Epigallocatechin-3-gallate ameliorates lipopolysaccharide induced acute thymus involution in mice via AMPK/Sirt1 pathway

Qing Su
The Second Affiliated Hospital of Zhengzhou University
Shu-ping Yang
Sanquan College of Xinxiang Medical University
Jun-ping Guo
Zhengzhou University
Yi-ren Rong
Zhengzhou University
Yun Sun
Zhengzhou University
Yu-rong Chai (yrchai@zzu.edu.cn)
Zhengzhou University

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Abstract

The thymus, a site to culture the naïve T lymphocytes, is susceptible to atrophy or involution due to ageing, inflammation and oxidation. Eepigallocatechin-3-gallate (EGCG) has been proven to possess the anti-inflammatory, anti-oxidant and anti-tumor activity. Here, we investigate the effects of EGCG on thymic involution induced by lipopolysaccharide (LPS), an endotoxin derived from gram-negative bacteria. Methodology included in vivo experiment on female kunming mice exposed to LPS and EGCG. Morphological assessment of thymic involution, the immunohistochemical detection, thymocyte subsets analysis by flow cytometry were further carried out to evaluate the potential role of EGCG on thymus. As a result, we found that EGCG alleviated LPS-induced thymic atrophy, increased mitochondrial membrane potential and SOD levels, and decreased MDA and ROS levels. In addition, EGCG pre-supplement restored the ratio of thymocyte subsets, the expression of autoimmune regulator (Aire), sex determining region Y-box2 (Sox2) and nanog homebox (Nanog), and reduced the number of senescent cells and collagen fiber deposition. Western blotting results indicated that EGCG treatment elevated LPS-induced decrease in pAMPK, Sirt1 protein expression. Collectively, EGCG relieved thymus architecture and function damaged by LPS via regulation of AMPK/ Sirt1 signaling pathway. Our findings may provide a new strategy on protection of thymus from involution caused by LPS by using appropriate adjuvant natural agents. EGCG might be considered as a potential agent for the prevention and treatment of thymic involution.

1. Introduction

The thymus is a primary lymphatic organ responsible for *de novo* production and maintenance of a diverse repertoire of immunocompetent T lymphocytes. The main components of the thymus include thymocytes of hematopoietic origin and thymic epithelial cells (TECs) of non-hematopoietic origin. The latter comprises thymic cortical epithelial cells (cTECs) and medullary epithelial cells (mTECs) [1] [2], that support thymocytes development and maturation and are responsible for positive and negative selection of them, respectively [3] [4]. Despite the requirement for lifelong replenishment of T lymphocytes, the thymus undergoes age-related progressive involution beginning as early as birth that is ascribed to disorganization of the thymic epithelial cell architecture and increased adiposity, impaired naive T lymphocytes production, accumulation of senescent cells, and adaptive immune senescence, termed “thymic involution or atrophy"” [5] [6] [7]. Also, thymic function is extremely sensitive to acute damage caused by everyday insults like stress and infection, as well as more profound injuries resulted from cytoreductive therapies [8] [9]. Consequently, there is a real need for strategies that can boost thymic function and enhance T lymphocytes immunity.

There is growing evidence that the thymus tissue is plastic and its degenerative process can be halted or reversed by intervention, even if the thymus is often considered to have insufficient ability to regenerate itself to re-establish complete thymic function [2] [10]. Regenerative therapies to boost thymic function after acute damage or to reverse age-related involution are suggested to be broadly stratified into four subgroups based on their cellular or molecular targets: the epithelial microenvironment that supports thymopoiesis, the precursors that provide the supply of developing thymocytes, modulation of hormones

and metabolism; and cellular therapies and bioengineering [9]. It has been demonstrated that the restoration of the thymic functions and the thymic re-growth may be achieved by some endocrinological (melatonin) or nutritional interventions (arginine or zinc), mediated by modulating neuroendocrine-thymus interaction in ageing [11] [12].

Flavonoids, a large group of phenolic compounds widely distributed in plants and fungus, have been well-known for their antioxidant, antimicrobial, and anti-inflammatory health benefits [2, 18, 19]. Genistein, a member of flavonoid family present in high quantities in soy, has been found to be able to induce thymic atrophy and suppress immune function [13] [14] [15] in humans and laboratory animals. Epigallocatechin-3-gallate (EGCG), also a member belongs to flavonoid family, is the most abundant and active compound in green tea, which possesses antioxidant, anti-inflammatory, anti-cancer and anti-bacterial effects, with therapeutic potentials for various human diseases [16]. It has been shown that EGCG can regulate a variety of cellular signaling pathways and inhibit tumorigenesis by suppressing the carcinogen activity [17] [18]. Diethylnitrosamine (DEN) -induced liver tumorigenesis in obese mice is prohibited by EGCG through inhibiting the IGF/IGF-1R axis, improving hyperinsulinemia, and reducing chronic inflammation [19]. As well, EGCG can restore natural immune homeostasis in many autoimmune diseases, and its pretreatment inhibits LPS-induced acute lung injury in mice [20] [21]. However, the effects of EGCG on thymic involution remain unknown. Therefore, in the current study we investigated the potential role of EGCG on thymic involution using a mouse model with acute thymic involution induced by lipopolysaccharide (LPS), which is usually used to establish the organ injury, such as liver, kidney and uterus [22] [23] [24], and further explored its associated mechanism of action.

2. Materials and methods

2.1. Animals and experimental design

Eight-week-old female Kunming mice were obtained from the Animal Experimental Center of Zhengzhou University. All the mice were housed at the animal facilities of the Center, with water and food free access in a 12-hour (h) light/12-h dark cycle, under cycled air ventilation and homeostasis of temperature and humidity. After recovering from transportation for one week, the mice were randomly distributed into three groups (n = 10 per group): LPS, EGCG and Control group. The mice in LPS and EGCG groups were orally gavaged normal saline and EGCG (40 mg/kg) (Yuanye Bio-Technology, China) respectively, once a day for 8 days. The dosage of EGCG used herein was based on the previous studies [25] [26]. On the fourth day, mice in the two groups were intraperitoneally injected with one dosage of LPS (2 mg/kg dissolved saline, Sigma-Aldrich, MO, USA). The mice in the control group were just orally gavaged with normal saline once a day for 8 days. Mouse body weight was measured every day before oral administration. At the end of experiments, mice were sacrificed by cervical dislocation, and the thymi were immediately removed, fixed for histological analysis and then stored at -80°C until use. The thymus index was calculated as thymus weight (mg) divided by body weight (g).

The mouse experimental procedures were reviewed and approved by the Medical Ethics Committee of Zhengzhou University (Ethics number: ZZUIRB 2018-017), in strict accordance with the Animals (Scientific Procedures) 1986 Act (UK) (amended 2013), as well as the Guide for the Care and Use of Laboratory Animals in China.

