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Original Article

EGFR promotes the apoptosis of CD4$^+$ T lymphocytes through TBK1/Glut1 induced Warburg effect in sepsis

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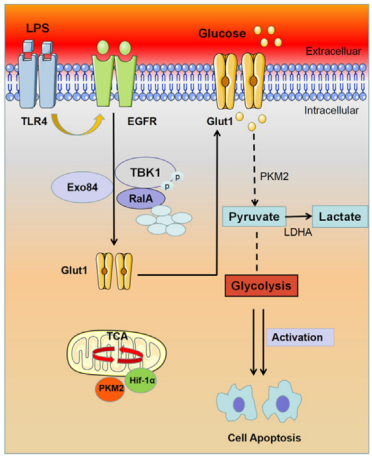
HIGHLIGHTS

- EGFR helps Glut1 translocate to CD4$^+$ T cells surface via the TBK1/Exo84/RalA in response to LPS.
- Glucose is transported by EGFR/Glut1 signal pathway and metabolized to lactate via the Warburg effect to supply energy for CD4$^+$ T cells activation.
- EGFR promotes activated CD4$^+$ T cells die via apoptosis which leading to cell depletion in sepsis.
- EGFR inhibitors effectively alleviated the decrease in total CD4$^+$ and naïve CD4$^+$ T cells in septic mice.
- The surface expression of EGFR and Glut1 and activation of human blood CD4$^+$ T cells increased in septic patients.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Sepsis-induced apoptosis leads to lymphopenia including the decrease of CD4$^+$ T cells thus favoring immunosuppression.
Objectives: Although epidermal growth factor receptor (EGFR) inhibitors significantly improve the survival rate of septic mice, the effect of EGFR on the function and metabolism of CD4$^+$ T cells in sepsis remained unknown.
Methods: CD4$^+$T cells from septic mice and patients were assessed for apoptosis, activation, Warburg metabolism and glucose transporter 1 (Glut1) expression with or without the interference of EGFR activation.
Results: EGFR facilitates CD4$^+$T cell activation and apoptosis through Glut1, which is a key enzyme that controls glycolysis in T cells. EGFR, TANK binding kinase 1 (TBK1) and Glut1 form a complex to facilitate Glut1 transportation from cytoplasm to cell surface. Both the levels of membrane expression of EGFR and Glut1 and the activation levels of CD4$^+$ T cells were significantly higher in patients with sepsis as compared with healthy subjects.

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Conclusion: Our data demonstrated that through its downstream TBK1/Exo84/RalA protein system, EGFR regulates Glut1 transporting to the cell surface, which is a key step for inducing the Warburg effect and the subsequent cellular activation and apoptosis of $CD4^+$ T lymphocytes and may eventually affect the immune functional status, causing immune cell exhaustion in sepsis.

Introduction

Sepsis can result in life-threatening symptoms that arise when the body's immune response to an infection leads to organ damage and even multiple organ dysfunction syndrome (MODS) [1]. The recent discoveries of the role of apoptosis and metabolism in T cell biology has contributed greatly to the understanding of immune cell regulation [2–4]. During sepsis, changes in metabolic pathways are critical to the activation and subsequent apoptosis of T cells [5,6]. Increasing evidence shows that inhibiting lymphocyte apoptosis can improve the survival rate of septic patients [7]. Therefore, preserving the number and function of $CD4^+$ T lymphocytes is important for the treatment of sepsis.

Patients with sepsis always have obvious glucose metabolism disorders, which mainly manifest as the cells' inability to oxidize glucose normally via the tricarboxylic acid cycle (TCA) and a switch to aerobic glycolysis (Warburg effect), which increases lactic acid levels. This kind of glucose metabolism disorder can directly affect the functional status of immune cells including $CD4^+$ T cells [8]. Metabolic changes of T cells directly affect their ability to proliferate, activate, differentiate and the ability to undergo apoptosis [9]. Usually, the TCA cycle and subsequent oxidative phosphorylation pathway provide the energy demand for T cells. Once activated, glucose transporter 1 (Glut1), a component of the facilitative glucose transporter family, transfers to the cell membrane, capturing glucose to transform it into lactate, even when abundant oxygen is available to conduct oxidative phosphorylation. Therefore, glucose uptake provides a pivotal metabolic control point for the activation of $CD4^+$ T cells through Glut1 [10]. The increased surface expression of Glut1 promotes the transport of a large amount of glucose into cytoplasm to supply sufficient energy for activated $CD4^+$ T cells through aerobic glycolysis [11]. Therefore, the transport of Glut1 from the cytoplasm onto the cell surface is a key step for the metabolic reprogramming and activation of $CD4^+$ T lymphocytes.

In 2015, we and other researchers reported that epidermal growth factor receptor (EGFR) inhibitors can significantly improve the survival rate of septic mice, indicating that EGFR plays a critical regulatory role in sepsis [12–14]. We further proved that EGFR phosphorylation leads to the activation of its substrates EPS8 and GRB2 which work together to coordinate the equilibrium of GTP and GDP-bound forms of Rab5a to secure the process of TLR4 internalization in response to lipopolysaccharide (LPS) in macrophages [15]. Although patients who survive severe sepsis often display the exhaustion of immune cells (especially $CD4^+$ T lymphocytes) and immune non-response, so far, few studies have discussed the role of EGFR in immune response, especially in the function of $CD4^+$ T cells during sepsis.

Here, we demonstrated that under LPS treatment, EGFR helps Glut1 to translocate onto the cell surface via the TBK1/Exo84/RalA signaling pathway. Then, glucose is captured by Glut1 and converted to lactate through the Warburg effect to meet the energy demand for the activation of $CD4^+$ T cells. Over activated $CD4^+$ T lymphocytes have a shorter life span and die via apoptosis, leading to $CD4^+$ T cell depletion in sepsis. Therefore, EGFR inhibitor erlotinib can effectively attenuate $CD4^+$ T lymphocyte depletion through down-regulating Warburg effect during sepsis. Our study therefore exposes a previously unidentified role of EGFR in the metabolic changes and activation of $CD4^+$ T lymphocytes during sepsis.

Materials and methods

Mice

Male 6- to 8-week-old C57BL/6 mice were purchased from Jiangsu Jicui Yaokang Bio-Tech Co., Ltd. (license number: SCXK (Su)2018-0008). The mice were housed in experimental Animal Center (SPF grade) of Guangdong Medical University under standardized conditions with a 12-hour light/dark cycle and free access to food and water. All animal experimental protocols were reviewed and approved by the Ethics Committee of Guangdong Medical University.

Murine splenic lymphocyte isolation

Spleens from C57BL/6 WT mice were harvested, ground and suspended in RPMI-1640 medium. Splenocyte suspensions were added with separation liquid (TBD Science, China) and centrifuged for 30 min, and the liquid was separated into four layers (from top to bottom: the diluent layer, ring-shaped milky white lymphocyte layer, transparent separating liquid layer, red blood cell layer). Erythrocytes were lysed with 2–3 mL of 1 × ACK lysis buffer for 3–5 min and lymphocytes were aquaired, subsequent experiments were performed.

$CD4^+$ T lymphocyte isolation and culture

$CD4^+$ T lymphocytes were negatively selected from splenic lymphocytes with EasySep™ Mouse $CD4^+$ T Cell Isolation Kit (Cat# 19852, Stemcell). 1×10^8 splenic lymphocytes were added 50 μL/mL of Rat Serum and Isolation Cocktail that incubated at RT for 10 min.. The cells were mixed and incubated with vortexed RapidSpheres™. The suspensions were mixed by gently pipetting up and down 2–3 times, the tube (without lid) was placed into the magnet and incubated at RT for 2.5 min. Picked up the magnet, and in one continuous motion inverted the magnet and tube, poured the enriched cell suspension into a new tube. Isolated $CD4^+$ T cells were ready and cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum(FBS) under standard conditions, and follow-up experiments were performed. We identified APC- CD4 purity by flow cytometry as shown in Supplementary Fig. S1a.

