IL-27 enhances peripheral B cell glycolysis of rheumatoid arthritis patients via activating mTOR signaling

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Research Article

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Abstract

Objective Our previous study found that increased serum IL-27 could promote rheumatoid arthritis (RA) B cell dysfunction via activating mTOR signaling pathway. This study aimed to explore the effects of IL-27 on B cell metabolism and clarify the mechanisms via which IL-27 enhancing glycolysis to induce B cells hyperactivation.

Methods Peripheral CD19^+ B cells were purified from healthy controls (HC) and RA patients and then cultured with or without anti-CD40/CpG and glycolysis inhibitor 2-deoxy-D-glucose (2-DG) or mTOR inhibitor rapamycin. Furthermore, the isolated CD19^+ B cells were treated by HC serum or RA serum in the presence and absence of recombinant human IL-27 or anti-IL-27 neutralizing antibodies or 2-DG or rapamycin. The B cell glycolysis level, proliferation, differentiation, and inflammatory actions were detected by qPCR, flow cytometry or ELISA.

Results Compared to HC B cells, glycolysis was increased significantly in RA B cells and glycolysis inhibition downregulated the proliferation, differentiation, and inflammatory actions of RA B cells. RA serum and IL-27 promoted B cell glycolysis, which could be obviously rescued by anti-IL-27 antibodies or mTOR inhibitor rapamycin.

Conclusion Enhanced cellular glycolysis of RA B cells induced by IL-27 might contribute to B cells hyperactivation through activating the mTOR signaling pathway.

1. Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease characterized by an increase in circulating auto-antibodies, inflammatory cytokines and chemokines, and altered metabolism, which leads to chronic inflammation and irreversible joint or systemic organ damage [1]. Immunologically, the identified risk factors for the disease are disrupted immune cell activation and cytokine production. Immunotherapeutic strategies have improved clinical outcomes and increased our understanding of RA pathogenesis, but there is no cure as of yet [2]. Further pathogenetic discoveries are necessary for the future of RA therapeutics.

The autoreactive B cells play an important role by secreting auto-antibodies and pro-inflammatory cytokines in the pathogenesis of RA, which largely depends on multiple cytokines and the interaction with T cells [3, 4]. The ability of activated lymphocytes to engage in inflammation may be determined by metabolic reprogramming to glycolysis enhancement [5]. Increasing evidence have shown that B cell development, differentiation, and function require specific metabolic adaptations in response to a complex signaling network in different environments [6]. According to the reports, the mammalian target of rapamycin (mTOR) is a key kinase for glucose uptake and glycolysis, which is essential for B cell proliferation, differentiation, and biological function [7-9]. Inhibition of mTOR with rapamycin markedly inhibits B cell proliferation and antibody responses in mice and humans [10]. PD-1^+ B cells showed increased activation of mTOR and glycolytic capacity and expression of proinflammatory cytokines in
Activated B cells differentiated into plasmablasts to produce Ig and proinflammatory cytokines, which were accompanied by enhanced mTOR activation as well as glycolysis [12-14]. Inhibition of glycolysis ameliorate arthritis in adjuvant arthritis rats [15]. Therefore, studying B-cell metabolism might provide new insights into the pathogenesis of RA.

Interleukin (IL)-27 is a soluble cytokine of the IL-12 family and plays pleiotropic roles in regulating inflammatory responses by activating JAK/STAT and p38 MAPK signaling pathways via binding to IL-27 receptor (IL-27R) [16]. Circulating IL-27 is elevated in RA patients and can influence RA development by regulating synovial fibroblasts and various immune cell responses [16-18]. Our previous study has demonstrated that increased IL-27 could promote RA B cell dysfunction via activating the mTOR signaling pathway [19], which indicates that IL-27 signaling may induce RA B cell hyperactivation by controlling cellular metabolism. However, further researches still need to be done.

In the present study, we aim to analyze the glycolysis levels in peripheral B cells of RA patients and investigate the mechanisms via which IL-27 enhancing glycolysis to regulate B cell biological function. Our results will provide new insight into the understanding of aberrant B cell response contributing to RA pathogenesis.

