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PKB/SGK-dependent GSK3-phosphorylation in the regulation of LPS-induced Ca^{2+} increase in mouse dendritic cells

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ABSTRACT

The function of dendritic cells (DCs) is modified by glycogen synthase kinase GSK3 and GSK3 inhibitors have been shown to protect against inflammatory disease. Regulators of GSK3 include the phosphoinositide 3 kinase (PI3K) pathway leading to activation of protein kinase B (PKB/Akt) and serum and glucocorticoid inducible kinase (SGK) isoforms, which in turn phosphorylate and thus inhibit GSK3. The present study explored, whether PKB/SGK-dependent inhibition of GSK3 contributes to the regulation of cytosolic Ca^{2+} concentration following stimulation with bacterial lipopolysaccharides (LPS). To this end DCs from mutant mice, in which PKB/SGK-dependent GSK3α,β regulation was disrupted by replacement of the serine residues in the respective SGK/PKB-phosphorylation consensus sequence by alanine (*gsk3*^{KI}), were compared to DCs from respective wild type mice (*gsk3*^{WT}). According to Western blotting, GSK3 phosphorylation was indeed absent in *gsk3*^{KI} DCs. According to flow cytometry, expression of antigen-presenting molecule major histocompatibility complex II (MHCII) and costimulatory molecule CD86, was similar in unstimulated and LPS (1 μg/ml, 24 h)-stimulated *gsk3*^{WT} and *gsk3*^{KI} DCs. Moreover, production of cytokines IL-6, IL-10, IL-12 and TNFα was not significantly different in *gsk3*^{KI} and *gsk3*^{WT} DCs. In *gsk3*^{WT} DCs, stimulation with LPS (1 μg/ml) within 10 min led to transient phosphorylation of GSK3. According to Fura2 fluorescence, LPS (1 μg/ml) increased cytosolic Ca^{2+} concentration, an effect significantly more pronounced in *gsk3*^{KI} DCs than in *gsk3*^{WT} DCs. Conversely, GSK3 inhibitor SB216763 (3-[2,4-Dichlorophenyl]-4-[1-methyl-1H-indol-3-yl]-1H-pyrrole-2,5-dione, 10 μM, 30 min) significantly blunted the increase of cytosolic Ca^{2+} concentration following LPS exposure. In conclusion, PKB/SGK-dependent GSK3α,β activity participates in the regulation of Ca^{2+} signaling in dendritic cells.

1. Introduction

The glycogen synthase kinase 3 (GSK3α,β) participates in the regulation of a wide variety of functions affecting metabolism, cell proliferation, cell differentiation and cell survival [1–3]. Accordingly, inadequate regulation of GSK3β contributes to the pathophysiology of diverse diseases such as diabetes, cancer, inflammation, mood disorders and neurodegeneration [4–6]. During inflammation GSK3 is critically important for the balance between pro- and anti-inflammatory cytokine production [7–12]. Along those lines, inflammatory responses could be attenuated by pharmacological GSK3 inhibition [8]. GSK3 inhibitors have proven effective in diverse inflammatory disorders including sepsis, arthritis, peritonitis, inflammatory bowel disease and experimental autoimmune encephalitis [8,13–17].

GSK3 activity is inhibited by serine phosphorylation, which suppresses kinase activity [18,19], an effect mediated by protein kinase B [20,21] and the serum and glucocorticoid inducible kinase SGK1 [22,23]. Both kinases are activated by the PI3 kinase pathway [24,25].