Flow cytometry analysis

A total of 1×10^6 lymphocytes were acquired and collected from mice spleen, and then 0.5 μg anti-mouse CD16/32 monoclonal antibody (Biolegend, USA) and 5 μL rat serum were used to block the Fc receptor for 10 min. For measuring the number of total $CD4^+$ T cell and naïve $CD4^+$ T cell, splenocytes were incubated with 1 μg CD3-APC-Cy7 (Biolegend), CD4-APC (eBioscience, Thermo Fisher Scientific, USA), TCRβ-PE and CD62L-FITC (eBio-

science, Thermo) for 30 min. For detecting cell activation, cells were stained with 1 μg CD25-PE and CD69-FITC (eBioscience, Thermo). For evaluating cell surface protein expression, EGFR (Thermo)-PE (eBioscience) and Glut1 (Proteintech, China) -FITC (eBioscience) were incubated for 30 min. To measure cell death, splenic cells were stained with PI and Annexin V (BD Biosciences, San Jose, CA, USA) for 15 min at room temperature. Cells were analyzed by FACSCanto II cytometer (BD Biosciences, San Jose, CA, USA). All data were analyzed by Flowjo10 software (Tree Star, Ashland, OR, USA).

Immunohistochemistry assay

For mouse spleen, heart, liver and lung tissue, formalin-fixed sections which were fixed with 4% paraformaldehyde were deparaffinized in Xylene, and endogenous peroxidase was blocked after antigen retrieval; and the specimens were blocked and stained with 1:500 CD4 and F4/80 antibodies (ZCi Bio, Shanghai, China) and secondary antibodies (1:200) sequentially. The sections were stained with 3-Diaminobenzidine 4-HCl (DAB) chromogenic solution and counterstained with Harris hematoxylin. The images were acquired and analyzed by microscopic examination. Further details are provided in the supplemental information.

Immunoblotting assay of $CD4^+$ T cells

Purified and cultured $CD4^+$ T cells were lysed for radioimmunoprecipitation assay (RIPA). After measuring the protein concentration, the protein samples were mixed with 1 × loading buffer and boiled. The protein samples were added onto 6%-12% gradient gels (Bio-Rad) and subjected to electrophoretic separation. The proteins were then transferred to PolyScreen PVDF Hybridization Transfer Membranes. The membranes were incubated with primary and secondary antibodies. We used luminous fluid and a Tanon 5200 imaging system to acquire and export images. The images were processed with ImageJ (autocontrast function).

Immunoprecipitation and immunoblotting

$CD4^+$ T cells were suspended in IP lysis buffer (Thermo, USA) with continuous mixing at 4℃. After centrifugation, 500 μL supernatant (500 μg protein) was precleared for 1 h with 20 μL of a 50% slurry of Protein A/G magnetic beads (Merck Millipore) at 4 °C. We used a magnetic stand to capture the beads and incubated the supernatants with 20 μL of antibodies against mouse EGFR at 4 °C overnight. Fifty microliters of Protein A/G magnetic beads were added to the solution and incubated for an additional hour. Thereafter, the beads were collected and washed 3 times with 1 mL PBS, and proteins were subjected to SDS-PAGE (6–15% gel) and then transferred to Immobilon-P membranes for western blotting.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from $CD4^+$ T cells using TRIzol RNA isolation reagent (Invitrogen, USA). Following the protocols, RNA (2.5 μg) was synthesized to DNA through a RevertAid First Strand cDNA Synthesis Kit and 1 × Maxima SYBR Green/ROX quantitative PCR Master Mix (TAKARA, Japan). RT-PCR was carried out on an ABI 7500 System (Applied Biosystems,USA). The fold changes in mRNA expression were calculated according to $2 - \Delta\Delta Ct$ equation which illustrated the difference in the number of between the target gene and internal control (β-actin). The gene-specific primers were listed as Supplementary information.

Immunofluorescence and proximity ligation assay

For immunofluorescence staining, $CD4^+$ T cells (1×10^4 cells) were collected in 5-mL centrifuge tubes, and fixed in 4% paraformaldehyde, followed by blocked with 5% BSA for 30 min. Then, the primary antibodies EGFR, Glut1, TBK1, Exo84 and RalA (1:50) were prepared with 5% BSA dissolved in 0.1% Triton-100, permeabilized and incubated with cells at 4 °C overnight. The cells were washded three times with PBS and incubated with fluorescent Fluor 488 and Alexa Fluor 647 labeled secondary antibodies (1:1000) at RT for 1 h in the dark.. The cells were washed and follow by counterstained with DAPI (1:1000), washed and then resuspended with 5 μL anti-fluorescence quencher; the mixture was added dropwise to the middle of clean glasses slide, covered with cover glass, sealed with resin at the edge and observed under a fluorescence microscope. Proximity Ligation Assay (PLA) was performed according to the manufacturer's protocol using the Duolink Detection Kit (Cambridge BioScience Ltd) [16,17]. Immunofluorescence staining of EGFR, Gut1 and TBK1 (same antibodies as above, all 1:50) for the Duolink was carried out following the above-described protocol for immunofluorescence detection. Probe incubation, ligation and amplification reaction were carried out according to the manufacturer instructions. Cells were examined with a confocal microscope (objective × 60, OLYMPUS).

TUNEL assay

1×10^4 $CD4^+$ T cells were collected and fixed with 4% paraformaldehyde for 10 min. To prevent the cells from clumping, they were fixed with slowly shaking on a side-swing shaker. Then, they were washed with PBS, followed by incubation with PBS containing 0.1% Triton X-100 for 5 min. The One Step TUNEL Apoptosis Assay Kit (BeyotimeChina) user's manual was followed to prepare an appropriate amount of 50 μL TUNEL test solution per tube. The samples were incubated with the solution for 1 h in the dark, washed and resuspended. Cells were observed under a microscope with florescent light after smearing (green fluorescence).

Lactate, pyruvic acid (PA) and fructose-1,6-diphosphate (FDP) content assays

The cells (1×10^4 cells) were collected after centrifugation, and the corresponding solutions were added in sequence according to the sample addition table in the Solarbio instructions. Lactate accumulation was assessed using a commercially obtained lactate estimation kit (Solarbio, China) according to the protocol. The cell was lysed with the extract, and the cells were disrupted by ultrasound in an ice bath, and the supernatant was collected after centrifugation at 12000 rpm for 10 min at 4 °C for assessing pyruvic acid and fructose-1,6-diphosphate content. The OD value was measured with a spectrophotometer after adding reagents according to the Solarbio kits user's instructions. The lactate, pyruvic acid and fructose-1,6-diphosphate content in Ctrl-, LPS-, PD-treated cells were pooled from three independent experiments as presented in the respective figures.

Patient study

Septic patients were those enrolled to the Affiliated Hospital of Guangdong Medical University ICU from January 2021 to June 2021 (in line with Sepsis 3.0) and healthy donors of the Guangdong Medical University physical examination center were recruited. The inclusion criteria: patients were over 18-year-old and legal representatives were able to sign ethical informed consent forms. Patients of both sexes were included. See supplementary Table 2 for detailed information.

Preparation of human peripheral blood mononuclear cells and flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation using Ficoll-PaqueTM Plus (GE Healthcare, Uppsala, Sweden) density gradient. Erythrocytes were then lysed using ACK lysis buffer. 1×10^6 cells were counted and resuspended in PBS. Then, 0.5–1 μg APC-H7-CD3, APC-CD4 and CD25-PE (BD Biosciences), EGFR (Thermo) and Glut1(Proteintech) stains were added. Both primary and secondary antibodies were stained at 4 °C for 30 min in the dark. Finally, cells were analyzed by a flow cytometer.

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Animal Ethics Committee of Guangdong Medical University (Approval no. GDY2002072). In case of human patient consent forms, all procedures were approved by Affiliated Hospital of Guangdong Medical University Ethics Committee. (ClinicalTrials. gov identifier ChiCTR2100042571).

Statistical analysis

All results are presented as the mean ± SD. Differences between two groups were analyzed by Student's *t* test. For multigroup comparisons, one-way ANOVA was performed. Post hoc analysis was made by using Tukey's post-hoc test following ANOVA. Statistical analysis was performed using GraphPad Prism 8.3 software (GraphPad software, CA, USA). $P < 0.05$ was considered as statistical significance.

