ORIGINAL ARTICLE

Inhibition of GSK-3 by Tideglusib Suppresses Activated Macrophages and Inflammatory Responses in Lipopolysaccharide-stimulated RAW 264.7 Cell Line

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ABSTRACT

Introduction: Glycogen synthase kinase-3 (GSK-3) is an important immune regulator that controls inflammation via inhibition of its protein kinase activities. Persistent inflammatory responses through the activation of immune cells and excessive production of immune mediators may cause tissue destruction and implicated in the development of chronic inflammatory diseases. The objective of this study was to examine the role of Tideglusib, a GSK-3 inhibitor, in inflammatory responses elicited through macrophage activation by investigating the expression of cell surface biomarkers and inflammatory molecule levels. **Method:** The effects of GSK-3 inhibition by Tideglusib on the expression of CD11b and CD40 and secretion of pro-inflammatory cytokines in the lipopolysaccharide (LPS)-activated macrophage-derived RAW 264.7 cells were determined by flow cytometry, while the presence of nitric oxide (NO) was determined by Griess assay. **Results:** Stimulation of RAW 264.7 cells with LPS increased substantial levels of CD11b and CD40 expressions, and secretion of NO, TNF-α, and MCP-1. However, the expression of these molecules was suppressed through inhibition of GSK-3. **Conclusion:** These findings suggest the significant role of Tideglusib to limit the upregulation of immune responses in activated macrophages, and as a potential anti-inflammatory drug for the intervention and treatment of inflammatory diseases.

Keywords: Glycogen synthase kinase-3 (GSK-3), Tideglusib, CD11b, CD40, Macrophages

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INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase involved in maintaining the balance between pro- and anti-inflammation in both innate and adaptive immunity through modulation of nuclear factor-κB (NFκB), a key transcription factor in regulating the inflammatory process (1). The mechanism by which GSK-3 signaling regulates immune response is possibly initiated by toll-like receptor 4 (TLR4) activation which activates the upstream molecule, phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt) signaling pathway (Fig.1) (2) . Activation of TLR4 by lipopolysaccharide (LPS) induces intracellular signalling which activates the transcription factor NFκB and production of various toxic metabolites including reactive oxygen species (ROS), nitric oxide (NO) and pro-inflammatory cytokines (3). The activation of PI3K causes Akt phosphorylation leading to activation of GSK-3 signaling (4). The activation of GSK-3 signaling induces the transcription of NFκB and subsequently promotes cytokine production such as TNF-α, interleukin (IL) -1, -6 and -8, and other molecules that are involved in immune responses (5).

There are many prevalent diseases which develop in the association between inflammation and GSK-3 signaling activation such as mood disorders, neurodegenerative diseases, diabetes, and cancer (6). In a bipolar mood disorder, GSK-3 activity is increased by enhancing the phosphorylation of NFκB, signal transducer, and activator of transcription 3, which results in increased production of pro-inflammatory cytokines and causing inhibition to cAMP response element-binding protein and activator protein 1 (7). Thus, inhibition of GSK-3 by lithium which is now used as mood stabilizers could lead to the resolution of the inflammatory state (7). The established relationship between GSK-3 and these disease conditions could be useful as one of potential therapeutic regimens to limit the immune

responses. Several studies have reported that inhibition of GSK-3 signaling greatly reduced the production of pro-inflammatory mediators such as prostaglandin E2, NO, and cytokines (8,9). Overactivation of GSK-3 in neurodegenerative diseases such as Alzheimer's disease (AD) inhibits the long term potentiation (LTP) and causes learning and memory deficits while changes in GSK-3 activity result in Aβ overproduction and tau hyperphosphorylation (10). A previous study showed that lithium treatment reduced tau phosphorylation, prevented Aβ toxicity, and inhibited GSK-3 activity in AD (11). GSK-3 also plays a role in the glucose transport activity through regulation of insulin action. Aberration in GSK-3 signaling was found to be associated with inability of insulin to activate glucose disposal and glycogen synthase in type 2 diabetes (12). Hence, selective inhibition of GSK-3 is crucial to enhance insulin-stimulated glucose transport activity. Increase in GSK-3 activity through phosphorylation by Akt and other kinases could also lead to abnormal growth and carcinogenesis (13). A previous study showed that persistent inhibition of GSK-3 may trigger an apoptotic response in cancer cells (14). Tideglusib, also known as NP-12, is a thiadiazolidinone derivative that acts as a non-ATP GSK-3 inhibitor. Tideglusib is one of many available GSK-3 inhibitors that currently under investigation for Alzheimer's disease clinical trials and treatment for progressive supranuclear palsy. It has been shown previously that Tideglusib can attenuate inflammatory responses in LPS-stimulated activated microglia cells (8).

