed that GSK3β inhibition upregulates IL-10 in BV2 cells as well as suppressing iNOS [57]. Most recently, it has been shown that the anti-inflammatory properties of lipoic acid on LPS-stimulated BV2 cells are mediated through GSK3β inactivation [52]. Furthermore, active (unphosphorylated) GSK3β is

important for adhesion molecule activation, endothelial interaction and monocytic crossing of the blood-brain barrier in response to bacteria and other inflammatory stimuli [58].

Heat-inactivated *S. aureus* induces TNF and NO production in microglia, with the production of both inflammatory mediators abrogated by GSK3β inhibition by LiCl or 6-bromo-indirubin-3′-oxime [59]. IL-10 is considered to be particularly important in protecting against *S. aureus*-mediated brain abscesses and is augmented by GSK3β inhibition [59]. *Francisella tularensis* induces pro-inflammatory cytokine production by increasing GSK3β activity. GSK3β is an attractive target for the treatment of tularemia, as LiCl suppresses inflammatory mediators (IL-6, IL-12p40 and TNF) induced by *F. tularensis* upon infection of macrophages promotes survival of *F. tularensis*-infected mice [55].

In addition to a role in controlling acute inflammatory responses to bacterial infection, GSK3β can also contribute to long term vaccine responses. Age-related immune senescence can reduce the efficacy of antibacterial vaccines, including the pneumococcal polysaccharide vaccine. It has been established that in aged animals stimulated with *Streptococcus pneumoniae*, the innate response is suppressed. In spleen-derived rat macrophages, this was associated with altered PI3K–Akt–GSK3β signal pathway activity and characterized by a reduced pro-inflammatory cytokine profile and elevated IL-10. Furthermore, inhibition of PI3K restored an appropriate innate response to *S. pneumoniae* as well as TLR1/2-, TLR2/6- and TLR4-specific agonists [60].

GSK3β is thus a potentially effective therapeutic target for bacterial shock-induced organ damage, ARDS, bone diseases, meningitis and tularemia. The development of more potent and specific GSK3β inhibitors, alongside deeper understandings of GSK3β-related signaling events, could lead to a new paradigm of treatment for bacterial diseases - one that aims prevents to prevent the multiple pathological consequences of robust inflammation and which is likely to be employed in conjunction with contemporary anti-bacterial regimens.

Exploitation of GSK3β and related molecules by pathogenic bacteria

Bacteria are known to circumvent aspects of the immune response by dysregulating kinase networks, as reviewed by Krachler *et al.* [61]. For example, the *Shigella* phosphothreonine lyase, OspF, inhibits ERK and, consequently, suppresses inflammation [61]; the *Yersinia* spp. acetyltrasferase, YopJ, inhibits MAPK, influencing apoptosis [61-62]; *Pseudomonas aeruginosa* ExoS and ExoT differentially influence Rac1 GTPase activity and strongly influence innate cell-specific invasion [63]; while the nucleoside-diphosphate-kinase, NDK, of *P. gingivalis* blocks ATP-induced reactive oxygen species (ROS) production in innate cells [64] and the serine phosphatase, SerB, dephosphorylates the p65 NF-κB subunit [65]. Thus, targeting of immune signaling molecules by bacteria is an established phenomenon. While it has long been known that multiple viruses, including adenoviruses and influenza viruses, specifically interfere with the PI3K–Akt–GSK3β axis in order to promote replication [66], increasing evidence suggests that one mechanism of immune evasion by bacterial pathogens is to also target GSK3β-related signaling events.

The phosphoinositide phosphatase, SigD (SopB), of *S. enterica* phosphorylates Akt and, subsequently, GSK3β and FoxO3a - a process thought to be important in the invasion of

enterocytes [67]. *P. aeruginosa* takes advantage of PI3K signaling in order to invade epithelial cells basolaterally. PI3K activation by *P. aeruginosa* results in phosphatidylinositol (3,4,5)-triphosphate (PIP_3) generation, Akt recruitment and the generation of PIP_3-rich membrane regions facilitating bacterial entry [68]. The role of the downstream regulator, GSK3β, in this invasion system has not been directly addressed. A similar requirement for PI3K in host cell invasion has been identified for other pathogens, including group A streptococci [69], *Helicobacter pylori* [70], *Chlamydia pneumoniae* [71] and *Listeria monocytogenes* [72].

