

RESEARCH ARTICLE

Over-expression of miR-223 induces M2 macrophage through glycolysis alteration and attenuates LPS-induced sepsis mouse model, the cell-based therapy in sepsis

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Citation: Dang CP, Leelahavanichkul A (2020) Over-expression of miR-223 induces M2 macrophage through glycolysis alteration and attenuates LPS-induced sepsis mouse model, the cell-based therapy in sepsis. PLoS ONE 15(7): e0236038. <https://doi.org/10.1371/journal.pone.0236038>

Editor: Partha Mukhopadhyay, National Institutes of Health, UNITED STATES

Received: March 11, 2020

Accepted: June 27, 2020

Published: July 13, 2020

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Data Availability Statement: All relevant data are within the paper.

Funding: Asada Leelahavanichkul was supported by Thailand Government Fund (RSA6080023), Thailand Government Fund 2016 to 2018 and Ratchadapisek Sompoch Endowment Fund Chulalongkorn University (760001-HR). Cong Dang Phi was supported the scholarship from The 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship.

Abstract

The attenuation of hyper-inflammation in sepsis with the administration of anti-inflammatory macrophages is an interesting adjuvant therapy for sepsis. Because the induction of anti-inflammatory macrophages by microRNA (miR), a regulator of mRNA, has been mentioned, the exploration on miR-induced anti-inflammatory macrophages was performed. The over-expression of miR-223 and miR-146a in RAW264.7 induced M2 macrophage-polarization (anti-inflammatory macrophages) as evaluated by the enhanced expression of *Arginase-1* and *Fizz*. However, miR-223 over-expressed cells demonstrated the more potent anti-inflammatory property against LPS stimulation as lesser *iNOS* expression, lower supernatant IL-6 and higher supernatant IL-10 compared with miR-146a over-expressed cells. Interestingly, LPS stimulation in miR-223 over-expressed cells, compared with LPS-stimulated control cells, demonstrated lower activity of glycolysis pathway and higher mitochondrial respiration, as evaluated by the extracellular flux analysis, and also down-regulated *HIF-1α*, an important enzyme of glycolysis pathway. In addition, the administration of miR-223 over-expressed macrophages with IL-4 pre-conditioning, but not IL-4 stimulated control cells, attenuated sepsis severity in LPS injected mice as evaluated by serum creatinine, liver enzymes, lung histology and serum cytokines. In conclusion, miR-223 interfered with the glycolysis pathway through the down-regulation of *HIF-1α*, resulting in the anti-inflammatory status. The over-expression of miR-223 in macrophages prevented the conversion into M1 macrophage polarization after LPS stimulation. The administration of miR-223 over-expressed macrophages, with IL-4 preconditioning, attenuated sepsis severity in LPS model. Hence, a proof of concept in the induction of anti-inflammatory macrophages through the cell-energy interference for sepsis treatment was proposed as a basis of cell-based therapy in sepsis.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Sepsis, a syndrome of organ dysfunction due to the dys-regulation of host responses to systemic infection, is a major cause of death in patients with clinically ill conditions and has been recognized as an important world-wide healthcare problem [1]. The imbalance of pro- and anti-inflammatory immune-responses is one of the important causes of death in sepsis and the anti-inflammation is an interesting adjunctive treatment of sepsis, especially during the pro-inflammatory state [2–4]. Indeed, compelling evidences indicate that robust innate immune responses, including macrophages, mainly accounts for sepsis pathophysiology [5].

Macrophages, heterogeneous immune cells with pleiotropic functions [6], are fundamentally divided into classically M1 and alternative M2 polarization with pro- and anti-inflammatory property, respectively [7]. Interestingly, macrophages in sepsis hyper-inflammation are predominant in M1 polarization and are responsible for the production of inflammatory mediators [8]. As such, the induction or the administration of anti-inflammatory M2 macrophages during sepsis hyper-inflammation is mentioned [9, 10]. In addition, microRNA (miR), a small non-coding single-strand RNA [11], regulates post-transcriptional messenger RNA (mRNA) and inhibits protein-translation [12]. Because one miR is able to silence multiple targets that are responsible for numerous biological processes [13], the manipulation of macrophage-polarization with miR is interesting [14]. Accordingly, miR-223 is essential for M2 polarization [15] and the down-regulation of miR-223 in patients with severe sepsis possibly induces predominant M1 polarization [16]. In parallel, miR-146a is well documented as an anti-inflammatory miR, preferentially augments M2 polarization [17], that attenuates sepsis-induced cardiac injury in a mouse model [18].

Interestingly, cellular metabolism is essential for immune cells [19], including macrophages [20], as the metabolic signatures of M1 and M2 macrophage-polarization are glycolysis and mitochondrial respiration, respectively [21]. Indeed, the induction of macrophage plasticity through the interference of cell energy and cell metabolism by miRs is mentioned as miR-33 augmented glycolysis and induced M1 macrophage-polarization in an atherosclerosis mouse model [22]. Although the impact of miRs upon malignant-cell metabolism is well-known [23], the information about influence of immune cells metabolism upon sepsis is still very little. Moreover, the anti-inflammatory potency of several miRs might be different, for example, miR-223 mediated anti-inflammation through HIF-1 α , PPAR γ and STAT3 [15, 24–26], while miR-146a inhibits IRAK1 and TRAF6 [27]. Here, the candidate miRs were over-expressed in macrophages and examined several cell characteristics, including cell energy, before using as a cell-based therapy in a sepsis LPS injection mouse model.

Materials and methods

Animal and animal model

Animal care and use protocol are based upon the National Institutes of Health (NIH), USA. The protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Male 8-wk-old mice on C57BL/6 background were purchased from Nomura Siam International (Pathumwan, Bangkok, Thailand) Mice were at rest for 1 wk in the animal facility before use and endotoxin (LPS) injection model was performed following previous publications [28–30]. Briefly, 4 mg/kg of endotoxin (LPS) from *Escherichia coli* 026:B6 (Sigma-Aldrich, St. Louis, MO, USA) was administered intra-peritonium (ip) at 1 h after the tail vein injection by miR over-expressed RAW264.7 preconditioning with IL-4 (detail later) at 1×10^6 CFU diluted in 0.3 ml phosphate buffer solution (PBS) as previously described [31–34]. Blood collection through tail vein was

performed at 3 days before LPS-injection (0 h) and at 2 and 4 h post-LPS. Mice were sacrificed at 6 h post LPS-injection by cardiac puncture under isoflurane anesthesia. Of note, some mice were sacrificed at these time-points for organ collection. Serum creatinine (Cr) by QuantiChrom Creatinine-Assay (DICT-500, BioAssay, Hayward, CA, USA), serum aspartate transaminase (AST) by EnzyChrom AST assay (EASTR-100, BioAssay), serum alanine transaminase (ALT) by EnzyChrom ALT assay (EALT-100, BioAssay) and serum cytokines by ELISA assays (ReproTech, Oldwick, NJ, USA) were analysed. In addition, blood leukocyte determination was performed as previous published [35]. In short, blood was mixed with 3% acetic acid, a hemolytic solution, with a ratio of blood: acetic acid at 6:100 by volume before counting with a hemocytometer. In parallel, Wright-stained blood smear was examined for the percentage of polymorphonuclear cells (PMN) and lymphocyte. The total number of these cells was calculated by the total count from hemocytometer multiplied by the percentage of cells from the Wright-stained slide.

Cell tracker and histological analysis

Macrophages (RAW264.7) at 3×10^6 cells per plate were incubated with cell-labeling Carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE; Sigma-Aldrich), at 25 μ M in PBS, for 30 minutes at 37°C according to the manufacturer's instructions. Then the buffer was gently aspirated and further incubated with warm media for 15 minutes before collecting the cells. Passively-diffused CFDA-SE in cytoplasm is cleaved by intracellular-esterase to form carboxyfluorescein succinimidyl ester with the fluorescent activity. CFDA-SE—labeled cells are confirmed under fluorescent microscope before the intravenous (iv) injection with 1×10^6 cells/ mouse. Then, mice were sacrificed with cardiac puncture under isoflurane anesthesia with organ collection in the optimal cutting temperature compound (OCT) at -80°C before cutting into 4 μ m-thick slides and DAPI (4', 6-diamidino-2-phenylindole) (Thermo fisher Scientific) was incubated for 10 minutes at room temperature for nuclear counter stain. Each slide was observed with fluorescein microscope at 10 random 200X fields for the enumeration of fluorescent-positive tracked-cells per field as the representatives for cell burden in organs.

