Activation of Toll-like receptor 2 via specific agonists promotes OXPHOS and glycolysis in gastric cancer cells rather than other Toll-like receptors

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Abstract

Background

Toll-like receptor (TLR)2 activation via microbial and host stimuli plays important roles in the regulation of metabolic reprogramming in immune cells. We recently showed that ligand-induced TLR2 activation enhanced OXPHOS and glycolytic activity of gastric epithelial (cancer) cells, thus promoting tumour progression.

Objective

To investigate the full spectrum of TLR family members whose ligand-dependent activation can cause metabolic reprogramming in gastric cancer (GC) cells.

Methods

The expression of TLR family members was measured by RT-qPCR and Western blotting in a panel of human GC cells. Metabolic changes in human GC cells which were induced by agonists for different TLRs (TLR2, 4, 9) were identified by performing the Seahorse bioenergetic assay, as well as by measuring L-lactate and ROS production. The expression of genes involved in oxidative phosphorylation and glycolysis was also profiled in stimulated GC cells by RT-qPCR. Western blot further characterized the expression of SOD2, a key downstream target of TLR2 signaling in GC.

Results

TLR2 signaling activated by either synthetic molecules or whole pathogen antigen enhanced glycolytic activity and mitochondrial respiration in TLR2-high expressing cells, whereas ligand-induced TLR4 and TLR9 activation inhibited mitochondrial respiration or extracellular acidification rate. Furthermore, genes implicated in the regulation of glucose metabolism and the redox system, such as *HIF1A*, *PFKFB3* and *SOD2*, were upregulated downstream of TLRs.

Conclusion

Our study reveals that ligand-induced activation of specific TLRs mediates diverse metabolic phenotype in human GC cells. TLR2 is the only family member that promotes both OXPHOS and glycolysis, which may result in tumor progression.

1. Introduction

Gastric cancer (GC) is one of the most lethal cancers worldwide, with a particularly high incidence rate in Eastern Asia¹. There is a strong link that dysregulation of the immune system following infection by pathogenic microbes (i.e. *Helicobacter pylori*) leads to a step-by-step procedure marked by gastritis, atrophy, intestinal metaplasia, dysplasia and ultimate adenocarcinoma². The involvement of pathogenic microbes in gastrointestinal inflammation and carcinogenesis is mainly associated with Toll-like receptors (TLRs), a key family of microbial sensors of the host innate and adaptive immune systems, in mediating chronic inflammatory responses that promote tumorigenesis³. Toll-like receptors (TLRs) have unique antigen-recognition domains that recognize pathogen-associated molecular patterns (PAMPs) as well as host-derived pathogens and initiate a pro-inflammatory response³⁻⁴.

Previous studies in this field are focused on immune cells in response to microbial components. However, epithelial cells especially of the gastrointestinal tract act as the first line host defense for sensing luminal pathogens or microbial components via TLRs, therefore participating innate immune response⁵. Aberrant signal transduction of TLRs in epithelial cells may also contribute on many progresses of diseases⁶⁻⁸. We previously showed that upregulation of TLR2 expression, one of the characteristics of intestinal type GC, promoted gastric epithelial cell survival and proliferation independent of inflammation. This oncogenic activity of TLR2 in gastric epithelial cancer cells is associated with a TLR2-induced gene signature enriched for anti-apoptotic genes, such as BCL2A1, BCL3, BIRC3, CFLAR, IER3 and TNFAIP3⁹⁻¹⁰. Furthermore, we provided the evidence that synthetic ligand induced TLR2 activation in human GC epithelial cells with high TLR2 expression enhanced both OXPHOS and glycolysis along with a strong STAT3 dependent transcriptional induction of SOD2. The augment of this redox mitochondrial protein expression enforced a more glycolytic metabolic phenotype as well as suppressed ROS production from TLR2-induced oxidation activity¹¹. In agreement with these findings, TLR2-SOD2 axis is associated with multiple clinical parameters of high malignancy and poor survival in gastric cancer patients¹¹. However, it is still unclear whether other microbial components induced TLR signaling activation leads to different metabolic alterations in GC epithelial cells, therefore influence cellular functions.

