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ARTICLE

CD74 Drives M1 Macrophage Polarization via STAT3 Signaling to Promote Antitumor Immunity in Breast Cancer

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ABSTRACT: Background: Immunosuppression contributes to breast cancer treatment failure, yet Cluster of Differentiation 74 (CD74) function in macrophages remains unclear. This study investigated how CD74 influences M1 macrophage polarization and its functional and expression profiles in breast cancer. **Methods:** We used bioinformatics analysis combined with *in vitro* cell experiments. The expression of CD74 in THP-1-derived M1 macrophages induced by Lipopolysaccharide/Interferon-gamma (LPS/IFN-γ) was knocked down by shRNA. Polarization markers were detected by WB, qPCR, and flow cytometry. Cytokines were detected by Enzyme-Linked Immunosorbent Assay (ELISA). The phagocytosis and killing effect of macrophages on MCF-7 cells were evaluated by a co-culture model combined with flow cytometry and Cell Counting Kit-8 (CCK-8). **Results:** CD74 is highly expressed in breast cancer and exerts a protective effect. Knocking down CD74 reduces the levels of M1 markers (iNOS, TNF-α) and inflammatory factors (TNF-α, IL-6), while simultaneously upregulating Signal Transducer and Activator of Transcription 3 (STAT3) tyrosine705 phosphorylation ($p < 0.01$). Functional experiments showed that CD74 deficiency impaired the ability of M1 macrophages to eliminate tumor cells via phagocytosis and induce tumor cell apoptosis ($p < 0.05$). **Conclusions:** CD74 has the potential to enhance macrophage immune function by inhibiting the STAT3 pathway and regulating M1 polarization. This offers a new perspective for researching the tumor immune microenvironment and presents CD74 as a potential target for breast cancer immunotherapy.

KEYWORDS: Macrophage polarization; cluster of differentiation 74; macrophage M1 phenotype; breast cancer; tumor microenvironment

1 Introduction

Breast cancer is the most common malignancy among women worldwide. Its high mortality is largely due to tumor invasion, metastasis, and resistance to treatment [1,2]. Research focus has recently shifted from solely studying tumor cells to also examining the surrounding tumor microenvironment (TME), which plays an indispensable role in the initiation, progression, immune evasion, and response to treatment of breast cancer [3,4]. Within the complex cellular network of the TME, immune cells are a vital component, with tumor-associated macrophages (TAMs) being one of the most prevalent types of infiltrating innate immune cells. Converting TAMs from the M2 phenotype to the M1 phenotype has been identified as an effective strategy for cancer treatment [5,6]. Depending on microenvironmental signals, macrophages can differentiate into either an antitumor M1 phenotype or a pro-tumor M2 phenotype [7]. The plasticity and heterogeneity of tumor-associated macrophages (TAMs) are widely recognized [8]. In breast cancer,

TAMs primarily exhibit an M2-like phenotype and drive disease progression by promoting angiogenesis, impairing T-cell function, and enhancing invasiveness [9,10]. Notably, M2 macrophages have been shown to predict poor prognosis in breast cancer patients [11]. Therefore, elucidating the molecular mechanisms that regulate the polarization of TAMs and promoting the conversion of these cells to an antitumor phenotype holds great promise for the immunotherapy of breast cancer.

CD74 chaperones MHC class II for antigen presentation [12,13] and, as a high-affinity macrophage migration inhibitory factor (MIF) receptor, drives inflammation, autoimmunity, and cancer via the MIF-CD74 axis [12,14]. In particular, CD74 is significantly upregulated in breast cancer and has been shown to modulate immune cells in the tumor microenvironment, making it a potential therapeutic target [15,16]. However, the expression and function of CD74 in breast cancer-infiltrating immune cells, especially macrophages, remain poorly understood. The mechanism by which CD74 modulates tumor immunity through regulating macrophage polarization remains unclear.

The JAK-STAT pathway is a crucial signaling network in breast cancer, acting as a central mediator of cytokine signal transduction [17,18]. Persistent activation of STAT3 promotes tumor cell proliferation and controls immune cell activity in the tumor microenvironment [19,20]. It is worth noting that STAT3 phosphorylation has a multifaceted and crucial impact on macrophage polarization. CD74 expression was positively correlated with M1 macrophage traits and STAT3 activation in our bioinformatics analysis. However, the functional linkages and mechanisms underlying this phenomenon have yet to be experimentally validated.

This study, therefore, seeks to clarify CD74's role in M1 polarization and its dependence on the STAT3 pathway.

2 Materials and Methods

2.1 Bioinformatics Analysis

To analyze CD74 expression, we utilized the UALCAN (https://ualcan.path.uab.edu/index.html) platform. Additionally, we performed survival and correlation analyses using the TCGA-BRCA dataset (dataset ID: TCGA-BRCA) on the GEPIA2 website (http://gepia2.cancer-pku.cn/#index). GSEA analysis was also conducted using the GSEA software (v4.0.2, Broad Institute, Cambridge, MA, USA). Furthermore, we employed TIMER2.0 (https://compbio.cn/timer2/) to analyze the correlation between CD74 and macrophages.

2.2 Immunofluorescence (IF)

Breast cancer and normal control tissues were obtained from the Department of Pathology, The First Huizhou Affiliated Hospital of Guangdong Medical University. All patients provided written informed consent, and all research was conducted in accordance with the relevant guidelines and regulations of the Institutional Review Board for Clinical Trials at the First Huizhou Affiliated Hospital of Guangdong Medical University. The ethics review number is KYLL-2024-009-01. The fresh tissue (n = 4) was fixed with formalin, and the paraffin-embedded sections were separated by grading, alcohol dewaxing, and rehydration. Antigen retrieval was performed with EDTA buffer (98°C, 20 min). Sections were blocked with solution (Beyotime, Shanghai, China) for 90 min, then incubated overnight at 4°C with anti-CD74 primary antibody (Proteintech, Rosemont, IL, USA) (Cat. No. 66390-1-lg, 1:400), followed by Alexa Fluor 555-conjugated donkey anti-mouse secondary antibody (Beyotime, China) (Cat. No. P0190, 1:1000) for 60 min at 37°C. Washing using an immunofluorescence special washing solution (Beyotime, China), each washing three times, each time for 10 min. Finally, the nucleus was stained with a drop of DAPI and

examined using an Olympus BX53 (Olympus Corporation, Tokyo, Japan) fluorescence inverted microscope. The microscope objective lens is observed 100 times.

