

Original Research

Regulation of Macrophage Efferocytosis by the CXCL10–CXCR3 Axis and CD36 in Acute Lung Injury

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Abstract

Background: Macrophage efferocytosis is the primary mechanism for clearing apoptotic cells (ACs) *in vivo* and is critical for maintaining tissue homeostasis. In acute lung injury (ALI), the C-X-C motif chemokine ligand 10 (CXCL10)–C-X-C motif chemokine receptor 3 (CXCR3) axis is upregulated and contributes significantly to pulmonary inflammation by regulating macrophage migration, invasion, polarization, and autophagy. However, whether this axis directly modulates macrophage efferocytosis remains unknown. **Methods:** ALI models, both *in vivo* and *in vitro*, models were used to assess the impact of CXCL10–CXCR3 axis on macrophage efferocytosis. Using the CXCR3 antagonist AMG487, we assessed efferocytic capacity and examined the cluster of differentiation 36 (CD36)-ubiquitin-like 4A (UBL4A) interaction under inflammatory conditions, revealing that UBL4A-mediated CD36 degradation regulates efferocytosis. **Results:** Efferocytosis was impaired in lung tissue and macrophages in ALI. Blocking the CXCL10–CXCR3 axis significantly enhanced efferocytosis *in vivo*. Mechanistically, inflammatory conditions promoted CD36 degradation by upregulating UBL4A. Treatment with AMG487 downregulated UBL4A expression, thereby preventing CD36 degradation and preserving efferocytosis *in vivo*, while attenuating UBL4A-mediated CD36 ubiquitination and degradation *in vitro*. **Conclusions:** The CXCL10–CXCR3 axis negatively regulates macrophage efferocytosis during ALI through UBL4A-mediated CD36 degradation. Targeting this axis with AMG487 preserves efferocytosis by restoring CD36 levels, suggesting that this approach represents a potential therapeutic strategy for ALI and establishing a theoretical foundation for future targeted interventions.

Keywords: macrophages efferocytosis; acute lung injury; chemokine CXCL10; CXCR3; CD36 antigens; UBL4A protein; receptors

1. Introduction

Apoptosis is an essential form of programmed cell death required for normal biological processes [1]. Inflammation triggers apoptosis in multiple cell types, such as infected and mechanically damaged cells [2]. Under physiological conditions, apoptotic cells (ACs) can scarcely be detected in tissues due to their rapid removal, which occurs through a process termed efferocytosis [3]. In many non-resolving chronic inflammatory diseases, efferocytosis is often functionally impaired, leading to the accumulation of ACs and subsequent secondary necrosis. This accumulation leads to pathological inflammation, tissue injury, and autoimmune diseases [4]. During caspase-dependent apoptosis, ACs fragment into smaller apoptotic bodies that are subsequently engulfed by macrophages, preventing the leakage of intracellular contents and pro-inflammatory factors. Conversely, the secondary lysis of accumulated uncleared ACs results in the release of damage-associated molecular patterns (DAMPs), including genomic and mitochondrial DNA, IL-1 α , IL-33, IL-36, ATP, and UTP, trig-

gering macrophage inflammatory responses [5]. This highlights the importance of efficient AC clearance for dampening the inflammatory response in acute lung injury (ALI).

Macrophages are central to efferocytosis *in vivo* [4]. This process occurs in three distinct stages [6]. First, ACs release early signals and chemokines, such as lysophosphatidylcholine, sphingosine-1-phosphate, and CXCL10, that direct macrophage migration toward them. Second, macrophages utilize secreted bridging factors (growth arrest-specific 6 [GAS6] and milk fat globule-EGF factor 8 [MFG-E8]) along with surface receptors (CD36) to recognize phosphatidylserine on ACs, driving recognition and binding [7,8]. Finally, macrophages engulf the ACs, forming a phagosome and completing their clearance [9,10]. Alveolar macrophages (AMs) function as the primary immune defense in the lungs, making up roughly 90% of local resident pulmonary immune populations [11]. Following the depletion of AMs, the levels of pro-inflammatory factors rise sharply, accompanied by decreased lung compliance and increased vascular permeability [12,13]. Over the subsequent 5 days, inflammatory cell infiltration con-



tinues to increase, expanding the damaged area within the lungs [14]. Furthermore, AMs undergo M1 polarization, releasing pro-inflammatory cytokines (TNF- α , IL-1, IL-6, and IL-12), nitric oxide, and reactive oxygen species [15]. Existing research confirms that GTS-21 acting as an $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) agonist can restrain M1 phenotypic shift of AMs and reduce the abundance of pro-inflammatory factors [16].

The CXCL10–CXCR3 axis is strongly linked to pulmonary inflammation, with both CXCL10 and its receptor, CXCR3, showing notably elevated expression across ALI models triggered by lipopolysaccharide (LPS), viral infections together with mechanical injury [17,18]. In patients with COVID-19, CXCL10 acts as a key chemokine that triggers the inflammatory cytokine storm [19]. The CXCL10–CXCR3 axis also regulates macrophage polarity, thereby influencing the ALI process [20]. CXCL10 is essential for macrophage responsiveness to inflammatory signals. During early stages of inflammation, macrophages migrate toward the inflamed area in response to CXCL10, driving a localized inflammatory response [21]. CXCL10 deficiency ameliorates disease severity by inhibiting macrophage infiltration, and CXCL10 induces macrophage migration via CXCR3 [22]. At the signaling level, CXCL10 activates intracellular pathways, including JAK and STAT1, which promote M1 macrophage polarization and induce pulmonary tissue inflammation. Conversely, inhibiting either CXCL10 or CXCR3 reduces the M1/M2 ratio in pulmonary macrophages and lowers the secretion of pro-inflammatory factors [23].

CD36 exerts a pivotal function during macrophage efferocytosis. In the peritonitis models, removing CD36 from the macrophage membrane leads to the accumulation of ACs, thereby inhibiting macrophage efferocytosis. Similarly, knocking down CD36 yields comparable results [24]. This indicates that impaired AC clearance directly drives AC accumulation and subsequent inflammatory cascades. Functioning as a surface receptor, CD36 participates in macrophage inflammatory signaling through Toll-like receptors (TLRs) and downstream mitogen-activated protein kinase (MAPK) cascades. Additionally, CD36 regulates macrophage migration, adhesion, and invasion *via* the focal adhesion kinase (FAK) signaling pathway [25,26]. Activation of CD36 also induces mitochondrial metabolic gene reprogramming in macrophages, driving a chronic inflammatory response [27]. Oxidized lipoproteins and lipids on ACs serve as activating stimuli for CD36, with oxidized phosphatidylcholine acting as a primary ligand [28]. Consequently, CD36 influences the entire process of macrophage efferocytosis.

In this study, we explored how the CXCL10–CXCR3 axis regulates macrophage efferocytosis and the underlying molecular mechanisms. Our findings showed that CD36 is a key mediator linking CXCL10–CXCR3 signaling to efferocytic function. We observed that inflam-

matory conditions impair efferocytosis in both lung tissue and macrophages, whereas inhibiting the CXCL10–CXCR3 axis protects efferocytosis across animal models and cellular experiments. These findings advance our understanding of how the CXCL10–CXCR3 axis modulates macrophage activities, offering new theoretical foundations for both ALI prevention and clinical intervention.