Results

EGFR inhibitor erlotinib alleviates the decrease in total CD4$^+$ and naïve CD4$^+$ T lymphocytes in septic mice

Compared with control group, the number of splenic CD4$^+$ T lymphocytes was significantly reduced at 6 h, 12 h and 24 h of LPS treatment. Furthermore, we found that the total and naïve CD4$^+$ T cells decreased most significantly at 12 h after LPS treatment, while there was no obvious difference between 12 h and 24 h, as shown in Supplementary Fig. S2a and S2b. Interestingly, EGFR inhibitor erlotinib pretreatment effectively alleviated the decrease in both the total and naïve CD4$^+$ T cells at 12 h after LPS treatment (Supplementary Fig. S2c and S2d).

EGFR inhibitors attenuates the LPS-induced apoptosis of CD4$^+$ T lymphocytes

The decrease of lymphocytes due to apoptosis is thought as one of significant pathogenic changes in the development of immunosuppression observed during sepsis progression [18–20]. As shown in Fig. 1a, CD4 positive cells decreased obviously in LPS induced septic mice models, and this effect was partly inhibited by erlotinib pretreatment. Its negative controls were shown in Supplementary Fig. S1b and S1c. We also assessed the apoptosis rate of CD4$^+$ T cells, and the levels of Bcl-2 and cleaved caspase-3 protein expression. Both in vivo and vitro, LPS induced cell apoptosis could be partially attenuated by erlotinib or PD168393 pretreatment (Fig. 1b and 1d). Moreover, compared with LPS group, PD168393 or erlotinib obviously decreased the expression of the proapoptotic cleaved caspase-3 protein and increased the expression of the anti-apoptotic protein Bcl-2 (Fig. 1c and 1e). In addition, these results were also verified by propidium iodide (PI) and TUNEL fluorescence staining (Fig. 1f and 1 g). All these results indicated that both in vitro and in vivo, LPS-induced apoptosis could be alleviated by the EGFR selective inhibitors PD168393 or erlotinib.

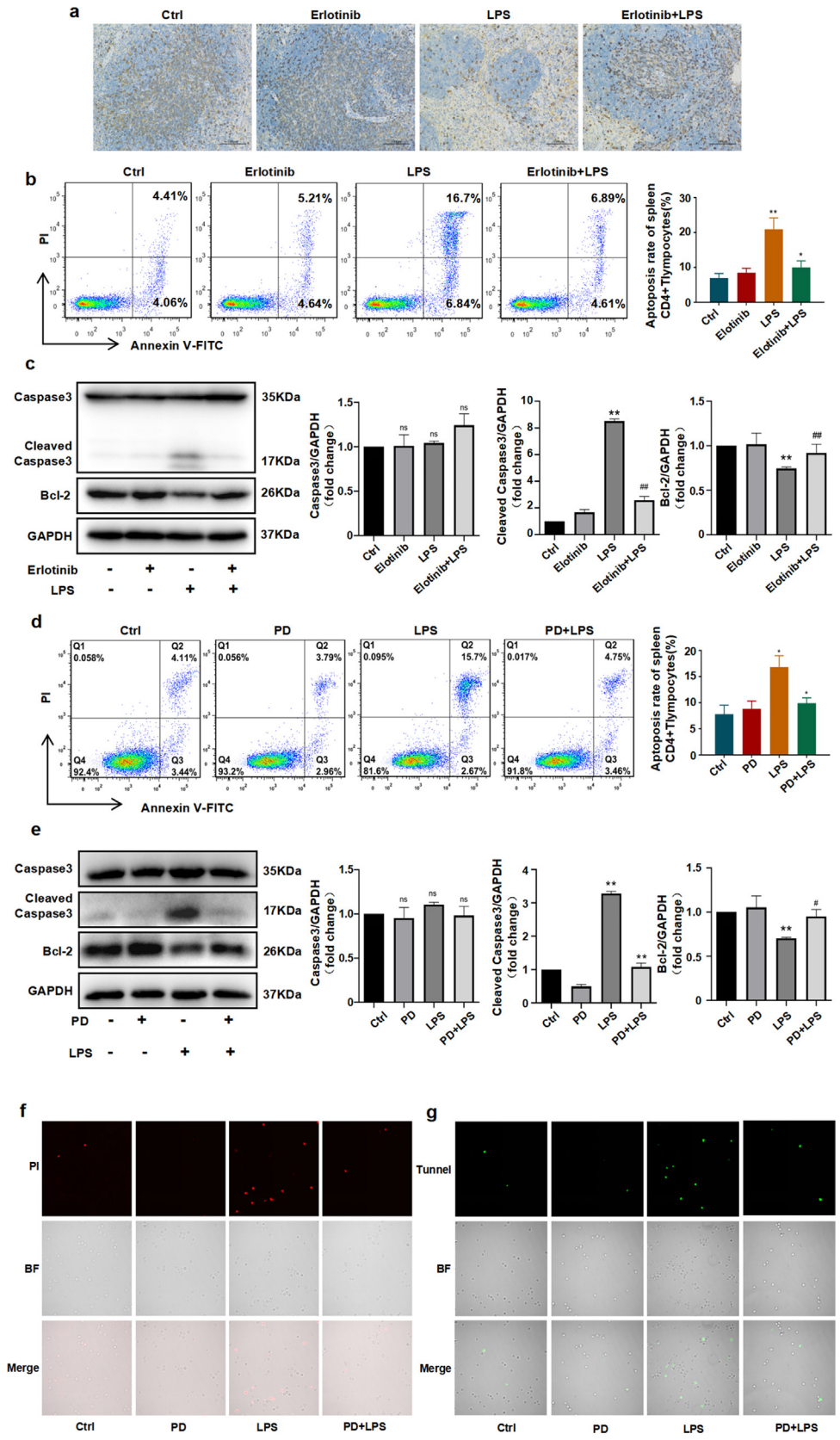
EGFR inhibitors reduce CD4$^+$ T lymphocytes activation in response to LPS

The excessively released inflammatory factors and their overactivation are the main reasons for the subsequent apoptosis of CD4$^+$ T lymphocytes in sepsis [21,22]. To investigate whether EGFR is participated in the regulation of CD4$^+$ T cell activation in sepsis, wild-type C57BL/6 mice were treated with LPS for 3, 6, 9, 12 and 24 h. The early response is represented by the up-regulation of the activation markers CD25 and CD69. We found that the CD4$^+$ T cell activation level increased significantly at 9 h, while it decreased gradually at 12 h and 24 h after LPS treatment (Fig. 2a and 2b). Interestingly, erlotinib obviously inhibited CD4$^+$ T cells activation compared with LPS group at 9 h after LPS treatment (Fig. 2c and 2d). To verify these results in vitro, lymphocytes were pretreated with PD168393 for 30 min before LPS treatment. As expected, PD168393 significantly inhibited the activation of CD25 and CD69 induced by LPS (Fig. 2e and 2f). All the above results indicated that EGFR activity is required for the activation of CD4$^+$ T cells in sepsis.

EGFR inhibitor suppresses the LPS induced Warburg effect in CD4$^+$ T lymphoytes

Glut1 is a key signaling molecule for the activation and metabolism of CD4$^+$ T lymphocytes [23]. PKM2 is associated with hypoxia-inducible factor 1α (HIF1α) and it activates transcription enzymes that is necessary for aerobic glycolysis in immune cells [24,25]. To verify the effect of EGFR inhibitors on glycolysis in CD4$^+$ T cells, we measured their metabolism-related enzymes and extracellular lactate production, which were primarily attributed to glycolysis. The WT C57BL/6 mice were pretreated with erlotinib for 2 h before initiating LPS treatment for 12 h and then splenic CD4$^+$ T cells were isolated. As shown in Supplementary Fig. S3a-S3d, LPS induced the expression of glycolytic pathway-related enzymes and the phosphorylation of the PKM2 and lactate dehydrogenase A (LDHA)

