Title: Characterizing the Role of Glycogen Synthase Kinase (GSK)-3α/β in Macrophage Polarization and the Regulation of Pro-Atherogenic Pathways

Authors and Affiliations:
Sarvatit Patel\textsuperscript{a,b} and Geoff H. Werstuck\textsuperscript{a,b,c,*}

\textsuperscript{a}Thrombosis and Atherosclerosis Research Institute, \textsuperscript{b}Department of Chemistry and Chemical Biology, \textsuperscript{c}Department of Medicine, McMaster University, Hamilton Ontario

Corresponding author:
Geoff H. Werstuck
Thrombosis and Atherosclerosis Research Institute
McMaster University
237 Barton Street East, Hamilton, Ontario, Canada
L8L 2X2
Tel: (905) 521-2100 ext. 40747
Fax: (905) 577-1427
Email: Geoff.Werstuck@taari.ca
Abstract

The molecular and cellular mechanisms that link cardiovascular risk factors to the initiation and progression of atherosclerosis are not understood. Recent findings from our laboratory indicate that endoplasmic reticulum (ER) stress signaling through glycogen synthase kinase (GSK)-3α/β induces pro-atherosclerotic pathways. The objective of this study was to define the specific roles of GSK3α and GSK3β in the activation of pro-atherogenic processes in macrophages. Bone marrow derived macrophages (BMDM) were isolated from low-density lipoprotein receptor knockout (Ldlr−/−) mice and Ldlr−/− mice with myeloid deficiency of GSK3α and/or GSK3β. M1 and M2 macrophages were used to examine functions relevant to the development of atherosclerosis, including polarization, inflammatory response, cell viability, lipid accumulation, migration, and metabolism. GSK3α deficiency impairs M1 macrophage polarization, and reduces the inflammatory response and lipid accumulation, but increases macrophage mobility/migration. GSK3β deficiency promotes M1 macrophage polarization, which further increases the inflammatory response and lipid accumulation, but decreases macrophage migration. Macrophages deficient in both GSK3α and GSK3β exhibit increased cell viability, proliferation, and metabolism. These studies begin to delineate the specific roles of GSK3α and GSK3β in macrophage polarization and function. These data suggest that myeloid cell GSK3α signaling regulates M1 macrophage polarization and pro-atherogenic functions to promote atherosclerosis development.
Introduction

Cardiovascular disease (CVD) is the leading cause of death in the world today\(^1\) and atherosclerosis is a major underlying cause of CVD. Macrophages are centrally involved in every stage of the development of atherosclerosis and they are the main cellular component within the atherosclerotic lesion\(^2,3\). Atherosclerosis initiates when endothelial cells (EC) respond to injury, which mediates the attachment and infiltration of monocytes. Monocytes invade the subintima and differentiate into macrophages. These macrophages take up modified-LDL particles and become foam cells, which form fatty streaks in the artery wall. Macrophage/foam cell apoptosis leads to the establishment of a necrotic core, which is a key feature of unstable plaques that are prone to rupture. Lesion rupture triggers atherothrombosis and can occlude the artery. This can lead to acute cardiovascular complications (myocardial infarction or stroke) and potentially death. The underlying molecular mechanisms that regulate macrophage function during the development of atherosclerosis are not completely understood.

Macrophages can be polarized into many different subtypes that have distinct characteristics and functions. The extreme phenotypes are pro-inflammatory (M1) macrophages and anti-inflammatory (M2) macrophages. M1 macrophages can be induced by exposure to T helper type 1 (Th1) cell products, such as interferon (IFN)-γ, or microbial products, such as lipopolysaccharide (LPS)\(^4\). In contrast, M2 anti-inflammatory macrophages can be induced by exposure to T helper type 2 (Th2) cell products, including interleukin (IL)-4 or tumor growth factor (TGF)-β. M1 macrophages produce pro-inflammatory cytokines (TNFα, IL-1β) and are believed to promote atherosclerotic lesion development and complexity\(^5,6\), whereas M2 macrophages produce anti-inflammatory cytokines (IL-10) and have tissue remodeling properties\(^7,8\). Other macrophage subtypes have been identified, including Mox, Mhem, and M4\(^9\). The roles and functions of these macrophages are less well understood. Macrophages are directly involved in a variety of processes during atherosclerosis including polarization, foam cell formation, apoptosis, cell viability/proliferation, and migration. The mechanism(s) and cellular signals that regulate macrophage polarization and other functions that contribute to the development of atherosclerosis are still unclear.