For organ injury analysis, the organs were fixed in 10% formalin before Hematoxylin and Eosin (H&E) staining process and the semi-quantitative analysis of organ injury. As such, lung injury was analyzed in accordance with previous publications [36, 37] through evaluation of area of the injury as determined by alveolar hemorrhage, alveolar congestion, neutrophil infiltration and alveolar wall thickness. Semi-quantitative scores were given in each observed field at 200x magnifications according to the following: 0 points, no injury in the observed field; 1 point, injury up to 25%; 2 points, injury up to 50%; 3 points, injury up to 75%; 4 points, injury in the entire field. The scores from 20 fields of each slide were calculated and were presented as the representative lung injury parameter.

Micro-RNA transfection and the preparation of IL-4 preconditioning cells

Murine macrophage cell line (RAW264.7) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and Penicillin-Streptomycin (Thermo fisher Scientific, Waltham, MA, USA) in 5% carbon dioxide (CO₂) at 37°C. Then the microRNA (miR) was transfected into 1.5×10^6 / well of macrophages in 24-well plate following a previous protocol [38]. Briefly, the transfection mixture consisted of 5 pmol of miR (miR146a, miR223 or mimic control) (Ambion, Austin, TX, USA) and 1.5 μ l Lipofectamin RNAimax reagent (Invitrogen, Carlsbad, CA, USA) in Opti-MEM (Gibco, Thermo Fisher Scientific) for one reaction was incubated with cells for 2 days before the verification of transfection-efficiency by quantitative polymerase chain reaction (qPCR) as demonstrated in Fig 1. In addition, M1 or M2

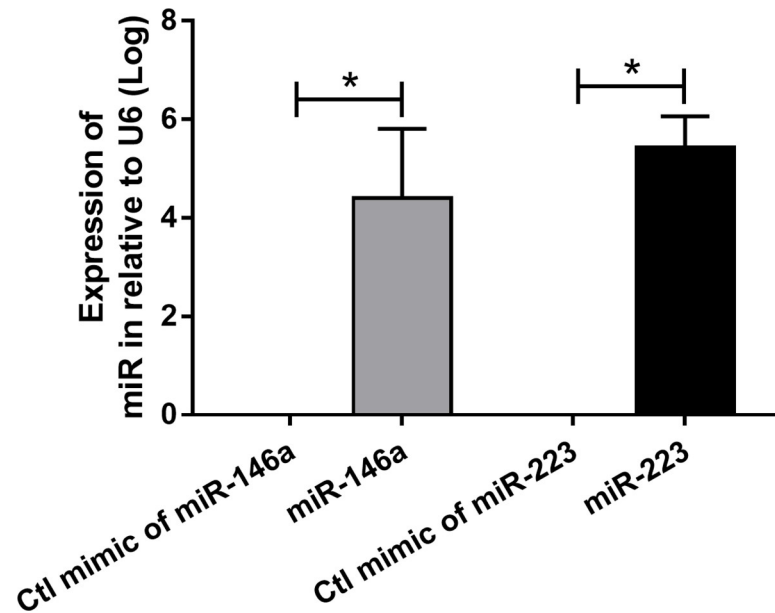


Fig 1. Transfection efficiency of cell culture. Transfection efficiency of RAW264.7 cell with miR-146a and miR-223 was determined by quantitative polymerase chain reaction using Taqman probe in relative to the expression of housekeeping gene U6.

<https://doi.org/10.1371/journal.pone.0236038.g001>

polarization was activated by LPS (10 ng/ml) or IL-4 (20 ng/ml), respectively, before cell collection and supernatant cytokines were measured by ELISA (ReproTech). For preparation of adoptive transfer, miR over-expressed RAW264.7 were treated with IL-4 (20 ng/ml) to activate M2 macrophage polarization, washed 2 times by PBS and intravenously administered into mice at 1 hour prior to LPS intra-peritoneal injection (4 mg/kg).

The determination of macrophage polarization by quantitative Polymerase Chain Reaction (qPCR)

The qPCR was performed following previous publications [29]. Briefly, total RNA was prepared from the cell culture by Trizol (Thermo fisher Scientific) and quantified by Nano drop ND-1000 (Thermo fisher scientific). The ratio of absorbance at optical density (OD) 260 divided by OD 280 more than 1.8 indicated the adequate purity for further testing. After that, RNA was converted into cDNA by Reverse Transcription System and quantitative polymerase chain reaction (qPCR) was performed using SYBR Green master mix (Applied biosystem, Foster city, CA, USA), cDNA template and target primers based on $\Delta\Delta\text{CT}$ method with β -actin as a housekeeping gene. Primers of miR-146a, miR-223 were hsa-miR-146a-5p (Ambion, Cat.4464066, ID.MC10722); mmu-miR-223, ID (Ambion, Cat.4464066, ID.MC14755). In addition, the nucleotide sequences of primers for the analysis of M1 macrophage polarization (*iNOS*, *TNF- α* and *IL-1 β*), M2 macrophage polarization (*Arginase-1*, *FIZZ-1* and *TGF- β*) and glycolysis pathway (Integrated DNA Technologies, Coralville, IA, USA) are demonstrated in Table 1.

Mitochondrial DNA (mtDNA), total cellular ATP and extracellular flux analysis

Total DNA from cells was extracted by Tissue Genomic DNA extraction mini kit (Favorgen Biotech, Wembley, WA, Australia) as previously published [39]. Briefly, DNA quantification

Table 1. List of primers for macrophage polarization and glycolysis pathway are demonstrated.

Primers		
Macrophage polarization		
β-actin	Forward	5' -CGGTTCGGATGCCCTGAGGCTCTT-3'
	Reward	5' -CGTCACACTTCATGATGGAATTGA-3'
Inducible nitric oxide synthase (<i>iNOS</i>)	Forward	5' -CCCTTCCGAAGTTTCTGGCAGCAGC-3'
	Reward	5' -GGCTGTGACAGCCCTCGTGGCTTTG-3'
Arginase 1 (<i>Arg-1</i>)	Forward	5' -CAGAAGAATG GAAGAGTCAG-3'
	Reward	5' -CAGATATGCA GGA GTCACC-3'
Tumor necrosis factor α (<i>TNF-α</i>)	Forward	5' -CCTCACACTCAGATCATCTTCTC-3'
	Reward	5' -AGATCCATGCCG TTGGCCAG-3'
Interleukin-1β (<i>IL-1β</i>)	Forward	5' -GAAATGCCACCTTTTGACAGTG-3'
	Reward	5' -TGGATGCTCTCATCAGGACAG-3'
Resistin-like molecule-α (<i>Fizz-1</i>)	Forward	5' -GCCAGGTCCTGGAACCTTTC-3'
	Reward	5' -GGAGCAGGGAGATGCAGATGAG-3'
Transforming growth factor-β (<i>TGF-β</i>)	Forward	5' -CAGAGCTGCGCTTGCAGAG-3'
	Reward	5' -GTCAGCAGCCGGTTACCAAG-3'
Glycolysis pathway analysis		
Solute carrier family 2, facilitated glucose transporter member 10 (<i>SLC2a10</i>)	Forward	5' -CAGTTCGGGTATAACACTGGTG-3'
	Reward	5' -GCCCCGACAGAGAAGATG -3'
Lactate dehydrogenase A (<i>LdhA</i>)	Forward	5' -CATGTCAAGTACAGTCCACACT -3'
	Reward	5' -TTCCAATTACTCGTTTTTGGGA -3'
Phosphofruktokinase (<i>PFK</i>)	Forward	5' -GGAGCGGAGAACATCAAGCC -3'
	Reward	5' -CGGCCTTCCCTCGTAGTGA -3'
Pyruvate dehydrogenase kinase-1 (<i>PDK1</i>)	Forward	5' -GGACTTCGGGTCAGTGAATGC -3'
	Reward	5' -TCCTGAGAAGATTGTCGGGGA -3'
Hypoxia-inducible factor 1α (<i>HIF-1α</i>)	Forward	5' -AGCTTCTGTTATGAGGCTCACC -3'
	Reward	5' -TGACTTGATGTTTCATCGTCCTC -3'