Fig. 1. The effect of EGFR inhibitors on apoptosis in CD4$^+$ T lymphocytes in sepsis. (a) Immunohistochemical staining for CD4$^+$ T cells. Spleens from septic mice or nonseptic mice were stained for CD4; DAB was used to stain the CD4$^+$ T cells (brown), and hematoxylin counterstain (blue) was used for background staining (scale bar, 100 μm). n = 3 per group. (b and d) C57BL/6 wt mice were treated with LPS (15 mg/kg, i.p.) for 12 h, while some groups were pretreated with erlotinib (100 mg/kg, gavage) 2 h prior to LPS treatment (i.p.). Flow cytometry detected the apoptosis rate of CD4$^+$ T cells in vivo (b). Splenic lymphocytes isolated from WT mice were treated with LPS (1 μg/mL, 12 h) in the presence or absence of PD168393(PD) pretreatment (10 μM) for 30 min followed by flow cytometry analysis of cell apoptosis in vitro (d) with quantification bar charts. n = 3 per group. (c) Representative western blot of cleaved caspase-3 and Bcl-2 protein expression level in CD4$^+$ T cells in mice treated with LPS (15 mg/kg.i.p.) and erlotinib or left untreated in vivo. n = 3 per group. (e) Western blot analyzed the above proteins in CD4$^+$ T cells treated with LPS (1 μg/mL) for 12 h with or without PD (10 μM) pretreatment for 30 min and the quantification bar charts. (f and g) PI staining (f), TUNEL staining (g) of cells under bright field observation under a fluorescence microscope. n = 3 per group. Data are expressed as the mean ± SEM and were analyzed by one-way ANOVA with Dunnett's multiple comparison post-test.. $^*P < 0.05$, compared with the control group; $^\#P < 0.05$, compared with the time-matched LPS alone group.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4.06%
5.21%
4.64%
16.7%
6.84%
6.89%
4.61%
Aptoposis rate of spleen CD4+Tlympocytes(%)
c
Caspase3
Cleaved Caspase3
Bcl-2
GAPDH
35KDa
17KDa
26KDa
37KDa
Erlotinib
LPS
Caspase3/GAPDH (fold change)
Cleaved Caspase3/GAPDH (fold change)
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d
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PI
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Q1 0.058%
Q2 4.11%
Q4 92.4%
Q3 3.44%
Q1 0.056%
Q2 3.79%
Q4 93.2%
Q3 2.96%
Q1 0.095%
Q2 15.7%
Q4 81.6%
Q3 2.67%
Q1 0.017%
Q2 4.75%
Q4 91.8%
Q3 3.46%
Aptoposis rate of spleen CD4+Tlympocytes(%)
e
Caspase3
Cleaved Caspase3
Bcl-2
GAPDH
35KDa
17KDa
26KDa
37KDa
PD
LPS
Caspase3/GAPDH (fold change)
Cleaved Caspase3/GAPDH (fold change)
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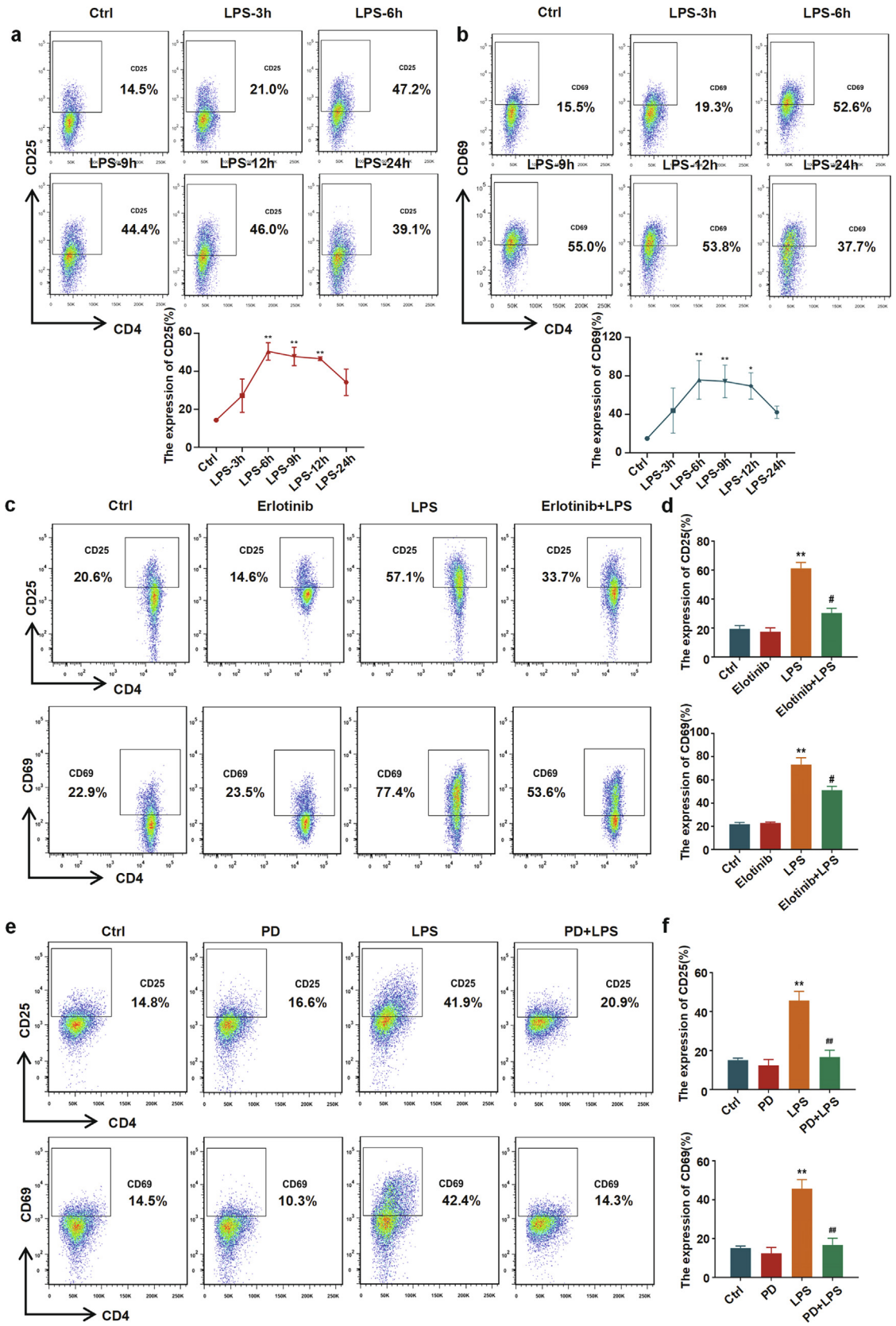


Fig. 2. EGFR inhibitors have an effect on CD4$^+$ T lymphocytes activation in sepsis. (a and b)WT (C57BL/6) mice were treated with LPS (15 mg/kg, i.p.) for 3, 6, 9, 12 and 24 h. (c) Some groups were gavaged with erlotinib (100 mg/kg) 2 h prior to LPS treatment. Single-cell suspensions of lymphocytes from the spleen were collected and identified with CD25 and CD69. The levels of the cell activation markers CD25 and CD69 in CD4$^+$ T cells were detected by flow cytometry. (d) The following are quantitative diagrams. n = 3 per group. (e) Spleen lymphocytes isolated from wild-type mice were exposed with LPS (1 μg/mL, 9 h) with or without PD (10 μM) pretreatment for 30 min followed by flow cytometry analysis of cell activation markers CD25 and CD69. n = 3 per group. (f) These are quantitative diagrams. Data are expressed as the mean ± SEM and were analyzed by one-way ANOVA with Dunnett's multiple comparison post-test.$^*P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the time-matched LPS alone group.

proteins, and these effects were suppressed by erlotinib. In addition, the enhanced $CD4^+$ T cells glycolysis in sepsis was verified by the increased extracellular lactate production, pyruvic acid (PA) and fructose-1,6-diphosphate (FDP) content, which was significantly inhibited by PD168393 pretreatment (Supplementary Fig. S3e-S3g). Furthermore, $CD4^+$ T cells cultured in vitro were pretreated with PD168393 for 30 min before LPS treatment. The increased mRNA levels of glycolysis pathway key enzymes (*Glut1*, *Pkm2*, *Pdk1, Pfpkb3, Ldha*) induced by LPS was also partly inhibited by PD168393 in $CD4^+$ T cells (Supplementary Fig. S3h). Similarly, 2-deoxyglucose (2DG) treatment decreased the expression levels of enzymes related to glycolysis after LPS treatment (Supplementary Fig. S3i). Thus, our results suggested that EGFR inhibitor PD168393 weakened LPS-induced upregulation of glycolysis-related factors in $CD4^+$ T cells.