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that plays an important role in many cellular pathways that regulate metabolism and viability. GSK3 has been linked to several disorders and diseases, including cancer\(^10\), bipolar mood disorder\(^11\), diabetes\(^12\), and Alzheimer’s disease\(^13\). There are two main forms of GSK3 in mammals: GSK3α (51 kDa) and GSK3β (47 kDa), as well as the splice variant of GSK3β, GSK3β\(^2\)\(^14\). Isoforms GSK3α and GSK3β are 98% homologous in the kinase domain and are expressed ubiquitously\(^15\). GSK3α/β is predominantly located in the cytoplasm, endoplasmic reticulum (ER), and nucleus\(^16\). GSK3α/β is a constitutively active kinase and its activity is directly regulated (inhibited) by the insulin and Wnt signaling pathways\(^17,18\). A study from our lab has shown that the presence of ER stress in Thp-1 derived macrophages activates the protein kinase R-like ER kinase (PERK) signaling branch of the unfolded protein response (UPR) to promote GSK3α/β activity\(^19\). Recent evidence suggests that GSK3α and GSK3β have distinct functions\(^20-22\). Whole-body GSK3α-deficient mice are viable and develop normally, while GSK3β deletion is embryonically lethal\(^23,24\). GSK3α and β play unique and independent roles in skeletal muscle cell insulin signaling\(^25,26,27\), cardiomyocyte development and proliferation\(^28\), and Th cell polarization\(^29\). Recent studies support a role for GSK3α/β in atherosclerosis. Results from our lab suggest that myeloid deletion of GSK3α, pharmacological mitigation of ER stress (by 4 phenylbutyrate), or inhibition of GSK3α/β (by valproate) attenuates the progression of atherosclerosis\(^29-31\). Together, these results suggest a role for myeloid-specific GSK3α in the development of atherosclerosis. However, the specific
roles of myeloid GSK3α and GSK3β in macrophage polarization and other pro-atherogenic functions are not known.

In this study we have isolated bone marrow derived macrophages from myeloid cell-specific GSK3α and/or GSK3β deficient Ldlr−/− mice to characterize the roles of GSK3α and GSK3β in specific cellular functions. Our results demonstrate that GSK3α and GSK3β play distinctive roles in defining macrophage phenotype and regulating atherogenic responses.
Results

Characterization of Myeloid Cell-Specific GSK3α and/or GSK3β deficiency in Ldlr−/− Mice

Myeloid GSK3α and/or GSK3β deletion did not significantly alter the number of monocytes or other cell types in whole blood (Supplementary Table II). To determine the effect of GSK3α and/or GSK3β deficiency on macrophage function, bone marrow was harvested from 8-10-week-old Ldlr−/− mice with myeloid-specific GSK3α and/or GSK3β deficiency. Bone marrow was cultured in the presence of MCSF to induce macrophage differentiation. Myeloid cell-specific deficiency of GSK3α and/or GSK3β was confirmed by RT-PCR and immunoblot. There was no detectable compensation in the gene expression (Figure 1a and 1b), or protein expression (Figure 1c and 1d, Supplementary Figure I), of the retained homolog when GSK3α or GSK3β was deleted.

To determine the efficiency of MCSF-induced differentiation, the macrophage (CD11b+ F4/80+ cells) number was assessed by flow cytometry. Results show that over 95% of cells were F4/80+ and CD11b+ (Figure 1e, Supplementary Figure I). Our gating method was able to capture all of CD11b+ and F4/80+ cells in one box. The results show that bone marrow from LMαKO, LMβKO, and LMαβKO mice is not significantly different than Lαβfl/fl (control) bone marrow in terms of its ability to differentiate into macrophages (Figure 1f). This suggests that GSK3α and/or β deficiency does not affect bone marrow cell number or the efficiency of bone marrow differentiation into M0 macrophages.

GSK3α deficiency impairs, and GSK3β deficiency enhances, inflammatory macrophage polarization

To examine the effect of GSK3α and/or GSK3β deletion on macrophage polarization, subsets of BMDM were exposed to 10 ng/ml LPS or 10ng/ml IL-4 for 24 hrs. Polarization efficiency was determined by analyzing gene expression of inducible nitric oxide synthase (iNOS) (Figure 2a) and arginase 1 (Arg1) (Figure 2b), as markers of inflammatory (M1) and anti-inflammatory (M2) macrophages, respectively. As expected, exposure of Lαβfl/fl (control) macrophages to LPS promotes iNOS expression, and exposure to IL-4 promotes Arg1 expression. LMdKO macrophages exhibit impaired LPS-induced iNOS expression. LMβKO and LMqβKO macrophages show significantly increased LPS-induced iNOS expression and decreased IL-4-induced Arg1 expression. Interestingly, LMdKO macrophages treated with LPS appear to be more polarized towards the anti-inflammatory (M2) phenotype, rather than an inflammatory (M1) phenotype, compared to the Lαβfl/fl (control) (Figure 2c and Figure 2d). LMβKO and LMqβKO macrophages treated with IL-4 were more polarized towards the M1 phenotype, rather than an M2 phenotype compared to the Lαβfl/fl (control) (Figure 2c and 2d). LMqβKO macrophages treated with LPS increased genes expression of heme oxygenase (HO1) and Txnd1 (Mox markers) compared to the Lαβfl/fl (control) (Supplementary Figure III). These results suggest that GSK3α and GSK3β play a central role in M1 and M2 polarization, respectively.

