

Studies of NDUFS4–CCL5 Axis Potentially Mediating Fibroblast–Macrophage Crosstalk in UVB-Induced Skin Damage

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Abstract

Objective

NDUFS4 (NADH: ubiquinone oxidoreductase iron-sulfur protein 4) is an isoform of mitochondrial complex I and plays a significant role in the regulation of skin photoaging. However, the function of NDUFS4 in UVB-induced immune cell crosstalk remains unclear. This study focuses on the role of NDUFS4 in the interactions between fibroblasts and macrophages.

Methods

UVB-treated BJ cells were utilized to establish a cellular photoaging model. Lentiviral vectors overexpressing NDUFS4 were transfected into BJ cells to construct an NDUFS4 overexpression cell model. Subsequently, culture supernatants from UVB-mediated BJ cells or overexpressed-NDUFS4 BJ cells were co-cultured with macrophages. CCK8, transwell, and flow cytometry were used to assess macrophage viability, migration ability, and polarization status. Finally, proteomic techniques were utilized to probe the downstream proteins regulated by NDUFS4 in order to investigate the molecular mechanism of NDUFS4.

Results

UVB-exposed BJ cells or BJ cells overexpressing NDUFS4 inhibited macrophage proliferation and migration, induced differentiation to an M1 macrophage phenotype, and activated interferon regulatory factor 3 (IRF3) pathways in macrophages. Notably, these effects could be reversed by the ferroptosis inhibitor Fer-1. Proteomic analysis revealed that NDUFS4 significantly increased the levels of CCL1 and CCL5, while inhibiting CXCL11. In contrast, Fer-1 significantly reversed the levels of all three chemokines. Notably, neutralization of CCL5 with a specific antibody reversed the NDUFS4 overexpression-induced M1 polarization and IRF3 activation in macrophages, confirming that CCL5 serves as a key mediator of the fibroblast-macrophage crosstalk driven by NDUFS4.

Conclusions

These findings suggest that NDUFS4 may promote a pro-inflammatory shift in macrophage phenotype under the present experimental conditions, as indicated by changes in selected polarization markers. NDUFS4 may serve as an effective therapeutic target for preventing skin damage; however, in vivo validation is required to confirm its translational relevance.

Introduction

Ultraviolet rays (UVR), a component of sunlight, pose a significant threat to the skin [1]. Among these, UVB rays (290–320 nm) have a high penetrating ability and are major contributors to skin damage [2]. Research has demonstrated that repeated exposure to UVB increases the levels of reactive oxygen species (ROS) in epidermal cells, which may promote carcinogenesis, cellular senescence, and other skin lesions [3–5]. Therefore, investigating the mechanisms of UVB-induced skin damage can aid in the development of therapeutic approaches for skin conditions such as photoaging.

After exposure to UVB radiation, neutrophils and macrophages successively infiltrate the dermis and then migrate to the epidermis [6–8]. For instance, exposing mouse skin to UVB for three weeks can enhance macrophage activation and infiltration in the epidermis, leading to an increased release of inflammatory factors that contribute to skin damage [9]. In response to pathophysiological stimuli, macrophages can differentiate functionally into various phenotypes, including pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages; M1 macrophages are characterized by the expression of CD86 and inducible nitric oxide synthase (iNOS), while M2 macrophages typically express CD206 and arginase-1 (Arg-1) [10, 11]. A study by Piia Karisola found that the proportion of pro-inflammatory M1 macrophages significantly increased at both time points following UVB exposure, as indicated by skin transcriptome analysis. Furthermore, the number of M1 macrophages correlated with the severity of skin erythema [12]. This suggests that UVB may exacerbate skin photodamage by promoting the M1 transition in macrophages.

NDUFS4 (NADH: ubiquinone oxidoreductase iron-sulfur protein 4) is an 18 kDa accessory subunit of mitochondrial complex I, which has been extensively studied in models of mitochondrial disease [13, 14]. NDUFS4 was expressed in primary skin fibroblasts, where its expression correlates with elevated levels of reactive oxygen species (ROS) and abnormal mitochondrial morphology [15]. Additionally, our research group identified NDUFS4 as a gene associated with UVB-mediated photoaging through RNA sequencing [16]. Inhibition of NDUFS4 expression was found to reduce UVB-induced ferroptosis in cells, indicating a close relationship between NDUFS4 expression and skin damage [16]. Furthermore, in macrophages, silencing NDUFS4 led to defects in mitochondrial complex I, which prevented the polarization of M1 macrophages induced by high glucose levels [17]. This suggests that NDUFS4 not only regulates ferroptosis in skin cells but also plays a role in stimulating the phenotypic differentiation of macrophages. However, the available studies lack reports on the role of NDUFS4 in skin cell scrambling with macrophages.

Among the downstream effectors of the cGAS-STING pathway, interferon regulatory factor 3 (IRF3) serves as a key transcription factor that orchestrates innate immune responses [18]. Upon activation, IRF3 is phosphorylated and translocates to the nucleus, where it drives the transcription of type I interferons and pro-inflammatory cytokines [18]. In the context of UVB-induced skin damage, accumulating evidence indicates that UVB irradiation triggers oxidative stress and leakage of double-stranded DNA from nuclei and mitochondria into the cytoplasm of skin cells, which activates the cGAS-STING pathway and subsequently leads to IRF3 phosphorylation [19]. Notably, previous studies have demonstrated that the supernatant from UVB-treated keratinocytes induces IRF3 activation in

macrophages, and this activation is closely associated with exacerbated skin inflammation [20]. Furthermore, IRF3 has been identified as a critical regulator of macrophage polarization, with its activation promoting the shift toward the pro-inflammatory M1 phenotype [18, 21]. The cGAS-STING-IRF3 axis therefore represents a crucial molecular link between UVB-damaged skin cells and macrophage-mediated inflammatory responses [19]. However, whether NDUFS4 in skin fibroblasts modulates macrophage function through the IRF3 pathway remains unclear.