2. Material And Methods

2.1 Patients and healthy controls

The blood samples in this study were collected from RA (n=52) patients in the rheumatology department of the Second Affiliated Hospital of Dalian Medical University. Patients with other autoimmune diseases and metabolic diseases were excluded. At the same time, physical examination subjects of matched gender and age were selected as the healthy controls (HC, n=45) in the physical examination Center of the hospital. Serum samples of RA patients and HC were stored at -80°C. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Dalian Medical University, and all participants signed informed consent. Clinical and laboratory characteristics of RA patients for study are listed in Table S1.

2.2 Sorting and stimulation of B cells

Peripheral blood mononuclear cells (PBMCs) were isolated with the Ficoll density-gradient centrifugation from RA patients and HC blood samples. In this study, the CD19⁺ B cells were purified from PBMCs (>95% purity) based on the manufacturer's instructions (Miltenyi, Germany). In order to study the impacts of human serum on B cells, CD19⁺ B cells (2.5*10⁵/well) from HC were cultured in RPMI 1640 medium supplemented with 2% fetal bovine serum (FBS), 2% HC serum, or 2% RA serum in the presence of 0.5 mg/ml anti-CD40 antibody (R&D, USA) and 0.1 mM CpG (Miltenyi, Germany) for 24 h. To explore the process of metabolic reprogramming, CD19⁺ B cells (2.5*10⁵/well) from HC or RA patients were stimulated with anti-CD40 antibody/CpG in the presence or absence of 50 ng/ml recombinant human IL-27 (Peprotech, USA) for 24 or 72 h in 48-well plates. In some experiments, 50 mg/ml anti-IL-27...
neutralizing antibodies (RD, USA), 1 mM 2-deoxy-d-glucose (2-DG, Solarbio, China), or 1 nM rapamycin (Solarbio, China) were added into culture system.

2.3 Flow cytometric analysis

Flow cytometric analysis was performed on CD19⁺ B cells. Briefly, cells were washed and suspended in 100 ml of PBS and stained with surface antigens for 30 minutes at 4°C. For intranuclear staining, cells were fixed and permeabilized with an intracellular fixation and permeabilization buffer set (eBioscience, USA) for 1 h followed by intranuclear staining for another 30 minutes at 4°C. The frequencies of staining-positive cells were detected by flow cytometry (Accuri C6, BD, USA). Fluorochrome conjugated anti-human antibodies were used as follows: APC-CD19 (Biolgend, USA), PerCp/Cy5.5-CD27 (Biolgend, USA), FITC-CD38 (Biolgend, USA), PE-HIF-1α (eBioscience, USA), PE-CD138 (Biolgend, USA), PE-CD86 (Biolgend, USA), PE-p-mTOR (eBioscience, USA), phospho-p70S6K and phospho-4E-BP1 (Cell Signaling Technology, USA). The second antibody anti-rabbit IgG (H+L) conjugated with APC and isotype-matched mouse IgG controls (eBioscience, USA).

2.4 Proliferation assays

CD19⁺ B cells were labeled with 2.5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE) (eBioscience, USA) in PBS for 10 minutes at room temperature. An excess of ice-cold RPMI 1640 medium with 10% FBS was added to the cells to quench the reaction and cells were washed extensively. CFSE-labeled cells (1*10⁵/well) were cultured according to above-mentioned methods. Following 5 days of culture, cells were collected and then stained with APC-CD19 antibody. B cell proliferation was determined by flow cytometry analysis of CFSE fluorescence intensity.

2.5 Glucose uptake measurement

Glucose uptake ability of PBMCs or CD19⁺ B cell treated as above-mentioned method were measured with fluorescent D-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG, Sigma, USA). Cells were incubated with 2-NBDG at a concentration of 50 mM for 30 minutes at room temperature. Then, cells were stained with APC-CD19 antibody for another 30 minutes. Cells were washed three times in PBS and acquired in a flow cytometry instrument, using the FITC channel to detect the signal from the fluorescent glucose uptake tracker.

2.6 RNA extraction and quantitative PCR (qPCR)

Total RNA was extracted from CD19⁺ B cells using Trizol reagent (Accurate Biotechnology Co., Ltd, China), and cDNA was transcribed using a SuperScript II RT kit (Applied Biosystems, China) according to manufacturers’ instructions. Expression levels of each gene were determined by qPCR using specific primers, and mRNA levels in each sample were normalized to the relative quantity of b-actin gene expression. The primers used in the study are listed in Table S2.
2.7 ELISA

CD19+ B cells were cultured accordingly, secreted IgG and IgM in the culture supernatants were quantitated by ELISA (SenBeiJia Bio, China) according to the instructions of the manufacturer. Meanwhile, the IL-6 and IL-10 in supernatants were also detected by ELISA kits (RD, USA).