2.2. Histological and immunohistochemistry examination

Thymus samples in each group were fixed in 4% formaldehyde solution followed dehydrated, embedded in paraffin, and serially sectioned (4 μm thickness). After dewaxing, the sections were conventionally stained with hematoxylin-eosin (H&E) and picro-sirius red (PSR) for collagen fiber examination. For immunohistochemical (IHC) analysis, following tissue sections deparaffinized and rehydrated, heat-induced epitope retrieval was then carried out by heating the sections in citrate buffer (10 mM, pH 6.0) for 20 min. Afterwards, sections were incubated with the following primary antibodies: goat polyclonal anti-Aire antibody (1:200, Santa Cruz, USA), rabbit polyclonal anti-Sox2 antibody (1:700, ThermoFisher, USA) and rabbit polyclonal anti-Nanog antibody (1:600, Servicebio, China) at 4℃ overnight. The next day, the sections were washed and followed by incubation with biotin-labeled secondary antibodies (30 min, room temperature) (ZSGB-bio, China) according to the manufacturer's instruction. Positive cells were observed with diaminobenzidine (DAB) solution, and finally, counter-staining was performed with hematoxylin. The stained slides were evaluated by three independent blinded readers. Quantification of IHC images was measured by analyzing immunostained areas by using Image J software (http://rsb.info.nih.gov/ij/) and data were presented as a proportion of DAB reactive area.

2.3. Senescence-associated β-galactosidase staining

Senescence-associatedβ-galactosidase (SA-β- Gal) staining was performed to assess the senescent cells in frozen section of thymus tissue according to Wei et al [27]. Briefly, the thymus tissue was fixed with 4% paraformaldehyde at 4℃ overnight, cut into 6 μm thickness slice. Frozen sections of thymus tissue were immersed in fresh SA-β-Gal staining solution and incubated in the darkness for 5 hours, 37℃, and finally, nucleus counter-staining was applied with neutral red. The slides were observed under light microscopy. The senescence positive cells were marked as blue area and the images were analyzed by Image J. Data were presented as mean staining intensity. We further examined the effects of LPS and EGCG on thymus senescence by SA-β-gal staining. Since SA-β-galactose is catalyzed by specific β-galactosidase in senescent cells, it produces a dark blue product and expresses a more active β-galactosidase, which can be used to label senescent cells.

2.4. Flow cytometric analysis

The thymus tissue samples were ground on ice immediately after collection. Thymocytes were separated by 400 mesh sieve and washed with PBS. Single cell suspensions of thymocytes were incubated with APC-conjugated anti-mouse CD3, PE-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD8 (Biolegend, San Diego, CA, USA), respectively. After icing for 40 min, the excess antibody was removed by washing three times with PBS and sodium azide, and vortex centrifugation. The cells were then resuspended in 5% fetal bovine serum. The samples were analyzed on a FACS instrument (BD

Biosciences, San Diego, CA, USA). Flow cytometry data analysis was performed using FlowJo 10.4 software.

2.5. Measurement of mitochondrial membrane potential, ROS, SOD and MDA

Mitochondrial staining with JC-1 dye (Solarbio, Beijing, China) was used to detect changes in mitochondrial membrane potential in the thymus according to the product manuals and Yang et al [28]. In brief, isolated thymocytes were gently suspended in JC-1 working solution and incubated in the darkness at 37°C for 30 min. After incubation, cells were precipitated by centrifugation at 600g for 4 min. Cells were then washed twice with JC-1 staining buffer and resuspended with 500 μl PBS. The red fluorescence of JC-1- aggregates (normally) was excited at 525 nm and emitted at 590 nm. The green fluorescence of JC-1 monomer (low Δψm, depolarized state) was excited at 485 nm and its emission was recorded at 530 nm. The reduction in the red/green FI ratio was indicated as thymus mitochondrial membrane depolarization. When JC-1 aggregates in the mitochondrial matrix, it produces red fluorescence, and at the case of the mitochondrial membrane potential is low, JC-1 can not aggregate and produces green fluorescence. The relative ratio of red to green fluorescence can measure the state of mitochondrial depolarization.

To evaluate the production of mitochondrial ROS in the thymus, the cell precipitates obtained were incubated with diluted DCFH- DA (Solarbio, Beijing, China) in the dark at 37℃ for 30 min. Intracellular ROS can oxidize non-fluorescent DCFH to fluorescent DCF, and the fluorescence intensity of DCF was measured using a fluorescence enzymelabeling instrument at excitation light 488 nm and emission light 525 nm, reflecting the level of intracellular ROS.

It has been shown that LPS-treated mice exhibit mitochondrial dysfunction, oxidative stress and inflammatory response [29]. To assess the activity of superoxide dismutase (SOD) and malondialdehyde (MDA), the thymus tissue was ground and homogenized with lysate, and the supernatant was collected after centrifugation at 10,000 g for 15 min. Then, the reagents were added to the supernatant and proceeded with the procedure according to manufacturer's instructions (Superoxide dismutase Assay Kit, Solarbio, Beijing, China). SOD activity was measured at 560nm using enzyme label instrument and expressed as U/g fresh tissue. MDA is the main product of lipid peroxidation. The supernatant was carried out with the procedures described in the specific kit used for dosing MDA activity (Micro Malondialdehyde Assay Kit, Solarbio, Beijing, China). The absorbance of thiobarbituric acid reactive substances in tissue homogenates was measured, using a spectrophotometer at 450 nm, 532 nm and 600 nm. MDA level was expressed as nmol/g fresh tissue.

2.6. Western blotting

Protein was extracted from thymus homogenates and subjected to SDS-PAGE gel electrophoresis. The bands were incubated with rabbit polyclonal anti-NF-κB antibody (1:1000, Wanleibio, China), rabbit polyclonal anti-Sirt1 antibody (1:1000, Wanleibio, China), mouse monoclonal anti-AMPK 1 antibody

(1:1000, Bioss, China), rabbit polyclonal anti-pAMPK antibody (1:1000, CST) and rabbit polyclonal anti-GAPDH antibody (1:2000, Beyotime, China). Finally, the ECL chromogenic agent was added to expose the image, and the images were analyzed by Image J.

2.7. Statistical analysis

All analyzes were performed using GraphPad Prism version 8.0 software (San Diego, California, USA). All values were expressed by Mean ± SEM, one-way analysis of variance (ANOVA) was used to analyze differences among the three groups with normally distributed data. $P < 0.05$ was considered as statistically significant in differences.

3. Results

3.1.EGCG improved the thymus structure and attenuated cellular senescence and fibrosis

The schematic diagram of animal experiment was indicated by Fig. 1. As shown in Fig. 2A and B, with LPS intraperitoneally injected, the thymus was significantly atrophied, along with the reduced thymus index; H&E sections indicated that a severe disorder of thymus cortex medulla and unclear boundary. However, pre-administration of EGCG restored thymus index and size with more clear cortex medulla area (Fig. 2C).