2.3 Cell Culture

THP-1 (TIB-202) was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-7 (CL-0149) human breast cancer cells were purchased from Pricella, Wuhan, China. THP-1 cells (ATCC) were maintained in RPMI-1640 medium (Cat. No. 11875093, GIBCO, Grand Island, NY, USA) supplemented with 100 mg/L penicillin, 100 mg/L streptomycin, 50 μM 2-mercaptoethanol. All cells have undergone Short Tandem Repeat (STR) identification and mycoplasma-free testing. Macrophage induction: THP-1 cells were treated with 100 ng/mL PMA (Cat. No. P8139, Sigma-Aldrich, St. Louis, MO, USA) at a density of 5×10^5 cells/mL, and then seeded into culture plates. After 2 days, the cells were cultured according to the assigned groups. MCF-7 cells were cultured in the same medium as THP-1, except that 10 μg/mL insulin was added instead of 2-mercaptoethanol.

For M1 polarization, THP-1-derived macrophages were exposed to 10 μg/L lipopolysaccharide (LPS, Cat. No. L2630, same source as PMA) and 20 μg/L interferon-gamma (IFN-γ, Cat. No. 300-02, PeproTech, Cranbury, NJ, USA) for 24 h. Macrophages were exposed to 20 ug/L IL-4 (Cat. No. 200-04, same source as IFN-γ) for 1 day to induce M2 polarization.

2.4 Construction of Knockdown Plasmids and Cell Transfection

Based on RNAi sequence design principles, target sequences were designed for the CD74 gene. A DNA sequence containing the target sequence and restriction sites was synthesized. The CD74 gene was cloned into the pENTR-U6 vector (NovoPro Bioscience, Shanghai, China) for knockdown. shRNA-CD74#1 plasmid (sequence: 5′-CCGCGCGACCTTATCTCCAACAATCTCGAGATTGTTGGAGATAAGGTCGCGTTTTT G-3′); shRNA-CD74#2 plasmid (sequence: 5′-CCGGCCACCGAAAGAGTCACTGGAACTCGAGTTCCAGTGAC TCTTTCGGTGGTTTTTG-3′); shRNA-CD74#3 plasmid (sequence: 5′-CCGGCCACCAAGTATGGCAACATG ACTCGAGTCATGTTGCCATACTTGGTGGTTTTTG-3′); shRNA-NC control plasmid (sequence: 5′-CCGG CAACAAGATGAAGAGCACCACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTG-3′). For transfection, THP-1 cells were seeded at 1×10^5 cells/well in 24-well plates, and MCF-7 cells were seeded at 8×10^4 cells/well and grown to ~70% confluence one day prior to transfection. Plasmid transfection reagent preparation: Plasmid DNA (500 ng) and Lipofectamine 3000 (1.5 μL, L3000015, Thermo Fisher Scientific, Waltham, MA, USA) were mixed in 150 μL OPTI-MEM (GIBCO, Invitrogen Corporation, USA) and incubated at 37°C in 5% CO_2.

2.5 qPCR

Total RNA was isolated from THP-1 and MCF-7 cells (Trizol). First digest with 0.25% trypsin-EDTA (Cat. No. 25200072, Gibco™, Thermo Fisher, Grand Island, NY, USA), then lyse with Trizol reagent (Cat. No. 9109, Takara, Kyoto, Japan). RNA was extracted by chloroform/isopropanol/ethanol and quantified by NanoDrop 2000 (Thermo Fisher, Wilmington, Delaware, USA). Take an appropriate amount of the extracted RNA and perform reverse transcription using the iScript™ cDNA Ready-to-Use Kit (Cat. No. 1708891, Bio-Rad, Hercules, CA, USA), which contains a mixture of random hexamers and dT-oligomer primers. qPCR reactions were performed on a CFX96 Touch qPCR system (same source as iScript™). Volumes: 20 μL; 10 μL reaction mixture (Cat. No. 1725121, same source as iScript™); 0.5 μL primers; 2 μL cDNA and water up to 20 μL. Subsequently, the amplification was analyzed to verify its specificity. Ct values were automatically calculated using CFX Manager software (same source as iScript™). Target gene expression

was normalized to β-actin and calibrated to the shRNA-NC + LPS/IFN-γ group using the $2^{-\Delta\Delta Ct}$ method (primers in Table 1).

Table 1: Primer and sequence information. The table lists the forward and reverse primer sequences for the target genes (iNOS, TNF-α, Arg1, CD206) and the reference gene (GAPDH).

Primers		Sequence (5′→3′)
iNOS	Forward	CGCATGACCTTGGTGTTTGG
	Reverse	CATAGACCTTGGGCTTGCCA
TNF-α	Forward	CCCGAGTGACAAGCCTGTAG
	Reverse	TGAGGTACAGGCCCTCTGAT
Arg1	Forward	ACTTAAAGAACAAGAGTGTGATGTG
	Reverse	ATTGCCAAACTGTGGTCTCC
CD206	Forward	CCAAACGCCTTCATTTGCCA
	Reverse	ACCTTCCTTGCACCCTGATG
GAPDH	Forward	GCAGGAGTACGATGAGTCCG
	Reverse	ACGCAGCTCAGTAACAGTCC