Inflammatory response is impaired in GSK3α deficient, and enhanced in GSK3β deficient, macrophages

The inflammatory response was determined by analyzing the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and NLR family pyrin domain containing 3 (NLRP3) in BMDM by immunofluorescence staining (Figure 2e and 2g). BMDM from Lαβfl/fl (control), LMdKO, LMβKO, and LMqβKO mice were cultured for 48 hrs. Macrophages were fixed and stained with antibodies against NF-κB p65 or NLRP3 and DAPI. Immunofluorescence was
and LMαβKO mice were fixed and immunostained with an antibody against the proliferation deficiency. Immunofluorescence staining was used to analyze the effects of GSK3α and/or GSK3β on macrophage proliferation markers. BMDM from Lαβfl/fl (control), LMαKO, LMβKO, and LMαβKO mice were fixed and immunostained with an antibody against the proliferation markers.

These results suggest that the presence of GSK3α enhances the inflammatory response and GSK3β represses the inflammatory response in M1 macrophages.

Lipid accumulation is impaired in GSK3α deficient macrophages and increased in GSK3β deficient macrophages

Macrophages are endocytic cells that readily take up lipoprotein particles and cell debris. To determine the roles of GSK3α and GSK3β in lipid accumulation, BMDM were isolated from Lαβfl/fl (control), LMαKO, LMβKO, and LMαβKO mice and cultured for 24 hrs. Cells were stained with Oil Red O and analyzed for lipid accumulation by quantifying ORO-stained area. The results indicate that M1 macrophages accumulate significantly more lipid compared to M2 macrophages (Figure 3a and 3b, Supplementary Figure V). In addition, LMαKO macrophages have a decreased tendency to accumulate lipids while LMβKO and LMαβKO macrophages have an increased ability to accumulate lipids (Figure 3b).

We next investigated the effect of GSK3α and/or GSK3β deletion on gene expression of scavenger receptor (SR)-A and CD36, two genes encoding proteins involved in lipid uptake (Figure 3c and 3d), and ATP-binding cassette transporter (ABCA1) and ATP binding cassette subfamily G member 1 (ABCG1), two genes encoding proteins involved in lipid efflux (Figure 3e and 3f). Results show that there is an increase in gene expression of (SR)-A, CD36, ABCA1, and ABCG1 in LMβKO and LMαβKO M1 macrophages. There is no difference in gene expression of these markers in LMαKO macrophages, relative to controls. Analysis of other genes involved in lipid accumulation, including SR-B1, liver X receptor alpha (LXRα), and lecithin–cholesterol acyltransferase (LCAT), revealed no significant differences in expression (Supplementary Figure V). The effect of GSK3α and/or GSK3β deficiency on lipid biosynthesis was determined by analyzing the gene expression of 3-hydroxy 3-methylglutaryl-CoA (HMG-CoA) and fatty acid synthase (FAS). Results show that LMβKO and LMαβKO macrophages have significantly reduced gene expression of HMG-CoA and FAS (Supplementary Figure V). Together, these findings suggest that the presence of GSK3α promotes lipid uptake and accumulation and GSK3β impedes lipid accumulation in M1 macrophages.

GSK3α and GSK3β play redundant roles in cell viability and proliferation

We next determined the effect of GSK3α and/or GSK3β deficiency on macrophage viability and function. BMDM were challenged with ER stress inducing agents, tunicamycin (Tm) or thapsigargin (Tg). Cell viability was measured using an alamarBlue cell viability assay (Figure 4a). As expected, Lαβfl/fl (control) M1 and M2 macrophages show a decrease in cell viability when treated with Tm or Tg. The viability of LMαKO and LMβKO macrophages was not significantly different than the Lαβfl/fl control. Cell viability was significantly increased in both M1 and M2 macrophages that were deficient in both GSK3α and GSK3β. These results suggest that GSK3α and GSK3β play redundant roles in the activation of pro-apoptotic pathways.

Immunofluorescence staining was used to analyze the effects of GSK3α and/or GSK3β deficiency on macrophage proliferation markers. BMDM from Lαβfl/fl (control), LMαKO, LMβKO, and LMαβKO mice were fixed and immunostained with an antibody against the proliferation markers.
marker Ki67 and DAPI (Figure 4b) and quantified (Figure 4c). The percentage of Ki67 positive cells was significantly increased in M2 macrophages in comparison to M1 macrophages (Figure 4c). LMaKO, LMBKO, and LMcβKO M2 macrophages had significantly increased expression of Ki67, compared to Lαβfl/fl controls. There was no significant difference found in the gene expression of proliferation marker cMyc (Supplementary Figure VI). These results suggest that GSK3α and GSK3β suppress M2 macrophage proliferation.

**Migration of M1 macrophages increase with GSK3α deficiency and decreases with GSK3β deficiency**

Macrophage migration was determined using a transwell plate assay. BMDM isolated from Lαβfl/fl (control), LMaKO, LMBKO, and LMcβKO mice were induced to migrate toward the chemokine, CCL19. As expected, M1 macrophages showed increased migratory activity towards CCL19, compared to M2 macrophages (Figure 5a). The results indicate that LMaKO macrophages had a greater tendency to migrate, whereas LMBKO and LMcβKO macrophages showed decreased migration.