2.8 Lactate assay

CD19+ B cells were cultured accordingly and the supernatants were collected. The serum of RA patients and HC as well as the culture supernatants were diluted properly for the measurement of lactate levels with a Lactate Assay Kit II (Abbkine Scientific, China) according to the protocol supplied by the manufacturer.

2.9 Statistical analysis

Data were summarized as means ± standard error of the mean (SEM). All statistical analyses were performed with the GraphPad Prism software (GraphPad, USA). All data within and between groups were compared using an independent samples t-test. A p-value<0.05 was considered significantly different.

3. Results

3.1 RA peripheral CD19+ B cells display enhanced glycolysis, which contributed to B cell hyperactivities

Metabolic reprogramming is crucial for the survival and specific functions of immune cells [20]. Our previous studies have shown altered peripheral B cell homeostasis and functions in RA patients [19]. Accordingly, in the present study, we will explore whether RA B cells have glycolysis changes, which induces aberrant RA B cells responses. CD19+ B cells from RA patients or HC were treated with or without anti-CD40/CpG and 2-DG for 24 h (Fig. 1a). The results indicated that RA CD19+ B cells had significantly increased expression of glycolysis-related molecules, including glucose transporter (Glut) 1, Glut3, hexokinase (HK) 2, HK3, lactate dehydrogenase (LDH), phosphate fructose kinase (PFK) 1/2 and pyruvate dehydrogenase kinase (PDK)-1 in comparison to HC B cells (Fig. 1b). RA B cells also showed higher glucose uptake capacity than HC B cells with or without anti-CD40/CpG stimulation (Fig. 1c). The levels of lactate in the B cell culture supernatant and plasma of RA patients were significantly higher than that of HC (Fig. 1d). Taken together, RA B cells display enhanced glycolysis compared to HC B cells.

To link glucose metabolism to RA B cell function, RA B cells were treated with glycolysis inhibitor 2-DG, a glucose analog, to block glycolysis by inhibiting HK2 activity. We found that 2-DG treatment obviously inhibited B cell proliferation and activation (Fig. 1e). More importantly, the frequencies of CD19+CD138+ plasma cells (Fig. 1f) and supernatant levels of IgM, IgG and cytokines IL-6 and IL-10 decreased significantly in the 2-DG-treated B cell culture system (Fig. 1g). These findings demonstrate that enhanced glycolysis may promote RA B cell hyperactivities, which can be restored by glycolysis inhibition.
3.2 IL-27 play a key role in RA inflammatory setting which can contribute to enhanced glycolysis of B cells

Our previous studies have showed that IL-27 could induce the imbalance of B cell subsets and B cell dysfunction via the activation of mTOR signal. Base on the fact of mTOR regulating glycolysis, in this study, we wonder whether IL-27 can promote the glycolysis of RA peripheral B cells. As expected, we found that IL-27 significantly promoted the expression of glycolysis-related molecules and glucose uptake capacity, as well as lactate production of B cells in vitro culture. And anti-IL-27 antibody treatment inhibited IL-27-induced enhanced B cell glycolysis (Fig. 2a-c).

Given that the activities of immune cells are related to the microenvironment, whether IL-27 is a key cytokine in RA inflammatory setting which can contribute to metabolic disorders of B cells. We cultured HC B cells in a medium containing RA or HC serum. The results showed that in comparison to HC serum-treated B cells, RA serum-treated B cells showed significantly increased expression of glycolysis-related molecules, enhanced glucose uptake capacity, and up-regulated lactate production in the supernatants (Fig. 3a-c). More importantly, in the presence of anti-IL-27 antibodies, the expression of glycolysis-related rate-limiting enzymes (Fig. 3d), the glucose uptake capacity (Fig. 3e) and supernatant lactate levels (Fig. 3f) were all significantly decreased in RA serum-treated B cells. The above results indicate that IL-27 play a vital role in RA inflammatory setting which can contribute to enhanced glycolysis of B cells.