Macrophages play significant roles in the immune response as a defence mechanism to protect the host against microbial infection. Activation of these phagocytic cells triggers inflammation through the release of cytokines, chemokines, and other toxic metabolites (15). Classically, activated macrophages express markers (i.e., CD68, CD86, CD80, F4/80, CD11b and CD40), predominantly release pro-inflammatory cytokines (i.e., IL-1, IL-6, IL-8, and IL-12, interferon gamma (IFN-γ), and TNF-α) and chemokines (16). Some of these cytokines play roles in regulating other cells including activation and proliferation of lymphocytes and involved in both acute and chronic inflammation (17). Activation of macrophages generally induces acute inflammation particularly during microbial invasion (17). However, damaging effects of activated macrophages such as septic shock can be detrimental due to excessive inflammatory responses driven by pro-inflammatory mediators which could lead to multi-organ dysfunction and death (15,18). Besides, failure of clearance of the infectious agent by acute inflammation, or persistent inflammatory response via excessive secretion of pro-inflammatory cytokines is a hallmark to the development of chronic inflammatory diseases (19,20). In this study, we aimed to investigate the effects of GSK-3 inhibition on activated macrophages through the evaluation of the expression of cell surface biomarkers and inflammatory molecules. We suggested that Tideglusib, a GSK-3 inhibitor can supress the upregulation of immune responses in activated macrophages and potentially be used as an anti-inflammatory drug for treatment of inflammatory diseases.

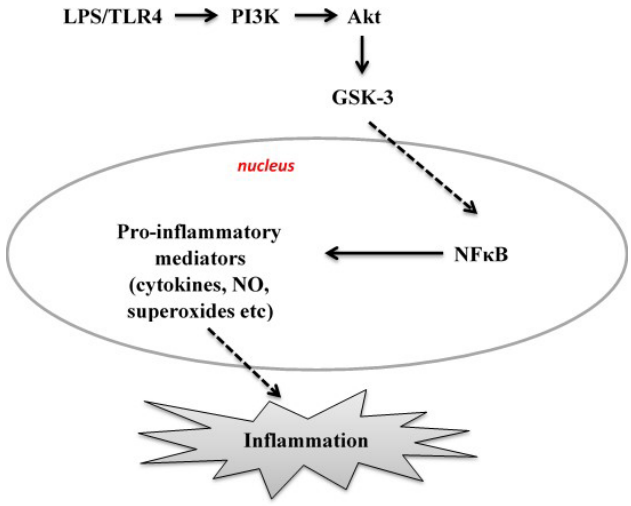

Fig.1 : Role of GSK-3 in inflammation. Activation of Toll-like receptor (TLR) through LPS stimulation promotes activation of PI3K/GSK-3 signaling. GSK-3 modulates inflammatory signaling in macrophages through regulation of NFκB activity which subsequently lead to release of pro-inflammatory mediators including cytokines, chemokines and reactive oxygen species.

MATERIALS AND METHODS

Cell culture

The murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured in a complete Dulbecco's Modified Eagle Media (DMEM) medium containing 10% fetal bovine serum (FBS), 100 µg/L streptomycin and 100 IU/mL penicillin (Gibco, Grand Island, NY, USA) and incubated at 37 ˚C in a 5% CO_2 incubator.

LPS stimulation and GSK-3 inhibition

The RAW 264.7 cells were seeded in 24-well plates at a density of $1x10^5$ cells/well. The medium of each well was aspirated and replaced with 1 ml of a fresh phenol red-free DMEM medium (Nacalai Tesque, Kyoto, Japan) supplemented with 5% FBS and 100 µg/L streptomycin and 100 IU/mL penicillin. The cells were stimulated with 1 µg/ml LPS extracted from Escherichia coli 0111: B4 (Sigma, St Louis, MO, USA) for 2 hours followed by a 24 hours treatment with 0, 5, or 10 µM Tideglusib (NP12), a Thiadiazolidinone class, selective, and non-ATP competitive GSK-3 inhibitor (Sigma, St Louis, MO, USA). The cells and cell culture medium were collected for subsequent experiments.