2.6 Western Blot

THP-1 and MCF-7 cells were lysed with RIPA buffer for 30 min and protein concentrations were measured using a BCA kit (both from KeyGEN BioTECH, Nanjing, China). Denatured protein samples (30 μg per lane, heated at 100°C for 10 min) were subjected to SDS-PAGE on a 5% stacking gel and a 10% separating gel. Electrophoresis was carried out at 80 V for 0.5 h, followed by 120 V for 1 h. Activate the PVDF membrane (Merck Millipore, Burlington, MA, USA) with methanol for 15 s, then perform blotting for 90 min at 100 V using a transfer buffer. After completion, incubate the membrane in TBST buffer containing 5% skim milk for 1 h. Upon completion, incubate overnight at 4°C with the following primary antibodies: The following primary antibodies were used: anti-CD74 (1:1000, ab108393), anti-iNOS (1:1000, ab178945), anti-Bax (1:1000, ab32503), anti-Bcl-2 (1:2000, ab182858), anti-p-STAT3 (Tyr705) (1:500, ab314450), and anti-GAPDH (1:10000, ab181602) from Abcam (Cambridge, UK); and anti-Arg1 (1:500, DF6657), anti-CD206 (1:500, DF4149), and anti-STAT3 (1:500, AF3293) from Affinity Biosciences (Cincinnati, OH, USA). After incubation, wash three times with TBST (10 min per wash), then incubate for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:10,000, catalog number ab205718, Abcam, Cambridge, UK). After washing (TBST, 3 × 10 min), membranes were incubated with HRP-goat anti-rabbit IgG (Cat. No. ab6721, Abcam, Cambridge, UK) for 1 h at room temperature. After incubation, the cells were washed three times with TBST again. Signal detection and analysis: ECL chemiluminescence substrate (Cat. No. WBKLS0500, Merck Millipore, Burlington, MA, USA) was incubated with the membrane for 2 min, and then placed in the ChemiDoc XRS + imaging system (Bio-Rad, Hercules, CA, USA) to collect signals. ImageJ Pro Plus 6.0 software (NIH, Bethesda, MD, USA) was used to semi-quantitatively analyze the gray value of the band. Relative expression = Target/GAPDH (grayscale ratio).

2.7 Flow Cytometry

Assessment of the polarization status of THP-1 cells by flow cytometry. The flow cytometer used was the Beckman DxFlex (Beckman Coulter, Brea, CA, USA). The following antibodies were incorporated: APC Mouse anti Human CD68 (1:50, Cat. No. MA5-23616, Thermo Fisher, Eugene, OR, USA), PerCP-Cy™5.5 CD86 (1:50, Cat. No. 561129, BD Biosciences, San Jose, CA, USA), and PE CD206 (1:50, Cat. No. 12-2069-42,

eBioscience, Thermo Fisher, San Diego, CA, USA). Incubate the cells for 20 min in the dark at 4°C. The fixation/membrane permeabilization solution used was Fixation/Permeabilization (Cat. No. 00-5523-00, same source as CD206). pHrodo-positive cell counts were detected using flow cytometry. To label the cells, pHrodo Green dye was added and incubated at 37°C in the dark for 1 h. Apoptosis levels were also detected using flow cytometry. To detect apoptosis levels, add Annexin V-FITC (1:50, catalog number 640906, BioLegend, San Diego, CA, USA) and incubate for 15 min at room temperature in the dark.

MCF-7 cells were labeled with pHrodo Green dye (Cat. No. P36013, Invitrogen, Eugene, OR, USA) for 1 h at 37°C. M1 macrophages were co-cultured with labeled MCF-7 cells for 2 h, then collected and analyzed by flow cytometry (Beckman DxFlex, Beckman Coulter, Brea, CA, USA). Phagocytosis was quantified as the percentage of $CD68^+$ macrophages that were pHrodo-positive. After co-culturing macrophages with MCF-7 cells at a 1:20 ratio for 24 h, MCF-7 cell apoptosis was assessed.

2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

Detect TNF-α and IL-6 levels in the culture supernatants of THP-1 and MCF-7 cells using a ready-to-use ELISA kit. TNF-α (Cat. No. DY210) and IL-6 (Cat. No. DY206) were detected using Human DuoSet ELISA kits, both from R&D Systems (Minneapolis, MN, USA). Collect the cell supernatant, add 100 μL of the standard or sample to the coated microplate, and incubate at 25°C for 2 h. Discard the supernatant, add 400 μL of wash buffer (PBS containing 0.05% Tween-20), and wash four times. Add an equal volume of secondary antibody and continue incubating at the same temperature for 2 h. After washing again, 100 μL streptavidin-HRP was added and incubated at room temperature in the dark for 20 min. After washing, add 100 μL of TMB substrate, incubate at room temperature in the dark for 20 min, then add 50 μL of stopping solution (2N H_2SO_4).

The standard sample was diluted by double dilution, and the concentration range was 0–500 pg/mL. Quantification was performed by fitting the standard curve using four-parameter logistic (4-PL) curve fitting [21]. Absorbance was measured at 450 nm using a microplate reader (SpectraMax iD3, Molecular Devices, San Jose, CA, USA), with correction at 570 nm. For STAT3 inhibition, macrophages were treated with 10 μM Stattic (Cat. No. S7024, Selleck Chemicals, Houston, TX, USA) simultaneously with LPS/IFN-γ for 24 h. Collect the cell supernatant after processing and perform an ELISA assay to detect TNF-α.

2.9 Cell Proliferation Assay

The CCK-8 ready-to-use assay kit (Cat. No. C6005, NCM Biotech, Suzhou, China) is used to detect cell proliferation (THP-1 and MCF-7). Add the CCK-8 reaction reagent according to the instructions; after a 2-h incubation, measure the results at a wavelength of 450 nm. (SpectraMax iD3, Molecular Devices, San Jose, CA, USA).

2.10 Statistical Analysis

All data are expressed as mean ± standard deviation (mean ± SD). Experiments were performed in at least three independent replicates, with three biological replicates per group (n = 3). Each independent experiment was conducted on separate days using independently prepared cells. Data normality was verified by the Shapiro-Wilk test and homogeneity of variances by Levene's test, both meeting the assumptions for one-way ANOVA ($p > 0.05$). Group comparisons were performed using one-way ANOVA with Tukey's post hoc test. Statistical significance was set at $p < 0.05$, with levels indicated as $^*p < 0.05$ and $^{**}p < 0.01$.