Through the senescent cells detected with the SA-β-Gal staining, we found that they were mainly distributed at the thymic cortical-medullary junction and stained blue. This increased number of senile cells induced by LPS was reduced by pre-administration of EGCG (Fig. 2E and G). The data suggested that EGCG has an inhibitory effect on thymocyte senescence. We further evaluated the accumulation of collagen fiber by PSR (picro-sirius red) staining. It indicated that LPS treatment increased the collagen fiber deposition which werestained red. Pre-administration of EGCG significantly diminished the number of collagen fiber deposition (Fig. 2D and F).

3.2.Effect of EGCG on expression of Aire, Sox2 and Nanog

Autoimmune regulator (Aire) is a unique transcriptional regulator that induces transcription of a broad range of tissue-specific antigens (TSAs) to promote self-tolerance and is expressed primarily in medullary thymic epithelial cells (mTECs) [30] [31]. IHC was used to investigate the effect of EGCG on the expression of Aire.

The Aire positive cells in the control and EGCG groups were higher than that in the LPS group, and the expression location was mainly distributed in the medullary region; after LPS treatment, the Aire positive cells were more dispersed and even found in the cortical region of the thymus. which suggested that LPS induced disorder in the thymic cortex (Fig. 3A and D) and EGCG administration restored the normal expression and location of Aire positive cells.

Nanog homebox (Nanog) is one of the transcription factors required for maintenance of embryonic stemcell pluripotency and self-renewal, and its enhanced expression confers self-renewal ability of embryonic stem cells [32] [33]. Sex determining region Y-box2 (Sox2) is essential for maintaining the stemness of embryonic cells and adult stem cell differentiation [34]. Through IHC, it showed that Sox2 and Nanog positive cells were mainly found in thymus cortical and medullary junction, LPS induced the expression of Nanog and Sox2 compared with the control group, and the expression location was mainly distributed in the marginal region of the thymus. EGCG administration seemed to normalized the expression of Nanog and Sox2 and the positive cells were mainly found in the corticomedullary junction (Fig. 3B, C and E, F).

3.3.EGCG benefited thymocyte subsets

It has been reported that LPS induced apoptosis of CD4 + CD8 + thymocytes in mice [35] [36]. Alterations of thymocyte subsets in the thymus examined by flow cytometry were shown in Fig. 4A. The percentage of double negative (CD4-CD8-) T cells were increased, while that of double positive (CD4 + CD8+) T cells were decreased in the LPS group. Pre-administration of EGCG reduced the increase in population of CD4-CD8- T cells, but elevated the reduction in CD4 + CD8 + T cell percentage (Fig. 4B-E). The results indicated that EGCG could recover the thymocyte subsets disorder.

3.4.Effect of EGCG on mitochondrial membrane potential, ROS, SOD and MDA

The mitochondrial membrane potential and ROS level thymus tissues were detected by the fluorescence enzyme labeling instrument, and the decrease of red to green fluorescence ratio represented the depolarization of mitochondrial membrane potential. As shown in Fig. 5A and B, compared to the control group, the mitochondrial membrane potential was reduced in the LPS group and the level of ROS was increased. Whereas, compared to the LPS group, the reduction of mitochondrial membrane potential was elevated by EGCG pre-administration, and EGCG administration significantly reduced ROS levels, suggesting that EGCG alleviates LPS-induced thymic mitochondrial depolarization and reduces ROS production, thus protects thymic mitochondria,

SOD defends against reactive oxygen species mediated tissue damage; whereas MDA, one of the final products of polyunsaturated fatty acid peroxidation, is known as a marker for oxidative stress [37]. As indicated in Fig. 5C and D, the SOD activity was decreased and the MDA content was increased in the LPS group, compared to the control group. EGCG pre-administration tremendously increased the SOD level and reduced the MDA content. The results suggested that EGCG treatment reduced oxidative stress damage and increased antioxidant levels in mice.

3.5.EGCG activated AMPK/Sirt1 pathways

The effects of EGCG on the expression of AMPK, pAMPK, Sirt1 and NF-κB proteins were further detected by western blotting (Fig. 6A). It showed that LPS treatment significantly downregulated pAMPK and Sirt1 protein expression levels, compared with the Control group, and there was no significant difference in

AMPK and NF-κB protein expression. Compared with the LPS group, EGCG pre-administration increased the expression of pAMPK and Sirt1, while the differences in the expression of AMPK and NF-κB were not changed (Fig. 6B-E). The results suggested that the AMPK/ Sirt1 signaling pathway play an important role in the protective effect of EGCG against LPS-induced acute thymic degeneration in mice.

4. Discussion

In this study, intraperitoneally injected LPS induced thymic atrophy characterized by reduced thymus index and lost boundary between the cortex and medulla, along with alterations in thymocyte subsets (CD4-CD8- T cells increased, CD4 + CD8 + T cells decreased), addition in senescent cells, collagen fiber, ROS level and MDA, and reduction in mitochondrial membrane potential and SOD. As well, the expression of transcriptional regulators Sox2 and Nanog were upregulated by the LPS, with a downregulated expression of Aire. Pre-administration of EGCG inhibited thymic atrophy, accompanied by normalized parameters including thymocyte subsets, senile cells, collagen fiber, SOD, ROS level, MDA, mitochondrial membrane potential, and expressions of Sox2, Nanog and Aire. In addition, EGCG treatment elevated LPS-induced decrease in pAMPK, Sirt1 protein expression.

The thymus, being vital for the differentiation and maturation of T lymphocytes [38], undergoes progressive age-related involution or physiological atrophy with impaired naive T lymphocytes production and a diminished adaptive immune response [5]. In addition, infectious diseases as well as inflammation and malnutrition may also cause pathological thymic atrophy [39] [40]. Lipopolysaccharide (LPS), a major cell surface mucopolysaccharide component of gram-negative bacteria [41], can cause cell damage to induce systemic inflammation [42], and has been used to induce thymic atrophy and thymocyte apoptosis [43] [44]. Herein, we revealed that LPS induced thymus to undergo acute atrophic degeneration with alterations in structure and associated parameters. Other studies have demonstrated that intraperitoneal injection of LPS is usually used to establish the organ injury, such as liver, kidney and uterus [22] [23] [24]. Also, LPS lead to thymic degeneration with reduced CD4 + CD8 + T cell subsets and thymus DNA breaks [45]. In neonatal mice, the thymus was atrophied and looked paler after LPS stimulation [46]. Another report indicated that intraamniotic LPS exposure resulted in a decreased thymic corticomedullary ratio within 24 hours [47].