GSK3 is required for adequate function of dendritic cells (DCs) [11,17,26–28], antigen-presenting cells crucial for the development of T cell immunity, for the initiation of primary immune responses and for the establishment of immunological memory [29,30]. GSK3 is constitutively active in DCs and suppresses their spontaneous maturation, as shown by increased expression of costimulatory molecules CD80, CD83 and CD86 and higher levels of IL-6 secretion upon pharmacological inhibition of GSK3 with LiCl [27]. Upon DC activation in response to a variety of TLR agonists, GSK3 attains a proinflammatory status mediating production of proinflammatory IL-12, IL-6, TNFα, IL-1β, and IFNγ and negatively regulating the production of anti-inflammatory IL-10 [11,17,26,27]. Accordingly, administration of a GSK3 inhibitor has been shown to suppress a Th1-mediated immune response against

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Leishmania major in a mouse model [26]. Moreover, GSK3 has been shown to counteract DC survival [31].

Various DC functions are regulated by cytosolic Ca^{2+} concentrations [32]. Nothing is known, however, on the putative participation of GSK3 in the regulation of Ca^{2+} signaling in DCs. To address this question, DCs were isolated from bone marrow of gene-targeted mice in which the serine residues within the respective PKB/SGK phosphorylation sites of GSKα and GSK3β had been replaced by alanine residues ($GSK3\alpha^{21A/21A}$, $GSK3\beta^{9A/9A}$). In those mice ($gsk3^{KI}$), GSKα and GSK3β are resistant against inactivation by PKB/SGK [33]. Along those lines, $gsk3^{KI}$ mice are resistant to the effect of insulin on muscle glycogen synthase [33]. Earlier studies disclosed altered renal function [34,35], gastric acid secretion [36], catecholamine release [37], and behavior [38] in those mice. Moreover, stimulation of IL-10 production following IFN-β treatment was blunted in $gsk3^{KI}$ mice [11].

2. Materials and methods

2.1. Mice

All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the care and use of laboratory animals and were approved by local authorities. Mice were generated in which the codon encoding Ser9 of the GSK3β gene was changed to encode nonphosphorylatable alanine ($GSK3\beta^{9A/9A}$), and simultaneously the codon encoding Ser21 of GSK3α was changed to encode the nonphosphorylatable $GSK3\alpha^{21A/21A}$ thus yielding the $GSK3\alpha/\beta^{21A/21A/9A/9A}$ double knockin mouse ($gsk3^{KI}$) as described previously [33]. The mice were compared to corresponding wild type mice ($gsk3^{WT}$).

2.2. Cell culture

Dendritic cells (DCs) were cultured from bone marrow of 7–12 week old mice. Bone marrow derived cells were flushed out of the cavities from the femur and tibia with PBS. Cells were then washed twice with RPMI and seeded out at a density of 2×10^6 -cells/10 ml per 60-mm dish. Cells were cultured for 8 days in RPMI 1640 (GIBCO, Carlsbad) containing: 10% FCS, 1% penicillin/streptomycin, 1% glutamine, 1% non-essential amino acids (NEAA) and 0.05% β-mercaptoethanol. Cultures were supplemented with GM-CSF (35 ng/mL, Preprotech Tebu) and fed with fresh medium containing GM-CSF on days 3 and 6. Experiments were performed on DCs at days 7–9.

2.3. Immunostaining and flow cytometry

Cells (10^6) were incubated in 200 μl PBS, containing 0.1% FCS and fluorochrome-conjugated antibodies at a concentration of 10 μg/ml. A total of 5×10^4 cells were analyzed in each individual experiment. The following antibodies (all from BD Pharmingen, Heidelberg, Germany) were used for staining: APC Hamster Anti-Mouse CD11c (Clone: HL3), PE-conjugated anti-mouse CD86, clone GL1 (Rat IgG_{2a}, κ) and PE-conjugated rat anti-mouse I-A/I-E, clone M5/114.15.2 (IgG2b, κ). Following incubation with the respective antibodies for 60 min at 4 °C, cells were washed twice and resuspended in the same buffer and subjected to flow cytometry.