3 Results

3.1 CD74 May Be a Potential Protective Factor for BRCA and Is Associated with the IL6/JAK/STAT3 Pathway

Analyze the expression of CD74 in breast cancer using UALCAN to investigate its role. The results revealed a significant overexpression of CD74 in breast cancer (Fig. 1A), which was further confirmed through immunofluorescence experiments (Fig. 1B). This suggests that CD74 may be functionally significant in this cancer type. Additionally, our survival analysis showed that higher CD74 expression predicted favorable outcomes in breast cancer (Fig. 1C). TIMER2.0 analysis revealed that CD74 expression significantly correlated with macrophage infiltration across multiple tumor types. Notably, CD74 levels significantly aligned with M1 macrophage abundance in breast cancer (Fig. 1D). Furthermore, our analysis revealed a significant positive correlation between CD74 and CD68 and CD86 (Fig. 1E,F). We conducted a GSEA analysis and discovered that the HALLMARK_INFLAMMATORY_RESPONSE was significantly enriched (Fig. 1G). High CD74 expression is significantly associated with widespread inflammatory activity, corroborating the findings of previous studies on immune cell infiltration. Furthermore, the IL6/JAK/STAT3 signaling pathway was also significantly enriched (Fig. 1H). This pathway is not only a well-known inflammatory signaling pathway, but also a key regulatory pathway for macrophage polarization. Additionally, the HALLMARK_INTERFERON_GAMMA_RESPONSE and HALLMARK_APOPTOSIS signaling pathways were enriched (Fig. 1I,J).

3.2 Knockdown of CD74 Inhibits M1 Macrophage Polarization and STAT3 Signaling Pathway Activation

To explore CD74 function in macrophage polarization, CD74 was knocked down in THP-1-derived macrophages (Fig. 2A), achieving substantial silencing efficiency. Subsequently, as described in the Materials and Methods section, THP-1-derived macrophages transfected with shRNA-NC or shRNA-CD74#2 plasmids were induced to adopt M1 or M2 polarization phenotypes. Compared to the blank control group, LPS/IFN-γ successfully induced M1 polarization, as shown by increased levels of iNOS (protein and mRNA) (Fig. 2B,C) and TNF-α (mRNA and supernatant secretion) (Fig. 2D,E), along with a decrease in tyrosine 705 phosphorylation of STAT3 protein (p-STAT3) (Fig. 2B). Importantly, CD74 knockdown markedly reduced M1 markers (iNOS, TNF-α) and inflammatory cytokine secretion (TNF-α, IL-6) compared to the LPS/IFN-γ control group (Fig. 2E,F). Additionally, p-STAT3 levels were markedly elevated in the CD74 knockdown group. Arg1 and CD206 mRNA levels remained unchanged (Fig. 2G,H). Collectively, Western blot, qPCR, and ELISA confirmed that CD74 is essential for M1 polarization.

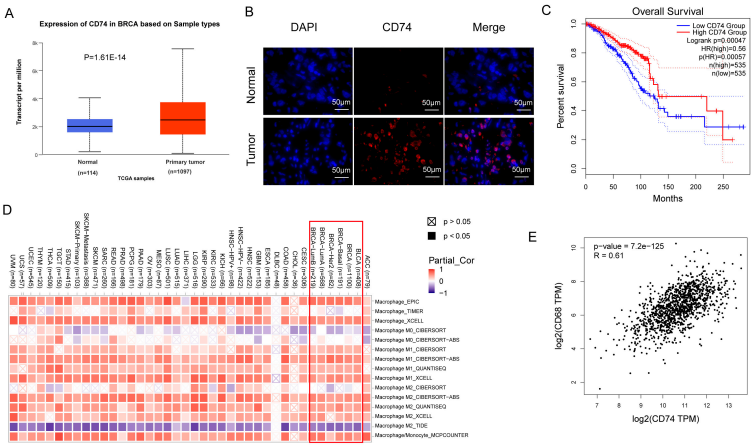


Figure 1: *Cont.*

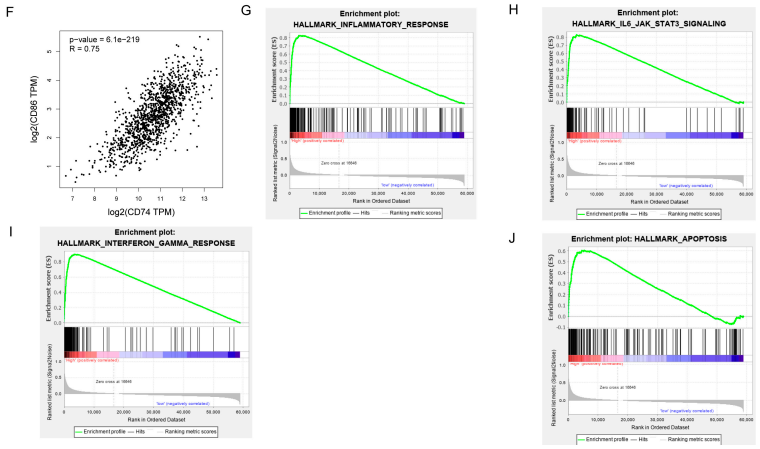
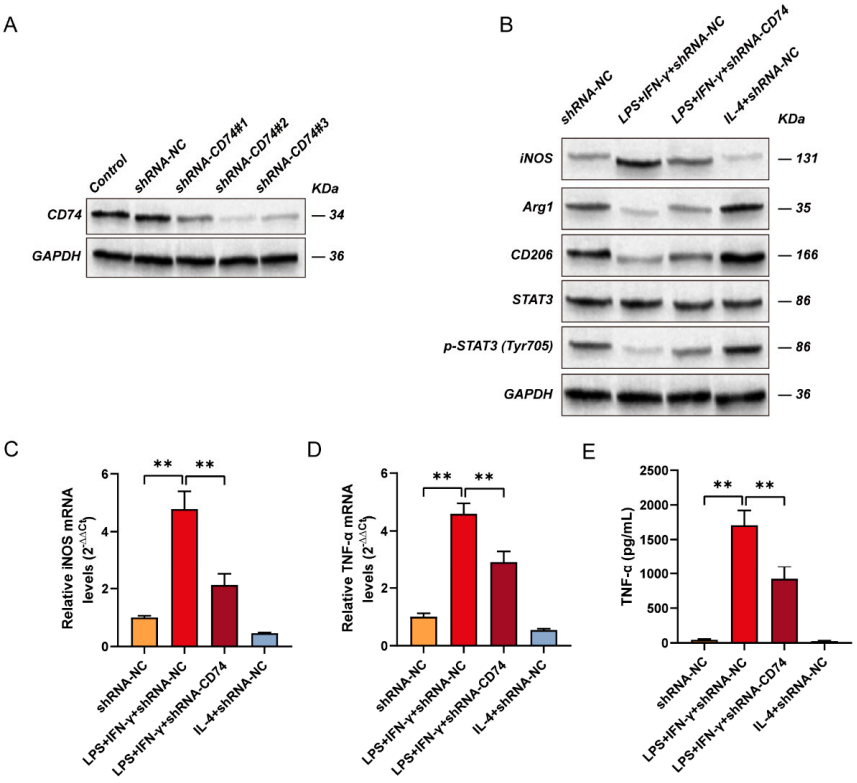