3.3 IL-27 enhance RA B cell glycolysis via activating mTOR signaling

To explore the relationship between mTOR signaling and glycolysis of RA B cells, we detected gene expressions of glycolysis-related molecules in RA B cells treated with or without mTOR specific inhibitor rapamycin. Our data showed that compared to untreated RA B cells, rapamycin-treated RA B cells exhibited remarkably reduced expression of glycolysis-related molecules (Fig. 4a), decreased glucose uptake capacity and lactate production (Fig. 4b-c). As a key transcription factor, hypoxia-inducible factor (HIF)-1α plays a crucial role in regulating cellular metabolism by inducing the expression of glycolytic enzymes. We found that rapamycin significantly reduced the level of HIF-1α in RA B cells (Fig. 4d). Taken together, mTOR signaling plays a critical role in enhancing RA B cell glycolysis.

We further explored whether IL-27 enhanced glycolysis by activating the mTOR signaling pathway, which ultimately leads to B cell dysfunction. mTOR inhibitor rapamycin was added to IL-27-stimulated B cells culture system. The results showed that the genes expression of glycolysis-related molecules (Fig. 4e), glucose uptake capacity (Fig. 4f) and lactate secretion (Fig. 4g), and HIF-1α expression (Fig. 4h) in B cells induced by IL-27 were significantly inhibited in the presence of rapamycin. These results suggest that IL-27 may enhance RA B cell glycolysis via activating the mTOR signaling pathway.

4. Discussion

In the present study, we found that B cell glycolysis of RA patients was significantly increased, which results in B cell dysfunction. In addition, IL-27 contributed to B cell glycolysis enhancement, which could
be rescued by the blockade of IL-27 or mTOR signaling pathway. All these results indicated that RA B cell dysfunction might be linked to the glycolysis enhancement induced by IL-27 via mTOR signaling pathway.

Studies have demonstrated that distinct immune cells can utilize different basic metabolic pathways to match their needs for specific functions depending on the cytokine environment [21]. Our previous study found that IL-27 in RA serum induced mTOR signaling activation in B cells [19]. mTOR is a master regulator of B cell development, survival, and function by promoting the shift from primarily oxidative metabolism to glycolytic metabolism [9, 22, 23]. Torigoe M and collages found that the enhanced mTOR1 activation and glycolysis is crucial for the differentiation of unswitched memory B cells into plasmablasts and the production of Ig and cytokines [12]. Rapamycin or 2-DG can inhibit the proliferation, differentiation into plasma/plasmablasts and production of IgG and IgM of B cells from Sjögren's syndrome patients [22]. Consistent with these findings, our present study found that the enhanced glycolysis contributed B cells hyperactivation. However, delicate metabolic reprogramming of different B cell subsets in the chronic inflammatory environment of RA should be investigated in the future.

Although it has been known that glycolysis is crucial for B cell expansion and antibody production, the molecular mechanisms underlying metabolic reprogramming to modulate B cell fate and function remain poorly defined. Studies reported that the specific immune responses may be linked to the metabolites-derived post-translational modification and specific enzyme inhibition [24, 25]. Lactate, the final product of glycolysis, has been shown to be proinflammatory by promoting Th17 cell differentiation in autoimmune disorders [25, 26]. A variety of metabolic cofactors, such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD), can be transcriptional regulators via post-translational modification of transcription factors [27]. Various metabolic enzymes, such as GAPDH and lactic dehydrogenase (LDH), have been shown to play non-canonical roles in regulating gene expression, inflammatory cytokine production, cell cycle progression, DNA damage repair, and cell apoptosis [28, 29]. Our present results showed that the increased glycolysis induced RA B cells to differentiate into antibody-producing cells and secrete high levels of IgM, IgG, and IL-6. Whereas, the underlying molecular mechanisms need further research.