2. Materials and Methods

2.1 Experimental Animals

C57BL/6 mice (male and female, 7–8 weeks old, 18–20 g weight; SKBex Biotech, Henan, China) were acclimatized for 7 days before experiments. Mice were randomly assigned to three experimental groups ($n = 4$ group, 2 males and 2 females per group; total $N = 12$) using a random number table: a control group, a poly(I:C) treatment group, and poly(I:C) combined with AMG487 group (APEX BIO, Houston, TX, USA). Mice were anesthetized with the inhalational anesthetic isoflurane (Ruiwode, Shenzhen, China). The trachea was then surgically exposed, followed by direct delivery of poly(I:C) (2.5 mg/kg) into the airway cavity. Six hours post-instillation, AMG487 (5 mg/kg) or an equivalent normal saline dosage was administered *via* intraperitoneal injection. Lung tissues were collected 18 hours afterward for follow-up assays. At the end of the experiment, mice were euthanized by inhalational anesthetic isoflurane followed by cervical dislocation to confirm death. All data collection and histological scoring were performed by investigators blinded to group allocation. Group identity was decoded only after all quantitative analyses were completed.

This study was performed following the China Council on Animal Care and Protocol guidelines. The procedures for animal care and use were approved by the Ethics Committee of Bengbu Medical University (approval number 2025-686). All applicable institutional and governmental regulations concerning the ethical use of animals were followed.

2.2 Cell Culture and Treatment

RAW264.7 cells (SCSP-5036, Cell Bank of Chinese Academy of Sciences, Shanghai, China) were incubated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA). Cells were kept at 37 °C within a humidified incubator filled with 5% CO₂. The passage 5–15 of the cells used in this study. The cells were plated in six-well plates and stimulated with CXCL10 (PeproTech, Rocky Hill, NJ, USA), AMG487 alongside poly(I:C) for 24 h, respectively. All cell lines were validated by short-tandem repeat (STR) profiling and tested negative for mycoplasma.

For CD36 and ubiquitin-like 4A (UBL4A) knock-down in RAW264.7 cells, target siRNAs were introduced using Lip8000 Transfection Reagent (Beyotime, Shanghai, China) when cells reached 80% confluence. Knock-

down efficiency was assessed by western blot at 24 h post-transfection. The siRNA sequence is shown in Table 1.

Table 1. Sequence of siRNA.

siRNA name	siRNA sequence
siRNA CD36	GGATTGGAGTGGTGATGTT
siRNA UBL4A	AGCTCAACCTAGTTGTAA

RAW264.7 cells were pretreated with selective inhibitors targeting major CXCR3 downstream cascades, including Bay 11-7082 (10 μ M, NF- κ B inhibitor), Ruxolitinib (10 μ M, JAK/STAT inhibitor), SB203580 (20 μ M, p38 MAPK inhibitor), and U0126 (10 μ M, ERK inhibitor) (Selleck, Houston, TX, USA) for 2 h, followed by stimulation with poly(I:C) (50 μ g/mL) for 24 h. Cells were then harvested for Western blot analysis.

2.3 Neutral Red Phagocytosis Experiment

Cells were seeded into a 96-well plate and cultured for 6 h. Next, drug-containing complete culture medium was prepared for the various experimental groups, with CXCL10 concentrations set at 0, 20, 100, 250, and 500 ng/mL, and AMG487 concentrations at 0.2, 1.0, 2.5, and 5.0 μ M. After 6 h, the 96-well plate was removed, and the medium was replaced with 100 μ L of drug-supplemented full growth medium per well. The plate was incubated at 37 °C, 5% CO₂ for 24 h. Subsequently, add 50 μ L of Neutral Red Staining Solution (Beyotime) to each well and incubate at 37 °C for 2 h. Following incubation, discard the old culture medium, wash the wells three times with PBS to eliminate excess neutral red dye, and add 100 μ L of the cell destaining solution to each well. Incubate at room temperature for 1 h. Measure the optical density of each sample at 540 nm.

2.4 RNA Isolation and Quantitative

Total RNA was isolated from RAW264.7 cells as well as mouse lung tissue with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Aliquots of 2 μ g total RNA per sample were reversely transcribed to generate single-stranded cDNA within a 10 μ L reaction system via the BeyoRT III First Strand cDNA Synthesis Kit (Beyotime). Quantitative real-time PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). Target gene expression was normalized to GAPDH, and relative fold changes were calculated using the $2^{-\Delta\Delta C_t}$ method. All experiments were performed in triplicate. Sequence information of the primers used is shown in Table 2.

2.5 Protein Extraction and Western Blot

Protein lysates were prepared by RIPA buffer lysis (Solarbio, Beijing, China) on ice followed by centrifugation (12,000 rpm, 10 min). Supernatant protein content was quantified via bicinchoninic acid (BCA) assay (Bey-

otime). Equal protein loads were resolved on 10% SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes for 2 h, and membrane sections corresponding to target molecular weights were probed with primary antibodies (anti-GAPDH cat. no. 60004-1-Ig, dilution 1:2000, anti-CD36 cat. no. 32731-1-AP, dilution 1:1000, anti-UBL4A cat. no. 14253-1-AP, dilution 1:1000, anti-AXL cat. no. 68108-1-Ig, dilution 1:500, anti-PROS cat. no. 16910-1-AP, dilution 1:500, anti-ubiquitin cat. no. 10201-2-AP, dilution 1:500; Proteintech, Wuhan, China) at 4 °C overnight. Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies (goat anti-rat IgG [H+L] and goat anti-rabbit IgG [H+L]) (Beyotime) for 2 h. After washing with Tween-20 (TBST), protein bands were visualized using an ultra-sensitive ECL chemiluminescence kit (Beyotime) and imaged on Tanon-5200 imaging system (Tanon, Shanghai, China).

For densitometric analysis, the band intensities of the target proteins were quantified using ImageJ 1.53e (National Institutes of Health, Bethesda, MD, USA) and normalized against the loading control GAPDH for each corresponding sample. The relative protein expression levels were expressed as the ratio of the target protein to GAPDH.

2.6 Co-Immunoprecipitation

RAW264.7 cells or lung tissue proteins were lysed using lysis buffer on ice for 15 min, followed by centrifugation to collect the cell or tissue lysate. The ubiquitination antibody, CD36 antibody, and control immunoglobulin IgG (Beyotime) were co-incubated with magnetic beads under ambient temperature for 1 h. Subsequently, the magnetic beads were blended with prepared cell lysate and kept rotating overnight at 4 °C. Following bead rinsing steps, isolated protein complexes were transferred onto gels to perform immunoblot detection.

2.7 Oil Red O Staining

CXCL10 (250 ng/mL) and AMG487 (2.5 μ M) were applied to RAW cells seeded in 6-well plates for 24 h. The cells were then washed with PBS and cultured in high-fat medium at 37 °C, 5% CO₂ for 12 h. After incubation, the 6-well plates were removed and washed with PBS. Cells were fixed (4% Paraformaldehyde (PFA), 20 min), rinsed briefly with staining wash buffer, and incubated with Modified Oil Red O solution (Beyotime) at 37 °C for 30 min. After discarding the dye, cells were rinsed with wash buffer (30 sec) and washed three times with distilled water (2 min each).