To investigate the underlying mechanism, the effect of GSK3α and/or GSK3β deficiency on C-C chemokine receptor type 7 (CCR7) expression was examined (Figure 5). M1 and M2 macrophages have similar levels of CCR7 expression (Figure 5b and 5c). GSK3α deficient macrophages displayed increased expression of CCR7 in M1 macrophages. LMBKO and LMcβKO macrophages displayed no difference in expression of CCR7 in M1 or M2 macrophages. These results suggest that GSK3α actively suppresses the expression of CCR7 expression. Analysis of other factors involved in macrophage migration, including sphingosine-1-phosphate receptor (S1PR) 1, S1PR3, and macrophage migration inhibitory factor (MIF), revealed no significant differences in gene expression (Supplementary Figure VII). Together these results suggest that GSK3α inhibits migration and GSK3β induces migration of M1 macrophages towards CCL19.

**Combined GSK3α and GSK3β deficiency increases metabolic activity**

Metabolic activity was determined by measuring the change in oxidative phosphorylation in BMDM using the Seahorse Analyzer XF24. The XF Cell Mito Stress analysis was used to measure mitochondrial activity. BMDM from Lαβfl/fl (control), LMaKO, LMBKO, and LMcβKO mice were cultured for 24 hrs after which mitochondrial activity was measured (Figure 6). As expected, M2 macrophages showed increased oxygen consumption rate (OCR) in comparison to M1 macrophages (Supplementary Figure VIII) and M1 macrophages showed increased extracellular acidification rate (ECAR) in comparison to M2 macrophages (Supplementary Figure VIII). Results suggest that LMcβKO macrophages have an increased OCR (Figure 6a and 6c) and ECAR (Figure 6e and 6f) compared to Lαβfl/fl (control) in both M1 and M2 macrophages. LMcβKO macrophages show a significant increase in basal, ATP-linked, spare respiratory capacity, and maximal OCR (Figure 6c and 6d). LMaKO and LMBKO macrophages showed no change in OCR in both M1 and M2 macrophages (Figure 6a and 6c). Together these results suggest that GSK3α and GSK3β play redundant roles in regulating metabolic activity.
GSK3α and GSK3β are highly homologous, constitutively active kinases that are expressed in most cells including macrophages. GSK3α and GSK3β function within central signal transduction pathways that regulate cell viability and metabolism and over 100 putative substrates have been identified. Dysregulation of GSK3α/β has been implicated in several metabolic disorders. Recent evidence suggests that GSK3α and GSK3β play distinct roles in sperm motility and fertility, amyloid production in the brain, cortical development, atherosclerosis development, and acute myeloid leukemia development. However, therapeutic targeting has been limited by our lack of understanding of homolog-specific functions as well as the lack of isoform-specific inhibitors. Here, we delineate the specific roles of GSK3α and GSK3β in macrophage polarization and pro-atherogenic functions. We show that myeloid GSK3α and GSK3β have distinct, opposing effects in the regulation of macrophage phenotype, inflammatory response, lipid accumulation, and migration. Conversely, GSK3α and GSK3β appear to play complementary, redundant roles in macrophage viability, proliferation, and metabolism.

The mechanisms regulating macrophage polarization involve specific signaling pathways. In M1 polarization, LPS and IFN-γ interact with cell surface receptors to promote the activation of NF-κB and hypoxia-inducible factor 1-alpha (HIF1α), which induce expression of pro-inflammatory cytokines (TNFα, IL-1β) and iNOS. Our results suggest that GSK3α deficient macrophages exhibit impaired LPS-induced expression of NF-κB and reductions in iNOS expression. This is consistent with our previous findings showing that GSK3α deletion suppresses M1 polarization in macrophages within the atherosclerotic lesion. GSK3β deficient macrophages have increased LPS-induced expression of NF-κB and NLRP3 and elevated iNOS expression. This result is consistent with previous reports showing the inactivation of GSK3β enhanced NF-κB activity and increases the inflammatory response. GSK3β deficient macrophages also have decreased IL-4-induced Arg1 expression, resulting in impaired M2 polarization. Together, these results suggest that GSK3α and GSK3β play opposing roles in the regulation of M1 and M2 polarization, with GSK3α promoting the inflammatory response and GSK3β supporting the anti-inflammatory response in macrophages.

Macrophages endocytose modified-LDL particles and become foam cells. Consistent with previous reports, we found that M1 macrophages accumulate significantly more lipid compared to M2 macrophages. Furthermore, we show that lipid accumulation in M1 macrophages is reduced with GSK3α deficiency and increased with GSK3β deficiency. To determine the mechanism(s) underlying these observations we assessed the expression levels of genes involved in lipid uptake, export, and biosynthesis. We found increased expression of genes encoding SR A, CD36, ABCA1, and ABCG1 in GSK3β deficient macrophages. This suggests that there is an overall increase in lipid transport in GSK3β deficient macrophages that results in increased lipid accumulation. Together, these findings suggest that GSK3α promotes lipid accumulation and GSK3β impedes lipid accumulation in M1 macrophages.