Griess assay

The presence of nitrite was determined in the cell culture media by using Griess reagent (1% sulphanilamide and 0.1% N-1-naphthylenediamine hydrochloride in 2.5% phosphoric acid). Briefly, 50 µl of cell culture media was incubated with an equal volume of freshly prepared

Griess reagent in a 96-well plate for 10 minutes at room temperature. The absorbance was measured at 540 nm in a microplate reader (Tecan Infinite® F50 Absorbance Microplate Reader, Switzerland). The sodium nitrite ($NaNO_2$) standard curve was generated to quantify the amount of nitrite in the samples.

CD11b and CD40 surface staining

The LPS-stimulated RAW 264.7 cells were harvested and washed with 1 ml of FACS buffer. The cells were then incubated with 3 µg/mL mouse Fc block (CD16/CD32: BD Biosciences) for 5 minutes at room temperature. Then, fluorescent dye Zombie NIR™ Fixable Viability Kit (1:1000; Biolegend, San Diego, CA) was added and incubated for another 15 minutes, followed by cell surface antibody staining (1:100; FITC Rat Anti-mouse CD11b and/or FITC Hamster Anti-mouse CD40, both from BD Biosciences, USA) or FITC Rat IgG2a, κ Isotype control antibody (1:100; BioLegend, San Diego, CA) staining for 30 minutes at 4°C in the dark. The percentages of cells were acquired using Facs DIVA software on a flow cytometer (BD FACS LSRFortessa™) and analyzed by using FlowJo V10 software.

Cytokine profile assay

The levels of secreted cytokines (TNF-α and MCP-1) from treated-RAW 264.7 cells were measured using a BD™ CBA Mouse Inflammation Kit (BD Biosciences, USA) following the manufacturer's protocol. Briefly, 50 µl of diluted (1:50 in assay diluent) cell culture supernatant was added into 50 µl of capture cytokine beads and 50 µl of PE-conjugated antibody, followed by 2 hours incubation at room temperature in the dark. The beads were acquired on a flow cytometer (BD FACS LSRFortessa™, BD Biosciences, USA) using BD FacsDIVA™ software (BD Biosciences, USA). The results were analyzed by FCAP Array software (BD Biosciences, USA).

Statistical analysis

The values expressed are means of two independent experiments ± standard error mean. The statistical analysis was performed by one-way or two-way ANOVA test. *$p < 0.05$ or **$p < 0.01$ is considered as statistically significant.

RESULTS

GSK-3 inhibitor reduces NO production in LPS-stimulated RAW 264.7 cells

To determine the effects of GSK-3 inhibition on NO production in macrophages, LPS-stimulated RAW 264.7 cells were treated with 5 µM and 10 µM Tideglusib. Previously, 5 µM Tideglusib (also known as NP12) and other GSK-3 inhibitors were screened for downregulation of immune responses in microglia (8). However, the use of >10 µM of Tideglusib in macrophages can cause toxicity to the cells (data not shown). The levels of NO in the culture medium was determined by the concentration of nitrite. The non-stimulated RAW 264.7 cells produced a very low amount of NO (0.935 ± 0.165 µM) (Fig. 2). Stimulation of RAW 264.7 cells with LPS increased a substantial amount of NO (2.400 ± 0.47 µM). However, presence of Tideglusib at 5 µM and 10 µM resulted in reduction of NO to 1.48 ± 0.250 µM and 1.795 ± 0.475 µM, respectively.

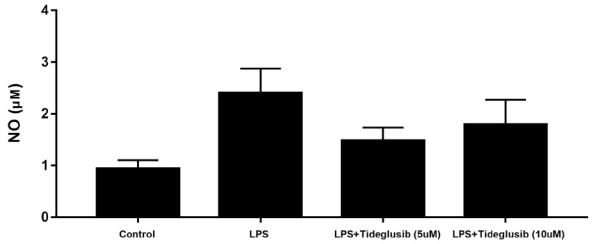

Fig. 2 : GSK-3 inhibitor reduces the NO production in RAW 264.7 cells. RAW264.7 cells were stimulated with LPS (1 µg/ml) for 2 hours prior to a treatment with Tideglusib (5 µM or 10 µM). The cell culture supernatants were collected at 24 hours post-treatment and the amount of NO production was determined by quantification of nitrite. Graph shows mean ± SEM with a one-way ANOVA analysis of three independent experiments. *$p<0.05$.