Figure 1: CD74 Bioinformatics analysis. (**A**) UALCAN analysis was used to examine the expression of CD74 in BRCA. (**B**) Immunofluorescence analysis revealed that CD74 was highly expressed in breast cancer tissues. Scale bar: 50 μm. (**C**) GEPIA2 analysis indicated that higher CD74 levels were linked to favorable prognosis in breast cancer (log-rank test). (**D**) TIMER2.0 assessed the correlation between CD74 and macrophage infiltration. (**E**) GEPIA2 analyzed CD74 correlation with CD68. (**F**) Correlation analysis between CD74 and CD86 was performed. (**G**–**J**) Gene Set Enrichment Analysis (GSEA) showed enrichment of (**G**) HALLMARK_INFLAMMATORY_RESPONSE, (**H**) HALLMARK_IL6_JAK_STAT3_SIGNALING, (**I**) HALLMARK_INTERFERON_GAMMA_RESPONSE, and (**J**) HALLMARK_APOPTOSIS in samples with high CD74 expression. Normalized enrichment score (NES) and false discovery rate (FDR) are indicated.



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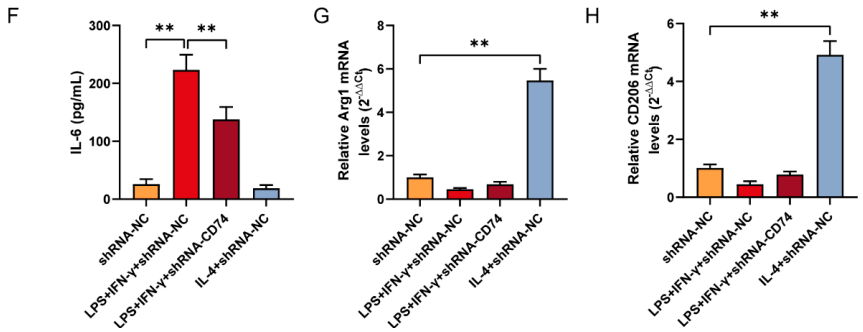

Figure 2: Knockdown of CD74 inhibits M1 polarization and promotes STAT3 activation. CD74 knockdown reduced M1 marker expression and cytokine secretion, while increasing STAT3 phosphorylation, indicating that CD74 promotes M1 polarization by suppressing STAT3 signaling. (**A**) Validation of CD74 knockout via Western blot. (**B**) Representative Western blot bands for iNOS, Arg1, CD206, STAT3, p-STAT3 (Tyr705), and GAPDH in each group. (**C**–**H**) mRNA expression levels of iNOS (**C**), TNF-α (**D**), Arg1 (**G**), and CD206 (**H**) were detected by qPCR in each group. (**E**,**F**) ELISA detection of TNF-α (**G**) and IL-6 (**F**) levels in cell culture supernatants. Data are presented as mean ± standard deviation. $^{**}p < 0.01$.

3.3 Knockdown of CD74 Reduces Expression of the M1 Macrophage Surface Marker CD86

Afterward, we conducted a flow cytometric analysis. LPS/IFN-γ stimulation significantly increased the proportion of $CD86^+$ M1 macrophages ($CD68^+$) compared to controls (Fig. 3A,B). However, CD74 knockdown reversed this effect, markedly reducing the percentage of positive cells. Additionally, the positivity rate for the M2 surface marker CD206 remained consistently low in all M1-induced groups, with no significant differences between them (Fig. 3C,D). This further supports the specific role of CD74 in M1 polarization.