2.8 Confocal Laser Scanning Microscope

Macrophages were seeded in confocal dishes at an appropriate density and cultured for 12 h. Macrophages were then labeled with Fluorescein isothiocyanate (FITC) (Beyotime), followed by two washes with complete medium. A549 cells in log-phase growth were cultured to 70–80% confluency, trypsinized, and exposed to UV irradiation

Table 2. Sequence of primers for RT-qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>CD36</i>	TTTCCTCTGACATTTGCAGGTCTA	AAAGGCATTGGCTGGAAGA
<i>GAS6</i>	TGCTGGCTTCCGAGTCTTC	CGGGGTCGTCTCGAACAC
<i>MFGE8</i>	CAGCCGTCCCCAATACTGC	GGGAGGCTAGGTTGTTGGAA
<i>AXL</i>	TGAGCCAACCGTGGAAGA	AGGCCACCTTATGCCGATCTA
<i>PROS</i>	CGCCGTGCAAATACCTTGTT	AATGAGCCAACACGGAATGC
<i>GAPDH</i>	CTCCGTGTTCTACCC	GCCCAAGATGCCCTTCA

(254 nm, 30 min) followed by 4 h incubation at 37 °C to induce apoptosis. ACs were labeled with Annexin V-PE (Beyotime) in Annexin V binding buffer for 15 min in the dark, washed once with binding buffer, and resuspended in serum-free DMEM. Labeled ACs were co-cultured with treated macrophages at a 5:1 ratio, incubated at 37 °C, 5% CO₂ for 2 h. After three PBS washes to remove unbound ACs, samples were treated with 0.04% trypan blue for 1 min to quench extracellular fluorescence, then fixed with 4% paraformaldehyde for 20 min. Following fixation, cells were permeabilized with 0.1% Triton X-100 for 5 min, blocked with 1% BSA for 30 min, and stained with Hoechst33342 for 5 min. Macrophage efferocytosis was evaluated using confocal laser scanning microscopy (CLSM), and the resulting micrographs were analyzed using ImageJ 1.53e software. For each condition, at least 5 random fields were selected. The efferocytic index was calculated as the ratio of phagocytosed ACs (Annexin V-PE positive signal within macrophage boundaries) to total macrophage count per field, expressed as a percentage.

2.9 HE Staining and Lung Scoring of Mouse Lung Tissue

Following fixation in 4% PFA, lung specimens underwent routine dehydration and paraffin embedding. Sections (4 µm) were cut and stained with hematoxylin-eosin for histological assessment. Lung injury was scored using a semi-quantitative system based on American Thoracic Society (ATS) criteria. The total score of each mouse was obtained by averaging values collected from ten independent visual fields. Elevated scores correspond to aggravated lung injury. To ensure consistency, all sections were scored on the same day by the same observer, with the intra-observer coefficient of variation being less than 5%.

2.10 Statistical Analysis

All experiments were performed in at least triplicate. Data are expressed as means ± standard deviation. Statistical analyses were conducted with GraphPad Prism 9.3.0 software (GraphPad Software, San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons. $p < 0.05$ was considered statistically significant.

3. Results

3.1 CXCL10–CXCR3 Activator CXCL10 Promotes Macrophage Efferocytosis

Studies have reported that CXCL10 paired with its cognate receptor CXCR3 shows elevated expression during inflammatory responses, with CXCL10 driving the chemotaxis of surrounding immune cells [17,29]. To evaluate how CXCL10 influences macrophage efferocytosis, RAW264.7 cells were stimulated with this chemokine. A neutral red phagocytosis assay was first performed to determine the optimal stimulation concentration. The results indicated that neutral red uptake by RAW264.7 cells increased significantly at CXCL10 concentrations of 100 and 500 ng/mL (Fig. 1A), Establishing 250 ng/mL as the optimal dose for subsequent experiments. Key molecules involved in AC clearance include the soluble bridging factors GAS6, MFG-E8, and Protein S (PROS1) [30,31], as well as receptor tyrosine kinases such as AXL (AXL receptor tyrosine kinase). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis revealed that CXCL10 treatment upregulated the mRNA levels of *Gas6*, *Mfge8*, *Axl*, and *Pros1* (Fig. 1B). Western blot analysis further demonstrated that CXCL10 elevated the cellular abundance of AXL and PROS (Fig. 1C). Finally, immunofluorescence staining confirmed that CXCL10 treatment enhanced the phagocytosis of ACs by macrophages. Quantitative analysis of the fluorescence intensity of Annexin V-PE signals co-localized with macrophages was performed using ImageJ software. Line-scan analysis of fluorescence intensity was further performed to verify the co-localization of macrophages (FITC, green trace) and engulfed ACs (Annexin V-PE, red trace). The left line-scan profile corresponds to the Ctrl group, while the right profile corresponds to the CXCL10-treated group. Greater overlap between the green and red fluorescence peaks in the CXCL10 group indicated increased internalization of ACs by RAW264.7 macrophages (Fig. 1D).

3.2 The CXCL10–CXCR3 Antagonist AMG487 Inhibits Macrophage Efferocytosis

AMG487 functions as a CXCL10–CXCR3 antagonist capable of restraining macrophage polarization toward the M1 phenotype, thereby alleviating autoimmune prostatitis [32]. Additionally, AMG487 regulates the expression levels of Arg-1, VEGF and TNF-α within macrophages, facil-