Therefore, this study focuses on the role and mechanism of NDUFS4. It was observed that skin fibroblasts overexpressing NDUFS4 led to a decrease in macrophage viability and promoted macrophage differentiation toward a pro-inflammatory M1 phenotype. This finding may be closely associated with UVB-induced skin damage. Therefore, exploring the role of NDUFS4 in fibroblast-macrophage crosstalk may provide a basis for understanding the molecular mechanisms of UVB-induced skin damage. Nevertheless, further validation in physiologically relevant models is warranted.

Materials and methods

Cell culture

In this study, macrophage THP-1 (CL-0233; Wuhan Procell Life Science & Technology Co., Ltd, China) and human skin fibroblasts BJ (CRL-2522; Wuhan Procell Life Science & Technology Co., Ltd, China) were used in this study. BJ cells were cultured in high-glucose Dulbecco 's Modified Eagle Medium (DMEM, 4.5 g/L glucose, PM150210, Procell, Wuhan, China) supplemented with 10% fetal bovine serum (FBS, 10270106, Gibco, Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (15140122, Gibco, USA). THP-1 cells were cultured in RPMI-1640 medium (PM150110, Procell, Wuhan, China) supplemented with 10% FBS, 0.05 mM β -mercaptoethanol (M3148, Sigma-Aldrich, St. Louis, MO, USA), and 1% penicillin-streptomycin. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells between passages 3 and 8 were used for all experiments.

Cell treatment

The THP-1 cells were treated with 100ng/ml Phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) for 24 hours in order to stimulate the cells to become M0-phase macrophages. BJ cells were treated with UVB radiation at a dose of 20mJ/cm² for 30 minutes to simulate skin cell photoaging [22–24]. For the Ferrostatin-1 (Fer-1) treatment group, 2, 5, 10, 20, and 50 μ M Fer-1 (HY-100579, Sigma-Aldrich, St. Louis, MO, USA) for 1 hour. Fer-1 was dissolved in DMSO at a concentration of 1 mg/mL to prepare a stock solution, which was then diluted with culture medium to the indicated working concentrations. Subsequently, cell culture supernatants from each group of BJ cells were co-cultured with PMA-treated THP-1 to explore macrophage function. For CCL5 neutralization experiments, BJ cells overexpressing NDUFS4 were treated with 1 μ g/mL anti-human CCL5 neutralizing antibody (MAB678,

R&D Systems, Minneapolis, MN, USA) or an isotype control IgG (MAB002, R&D Systems) for 24 hours prior to UVB exposure. The culture supernatants were then collected and used to treat macrophages.

For the co-culture assay, THP-1 cells were seeded into Transwell inserts (3413, Corning, Somerville, MA, USA) at 80% confluence and treated with 100 ng/mL PMA for 24 hours to induce differentiation into M0 macrophages. Meanwhile, BJ cells were seeded into 24-well plates and infected with lentiviral vectors overexpressing NDUFS4 or negative control lentivirus. After 24 hours of infection, the THP-1 cells in the inserts were washed twice with PBS, and 100 μ L of complete medium was added to each insert. The inserts were then placed into the 24-well plates containing the BJ cells and co-cultured for 2 hours. Following co-culture, the inserts were removed, and the THP-1 cells were harvested by trypsinization for subsequent experiments.

MTT assay

To determine the optimal concentration of Fer-1 without causing cytotoxicity, BJ cells were seeded into 96-well plates at a density of 5×10^3 cells per well and cultured overnight to allow attachment. The cells were then treated with various concentrations of Fer-1 (2, 5, 10, 20, and 50 μ M) for 12, 24, and 48 hours. Following treatment, the culture medium was replaced with 100 μ L of fresh medium containing 0.5 mg/mL methyl thiazolyl tetrazolium (MTT, YEASEN, Shanghai, China) and incubated at 37°C for 4 hours. After incubation, the medium was carefully removed, and 150 μ L of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formed formazan crystals. The plates were gently shaken for 10 minutes in the dark, and the absorbance was measured at 490 nm using a microplate reader (Multiscan MK3, Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was calculated as the percentage of absorbance relative to the untreated control group. All experiments were performed in triplicate.

Cell transfection

NDUFS4 overexpression lentiviral vector and negative control NC were synthesized by GeneChem (Shanghai, China). BJ cells were inoculated in 24-well plates at a density of 3×10^4 cells/well 1 day before infection. 24 hours later, the medium was changed to DMEM medium containing hexadimethrine bromide (5 μ g/mL; Sigma-Aldrich, St. Louis, MO, USA). Then BJ cells were infected with 100 μ L of viral stock solution. From the next day, BJ cells were cultured in DMEM medium containing 1 μ g/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA) to selectively screen the medium for subclones to obtain NDUFS4 overexpressing cell lines.

Cell Counting Kit 8 (CCK-8)

THP-1 cells (5×10^3 /well) were inoculated into 96-well plates and cultured in 100 μ L medium. One to five days after co-treating THP-1 cells using BJ cell culture supernatant, the cells were incubated with 10 μ L CCK-8 reagent (Beyotime, Shanghai, China)/well for 4 hours. All experiments were repeated three

times. Absorbance was analyzed at 450 nm using a spectrophotometer (Multiscan MK3, Thermo Fisher Scientific, Waltham, MA, USA), and medium wells were used as blanks.