To build on this, we examined TLR expressions in a panel of human gastric cancer cells. Cells were stimulated with a variety of TLR agonists to illustrate the alteration of metabolic phenotype in these cells in order to figure out whether their capacity of glycolysis and oxidative phosphorylation will increase. Furthermore, these genres of phenomenon were related to ROS reduction and whether SOD2 would contribute to the mechanism depended on the cell types. Genes related to glycolysis, OXPHOS and mitochondrial protein were also included.

2. Materials And Methods

2.1 Cell lines culture

Human cell lines AGS, AZ521, MKN28, N87, SNU16, TMK1(American Type Culture Collection, ATCC), MKN1, MKN7, NUGC4(Japanese Collection of Research Bioresources Cell Bank) and SNU601(Korean Cell

Line Bank) were grown in Gibco RPMI 1640 medium containing 10% fetal calf serum (FCS), 1% penicillin-streptomycin and 1% L-glutamine (Thermo Fisher Scientific). Cell lines were characterized/authenticated by short tandem repeat profiling (PowerPlex HS16 System kit, Promega) and passaged for under 6 months after receipt. Cell lines were routinely tested for mycoplasma contamination (MycoAlert PLUS Mycoplasma Detection Kit, Lonza).

2.2 Protein extraction and immunoblotting

The cell lysates were extracted using RIPA lysis buffer with phosphatase and protease inhibitor cocktails (Roche). Reduced and denatured lysates were electrophoresed on 10–12% SDS-PAGE gels followed by transferring to PVDF membranes (Millipore). Immunoblotting was performed with antibodies against TLR2, TLR9, SOD2 (Cell Signaling Technology) and TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA). Actin (Sigma-Aldrich) and tubulin (Abcam) were also used. Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR) and quantified using the ImageJ software.

2.3 RNA isolation and gene expression analysis

Total RNA was isolated from human GC cell lines using RNeasy Mini Kit (Qiagen) with DNase treatment (Qiagen). RNA was then transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Quantitative real-time PCR (qPCR) was performed using QuantStudio™ 6 Flex System (ThermoFisher). Gene expression was normalized to the expression of housekeeping gene 18S. Relative fold changes were transformed using the comparative threshold cycle (CT) method (the $2^{-\Delta\Delta CT}$ method) with the QuantStudio™ Real-Time PCR Software. Primer sequences are provided in Supplementary Table S1.

2.4 TLRs stimulations and assay reagents

GC cell lines were grown in 12-well plates in triplicate (1×10^5 cells/well). After serum-free starvation overnight, cells were treated with phosphate-buffered saline (PBS control), Pam3CSK4 (P3C, InvivoGen) 10 µg/ml, Lipopolysaccharides from Escherichia coli O111:B4 (LPS, Sigma) 10 ng/ml, FSL-1 (Pam2CGDPKHPKSF, InvivoGen) 1 µg/ml, Heat Killed Listeria monocytogenes (HKLM, InvivoGen) 10^7 - 10^8 cells/ml, ODN2006 (ODN7909, Stimulatory CpG ODN, Class B, InvivoGen) 2 µM for 24 h. Cell viability assay in response to TLR ligands for cell number normalization was also conducted using the MTT assay (Invitrogen) in 96-well plate following the manufacturer's instructions.

2.5 Bioenergetic assays

Real-time OCAR (oxygen consumption rate) and ECAR (extracellular acidification rate) were measured using an XFe-96 Extracellular Flux Analyzers (Seahorse Bioscience, Agilent). Cells were plated at different numbers respectively (MKN1, 1.5×10^4 per well; NUGC4, 2.5×10^4 per well; AGS, 1×10^4 per well). After overnight starvation, cells were stimulated with various TLR ligands. On the day of assay, the normal RPMI 1640 medium was changed into Seahorse assay medium with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose (adjust the pH to 7.4) and incubated in a 37°C non-CO2 incubator for 1 h prior to assay. Pharmaceutical compounds in the assay kit were used at the indicated concentration (oligomycin, 1 µM; FCCP, 0.2 µM for MKN1, 0.5 µM for NUGC4 and AGS, antimycin A and rotenone mix 0.5 µM).