Targeting GSK3β appears to increase the virulence of some pathogens. *Bacillus anthracis* influences GSK3β phosphorylation in pulmonary epithelial cells with both the lethal (LeTx, a MAPK inhibitor) and edema toxins (EdTx, an adenylate cyclase) produced by this bacterium inhibiting AKT phosphorylation. Furthermore, the delivery of wortmannin, a PI3K–AKT inhibitor, hastens death in toxin-producing *B. anthracis*-infected mice [73]. *S. aureus* similarly exploits GSK3β for virulence, with PI3K–AKT-mediated GSK3β phosphorylation at Ser9 a key event during endothelial invasion [74].

Early in macrophage infection, *Yersinia enterocolitica* phosphorylates GSK3β, along with Akt and FKHRL1, in a PI3K-dependent manner, with these phosphorylation events reversed later by virulent - but not avirulent - strains. These events are mediated by the *Y. enterocolitica* YopH protein. It has been hypothesized that one key consequence of such YopH activity is immunoprotection via the downregulation of MCP-1 and the subsequent inhibition of pathogen recruitment to lymph nodes [75]. YopH also inhibited T cell proliferation and the PI3K-dependent secretion of IL-2 [75].

Group B streptococci are important etiological agents of pneumonia, sepsis and – particularly in newborns – meningitis. As epithelial infection by *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) leads to bacterial SopB-mediated activation of Akt and suppression of caspase 3-induced apoptosis [76], Burnham *et al.* examined the importance of GSK3β in group B streptococcal infection and found that infection-elicited GSK3β phosphorylation which protected against camptothecin-induced epithelial apoptosis for at least 18 hours [77]. Presumably, this is a mechanism that promotes bacterial persistence inside target eukaryotic cells. Inhibitors of the Akt–GSK3β signaling cascade, which is also important in actin dynamics, have been used to effectively block bacterial invasion of, but not attachment to, epithelial cells [77]. Similar data have been found with group A streptococci [53].

Direct or indirect inactivation of GSK3β by bacteria presumably suppresses the innate response, providing immune protection to the infecting microbe. Indeed, non-typeable *Haemophilus influenza* has been shown to activate the PI3K–Akt pathway in epithelial cells, leading to p38 MAP kinase inhibition and the downregulation of TLR2 [78]. *S.* Typhimurium infection leads to reduced phosphorylation, thus enhanced activity, of GSK3β and degradation β-catenin, thus augmenting the production of pro-inflammatory cytokines IL-6 and IL-8 and colonic inflammation [38].

Virulent strains of *H. pylori*, associated with gastric ulcers and cancer, secrete high amounts of vacuolating toxin, VacA - a promising *H. pylori* vaccine component. VacA, which exerts influence on multiple eukaryotic signaling events, inactivates GSK3β in a PI3K-dependent manner [79–80]. *H. pylori* isolates from subjects exhibiting regression of gastric intestinal metaplasia following *H. pylori* eradication, more efficiently phosphorylate GSK3β in gastric adenocarcinoma (AGS) cells than strains from patients with persistent intestinal metaplasia [81]. Furthermore, the *Chlamydomonas*-derived peptide, H-P-6 (Pro-Gln-Pro-Lys-Val-Leu-Asp-Ser), has been shown to suppress *H. pylori*-induced hyperproliferation and migration of AGS cells by a mechanism that involves epidermal growth factor receptor (EGFR) activation of the PI3K–Akt pathway and GSK3β inactivation [82]. Thus GSK3β represents a potential therapeutic target for the prevention of *H. pylori*-driven gastric cancer.

In mice, pulmonary infection with *Klebsiella pneumonia* also leads to GSK3β phosphorylation, along with multiple other effects on eukaryotic kinases, that may well be relevant to immune suppression and avoidance [83].