EGFR and Glut1 coordinated glycolysis and participated in the activation and apoptosis of $CD4^+$ T lymphocytes in sepsis

Glut1 activation is an important indicator of $CD4^+$ T lymphocytes activation, and it is essential for cell growth, proliferation and immune function [26]. Similar to the effects of EGFR inhibitors both Glut1 inhibitor WZB117 and glycolysis inhibitor 2DG respectively suppressed the LPS-induced apoptosis (Fig. 3a) and activation (Fig. 3d and 3e) of $CD4^+$ T cells. In addition, EGFR/Glut1/glycolysis inhibitors down-regulated apoptosis-related protein expression such as cleaved caspase-3, HIF-1α, LDHA and PKM2 (Fig. 3b). On the other hand, PD168393, WZB117 and 2DG could also respectively inhibited the activation and apoptosis of $CD4^+$ T cells at 9 h and 12 h after LPS treatment (Fig. 3f-3i). All these results indicated that EGFR, and Glut1 coordinated glycolysis jointly and participated in the activation and subsequent apoptosis of $CD4^+$ T cells in sepsis.

EGFR inhibitors inhibited LPS-induced surface expression of Glut1 in $CD4^+$ T lymphocytes

The translocation of Glut1 to cell membrane to capture glucose for aerobic glycolysis is indispensable for $CD4^+$ T lymphocytes activation [27]. We found that the EGFR inhibitors erlotinib or PD168393 could inhibit LPS-induced expression of EGFR and Glut1 on $CD4^+$ T cells surface both in vitro and in vivo (Fig. 4a-4d). Coimmunoprecipitation experiments demonstrated that PD168393 partially suppressed the interaction between EGFR and Glut1 at 3 h after LPS treatment (Fig. 4e). With immunofluorescence imaging we observed the colocalization between EGFR and Glut1 at $CD4^+$ T cell surface in response to LPS and this colocalization could also be partially blocked by PD168393 pretreatment (Fig. 4f).

EGFR and TBK1 participated in the regulation of LPS-induced cell surface expression of Glut1 in $CD4^+$ T lymphocytes

Recent study reported that phosphorylation of the exocyst protein Exo84 by TBK1 promotes insulin-stimulated Glut4 trafficking [28]. We wondered whether TBK1 could act through Glut1 to regulate $CD4^+$ T cell activation in sepsis. As expected, TBK1 inhibitor amlexanox pretreatment effectively inhibited the $CD4^+$ T cell surface expression of Glut1 at 12 h after LPS treatment (Supplementary Fig. S4e and S4f). Meanwhile, amlexanox also inhibited CD25 and CD69 expression on $CD4^+$ T cells in response to LPS treatment (Supplementary Fig. S4c and S4d, S4g and S4h).

We also observed the co-localization between Glut1 and TBK1 in $CD4^+$ T cells at 3 h after LPS treatment, and this co-localization was inhibited by both EGFR phosphorylation inhibitor PD168393 and the TBK1 inhibitor amlexanox (Fig. 5a). Co-immunoprecipitation experiments indicated that EGFR, Glut1 and TBK1 might bind to each other in response to LPS treatment and these binding could be inhibited by PD168393 (Fig. 5b). It also induced co-localization of EGFR, Glut1 and TBK1 in $CD4^+$ T cells at 3 h after LPS treatment (Fig. 5c). In addition, to visualize the interaction among EGFR, Glut1 and TBK1 in intact $CD4^+$ T lymphocytes, we applied in situ proximity ligation assay (PLA). We obtained discrete proximity labeling of EGFR and TBK1 at 3 h after LPS treatment and this signal was blocked by PD168393 or amlexanox pretreatment (Fig. 5d). EGFR physically interacts with TBK1 in $CD4^+$ T cells. Meanwhile, LPS-induced proximity labeling between EGFR and Glut1 was markedly reduced by PD168393 and amlexanox (Fig. 5e). PLA supported the association of EGFR with Glut1 in situ. Likewise, enhanced TBK1-Glut1 PLA signals were detected upon LPS priming, which were inhibited by PD168393 and amlexanox (Fig. 5f). Collectively, our results indicated that EGFR/TBK1/Glut1 may interact to regulate LPS-induced intracellular transport of Glut1 in $CD4^+$T cells.

EGFR faciliated Glut1 cell surface expression through TBK1/Exo84/RalA protein systems

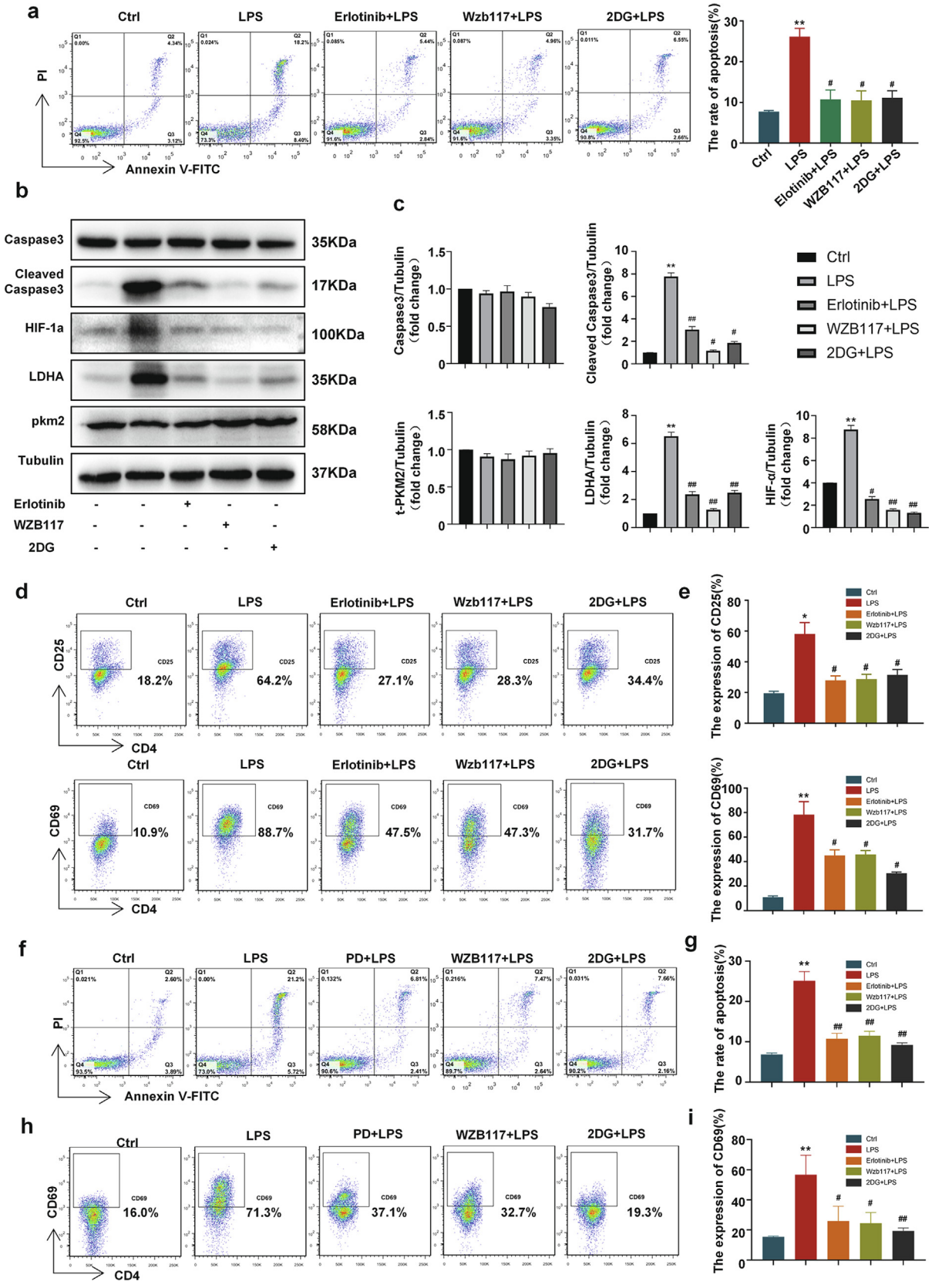
Since EGFR inhibitors decreased cell surface expression of Glut1/TBK1, we hypothesized that EGFR phosphorylation might be related to TBK1/Exo84/RalA protein system, which plays an important role in signal transduction and immune response. As shown in Fig. 6a and 6b, PD168393 pretreatment attenuated the co-localization and co-immunoprecipitation between Exo84 and Glut1 at 3 h after LPS treatment. More interestingly, PD168393 also effectively inhibited the LPS-induced decrease in the co-localization between TBK1, Glut1 and RalA (Fig. 6c). To further illustrate the role of EGFR in the TBK1/Exo84/RalA mediated Glut1 membrane transport, $CD4^+$ T cells were, respectively, pretreated with EGFR inhibitor PD168393, Glut1 inhibitor WZB117, glycolysis inhibitor 2DG, or TBK1 inhibitor amlexanox for 30 min. Then, we observed that the co-localization of EGFR, Glut1 and RalA on $CD4^+$ T cells membrane at 3 h after LPS treatment could be destroyed by any one of those three kind of inhibitors (Fig. 6d). These results indicated that EGFR phosphorylation is needed for TBK1/Exo84/RalA protein systems mediated Glut1 membrane transport in sepsis.