It is noteworthy that the thymus is adaptable to ageing and environmental factors [48] and several intervention or therapeutic approaches for boosting thymus function have been suggested [9]. To our knowledge, the current study firstly investigated the association of EGCG and thymic involution or atrophy, and found that pre-administration of EGCG had an inhibitive role in thymic structure damage induced by LPS injection, and normalized alterations of the associated parameters. The beneficial effects of EGCG on health and diseases have been reported. For example, in a murine model of dextran sulfate sodium (DSS) -induced colitis, following DSS supplementation, oral EGCG delivery enriches short-chain fatty acid producing bacteria, thereby reducing DSS-induced murine colitis and enhancing colonic barrier integrity, and pre-delivery of EGCG produces similar effects [49]. Also, extensive clinical evidence indicates that EGCG has potential benefits in the prevention and treatment of vascular inflammation in rheumatoid

arthritis, some of these benefits are achieved through inhibition of markers/mediators such as interleukin-6, C-reactive protein and tumor necrosis factor-α [50]. Furthermore, it is reported that EGCG and other catechins bind to the S1 ubiquitin-binding site of papain-like protease protein, and thus inhibit its protease function and disable the inhibitory function of SARS-CoV-2 of the ubiquitin proteasome system and the interferon-stimulated gene system, suggesting EGCG as a potential therapeutic agent for SARS-CoV-2 infection [51].

Changes in the peripheral T cell pool and function and phenotype of T cells, and reduction in the output of naive T cells occur with the thymic degeneration induced by ageing, infection and etc. [52]. Herein, the percentage of CD4 + CD8 + lymphocytes was reduced and the CD4-CD8- lymphocytes percentage was increased by LPS injection, and pre-administration of EGCG normalized these lymphocytes' alteration, suggesting that EGCG could ameliorate the LPS-induced disturbance in thymocyte subsets. Similarly, it has been reported that, in extra-thymus tissues, EGCG not only suppresses expansion and cell cycle progression of naïve CD4 + lymphocytes by modulating cell cycle-related proteins, but also inhibits naive CD4 + lymphocytes differentiation into Th1 and Th17 effector subsets by impacting their respective signaling transducers and transcription factors, thus improving T-cell-mediated autoimmune diseases [53]. Also, EGCG leads to an increased proportion of CD8 + lymphocytes by favoring secretion of Th1 cytokines (e.g., IL-2, INF-γ) [54]. Furthermore, EGCG reduces the body weight and fat infiltration in liver tissue while improving serum lipid profiles, with a higher Treg/Th17 cell ratio in CD4 + lymphocytes differentiation by decreasing the ratio of STAT3/STAT5 expression in DIO mice [55]. Additionally, in mouse autoimmune arthritis, the proportion of forkhead box protein 3-positive regulatory lymphocytes is increased in the spleens treated with EGCG compared with control mice, whereas the proportion of T helper 17 cells is reduced, probably mediated by the increased expression of nuclear respiratory factor 2, heme oxygenase-1, and extracellular signal-regulated kinase [56].

Besides triggering inflammatory responses, the level of p16 and p21, key markers of senescence, is elevated in the LPS-induced acute lung injury animal model [57]. Moreover, senescence-associated β-galactosidase (SA-β-gal), a hypothetical hydrolase enzyme in the senescent cells, is considered as the widely used biomarker for senescent and aging cells [58]. Our data further demonstrated that the augmented SA-β-Gal positive cells in the thymus by LPS were reduced by pre-administration of EGCG, implying its inhibitory impact on the thymocyte senescence. Studies on the anti-senescence effects of EGCG have demonstrated that polyphenols including EGCG can maintain autophagic equilibrium, thereby providing various health benefits in mediating neuroprotection and exhibiting anticancer and antidiabetic properties [59]. Mechanistic study demonstrates that, by activating PI3K/Akt/eNOS signaling pathway, EGCG can down-regulate the expression of caspase-3 and Bax, increase the expression of Bcl-2, reduce neuronal apoptosis and protect against brain injury [60].

As well, abnormal tissue collagen deposition and fibrosis can be driven by the aging process, chronic inflammation and the accumulation of molecular damage [61] [62] [63]. Similar to pulmonary and liver fibrosis induced by LPS [64] [65], in the current study, LPS injection accelerated the thymus tissue fibrosis, and this alteration was correctly reversed by EGCG pre-administration. The anti-fibrosis effects of EGCG

have been verified widely. Besides some antiviral and anti-sepsis actions, EGCG benefits lie in its anti-fibrotic effect and in the ability to simultaneously downregulate expression and signaling of many inflammatory mediators, thus counteracting COVID-19 infection [66]. EGCG has a protective role in chronic kidney disease including fibrosis, based on available preclinical evidence, particularly its antioxidant property through preservation of mitochondrial function and activation of nuclear factor erythroid 2-related factor 2 and heme oxygenase-1 signaling, anti-inflammatory activity, and protective effect against epithelial mesenchymal transition [67]. Also, EGCG treatment has beneficial effects on NAFLD and its related fibrosis and carcinoma because of its effect of anti-oxidation, anti-inflammation, and its effect on energy metabolism through up-regulation of LDL-R, activation of AMPK, regulating SREBPs, increasing lipid oxidation and improving insulin resistance, etc. [68].

Studies show that in LPS-induced lung injury, an imbalance between the antioxidant system and excess free radical production leads to membrane lipid peroxidation and severe oxidative stress injury[69]. In the present study, LPS-treated mouse thymus tissue had increased ROS level and MDA level, and decreased mitochondrial membrane potential and SOD level. However, EGCG administration normalized alterations in mitochondrial membrane potential, a key indicator for cell health and viability [70] [71], ROS level and oxidative and anti-oxidative indexes MDA and SOD [72] [73], induced by LPS injection. It was suggested that EGCG could alleviate LPS-induced oxidative stress in mouse thymus and protect mitochondrial function.

The main components of the thymus include thymocytes of hematopoietic origin as well as thymic epithelial cells (TECs) of non-hematopoietic origin. The thymic epithelium starts to decrease as a main feature of age-related thymic involution from as early as the first year of human life, paralleling with a perivascular expansion filled with adipocytes and peripheral lymphocytes [74]. Both Nanog and Sox2 are transcription factors essential for maintaining the phenotype of pluripotent embryonic stem cells [75] [76] and pluripotency and self-renewal in these stem cells [77] [78]. Aire is an autoimmune regulator that participates in immune tolerance and prevents autoimmune diseases by regulating the expression of tissue-specific antigens mixed in TECs [79] [80]. In this study, the elevated expression of Sox2 and Nanog in the thymus after LPS induction was found, which is in keeping with the report that LPS-induced acute uterine injury leads to a rapid increase in the levels of transcription factors Sox2, Nanog mRNA and protein in utero [23]. Whereas, EGCG pre-administration inhibited the upregulated expression of Sox2 and Nanog with LPS injection. The downregulated expression of Aire induced by LPS was elevated by EGCG administration. Therefore, we speculated that Nanog and Sox2 were involved in tissue repair and regeneration after acute injury through activation of thymic epithelial progenitor cells. In cancer studies, EGCG is found to be able to inhibit the self-renewal capacity of cancer stem cells by suppressing their sphere forming capacity, and attenuates the expression of stem cell markers, such as Oct4, Sox2, Nanog and CD44 [81] [82]. Nonetheless, the detailed effects of EGCG on thymocytes of hematopoietic origin and epithelial non-hematopoietic origin remain to be elucidated in the future.

