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Original Article

USP18-driven macrophage M2 polarization through fatty acid β-oxidation inhibits immune responses induced by RSV infection

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Abstract

Introduction: Respiratory syncytial virus (RSV) is a leading cause of severe lower respiratory infections in infants under six months, with limited treatment options available. Macrophages play a pivotal role in antiviral immunity, where their polarization state directly modulates immune responses.

Methodology: The expression changes of ubiquitin-specific protease 18 (USP18) in RSV-infected samples were analyzed through the Gene Expression Omnibus database. An RSV infection model was established in C57BL/6 mice, and USP18 expression in lung tissues was measured by quantitative polymerase chain reaction (qPCR). Adenovirus-mediated USP18 knockdown was performed via tail vein injection, and its effects on immune cells were assessed using enzyme-linked immunosorbent assay (ELISA) and qPCR. THP-1 macrophage models with USP18 overexpression and knockdown were constructed. The effects of USP18 on macrophage polarization, immune function, and fatty acid β-oxidation during RSV infection were evaluated using immunofluorescence, ELISA, fatty acid oxidation (FAO) assays, boron-dipyrromethene (BODIPY) staining, and Western blot.

Results: USP18 was markedly overexpressed in RSV-infected samples. USP18 knockdown in mice alleviated lung damage, reduced M2 macrophage markers, and enhanced CD8+ T cell activity. Cellular experiments demonstrated that USP18 promotes M2 macrophage polarization by enhancing fatty acid β-oxidation and regulating the expression of related enzymes, including ACLY, FASN, and ACC1. Elevated USP18 in macrophages during RSV infection inhibits CD8+ T cell activation, contributing to immune suppression. These effects were reversible with FAO inhibitors.

Conclusions: USP18 upregulation in RSV-infected cells induces M2 macrophage polarization via fatty acid β-oxidation, thereby promoting immune suppression.

Key words: fatty acid β-oxidation; macrophages; respiratory syncytial virus; USP1.

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Introduction

Respiratory syncytial virus (RSV), a significant pathogen within the Pneumoviridae family, is the leading cause of lower respiratory infections in infants under 6 months of age, including bronchiolitis and pneumonia. Hospitalizations due to RSV are particularly high among young children, with the most severe cases occurring in premature infants and those with congenital heart disease or primary immunodeficiencies [1]. Current management of RSV infection largely relies on supportive care. While palivizumab, a monoclonal antibody for prophylaxis, has been approved for high-risk infants, its high cost significantly limits its accessibility [2]. Meanwhile, the development of RSV vaccines faces challenges, including viral immune evasion and safety concerns [3].

During RSV infection, the host immune response is marked by sophisticated regulatory mechanisms. The innate immune response is activated early in the infection process, with pattern recognition receptors (PRRs) triggering the production of interferons (such as IFNγ) and the release of inflammatory cytokines [3]. This initial response is not designed to completely prevent viral infection, but rather to reduce its impact and buy time for the adaptive immune system to engage. Subsequently, T cells within the adaptive immune system take action by recognizing and eliminating virus-infected cells [4]. Macrophages, which are key effectors of the innate immune system, are integral to the immunopathology of RSV infection. Research has shown that RSV infection can trigger an increase in *GAS6* expression, which in turn drives macrophages to adopt an M2 phenotype [5]. While this shift can be beneficial for tissue repair in certain contexts, it may contribute to adverse outcomes during RSV infection. Specifically, M2 macrophages secrete matrix metalloproteinase-12 (MMP12), which can recruit neutrophils and worsen allergic airway

inflammation [6]. In contrast, M1-like macrophages have been reported to reduce RSV infection in bronchial epithelial cells through a mediator-dependent mechanism [7]. Given these insights, targeting the polarization of macrophages could be a promising therapeutic avenue.

The focus on macrophage metabolic reprogramming in immune responses has intensified over the past few years. Lipid metabolism, a significant pathway in cellular energy metabolism, has been a key area of investigation. The functional state of immune cells is heavily impacted by fatty acid β-oxidation, which regulates intracellular acetyl-CoA levels and mitochondrial function; these regulatory actions have a direct effect on histone acetylation and the production of metabolic intermediates [8]. Fatty acid β-oxidation is a critical pathway in the function of various immune cells. For instance, excessive lipid accumulation can blunt the functionality of T cells and dendritic cells, thereby facilitating immune evasion by tumor cells within the tumor microenvironment [9]. The metabolic profiles of macrophages are a key determinant of their polarization. M2 macrophages typically generate energy through fatty acid β-oxidation, while M1 macrophages rely more on glycolysis [10]. This metabolic difference is essential for defining the functional phenotypes of these macrophage subsets. Macrophage fatty acid β-oxidation is regulated by multiple pathways and key genes. Notably, *FABP5* knockout or *FSP1* overexpression can both activate this metabolic pathway in a PPARγ-dependent manner, promoting the M2 phenotype [11,12]. Despite these insights, the specific mechanisms underlying fatty acid β-oxidation during RSV infection and its impact on macrophage polarization remain unclear.