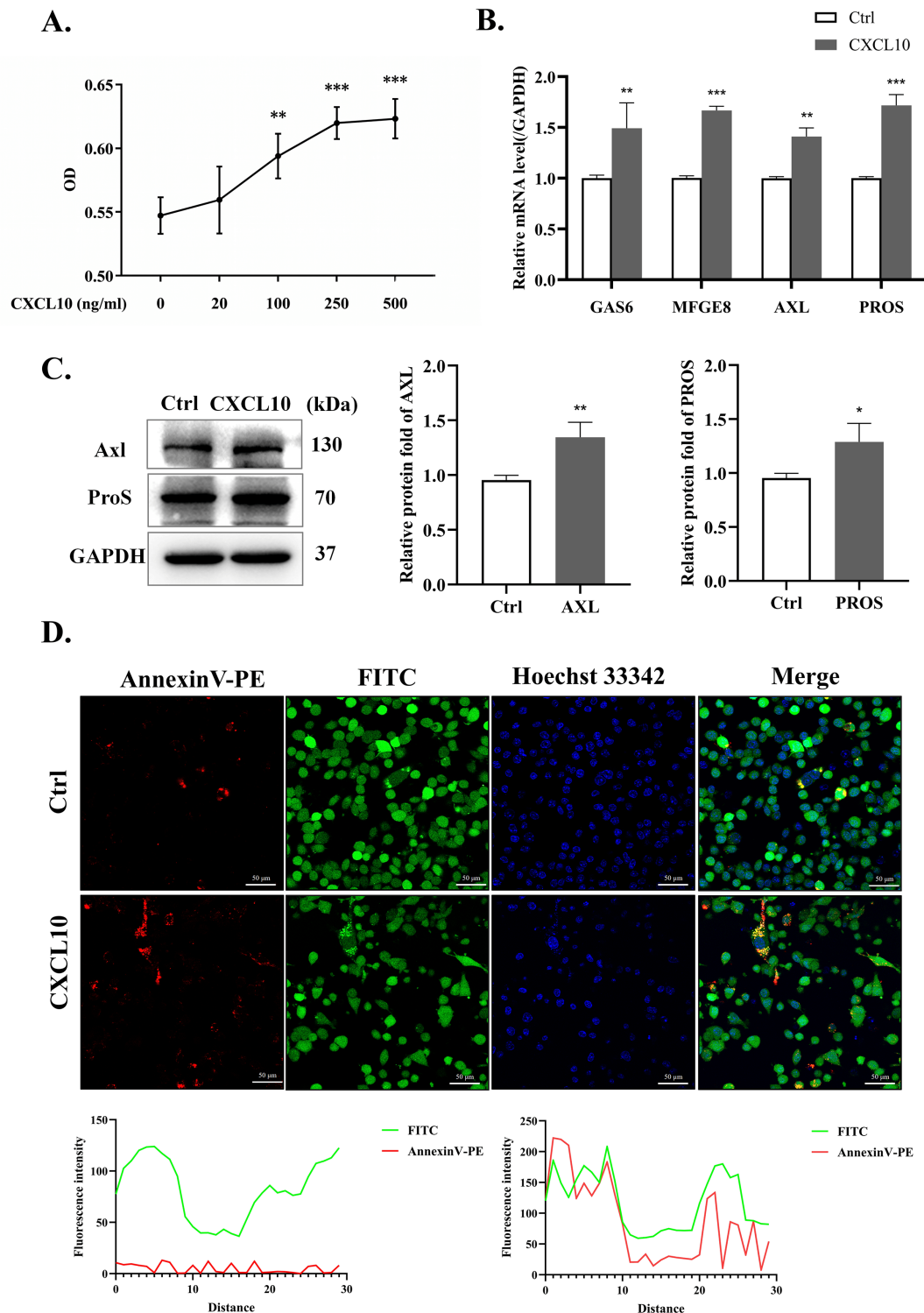


Fig. 1. CXCL10 promotes macrophage efferocytosis. (A) Neutral red phagocytosis assay for assessing the phagocytic capacity of RAW264.7 cells stimulated with CXCL10 (20, 100, 250, and 500 ng/mL; absorbance at 540 nm, $n = 6$). (B) Relative mRNA levels of *Gas6*, *Mfge8*, *Axl*, and *Pros* in RAW264.7 cells following CXCL10 (250 ng/mL) treatment, determined by RT-qPCR. (C) Western blot analysis of AXL and PROS proteins expression in RAW264.7 cells. (D) Confocal laser scanning microscopy of the phagocytosis of ACs by RAW264.7 cells. Macrophages (green) were labeled with FITC, and ACs (red) were labeled with Annexin V-PE ($n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). CXCL10, C-X-C motif chemokine ligand 10; RT-qPCR, Reverse transcription quantitative polymerase chain reaction; AXL, AXL receptor tyrosine kinase; PROS, Protein S; scale bar = 50 μm .

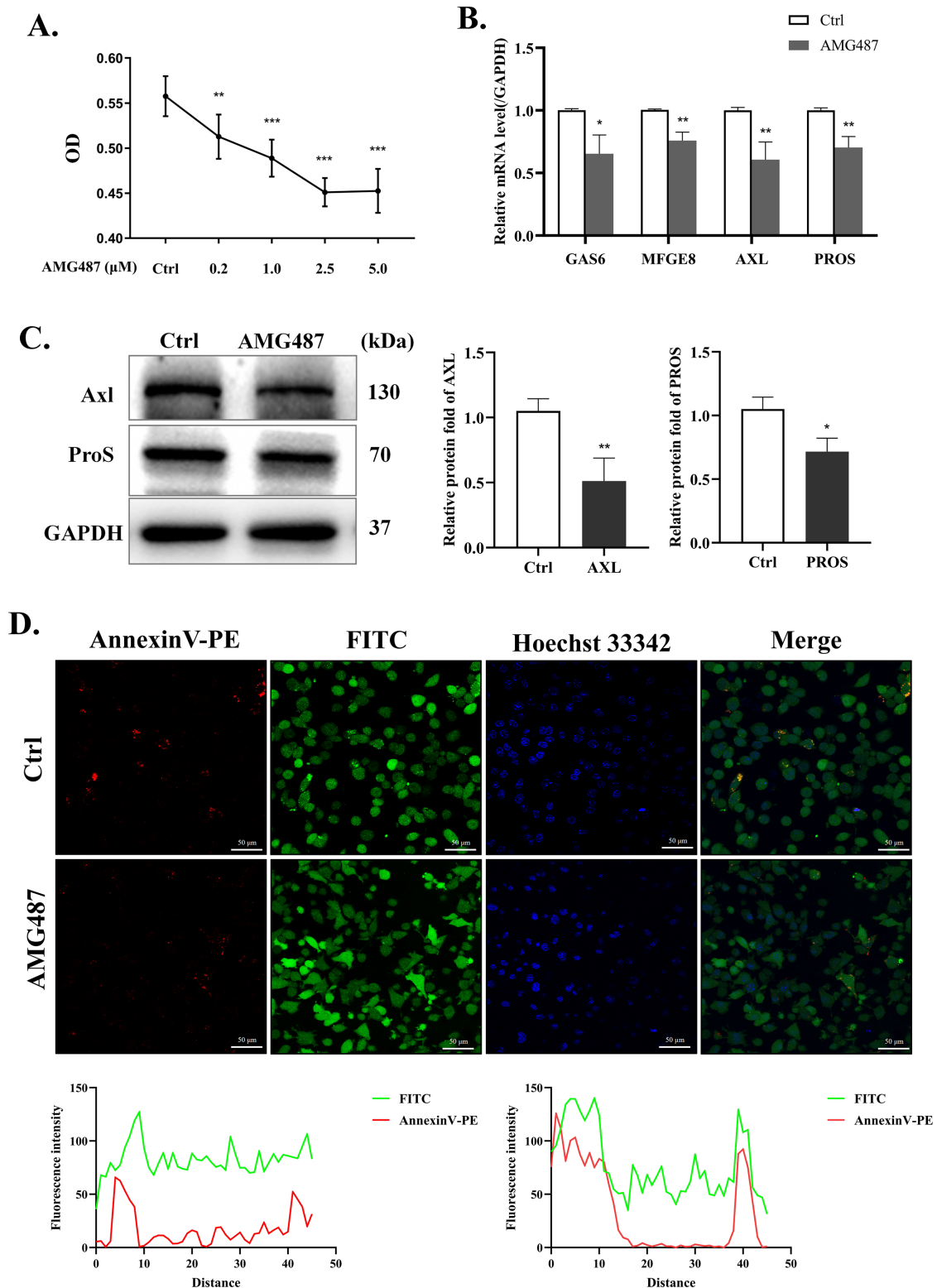


Fig. 2. The CXCL10–CXCR3 antagonist AMG487 inhibits macrophage efferocytosis. (A) Neutral red phagocytosis assay for assessing the phagocytic capacity of RAW264.7 cells treated with AMG487 (0.2, 1.0, 2.5, and 5.0 μM; absorbance at 540 nm, $n = 6$). (B) Relative mRNA levels of Gas6, Mfge8, Axl, and Pros in RAW264.7 cells following AMG487 (2.5 μM) treatment, determined by RT-qPCR. (C) Western blot analysis of AXL and PROS protein expression in RAW264.7 cells. (D) Confocal laser scanning microscopy of AC by RAW264.7 cells. Macrophages (green) were labeled with FITC, and ACs (red) were labeled with Annexin V-PE ($n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; scale bar = 50 μm).

itating endothelial cell recovery and tissue restoration [33]. RAW264.7 cells were treated with AMG487 to investigate its effect on macrophage efferocytosis. A marked decrease in neutral red uptake was observed in the 0.2, 1.0, 2.5 and 5.0 μM treatment groups (Fig. 2A), with 2.5 μM selected as the working concentration. Subsequent analysis indicated that AMG487 suppressed the mRNA level of *Gas6*, *Mfge8*, *Axl*, and *Pros* (Fig. 2B) and downregulated the protein expression of AXL and PROS (Fig. 2C). Furthermore, immunofluorescence staining confirmed that AMG487 treatment reduced macrophage phagocytosis of ACs. Line-scan analysis of fluorescence intensity was further performed to verify the co-localization of macrophages (FITC, green trace) and engulfed ACs (Annexin V-PE, red trace). The left line-scan profile corresponds to the Ctrl group, while the right profile corresponds to the AMG487-treated group. Reduced overlap between the green and red fluorescence peaks in the AMG487 group indicated diminished internalization of ACs by RAW264.7 macrophages (Fig. 2D).