$CD4^+$ T lymphocytes activation and surface expression of EGFR and Glut1 increased in septic patients

$CD4^+$ and $CD8^+$ T cells depletion from septic patients blood has been shown to be closely interrelated with the clinical findings of lymphocyte apoptosis [29,30]. Based on the above results, we wondered whether these phenomena could also be observed in septic patients. Septic patients were included according to the diagnostic criteria of sepsis (Sepsis 3.0) [31], and $CD4^+$ T lymphocyte were isolated from blood samples. We found that $CD4^+$ T lymphocyte depletion and activation (as indicated by markers such as CD25) were significantly promoted in septic patients relative to that seen in healthy donors (Supplementary Fig. S5a-S5c), and cell surface expression of EGFR and Glut1 were also significantly increased (Supplementary Fig. S5d-S5f). These results indicated that EGFR and Glut1 may be an effective new target for the prevention of septic immunosuppression..

Discussion

$CD4^+$ T cells are crucial in coordinating the immune response against pathogens and act by arousing other immune cells, such as $CD8^+$ T lymphocytes [32]. Sepsis rapidly stimulates severe apoptosis in $CD4^+$ T cells, leading to a decrease in not only the number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2DG+LPS
b
Caspase3
Cleaved Caspase3
HIF-1a
LDHA
pkm2
Tubulin
35KDa
17KDa
100KDa
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Erlotinib
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Caspase3/Tubulin (fold change)
Cleaved Caspase3/Tubulin (fold change)
t-PKM2/Tubulin (fold change)
LDHA/Tubulin (fold change)
HIF-α/Tubulin (fold change)
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d
CD25
CD4
18.2%
64.2%
27.1%
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CD69
10.9%
88.7%
47.5%
47.3%
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e
The expression of CD25(%)
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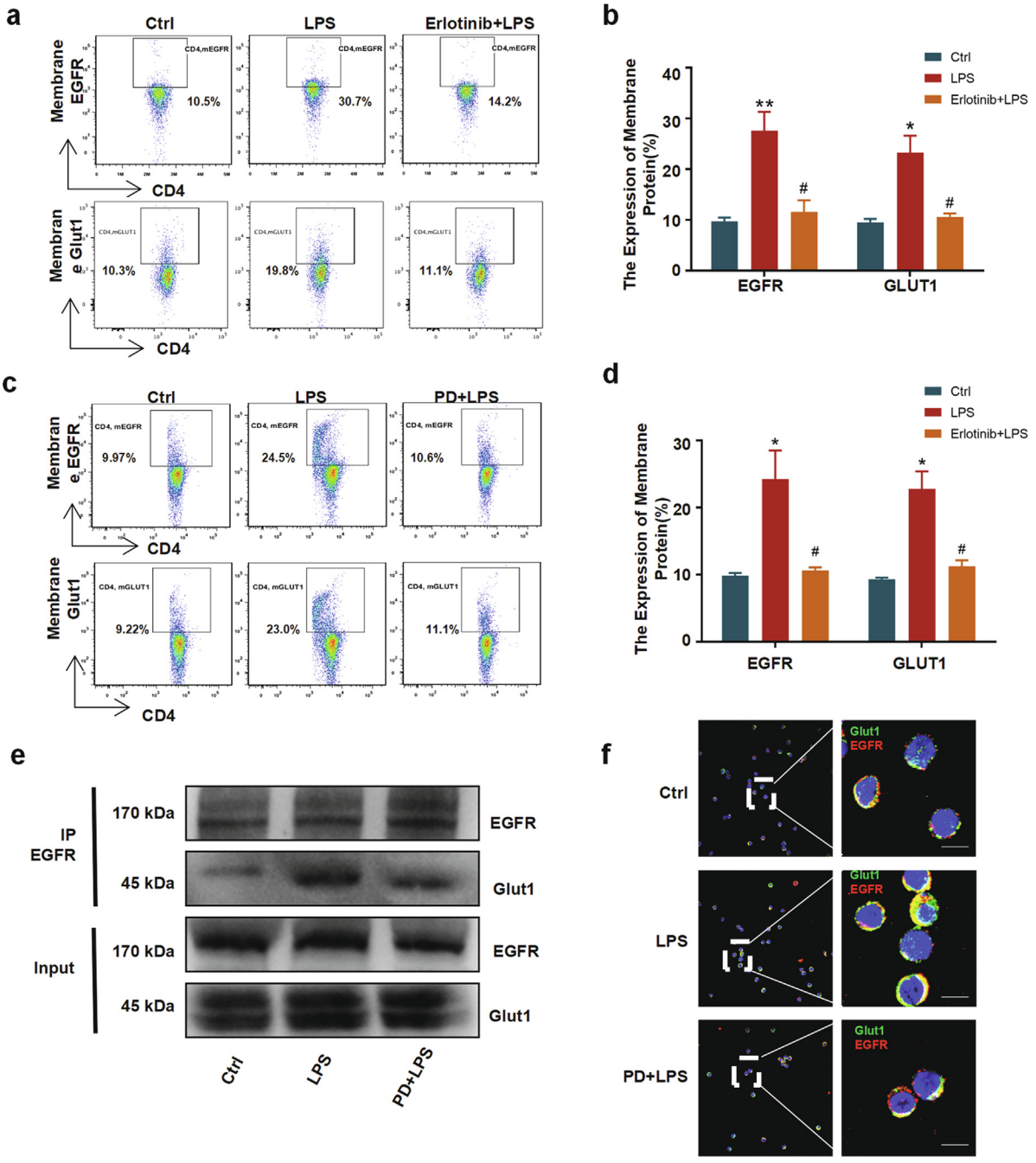

Fig. 4. The effect of EGFR inhibitors on the surface expression and localization of Glut1 in CD4$^+$ T cells in response to LPS. (a and b) Wild-type mice were treated with LPS while some cells were pretreated with erlotinib. (a) Flow cytometry analyzed the surface expression of EGFR and Glut1 in CD4$^+$ T cells. (b) Representative quantification bar charts. n = 3 per group. (c and d) CD4$^+$ T cells were treated with LPS (1 μg/mL, 12 h) with or without PD (10 μM) pretreatment for 30 min followed by flow cytometry analysis of cell surface EGFR and Glut1 intensity. (d) Quantification bar charts. n = 3 per group. (e) Representative coimmunoprecipitation results for EGFR and Glut1 in CD4$^+$ T cells.(f) Representative immunofluorescence results for EGFR and Glut1 in CD4$^+$ T cells treated with LPS (1 μg/mL, 3 h) with or without PD pretreatment. Colocalization of Glut1 (green), EGFR (red), and DAPI (blue). (scale bar, 10 μm). n = 3 per group. Data are expressed as the mean ± SEM and were analyzed by one-way ANOVA with Dunnett's multiple comparison post-test. $^*P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the time-matched LPS alone group.