2.6 L-lactate assay and ROS measurement

The production of lactate was analyzed to determine the rate of aerobic glycolysis. L-lactate was measured by L-Lactate Assay Kit (Colorimetric) (Abcam) following the manufacturer's instructions. The lactate production was assessed by measuring the absorbance at 450 nm and calculated using the equation obtained from the linear regression of the standard curve. Total cellular ROS were measured using the ROS detection assay kit (Abcam) following the kit protocol. The assay was incubated for 60 min at 37°C in dark. The fluorescence of ROS was measured on a 96-well a FLUOstar Omega microplate reader.

2.7 Statistical analysis

All data were analyzed by SPSS 21.0 and GraphPad Prism 7 and presented as the mean $\pm$ SD. Statistical comparisons were determined using student's t-test. Univariate and multivariate Cox regression models were used to analyze independent indicators of patient prognosis. Kaplan-Meier plots and log-rank tests were also performed for survival analysis¹². A p value < 0.05 was considered statistically significant.

3. Results

3.1 The differential expressions of TLR2, TLR4 and TLR9 in gastric cancer cell lines

As RNA and protein expression of TLR2, TLR4 and TLR9 were previously shown highly expressed in gastric tissue human GC tissue¹³⁻¹⁴ and tumor tissue from gp130^{F/F} mouse stomach, a mouse model of gastric cancer that spontaneously develops gastric intestinal type hyperplasia at early 6 weeks of age¹⁵ compared with matched adjacent nontumoral or wild-type stomach tissues, we firstly examined mRNA and protein expression level of TLR2, TLR4 and TLR9 in a panel of human gastric cancer cell lines. Indeed, TLR2, TLR4 and TLR9 were differentially expressed in the panel of GC cells. MKN1 and MKN7 had the most TLR2 mRNA expression while TLR4 expression was abundantly presented in AGS, MKN1 and N87 (Fig. 1A, B). Most GC lines exhibited TLR9 expression at transcriptional level whereas the expression in MKN28 and SNU601 was higher than that of other lines (Fig. 1C). In addition, the levels of all TLRs protein expression were confirmed by Western blot using specific antibodies as shown in Fig. 1D-F. We then selected MKN1 and AGS as the representatives of lines with high TLR2 and TLR4 expression respectively. NUGC4 was chosen to stand for the line with high TLR9 expression in the following experiments.

3.2 TLRs activation in GC cell lines mediates OXPHOS and glycolysis

To evaluate whether TLRs regulates metabolic alterations in human GC epithelial cells, representative lines mentioned above were stimulated with TLR ligands (P3C (Pam3CSK4), LPS or CpG) indicated for

24 h and subjected to bioenergetic functional assays. The high TLR2 expressing line, MKN1 had an increased basal oxygen consumption rate (OCR) (Fig. 2B, column 1) and the OCR induced by the proton ionophore (uncoupler) FCCP (measuring maximal respiration capacity) in response to P3C (Fig. 2A). Spare respiration capacity (SRC), the parameter calculated by the difference between basal and maximal OCR was also elevated, reflecting the enhanced ability of cells dealing with increased energy needs (Fig. 2B, column 3). Furthermore, the extracellular acidification rate (ECAR), representing glycolytic activity (Fig. 2B, column 2) and lactate production, which measures acid production in cell media (Fig. 2G, column 1) were consistently increased in response to P3C. Unlike TLR2 activation, the ECAR in LPS treated AGS with high expression of TLR4 was downregulated with no change in L-lactate production. LPS induced TLR4 activation had no effect on OXPHOS and lactate despite of considerable changes of ECAR and FCCP induced SRC (Fig. 2C, D, G). In contrast, both OXPHOS and glycolysis were strongly inhibited in NUGC4, the line with high expression of TLR9. As shown in Fig. 2E-G, basal and maximal OCR, basal ECAR and SRC were all downregulated in NUGC4 cell after 24 h stimuli of CpG. We then examined the ROS production following TLR activation using fluorescence signals. Surprisingly, the reduction of ROS production was observed in P3C treated MKN1 regardless of the augmented mitochondrial activity whereas ROS in NUGC4 was decreased in the presence of CpG (Fig. 2H). We confirmed that these observations were not due to any difference in cell viability (Supp Fig. 1A). Therefore, these data suggest diverse metabolic phenotypes induced following the activation of different TLRs signaling pathways.