3.3 CXCL10–CXCR3 Regulate Macrophage Efferocytosis Via CD36

CD36 functions as a key scavenger receptor and recognition molecule during macrophage efferocytosis [8]. To determine whether CD36 mediates the effects exerted by the CXCL10–CXCR3 axis upon macrophage efferocytosis, its levels were evaluated following axis activation or inhibition. RT-qPCR and Western blot analyses indicated that CD36 expression is regulated by the CXCL10–CXCR3 axis, with CXCL10 inducing the upregulation of CD36 and AMG487 suppressing its expression (Fig. 3A–C). Given that CD36 mediates lipid uptake, intracellular lipid buildup was examined using Oil Red O staining. CXCL10 treatment increased intracellular lipid droplet accumulation, whereas AMG487 treatment exerted the opposite effect (Fig. 3D).

To evaluate whether CD36 is required for CXCL10–CXCR3-driven efferocytosis, we knocked down CD36 expression in RAW264.7 cells using siRNA (Fig. 3E). The knockdown of CD36 resulted in reduced baseline protein levels of the efferocytosis receptors AXL and PROS (Fig. 3F). Furthermore, in CD36-deficient macrophages, CXCL10 failed to upregulate the mRNA level of *Gas6*, *Mfge8*, *Axl*, and *Pros*, while AMG487 lost its ability to downregulate these transcripts (Fig. 3G). Western blot analysis confirmed that neither CXCL10 nor AMG487 altered AXL and PROS protein expression in the absence of CD36 (Fig. 3H,I).

3.4 CD36 Ubiquitination in Macrophages Impairs Efferocytosis During Inflammation

Viral infections are major drivers of inflammatory responses, with double-stranded RNA acting as a classic danger signal [34]. As an artificial double-stranded RNA mimic, poly(I:C) is widely used in ALI models to reca-

pitulate virus-triggered pulmonary inflammation [35]. Exposure to gradient doses of poly(I:C) triggered the upregulation of *Cd36* mRNA level (Fig. 4A). Conversely, the protein levels of CD36, AXL, and PROS significantly decreased following treatment with 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ poly(I:C) (Fig. 4B), with 50 $\mu\text{g}/\text{mL}$ selected as the optimal working concentration. This inverse relationship between mRNA and protein levels suggests that poly(I:C) promotes the post-translational degradation of CD36 [36].

Studies have indicated that the ubiquitin-proteasome system regulates CD36 stability, with polyubiquitination targeting it for proteasomal degradation [37]. Co-immunoprecipitation assays verified that cells treated with poly(I:C) showed a significant increase in the ubiquitination level of CD36 (Fig. 4C). To elucidate the pathway driving this degradation, UBL4A, an essential component involved in proteasomal targeting was evaluated [38,39]. Western blot analysis indicated that poly(I:C) stimulation upregulated UBL4A protein levels (Fig. 4D). Furthermore, treatment with the proteasome inhibitor MG132 prevented poly(I:C)-induced CD36 degradation, restoring CD36 protein levels in macrophages (Fig. 4E). Similarly, the siRNA-mediated knockdown of UBL4A prevented CD36 degradation following poly(I:C) stimulation (Fig. 4F,G).

3.5 AMG487 Inhibits the Ubiquitination of CD36 in Macrophages During Inflammation

Studies have demonstrated that the CXCL10–CXCR3 antagonist AMG487 alleviates inflammatory responses across various pathological conditions [40,41]. To explore the effect of AMG487 on efferocytosis during inflammation, the CXCL10–CXCR3 axis was inhibited using AMG487 in poly(I:C)-stimulated macrophages. Following poly(I:C) stimulation, AMG487 treatment downregulated UBL4A protein levels while simultaneously restoring CD36 protein levels (Fig. 5A,B). Co-immunoprecipitation analysis revealed that AMG487 markedly suppressed CD36 ubiquitination under inflammatory conditions (Fig. 5C).

During ALI, the number of ACs in lung tissue, including pulmonary endothelial cells and neutrophils, has been reported to increase significantly [42,43]. Poly(I:C) induced ALI in mice, resulting in notable pathological changes [35]. To model ALI *in vivo*, mice were treated with poly(I:C) across four time points (0, 12, 24, and 48 h). The results indicated that efferocytosis in mouse lungs is impaired during acute inflammation. Western blot analysis showed that CD36, AXL, and PROS protein levels decreased while UBL4A protein levels increased in ALI mice (Fig. 5D). Additionally, co-immunoprecipitation assays demonstrated that CD36 ubiquitination was increased in lung tissue of these mice (Fig. 5E). At 12 h post-poly(I:C) treatment, CD36, AXL, and PROS1 protein expression was significantly reduced, whereas UBL4A protein expression was increased (Fig. 5F). Treatment with AMG487 mitigated lung injury triggered by poly(I:C) (Supplementary