<https://doi.org/10.1371/journal.pone.0236038.t001>

was determined by Nano drop ND-100 (Thermo scientific) and mtDNA copy number was measured by qPCR with cellular DNA template, Mastermix 1xKAPA fast SYBR Green (KAPA Biosystems, Wilmington, MA, USA) and primers of mitochondrial encoded mtDNA (Favorgen Biotech). Quantification of mtDNA was analyzed by $\Delta\Delta CT$ method normalized by the expression of house-keeping gene $\beta 2$ -microglobulin ($\beta 2M$) with the following sequence, forward, 5' -TTCTGGTGCTTGTCTCACTGA-3', reverse, 5' -CAGTATGTTTCGGCTTCCCA TTC-3'. In addition, ATP content in 2×10^4 cells/ well was determined by the Luminescent ATP Detection Assay (Abcam, Cambridge, UK) according to the manufacturer's protocol [39]. In addition, energy metabolism profiles with estimation of glycolysis and mitochondrial oxidative phosphorylation with extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were performed, respectively, by Seahorse XFp Analyzers (Agilent, Santa Clara, CA, USA) upon the miR-transfected RAW264.7 at 1×10^4 cells/ well by Seahorse Wave 2.6 software as previously described [29]. Glycolysis and mitochondrial parameters was calculated using the generator program report of XF Glycolysis stress test and XF Cell mito stress test, respectively, based on these following equations; Glycolysis = ECAR between glucose and oligomycin-ECAR before glucose administration, glycolysis capacity = ECAR between oligomycin and 2-Deoxy-d-glucose (2-DG)-ECAR before glucose administration, glycolysis reserve = ECAR between oligomycin and 2-DG-ECAR between glucose and oligomycin, basal respiration = OCR before oligomycin-OCR after antimycin A/ rotenone, respiratory

capacity (maximal respiration) = OCR between Carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and antimycin A/ rotenone–OCR after antimycin A/ rotenone and respiratory reserve = OCR between FCCP and antimycin A/ rotenone–OCR before oligomycin.

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) was used for all statistical analyses. Differences between groups were calculated by Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's analysis for the comparison of two or more experimental groups, respectively. *In vitro* data were based on independent triplicate experiments and represented by mean \pm standard error (SE). A p value less than 0.05 was considered as statistically significant.

Results

The over-expression of miR-223 facilitated M2 macrophage polarization in macrophages (RAW 246.5) through the inhibition of several genes in glycolysis pathway. In addition, the adoptive transfer of miR-223 over-expressed macrophages with IL-4 pre-conditioning attenuated LPS-induced sepsis mouse model.

The over-expression of miR-223 in macrophages reduced the characteristics of M1 polarization and decreased pro-inflammatory cytokines in cell-supernatant

Because of the known anti-inflammatory effect of both miR-223 and miR-146a in macrophages [27], both miRs were transfected in RAW264.7 before the induction of M1 and M2 polarization by LPS and IL-4, respectively. As such, the overexpression of miR-223 down-regulated the gene expression of M1 biomarkers including *iNOS* and *IL-1 β* , but not *TNF- α* , while the over-expressed miR-146a down-regulated only *IL-1 β* (Fig 2A–2C). In parallel, the overexpression of miR-223 induced M2 polarization as demonstrated by the up-regulation of *Arg-1* and *Fizz*, but not *TGF- β* , and the over-expressed miR-146a up-regulated only *TGF- β* (Fig 2D–2F). However, the expression of both miRs did not further enhance the characteristics of IL-4 activated M2 polarization, as determined by the expression of several biomarkers, in comparison with IL-4 induced control mimic cells (Fig 2D–2F). In addition, the anti-inflammatory property of miR-223 was demonstrated by i) the reduction of supernatant IL-6, but not TNF- α , after LPS activation (Fig 2G and 2H) and ii) the induction of supernatant IL-10 after IL-4 stimulation (Fig 2I). On the other hand, the over-expression of miR-146a did not demonstrate an anti-inflammatory effect as evaluated by supernatant cytokines (Fig 2G–2I). Because the conversion from IL-4 activated M2 polarization into pro-inflammatory macrophages by LPS was previously mentioned [40, 41], the property to maintain M2 polarization after IL-4 stimulation in miR-223 overexpressed macrophages was tested. Accordingly, while IL-4 stimulation alone induced very low expression of *iNOS*, *TNF- α* and *IL-1 β* compared with LPS stimulation (Fig 2A–2C), LPS activation in IL-4 pre-conditioning macrophages (IL-4/ LPS) demonstrated a similar level of these genes in comparison with LPS stimulation alone (media/ LPS) (Fig 3A–3C). However, miR-223 over-expressed macrophages with IL-4 pre-conditioning (IL-4/ LPS) enhanced only some pro-inflammatory characteristics but still demonstrated lower *iNOS* expression, higher *Arg-1* expression and lower supernatant IL-6 (Fig 3) compared with the miR-146a over-expressed cells.

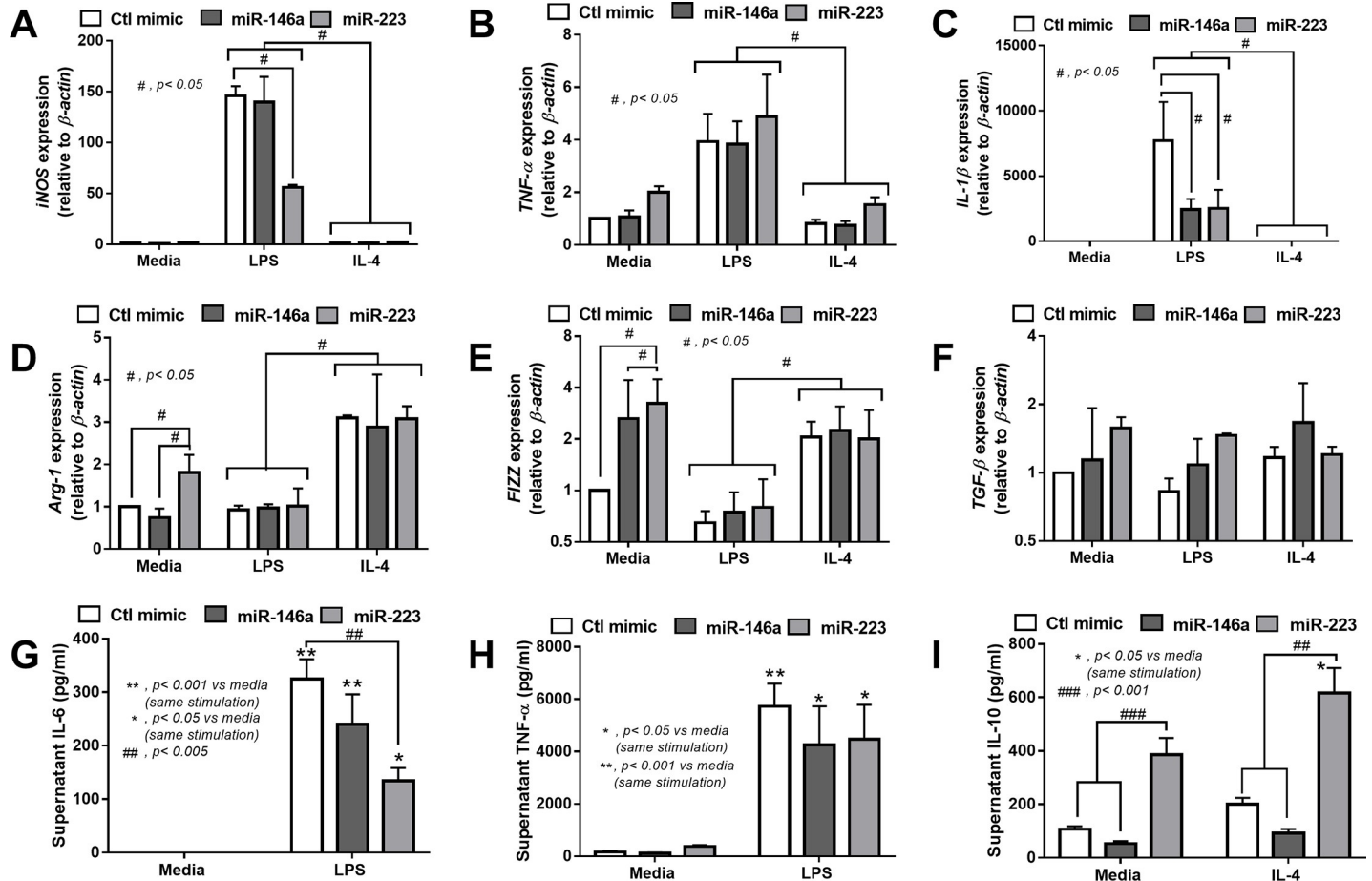


Fig 2. Macrophage anti-inflammatory property after the over-expression of miR-223 is higher than miR-146a. The characteristics of M1 macrophage polarization as determined by the expression of *iNOS*, *TNF- α* and *IL-1 β* (A–C), and M2 macrophage polarization as evaluated by the expression of *Arg-1*, *Fizz2* and *TGF- β* (D–F), of macrophages with the over-expression of control mimic (Ctl mimic), miR-146a and miR-223 after the stimulation with LPS and IL-4, the stimulator of M1 and M2 macrophage polarization, respectively, are demonstrated. In addition, the supernatant pro-inflammatory cytokines, IL-6 and TNF- α (G, H), after LPS stimulation and an anti-inflammatory cytokine, IL-10 (I), after IL-4 activation, from these macrophages are demonstrated.