2.4. Western blotting

The expression levels of each protein were analyzed by Western blotting. In brief, 4×10^7 DCs from $gsk3^{KI}$ or $gsk3^{WT}$ mice were washed with ice cold phosphate-buffered saline (PBS) and cells were lysed with cell lysis buffer (Cell Signaling Technology, Inc., New England Biolabs). The extracts were centrifuged at 13,000 rpm for 20 min at 4 °C and the protein concentration of the supernatant was determined. Total protein (30 μg) was subjected to 10% SDS–PAGE. Proteins were transferred to a nitrocellulose membrane (VWR) and the membranes were then blocked for 1 h at room temperature with 10% non- fat dried milk in tris-buffered saline (TBS) containing 0.1% Tween 20. For immunoblotting the membranes were incubated overnight at 4 °C with GSK3α/β (D75D3) XP™ antibody (1:1000, Cell Signaling Technology, Inc., New England Biolabs, 46, 51 kDa) or with phospho-GSK3α/β (Ser21/9) antibody (1:1000, Cell Signaling Technology, Inc., New England Biolabs, 46, 51 kDa). A GAPDH antibody (1:1000, Cell Signaling Technology, Inc., New England Biolabs) was used as a loading control. Specific protein bands were visualized after subsequent incubation with a 1:3000 dilution of anti-rabbit IgG conjugated to horseradish peroxidase and a Super Signal Chemiluminescence detection procedure (GE Healthcare, UK). Specific bands were quantified by Quantity one software (Bio rad gel doc system, Chemidoc XRS). Levels of each protein were expressed as the ratio of signal intensity for the target protein relative to that of GAPDH or target phospho-protein relative to the total protein.

2.5. Measurement of intracellular Ca^{2+}

To determine cytosolic Ca^{2+} concentration, the cells were loaded with Fura-2/AM (2 μM, Molecular Probes, Goettingen, Germany) for 15 min at 37 °C. Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Cells were excited alternatively at 340 or 380 nm and the light was deflected by a dichroic mirror into either the objective (Fluar 40×/1.30 oil, Zeiss, Oberkochen, Germany) or a camera (Proxitronic, Bensheim, Germany). Emitted fluorescence intensity was recorded at 505 nm and data acquisition was accomplished by using specialized computer software (Metafluor, Universal Imaging Downingtown, USA). As a measure for the increase of cytosolic Ca^{2+} concentration, the slope and peak of the changes in the 340/380 nm ratio were determined in each experiment. Intracellular Ca^{2+} was measured prior to and following addition of lipopolysaccharides (LPS) from *Escherichia coli* (1 μg/ml, Enzo Life Sciences, Lausen, Switzerland) to the Ringer solution. In some experiments DCs were treated with GSK3 inhibitor 3- [2,4-Dichlorophenyl]-4-[1-methyl-1H-indol-3-yl]-1H-pyrrole-2,5-dione (SB216763, 10 μM, Enzo Life Sciences, Lausen, Switzerland) for 30 min before the experiment. The Ringer solution contained (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 32.2 Hepes, 2 Na_2HPO_4, 2 $CaCl_2$, and 5 glucose at pH 7.4.

2.6. IL-6, IL-10, IL-12 and TNFα measurements

IL-6, IL-10, IL-12 and TNFα concentrations in DC culture supernatants was determined by using OptEIA ELISA kits (BD Pharmingen) according to the manufacturer's protocol.

2.7. Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using unpaired Student *t*-test or ANOVA. GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, USA) was used. Only results with $p < 0.05$ were considered statistically significant.