Differential expression of CD11b and CD40 by inhibition of GSK-3

Next, we evaluated the effects of GSK-3 inhibition on macrophages activation status by assessing the percentage of cells expressing CD11b and CD40 (Fig. 3). In the absence of LPS, the total percentage of macrophages expressing basal level of CD11b was determined at 25.65 ± 5.05% (Fig. 3A). Treatment of Tideglusib slightly reduced the number of cells to 21.95 ± 0.75% (5 µM Tideglusib) and 19.65±10.16% (10 µM Tideglusib). In contrast, increased expression of CD11b in LPS-stimulated macrophages (43.55 ± 5.95%) was suppressed by approximately 15% when treated with 10 µM Tideglusib (Fig. 3B). Similarly, expression of CD40 in the resting cells was relatively low (38.1 ± 0.80%), but upon LPS stimulation, the percentage of CD40-expressing cells was significantly increased (93.5 ± 1.7%) (Fig. 3A). However, treatment with 10 µM Tideglusib significantly reduced the CD40-expressing cells to 66.4 ± 4.0% ($p<0.01$). In contrast, although it was not statistically significant, inhibition of GSK-3 in unstimulated RAW 264.7 cells increased the CD40 expressing cells in a dose-dependent manner of 43.4 ± 1.1% (5 µM Tideglusib) and 67.4 ± 2.3% (10 µM Tideglusib) (Fig. 3B).

Inhibition of GSK-3 reduces TNF-α and MCP-1 production in LPS-stimulated RAW 264.7 cells

We then determined the potential effects of GSK-3 inhibitor on cytokines release in LPS-stimulated RAW 264.7 cells using a CBA assay. The amounts of TNF-α and MCP-1 are shown in Figure 4. The level of TNF-α maintained low in unstimulated RAW 264.7

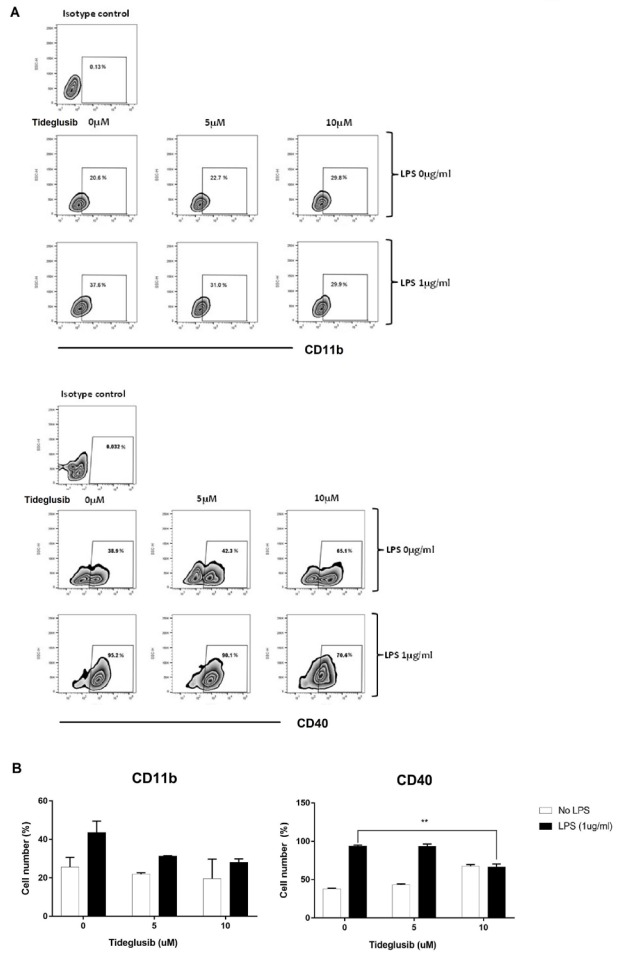

Fig. 3 : GSK-3 inhibitor hinders the expression of CD11b and CD40 in LPS-stimulated RAW 264.7 cells. Staining of CD11b (A) and CD40 (B) on the RAW 264.7 cell membrane was carried out following 24 hours treatment with or without LPS (1 μg/ml) and Tideglusib (5 or 10 μM). The histogram depicts the representative of CD11b and CD40 expression from two independent experiments that normalized with isotype control. Cumulative expression of CD11b and CD40 are shown mean ± SEM with a two-way ANOVA analysis. *p<0.05 or **p<0.01.