NF-κB is an important nuclear protein factor that can regulate gene expression, and as a common transcriptional regulator, it is widely involved in various responses in the body, such as inflammatory

response, cell proliferation and apoptosis, immune response, etc [83]. AMPK plays an important role in the regulation of cellular energy homeostasis, mainly maintaining intracellular energy balance and regulating systemic energy metabolism [84]. Sirt1 is an NAD+-dependent deacetylase that affects a variety of biological processes including regulation of inflammation and aging metabolism through deacetylation of multiple proteins [85]. AMPK enhances Sirt1 activity by increasing cellular NAD + levels, and when AMPK, Sirt1 is activated, acetylation of NF-κB p65 increases the transcriptional activity of the NF-κB complex and negatively regulates downstream NF-κB transcriptional activity, thereby suppressing the inflammatory response [86]. It has been suggested that irisin may ameliorate LPS-induced acute lung injury in rats by inhibiting macrophage infiltration and inflammatory response through activation of AMPK/Sirt1/NF-κB pathway [87]. Whereas LPS induces inflammation, leading to cell and organ damage, we speculate that the AMPK/Sirt1/NF-κB pathway may mediate the protective effect in EGCG against LPS-induced acute thymic degeneration. In this experiment, we detected the expression of AMPK, Sirt1 and NF-κB in mouse thymus tissue using WB, and found that the expression of pAMPK and Sirt1 was decreased in the thymus of LPS-treated mice compared with the Control group while the expression of pAMPK and Sirt1 was increased after EGCG administration. It is suggested that EGCG alleviates and protects against LPS-induced acute thymic degeneration in mice, which may be related to the activation of AMPK/Sirt1 pathway and attenuates the inflammatory response.

In summary, we elucidated the beneficial effects of EGCG on thymic degeneration caused by LPS-induced inflammatory responses and explored the potential mechanisms by which EGCG protected against thymic degeneration. Pre-administration of EGCG inhibited thymic atrophy, accompanied by normalized T cell subsets, inhibition of cell senescence, collagen fiber and mitochondrial oxidation, reduced oxidative stress in thymus, and altered expressions of Sox2, Nanog and Aire associated with epithelial progenitor cells, and the mechanism may be related to the activation of AMPK/Sirt1 pathway. The data suggested that EGCG might be a potential agent for prevention and treatment of thymic involution.

Declarations

Author Contributions: Su Q.: Writing-original draft, Formal analysis, Methodology, Data curation. Yang S.P.: Investigation, Writing-review & editing. Guo J.P. and Rong Y.R: investigation. Sun Y.: Supervision. Chai Y.R.: Conceptualization, Funding acquisition, Project administration, Methodology, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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Figures

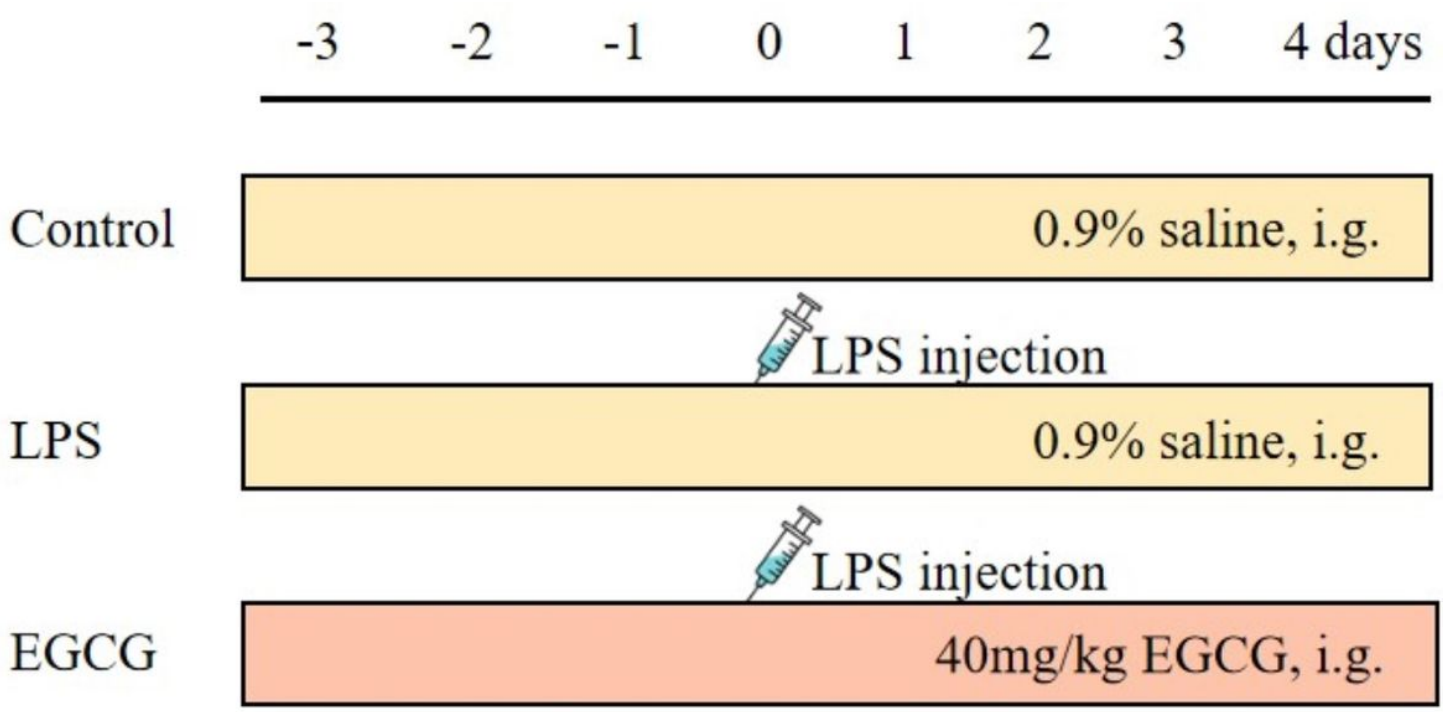

Figure 1

Schematic diagram of animal experiment. LPS: Lipopolysaccharide, EGCG: Epigallocatechin-3-gallate.