Fig. 3. EGFR/Glut1/ glycolysis inhibitors decreased activation and apoptosis of CD4$^+$ T cells induced by LPS. (a-d) Wild type mice were treated with LPS (15 mg/kg.i.p.). In several groups, mice were pretreated with erlotinib (100 mg/kg by gavage), WZB117 (30 mg/kg, i.p.) and 2DG (500 mg/k, i.p.) 2 h prior to LPS treatment. Single-spleen-cell suspensions were collected and analyzed by flow cytometry. (a) Flow cytometry analysis of the apoptosis rates in vivo, and the quantification bar charts. (b) Representative western blots of cleaved caspase-3 and PKM2, LDHA, Hif-1a expression in CD4$^+$ T cells and the quantification bar charts. (c)The expression of CD25 and CD69 in CD4$^+$ T cells were detected. (d) Quantification bar charts. n = 3 per group. (e-h) CD4$^+$ T cells were treated with LPS (1 μg/mL) in the presence or absence of pretreatment for 30 min of PD (10 μM), WZB117 (5 μM), 2DG (1 mol/L). (e) Flow cytometry to detect cells death. (f) Quantification bar charts. (g) The expression of CD69 of CD4$^+$ T cells was detected. (h) Quantification bar charts. n = 3 per group. Data are expressed as the mean ± SEM and were analyzed by Student's *t* test or two-way ANOVA with multiple-comparison testing. $^*P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the time-matched LPS alone group.

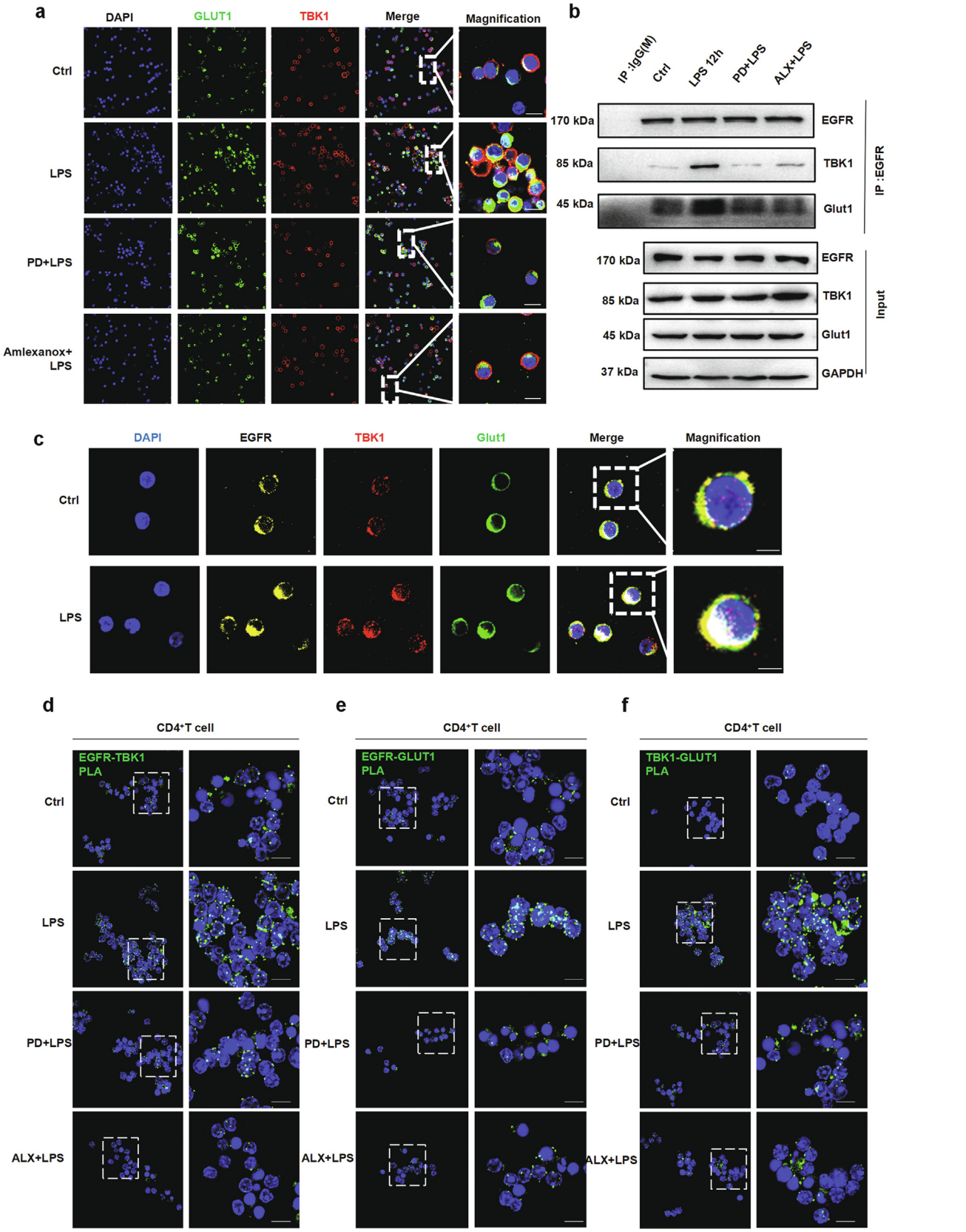

Fig. 5. The interactions of Glut1, EGFR and TBK1 in $CD4^+$ T lymphocytes. (a-f) $CD4^+$ T cells were treated with LPS (1 μg/mL, 3 h)with or without PD (10 μM) or TBK1 inhibitor amlexanox (5 μM) pretreatment for 30 min. (a) Colocalization of cell surface proteins TBK1 and Glut1 according to immunofluorescence assays. Scale bar, 10 μm. (b) co-immunoprecipitation of EGFR, Glut1 and TBK1 in $CD4^+$ T cells. (c) Immunofluorescence of EGFR, Glut1 and TBK1 in $CD4^+$ T cells. Scale bar, 5 μm. (d-f) PLA of EGFR-TBK, EGFR-Glut1, TBK1-Glut1 in $CD4^+$ T cells. PLA puncta, green dots; nuclei, blue (DAPI). Scale bar, 100 μm. n = 3 per group.