IL-27 plays diverse regulatory roles in different immune disorders [30]. Studies indicated that IL-27R deficiency delayed the development and reduced the severity of arthritis in mice by decreasing IFN-γ secretion and the increased IL-27 can contribute to RA development by activating synovial fibroblast to express high levels of adhesion molecules and chemokines [18, 31]. On the contrary, exogenous IL-27 treatment significantly attenuated collagen-induced arthritis by decreasing serum IL-6 and IgG2a levels and blocking IFN-γ and IL-17 production of CD4+ T cells [32]. In addition, studies reported that IL-27 signaling could limit Th1 cell-mediated host tissue damage by inhibiting glycolysis [33]. And IL-27R deficiency could lead to severe liver pathology by exacerbating Th1-mediated immune responses via increasing glycolytic metabolism in CD4+ T cells during infection [34, 35]. While, the other study found that loss of IL-27 signaling resulted in decreased mitochondrial function, with no corresponding increase
in glycolysis of T cells during vaccination [36]. In this study, we found that the serum IL-27 enhanced the glycolysis of B cells via activating mTOR signaling pathway, which led to B cell hyperactivities in RA patients. All these data indicate that it is too simple to define a clear-cut role for a specific cytokine underlying distinct inflammatory situations. The exact role of IL-27 in B cell metabolism and biological effects during the evolution of RA requires more investigation.

**Declarations**

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**Author Contributions**

Y. T and X. L designed the experiments and revised the manuscript; J. Q and J. L performed the experiments and analyzed the data; X. Z and H. H contributed to reagents, materials, and analysis tools. All the authors have read, reviewed, and edited the manuscript and agreed for submission to this journal.

**Conflict of interest statement**

The authors report no conflicts of interest in this work.

**References**


Figures
Figure 1

Enhanced glycolysis contributed to RA B cell hyperactivities. (a) CD19⁺ B cells were sorted with magnetic beads from PBMCs of RA patients and HC and treated with or without anti-CD40/CpG. (b) Genes expression of the key molecules in glycolysis were quantified by qPCR. Flow cytometry was used to detect Glut1, HK2 and LDH levels in B cells of RA patients and HC. (c) Glucose uptake capacity of CD19⁺ B cells from RA patients and HC were determined by flow cytometry. (d) Lactate levels in RA and HC serum were measured with the Lactate Assay Kit II (n=20). (e-f) RA CD19⁺ B cells were treated with DMSO or 2-DG, and CFSE-labelled B cells were analyzed by flow cytometry after 5 days. CD86 expression and
CD19⁺CD138⁺ plasma cells were quantified by flow cytometry after 72 h. (g) The production of IgM, IgG, IL-6 and IL-10 in the supernatant was measured by ELISA after 72 h. n=5, *p<0.05, **p<0.01, ***p<0.001.

Figure 2

IL-27 contributes to enhanced glycolysis of RA CD19⁺ B cells. RA CD19⁺ B cells were sorted with magnetic beads and stimulated by anti-CD40/CpG and rIL-27 with or without anti-IL-27 antibody. (a) Genes expression of the key molecules in glycolysis were quantified by qPCR after 24 h. (b) The capacity of glucose uptake was determined by flow cytometry. (c) The lactate level in the supernatants was detected with the Lactate Assay Kit II after 72 h. n=5, *p<0.05, **p<0.01, ***p<0.001.
Figure 3

Blocking IL-27 can inhibit RA serum-induced B cell glycolysis enhancement. CD19+ B cells were sorted with magnetic beads from PBMCs of HC, which were cultured in RPMI 1640 supplemented with 2% FBS, 2% RA or HC serum, and anti-CD40/CpG with or without anti-IL-27 neutralizing antibody (50 mg/ml). (a, d) Genes and protein expression of the key molecules in glycolysis were quantified by qPCR and flow cytometry. (b, e) The capacity of glucose uptake was determined by flow cytometry. (c, f) The lactate level in the supernatants was measured with the Lactate Assay Kit II. n=5, *p<0.05, **p<0.01, ***p<0.001.
Figure 4

Involvement of mTOR signaling pathway in IL-27-induced glycolysis enhancement of RA B cells. CD19+ B cells were sorted with magnetic beads from PBMCs of RA patients, which were stimulated by anti-CD40/CpG with or without rapamycin or rIL-27. (a, e) Genes expression of the key molecules in glycolysis were quantified by qPCR and flow cytometry after 24 h. (b, f) The glucose uptake capacity was determined by flow cytometry. (c, g) The lactate level in the supernatants was determined with the Lactate Assay Kit II. (d, h) HIF-1α expression was quantified by flow cytometry after 72 h. n=5, *p<0.05, **p<0.01, ***p<0.001.

Supplementary Files

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