Finally, there may be scope to exploit bacterial GSK-targeting products therapeutically. For example, the *Streptomyces albus*-derived livestock antibiotic, salinomycin, that is being developed as an anti-cancer drug clearly phosphorylates Akt, GSK3β and mammalian target of rapamycin (mTOR) in head and neck squamous cell carcinoma stem cells [84]. In a similar vein, S632A3, a new glutarimide antibiotic isolated from *Streptomyces hygroscopicus*, is a potent inhibitor of inflammation in LPS-stimulated macrophage-like RAW cells. This inflammatory suppression occurs via inactivation of GSK3β and the differential promotion of CREB–CBP interactions over NF-κB which, subsequently, leads to increased IL-10 production [85]. Several other antibiotics have also been shown to influence GSK3β activity [86]. There may also be occasion to employ GSK3β-inhibitors as an adjunct to antibiotic treatment. *Streptomyces verticillus*-derived bleomycin, used in the treatment of various cancers, can induce lung injury. In a murine model, bleomycin-induced pulmonary inflammation, characterized by neutrophil infiltration, edema and the accumulation of inflammatory mediators (myeloperoxidase, iNOS, TNF and IL-1β), is dramatically reduced upon GSK3β inhibition by TDZD8 [87].

We are beginning to understand that several bacteria exploit GSK3β and related signaling molecules in order to subvert the immune response. It is not yet clear whether or not this is a widespread strategy among human pathogens. Furthermore, the molecular mechanisms of GSK3β targeting by microbes remain to be more fully elucidated. Understanding such pathogen-GSK3β interactions could lead to novel antibacterial therapeutics.

Concluding remarks

GSK3β plays a critical role in the regulation of pathogen-induced inflammatory responses through the regulation of pro- and anti-inflammatory cytokine production. GSK3β has also been aggressively pursued as a therapeutic target for the control of infectious diseases. Furthermore, GSK3β is exploited by pathogenic bacteria seeking to avoid the immune system. Further molecular understandings of complex GSK3β signaling events (Box 1),

combined with the emergence of better and more specific inhibitors, is likely to lead to more effective interventions.

Box 1

Outstanding questions

- Can more specific, pharmacologically desirable GSK3β inhibitors be developed?
- What pro- and anti-inflammatory signaling pathways crosstalk with GSK3β?
- Will conjunctive anti-bacterial (antibiotics) and anti-inflammatory (GSK3β) therapies prove more efficacious against shock than individual therapies?
- Is targeting of GSK3β a common immune evasion strategy of pathogenic bacteria?

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Highlights

- GSK3β is a constitutively active serine/threonine (S/T) kinase with multiple Toll-like receptor (TLR) signal-related targets.
- Inhibition of GSK3β is an effective treatment for septicemic shock.
- Some important bacterial pathogens target GSK3β to enable intracellular infection and to evade host responses.
- Some antibiotics function as GSK3β inhibitors.