Our previously published findings have suggested that cardiovascular risk factors promote atherogenesis by a mechanism involving ER stress-induced activation of GSK3α/β. This pathway promotes lipid accumulation and apoptosis of macrophage/foam cells in vitro. We investigated the effect of ER stress on macrophage viability in GSK3α or GSK3β deficient cells. We found that myeloid deficiency of either the GSK3α or GSK3β isoform did not have any effect on cell viability. Deficiency of both isoforms significantly increased cell viability in both M1 and
M2 macrophages. These results suggest that GSK3α and GSK3β are both required to facilitate ER stress-induced cell death pathways.

It has recently become evident that lesional macrophage proliferation may play an important role in atherosclerotic plaque development\textsuperscript{45,46}. The complete understanding of the molecular and cellular mechanisms that regulate macrophage proliferation in atherosclerotic lesions is still unknown. Our results show that there were significantly more Ki67 positive cells when macrophages were polarized to an M2 phenotype relative to an M1 phenotype. A previous study has shown that exposure to Th2 cytokines stimulates adipose tissue macrophage (M2) proliferation and inhibits M1 proliferation\textsuperscript{47}. Deficiency of GSK3α and/or GSK3β significantly increases the % of Ki67 positive cells. These results suggest that GSK3α and/or GSK3β suppress M2 macrophage proliferation.

To effectively carry out their phagocytic role within the artery wall, macrophages are required to migrate toward chemotactic signals within the lesional environment. Macrophage migration is known to be regulated by several factors including intracellular cholesterol content\textsuperscript{48-50}. We investigated the effect of GSK3α and/or β deficiency on CCL19-CCR7 stimulated migration using a transwell assay\textsuperscript{51}. Our results show that GSK3α deficient macrophages are more proficient at migrating, whereas GSK3β deficient and GSK3αβ deficient macrophages are less proficient at migrating, towards a CCL19 chemokine signal. To determine if migration ability was regulated at the level of chemokine receptor expression, we determined the expression of CCR7 in these GSK3α/β deficient macrophages\textsuperscript{51}. As expected, we found that both M1 and M2 macrophages have similar CCR7 expression levels. GSK3α deficient macrophages, but not GSK3β deficient macrophages, had increased expression of CCR7 in M1 macrophages. Together, these results suggest that GSK3α inhibits the migration of M1 macrophages towards CCL19 by regulating CCR7 expression.

M1 macrophages are known to have enhanced glycolytic metabolism and reduced mitochondrial activity, whereas M2 macrophages rely upon mitochondrial oxidative phosphorylation (OXPHOS). The effect of metabolic changes in macrophages on the development of atherosclerotic plaque is poorly defined. Recent evidence suggests that a high degree of anaerobic glycolysis\textsuperscript{52} and mitochondrial oxidative stress\textsuperscript{53} in macrophages play an important role in the development of advanced atherosclerosis lesions. Our results indicate that deficiency of both GSK3α and GSK3β increases the mitochondrial activity (OCR) in both M1 and M2 macrophages. These results suggest that GSK3α and GSK3β play a redundant role in the mitochondrial activity of macrophages.

In summary, GSK3α and GSK3β play distinct, and often opposing roles in the signaling pathway of atherogenic functions. Consistent with previous findings, myeloid GSK3α signaling appears to play an important pro-atherogenic role, inducing M1 macrophage polarization and activating pro-atherogenic pathways to accelerate plaque development. Further investigations are needed to delineate the downstream substrates and pathways through which GSK3α acts and to explore the potential for targeting GSK3α with isoform specific small molecule inhibitors as an anti-atherogenic therapy.
Methods

Mouse models

Myeloid-specific GSK3α and/or GSK3β deficient mice were created in an Ldlr−/− genetic background, as previously described29. Myeloid specific GSK3α knockout mice (Ldlr−/−LyzMCre+/−GSK3αfl/fl or LMαKO), myeloid-specific GSK3β knockout mice (Ldlr−/−LyzMCre+/−GSK3βfl/fl or LMβKO), and myeloid-specific GSK3α and β knockout mice (Ldlr−/−LyzMCre+/−GSK3αfl/flGSK3βfl/fl or LMαβKO) were utilized in this study. Ldlr−/−GSK3αfl/flGSK3βfl/fl (Lαβfl/fl) mice were used as controls. All experimental mice had unlimited access to food and water and were maintained on a 12-hour light/dark cycle. All animal experiments were conducted with pre-approval of the McMaster University Animal Research Ethics Board. All experiments conform with the guidelines and regulation of the Canadian Council on Animal Care.