Ubiquitin-specific protease 18 (USP18, also known as UBP43) is a pivotal enzyme that modulates the post-translational modification of interferon-stimulated gene 15 (*ISG15*), a critical pathway in cellular regulation. USP18 has been implicated in the pathogenesis of numerous human diseases, including infections, neurological disorders, and cancer [13]. Elevated USP18 expression appears to drive lipolysis and fatty acid oxidation (FAO), processes that may accelerate lung cancer progression [14]. The absence of USP18 in tumor-associated macrophages impacts their polarization and modulates their immunosuppressive functions [15]. But how it affects RSV infection is still being explored. USP18 is an important player in immune regulation and metabolic reprogramming. Thus, investigating its role and underlying mechanisms in RSV infection could be highly significant, both theoretically and clinically.

With this background, this research delves into the role of USP18 in RSV infection. Mouse models and cell experiments were used to determine that USP18 promotes fatty acid β-oxidation, which in turn fosters M2 macrophage polarization and dampens the adaptive immune response of the host. These results provide valuable insights into RSV infection mechanisms and support the development of innovative USP18-targeted therapies.

Methodology

Bioinformatics

The Gene Expression Omnibus (GEO) dataset (GSE188427) was used to compare *USP18* expression in blood samples from RSV-infected infants under 2 years old, and differences were flagged with false discovery rate (FDR) < 0.05 and |log2 Fold Change| > ± 1.

Cell culture

Human macrophage THP-1 cells and human alveolar epithelial cells (HPAEpiC) were obtained from Procell (Wuhan, China). CD8+ T cells, sourced from IMMOCELL (Xiamen, China), were cultured in a specialized medium. These T cells were activated with IL-2 prior to co-culture. THP-1 cells were maintained in RPMI-1640 medium, while HPAEpiC cells were cultured in their dedicated medium. All media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated at 37 °C in a 5% CO_2 incubator (Thermo Fisher, Waltham, MA, USA). In order to generate RSV-infected macrophages, THP-1 cells were first differentiated into M0 macrophages with a 24-hour PMA treatment (MCE, Monmouth Junction, NJ, USA) and then infected with RSV for an additional 24 hours. FBS and antibiotics were purchased from Gibco (Grand Island, NY, USA). The fatty acid metabolism inhibitor etomoxir was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell transfection

The pcDNA3.1 empty vector (oe-NC), the pcDNA3.1-USP18 plasmid (oe-USP18), the shRNA plasmid for USP18 knockdown (sh-USP18), and the pLKO.1-puro empty vector (sh-NC) were sourced from Genepharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was mixed with the plasmids at a 1:1 ratio in serum-free medium for transfection. After a 30-minute rest to form complexes, the mixture was added to cells in a 6-well plate when the cell confluence was about 80%. The cells were

incubated at 37 °C for 24 hours, after which the medium was replaced with serum-containing medium for an additional 48 hours. RNA was extracted to detect *USP18* mRNA expression and assess the knockdown efficiency.

In the case of the RSV infection experiments, the cells were infected with the RSV A2 strain (ATCC, USA) 24 hours after plasmid transfection. A viral concentration of 4.65×10^6 TCID50 particles per mL was used and the cells were incubated at 33 °C for 2 hours to facilitate the initial stages of infection. Following this, the medium was refreshed and the cells were cultured for another 24 hours to allow the infection to progress.

RNA extraction and quantitative polymerase chain reaction (qPCR)

The cells were lysed with trizol reagent (Solarbio, Beijing, China) and the lysate was collected into 1.5 mL centrifuge tubes. The lysate was treated with chloroform, isopropanol, and 75% ethanol, and then the RNA was pelleted by centrifugation. The supernatant was discarded, the pellet was dried, and the RNA was resuspended in DEPC-treated water. RNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher, Waltham, MA, USA). The RNA was reverse-transcribed into cDNA using the HiScript IV RT Kit (Vazyme, Nanjing, China) and stored at – 20 °C.

The qPCR reactions were prepared according to the kit protocol, using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and β-actin as the reference gene. The ABI7500 Real-Time PCR System (Thermo Fisher, Waltham, MA, USA) was used for detection. The relative expression of the genes was analyzed using the 2-△△Ct method. The primer information is provided in Table 1.

Western blot (WB)

Total protein was extracted from the cells using a total protein extraction kit (Solarbio, Beijing,China). The samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking with skimmed milk, the membrane was incubated with primary antibodies overnight at 4 °C. The primary antibodies used were anti-ACC1 (Proteintech, 21923-1-AP, Wuhan, China), anti-FASN (Proteintech, 10624-2-AP, Wuhan, China), anti-ACLY (Proteintech, 15421-1-AP, Wuhan, China), and anti-β-actin (Proteintech, 20536-1-AP, Wuhan, China). Following washing, the membrane was incubated with a secondary antibody IgG (Proteintech, SA00001-2, Wuhan, China) for 1 hour. Protein bands were detected using an enhanced ECL chemiluminescent detection kit (Vazyme, Nanjing, China) and visualized with a chemiluminescent imaging system (Tanon, Shanghai, China).

Table 1. Primers used in the analysis.