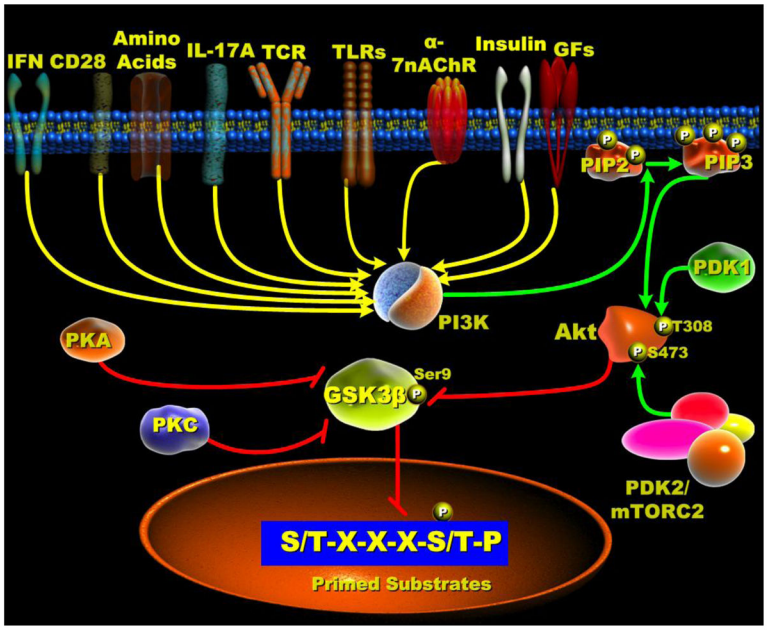

Figure 1. Mediators of GSK3β phosphorylation

In its active state, GSK3β exhibits a 100–1000-fold increased efficiency in substrate specificity for pre-primed (pre-phosphorylated) substrates, as compared to non-primed substrates. However, multiple agonists including growth factors, insulin, α7nAchR, TLRs, TCR, cytokines, CD28, and amino acids phospho-activate PI3K, which results in the generation of PIP_3 that allows for the recruitment of Akt via its pleckstrin homology domain. Full activation of Akt occurs through phosphorylation at threonine 308 by PDK1 and serine 473 by PDK2/mTORC2. Upon activation, Akt can phosphorylate GSK3β (Ser9) resulting in GSK3β inactivation. PKC and PKA also can phospho-inactivate GSK3β.

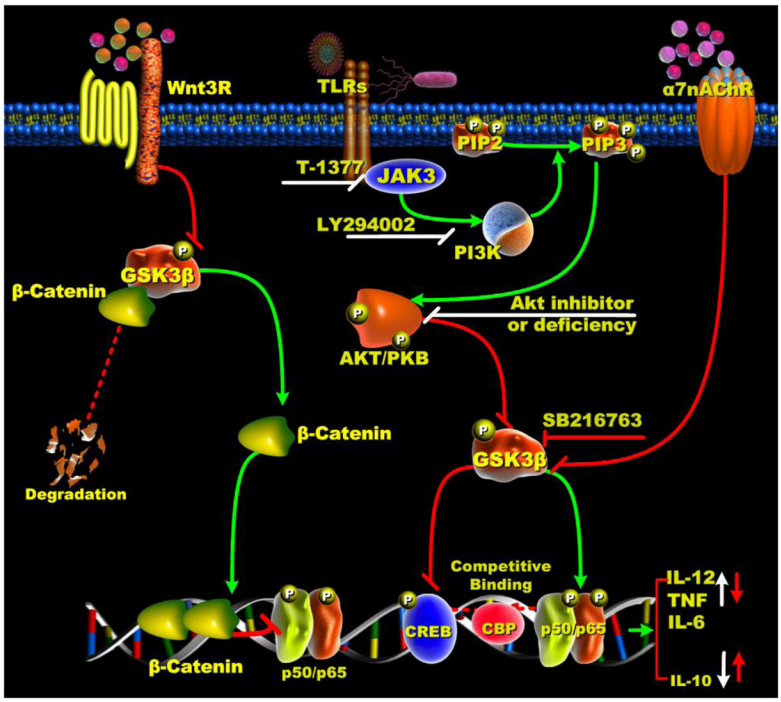

Figure 2. GSK3β is a central player in Wnt, α7nAChR- and PI3K-mediated anti-inflammatory signaling