3. Results

The present study explored whether protein kinase B (PKB/Akt) and serum & glucocorticoid inducible kinase (SGK) dependent phosphorylation of glycogen synthase kinase 3 (GSK3α,β) participates in the regulation of Ca^{2+} signaling in bone marrow derived dendritic cells (DCs). To this end, DCs were isolated from bone marrow of mice expressing GSK3α,β rendered PKB/Akt and SGK resistant by replacement of the serine residue in the respective SGK/PKB/Akt-phosphorylation consensus sequence by alanine (*gsk3*^{KI}). For comparison, DCs were isolated from respective wild type mice (*gsk3*^{WT}). As illustrated in Fig. 1, the surface abundance of the costimulatory molecule CD86 and antigen-presenting molecule MHC II was similar on CD11c-positive immature and on lipopolysaccharide (LPS, 1 μg/ml, 24 h)-matured DCs from *gsk3*^{KI} and *gsk3*^{WT} mice. The release of interleukin (IL)-12, IL-6, and TNFα, as well as the release of IL-10 were again similar in LPS-matured DCs from *gsk3*^{KI} and *gsk3*^{WT} mice (Fig. 1C).

Western blotting was employed to determine GSK phosphorylation. As illustrated in Fig. 2, phosphorylated GSK3 was observed in DCs from *gsk3*^{WT} mice but not in DCs from *gsk3*^{KI} mice. The mutation thus indeed disrupted GSK3α/β phosphorylation. Additional Western blots were performed to elucidate whether GSK3 phosphorylation in DCs from *gsk3*^{WT} mice could be modified by exposure to LPS (1 μg/ml). Similar to previously published studies [17,26], addition of LPS was followed by a rapid and transient phosphorylation of GSK3 in *gsk3*^{WT} DCs (Fig. 2B and C).