<https://doi.org/10.1371/journal.pone.0236038.g002>

The inhibition of enzyme in glycolysis pathway by the miR-223 over-expression induced resistance against LPS-enhanced M1 polarization

Because the alteration of immunometabolism is, at least in part, responsible for the expression of pro- or anti-inflammatory characteristics of macrophages [42], the metabolism profiles are explored by the extracellular flux analysis. As such, LPS induced the glycolysis pathway as indicated by increased glycolysis area, glycolysis capacity and glycolysis reserve (Fig 4A; Ctl mimic-LPS vs. Ctl mimic-media; 4E–4G). In parallel, LPS reduced the mitochondrial pathway as indicated by reduced basal respiration, respiratory capacity and respiratory reserve (Fig 4B; Ctl mimic-LPS vs. Ctl mimic-media, 4H–4J). However, the patterns of glycolysis and mitochondrial responses in miR-223 over-expressed macrophages after LPS stimulation was similar to control (Fig 4A and 4B; Ctl mimic-media vs. miR-223-LPS, 4E–4J) implying the resistance of miR-223 over-expressed cells against the direction of LPS stimulation. On the other hand, IL-4 (Ctl mimic IL-4) also increased glycolysis activity compared with control (Ctl mimic-media) (Fig 4C and 4E–4G) without the alteration in mitochondrial activity (Fig 4D and 4H–4J). Moreover, LPS also reduced mitochondrial number, as determined by mitochondrial DNA analysis, and decreased total ATP while IL-4 did not change these parameters (Fig

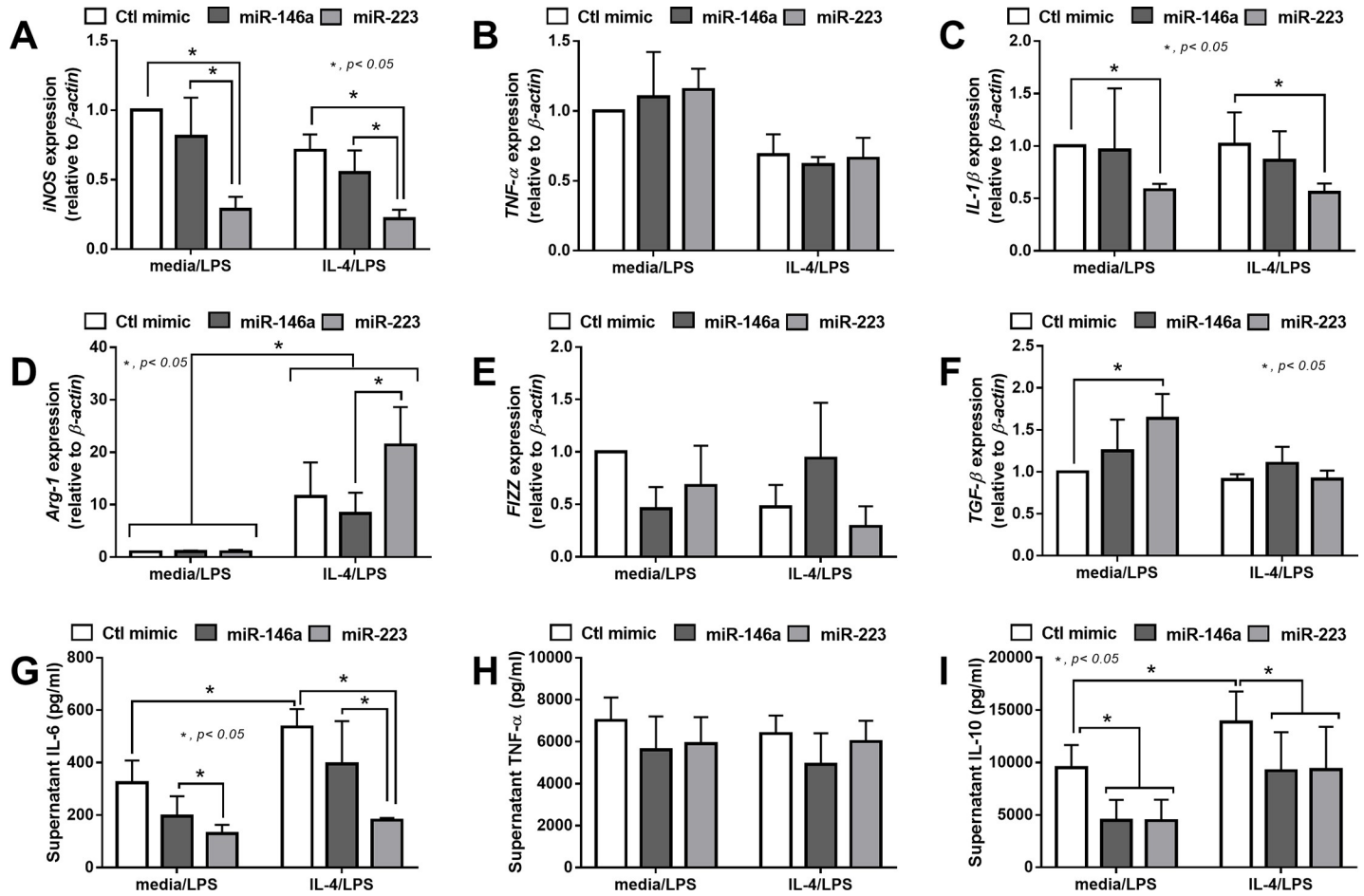


Fig 3. Over-expression of miR-223 maintains IL-4 induced anti-inflammatory characteristics of macrophages after LPS-induction better than miR-146a over-expression. The characteristics of M1 macrophage polarization as determined by the expression of *iNOS*, *TNF- α* and *IL-1 β* (A-C), and M2 macrophage polarization as evaluated by the expression of *Arg-1*, *Fizz* and *TGF- β* (D-F), of macrophages with the over-expression of control mimic (Ctl mimic), miR-146a and miR-223 that stimulated with IL-4, the M2 macrophage polarization stimulator, prior to the stimulation with LPS, M1 macrophage-polarization stimulator, (IL-4/LPS) or control media prior to LPS (media/LPS) are demonstrated. In addition, the supernatant cytokines, IL-6, TNF- α and IL-10 (G-I), of these macrophages are demonstrated.

<https://doi.org/10.1371/journal.pone.0236038.g003>

4K and 4L). Interestingly, mitochondrial DNA and ATP production in miR-223 over-expressed cells with LPS stimulation did not differ from the control macrophages (Fig 4K and 4L). Of note, the profile of extra-cellular flux analysis in miR-223 over-expressed macrophages without LPS (miR-223-media) did not differ from the control mimic (Ctl mimic-media). These data supported that LPS induced glycolysis, suppressed mitochondrial function and reduced ATP production in macrophages but were attenuated by miR-223 over-expression.