Primer name	Primers (5'—3')
Arg1-F (mouse)	TTTTAGGGTTACGGCCGGTG
Arg1-R (mouse)	TTTGAGAAAGGCGCTCCGAT
IL-10-F (mouse)	CAGTACAGCCGGGAAGACAA
IL-10-R (mouse)	CCTGGGGCATCACTTCTACC
USP18-F (human)	TTGCATGGCGCTTGAGAGAT
USP18-R (human)	CCCAAACGCCTTGCTCATTC
USP18-F (mouse)	CTGCAGGGTCTGTTCACCAT
USP18-R (mouse)	GGGCTGGACGAAACATCTCA
β-actin-F (human)	CTTCGCGGGCGACGAT
β-actin-R (human)	CCACATAGGAATCCTTCTGACC
β-actin-F (mouse)	GACTGTTACTGAGCTGCGTTT
β-actin-R (mouse)	AGGGTGAGGGACTTCCTGTA

Animal assay

In order to establish the RSV infection model, C57BL/6 neonatal mice were obtained from SPFBIOTECH (Beijing, China) and housed in a standard laboratory environment with free access to food and water. After a one-week acclimation period, the mice were randomly assigned into two groups: a control group (n = 3) and an RSV-infected group (n = 10). RSV infection was induced by intranasal inoculation. Mice were anesthetized with 3~4% sevoflurane, then inoculated intranasally with RSV long strain virus suspension at a volume of 0.1 mL per mouse, once daily. The control group received an equal volume of 0.9% sodium chloride solution without RSV long strain virus, 0.1 mL per mouse, once daily. The inoculation was performed for 3 consecutive days. Signs of successful infection included fever, nasal discharge, wheezing, rapid breathing, anorexia, hunched posture, and ruffled fur.

After the model was set up, adenovirus-packaged *USP18* shRNA or adeno-associated virus (AAV)-packaged sh-NC (5×10^9 particles/100 μL) was administered to the RSV group mice via tail vein injection. These groups were termed the sh-NC (n = 5) and sh-USP18 (n = 5) groups. This study was approved by the Animal Ethics Committee of Guangdong Medical Laboratory Animal Center (approval no. D202508-3).

Hematoxylin and eosin (H&E) staining

H&E staining was carried out using a kit from Solarbio (Beijing, China). Sections were deparaffinized with xylene and rehydrated through a graded ethanol

series. They were stained with hematoxylin for 5 minutes at room temperature, differentiated in hydrochloric acid–ethanol solution for a few seconds, and stained with eosin for 5 minutes at room temperature. The sections were then dehydrated in anhydrous ethanol for 5 minutes (3 times), cleared in xylene for 2 minutes at room temperature, mounted with neutral gum, and examined under an optical microscope.

Enzyme-linked immunosorbent assay (ELISA)

In order to determine the activation levels of T cells, the protein levels of IFN-γ, TNF-α, and IL-2 in tissue and cell samples were measured using ELISA kits from Elabscience (Wuhan, China). The cell supernatants or tissue homogenates were collected, 50 µL of each sample was added to 50 µL of horseradish peroxidase (HRP) solution, and incubated at 37 °C for 1 hour. After incubation, the samples were washed 3 times, substrate solution was added, and the mixture was incubated in the dark for 20 minutes. The reaction was terminated with a stop solution, and absorbance was measured at 450 nm using a microplate reader (Thermo Fisher, Waltham, MA, USA).