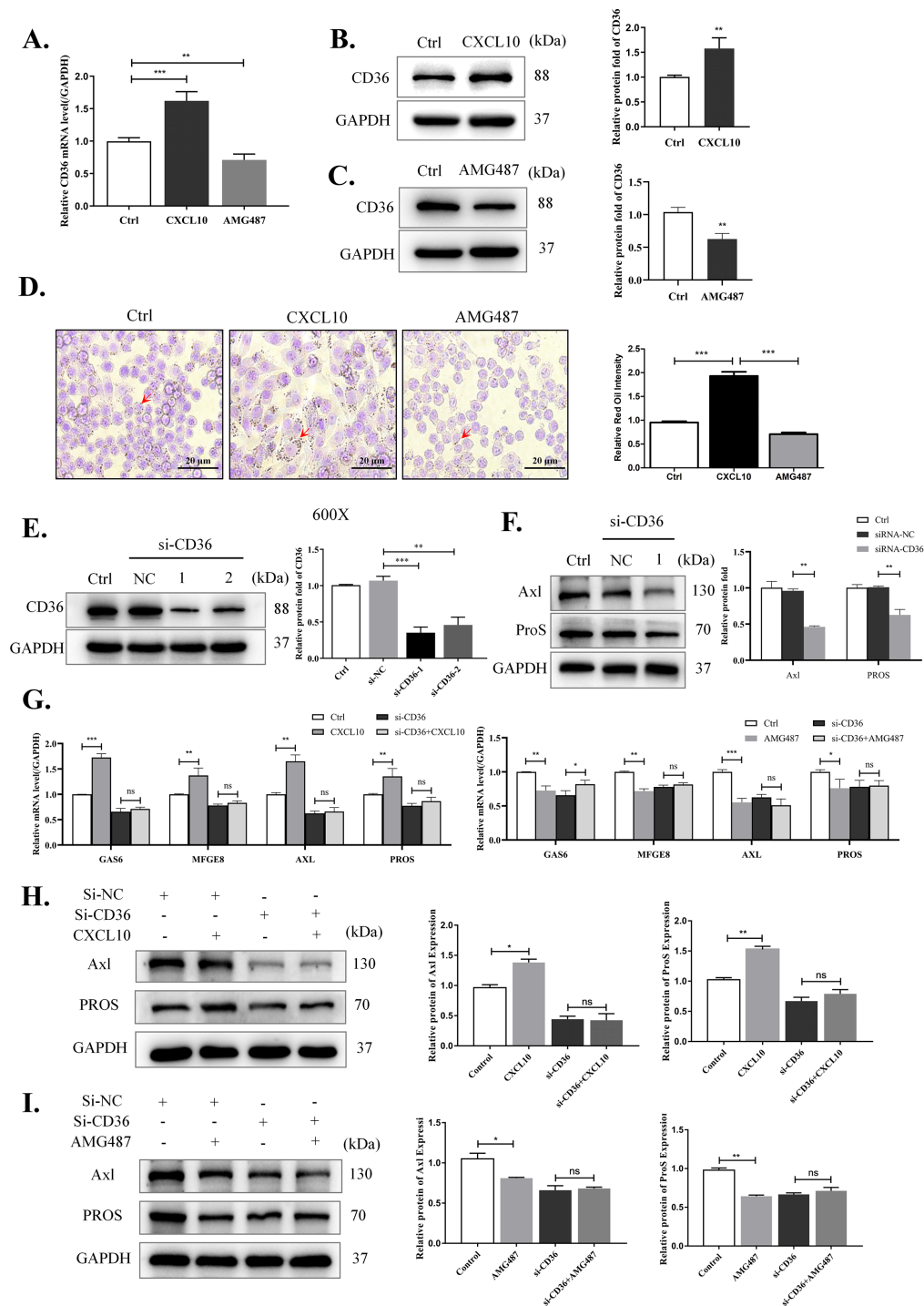


Fig. 3. The CXCL10–CXCR3 axis regulates macrophage efferocytosis via CD36. (A) Relative mRNA level of *Cd36* in RAW264.7 cells treated with CXCL10 or AMG487, determined by RT-qPCR. (B,C) Western blot analysis and corresponding quantification of CD36 protein levels in RAW264.7 cells treated with CXCL10 or AMG487. (D) Representative light microscopy images of Oil Red O staining evaluating intracellular lipid accumulation in RAW264.7 cells following CXCL10 or AMG487 treatment. Red arrows indicate intracellular lipid droplets; scale bar = 20 μ m. (E) Western blot analysis validating siRNA-mediated CD36 knockdown efficiency in RAW264.7 cells. (F) Western blot analysis of AXL and PROS protein expression in RAW264.7 cells following CD36 knockdown. (G) Relative mRNA level of *Gas6*, *Mfge8*, *Axl*, and *Pros* in CD36-knockdown RAW264.7 cells treated with CXCL10 or AMG487, determined by RT-qPCR. (H,I) Western blot analysis and quantification of AXL and PROS1 protein levels in CD36-knockdown RAW264.7 cells treated with CXCL10 or AMG487 ($n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns, no significant).

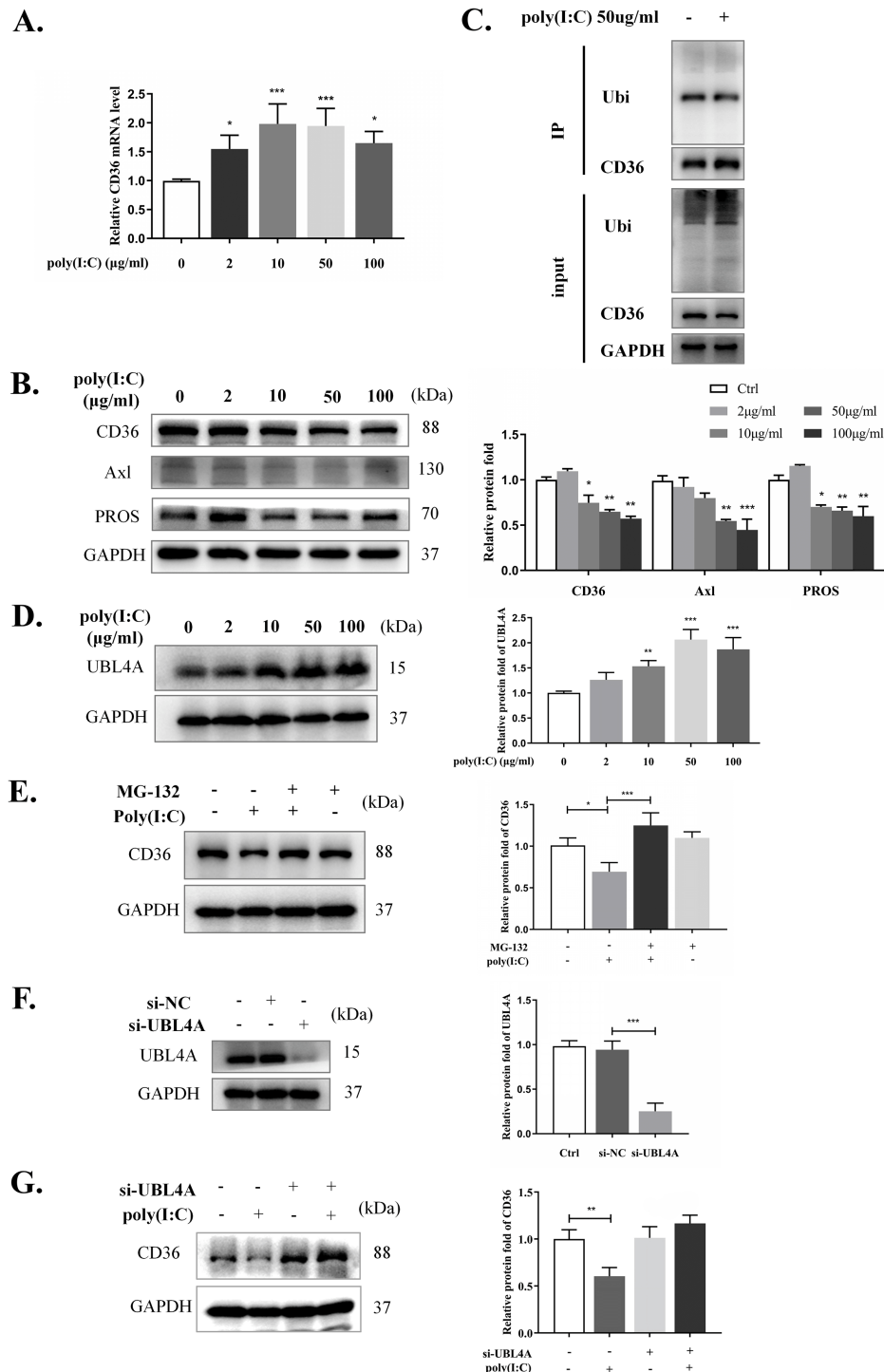


Fig. 4. CD36 ubiquitination in macrophages impairs efferocytosis during inflammation. (A) Relative mRNA level of *Cd36* in RAW264.7 cells following poly(I:C) stimulation, determined by RT-qPCR. (B) Western blot analysis of CD36, AXL, and PROS protein expression in RAW264.7 cells following poly(I:C) stimulation. (C) Co-immunoprecipitation analysis assessing the ubiquitination level of CD36 in RAW264.7 cells. (D) Western blot analysis of UBL4A protein levels in RAW264.7 cells following poly(I:C) treatment. (E) Western blot analysis of CD36 protein expression within RAW264.7 cells exposed to poly(I:C), with or without MG132 treatment. (F) Western blot analysis validating siRNA-mediated UBL4A knockdown efficiency in RAW264.7 cells. (G) Western blot analysis of UBL4A and CD36 protein expression in RAW264.7 cells following UBL4A knockdown and poly(I:C) stimulation ($n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