Due to the inhibitory property of miR against the mRNA functions [12], we hypothesize that miR-223 might inhibit some mRNAs in glycolysis pathway. Accordingly, hypoxic inducible factor-1 α (HIF-1 α) is an enzyme responsible to enhance glycolysis pathway through several mediators including Solute carrier family 2, facilitated glucose transporter member 10 (SLC2a10), phosphofructokinase (PFK), Lactate dehydrogenase A (LdhA) [43] (Fig 5A). Furthermore, HIF-1 α also reduces the mitochondrial function through the expression of pyruvate dehydrogenase kinase (PDK1) [44]. Of note, increased PDK1 inhibited pyruvate dehydrogenase (PHD), an enzyme that converts pyruvate into acetyl CoA supplying for Krebs cycle, leads to the reduction of mitochondrial function [44]. As such, the exploration of glycolysis-related genes in macrophages demonstrated that LPS induced only *HIF-1 α* expression (Fig

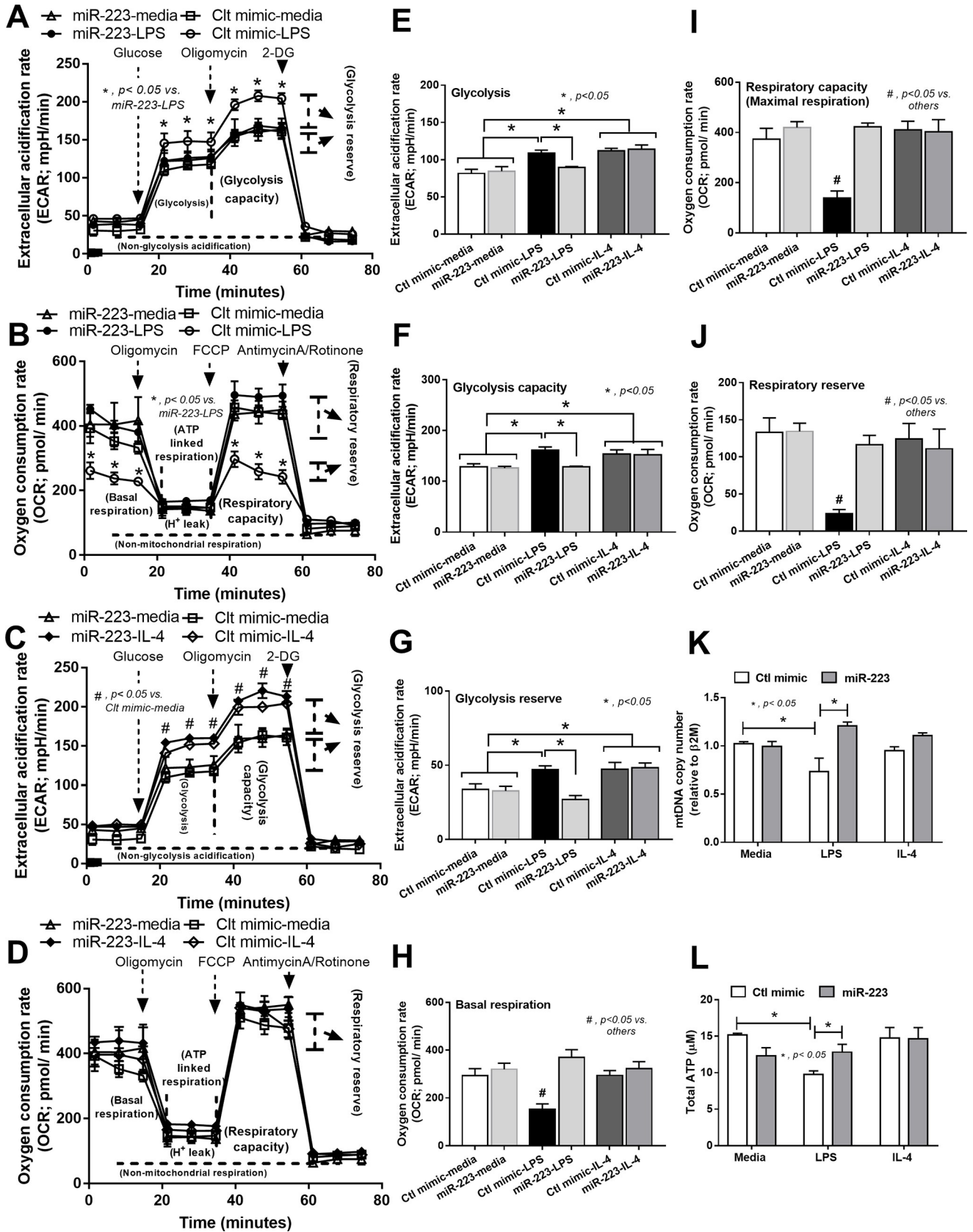


Fig 4. Over-expression of miR-223 in LPS-activated macrophages reduces glycolysis activity and improves mitochondrial function as determined by extracellular flux analysis. The extracellular flux analysis pattern of macrophages over-expressed by miR-223 or control mimic (Ctl-mimic) as evaluated by extracellular acidification rate of glucose stress test for glycolysis pathway analysis and oxygen consumption rate of mitochondrial stress test for mitochondrial pathway analysis after the stimulation by LPS versus control media (A, B) or IL-4 versus control media (C, D) with the comparison in column bar graph of glucose stress test parameters (glycolysis, respiratory capacity and glycolysis reserve) (E-G) and mitochondrial stress test parameters (basal respiration, respiratory capacity and respiratory reserve) (H-J) are demonstrated. In addition, the mitochondrial content as analyzed by the copy number of mitochondria (mtDNA) (K) and the ATP production (L) of macrophages over-expressed with Ctl-mimic or miR-223 after the stimulation with LPS, IL-4 or control media are demonstrated. Of note, the extracellular flux analysis pattern of miR-223-media is not showed due to the similarity to Ctl-mimic-media.

<https://doi.org/10.1371/journal.pone.0236038.g004>

5B), while IL-4 enhanced the expression of several genes including; *HIF-1 α* , *SLC2a10*, *PDK1*, *PKF* and *ldhA* (Fig 5C–5F). Although miR-223 over-expression reduced only *HIF-1 α* in LPS-activated macrophages (Fig 5A), the LPS-induced glycolysis was inhibited (Fig 4A), implying the major role of *HIF-1 α* in the glycolysis pathway. In contrast, miR-223 inhibited both *PDK-1* and *PFK* in IL-4 stimulated-cells (Fig 5E and 5F) without any impact upon extracellular flux pattern (Fig 4C and 4D), suggesting less importance of these enzymes in glycolysis pathway.

The administration of IL-4 preconditioning macrophages with miR-223 over-expression attenuated sepsis in LPS injection mice

Because preconditioning with IL-4 in miR-223 over-expressed macrophages demonstrated anti-inflammatory state (Fig 2) that resisted the LPS induced pro-inflammation (Fig 3) possibly through the interference of *HIF-1 α* (Figs 4 and 5), these cells were used *in vivo* following previous publications [31–34]. As such, the intravenous cells administration demonstrated the highest cell accumulation in kidney and in liver at 1 h and 6 h post-injection, respectively (Fig 6). The administration of IL-4 pre-conditioning macrophages with miR-223 over-expression, but not IL-4 stimulated control cells, attenuated the severity of LPS injection model as determined by renal injury (serum creatinine), liver injury (aspartate transaminase and alanine transaminase) (Fig 7A–7C) without therapeutic effect on leukocyte count (Fig 7D–7F). In addition, treatment with miR-223-manipulated cells also reduced pro-inflammatory cytokine (TNF- α), but not IL-6, increased anti-inflammatory cytokine (IL-10) (Fig 7G–7I) and improved lung pathology (Fig 8).

Discussion

The over-expression of miR-223 in macrophages induced anti-inflammatory responses against LPS stimulation possibly due to the glycolysis interference through reduced *HIF-1 α* . The administration of IL-4 stimulated miR-223 over-expressed macrophages attenuated the severity of LPS injection sepsis mouse model.

Anti-inflammatory property of miR-223 over-expressed macrophages

The hyper-inflammatory responses induced sepsis mortality and the attenuation by anti-inflammatory macrophages are well-known [9, 10]. Due to the multiple inhibitory targets, either complementary or non-complementary sequence mRNA, is possible from a single miR [13], miR is an interesting method to induce anti-inflammatory macrophages. Indeed, both miR-223 and miR-146a (without IL-4) are associated with M2 macrophages polarization [45, 46] through, at least in part, targeting PPAR γ [15, 47]. With LPS stimulation, the anti-inflammatory property of miR-223 over-expressed macrophages was more potent than miR-146a over-expressed cells as determined by the reduced expression of *IL-1 β* and *iNOS* with decreased supernatant IL-6 and increased supernatant IL-10. Different effects of these miRs suggest the inhibition in different target-molecules. However, previous studies report that IL-