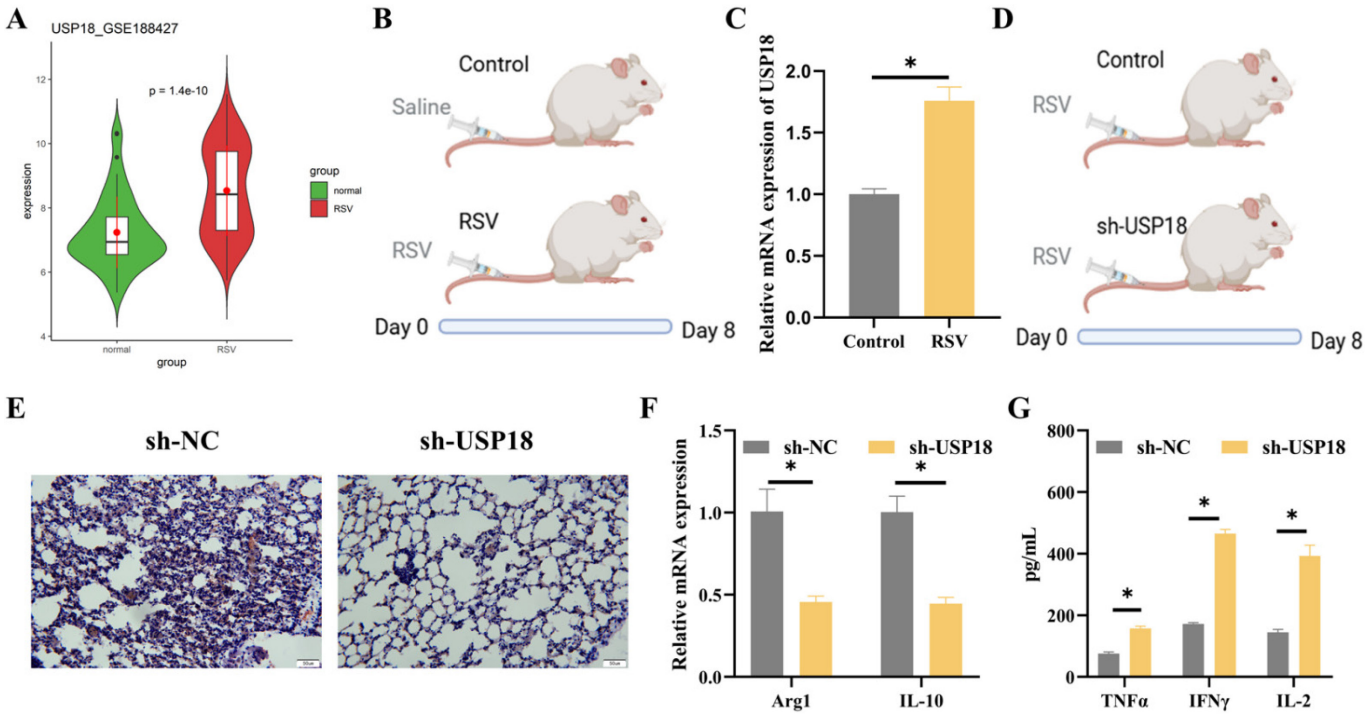
Detection of FAO

The FAO rate was measured using an FAO Assay Kit from Elabscience (Wuhan,China). After collecting and centrifuging 1×10^6 cells, the working solution was prepared according to the kit's instructions. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher, Waltham, MA, USA).

Boron-dipyrromethene (BODIPY) staining for lipid droplet quantification

A lipid droplet green fluorescence detection kit (BODIPY 493/503) from Beyotime (Shanghai, China) was used to detect lipid droplet accumulation in cells. The cells were cultured in a 6-well plate for 24 hours. The medium was then removed, and the cells were gently rinsed with phosphate-buffered saline (PBS) to clear any remaining medium. Subsequently, 1 mL of the staining solution was added to each well and incubated at room temperature in the dark for 20 minutes. After staining, the cells were washed with PBS to remove excess dye. Lipid droplets were observed under a fluorescence microscope by detecting green fluorescence.

Figure 1. Macrophage ubiquitin-specific protease 18 (USP18) expression enhances respiratory syncytial virus (RSV) infection and induces M2 polarization.



A: USP18 expression levels in normal and RSV-infected samples were analyzed using the Gene Expression Omnibus (GEO) database; **B:** An RSV-infected mouse model was established; **C:** USP18 mRNA expression levels were detected by quantitative polymerase chain reaction (qPCR); **D:** USP18 knockdown adenovirus was injected intravenously into RSV-infected mice; **E:** Lung damage was observed via hematoxylin and eosin (H&E) staining; **F:** Expression of M2 markers (Arg1, IL-10) in lung tissues was detected by qPCR; **G:** Levels of CD8+ T cell markers (TNFα and IFNγ) and CD4+ T cell cytokines (IL-2) in the lungs were measured by ELISA. *$p < 0.05$.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Cell proliferation was monitored using the MTT assay kit (Elabscience, Wuhan, China). The cells were seeded at 5,000 cells per well in a 96-well plate and allowed to settle. Post-treatment, cells were incubated with 50 μL of 1 × MTT solution for 4 hours. The supernatant was removed, and 100 μL of formazan dissolution solution was added to dissolve the crystals over a 2-hour period. Absorbance was subsequently measured at 570 nm using a microplate reader (Thermo Fisher, Waltham, MA, USA).

Immunofluorescence (IF)

The cells cultured in 6-well plates were fixed with 4% paraformaldehyde, permeabilized with 0.25% triton X-100 (Beyotime, Shanghai, China), and blocked with 3% BSA in PBS. They were then incubated with the primary antibody anti-CD206 (Proteintech, CL488-18704, Wuhan, China) at 4 °C for 1 hour, followed by a 30-minute incubation with the secondary antibody anti-IgG (Proteintech, SA00013-4, Wuhan, China) at room temperature. After 4′,6-diamidino-2-phenylindole (DAPI) staining, the cells were mounted on slides and imaged under a fluorescence microscope (Carl Zeiss, Jena, Germany).

Statistical analysis

The experiments were repeated at least 3 times to ensure reliability. Data are presented as mean ± standard deviation (SD). GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for data analysis and figure preparation. Student's t-test was used to determine significance, with $p < 0.05$ indicating a significant difference.

Results

Macrophage USP18 expression enhances RSV infection and induces M2 polarization

USP18 expression in healthy and RSV-infected samples were compared using the GEO database, revealing a substantial rise in *USP18* in RSV-infected blood (Figure 1A). This finding led to conducting an in vivo experiment using C57BL/6 mice. The mice were divided into control and RSV-infected groups. After 8 days, the mice were euthanized, and their lungs were collected for analysis (Figure 1B). qPCR analysis showed that *USP18* expression was notably higher in the lungs of RSV-infected mice (Figure 1C). An adenovirus was used to knock down *USP18* specifically in macrophages to explore the functional role of *USP18* in RSV infection, resulting in sh-USP18 and control sh-NC groups, which were then infected with the RSV A2 strain (Figure 1D). H&E staining showed that RSV infection induced marked inflammatory cell infiltration, interstitial congestion, and alveolar wall thickening in control lungs; strikingly, these pathological alterations were substantially alleviated when *USP18* was knocked down (Figure 1E).