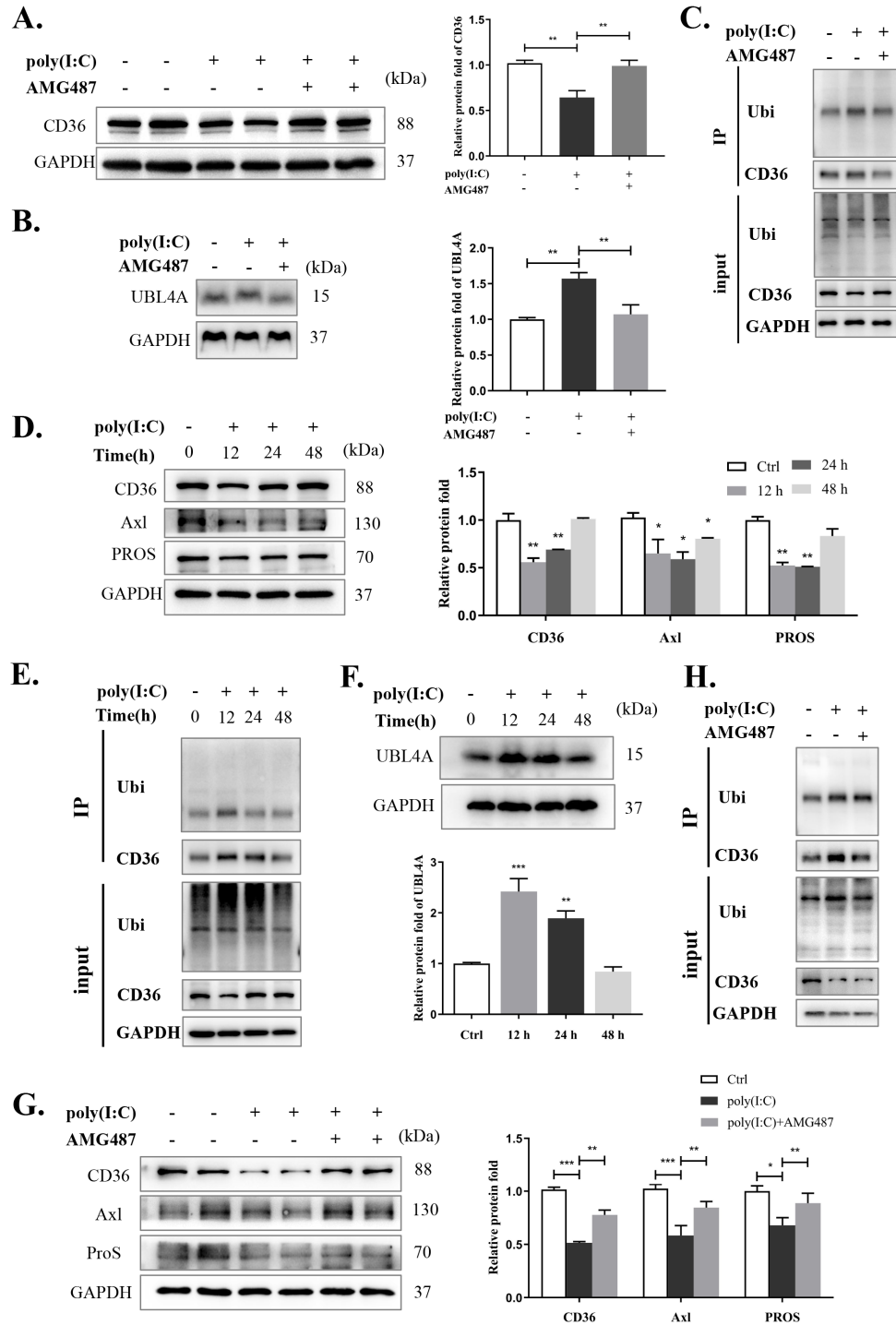


Fig. 5. AMG487 inhibits CD36 ubiquitination in macrophages and lung tissue during inflammation. (A) Western blot analysis of CD36 protein expression in RAW264.7 cells treated with poly(I:C) and AMG487. (B) Western blot analysis of UBL4A protein expression in RAW264.7 cells treated with poly(I:C) and AMG487. (C) Co-immunoprecipitation analysis assessing CD36 ubiquitination levels in RAW264.7 cells receiving poly(I:C) alongside AMG487 treatment. (D) Western blot analysis of CD36, AXL and PROS protein expression in lung tissues from ALI model mice across a time course. (E) Co-immunoprecipitation analysis of CD36 ubiquitination levels in lung tissues from ALI mice. (F) Western blot analysis of UBL4A protein levels in lung tissues from ALI mice. (G) Western blot analysis of CD36, AXL and PROS protein levels in lung tissues from AMG487 treated ALI mice. (H) Co-immunoprecipitation analysis of CD36 ubiquitination in lung tissues from AMG487 treated ALI mice ($n = 4$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Fig. 1) and restored CD36, AXL, and PROS protein expression (Fig. 5G). Co-immunoprecipitation confirmed that AMG487 inhibits CD36 ubiquitination in inflamed lung tissue, similar to its effect in macrophages (Fig. 5H). To dissect the upstream signaling cascades linking CXCR3 engagement to UBL4A transcription, we performed a pharmacological inhibitor screening in RAW264.7 cells under poly(I:C) stimulation (**Supplementary Fig. 2**). Pretreatment with Bay 11-7082 (NF- κ B inhibitor) or Ruxolitinib (JAK/STAT inhibitor) attenuated UBL4A upregulation and restored CD36 expression, indicating that both NF- κ B and JAK/STAT pathways are major transducers of CXCR3 signals to UBL4A [44,45,46,47]. Interestingly, inhibition of p38 MAPK (SB203580) or ERK (U0126) also partially reversed the poly(I:C) induced changes, suggesting additional involvement of MAPK cascades. Collectively, these data imply that CXCR3 orchestrates UBL4A transcription through a cooperative network encompassing NF- κ B, JAK/STAT, and MAPK axes, rather than a single linear pathway.

4. Discussion

Accumulating evidence positions the CXCL10–CXCR3 signaling pair as a critical regulator of inflammation-driven pathology [48]. CXCL10–CXCR3 signaling affects macrophage morphology and function, inducing macrophage migration to inflammatory site. CXCL10–CXCR3 also regulates macrophage polarization. CXCL10 promotes M1 polarization and drives tissue inflammatory responses [20,41]. We found that the CXCL10–CXCR3 pathway directly regulates macrophage efferocytosis. Under homeostatic, non-inflammatory conditions, CXCL10 stimulated efferocytosis to clear ACs and maintain tissue stability, whereas blockade of the axis with AMG487 reduced AC uptake. These observations indicate that CXCL10–CXCR3 signaling modulates inflammatory responses by directly tuning macrophage efferocytic capacity.

As a multifunctional scavenger receptor, CD36 integrates lipid uptake with downstream signaling through Src family kinases (SFKs), MAPKs, AMPK, and NADPH oxidase (NOX) complexes [27,49,50]. CD36 is also essential for efferocytosis; for instance, CD36 deficiency in bleomycin-induced ALI leads to AC accumulation in pulmonary tissue and subsequent fibrosis [8]. In this study, we confirmed that CD36 is a central mediator of CXCL10–CXCR3-driven efferocytosis. Under physiological conditions, ACs trigger CXCL10 release, which enhances CD36-mediated efferocytosis to complete cell clearance. Under severe inflammatory conditions, however, macrophage efferocytosis becomes impaired. Despite high CXCL10 expression during acute inflammation, CD36 undergoes UBL4A-dependent ubiquitination and subsequent proteasomal degradation. In viral ALI models, this inflammation-mediated degradation of CD36 disrupts pulmonary effe-

rocytosis, leading to AC accumulation and the exacerbation of tissue injury. Results from this work revealed that pharmacological inhibition of the CXCL10–CXCR3 signaling axis using AMG487 suppresses UBL4A expression, thereby preventing CD36 ubiquitination and restoring efferocytic clearance in both macrophages and inflamed lung tissue. This suggests that targeting CXCL10–CXCR3 signaling offers a viable therapeutic strategy for preserving CD36 stability and resolving tissue inflammation. A limitation of our work was the reliance on RAW264.7 cells for *in vitro* mechanistic evaluations. Future research should consider isolating primary bone marrow-derived macrophages (BMDMs) or resident AMs to further validate our findings.