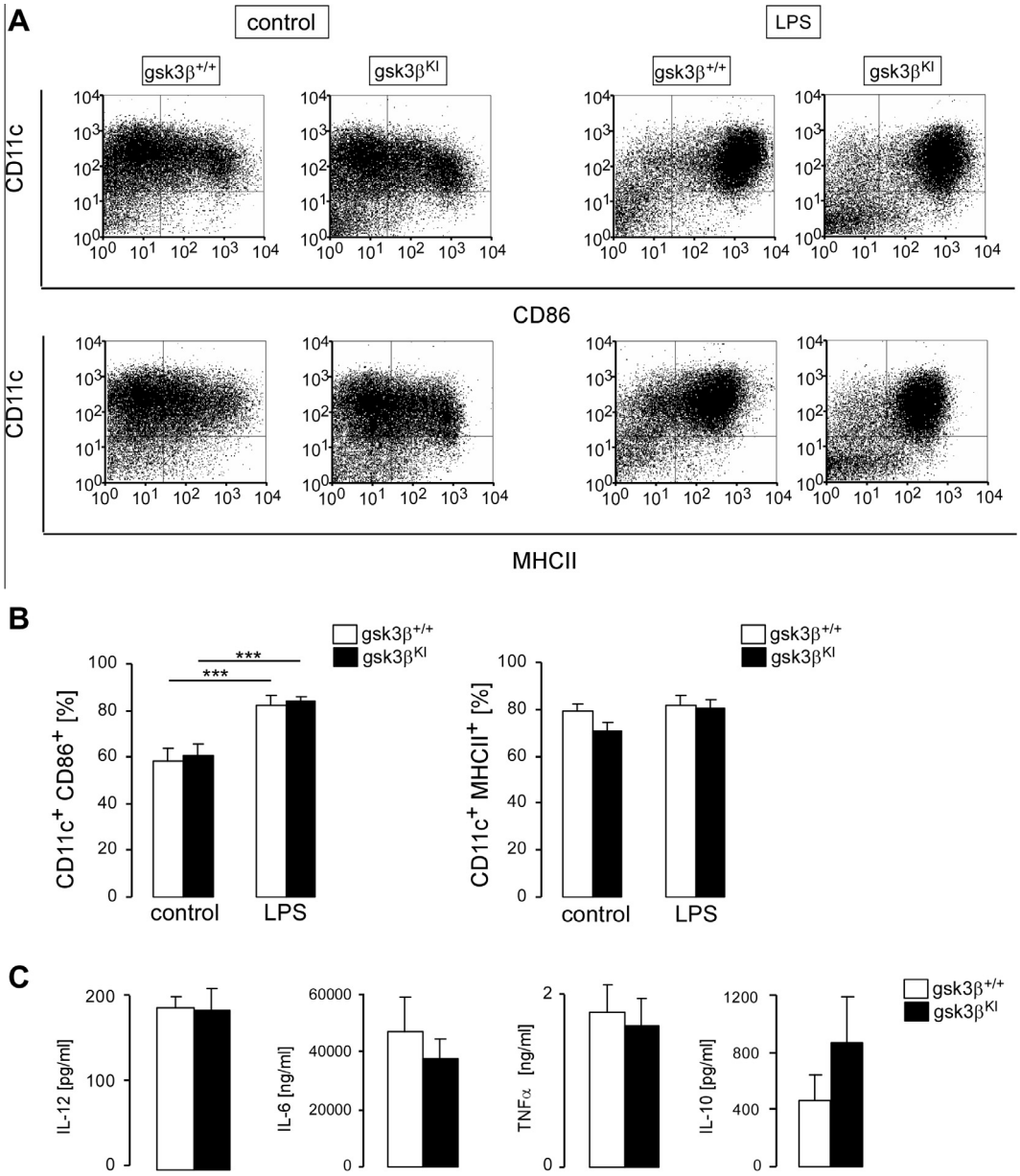

Fig. 1. Differentiation and maturation of bone marrow derived dendritic cells (DCs) from *gsk3*^{WT} and *gsk3*^{KI} mice. (A) Original dot plots of $CD11c^+CD86^+$ (above) and $CD11c^+MHC\ II^+$ (below) DCs at the basal level (control, 1st and 2nd panels) and stimulated with LPS (LPS, 1 μg/ml, 24 h, 3rd and 4th panels) from wild type mice (*gsk3*^{WT}, 1st and 3rd panels) and GSK3α/β$^{21A/21A/9A/9A}$ double knockin mouse (*gsk3*^{KI}, 2nd and 4th panels) mice. (B) Arithmetic means ± SEM (n = 11) of the percentage of $CD11c^+CD86^+$ (left) and $CD11c^+MHC\ II^+$ (right) DCs under control and 24 h after LPS stimulation in primary cultures from *gsk3*^{WT} (open bars) and *gsk3*^{KI} (closed bars) mice. $^{***}(p < 0.001)$ indicates significant difference, ANOVA. (C) Arithmetic means ± SEM (n = 7–10) of IL-12, IL-6, TNFα, and IL-10 secretion in cultured *gsk3*^{KI} DCs (closed bars) and corresponding wild-type mice *gsk3*^{WT} (open bars) after stimulation with LPS (1 μg/ml, 24 h).