When a virus infects a host, it often sets off a cascade of immune responses, including both innate and adaptive immunity. M2 macrophages are particularly important for tissue repair and immune suppression during viral infections, while cytokines including TNFα, IFNγ, and IL-2 from T cells help fine-tune the adaptive immune response. To understand how *USP18* affects these processes, the expression of M2 macrophage markers in lung tissue after knocking down *USP18* was investigated. qPCR revealed that the expression of these markers was remarkably reduced after *USP18* knockdown (Figure 1F). ELISA revealed that the secretion of TNFα, IFNγ, and IL-2 from CD8+ and CD4+ T cells in lung tissues appreciably increased after USP18 was knocked down (Figure 1G). Collectively, these results suggest that RSV infection triggered a marked upregulation of *USP18* in the lungs, and that *USP18* appeared to drive M2 macrophage polarization and dampen adaptive immune responses.

USP18-mediated M2 polarization of macrophages suppresses antiviral immune responses following RSV infection

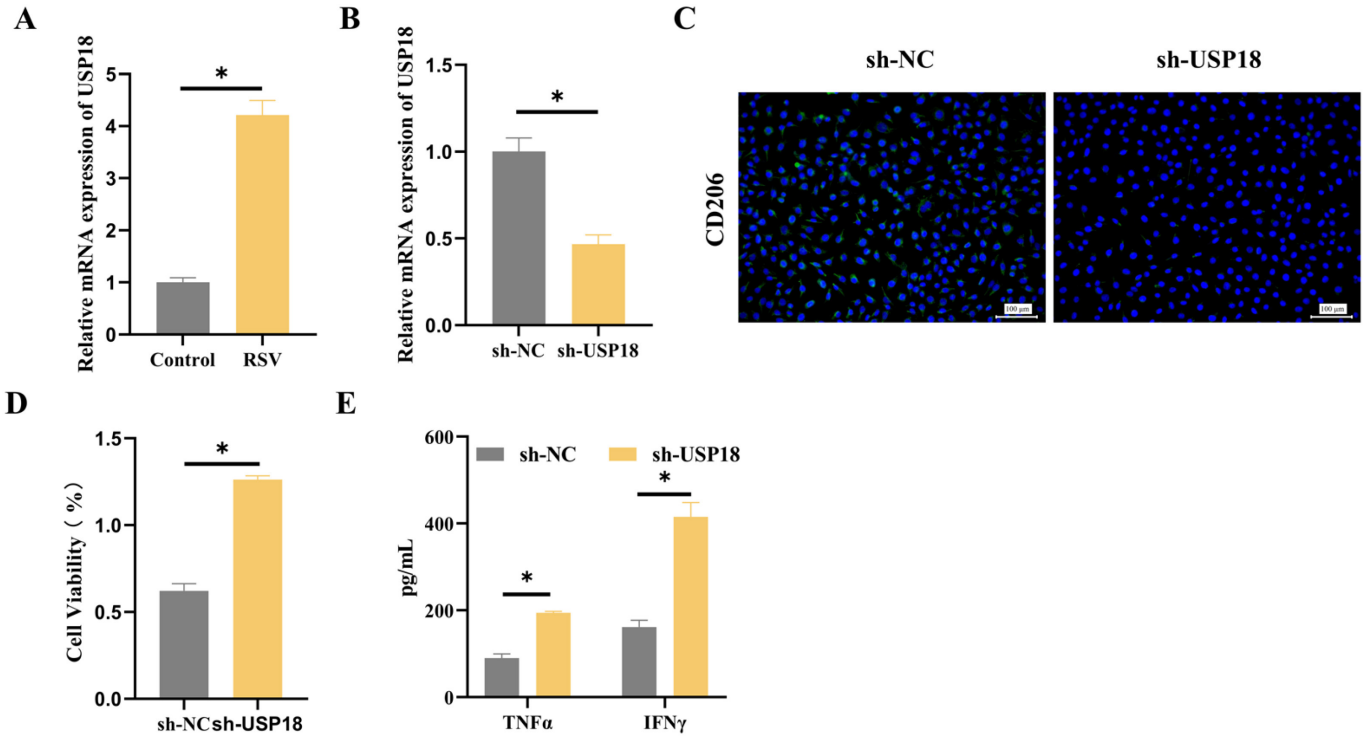
Previous research has shown that USP18 deficiency in macrophages can shift their polarization and suppress immune responses [15]. The mechanism by which RSV infection affects *USP18* expression in macrophages was first examined. qPCR results revealed that RSV infection markedly boosted *USP18* levels in these cells (Figure 2A). Thus, it was hypothesized that the high *USP18* expression in macrophages following RSV infection might alter their polarization and, in turn, modulate the immune response. The human THP-1 monocyte model was used to test this hypothesis. The THP-1 cells were differentiated into M0 macrophages with PMA for 24 hours, then infected them with RSV for another 24 hours to mimic the infection state. After knocking down *USP18*, efficient knockdown was confirmed via qPCR (Figure 2B). It showed reduced CD206 fluorescence intensity following *USP18* knockdown (Figure 2C), suggesting that *USP18* promotes M2 polarization in macrophages. To elucidate the impact of macrophage *USP18* on antiviral immunity, a co-culture system was established with human alveolar epithelial cells (HPAEPIC) and two