EdU proliferation assay

To evaluate macrophage proliferation, THP-1 cells were seeded into 96-well plates at a density of 5×10^3 cells per well and co-cultured with BJ cell culture supernatants as described above. After co-culture, cells were incubated with 10 μ M 5-ethynyl-2'-deoxyuridine (EdU, Beyotime, Shanghai, China) for 2 hours at 37°C under standard culture conditions. Following EdU labeling, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then permeabilized with 0.5% Triton X-100 for 20 minutes. Subsequently, the incorporated EdU was detected by incubation with a Click reaction mixture containing fluorescent azide, CuSO_4 , and reducing agent for 30 minutes at room temperature, protected from light, according to the manufacturer's instructions (Beyotime, Shanghai, China). Cell nuclei were counterstained with Hoechst 33342 or DAPI for 10 minutes. Proliferating cells (EdU-positive) were visualized under a fluorescence microscope (Olympus IX73, Tokyo, Japan).

Transwell Assay

Transwell assays were performed using Costar chambers (Corning, Somerville, MA, USA) to assess THP-1 cells migration. The upper chamber was inoculated with THP-1 cells (5×10^4) suspended in 200 μ l of serum-free medium, while the bottom chamber was filled with RPMI-1640 media that included 10% FBS. The cells were incubated for 2 days and then removed from the upper chamber using cotton swabs. After 30 minutes of 4% paraformaldehyde fixation, migrating cells were stained for 20 minutes with 0.1% crystal violet. A microscope (Olympus IX73, Tokyo, Japan, $\times 40$) was used to count the cells..

Real-time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted with Trizol (TIANGEN, Shanghai, China) lysis buffer and 4 μ g of total RNA was converted to cDNA using MultiScribe™ reverse transcriptase (Applied Biosystems, Waltham, MA, USA). cDNA was amplified using Applied Biosystems' Fast SYBR® Green Master Mix. RT-qPCR was used to quantify the amount of RNA extracted using the StepOnePlus™. Primer sequences used for RT-qPCR are listed in Table 1.

Table 1
List of all primers used RT-qPCR.

Species	Primer	Sequence (5'-3')
Homo sapiens	GAPDH_F	TGACTTCAACAGCGACACCCA
	GAPDH_R	CACCCTGTTGCTGTAGCCAAA
	CD86-F	ATCTTCTTAGGTTCTGGGT
	CD86-R	GGCTTGTATCAATGTATCAT
	iNOS-F	GGACTCACAGCCTTTGGA
	iNOS-R	GGATGTCGGACTTTGTAGATT
	Arg1-F	CATAGGGATTATTGGAGC
	Arg1-R	TCATTAGGGATGTCAGCA
	CD206-F	GAAAGGTGACCCTACTATGT
	CD206-R	AACTGGTGGATTGTCTTG
	NDUFS4-F	CTCTCTGAATCGCTGGAAGG
	NDUFS4-R	GATGGTCGAACTGGTCTGAAT
	I-309-F	CAGCTCCATCTGCTCCAAT
	I-309-R	CCTCTGAACCCATCCAAC
	I-TAC-F	AGCAGTGAAAGTGGCAGAT
	I-TAC-R	GATTTAGGCATCGTTGTCC
	RNATES-F	CCCTCGCTGTCATCCTCAT
	RNATES-R	ACACTTGGCGGTTCTTTTCG

Flow cytometry

The distribution of CD86 and CD206 in THP-1 cells was analyzed in this study using flow cytometry. Briefly, 100 µm filters (Corning Glass Works, Corning, NY, USA) were processed using ACK buffer (Thermo Fisher Scientific, Waltham, MA, USA) to generate single cell suspensions. Antibodies to CD206-FITC (321104, Biolegend, San Diego, CA, USA) and CD86-APC (381004, Biolegend, San Diego, CA, USA) were incubated on the cells for 30 minutes. The amounts of APC and FITC were quantified using a FACScan flow cytometer (BD Biosciences, New Jersey, USA). The flow cytometry gating strategy is shown in Supplementary Material 1.

Western blotting (WB)

RIPA buffer (Beyotime, Shanghai, China) was used to lyse proteins. A PVDF membrane was utilized to retain the proteins following their separation (50 µg) by 10% SDS-polyacrylamide gel electrophoresis. After sealing the membranes with 5% skim milk powder, the primary antibodies were incubated for 12 hours. The membrane was then treated with second antibody for 1 hour followed by three TBST washes. After one more wash, the membranes were analyzed using an ECL protein blotting assay (Beyotime, Shanghai, China). GAPDH or β-actin was used as the internal loading control to normalize protein expression levels. The antibody information used in this study is displayed in Table 2.

Table 2
Antibody Information.

Gene name	Catalog Number	Manufacturer	Dilution ratios
IRF3	Ab68481	Abcam	1:1000
P-IRF3	07-582-I	sigma	1:3000
NDUFS4	abcam	Abcam	1:1000
GAPDH	AF7021	Affinity	1:3000
Goat Anti-Rabbit	S0001	Affinity	1:3000

Proteomics sequence and data analysis

The downstream protein expression of NDUFS4 was measured according to the previous studies of our group [25]. Briefly, for each sample, 20 mg of protein was mixed with five times the amount of up-sampling buffer and then separated. After desalting and concentrating the digested peptides for each sample using vacuum centrifugation, they were reconstituted in 40 µL of 0.1% formic acid. The peptide mixture for each sample was labeled with TMT (Thermo Fisher Scientific, Waltham, MA, USA) reagent according to the manufacturer's instructions. LC-MS/MS analysis was performed using a Q Exactive HF mass spectrometer (Thermo Scientific, Waltham, MA, USA). Differentially expressed proteins (DEPs) were identified using a fold change greater than 1.5 and a P-value of less than 0.05. Volcano plots of DEP were drawn using ggplot2 in R; Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO) enrichment of DEP was implemented using ClusterProfiler (v3.8.1).

Enzyme-Linked Immunosorbent Assay (ELISA)

In according with the kit instructions, the commercially available Human CCL5/RANTES ELISA Kit (EK1129–96, MultiSciences Biotech Co., Ltd., Hangzhou, China), Human CXCL11/I-TAC ELISA Kit (EK1207–96. MultiSciences Biotech Co., Ltd., Hangzhou, China) and Human CCL1 (I-309) ELISA Kit (EHCCL1, invitrogen, Shanghai, China) were used to determine the concentrations of CCL5, CXCL11, and CCL1 in the supernatants of BJ cell cultures using a spectrophotometer (Multiscan MK3, Thermo Fisher Scientific, Waltham, MA, USA) to determine the OD value of the samples at 450 nm.