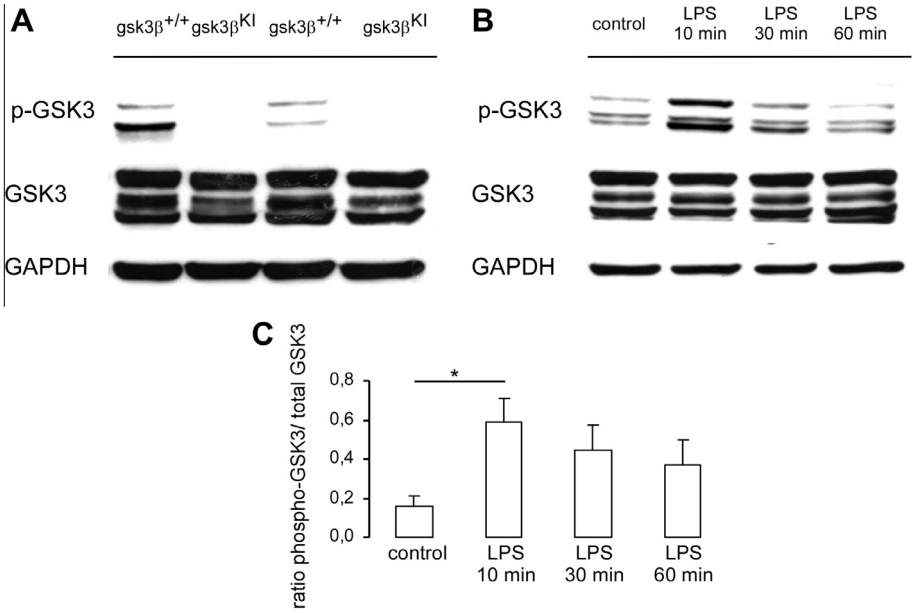

Fig. 2. GSK phosphorylation in DCs from *gsk3*^{KI} and *gsk3*^{WT} mice. (A) Original Western blot of the expression of phosphorylated GSK3 (upper panel), of total GSK3 protein (middle panel) and of GAPDH protein (lower panel) in DCs derived from bone marrow of *gsk3*^{KI} and *gsk3*^{WT} mice. (B) Original Western blot of the expression of phosphorylated GSK3 protein (upper panel), of total GSK3 protein (middle panel), and of GAPDH protein (lower panel) in *gsk3*^{WT} DCs prior to (con) and 10–60 min (10′, 30′, 60′) following acute addition of lipopolysaccharide (LPS, 1 μg/ml). (C) Arithmetic means ± SEM ($n = 6$ independent experiments) of phosphorylated over total GSK3 protein abundance in DCs from *gsk3*^{WT} mice prior to (white bar) and 10–60 min (10′, 30′, 60′) following acute addition of lipopolysaccharide (LPS, 100 ng/ml; black bars. $^{*}(p < 0.05)$, ANOVA.

In subsequent experiments, Fura2-fluorescence has been employed to determine whether PKB/Akt and SGK dependent phosphorylation of GSK3α/β participates in the regulation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Measurements were performed prior to and following stimulation of DCs with LPS. As illustrated in Fig. 3, both slope and peak of LPS-induced $[Ca^{2+}]_i$ increase were significantly more pronounced in DCs from *gsk3*^{KI} mice than in DCs from *gsk3*^{WT} mice.

Further studies were performed to determine whether the LPS induced increase of $[Ca^{2+}]_i$ was modified by pharmacological inhibition of GSK3 with the GSK3 inhibitor SB216763 (3-[2,4-Dichlorophenyl]-4-[1-methyl-1H-indol-3-yl]-1H-pyrrole-2,5-dione, 10 μM, 30 min). As illustrated in Fig. 4, both slope and peak of LPS-induced increase of $[Ca^{2+}]_i$ were significantly reduced by SB216763.

4. Discussion

The present observations disclose a completely novel role of PKB/Akt and SGK dependent GSK3α,β phosphorylation, i.e. the regulation of cytosolic Ca^{2+} concentration in dendritic cells (DCs). PKB/Akt [20,21] and SGK [22,23] inhibit GSK3 by phosphorylation of the enzyme. Thus, disruption of the PKB/Akt and SGK dependent phosphorylation prevents the inhibition of GSK3 following stimulation of the phosphoinositide (PI) 3 kinase pathway [33]. The present observations confirm earlier studies that GSK3 is constitutively active in immature DCs [27] and becomes transiently phosphorylated upon LPS stimulation [17,26]. Despite partial functional inhibition via phosphorylation, GSK3 acquires a novel proinflammatory task in the context of DC activation [27].