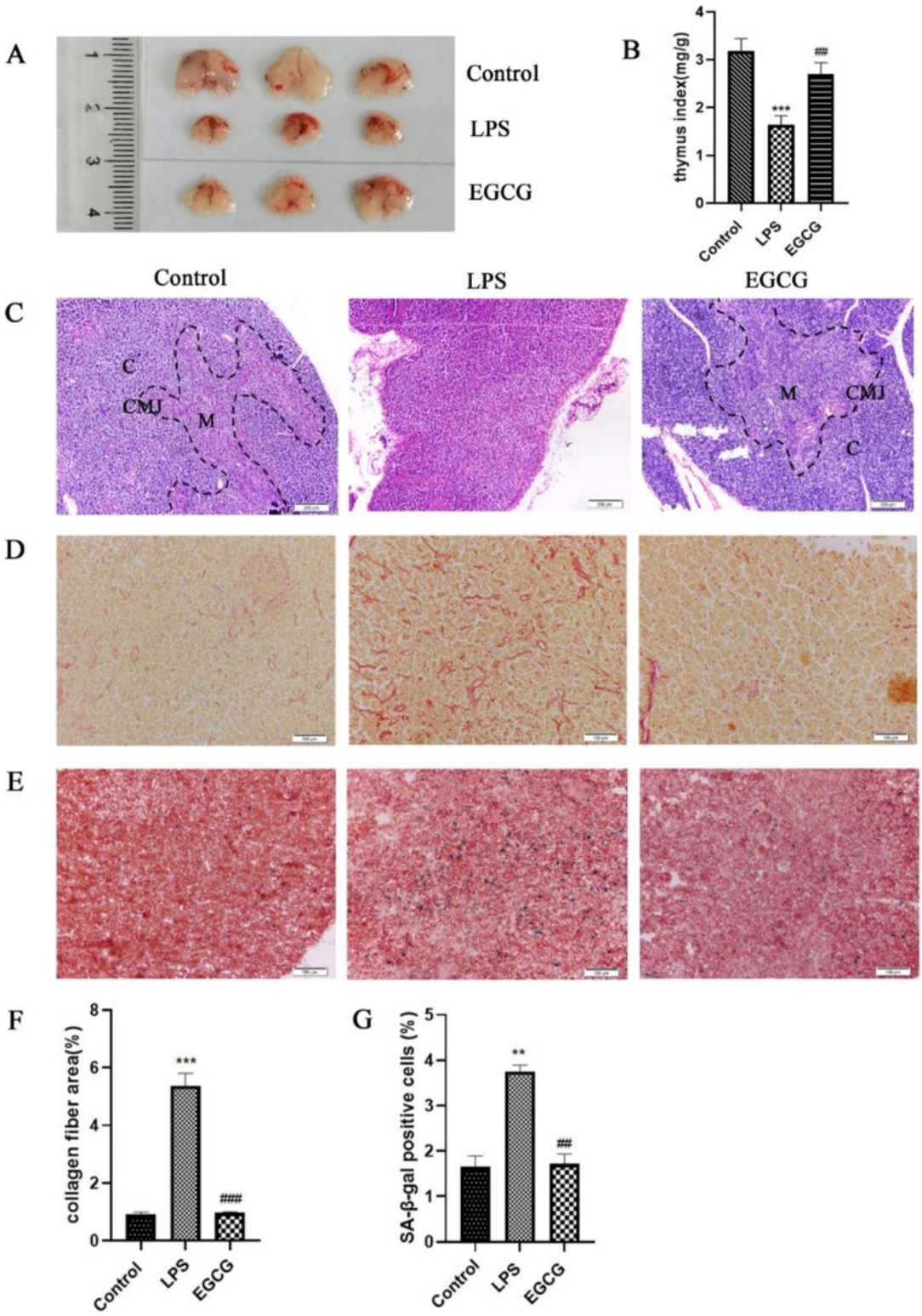

Figure 2

Thymus morphological alterations with LPS and EGCG administration. (A) Thymus size and (B) thymus index were measured. The thymus index was calculated as follows: thymus index (mg/g) = thymus weight (mg) / body weight (g). (C) Representative thymus tissues H&E sections of different groups. (D) Picro-sirius red staining for fibrosis in thymic tissue. (E) SA-β-Gal staining for senescent cells with frozen thymus sections. SA-β-Gal positive senescent cells were shown in blue, and nuclei were red with neutral

red staining. (F-G) Quantitative analysis of SA-β-Gal and Picro-sirius red staining data, respectively. C, cortex; M, medulla; CMJ, cortex-medulla junction; Black dotted line, cortex-medulla junction. Scale bar: (C) 200 μm, (D-E) 100 μm. The data were expressed as mean ± SEM, compared to the control group, $^{**}P < 0.01$, $^{***}P < 0.001$; compared to the LPS group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