Statistical analysis

All data were analyzed using GraphPad Prism 8.0 software. Each experiment was biologically replicated three times and results are expressed as mean $\pm$ standard deviation (SD). One-way ANOVA combined with Dunnett's multiple comparison test was used to determine whether statistically significant differences existed in multiple comparisons. P-values less than 0.05 indicated statistical significance.

Results

UVB-exposed human skin fibroblasts mediate macrophage polarization

To evaluate the cytotoxic effects of Fer-1 on BJ cells, cells were treated with various concentrations of Fer-1 (2, 5, 10, 20, and 50 μ M). As shown in Fig. 1A, Fer-1 had no significant effect on BJ cell viability at concentrations up to 10 μ M compared with the control group, whereas concentrations of 20 μ M and 50 μ M significantly reduced cell viability. Therefore, 10 μ M Fer-1 was selected for subsequent experiments to avoid potential cytotoxicity while maintaining its ferroptosis-inhibitory effect. UVB-exposed human skin fibroblast BJ culture supernatants were collected and used to treat macrophages in order to investigate fibroblast-macrophage interactions. The results indicated that UVB-treated BJ culture supernatants significantly reduced macrophage viability (Fig. 1B). In contrast, the treatment of culture supernatants from UVB-irradiated BJ cells with the ferroptosis inhibitor Fer-1 resulted in an increase in macrophage viability (Fig. 1B). EdU staining results showed that the culture supernatant from UVB-treated BJ cells significantly inhibited macrophage proliferation, whereas treatment with a ferroptosis inhibitor reversed this effect (Fig. 1C). The capacity of macrophage migration was assessed using a Transwell assay. We found that UVB-irradiated BJ culture supernatants inhibited migration of macrophage compared to the control group, while Fer-1-treated supernatants restored the macrophage migration ability (Fig. 1D). In this study, macrophage polarization was assessed using a panel of well-established M1 (iNOS and CD86) and M2 (Arg-1 and CD206) markers at both the mRNA and protein levels. Results from RT-qPCR indicated that UVB-irradiated BJ culture supernatants increased the expression of M1 phenotypic genes (iNOS/CD86) and decreased the expression of M2 phenotypic genes (Arg-1/CD206) in macrophages (Fig. 1E). In contrast, Fer-1-treated BJ culture supernatants inhibited the expression of M1 phenotype genes (iNOS/CD86) and increased the expression of M2 phenotype genes (Arg-1/CD206) in macrophages (Fig. 1E). Flow cytometry results corroborated the PCR findings, showing that UVB-irradiated BJ culture supernatants increased the number of CD86-positive macrophages, whereas Fer-1 treatment increased the number of CD206-positive macrophages (Fig. 1F). Furthermore, we used WB to detect the expression of cyclic GMP-AMP synthase (cGAS)/interferon gene stimulating factor (STING) pathway proteins interferon regulatory factor 3 (IRF3) and pIRF3 in macrophages. The results showed that UVB-irradiated BJ culture supernatants increased pIRF3 expression, while Fer-1 treatment demonstrated the opposite result (Fig. 1G). Taken together, the results indicate the culture supernatant from UVB-irradiated BJ cells activated the cGAS/STING pathway in macrophages and inhibited macrophage proliferation, migration, and M2 phenotypic status, which was reversed when BJ cells were treated with Fer-1.

Overexpression of NDUFS4 (NADH: ubiquinone oxidoreductase iron-sulfur protein 4) in skin fibroblasts induced macrophage transition to M1 phenotype

NDUFS4 (NADH: ubiquinone oxidoreductase iron-sulfur protein 4) regulates macrophage polarization [26]; however, the impact of NDUFS4 expression in skin fibroblasts on macrophage function remains unclear. To investigate this, we collected culture supernatants from fibroblasts following the overexpression of NDUFS4 and UVB treatment for further incubation with macrophages. Our findings revealed that NDUFS4 overexpression (NDUFS4-OE) significantly increased NDUFS4 expression in BJ cells (Fig. 2A-B). In contrast, the expression of this gene was suppressed when BJ cells were treated with Fer-1 (OE + Fer-1) (Fig. 2A-B). Additionally, the proliferation and migration abilities of macrophages in the NDUFS4-OE group were downregulated compared to the negative control (NC) group (Fig. 2C-D). Conversely, the proliferation and migration abilities of macrophages in the OE + Fer-1 group were higher than those observed in the NDUFS4-OE group (Fig. 2C-D). Additionally, culture supernatant from BJ cells after overexpression of NDUFS4 promoted the expression of M1 phenotypic genes (iNOS/CD86) and the number of CD86-positive macrophages, while decreasing the expression of M2 phenotypic genes (Arg-1/CD206) and the number of CD206-positive macrophages, whereas the phenotypic changes of the macrophages were reversed by treatment of the BJ cell cells with Fer-1 (Fig. 2E-F). Furthermore, we also found that NDUFS4-OE BJ cells culture supernatants increased pIRF3 expression, while Fer-1 treatment demonstrated the opposite result (Fig. 2G).

Subsequently, BJ cells overexpressing NDUFS4 were co-cultured with macrophages to investigate their effects on t macrophages. Consistent with the findings from the co-culture of BJ cell supernatants, NDUFS4-overexpressing BJ cells not only inhibited macrophage migration but also promoted the M1 phenotype while suppressing the M2 phenotype of macrophages (Fig. 3A-C). Notably, these changes were reversed when BJ cells were treated with Fer-1 (Fig. 3A-C). Additionally, NDUFS4-overexpressing BJ cells increased the expression of pIRF3 in macrophages, whereas BJ cells treated with Fer-1 inhibited pIRF3 expression (Fig. 3D). These results suggest that fibroblast overexpression of NDUFS4 exacerbated macrophage injury and induced a switch to the M1 phenotype in macrophages.