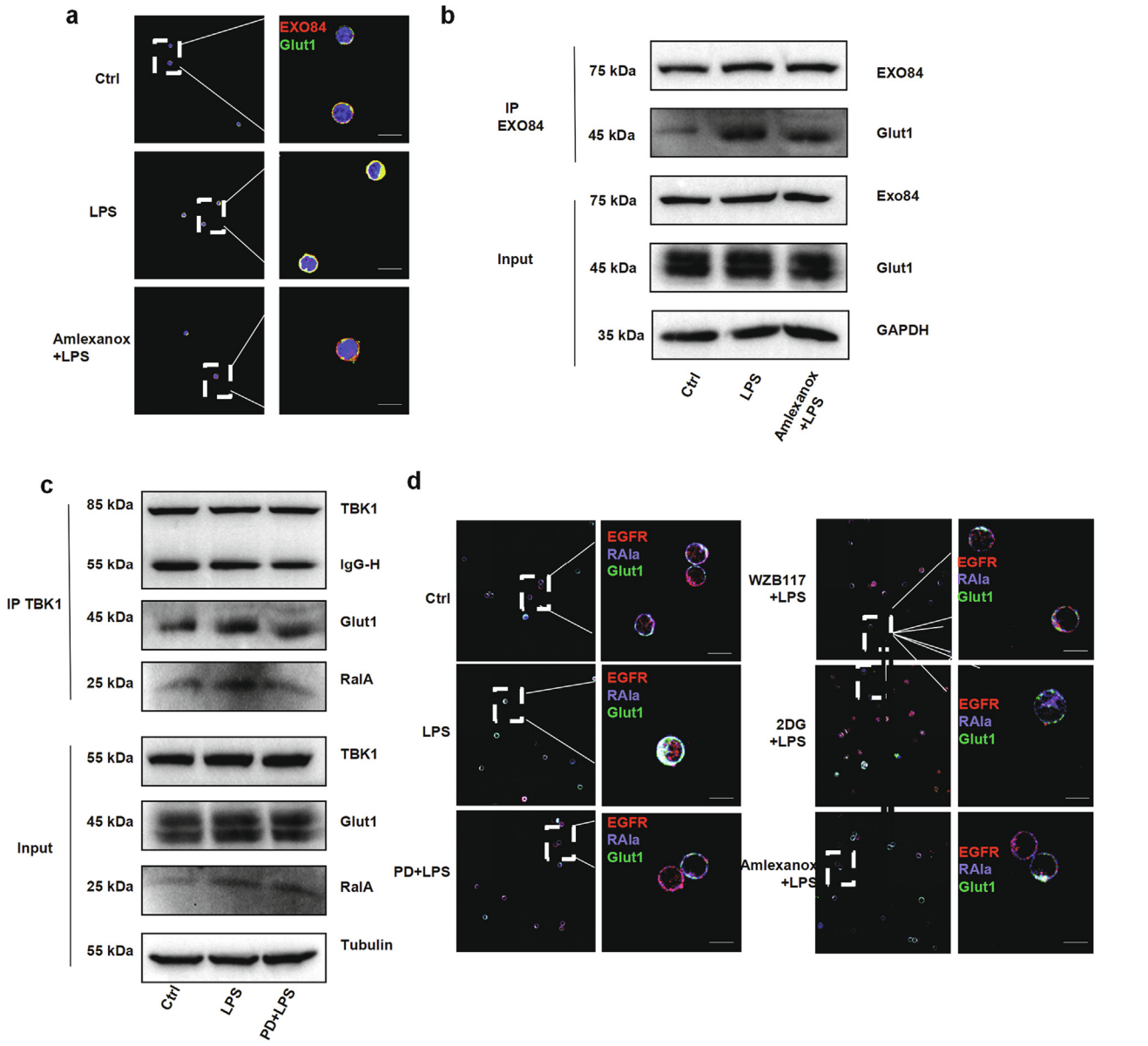

Fig. 6. EGFR participates in the regulation of Glut1 membrane transport through the TBK1/Exo84/RalA protein systems. (a-d) $CD4^+$ T cells were treated with LPS (1 μg/mL, 3 h) with or without PD or amlexanox pretreatment for 30 min. (a and b) The colocalization and coexpression of Exo84 and Glut1 in $CD4^+$ T cells were obsearved by confocal immunofluorescence and immunoprecipitation assays. (c) The coexpression of TBK1, RalA and Glut1 in $CD4^+$ T cells was detected by immunoprecipitation. (d) The colocalization of EGFR, RalA and Glut1 in $CD4^+$ T cells was obsearved by confocal immunofluorescence analysis. scale bar, 10 μm. n = 3 per group.

but also the function of remained lymphocytes. Our study highlights a promising role for EGFR inhibitors during sepsis to restore the immune functions of $CD4^+$ T lymphocytes through attenuating the nunber of both total $CD4^+$ and naïve $CD4^+$ T cells. It is well known that In most cases, the reduction of $CD4^+$ T cells is a consequence of increased apoptosis, which signifies the main mechanism of $CD4^+$ T cell depletion [33,34]. With multiple experiments, such as flow cytometry, western blot and TUNEL staining, we proved that the EGFR inhibitor erlotinib can inhibit sepsis-induced apoptosis and the activation levels of spleen-derived $CD4^+$ T cells.. It has known that $CD4^+$ T cell activation shortens the life span and contributes to cellular apoptosis [5]. We selected CD25 and CD69 as activation indicators and observed that erlotinib significantly inhibited $CD4^+$ T cell activation. All these results indicated that EGFR activation has a vital role in the lymphopenia during LPS-induced sepsis.

Glut1, which facilitates the diffusion of glucose across the cell membrane, is an important enzyme involved in activated T lymphocytes during glycolysis process [27,35,36]. LDHA enzymatic activity is essential to sustain IFN-γ production by human $CD4^+$ T cells through inducing Warburg metabolism [37]. We found that EGFR plays a critical role in Glut1-driven lactate uptake, supporting the proinflammatory response of $CD4^+$ T cells. Growing evidence shows that activated $CD4^+$ T cell participates in the glycolytic pathway to provide energy for activated T cells [11,38,39]. In addition, the phosphorylation of PKM2 and the production of lactic acid are other important features related to increased glycolysis. EGFR regulates the phosphorylation of PKM2 in the LPS-induced Warburg effect [40]. Here, we found that not only the glycolysis inhibitor 2DG but also EGFR inhibitors reduced the levels of glycolysis-related enzymes, lactic acid, pyruvic acid, fructose-1,6-diphosphate and phosphorylated PKM2 and LDHA in LPS-treated

$CD4^+$ T cells. Besides EGFR inhibitor erlotinib, both the Glut1 inhibitor WZB117 and the glycolysis inhibitor 2DG also reduced $CD4^+$ T cell activation and apoptosis in LPS-induced septic mice, indicating that Glut1 may play a role in EGFR's effect on $CD4^+$ T cell glycolysis and apoptosis in sepsis. In response to LPS treatment, the cell surface expression of both EGFR and Glut1 increased. Inhibiting the phosphorylation of EGFR with erlotinib or PD168393 decreased both EGFR and Glut1 cell surface expression. In addition, we also observed the co-localization and coimmunoprecipitation between EGFR and Glut1. Their co-localization together with the fact that either the EGFR inhibitor erlotinib or the Glut1 inhibitor WZB117 similarly reduced $CD4^+$ T cell activation and apoptosis in LPS-induced septic mice collectively indicate that EGFR may interact directly with Glut1 to regulate the cellular transport of Glut1 and affect the glucose metabolism, activation and apoptosis of $CD4^+$ T cells in response to LPS.

A study about development and progression of breast tumor has demonstrated EGFR recruits TBK1 to the transcription factor IRF3 and forming a ternary complex [41]. Interestingly, Here we found that EGFR forms a complex with TBK1 and Glut1 to facilitate Glut1 transportation from the cytoplasm to the cell surface. Furthermore, this complex physically interacts with RalA and Exo84. Amlexanox, a TBK1 inhibitor, can not only inhibit the expression of Glut1 on the membrane surface but can also disrupt the co-localization between Glut1 and Exo84 in $CD4^+$ T cells after LPS treatment. Exo84, one of the subunits of exocyst complex that can bind to the small GTPase RalA, is phosphorylated by the protein kinase TBK1 when RalA is activated [42]. Moreover, RalB GTPase-mediated activation of TBK1 cause the transduction of innate immune signals [43]. Our research first proved the interactions between EGFR and TBK1, Glut1, and RalA revealing that the EGFR inhibitor erlotinib suppressed the coexpression of TBK1, Glut1, and RalA in $CD4^+$ T cells after LPS treatment. In addition, any of the EGFR inhibitor erlotinib, Glut1 inhibitor WZB117, glycolysis inhibitor 2DG and TBK1 inhibitor amlexanox decreased the colocalization of EGFR, Glut1 and RalA, which demonstrated that EGFR participates in the regulation of cell surface trafficking of Glut1 through the TBK1/Exo84/RalA protein system.

In addition, Compared with healthy donors, peripheral blood from sepsis patients not only had increased membrane expression levels of EGFR and Glut1 but also had increased activation levels of $CD4^+$ T lymphocytes. However, a small number of patients with severe sepsis had severe lymphocyte depletion and decreased activation, resulting in a decrease in the expression levels of membrane proteins. The exact reasons need to be further explored.

Conclusions

Our study proposed that EGFR facilitates Glut1 to translocate to the cell surface via the TBK1/Exo84/RalA signaling pathway in response to LPS treatment. Then, glucose is captured by Glut1 and metabolized to lactate via the Warburg effect to supply energy for $CD4^+$ T cells activation. Activated $CD4^+$ T cells have a shorter lifespan and die via apoptosis, leading to $CD4^+$ T cell depletion in sepsis. These results provide new evidence to explain a potential new role of EGFR in sepsis regarding $CD4^+$ T lymphocyte apoptosis and depletion.

Compliance with Ethics Requirements

All Institutional and National Guidelines for the care and use of animals were followed. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

CRediT authorship contribution statement

Li Huang: Conceptualization, Methodology, Writing – original draft. **Xuedi Zhang:** Validation, Methodology. **Junyu Fan:** Investigation, Visualization. **Xiaolei Liu:** Software, Formal analysis. **Shuhua Luo:** Investigation. **Dianqing Cao:** Project administration. **Youtan Liu:** Project administration. **Zhengyuan Xia:** Supervision, Visualization. **Hanhui Zhong:** Software, Investigation. **Cuiping Chen:** Resources. **Liangqing Zhang:** Supervision. **Zhifeng Liu:** Conceptualization, Writing – review & editing. **Jing Tang:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.04.010.

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