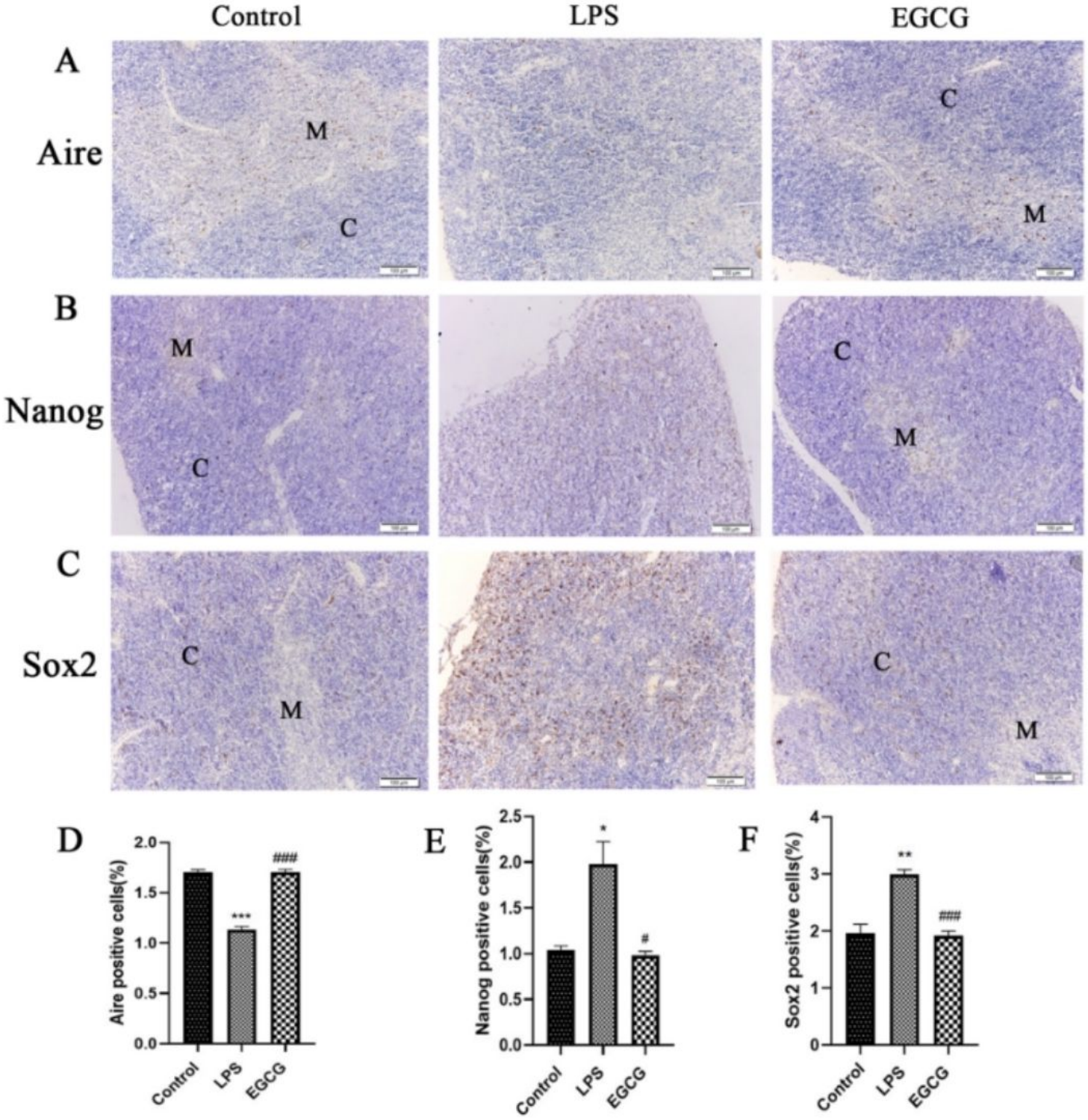

Figure 3

Immunohistochemical evaluation of Aire, Nanog and Sox2 with EGCG addition. (A-C) Representative immunohistochemical images of Aire, Nanog and Sox2. (D-F) Image J software was used to measure the density of brown-stained positive areas, and GraphPad Prism software was used to analyze the data. Scale bar: 100 μm. The data were expressed as mean ± SEM, compared to the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; compared to the LPS group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$.

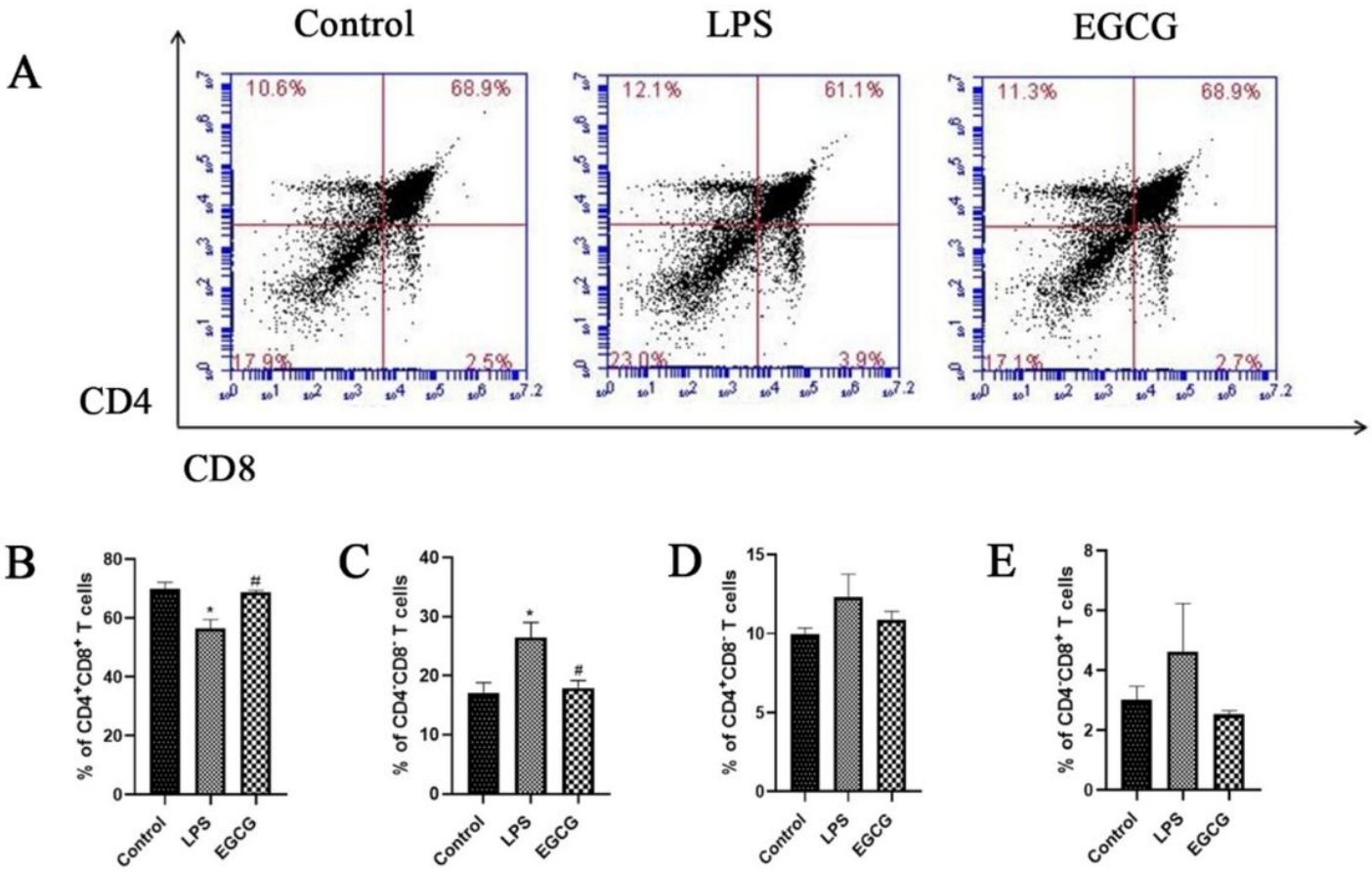

Figure 4

EGCG adjusted thymocyte subsets. The percentage of thymocyte subsets were detected by flow cytometry. (A) A representative flow cytometry dot plot. (B-E) Quantitative analysis the of CD4+CD8+, CD4-CD8-, CD4+CD8- and CD4-CD8+ thymocytes proportion. The data were expressed as mean ± SEM, compared to the control group, $^{*}P < 0.05$; compared to the LPS group, $^{\#}P < 0.05$.