3.3 TLR2 promotes OXPHOS and glycolysis in GC cell lines rather than other TLRs

Besides the effect of these TLR ligands in respective TLR high expressing cells, whether these cell lines were influenced by other two TLR agonists was considered as well. All these ligands were used in the same concentration indicated before. In MKN1, LPS and CpG reduced the ability of OXPHOS and glycolysis based on OCR, ECAR and SRC (Fig. 3A-B). In AGS, P3C and CpG only had impact on SRC and showed an opposite alteration (Fig. 3C-D). P3C greatly stimulated the ability of OXPHOS and glycolysis in NUGC4 cell, another line with detectable expression of TLR2 (Fig. 1A, D) while LPS had no effect (Fig. 3E-F). Lactate and ROS production were shown in Fig. 3G-H which indicated that TLR2 agonist P3C had greatest change in these cell lines.

3.4 TLR2 agonists from different origins induced comparable metabolic phenotypes in GC cells

As Pam3CSK4 induced greater metabolic changes in GC cells, we further tested whether different TLR2 ligands derived from whole pathogen lysates or synthetic lipoproteins give rise to similar effects on MKN1 cell. FSL-1, a synthetic diacylated lipopeptide recognized by a TLR2/6 heterodimer, or P3C, mainly recognized through a TLR1/2 heterodimer were added at the indicated concentration (FSL1: 1 µg/ml; P3C: 10 µg/ml) for 24 h. Consistently, FSL-1 or P3C induced TLR2 activation promoted both

mitochondrial activity and glycolysis as OCRs, SRC, ECAR as well as lactate production were all increased as shown in Fig. 4A-B, whereas the significant reduction of ROS was detected by fluorescent signal in MKN1 supernatant following FSL-1 or P3C stimulation (Fig. 4F). In addition, we had comparable observations of the changes of metabolic phenotypes for NUGC4 in response to P3C and FSL-1 treatment (Supp Fig. 2A-B).

We next evaluated the influences of whole pathogen lysate induced TLR2 activation on cellular levels of oxidative stress and glycolytic activity. Heat Killed *Listeria monocytogenes* (HKLM), an intracellular Gram-positive bacterium prepared in a specific approach which was mainly indicated to target TLR2 according to the literature¹⁶. Two doses (10^7 cells/ml and 10^8 cells/ml) are used most commonly in the literature and recommended by the manufacturer. Consistent with those two synthetic TLR2 agonists, OCR, ECAR, SRC and lactate production were all significantly increased in MKN1 cell in a dose-dependent manner (Fig. 4C-D). Furthermore, cellular ROS levels were markedly reduced in both HKLM-treated MKN1 cells (Fig. 4H). In addition, similar observations were detected in NUGC4 in response to higher dose of HKLM (Supp Fig. 2C-D). Lactate production (Supp Fig. 2E, G) and cellular ROS levels (Supp Fig. 2F, H) of HKLM-treated NUGC4 cells showed a similar trend to MKN1. However, cell viability was unchanged in any agonists stimulated cell lines compared to non-stimulated control group (Supp Fig. 1A-D). Collectively, these data suggest that activation of conserved TLR2 signaling by either synthetic ligands or bacteria derived agonist in GC cell lines with detectable TLR2 expression causes comparable metabolic alterations, including enhanced OXPHOS and glycolysis, increased acid production, and reduced ROS levels.

3.5 TLR2 activation promotes the expression of key genes in control of bioenergetic processes and redox systems

To explore the potential mechanism of TLRs activation in metabolic reprogramming¹¹, we examined the expression of key genes involving in mitochondrial function, glycolysis and oxidative phosphorylation etc. As shown in Fig. 5A, qPCR analyses of synthetic ligand-treated MKN1 cells revealed that genes involving in glycolysis, such as pyruvate kinase isozyme M2 (*PKM2*) and 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (*PFKFR3*) were significantly elevated. P3C stimulation appeared to have greater effects on OXPHOS genes such as cyclic AMP-responsive element-binding protein 1 (*CREB1*) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*) (Fig. 5B). Furthermore, genes located on mitochondrial chromosome such as mitochondrially encoded ATP synthase 6 (*MT-ATP6*), mitochondrial cytochrome b (*MT-CYTB*) and mitochondrially encoded NADH dehydrogenase 1 (*MT-ND1*) were elevated as well (Fig. 5C).