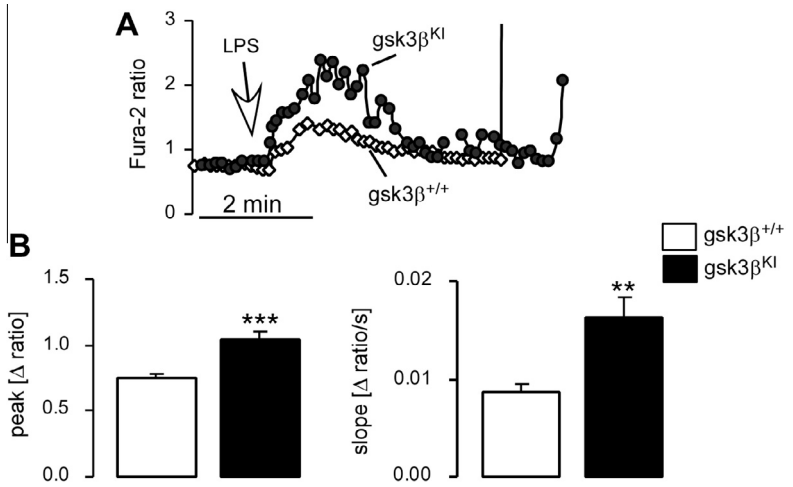

Fig. 3. LPS-induced increase of $[Ca^{2+}]_i$ in DCs from *gsk3*^{KI} and *gsk3*^{WT} mice. (A) Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded *gsk3*^{KI} and *gsk3*^{WT} DCs prior to and following acute addition of lipopolysaccharide (LPS, 1 μg/ml; white arrow). For quantification, peak (Δ ratio) and slope (Δ ratio/s) of $[Ca^{2+}]_i$ increase following addition of LPS were calculated. (B) Arithmetic means ± SEM ($n = 43$–65) of the peak value (left) and slope (right) of the change in intracellular Ca^{2+} concentrations following addition of LPS to *gsk3*^{WT} DCs (white bars) and *gsk3*^{KI} DCs (black bars). $^{***}(p < 0.001)$, unpaired *t*-test.

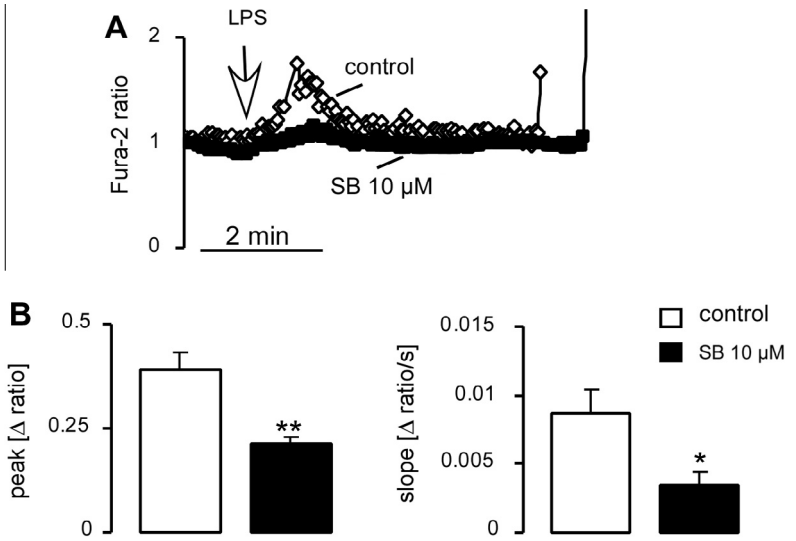

Fig. 4. LPS-induced increase of $[Ca^{2+}]_i$ in DCs from *gsk3*^{WT} mice in the absence and presence of GSK3 inhibitor SB216763. (A) Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded *gsk3*^{WT} DCs prior to and following acute addition of LPS (1 μg/ml; white arrow) in the absence (control, white symbols) and presence (SB, black symbols) of the GSK3 inhibitor SB216763 (10 μM, 30 min pre-treatment before the experiment and then throughout the experiment). (B) Arithmetic means ± SEM (n = 48–68) of the peak value (left) and slope (right) of the change in intracellular Ca^{2+} concentrations following addition of LPS to *gsk3*^{WT} DCs in the absence (control, white bars) and presence (SB, black bars) of the GSK3 inhibitor SB216763.*($p < 0.05$), **($p < 0.01$), unpaired t-test.