groups of macrophages. Following 24 hours of RSV infection, these cells were co-cultured with activated CD8+ T cells. MTT assays demonstrated that USP18 knockdown in macrophages dramatically enhanced HPAEPIC viability (Figure 2D). ELISA analysis revealed that TNFα and IFNγ secretion from CD8+ T cells was appreciably higher in the USP18-knockdown group compared to the control group (Figure 2E). These results suggest that USP18 expression in macrophages impairs CD8+ T cell function by promoting M2 polarization, thereby weakening antiviral immunity.

USP18 promotes M2 polarization of macrophages via fatty acid β-oxidation

USP18 levels can surge, triggering increased fat breakdown and FAO, which in turn fuels lung cancer growth [14]. In macrophages, a comparable spike in fatty acid β-oxidation can drive them into the M2 state and amp up allergic asthma severity [11]. Based on the findings from these studies, it was speculated that USP18 might mediate the M2 polarization of macrophages through the activation of fatty acid β-oxidation. Hence, macrophages overexpressing *USP18* were constructed and rescue experiments using the fatty acid metabolism inhibitor etomoxir (100 μM) were conducted. The expression of *USP18* in each group of cells was measured using qPCR, and it was found *USP18* expression was remarkably elevated in the overexpressing cells, and that the inhibitor did not affect *USP18* expression (Figure 3A). IF was employed to detect the M2 macrophage marker CD206. The results revealed that overexpression of *USP18* increased fluorescence intensity, whereas treatment with the inhibitor led to weaker fluorescence intensity (Figure 3B). Further evidence indicates that *USP18* regulates macrophage polarization through fatty acid metabolism. To further explore the impact of fatty acid oxidation (FAO) on macrophage polarization, the FAO levels were measured in macrophages from different groups using an FAO detection kit. *USP18* overexpression significantly increased FAO levels, while etomoxir treatment effectively reduced them (Figure 3C). BODIPY staining showed that *USP18* overexpression decreased lipid droplet content, whereas inhibitor treatment increased lipid droplet fluorescence intensity (Figure 3D). These findings provide further evidence that *USP18* regulates macrophage polarization through fatty acid metabolism. Western

Figure 2. Ubiquitin-specific protease 18 (USP18)-mediated M2 polarization of macrophages suppresses antiviral immune responses following respiratory syncytial virus (RSV) infection.



A-B: USP18 mRNA expression was quantified by quantitative polymerase chain reaction (qPCR); **C:** The macrophage marker CD206 was detected by immunofluorescence (IF); **D:** Epithelial cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; **E:** CD8+ T cell markers (TNFα and IFNγ) were measured by enzyme-linked immunosorbent assay (ELISA). *p < 0.05.