Bone marrow-derived macrophage (BMDM) isolation and polarization

At the age of 8-10 weeks, tibias and femurs were harvested and bone marrow was collected from Lαβfl/fl (control), LMαKO, LMβKO, and LMαβKO mice using a 70 μm nylon mesh passing through the medullary cavity. Cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 15% (v/v) fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, 1X MEM non-essential amino acids, and 20 ng/ml macrophage colony stimulating factor (MCSF, Cell Signaling). Cells were counted using a hemocytometer and 5 x 10^6 cells were seeded onto a 10 cm plate containing 10 ml of medium. After six days in a humidified 37°C incubator (5% CO2), cells were washed twice with warm, sterile 1X Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium. Macrophages were detached using accutase (Cedarlane), and cells were counted with a hemocytometer and replated for subsequent experiments. Subsets of cells were polarized to M1 macrophages by exposure to 10 ng/ml lipopolysaccharide (LPS), or M2 macrophages by exposure to 10ng/ml IL-4 for 24 hours or left unstimulated as M0 macrophages.

Flow cytometry

Plated cells were detached with accutase and incubated with an Fc-receptor blocking antibody (anti-CD16/32 (1:100), eBioscience) for 30 min. Macrophage-specific surface markers were identified by incubating with fluorescently labeled antibodies against CD11b (1:50, Life Technologies) and F4/80 (1:50, BD Pharmagen) for 1 hour. Unbound antibodies were washed off in FACS buffer (PBA, 0.1% BSA, 0.1% sodium azide). Flow cytometry was performed using a BD LSR II flow cytometer (BD Biosciences).

Gene expression

BMDM were seeded onto 12-well tissue culture plates at a density of 4 x 10^5 cells/well in 1 ml medium and polarized as described above. Total RNA was isolated using TRIzol® Reagent (Invitrogen), as previously described19,29. Purified total RNA was resuspended in DNase/RNase-free water and RNA concentration and purity were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific). DNA was prepared from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using 1 μl of resulting cDNA, 12.5 μl SensiFAST SYBR-Rox (Thermo Fisher Scientific), 1.25 μl of forward and reverse primers (500 nM, IDT) (Supplementary Table I), and 8 μl of RNase-water in a total volume of 24 μl/well. The following conditions were used to amplify cDNA: 10-minute hold at 95 °C, followed
by 40 cycles consisting of a 15-second melt at 95 °C, followed by 1-minute annealing at 60 °C. Relative quantitative analysis (2-ddCt) was performed by normalizing data to the β-actin reference gene.

**Characterization of macrophages**

BMDM were cultured and resuspended in lysis buffer (4x SDS PAGE sample buffer). Cell extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were blocked with 5% non-fat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 45min and then incubated overnight with primary antibody against GSK3α/β (1:1000, Cell signaling) or β-Actin (1:3000, Sigma) at 4 °C. Membranes were washed and incubated with a horseradish peroxidase-conjugated anti-rabbit antibody (1:200, Dako) or anti-mouse antibody (1:200, Dako) for 1 h. Blots were washed with TBST three times for 5 min each and developed with the ECL system (Millipore). Images were captured using a Molecular Imager ChemiDoc XRS+ (Bio-Rad).

**AlamarBlue cell viability assay**

BMDM were cultured in 96 well tissue culture plates at a density of 1 x 10^5 cells/well/100 μl medium. Cells were polarized as described above and then treated with 10 μM thapsigargin (Tg), or 10 μg/ml tunicamycin (Tm), or left untreated for 24 hours. Cell viability was determined using the alamarBlue™ assay (Bio-rad). Cells were washed and alamarBlue™ reagent (Bio-Rad) was added. Absorbance was determined at 570 nm (reduction) and 600 nm (oxidation) to calculate cell viability.

**Immunofluorescent staining**

BMDM were seeded onto 8 chamber slides (Thermo Fisher Scientific) at a density of 1 X 10^5 cells/200ul/chamber and incubated at 37°C. Immunostaining was performed as previously described^{19,29}. Cells were washed with 1x PBS, fixed with 4% paraformaldehyde (PFA) for 15 min. Cells were permeabilized with 0.5% Triton X-100 for 5 min and then incubated in blocking solution (3% goat serum, 0.5 % BSA, 1X PBS) for 1hr. Primary antibodies against the proliferation marker Ki67 (1:200, Abcam), NF-κB p65 (1:50, Santa Cruz), NLRP3 (1:50, Abcam), or CCR7 (1:100, Abcam) were added. After 24 hours, cells were washed and then incubated with secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG (1:250, Thermo Fisher Scientific) or Alexa Fluor 488 goat anti-rabbit IgG (1:250, Thermo Fisher Scientific), for 2 hrs. Separate slides of cells were stained with pre-immune IgG instead of primary antibodies to control for non-specific staining (Supplementary Figure IX). Cells were washed and stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:5000, Invitrogen). The slides were mounted using Fluoromount Aqueous Mounting Medium (Sigma) and stored at 4°C in the dark. Images of the stained sections were collected using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera. ImageJ 1.52q software was used to quantify immunofluorescent staining.