Proteomics-based screening of downstream proteins regulated by NDUFS4

To investigate the mechanism by which fibroblasts with differential NDUFS4 expression regulate macrophage polarization, we collected supernatants from fibroblasts overexpressing NDUFS4 and from control UVB-induced fibroblasts. Then, proteomics was performed to screen for downstream proteins associated with NDUFS4. The results showed that NDUFS4 overexpression resulted in drastic protein alterations, and a total of 22 proteins were significantly altered (17 down-regulated proteins and 5 up-regulated proteins) (Fig. 4A; Table S1). These differentially expressed proteins were mainly enriched in some biological processes, such as “taxis”, “response to chemokine”, “neutrophil migration” and so on (Fig. 4B), most of which are closely related to the regulation of chemokines. KEGG enrichment analysis showed that these differentially expressed proteins were mainly enriched in “Viral protein interaction

with cytokine and cytokine receptor”, “Toll-like receptor signaling pathway”, “TNF signaling pathway” and other pathways (Fig. 4C).

NDUFS4 regulated the expression of chemokines

Since the differentially expressed proteins regulated by NDUFS4 were primarily enriched in chemokine-related regulatory pathways, we screened and identified three chemokines (CCL1, CXCL11, and CCL5) that were significantly regulated by NDUFS4 based on the proteomics results. ELISA and RT-qPCR were employed to validate their expression in the supernatants from the BJ cell culture. The results indicated that overexpression of NDUFS4 significantly increased the levels of CCL1 and CCL5 while decreasing the level of CXCL11 compared to the NC group (Fig. 5A-B). Additionally, Fer-1 significantly elevated the levels of CXCL11 while reducing the levels of CCL1 and CCL5 (Fig. 5A-B). These findings suggest that NDUFS4 may significantly inhibit the anti-inflammatory function of macrophages during skin photoaging by regulating the expression of CCL1, CXCL11, and CCL5 in fibroblasts.

Overexpression of NDUFS4 in skin fibroblasts induced macrophage M1 polarization by promoting CCL5 release

Since NDUFS4 overexpression most significantly affected CCL1 release, we investigated whether CCL1 expression mediates the effects of NDUFS4. A CCL1 neutralizing antibody (anti-CCL1) was introduced to block CCL1 release. Conditioned media from UVB-treated BJ cells overexpressing NDUFS4 or treated with anti-CCL1 were collected and used to culture macrophages. The results showed that NDUFS4 overexpression reduced macrophage migration, and this effect was reversed by anti-CCL1 (Fig. 6A). In addition, conditioned media from BJ cells overexpressing NDUFS4 promoted the expression of M1 phenotype genes (iNOS/CD86) and increased the percentage of CD86-positive macrophages, while decreasing the expression of M2 phenotype genes (Arg-1/CD206) and the percentage of CD206-positive macrophages; these phenotypic changes in macrophages were reversed by anti-CCL1 treatment (Fig. 6B-C). Moreover, conditioned media from NDUFS4-OE BJ cells increased p-IRF3 expression, whereas anti-CCL1 treatment produced the opposite result (Fig. 6D). In summary, these results demonstrate that overexpression of NDUFS4 in skin fibroblasts induces macrophage M1 polarization by promoting CCL5 release.

Discussion

High-energy UVB radiation penetrates the epidermis upon exposure to sunlight and is primarily absorbed by epidermal cells [27]. Furthermore, UVB-affected epidermal cells play a crucial role in regulating macrophage infiltration, migration, and phenotypic differentiation, which disrupts the skin barrier [28, 29]. In the present study, we investigated the crosstalk between skin fibroblasts and macrophages. The results indicated that UVB-exposed fibroblasts, or fibroblasts overexpressing NDUFS4, inhibited macrophage proliferation and migration, induced differentiation into the M1 macrophage phenotype, and activated the IRF3 pathway in macrophages. Notably, these effects could be reversed by the ferroptosis

inhibitor Fer-1. Proteomic analyses revealed that NDUFS4 significantly increased the levels of CCL1 and CCL5 while inhibiting the levels of CXCL11. Conversely, Fer-1 markedly reversed the levels of these three chemokines. These findings suggest that NDUFS4 may promote a pro-inflammatory shift in macrophage phenotype under the present experimental conditions, as reflected by changes in the selected polarization markers (iNOS, CD86, Arg-1, and CD206). This effect was associated with the modulation of CCL1, CCL5, and CXCL11 secretion and may contribute to the progression of UVB-related cellular dysfunction. However, we acknowledge that our current analysis is limited to a selected panel of markers, and a more comprehensive profiling of cytokines and surface markers would be required to fully establish the phenotypic spectrum.