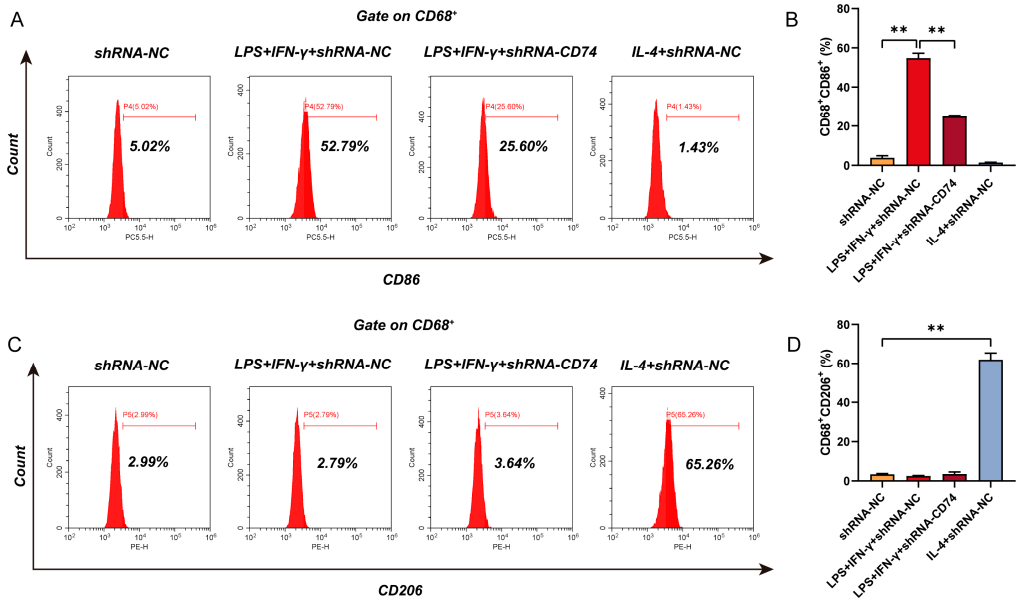

Figure 3: Knockdown of CD74 reduces CD86 expression in M1 macrophages. Flow cytometry showed that CD74 knockdown decreased the proportion of $CD86^+$ cells without affecting CD206 expression, confirming the specific role of CD74 in M1 polarization. (**A**,**C**) Flow cytometry plots showing the percentage of $CD86^+$ (**A**) and $CD206^+$ (**C**) cells within the $CD68^+$ macrophage population. (**B**,**D**) Determine the proportions of $CD68^+CD86^+$ (**B**) and $CD68^+CD206^+$ (**D**) cells. The data is presented as mean ± standard deviation. $^{**}p < 0.01$.

3.4 Knockdown of CD74 Impairs the Phagocytic and Pro-Apoptotic Capabilities of M1 Macrophages against Breast Cancer Cells

Phagocytosis assays (see Materials and Methods) revealed that M1 macrophages exhibited markedly enhanced phagocytic activity (Fig. 4A,B). In contrast, CD74 knockdown markedly reduced M1 macrophage phagocytosis, indicating CD74 is essential for this function. Additionally, we examined the killing ability of macrophages on tumor cells. Annexin V/PI staining showed that MCF-7 apoptosis increased after co-culture with LPS/IFN-γ-stimulated M1 macrophages (Fig. 4C,D). However, CD74 knockdown in M1 macrophages markedly reduced this effect. This demonstrates the essential role of CD74-mediated M1 polarization in the function of macrophages in killing tumor cells.

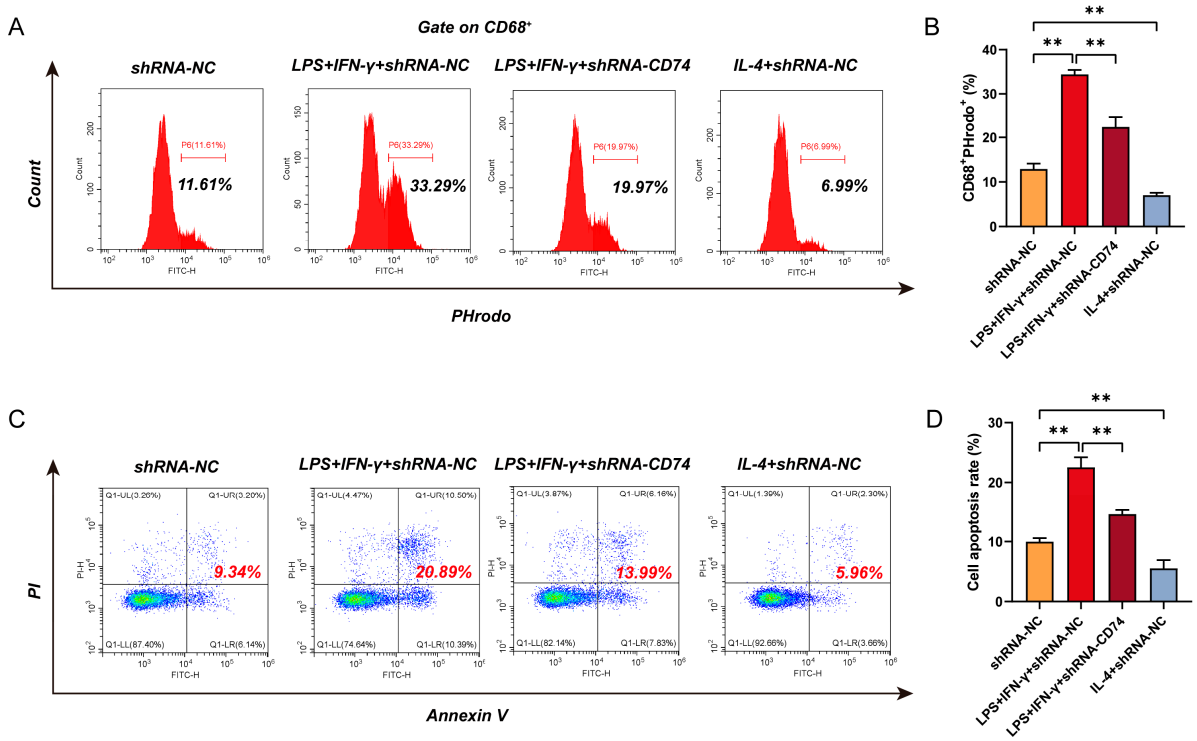

Figure 4: Knockdown of CD74 impairs the phagocytic and pro-apoptotic functions of M1 macrophages. CD74 deficiency significantly reduced the phagocytic capacity of M1 macrophages and their ability to induce tumor cell apoptosis, demonstrating the essential role of CD74 in M1 effector functions. (**A**) Flow cytometry plots showing the proportion of pHrodo-positive cells within the $CD68^+$ macrophage population. CD68 was used as a pan-macrophage marker; however, before the assay, these cells were confirmed to be M1-polarized based on increased CD86 expression (Fig. 3) and M1 marker expression (Fig. 2). (**B**) Statistical analysis of the proportion of CD68 pHrodo ∂ cells. (**C**) The representative flow cytometry diagram showed the apoptosis of MCF-7 cells detected by Annexin V-FITC/PI double staining. (**D**) Quantification of total (early + late) MCF-7 apoptosis. $^{**}p < 0.01$.

3.5 CD74 Knockdown in M1 Macrophages Altered Apoptotic Protein Levels and Promoted Co-Cultured Tumor Cell Viability

To explore the molecular basis of apoptosis, apoptosis-related protein levels were examined in co-cultured tumor cells. Western blot revealed that co-culture with CD74-knockdown M1 macrophages reduced pro-apoptotic Bax while elevating anti-apoptotic Bcl-2 in MCF-7 cells (Fig. 5A–C). Additionally, CCK-8 assays confirmed that the survival rate of MCF-7 cells significantly increased under these conditions (Fig. 5D).