Similarly, low or high dose of HKLM stimulations in MKN1 cells led to significant elevated expression for genes encoding hypoxia-inducible factor 1 alpha (*HIF1a*), pyruvate kinase isozyme M2 (*PKM2*), hexokinase 2 (*HK2*) (Fig. 5D). Likewise, *PPARGC1A*, the gene promoting OXPHOS, were uniquely increased in two doses of HKLM treated cells (Fig. 5E). And the expression of all mitochondrially encoded genes we studies was dramatically upregulated in the presence of HKLM (Fig. 5F). However, the only

expression of HIF1A and PFKFB3 were increased in AGS, the high TLR4 expressing cell line or NUGC4, the high TLR9 expressing cell line treated with LPS and CpG respectively (Supp Fig. 3A, B) regardless of any alteration in bioenergetic assays observed (Fig. 2C-F).

To delineate the intricate antioxidant system contributing to the reduction of ROS following TLR2 activation, we also analyzed the key genes implicated in other redox systems including manganese-dependent SOD2 gene, glutamate-cysteine ligase catalytic subunit (GCLC) and glutathione synthetase (GSS). qPCR showed that 24-hour stimulation from all agonists had strong transcriptional SOD2 induction in high TLR2 expressing lines, MKN1 cells (Fig. 5G). Consistently, SOD2 protein exhibited a significant increase in MKN1 and NUGC4 upon various ligands-induced TLR2 activation but not in response to any other TLR agonists (Fig. 5G-H). By contrast, SOD2 expression displayed no change in AGS regardless of any TLR activations (Fig. 5H). Taken together, key genes from multiple redox systems involves in mediating TLR2 dependent bioenergetic alterations.

4. Discussion

The findings of metabolic reprogramming between OXPHOS and glycolysis in immune cells provide valuable insight into the nature of immune activation in response to microbial-derived components.¹⁷⁻¹⁸. Our previous studies demonstrated that overexpression of TLR2 is associated with multiple clinical parameters of advanced stage disease including distant metastasis, microvascular invasion and stage, as well as poor survival in gastric cancer. Activation of TLR2 augments both oxidative phosphorylation (OXPHOS) and glycolysis in GC cells. However, there still remains unanswered questions concerning the interplay between other TLRs signaling and metabolic alteration on cancer epithelial cells and underlying mechanism how aberrant activation of TLRs in epithelial cells impacts the progress of diseases⁹⁻¹⁰.

In this study, we provided evidence that among activation of various TLRs, TLR2 yielded greatest effects on metabolic phenotypes. Regardless of synthetic ligands or pathogen components, TLR2 activation promoted both glycolysis and oxidative phosphorylation. However, there were still differences of observations in bioenergetic assays in a specific ligand dependent manner. For instance, FSL-1 treatment resulting in TLR2/TLR6 heterodimers exhibited a less significant induction of metabolic phenotype than P3C, rendering the formation of TLR1/TLR2 heterodimers¹⁹. Furthermore, the effect of HKLM seemed to be more significant than using the synthetic ligands on metabolic alterations. This could be due to multiple targets other than TLR2 in cells in the presence of bacteria derived agonist¹⁶. It is therefore worth considering the notion that this structural-based specificity may contribute to the diverse bioenergetic signatures of cancer cells in response to host defence responses, thus impacting on the pathogenesis of infection-associated cancers, such as gastric cancer, which may lead to a diverse intensity of TLRs stimulation and subsequent complex biological consequences¹⁶.