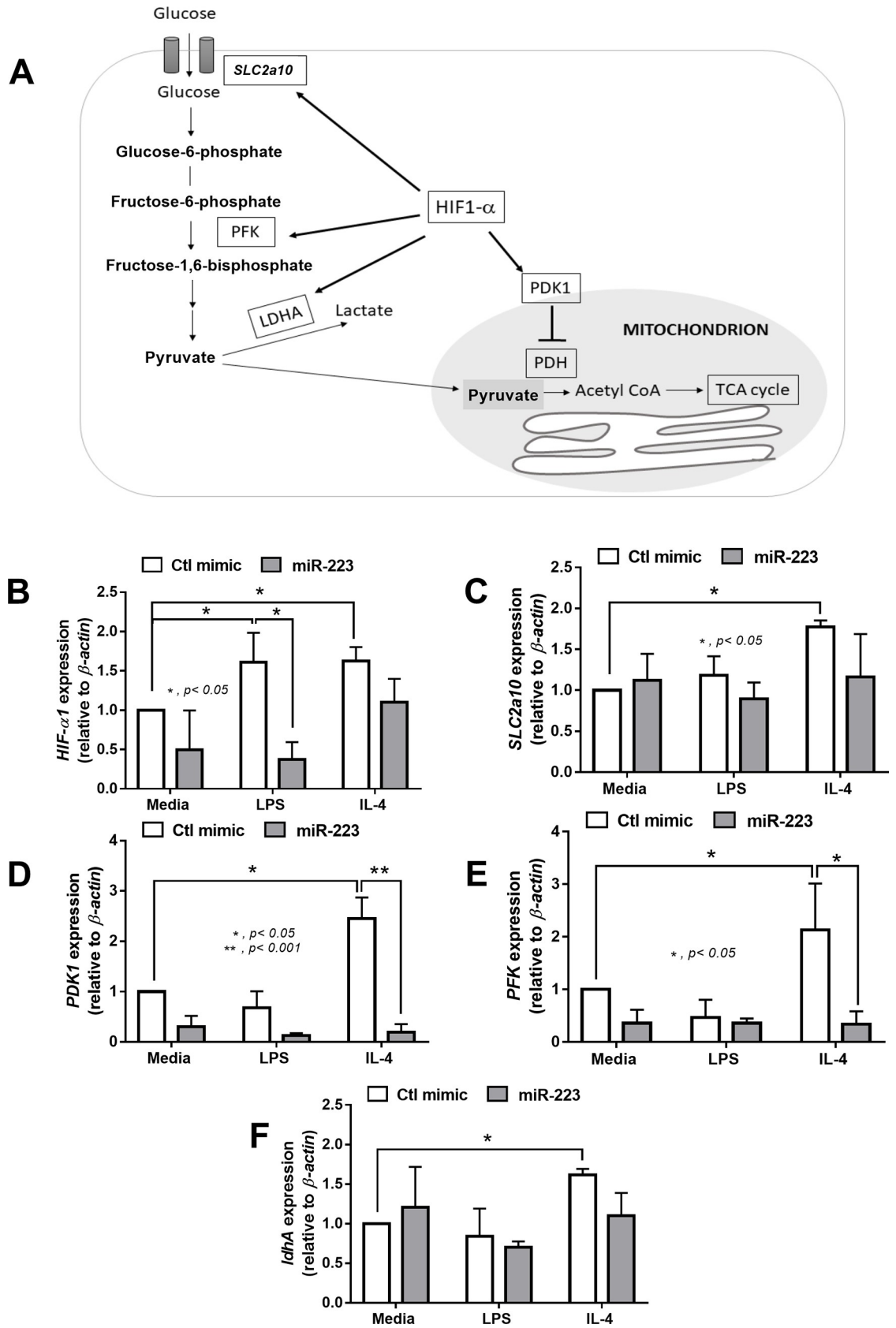


Fig 5. Over-expression of miR-223 in LPS-activated macrophages reduces *HIF-1 α* expression, while miR-223 over-expression in IL-4-activated macrophages decreases expression of *PDK-1* and *PFK*. The diagram demonstrates enzymes that associated with glycolysis pathway (A) and the gene expression of these enzymes in macrophages with the over-expression of miR-223 or control mimic (Ctl mimic) after the stimulation with LPS or IL-4 (B-F) are demonstrated. *HIF-1 α* , hypoxia-inducible factor-1 α ; *SLC2a10*, solute carrier family 2, facilitated glucose transporter member 10; *PFK*, phosphofructokinase; *LDHA*, lactate dehydrogenase A; *PDK1*, pyruvate dehydrogenase kinase 1, *PDH*: pyruvate dehydrogenase; Acetyl-CoA, Acetyl Coenzyme A; TCA cycle, tri-carboxylic acid cycle or Krebs cycle.

<https://doi.org/10.1371/journal.pone.0236038.g005>

4-primed macrophages are easily converted back into the pro-inflammatory macrophages after the subsequent LPS challenge due to the macrophage plasticity [40, 41]. Here, miR-223 over-expression prevented the conversion back into the pro-inflammatory state with the subsequent LPS stimulation but miR-223 did not enhance the expression of IL-4 induced anti-inflammatory genes.

The interference of glycolysis pathway by miR-223 induced anti-inflammatory macrophages

The signatures of immunometabolic pathway of M1 and M2 polarized macrophages are glycolysis and mitochondrial oxidative phosphorylation, respectively [21]. As such, the induction of macrophage polarization by immunometabolic alteration using miRs is mentioned [22]. Here, LPS down regulated mitochondrial oxidative phosphorylation, along with the reduction of mtDNA and ATP production, with glycolysis enhancement while IL-4 stimulated only the glycolysis pathway supporting previous publications [48–50]. While glycolysis is not a major metabolic pathway in M2 macrophages polarization [51], glycolysis is required for the regular M2 macrophage functions [48] and the interference on glycolysis inhibits M2 macrophage polarization [49]. Because i) LPS increased only *HIF-1 α* while IL-4 enhanced *HIF-1 α* with other genes and ii) miR-223 over-expression, that mainly inhibited *HIF-1 α* , turned the immunometabolic characteristics of LPS-stimulated cells into the neutral state but no effect on IL-4-stimulated cells. These data implied a possible key role of *HIF-1 α* in glycolysis pathway after LPS activation [52]. Indeed, the impact of *HIF-1 α* upon glycolysis and M1 polarization is previously mentioned including; i) *HIF-1 α* expression up-regulates several enzymes in glycolysis [43], ii) *HIF-1 α* overexpression enhances glycolysis and increases inflammatory cytokine production in macrophages [53] and iii) *HIF-1 α* deficient macrophages did not produce cytokines after LPS stimulation [54]. Thus the interference of *HIF-1 α* expression might be one of the important mechanisms of miR-223-induced anti-inflammatory macrophages.

In addition, from the previous reports, miR-223 also induced anti-inflammatory properties through the interference of other mechanisms such as STAT3, Nfat5 and PPAR γ [15, 26] implying the multiple targets of miR-223 for anti-inflammatory induction. On the other hand, miR-223 over-expression reduced the expression of *PFK* and *PDK-1*, but did not alter immunometabolic characteristics, of IL-4 stimulated cells implying that these enzymes are not the main enzymes used for cell-energy generation during IL-4 induced M2 macrophage polarization.

Of note, miR-223 regulation on *HIF-1 α* translation is nicely mentioned though the analysis of 3-prime un-translated region (3' UTR) [24, 25]. In addition, LPS and IL-4 possibly mediate *HIF-1 α* expression differently. Our working hypothesis is that while LPS enhances *HIF-1 α* through LPS-induced reactive oxygen species [55], IL-4 activates *HIF-1 α* via Akt-mTORC [48, 49], an upstream signaling of *HIF-1 α* [56]. As such, LPS and IL-4 enhanced glycolysis as demonstrated by extracellular flux analysis and genes of glycolysis pathway mainly through *HIF-1 α* and *HIF-1 α* with other several genes, respectively (Fig 4). It seems that miR-223 targets only on *HIF-1 α* in LPS-enhanced glycolysis, but binds to *HIF-1 α* together with other several genes