Several studies have demonstrated that macrophages play a significant role in the process of skin damage [29–31]. In skin tissues exposed to UVB radiation, macrophages are the first immune cells to infiltrate the site of injury, undergoing phenotypic differentiation to regulate skin barrier function. Previous research has shown that the supernatant from UVB-treated keratinocytes enhanced the activation of IRF3 in macrophages, which exacerbated skin damage [20]. Additionally, UVB-exposed keratinocytes have been found to promote M1 polarization in macrophages [32]. These findings align with the results of the present study, which also revealed that UVB-exposed skin fibroblasts decreased macrophage viability and migration while promoting M1 polarization. STING is a crucial innate immune adapter. In macrophages, the activation of the STING pathway enhances macrophage migration [33]. IRF3 is a key kinase for STING, and activation of IRF3 promotes macrophage communication [33]. This study found that supernatants from UVB-exposed skin fibroblasts increased pIRF3 levels in macrophages. These findings suggest that UVB-exposed skin fibroblast supernatants facilitated fibroblast-macrophage crosstalk by activating the STING pathway. Notably, treatment of fibroblasts with the ferroptosis inhibitor Fer-1 reversed macrophage function. Specifically, it enhanced macrophage proliferation and migration while simultaneously promoting M2 polarization of macrophages. Previous studies have demonstrated that pro-inflammatory cytokines and chemokines secreted by keratinocytes undergoing regulated cell death (RCD) play a crucial role in the initiation and exacerbation of skin inflammation [34]. In addition, cytokines and chemokines can recruit macrophages and induce macrophage differentiation to different phenotypes in response to external damage [35]. For instance, interferon-gamma (IFN- γ) secreted by keratinocytes undergoing UVB-induced ferroptosis promoted M1 polarization of macrophages, thereby worsening UVB-induced skin inflammation [32]. Accordingly, inhibiting ferroptosis improves skin cell function, reduces the inflammatory phenotype of macrophages, and ultimately protects the skin barrier from photodamage.

NDUFS4 is essential for the assembly of mitochondrial complex I [36, 37] and has been identified as a mutational hotspot [38]. NDUFS4 has been extensively studied in various models of mitochondrial disease; for instance, NDUFS4 deficiency led to systemic inflammation and osteosclerosis [39]. Deletion of NDUFS4 in the mouse brain resulted in impaired glutamate/alpha-ketoglutarate metabolism, exacerbating the mitochondrial disease known as Leigh syndrome (LS) [40]. However, in contrast to these diseases, high expression levels of NDUFS4 have been strongly associated with skin damage in studies of photoaging. Our previous research indicated that inhibiting NDUFS4 expression reduced UVB-

induced cellular ferroptosis, suggesting a positive correlation between NDUFS4 expression and skin damage [16]. In the present study, we discovered that supernatants from overexpressing NDUFS4 fibroblast inhibited macrophage activity and promoted M1 polarization of macrophages, which was linked to the activation of the STING pathway, an important signaling mechanism in macrophages. Furthermore, the compound Fer-1 was found to reverse these phenotypes in macrophages. In line with previous studies, this may be attributed to the overexpression of NDUFS4 promoting ferroptosis in fibroblasts, which in turn enhances M1 polarization in macrophages [32] and ultimately exacerbates UVB-induced skin photodamage.

Numerous studies have demonstrated that chemokines released by fibroblasts can facilitate communication with macrophages, including the regulation of macrophage polarization status [35, 41, 42]. In the present study, differentially expressed proteins (DEPs) were identified in fibroblast supernatants using proteomic techniques. A series of DEPs were discovered in fibroblasts overexpressing NDUFS4, and notably, these DEPs were primarily enriched in chemokine-related pathways. This finding indirectly suggests that NDUFS4 in fibroblasts promotes fibroblast-macrophage crosstalk by modulating chemokine release. Specifically, the overexpression of NDUFS4 significantly influenced the release of three chemokines (CCL1, CXCL11, and CCL5): it increased the release of CCL1 and CCL5 while decreasing the release of CXCL11. CCL1 and CCL5 are pro-inflammatory mediators, and the upregulation of CCL5 has been shown to promote M1-like macrophage polarization [43, 44]. Additionally, the expression of CXCL11 facilitated macrophage migration [45]. Furthermore, the relationship between IRF3 activation and chemokine production in macrophages has been elucidated in several contexts. IRF3 serves as a key transcription factor that directly regulates the expression of a distinct set of chemokines and cytokines [46]. Studies have demonstrated that IRF3 deficiency in macrophages leads to complete inhibition of CCL5 production in response to pathogenic stimuli, with partial or no effects on other cytokines, suggesting that IRF3 selectively governs the expression of specific chemokines rather than functioning as a global inflammatory regulator [46]. Furthermore, IRF3 has been identified as a crucial transcription factor in the non-MyD88 pathway, capable of transactivating genes including CCL5, CXCL10, and arginase II [47]. This regulatory axis is not unidirectional; IRF3 itself can be activated downstream of chemokine receptor engagement. For instance, the PP4-TBK1-IRF3 axis has been shown to directly regulate macrophage CCL5 production, with TBK1-mediated IRF3 phosphorylation serving as a critical step in controlling CCL5 synthesis [48]. More broadly, the STING-IRF3 signaling axis has been demonstrated to epigenetically program macrophages toward a pro-inflammatory phenotype, orchestrating coordinated chromatin remodeling that promotes pro-inflammatory gene programs while suppressing phagocytic/anti-inflammatory gene expression. These findings collectively suggest a reciprocal regulatory relationship: chemokine signals can trigger IRF3 activation, while activated IRF3 in turn drives the transcription of chemokines including CCL5, forming a positive feedback loop that amplifies inflammatory responses.

Importantly, to establish a causal link between NDUFS4-regulated chemokine release and macrophage phenotypic changes, we performed functional neutralization experiments. Treatment with an anti-CCL5 neutralizing antibody effectively reversed NDUFS4-induced macrophage M1 polarization, as evidenced

by the decreased expression of M1 markers (iNOS/CD86) and increased expression of M2 markers (Arg-1/CD206), along with restored macrophage migration and attenuated IRF3 phosphorylation. These findings collectively demonstrate that CCL5 serves as a critical functional mediator of the NDUFS4-driven fibroblast-macrophage crosstalk, directly linking altered fibroblast chemokine secretion to impaired macrophage activity. However, it remains to be determined whether the observed inhibition of macrophage proliferation and migration is exclusively attributable to CCL5 or involves cooperative effects with other NDUFS4-regulated chemokines (e.g., CCL1 and CXCL11). In addition, the temporal and functional relationship between chemokine-mediated signaling and IRF3 activation within macrophages, i.e., whether IRF3 phosphorylation occurs downstream of chemokine receptor engagement or through parallel pathways, warrants further mechanistic dissection.