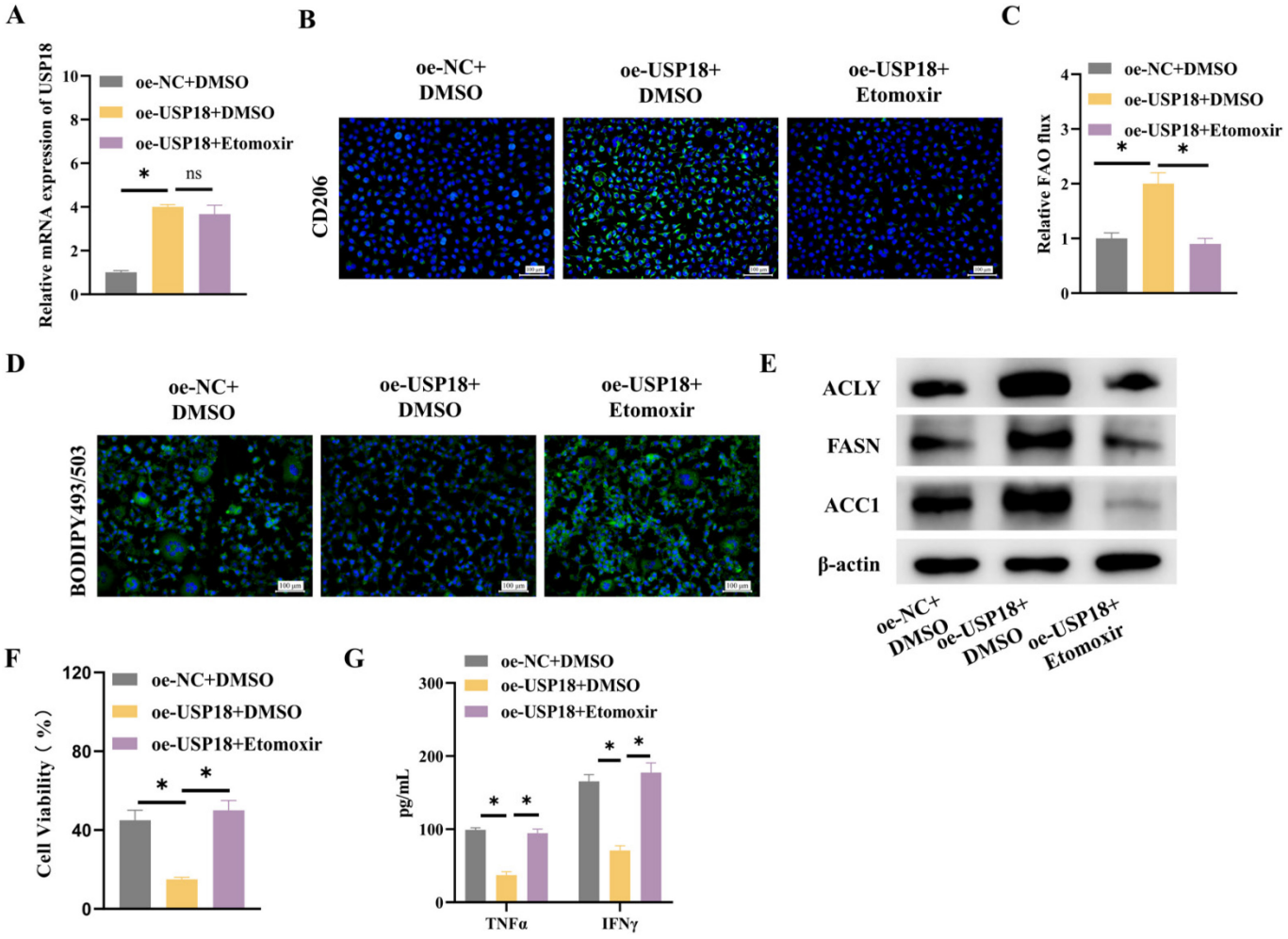
blot (WB) showed that key proteins involved in FAO, including ATP citrate lyase (ACLY), fatty acid synthase (FASN), and acetyl-CoA carboxylase 1 (ACC1), were upregulated in *USP18*-overexpressing cells and downregulated with the inhibitor (Figure 3E). Collectively, these findings suggest that *USP18* promotes M2 polarization of macrophages by activating FAO, thereby modulating immune responses.

Ultimately, the mechanisms that influence the immune response to RSV infection were analyzed. Macrophages were co-cultured with HPAEPIC cells and infected with RSV for 24 hours. After that, activated CD8+ T cells were introduced into the mix. The MTT assay was used to measure cell viability, and it was found that overexpression of *USP18* had a pronounced inhibitory effect on the viability of HPAEPIC cells. However, this inhibition could be effectively counteracted by treating the cells with a FAO inhibitor (Figure 3F). ELISA detected considerably lower levels of TNFα and IFNγ in the supernatant when *USP18* was overexpressed. These cytokines are vital for the proper functioning of CD8+ T cells. However, this reduction was effectively reversed by treatment with a FAO inhibitor (Figure 3G). These findings indicate that *USP18* could drive macrophage polarization towards the M2 phenotype through fatty acid β-oxidation, and subsequently inhibit CD8+ T cell activation and weaken the immune response to RSV infection.

Discussion

This study has revealed a previously unknown

Figure 3. Ubiquitin-specific protease 18 (USP18) promotes M2 polarization of macrophages via fatty acid β-oxidation (FAO).



A: USP18 mRNA expression was detected by quantitative polymerase chain reaction (qPCR); **B:** Expression of the macrophage marker CD206 was analyzed by immunofluorescence (IF); **C:** FAO rate was evaluated using a FAO assay kit; **D:** Intracellular lipid droplet content was measured using boron-dipyrromethene (BODIPY)493/503; **E:** Protein expression related to fatty acid β-oxidation (ACLY, FASN, and ACC1) was detected by Western blot (WB); **F:** Epithelial cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; **G:** CD8+ T cell markers (TNFα and IFNγ) were measured by enzyme-linked immunosorbent assay (ELISA). *$p < 0.05$.

mechanism by which RSV infection influences macrophage polarization. It was shown that in a mouse model, RSV infection triggers an evident increase in *USP18* expression in lung tissue. RSV-induced lung damage could be reduced by knocking down *USP18*. This intervention also led to decreased macrophage marker expression and increased levels of CD8+ and CD4+ T cell effector molecules, indicating a more effective immune response. When *USP18* was knocked down in macrophages, the expression of M2 macrophage markers was accordingly reduced. An RSV infection model was established using alveolar epithelial cells in co-culture. The cells in the knockdown group showed stronger viability. When co-cultured with CD8+ T cells, the supernatants from the knockdown group exhibited increased levels of IFNγ and TNFα. Meanwhile, macrophages overexpressing USP18 had higher FAO rates, with fewer lipid droplets and elevated expression of β-oxidation-related proteins. Co-culturing with alveolar epithelial cells enhanced the cytotoxicity of RSV against epithelial cells, while co-culturing with CD8+ T cells considerably reduced the expression of effector molecules. These changes were all reversed by a FAO inhibitor.