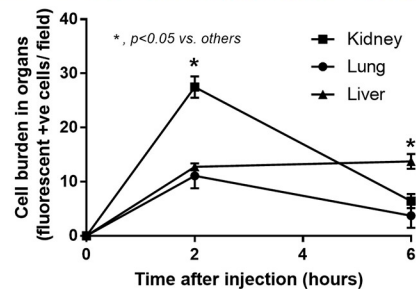
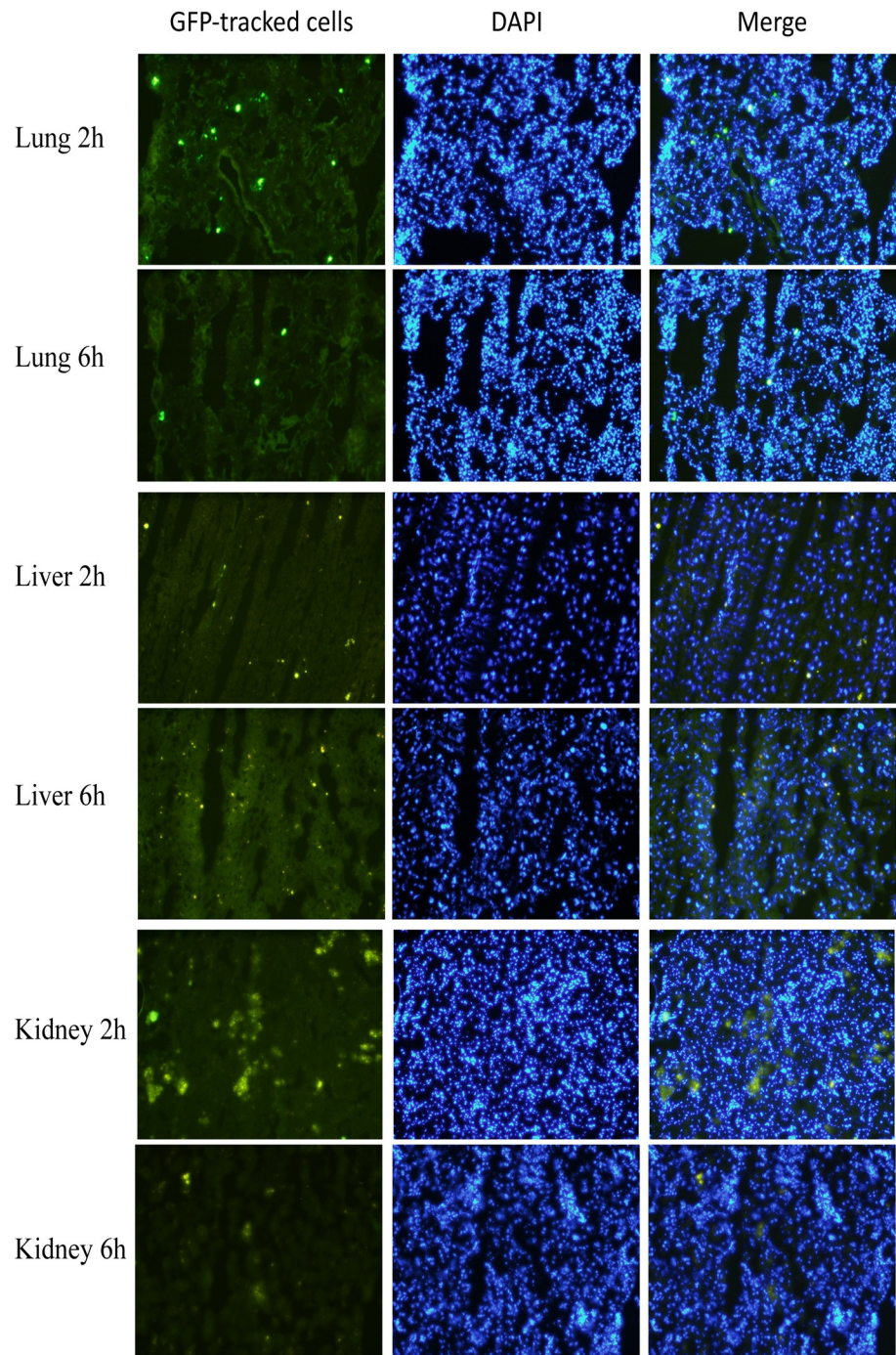


Fig 6. Macrophages are demonstrated in kidney and liver at 2 h and 6 h after administration. The representative pictures of the kinetics of tagged green fluorescent protein (GFP) macrophages after intravenous injection in different organs at different time-points and the analysis of cells burdens in organs are demonstrated. DAPI, 4', 6-diamidino-2-phenylindole (a DNA fluorescence staining color).

<https://doi.org/10.1371/journal.pone.0236038.g006>

in IL-4-enhanced glycolysis. Indeed, the predicted-targets of miR-223 from the database of miRNAWalk 2.0 and TargetScan.org version 7.2, the comprehensive database on the predicted and validated targets of human and murine miR [57], was including both *HIF-1α* and *PFK*. Here, we demonstrated that LPS increased *HIF-1α* while IL-4 induced *HIF-1α*, *PDK-1* and *PFK*. Hence, miR-223 blocked only *HIF-1α* in LPS stimulation but targeted on *HIF-1α*, *PDK-1* and *PFK* in IL-4 induction, resulted in the higher inhibitory potency on LPS-derived *HIF-1α* compared with IL-4 induced *HIF-1α*. More studies in this topic are interesting.

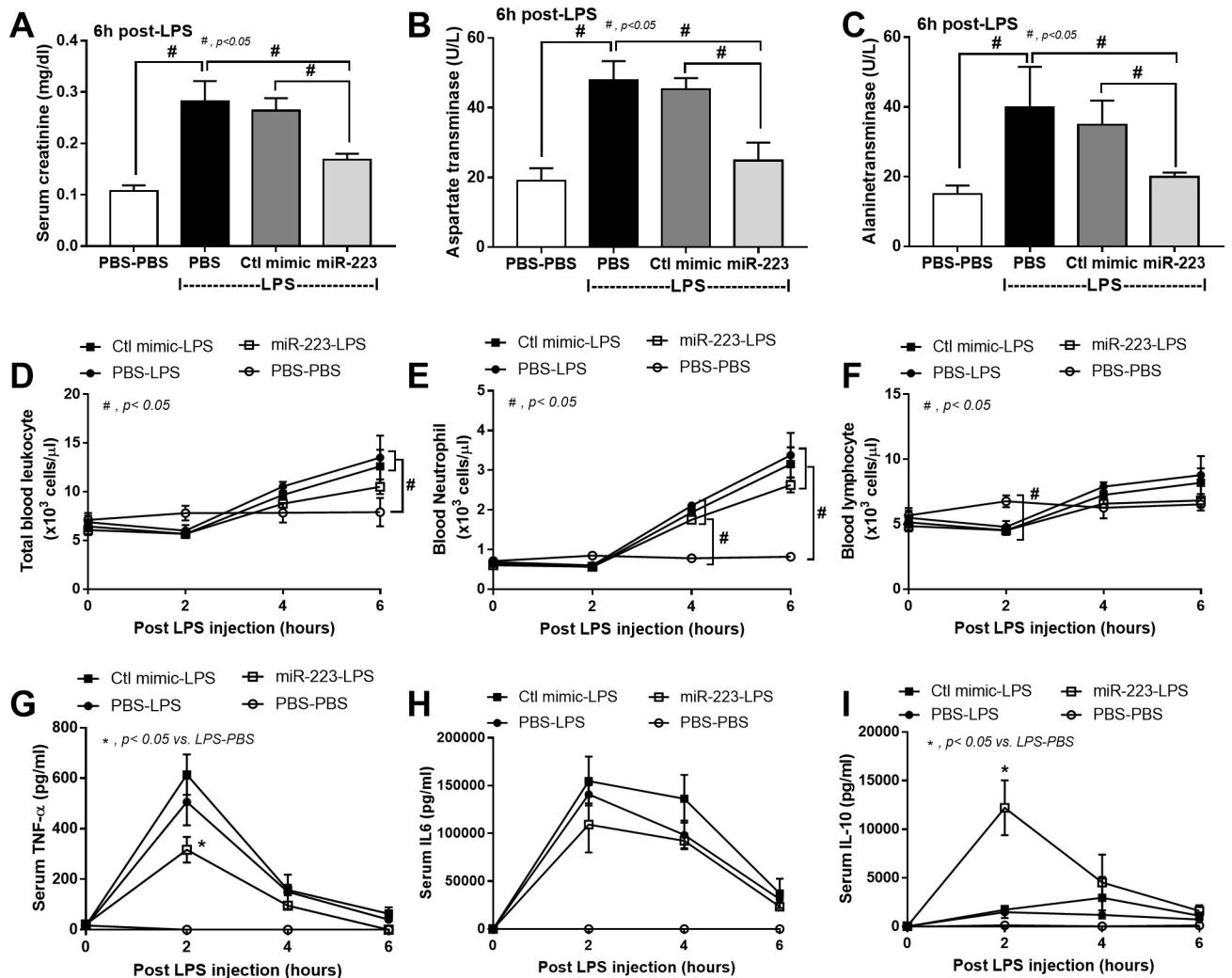


Fig 7. Administration of miR-223 over-expressed macrophages attenuates sepsis in LPS injection mouse model. The characteristics of LPS injection mice after the administration with IL-4-activated-macrophages without gene over-expression (PBS-LPS) or with control mimic over-expression (Ctl mimic-LPS) or with miR-223 over-expression (miR-223-LPS) as evaluated by serum creatinine (A), aspartate transaminase (B), alanine transaminase (C), blood leukocyte evaluation (D-F) and serum cytokines (G-I) are demonstrated (n = 5-7/ group). Of note, only the control mice injected with phosphate buffer solution (PBS) group without LPS stimulation (PBS-PBS) is presented, but not Ctl mimic-PBS and miR-223-PBS, due to the similar values among these 3 control groups.