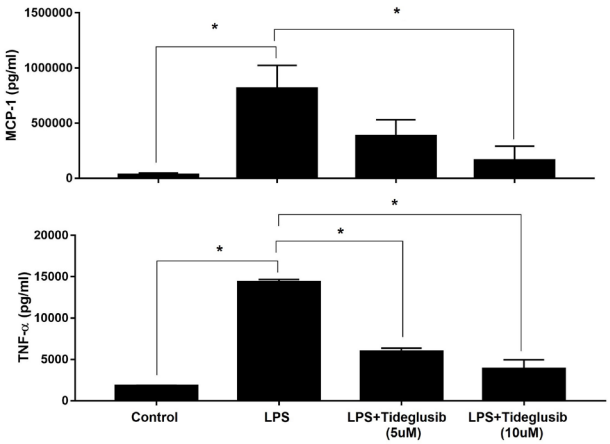

Fig. 4 : GSK-3 inhibitor decreases the production of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. The cell culture supernatants of RAW264.7 cells were collected following 24 hours incubation with LPS (1 μg/ml) and Tideglusib (5 μM or 10 μM). The amount of TNF-α and MCP-1 (IL-8) was determined using cytokine bead array. Graph shows mean ± SEM with a one-way ANOVA analysis of three independent experiments with significant increased or decreased, *p<0.05.

cells (1825.89 ± 48.50 pg/ml) but peaked significantly ($p < 0.05$) when stimulated with LPS (14366.09 ± 316.95 pg/ml). However, the inhibition of GSK-3 in LPS-activated RAW 264.7 cells treated with either 5 μM or 10 μM Tideglusib caused significant reduction ($p < 0.05$) in TNF-α production, 5950.98 ± 428.72 pg/ml and 3835.99 ± 1135.31 pg/ml, respectively. In addition, the low level of MCP-1 in unstimulated RAW 264.7 cells (32117.22 ± 15503.38 pg/ml) was increased significantly ($p < 0.05$) in the presence of LPS (813887.05 ± 210770.95 pg/ml). Conversely, treatment with Tideglusib at 5 μM reduced the production of cytokines to 382876.05 ± 149581.25 pg/ml and treatment with Tideglusib at 10 μM caused significant reduction of cytokines production to 164536.49 ± 127838.61 pg/ml ($p < 0.05$).

DISCUSSION

This study focused on the effects of GSK-3 inhibitor in promoting anti-inflammatory responses. One of the key threads that emerged was the potential roles of GSK-3 signaling in activated macrophages. NO is a multifunctional reactive metabolite secreted by phagocytes through inducible nitric oxide synthase (iNOS) activation, critically involved in the host defense against intracellular pathogens (21). This immune mediator plays a role as a neurotransmitter and a vasodilator as well as in immune protection against tumor cells and pathogens through stimulation of inflammatory signaling (22). Activation of RAW 264.7 cells by LPS showed a mark increase in the production of NO. Previous findings reported that stimulation of BV-2 cells, resident brain macrophages, by LPS induced production of NO (8). Other in vitro studies showed the effects of LPS in bovine mammary epithelial cells that regulate inflammatory responses through the release of cytokines and the production of NO. The data also showed that the activity of iNOS and the concentrations of NO were significantly higher in LPS-treated groups compared to the control group in a dose-dependent manner (23). However, excessive production of NO especially during chronic infections can be detrimental and implicated in the chronic inflammation and tissue damage (24). Our data showed that the stimulation of macrophages with LPS in RAW 264.7 cells increased the amount of NO as compared to the control group. The high level of NO production in the cells could indicate the occurrence of chronic inflammation. Thus, the ability of Tideglusib in reducing the production of NO in LPS-activated RAW 264.7 cells suggests a potential role of GSK-3 inhibitor to limit inflammation and tissue damage.