We also reported the opposite findings of metabolic changes in response to TLR4 and TLR9 ligands in cell lines. It has been shown that TLR4 localizes on the surface of the cells whereas TLR9 is detected within endosomes and ER²⁰. Thus, TLR9 requires stimulants such as microbial DNA to translocate into

endosome to trigger the action²¹, which is quite different from TLR4. Besides, both of TLR4 and TLR9 can trigger MyD88-dependent pathway but TLR4 can also trigger MyD88-independent one, which is through TIR-domain-containing adapter-inducing interferon- β (TRIF), resulting in interferon regulatory factor (IRF) activation and anti-viral responses²². This indicates that TLRs may differ in downstream modulators and thus express diverse biological characteristics. In addition, the involvement of JAK-STAT3, JNK MAPK and NF- κ B cascades contribute to this metabolic change¹¹, which can be considered as the cell-autonomous effects as well as the mutations of AGS and NUGC4 cell lines could cause the difference. In summary, we discovered that TLR2 activation mainly promotes mitochondrial respiration and reduced ROS production in order to protect cells while CpG suppressed the function of OXPHOS and glycolysis, may provide insights in different roles of TLR2 and TLR9.

Furthermore, we selected several typical genes encoding proteins that controlling OXPHOS, glycolysis and genes regulating mitochondrial function. We showed that most of the genes encoding mitochondrial protein were upregulated following TLR2 activation, indicating that components of the electron transport chain and subsequent ATP synthesis may be predominantly influenced by TLR2 activation rather than other TLRs²³⁻²⁴. Intriguingly, LPS and CpG strongly induced HIF1A and PFKFB3 transcription (Supp Fig. 2A, B). These two molecules have been extensively reported as key regulators in controlling glucose metabolism in various cell types. In the transformation of human normal melanocytes exposed to chronic insulin and glucose supplementation, glycolysis was modified with increased transcription of HIF1A and decreased transcription of PFKFB3²⁵. In cancer cells, HIF1A and PFKFB3 control two different modulation pathways in driving a rearrangement of glucose metabolism that contributes to limiting ROS production and autophagy activation in condition of nutrient deprivation via MAPK14/p38 α ²⁶. In conclusion, HIF1A and PFKFB3 may play different roles in glucose metabolism in the context of cellular conditions and cell types.

In addition, TLR2 signaling but not TLR4 and TLR9, is a *de facto* inducer for SOD2 expression according to Figure S2A-D. This is due to the causal effect for SOD2 upregulation for maintaining cellular redox balance following TLR2 induced metabolic reprogramming as we previously showed¹¹. Elevated SOD2 expression has also been associated with increased aggressiveness and metastasis in gastric and colorectal cancers²⁷⁻²⁸. Upregulation of SOD2 by a variety of modifications has a direct impact on mtH₂O₂ generation, accumulation and clearance in the mitochondria, indicating that SOD2 can be a primary generator of H₂O₂ leaving the mitochondria to stimulate signaling pathways that likely promote more aggressive cancer phenotypes²⁹⁻³¹. Our previous study elucidated an indispensable role of SOD2 in both oxidative respiration and glycolysis which is highly likely attributed to SOD2 regulation of the cellular redox balance in gastric cancer¹¹. Hence, TLR2-SOD2 axis may be a specific pathway which cannot be copied in other TLRs in GC cells.

Despite that there is no evidence that any TLRs activation by ligands tested promotes GC proliferation or cell viability in this study which is shown in supplementary Figure S1A-D, we still cannot exclude the possibilities that TLRs signaling may indirectly exert oncogenic activities on cancer epithelial cells by the

complex interplay with different cell types in tumor microenvironment. Noninflammatory role of TLRs in tumor progression and initiation such as the enhancement of proliferation is required to be further elucidated.

In summary, our current study reveals that diverse metabolic phenotypes may be induced by different TLR ligands in gastric epithelial cancer cells. Not only synthetic ligands but also heat killing bacterial components give rise to strong cellular OXPHOS and glycolysis in a TLR2 dependent manner. The expression of many key genes mediating multiple metabolic functions are upregulated in the presence of various ligands.

Abbreviations

TLR: Toll like receptor; SOD2:Superoxide dismutase 2; ROS:Reactive oxygen species; SRC:Spare respiratory capacity; OCR:Oxygen consumption rate; ECAR:Extracellular acidification rate; OXPHOS:oxidative phosphorylation.