<https://doi.org/10.1371/journal.pone.0236038.g007>

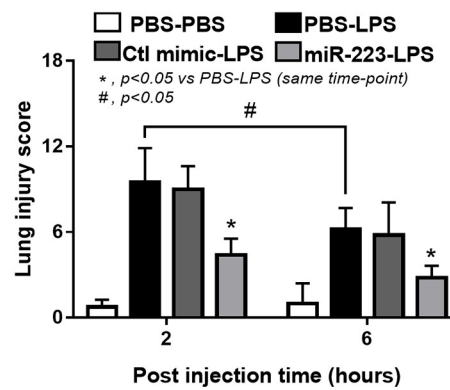
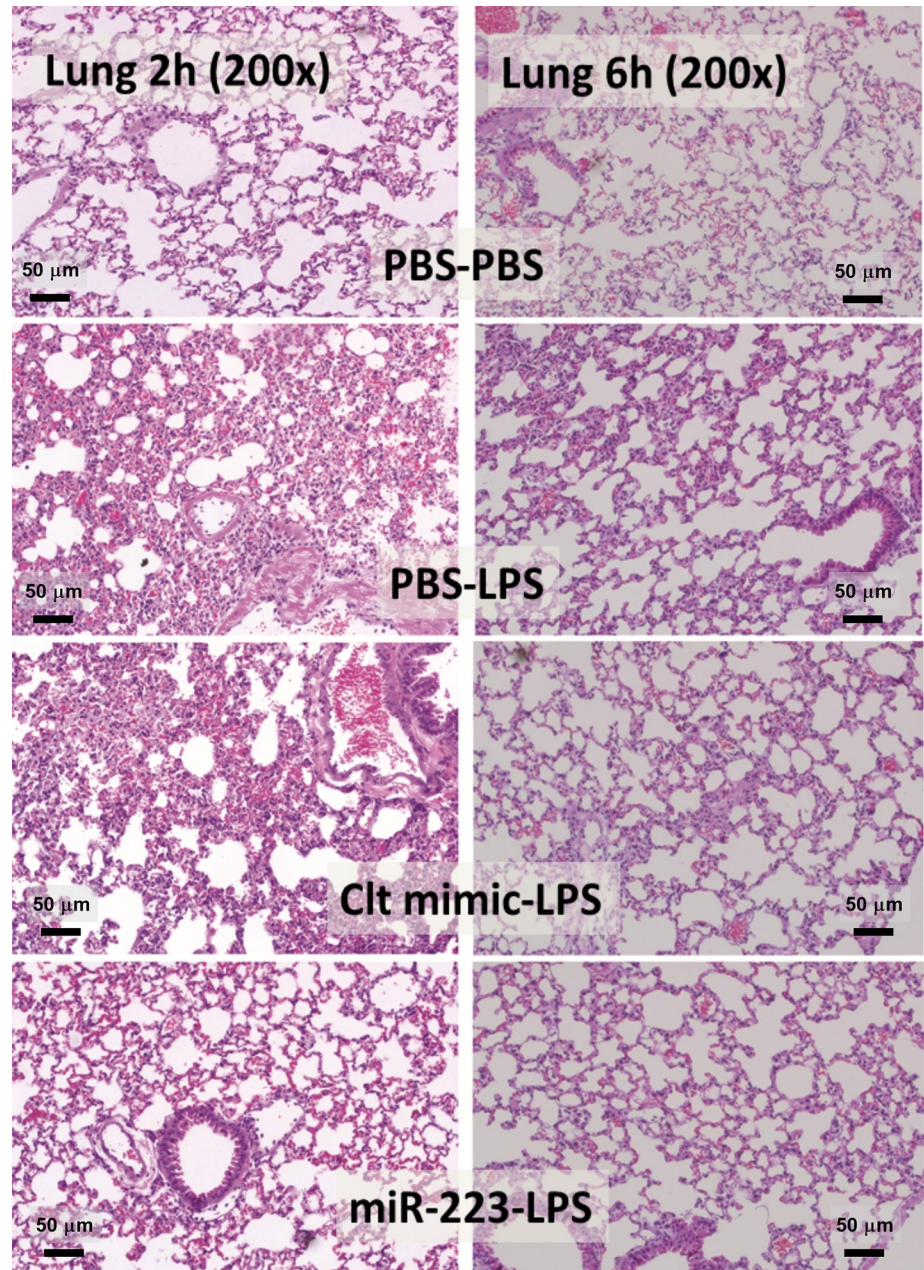


Fig 8. Administration of miR-223 over-expressed macrophages attenuates sepsis-induced lung injury in LPS injection mouse model. The representative of lung histology (A) of LPS administered mice preconditioning with IL-4-activated-macrophages without gene over-expression (PBS-LPS) or with control mimic over-expression (Ctl mimic-LPS) or with miR-223 over-expression (miR-223-LPS) by the semi-quantitative histology score (B) are demonstrated (n = 5-7/ group). Of note, only the control mice injected with phosphate buffer solution (PBS) group without LPS stimulation (PBS-PBS) is presented, but not Ctl mimic-PBS and miR-223-PBS, due to the similar pictures among these 3 control groups.

<https://doi.org/10.1371/journal.pone.0236038.g008>

The administration of IL-4 stimulated miR-223 over-expressed macrophages, a proof of concept for anti-inflammatory treatment in sepsis

Because of the potent anti-inflammatory property and the resistance against LPS induced macrophage-conversion of miR-223 over-expression, IL-4 stimulated miR-223 over-expressed macrophages were tested in LPS injection mouse model as a proof of concept for sepsis treatment. The injected cells distributed in lung, liver and kidney for at least 6 h after the administration. Immune-mediated rejection of C57BL/6 mice toward the administered RAW264.7 cell, a cell line from BALB/c mice, might be responsible for the cell depletion which could be improved in the clinical translation. Nevertheless, the treatment attenuated the injury in several organs as evaluated by lung histology, liver enzymes and serum creatinine with decreased systemic inflammation as demonstrated by decreased TNF- α and increased IL-10 in serum at 6 h post LPS administration, a time-point with the highest injury of the model. Interestingly, administration of IL-4 stimulated macrophages without miR-223 over-expression did not demonstrate any beneficial effects in LPS injection model, perhaps due to the conversion back into pro-inflammatory state of the injected-cells by LPS stimulation as previously mentioned [40, 41, 58]. In addition, the higher pro-inflammatory responses by LPS stimulation in IL-4 pre-conditioning macrophages in comparison with LPS-stimulated control macrophages is demonstrated here (Fig 4G) supporting several previous publications [40, 59, 60], possibly through IL-4 activated STAT-6 [41], which is a possible limitation of cell therapy in sepsis. Hence, the plasticity of macrophages in cell-based therapy in sepsis is an important issue for further studies. In translational aspect, the injected cells that distribute in several organs might be responsible for direct local inflammatory control within the organs and is possibly an interesting adjunctive therapy in sepsis. Cell-based therapy has been currently developed for cancer treatment [61, 62] and some of these therapies might also be appropriated for use in sepsis. More studies are interesting.

In conclusion, miR-223 over-expression induced anti-inflammation through the down-regulation of *HIF-1 α* that inhibited the glycolysis pathway. The anti-inflammatory induction by IL-4 in miR-223 over-expressed macrophages prevented the back-conversion into pro-inflammatory macrophage after LPS stimulation. These cells are proposed as a candidate for cell therapy during the pro-inflammatory phase of sepsis.

Acknowledgments

We would like to thank to Peerapat Visitchanakul and Jiraphorn Issara-Amphorn for technical assistance.

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Resources: Cong Phi Dang.

Supervision: Asada Leelahavanichkul.

Writing – original draft: Cong Phi Dang.

Writing – review & editing: Asada Leelahavanichkul.

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