However, this study has several limitations. First, the mechanistic details by which fibroblast-derived chemokines regulate macrophage function have not been fully elucidated, and the downstream signaling pathways involved require further investigation. Second, this study is primarily based on immortalized BJ fibroblasts and THP-1-derived macrophages. While these cell lines are widely employed and well-validated in vitro models for photoaging and macrophage biology, they may not fully recapitulate the complex cellular and tissue microenvironment of native human skin. Third, while we observed consistent changes in representative M1/M2 markers (iNOS, CD86, Arg-1, and CD206) and chemokine profiles, a comprehensive characterization of the macrophage inflammatory phenotype, including a broader panel of cytokines, surface markers, and functional assays, was beyond the scope of the present study. Therefore, our conclusions regarding phenotypic polarization are limited to the markers examined and should be considered as indicative rather than definitive. Fourth, skin photodamage is a complex process involving multiple immune cell types; beyond macrophages, other immune cells such as T cells and natural killer (NK) cells may also participate in the inflammatory response to UVB exposure. Consequently, the conclusions drawn from this study are limited to cellular mechanisms observed in vitro, and the translational relevance of targeting NDUFS4 for therapeutic intervention requires further validation in more physiologically relevant models, including primary cell cultures, ex vivo skin tissues, and animal models of UVB-induced photodamage. Nonetheless, we believe that our findings provide novel mechanistic insights into fibroblast-macrophage crosstalk via the NDUFS4-chemokine axis, which may serve as a foundation for future in-depth investigations.

In conclusion, the results of this study suggest that NDUFS4 mediates the crosstalk between fibroblasts and macrophages, exacerbating skin damage by regulating chemokine release. This research elucidates some of the pathomechanisms underlying sunburn and provides a preliminary mechanistic basis that may inform future translational studies.

Declarations

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Ethical Approval: Not required.

Ethics statement: Not applicable

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution

Yan Teng: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. Qiong Gao: Investigation, Formal analysis, Visualization. Xiaohua Tao: Investigation, Resources. Danfeng Xu: Investigation, Data curation. Youming Huang: Resources, Supervision. Yibin Fan: Conceptualization, Supervision, Project administration, Writing – review & editing, Funding acquisition.

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Figures

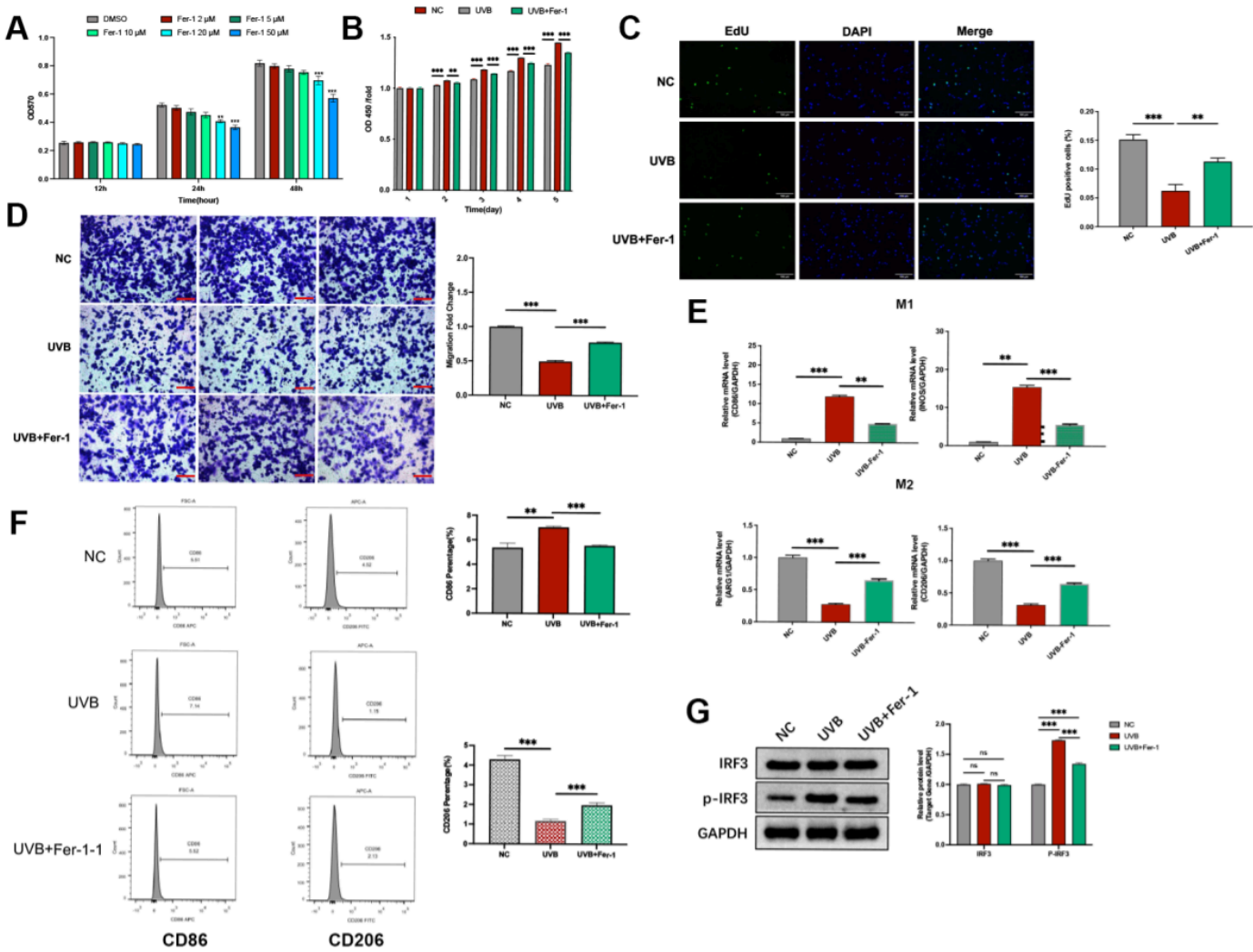


Figure 1

UVB-exposed human skin fibroblasts mediate macrophage polarization.