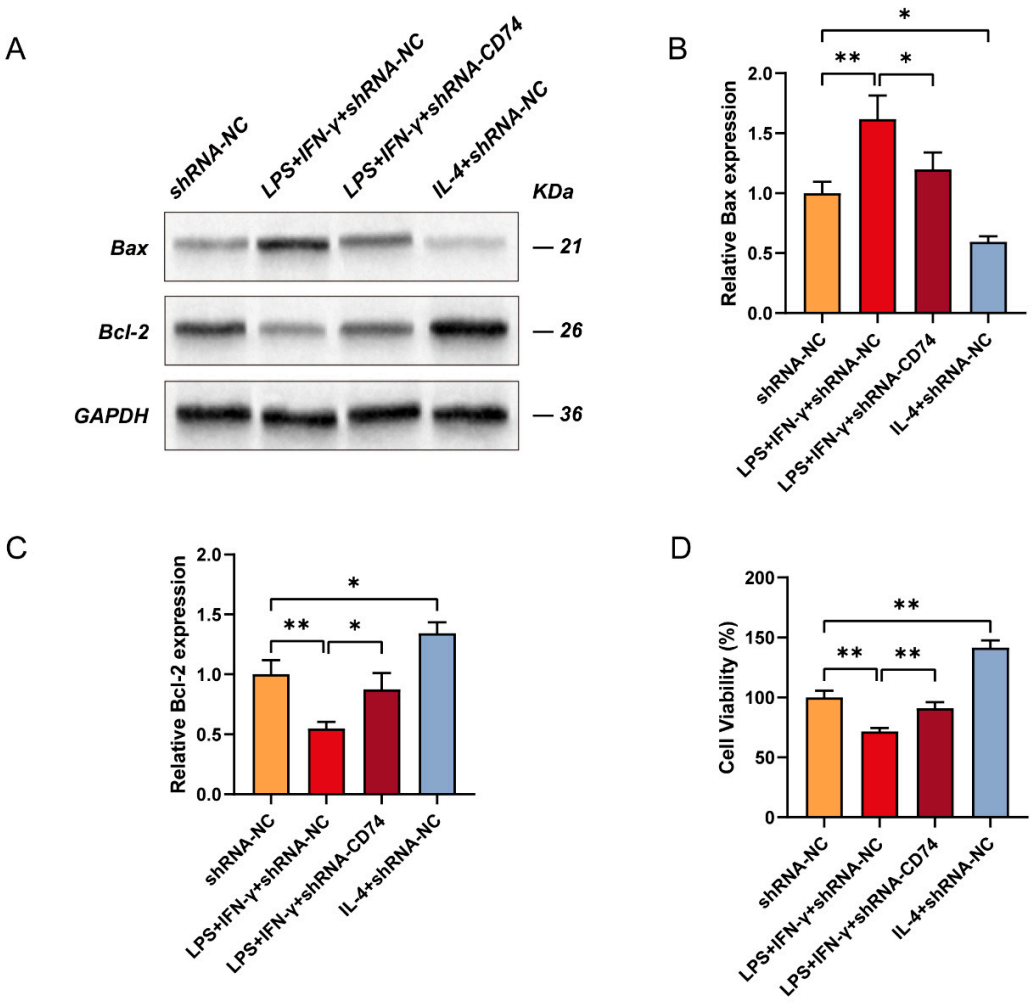

Figure 5: Knockdown of CD74 alters apoptotic protein expression and enhances tumor cell viability. MCF-7 cells co-cultured with CD74-knockdown M1 macrophages showed decreased Bax, increased Bcl-2, and enhanced viability, indicating that CD74-mediated M1 polarization suppresses tumor cell survival. (**A**) Representative Western blot bands of Bax, Bcl-2, and GAPDH proteins in MCF-7 cells after co-culture. (**B**) Quantitative analysis of Bax protein levels and (**C**) Bcl-2 protein levels, with GAPDH as the internal reference. (**D**) The viability of MCF-7 cells after co-culture was measured using the CCK-8 method. $^{*}p < 0.05$, $^{**}p < 0.01$.

3.6 Inhibition of STAT3 Restores M1 Function in CD74-Knockdown Macrophages

To determine whether STAT3 is a downstream effector molecule of CD74, we treated CD74-knockdown M1 macrophages with STAT3 inhibitor Stattic. CD74 knockdown significantly lowered TNF-α secretion versus controls (Fig. A1, $p < 0.05$). Importantly, STAT3 inhibitor treatment significantly restored the secretion of TNF-α in CD74 knockdown cells ($p < 0.05$ vs. CD74 knockdown alone). These findings identify STAT3 as a key effector of CD74 in promoting M1 polarization.

4 Discussion

Macrophages exhibit plasticity in the tumor microenvironment (TME), and their functional status (pro-inflammatory M1 or anti-inflammatory M2) largely determines the progression or regression of tumors [10]. Therefore, identifying key molecules that regulate macrophage polarization has become a

crucial strategy for cancer immunotherapy. In this study, CD74 was identified as a core molecule that drives macrophages to polarize towards the anti-tumor M1 phenotype. The underlying JAK-STAT3 pathway was also elucidated, providing a potential new target for immunotherapy. While previous studies have shown that STAT3 activation can stimulate M2 polarization of macrophages [22–24], our study focused on the unique role of CD74 in promoting M1 polarization by inhibiting the phosphorylation of STAT3 (Tyr705).

Our research aligns with previous studies on CD74's role as a regulator of immune cells and inflammatory signaling [13,25,26]. CD74 not only serves as a chaperone protein for MHC class II molecules but also acts as a high-affinity receptor for MIF, participating in various inflammatory signaling pathways [27]. The MIF-CD74 signaling axis is known to activate several pro-inflammatory pathways, including ERK, PI3K/AKT, and NF-κb [28–30]. This study is the first to establish a clear connection between CD74 and the JAK-STAT3 pathway in M1 polarization. Our data indicate that CD74 serves as a key activation point for this pathway, and silencing CD74 leads to an increase in p-STAT3 levels and a decrease in downstream M1 gene expression. This discovery provides a crucial link in understanding the signaling network of M1 polarization.