Microbial pathogens and their components initiate the innate immune response through activation of multiple signaling pathways, most importantly the TLR-NF-κB axis, driving the production of multiple pro-inflammatory cytokines. Unphosphorylated GSK3β is an essential and controlling mediator of this inflammatory response to bacteria. Likely in order to prevent an overly robust response to infection, several endogenous mechanisms serve to dampen bacterial-driven inflammation. The cholinergic anti-inflammatory pathway, triggered upon α7nAChR engagement by endogenous (acetylcholine) and exogenous (e.g., nicotine) agonists, is a classic example. α7nAChR-mediated activation of PI3K leads to phospho-inactivation of GSK3β at Ser9 (through Akt and PIP_3) resulting in increased activity of CREB, displacement of NF-κB p65 from the co-activator of transcription, CBP, and lowered transcriptional activity of NF-κB p65-driven pro-inflammatory genes. Increased chromosomal access to CREB also promotes production of anti-inflammatory IL-10. Inhibitors of TLR pathway molecules (JAK3; PI3K; Akt) that normally drive GSK3β Ser9 phosphorylation (white lines) exacerbate bacterial-driven inflammation, while promoters of GSK3β Ser9 phosphorylation (α7nAChR pathway) or pharmaceutical GSK3β inhibitors (e.g., SB216763) suppress pro-inflammatory mediators while promoting anti-inflammatory IL-10 in response to infection (red lines). Perhaps independently, activation of the canonical Wnt (Wnt/β-catenin) pathway also leads to phospho-inactivation of GSK3β and the stabilization of β-catenin which suppresses inflammation by interference with NF-κB. There is much current emphasis on the development of pharmacological inhibitors of GSK3β that alter the magnitude of the inflammatory response through differentially regulating pro- and anti- inflammatory cytokines. Therefore, it is possible that we will eventually be able to up- or down-regulate the intensity of the innate response to bacterial infection depending on the clinical necessity.

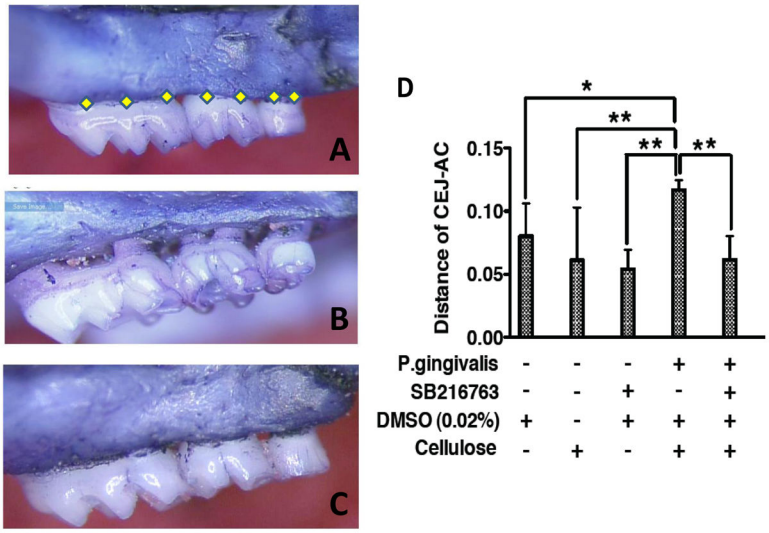

Figure 3. GSK3β inhibition abrogates pathogen-induced bone loss

8–12 week old B6129SF2/J mice were randomly divided into three control groups and two experimental groups (n = 5 per group). The control groups were treated with cellulose (sham infected), 0.02% DMSO, or SB216763 (10 mg/kg) respectively. The experimental groups were orally infected with *P.gingivalis 33277* with or without pretreatment of the GSK3 inhibitor, SB216763 (10 mg/kg). Alveolar bone loss was visualized by methylene blue / eosin staining six weeks later. Typical maxillae from (A) sham-infected, (B) *P. gingivalis*-infected, and (C) SB216763-treated, *P. gingivalis*-infected mice are presented. (D) The distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured 6 weeks post infection at 14 predetermined maxillary buccal sites, marked by yellow diamonds, in 8–12 week old B6129SF2/J mice divided into three control (cellulose; DMSO; or SB216763 treated) and two experimental (*P. gingivalis*-infected; and SB216763-treated, *P. gingivalis*-infected mice) groups. Data are presented as mean distance CEJ-ABC in mm ± s.d. where there are 5 mice per group. Symbols: *, $p < 0.05$ compared to *P. gingivalis* treated group; ** $p < 0.01$ compared to *P. gingivalis* treated group. Reproduced, with permission, from Adamowicz *et al*. [16].