Whereas pharmacological inhibition of GSK3 attenuates IL-12 [17,26,27], IL-6 and TNFα [27,39], and enhanced IL-10 [17,26] production, and GSK3 blockers inhibit the wortmannin-enhanced IL12p70 secretion of monocyte-derived DCs [27], we could not observe that resistance of GSK3 to PKB/Akt/SGK-dependent phosphorylation leads to significant changes in IL-12, IL-6, TNFα, and IL-10 production in mouse DCs. The effect of LPS on GSK3 phosphorylation is in large part transient and looses statistical significance within 30 min. Thus, PKB/Akt and SGK dependent phosphorylation of GSK3 may delay rather than prevent GSK3 sensitive functions of DCs. In contrast to LPS stimulation, stimulation of DCs with INF-β leads to a prolonged inhibition of GSK3 and accordingly INF-β-stimulated DCs expressing an active GSK3 knockin produced strongly reduced amounts of IL-10 [11]. Moreover, upon LPS stimulation inhibition of GSK3 through phosphorylation can be overcome by the emergence of high concentrations of primed GSK3 targets (proteins phosphorylated by other kinases), since GSK3 phospho-Ser21/9 moiety acts as a competitive inhibitor to the phospho-binding pocket [27]. Primed GSK3 targets may therefore successfully compete for access to GSK3 [27], resulting in a sufficient activity of this kinase in wild type cells. It is also worth noting that GSK3 inhibitors did not abrogate the stimulation of IL-6 and TNFα secretion in human monocyte-derived DCs following inhibition of PI3K with wortmannin [27]. Thus, inhibitory effects of the PI3K-pathway may not be fully dependent on GSK3 inhibition.

Whereas expression of MHCII, CD86 and cytokine production were not affected in *gsk3*^{KI} DCs, resistance of GSK3 to PKB/Akt and SGK dependent inhibition increases the LPS induced increase of $[Ca^{2+}]_i$. Accordingly, activated GSK3 apparently augments the effect of LPS stimulation on $[Ca^{2+}]_i$. Along those lines, LPS induced increase of $[Ca^{2+}]_i$ is almost abolished in the presence of GSK3 inhibitor SB216763. In theory, GSK3 could modify the increase of $[Ca^{2+}]_i$ by increasing Ca^{2+} release or/and entry, by decreasing Ca^{2+} buffering or by inhibiting Ca^{2+} extrusion.

In theory, GSK3 could stimulate Ca^{2+} release/entry by stimulating the expression of ER/cell membrane Ca^{2+} channels or positive regulators thereof. However, the GSK3 inhibitor SB216763 was effective within a few minutes indicating that GSK3 is at least partially effective by directly or indirectly triggering activation or inactivation of existing proteins. Similarly, decreased expression of buffering proteins or Ca^{2+} extruding proteins cannot explain the rapid effect of the inhibitor.

Differences between DCs from *gsk3*^{KI} mice and DCs from *gsk3*^{WT} mice could not only result from direct action of GSK3 in DCs, or from influence of GSK3 in DCs on gene expression but as well from effects of GSK3 activity in other cell types which indirectly influence gene expression in DCs. According to previous observations, PKB/Akt and SGK1 resistance of GSK3 could impact on steroid hormone release [34], catecholamine release [37], and function of lymphocytes [40]. At least in theory, those alterations could exert imprinting effects on DCs, which are maintained following isolation and subsequent culture. The direct effect of GSK3 inhibition, however, strongly suggests that the activity of GSK3 expressed in DCs influences Ca^{2+} signaling of those cells.

In conclusion, the present observations reveal a novel, powerful regulator of Ca^{2+} signaling in DCs. Disrupting PKB/Akt and SGK dependent phosphorylation of GSK3 augments, and pharmacological inhibition of GSK3 blunts the LPS induced increase of cytosolic Ca^{2+} concentration in dendritic cells.

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