A prominent elevation in *USP18* expression in lung tissues was observed in the RSV-infected mouse model. Moreover, knocking down *USP18* notably alleviated pathological damage. Similar findings have been reported in human immunodeficiency virus (HIV) infection cases. *USP18* acts as a pleiotropic enhancer of HIV-1 replication. HIV-1 infection induces *USP18*, which robustly enhances HIV-1 replication by eliminating the antiviral function of p2116. *USP18*, a type I interferon-induced gene, is involved in the removal of ISG15—a ubiquitin-like protein—from target proteins [17]. ISG15 is a key antiviral factor in RSV infection. It restricts RSV replication and modulates the innate immune response through a process called ISGylation, where ISG15 is attached to other proteins [18]. This research has uncovered a novel role for *USP18* in viral infections, specifically through its impact on macrophage polarization. Following RSV infection, *USP18* expression in macrophages was notably upregulated, coinciding with an increase in M2 macrophage markers. These data suggest that *USP18* may promote a shift toward an anti-inflammatory macrophage phenotype. Additionally, it was observed that the levels of key pro-inflammatory cytokines, including TNFα, IFNγ, and IL-2, were negatively correlated with *USP18* expression.

The role of *USP18* in different types of viral infections is highly varied. Hou *et al*. demonstrated that *USP18* can bolster antiviral defenses against the Sendai virus and the encephalomyocarditis virus, hinting at its potential as a positive regulator in certain infection contexts [19]. However, the preponderance of evidence points to a more nuanced role for *USP18*, one that often involves immune suppression. To illustrate, the deficiency of *USP18* can lead to boosted IFNγ secretion from CD8+ T cells, thus accelerating the progression of autoimmune diseases [20]. Meanwhile, *USP18* overexpression can ramp up dengue virus resistance to IFNα by suppressing the interferon signaling pathway [21]. It was discovered that knocking down *USP18* in macrophages dramatically reduced the expression of *CD206*, a hallmark of M2 macrophages. In subsequent co-culture experiments, it was observed that this reduction greatly improved the survival of epithelial cells during RSV infection and enhanced the effector functions of CD8+ T cells. These results align with recent findings in the field of oncology. For example, in the study of tumor microenvironments, *USP18* has been shown to potently drive macrophages toward the M2 phenotype by inhibiting the *STAT1* signaling pathway, thereby facilitating the tumor to evade the immune system [15]. Li *et al*. further demonstrated that *USP18* deficiency in macrophages can regulate glycolysis-related genes and impact mitochondrial activity, ultimately steering macrophages toward the M1 phenotype [22]. Together, these studies suggest the critical role of *USP18* in modulating cellular metabolism and immune cell polarization.

In this study, it was found that *USP18* had a profound effect on FAO in macrophages. FAO detection assays and BODIPY staining revealed that *USP18* overexpression notably elevated FAO while diminishing lipid droplet accumulation within cells. This mechanism has been previously discussed in lung cancer, where *USP18* promotes tumor growth by enhancing fatty acid metabolism [14]. The findings establish a direct link between this mechanism and macrophage immune function in viral infections for the first time, suggesting a broader role for *USP18* in immune regulation. Additionally, it was noted that overexpression of *USP18* led to increased expression of M2 markers such as *CD206*, which subsequently reduced the viability of epithelial cells and the activation of CD8+ T cells. These changes could be restored using a fatty acid β-oxidation inhibitor. Viral infections generally induce metabolic reprogramming in host immune cells, which substantially impacts their functional states and immune response capabilities. Srikanth *et al.* observed that RSV infection leads to elevated *ACC1* expression in dendritic cells (DCs), a

key enzyme involved in fatty acid metabolism [23]. By targeting *ACC1*, they were able to improve mitochondrial function and enhance the antiviral Th1 response, effectively mitigating the pathogenic effects of RSV infection in both experimental and clinical settings. This study further supports the idea that enhanced fatty acid β-oxidation is a hallmark of RSV infection across various immune cells. This metabolic alteration appears to be crucial for the ability of the virus to evade immune detection and thus impacts the overall immune response of the host.

Conclusions

This study has illuminated how *USP18* orchestrates M2 macrophage polarization via fatty acid β-oxidation, thereby dampening the adaptive immune response during RSV infection. This insight not only enriches our knowledge of the function of *USP18* and the pathogenesis of RSV, but lays the groundwork for developing novel therapeutic approaches to combat RSV, offering promising targets for intervention.

However, the present research is not without its limitations. Initially, the study was largely based on cell and mouse models, and the correlation and significance of the *USP18*-fatty acid β-oxidation-M2 polarization axis require further substantiation in clinical samples. Moreover, it is still uncertain whether the regulation of M2 polarization by *USP18* is entirely dependent on fatty acid metabolism, as other downstream pathways may also contribute.

Funding

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Data availability

The data and materials in the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was approved by the Animal Ethics Committee of Guangdong Medical Laboratory Animal Center (approval no. D202508-3).

Authors' contributions

DY, LL: study design; LZ: literature search; JZ: data acquisition; DY: manuscript writing; LZ: data analysis, manuscript draft; LL: manuscript revision. All authors approved the final version of the manuscript.

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Conflict of interest

No conflict of interest is declared.

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