**Oil Red O staining**

Chamber slides with 8 chambers (wells) were used for the lipid accumulation assay. Cells were plated in 8 chamber slides (Thermo Fisher Scientific) at a density of 1.5 X 10^5 cells/200ul/chamber and incubated at 37°C for 24 hrs in DMEM. Oil Red O stock solution was prepared by dissolving 2.5 g Oil Red O powder (Sigma) in 500 ml isopropanol (100%) in a water bath at 70°C for 10 min. The stock solution was filtered while it was warm. Before staining, the
working solution was prepared by diluting stock solution 3:2 within ddH$_2$O. Cells were fixed with 4% PFA for 15 min. and permeabilized with 0.5% Triton X-100 for 5 min. Cells were stained with filtered oil red O working solution at 37°C for 15-20 min. Cells were washed in 60% isopropanol for 15-30 sec. and PBS 2 times, then stained with DAPI (1:5000, Invitrogen) for 2-5 min. The cells were mounted by using Fluoromount Aqueous Mounting Medium (Sigma) and stored at 4°C in the dark. Lipid content was visualized by a bright-field or fluorescent microscope (Olympus BX41 microscope connected to a DP71 Olympus camera) and quantified.

Migration Assay

Cells were seeded at a density of 0.8 X 10$^5$ cells/200ul/insert onto Transwell inserts (pore size of 3 µm, Corning Costar) that were pre-coated with rat tail collagen I (4mg/ml, Millipore). Cells were added in the upper chamber and incubated at 37°C for 1 hr in serum-free media. These filter inserts were placed in wells containing the serum-free media with 0.5ug/ml chemokine ligand 19 (CCL19, R&D Systems) and incubated at 37°C. After 4 hrs, inserts with the cells were removed and washed with 1x PBS. Cells were fixed with 4% PFA for 15 min. After washing with 1x PBS, cells were stained with DAPI (1:5000, Invitrogen) for 2-5 min. Filters were then rinsed twice with 1x PBS. The cells on the upper surface, that had not migrated, were removed by carefully scraping with a cotton swab. Migrated cells, on the lower surface, were visualized and quantified using a fluorescent microscope (Olympus BX41 microscope connected to a DP71 Olympus camera, with 4x objective).

Extracellular Flux Analysis

BMDM were plated in Seahorse XF24 plate (Agilent) at a density of 40,000/well in DMEM and cultured for 24 hours before the medium was replaced with a fresh DMEM. The assay was performed as described previously$^{54}$. One hour before the assay, media were exchanged for XF24 media. Mito Stress assay was performed by sequential addition of Oligomycin (inhibitor of ATP synthesis), carbonyl cyanide 4-((trifluoromethoxy) phenylhydrazone (FCCP, uncoupling agent), and rotenone/antimycin A (inhibitors of complex I and complex III of the respiratory chain, respectively) were diluted into XF24 media and loaded into the accompanying cartridge to achieve final concentrations of 1 µM, 2 µM, and 0.5 µM, respectively. Injections of the drugs into the medium occurred at the time points specified. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were monitored using a Seahorse Bioscience XF24 Extracellular Flux Analyzer (Agilent). Data are analyzed using Wave Desktop Software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 software. Data were analyzed by one- or two-way ANOVA, followed by the Bonferroni multiple comparison test between all groups. Error bars represent the standard error of the mean (SEM). For all experiments, a p-value lower than 0.05 was considered statistically significant. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.
References


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Author contributions

G.H.W. and S.P. conceived and designed the study. S.P. conducted and analyzed all experiments except Extracellular Flux assay. All authors reviewed the manuscript.

Additional Information

The author(s) conformed with ARRIVE guidelines in the completion of this study.

Competing interests

The author(s) declare no competing interests.
Figure Legends

Figure 1. Effect of GSK3α and/or GSK3β deficiency on macrophage differentiation and characterization of myeloid cell–specific GSK3α and/or GSK3β knockout in BMDM. BMDM isolated from Lqβfl/fl(control), LMaKO, LMβKO and LMcβKO mice. BMDMs were exposed to 10 ng/mL LPS or 10 ng/mL IL-4 for 24 hours to induce M1 or M2 macrophage polarization respectively. Quantification of (a) GSK3α and (b) GSK3β gene expression in BMDM by RT-PCR. Data are normalized to the βactin reference gene. (c) Whole tissue lysates from control and macrophage specific GSK3α and/or GSK3β knockout mice were resolved by SDS-PAGE and probed with antibodies against GSK3α, GSK3β and βactin. Representative images are shown (full-length blots are presented in Supplementary Figure 1) (d) Quantified GSK3α and GSK3β protein levels in BMDM determined by densitometry analysis. To determine the bone marrow progenitor cells differentiation into macrophages, cells were labelled with antibodies against the macrophage-specific surface markers, CD11b and F4/80. Cells from each experimental group were examined on a BD FACS Calibur flow cytometer. (e) Contour diagram of CD11b/F4/8 of BMDM. (f) Comparison of number of macrophages differentiated in various groups. Results are reported as the fold change relative to control UT. n=3-4; mean ± SEM; * is the comparison between UT, LPS(M1), and IL-4(M2) treatments; # is the comparison between control and KOs; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. GSK3 indicates glycogen synthase kinase 3; BMDM, bone marrow derived macrophages; UT, untreated; LPS, lipopolysaccharide; IL-4, interleukin-4.