Declarations

Conflict of interest

The authors have no conflict of interest

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Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Authors' contributions

Study concept and design: LJK and YL. Acquisition of data: LYZ, JCB, LYD, GQS ,and BJJ. Analysis and interpretation of data: LYZ, JCB, and YL. Drafting of the manuscript: LJK and YL. Critical revision of the

manuscript for important intellectual content: LYZ and JCB. Statistical analysis: JCB. Obtained funding: LJK and YL. Administrative, technical, or material support: LJK and YL. Study supervision: LJK. Writing, reviewing, and editing of the manuscript: LYZ and JCB. All authors read and approved the final manuscript.

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Supporting Information

Activation of Toll-like receptor 2 via specific agonists promotes OXPHOS and glycolysis in gastric cancer cells rather than other Toll-like receptors.

Supplementary Table S1. Primer sequences used for SYBR qPCR gene expression assays.

Supplementary Fig. 1. Seahorse cell number normalization was performed by MTT assay.

Supplementary Fig. 2. Metabolic alteration of NUGC4 treated with TLR2 ligands.

Supplementary Fig. 3. Alterations of metabolism related genes were shown by qPCR.

Figures

Figure 1

Expression of TLR2, TLR4 and TLR9 in GC tissues and cell lines. (A-C) Relative mRNA expression of TLR2(A), TLR4(B) and TLR9(C) in a diversity of human gastric cancer cell lines. Protein expression of TLR2 (D), TLR4 (E) and TLR9 (F) in GC cell lines were detected by Western blot.

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Figure 2

Alteration of the metabolic phenotype and ROS production in human GC cells stimulated by various TLR ligands. (A, C and E) Oxygen consumption rate was assayed using the Seahorse XF analyzer (Seahorse Bioscience) in GC cell lines, MKN1, AGS and NUGC4, stimulated with PBS, TLR2 ligand P3C (10 μ g/ml), TLR4 ligand LPS (10 ng/ml), TLR9 ligand CpG (2 μ M/ml) for 24 h following sequential treatment with compounds oligomycin, FCCP and a mix of antimycin A and rotenone. (B, D and F) basal oxygen

consumption rate (basal OCR), basal extracellular acidification rate (basal ECAR) and spare respiratory capacity (SRC) were measured in GC cells. (G) L-lactate production was measured in cell supernatant treated with PBS or different TLR ligands respectively. (H) Total cellular ROS were measured using fluorescence-based ROS detection assay kits in two GC cell lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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Figure 3

Cell lines were stimulated by non-specific TLR agonists and several metabolic parameters were shown. (A-F) All those three cell lines MKN1, AGS and NUGC4 were stimulated by two of the TLR2, 4 and 9 agonists in the same manner as the specific TLR ligand did. OCR, ECAR and SRC were measured and compared in bar charts. (G-H) L-lactate and ROS production were detected in these cell lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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Figure 4

TLR2 ligands stimulation changed metabolic phenotype and ROS production in human GC cells. (A) MKN1 were stimulated with PBS, TLR2 ligand P3C (10 μ g/ml) and FSL-1 (1 μ g/ml) for 24 h following sequential treatment with compounds oligomycin, FCCP and a mix of antimycin A and rotenone. (B) basal oxygen consumption rate (basal OCR), basal extracellular acidification rate (basal ECAR) and spare respiratory capacity (SRC) were measured in MKN1. (C) MKN1 was stimulated with PBS, HKLM (107 and

108 cells/ml) for 24 h then were measured by Seahorse XF96 analyzer in previous settings. (D) basal oxygen consumption rate (basal OCR), basal extracellular acidification rate (basal ECAR) and spare respiratory capacity (SRC) were measured in MKN1 cells. (E, G) L-lactate production was measured in cell supernatant treated with PBS, P3C, FSL-1 and HKLM. (F, H) Total cellular ROS were measured using fluorescence-based ROS detection assay kits in MKN1 cell lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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Figure 5

Metabolism related genes were changed due to TLR2 stimulation. (A-F) qPCR expression analyses of several genes mediating glycolysis, oxidative phosphorylation and mitochondrial function in MKN1 cell treated by P3C, FSL-1 (A-C) and HKLM (D-F). (G) mRNA level of SOD2, GCLC and GSS in MKN1 treated with P3C, FSL-1 and HKLM. (H) Protein level of SOD2 in MKN1, AGS and NUGC4 cells treated with different TLR ligands. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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