CD11b/CD18 is a cell surface protein of αMβ2 integrin (Mac-1) involved in cell adhesion and abundantly expressed on macrophages. The upregulation of CD11b expression in macrophages can indicate the acute and chronic inflammation status (25) and cell phenotypic profiles (26). In our study, the expression of CD11b in the

resting RAW 264.7 cells was relatively low. Stimulation of the cells with LPS induced cell activation which was determined by an increase in the expression of CD11b. Interestingly, the expression of CD11b in activated macrophages was downregulated in the presence of Tideglusib, which demonstrated the involvement of GSK-3 in the activation process of macrophages. This finding is consistent with the previously reported study which found that the expression of CD11b upon stimulation with LPS in microglia was diminished when treated with lithium, another GSK-3 inhibitor (27), and suggested that increased activation of macrophage by LPS stimulation was attenuated with GSK-3 inhibitor.

CD40, a molecule found on antigen presenting cells including dendritic cells and macrophages, plays a vital role in immune regulation and highly expressed upon cell activation (28). The expression of CD40 in macrophages was highly upregulated by LPS stimulation, but significantly reduced in the presence of Tideglusib at a higher concentration (10 μM). Interestingly, this reduction was relatively stable and CD40 expression maintained at marginal levels (app. 66%), suggesting a constant expression of CD40 in activated macrophage post-GSK-3 inhibition. It has been reported earlier that CD40 signaling played a protective role against pathogens in macrophages (29). Expression of CD40 in macrophages and binding to its ligand (CD40L) on T cells promote the release of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1 (α and β), IL-6, IL-8 and IL-12 (20). Thus, constant expression of CD40 is crucial for maintaining the host immune defense, especially to human macrophages that frequently exposed to a large repertoire of antigens in the environment. On the other hand, our data revealed that inhibition of GSK-3 in the absence of LPS resulted in increased CD40 expression in RAW 264.7 cells. In other reports, deficiency of GSK-3 in mice was found to enhance CD40-stimulated B cell proliferation (31). This finding was possibly due to the regulatory roles of GSK-3 in maintaining the balance between pro- and anti-inflammation through a specific condition. Altogether, these findings led us to postulate that persistent expression of CD40 during inhibition of GSK-3 signaling could sustain the protective role of macrophages while promoting anti-inflammatory effects to limit the possible damage caused by excessive inflammation.

Activation of macrophages by LPS is associated with the production of pro-inflammatory cytokines, IL-1β, IL-6, IL-8, IL-12, TNF-α and MCP-1 (IL-8), along with other mediators such as NO (8,16). Typically, these cytokines-producing macrophages can mediate inflammation associated with tissue damage and diseases in worse cases (32). Inhibition of GSK-3 may play in part in downregulating inflammation. Previously, Norma et al., 2016 reported that the production of prostaglandin E2 (PGE2), a potent inflammatory mediator, induced by upregulation of cyclooxygenase-2 (COX-2) in LPS-stimulated macrophage-like cells was inhibited by the presence of GSK-3 inhibitor (9). Besides, inhibition of GSK-3 signaling by several types of GSK-3 inhibitor resulted in the reduction of TNF-α, IL-6, and MCP-1 in LPS-stimulated microglia (8). These data supported our study which showed that inhibition of GSK-3 by Tideglusib caused reduction in the production of TNF-α and MCP-1 in LPS-activated RAW 264.7 cells. Thus, this finding suggests the involvement of GSK-3 signaling in downregulating inflammatory responses in previously activated macrophages and it could be a potential strategy for the attenuation of acute or chronic inflammation. Nevertheless, a cross-reaction in the cytokine profile of the CBA assay may occur and affect the data obtained. This could be due to interactions between multiple different antibodies and cytokines (antigens) in the sample solution (33). Thus, an evaluation of the cytokine gene expression could be useful to validate the data.

CONCLUSION

GSK-3 signaling is crucial for many biological processes including inflammation. Excessive production of inflammatory mediators by activated macrophages, however, often causes damage to the host. The present finding shows that inhibition of GSK-3 signaling leads to a reduction of macrophages activation and induces the suppression of inflammatory responses. Interestingly, despite the phenotypic changes of macrophages by blockade of GSK-3 signaling, constant expression of CD40 indicates the sustainability of macrophages to hold its protective functions. Thus, inhibition of GSK-3 signaling could provide a useful strategy to limit chronic inflammation and improve immune protection at the same time.

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