Figure 2. M1 macrophage polarization is impaired with a GSK3α deficiency and enhanced with a GSK3β deficiency. BMDM were isolated from Lqβfl/fl(control), LMaKO, LMβKO and LMcβKO mice and exposed to 10 ng/mL LPS or 10 ng/mL IL-4 for 24 hours to induce M1 or M2 macrophage polarization respectively. Polarization efficiency were examined by quantify the transcription expression of the gene associated with M1 and M2 macrophage polarization by using RT-PCR. (a) iNOS (b) Arg1 (c) iNOS/Arg1 (d) Arg1/iNOS. Data are normalized to the βactin reference gene. NF-κB and NLRP3 was stained using primary antibody, anti-NF-κB p65 and anti-NLRP3. Representative images of (e) NF-κB p65 and (g) NLRP3 staining of control, GSK3α and/or GSK3β deficient macrophages. (f) NF-κB p65 and (h) NLRP3 stained area per cell was quantified. Results are reported as the fold change relative to control UT. n=3-4; mean ± SEM; * is the comparison between UT, LPS(M1), and IL-4(M2) treatments; # is the comparison between control and KOs; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. GSK3 indicates glycogen synthase kinase 3; BMDM, bone marrow derived macrophages; UT, untreated; LPS, lipopolysaccharide; IL-4, interleukin-4; iNOS, inducible nitric oxide synthases; Arg1, arginase 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, NLR family pyrin domain containing 3.

Figure 3. M1 Macrophage lipid accumulation is impaired by GSK3α deficiency and enhanced with GSK3β deficiency. Lipid accumulation of these macrophages were determined by Oil Red O staining. (a) Oil red O staining of GSK3α and/or GSK3β deficient macrophages and lipid accumulation was quantified by measuring (b) ORO-stained area/cell. Lipid accumulation were also measure by determining gene expression of lipid transport proteins such as (c) SR A (d) CD36 (e) ABCA1 (f) ABCG1. Results are reported as the fold change relative to control UT. n=3-4; mean ± SEM; * is the comparison between UT, LPS(M1), and IL-4(M2)
treatments; # is the comparison between control and KOs; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. GSK3 indicates glycogen synthase kinase 3; ORO, oil red o; UT, untreated; LPS, lipopolysaccharide; IL-4, interleukin-4; SR A, scavenger receptor A; ABCA1, ATP-binding cassette transporter; ABCG1, ATP Binding Cassette Subfamily G Member 1.

Figure 4: GSK3α and GSK3β have complementary roles in the regulation of macrophage viability. Cytotoxic effects of Tg or Tm was assessed by determining the cell viability of the macrophages. (a) Cell viability was quantified by normalizing the reduced alamarBlue values to untreated control. n=4-8. Proliferation of these macrophages were determined by (b) Ki67 staining of proliferated macrophages and proliferated cells were quantified by counting (c) the no. of cells proliferated. n=4; mean ± SEM; * is the comparison between UT, LPS(M1), and IL-4(M2) treatments; # is the comparison between control and KOs; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. GSK3 indicates glycogen synthase kinase 3; UT, untreated; LPS, lipopolysaccharide; IL-4, interleukin-4; Tg, thapsigargin; Tm, tunicamycin; ER, endoplasmic reticulum.

Figure 5. Migration of M1 macrophages enhanced with a GSK3α deficiency and impaired with a GSK3β deficiency. Migration of these macrophages towards CCL19 were determine by using transwell plates. (a) migrated cells were quantified by counting the no. of cells migrated. (b) CCR7 staining of GSK3α and/or GSK3β deficient macrophages and CCR7 expression was quantified by measuring (c) CCR7 stained area/cell. Results are reported as the fold change relative to control UT. n=4; mean ± SEM; * is the comparison between M0, M1, and M2; # is the comparison between control and KOs; ***p<0.001, ****p<0.0001. GSK3 indicates glycogen synthase kinase 3; UT, untreated; LPS, lipopolysaccharide; IL-4, interleukin-4; CCR7, C-C chemokine receptor type 7.

Figure 6. GSK3α and GSK3β both together play a role metabolic activity of macrophages. Metabolic activity of macrophages were determine by using seahorse extracellular flux analysis. OXPHOS was measure by analysing OCR (normalized to protein content) in GSK3α and/or GSK3β deficient (a) M1 macrophages and (b) M2 macrophages. Glycolysis was measure by analysing ECAR (normalized to protein content) in GSK3α and/or GSK3β deficient (c) M1 macrophages and (d) M2 macrophages. n=4; mean ± SEM; * is the comparison between control and KOs; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. GSK3 indicates glycogen synthase kinase 3; OXPHOS, oxidative phosphorylation; OCR, oxygen consumption rate; ECAR, extracellular acidification rate.

Figure 7: Specific role of myeloid GSK3α/β in proatherogenic functions. Myeloid GSK3α and GSK3β have opposing roles in macrophage polarization, inflammatory response, lipid accumulation, and migration of macrophages. Myeloid GSK3α and GSK3β play redundant roles to increase macrophage(M2) proliferation and metabolism of macrophages.