STAT3 is activated by upstream kinases (such as JAK1, JAK2, and Src) [31] and inhibited by protein tyrosine phosphatases (such as SHP-1 and SHP-2) [32,33]. Given that CD74 has been reported to interact with Src family kinases and PI3K/AKT pathway [34], it is speculated that CD74 may limit the phosphorylation of STAT3 by interfering with these upstream kinases. In the future, it is necessary to use co-immunoprecipitation and kinase activity detection to further clarify whether CD74 directly or indirectly regulates the activation of STAT3 in macrophages.

In addition to the STAT3 pathway, CD74 has been reported to activate multiple signaling pathways in monocytes, including ERK, AMPK, and NF-κB [12]. These pathways also regulate macrophage polarization and inflammation. The MAPK/AP-1 axis, for instance, drives pro-inflammatory cytokine production [35], while the PI3K/AKT/mTOR pathway affects the metabolic reprogramming and polarization of macrophages [36]. Although this study focused on the STAT3 pathway, CD74 likely coordinates multiple signaling cascades simultaneously to regulate the functional status of macrophages [12]. Further investigation of these pathways in CD74-mediated M1 differentiation will broaden our understanding of the molecular network through which CD74 regulates macrophage polarization.

CD74's involvement in tumorigenesis and progression has been well documented across multiple cancer types. For example, in hepatocellular carcinoma, CD74 mediates tumor-immune cell interactions to drive malignant behavior [26]. In triple-negative breast cancer, CD74 may foster an immunosuppressive microenvironment by expanding regulatory B cells and dendritic cells [16]. In pancreatic cancer, CD74 drives the TRAF6-NF-κB axis, promoting pro-inflammatory cytokine release and modulating tumor inflammation [37].

Functionally, CD74 not only regulates M1 polarization but also governs its key effector functions. Phagocytosis and induction of tumor cell apoptosis are essential mechanisms through which M1 macrophages exert their anti-tumor effects [38–40]. Our data clearly demonstrate that both of these functions are significantly impaired after CD74 knockdown. Furthermore, we have shown the impact of this functional change on tumor cells themselves, at two levels: apoptosis-related proteins (decreased Bax/Bcl-2 ratio) and cell viability. These results provide initial evidence of the relationship between CD74 expression and STAT3 phosphorylation levels, M1 marker expression, and functional phenotype. They also suggest a potential effect of CD74 on tumor cell behavior, laying the groundwork for further research on its role in regulating the anti-tumor function of macrophages.

This study has several limitations. First, all experiments were conducted *in vitro* using cell lines, and there was no *in vivo* validation using animal models. Second, the exact mechanism by which CD74 regulates STAT3 phosphorylation remains unclear and requires further investigation. Third, other potential signaling pathways (such as ERK, AMPK, and NF-κB) were not examined in the experiments.

This study is primarily focused on the *in vitro* cell model, and while the mechanism is well understood, it lacks confirmation through *in vivo* experiments. Future *in vivo* studies should determine whether CD74 knockdown promotes breast cancer growth by suppressing TAM M1 polarization in mouse models. Additionally, it would be valuable to investigate the role of specific ligands upstream of CD74 (such as MIF) in this process, as well as the potential cross-talk between CD74 and TLR4 signals in coordinating M1 polarization. Finally, to further the translational medicine value of this research, it would be beneficial to perform immunohistochemistry or bioinformatics analysis on clinical breast cancer tissue samples to explore the relationship between CD74 expression levels, macrophage M1/M2 polarization status, and patient prognosis.

5 Conclusions

In summary, CD74 may promote M1 polarization of macrophages *in vitro* by regulating the JAK-STAT3 pathway. This may also enhance their ability to engulf and kill tumor cells. CD74 may be involved in immune activation within the breast cancer microenvironment; the underlying mechanisms require further investigation. Targeting CD74 could potentially serve as a strategy for regulating the function of tumor-associated macrophages and enhancing anti-tumor immunity.

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Availability of Data and Materials: CD74 expression was analyzed using the UALCAN platform (Direct web link: https://ualcan.path.uab.edu/cgi-bin/TCGAExResultNew2.pl?genenam=CD74&ctype=BRCA). Survival and correlation analyses of the BRCA dataset from TCGA were performed via the GEPIA2 website (Direct web link: http://gepia2.cancer-pku.cn/#survival). The association between CD74 and macrophages was assessed using TIMER2.0 (Direct web link: https://compbio.cn/timer2/). No other data sources require disclosure. All the experimental data of this manuscript can be obtained from the corresponding authors according to reasonable requirements.

Ethics Approval: The Clinical Trial Ethics Committee of the First Huizhou Affiliated Hospital of Guangdong Medical University has approved this study. The Ethics Review number is KYLL-2024-009-01. All experiments were carried out in accordance with the relevant guiding principles and regulations of the Clinical Trial Ethics Committee of The First Huizhou Affiliated Hospital of Guangdong Medical University. All patients signed the informed consent form.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Appendix A Fig. A1:

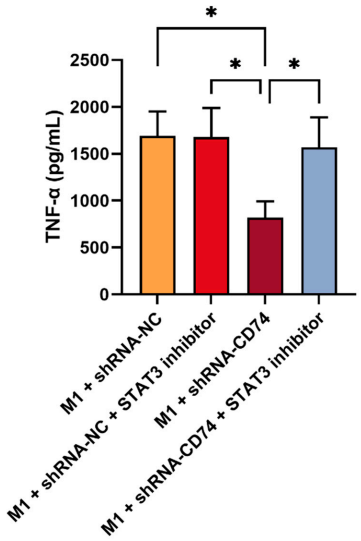

Figure A1: STAT3 inhibition restores TNF-α secretion in CD74-knockdown M1 macrophages. THP-1-derived macrophages were transfected as indicated and polarized to M1 with LPS/IFN-γ for 24 h, with or without Stattic (10 μM). TNF-α levels were measured by ELISA. Data are mean ± SD from three independent experiments. $^{*}p < 0.05$.

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