In our study, CXCL10 promoted lipid droplet accumulation in macrophages, whereas AMG487 reduced it, consistent with the role of CD36 as a scavenger receptor mediating lipid uptake. Under homeostatic conditions, CD36-mediated lipid handling supports efferocytosis by enabling clearance of apoptotic cell-derived lipids. However, under inflammatory conditions, CD36 degradation creates a dual impairment: efferocytic failure drives retention of uncleared apoptotic cells undergoing secondary necrosis and release DAMPs, perpetuating inflammation [5]. Simultaneously, residual CD36 activity drives lipid overload, triggering mitochondrial metabolic reprogramming, mtROS production, and NF- κ B activation [27]. Moreover, cholesterol crystals internalized via CD36 activate the NLRP3 inflammasome, elevating the output of IL-1 β and IL-18 [51,52]. Lipid-laden macrophages (foam cells) further promote fibrotic remodeling, as observed in viral lung injury models [53]. Thus, the CXCL10–CXCR3–CD36 axis drives sustained pulmonary inflammation through convergent mechanisms of impaired efferocytosis and lipid overload. AMG487 interrupts both arms by preserving CD36. Future studies should characterize lipid species via lipidomics, validate NLRP3 involvement using knockout models, and assess the capacity of AMG487 to prevent the transition from ALI to fibrosis.

Poly(I:C) can be sensed by Toll-like receptor 3 (TLR3) and triggers inflammatory cascades via type I interferons [54]. For this reason, poly(I:C) exposure is extensively applied to establish viral ALI experimental models. In our poly(I:C)-induced ALI model, CD36 protein expression in macrophages decreased, and efferocytosis was impaired, even though *Cd36* mRNA level increased. A similar uncoupling has been reported with USP14 deficiency, where the loss of USP14 accelerates CD36 protein degradation without reducing *Cd36* mRNA abundance [36]. We reasoned that post-translational ubiquitination drives the rapid degradation of CD36 protein during acute inflammation, resulting in defective efferocytosis. UBL4A is recognized as a direct mediator of proteasomal targeting for specific protein substrates [38,39]. In ALI, UBL4A mediates macrophage CD36 degradation, with UBL4A protein levels rising markedly across cellular assays and ani-

mal models. Silencing UBL4A elevates CD36 expression levels. Beyond its role in proteostasis, UBL4A also regulates cell motility, as UBL4A deficiency significantly impairs macrophage migration [55]. We hypothesize that elevated UBL4A expression enhances macrophage recruitment to the lungs following viral infection. However, the simultaneous UBL4A-driven loss of CD36 weakens efferocytosis, thereby facilitating early viral replication. As viral activity progresses, this defect in AC clearance triggers an exaggerated inflammatory response in the lungs. Our pharmacological dissection reveals that CXCR3 employs multiple convergent signaling modules, NF- κ B, JAK/STAT, and MAPK, to elevate UBL4A expression under inflammatory conditions. This pleiotropic regulation may underlie the robust and sustained upregulation of UBL4A within the inflammatory milieu and may also explain why targeting a single kinase is insufficient to fully abrogate CD36 degradation. Future studies utilizing genetic deletion of individual transcription factors or ChIP-seq analysis will be essential for mapping the cis-regulatory elements of the UBL4A promoter that respond to each pathway. Although existing studies note that poly(I:C) can strengthen macrophage phagocytic uptake of tumor cells through the CD47-SIRP α axis [56], this process is mechanistically distinct from efferocytosis, which depends on CD36 and TAM receptors. Moreover, poly(I:C) exerts time-dependent effects, acute TLR3 activation transiently enhances innate immunity, whereas sustained stimulation (24–48 h) triggers UBL4A mediated CD36 degradation. Notably, although poly(I:C) upregulates CD36 mRNA, UBL4A driven ubiquitination degrades CD36 protein, functionally uncoupling IP-10 production from efferocytic capacity.

The CXCL10–CXCR3 antagonist AMG487 preserved efferocytosis in both lung tissue and macrophages during acute inflammation. Mechanistically, AMG487 downregulates UBL4A expression, thereby inhibiting CD36 ubiquitination and restoring CD36 protein levels. Studies have reported that AMG487 alleviates inflammation *in vivo*. In rheumatoid arthritis models, AMG487 attenuates disease severity by dampening NF- κ B activation and curtailing the production of inflammatory cytokines such as MCP-1, TNF- α and IFN- γ [57]. In ALI, AMG487 upregulates CD36 protein expression to protect efferocytosis in the lung, similar to its effects in macrophages. CXCL10 concentrations are elevated during inflammation [58]. AMG487 can also inhibit CXCR3 activation and prevent excessive immune cell activation *in vivo*. The changes in CD36 and efferocytosis were consistent across models. These findings suggest that AMG487 may regulate alveolar macrophage efferocytosis to protect against ALI. Future experiments will require further knockout of CD36 in mouse lung tissue to validate the above conclusions. Notably, while we provide molecular evidence demonstrating that AMG487 reduces CD36 ubiquitination and restores CD36 expression within RAW264.7 cells treated with poly(I:C) *in*

vitro, a direct functional efferocytosis assay with AMG487 under poly(I:C) induction was not conducted *in vitro*. Nevertheless, such molecular rescue is consistent across cellular assays and animal model settings. The mechanistic pathway, which involves poly(I:C) leading to UBL4A upregulation, followed by CD36 degradation and ultimately resulting in impaired efferocytosis, is supported by multiple lines of evidence, including siRNA knockdown, proteasome inhibition, and co-immunoprecipitation experiments.

5. Limitations

Several limitations warrant consideration. First, although AMG487 treatment preserved lung tissue efferocytosis *in vivo*, we did not perform cell-type-specific knockout of *Cd36* or *Cxcr3* in AMs or other resident pulmonary cells. Conditional knockout models are essential for attributing these efferocytic changes directly to macrophage-intrinsic CD36 regulation, rather than to indirect contributions from other CXCR3-expressing cells, such as endothelial cells or neutrophils, within the inflammatory milieu. Second, cell-based mechanistic investigations adopted the RAW264.7 cell line, an immortalized model that does not capture the receptor profiles or functional heterogeneity of primary cells. Isolating primary AMs or BMDMs, including distinct tissue-resident and recruited macrophage populations, will improve the translational relevance of our findings during ALI. Third, although we found that UBL4A is a key mediator of CD36 ubiquitination downstream of CXCL10–CXCR3 signaling, the pathway connecting CXCR3 engagement to *Ubl4a* transcriptional upregulation remains undefined. Elucidating upstream molecular events could provide a comprehensive understanding of how the chemokine axis orchestrates CD36 degradation.

6. Conclusions

In this study, we elucidated how CXCL10–CXCR3 regulates macrophage efferocytosis amid ALI progression. Our findings lay theoretical groundwork to elucidate the pathogenic mechanisms of viral-triggered ALI, identifying this chemokine axis as a promising therapeutic target for ALI prevention and treatment.

Availability of Data and Materials

The datasets and raw data used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

WG developed the concept and designed the studies. TM, YH, and HK analyzed the *in vivo* and *in vitro* studies and performed statistical analysis. TM and LL contributed to the experimental studies and data acquisition. WG wrote the initial draft of the manuscript. TM, YH, HK, and WG assisted in the preparation of the manuscript and reviewed

the draft. WG and YH supervised and participated in the writing and revision of this manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal studies were conducted in accordance with the ARRIVE guidelines and the guidelines approved by the Ethics Committee of Bengbu Medical University (approval number 2025-686). This study was performed following the China Council on Animal Care and Protocol guidelines and adhered to the relevant ethical regulations for animal experimentation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL55164>.

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