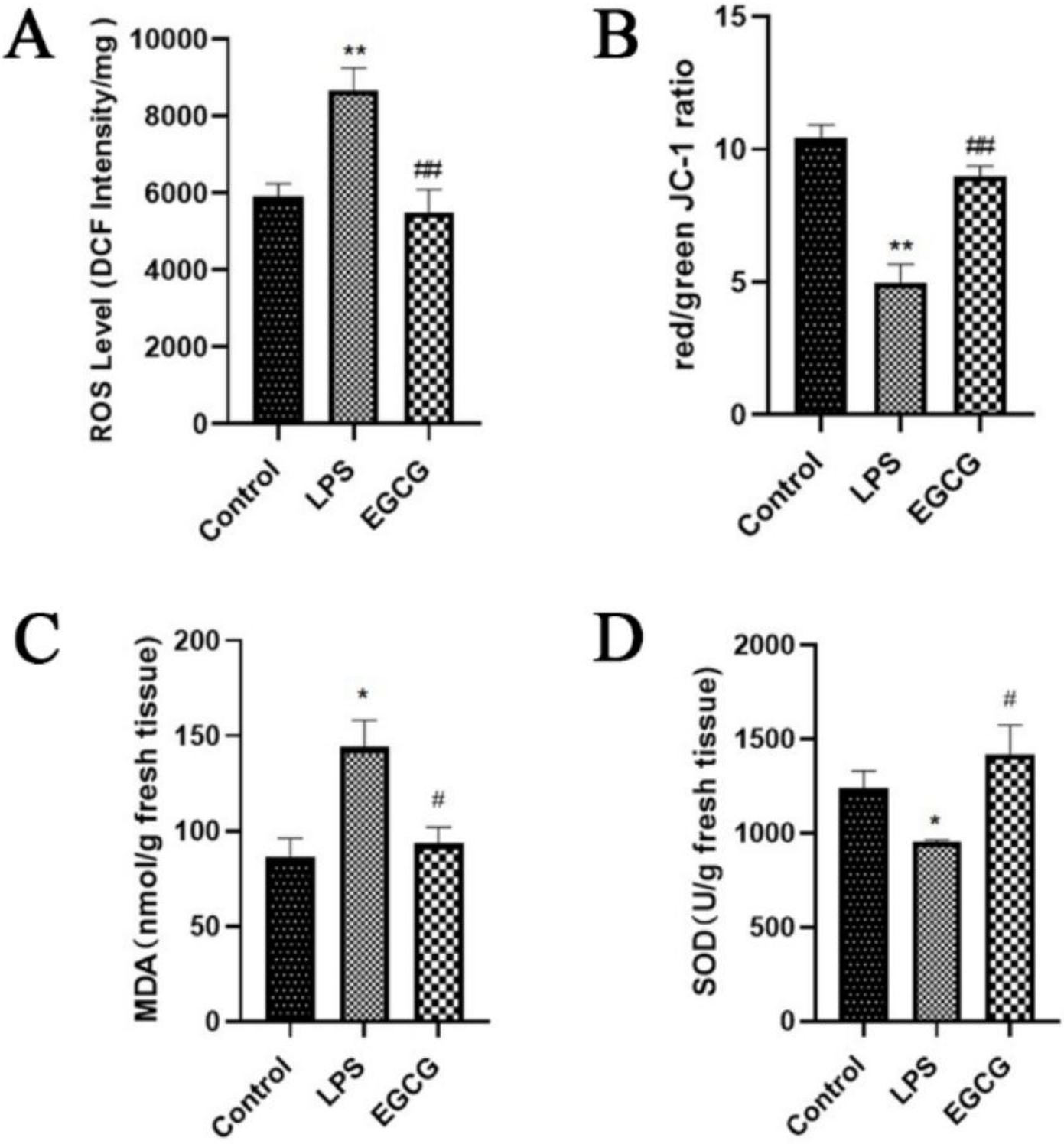

Figure 5

ROS level, mitochondrial membrane potential, MDA and SOD with EGCG addition. (A) The effects of EGCG administration on ROS level, (B) mitochondrial membrane potential (red/green JC-1 ratio), (C, D) MDA content and SOD activity. Data are the means ± SEM, compared to the control group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared to the LPS group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

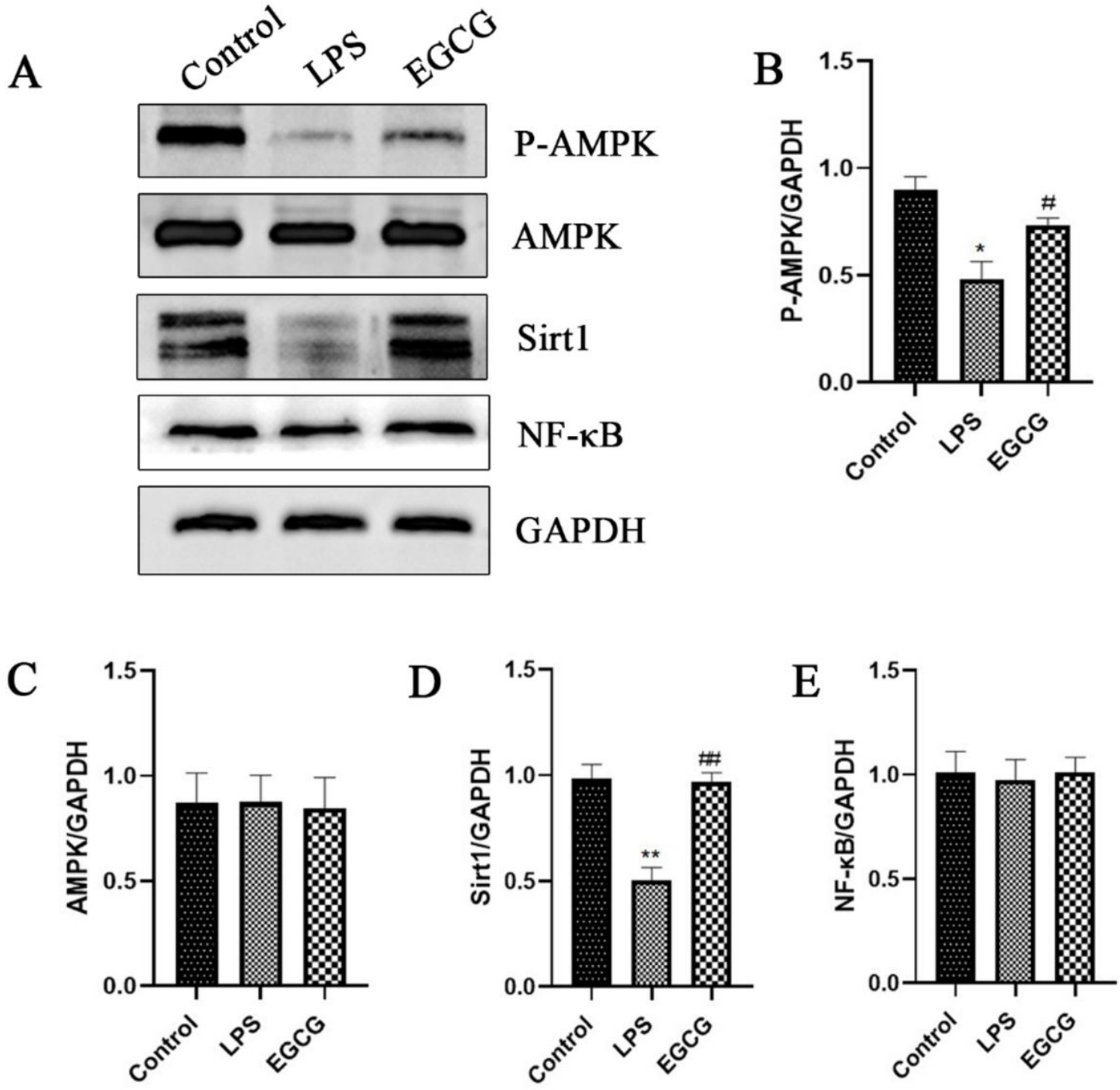

Figure 6

Western blotting evaluation of AMPK/Sirt1/NF-κB with EGCG addition. (A) The expression of AMPK, pAMPK, Sirt1 and NF-κB was detected by western blotting. (B-E) The density of bands was analyzed by Image J. The data were expressed as mean ± SEM, compared to the control group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared to the LPS group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.