(A) BJ cell viability after treatment with various concentrations of Fer-1 (2, 5, 10, 20, and 50 μ M) assessed by MTT assay. BJ cells were treated with UVB (20 mJ/cm²) or Fer-1 (10 μ M). Subsequently, culture supernatants of these BJ cells were co-cultured with THP-1 macrophages. (B) Macrophage viability was examined using the CCK8 assay. (B) Macrophage proliferation was examined using the EdU

staining. (C) The migratory capacity of macrophages was probed using the Transwell assay. Representative images are shown at a total magnification of $\times 40$ (scale bar: 100 μm). (D) The mRNA expression of iNOS, CD86, Arg-1, and CD206 in macrophages was detected by RT-qPCR. (E) CD86 and CD206 positive macrophage counts analyzed by flow cytometry. (F) The protein expression of IRF3 and pIRF3 in macrophages was detected by WB. All experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

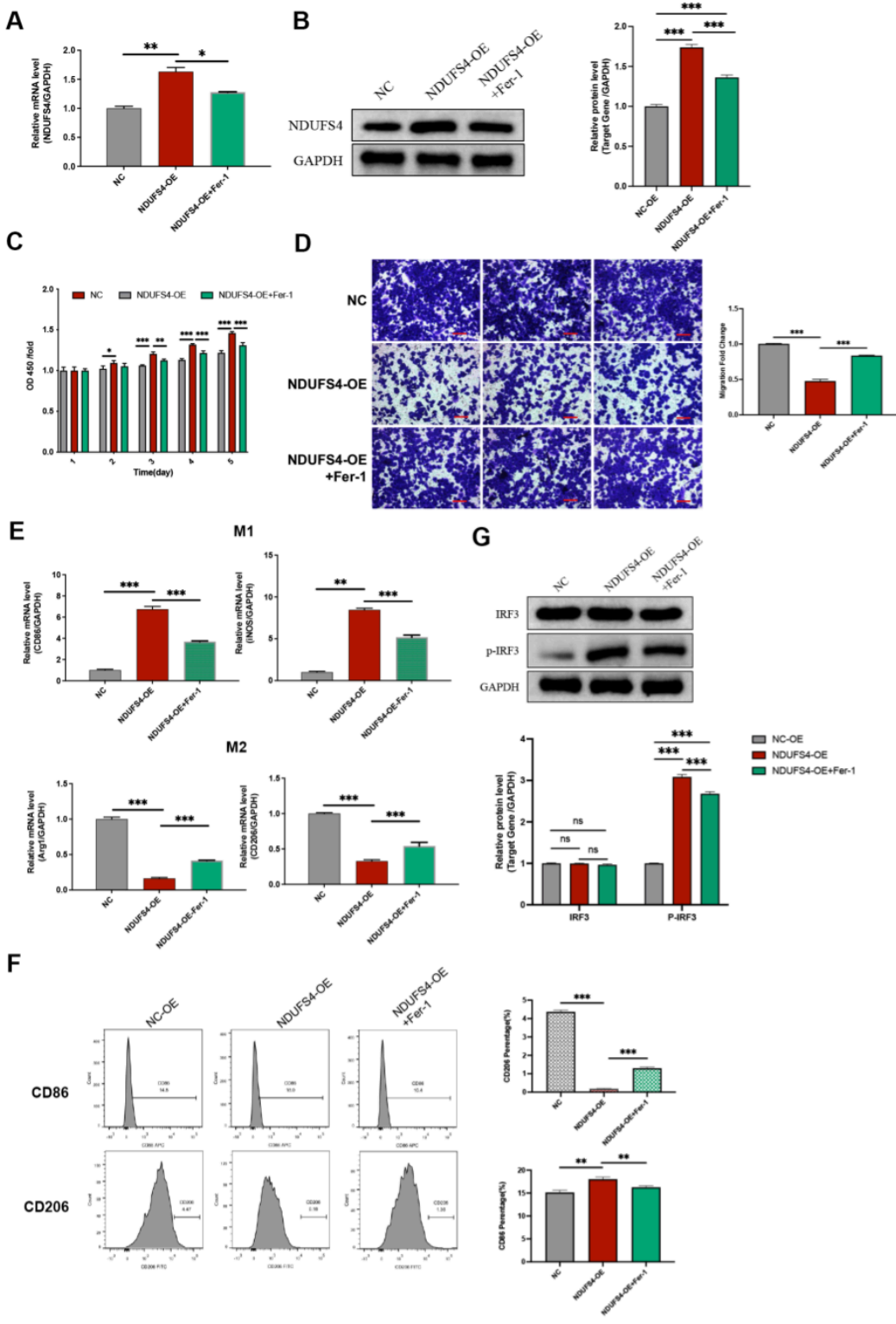


Figure 2

Culture supernatant from NDUFS4-overexpressed skin fibroblasts induced macrophage transition to M1 phenotype.

BJ cells were transfected to overexpress NDUFS4 or treated with Fer-1 (10 μ M) and then exposed to UVB irradiation. The culture supernatants from these BJ cells were collected and used to co-culture with THP-1-derived macrophages. (A) The mRNA expression of NDUFS4 in BJ cells was detected by RT-qPCR. (B) The protein expression of NDUFS4 in BJ cells was detected by WB. (C) Macrophage viability was examined using the CCK8 assay. (D) The migratory capacity of macrophages was probed using the Transwell assay. Representative images are shown at a total magnification of $\times 40$ (scale bar: 100 μ m). (E) The mRNA expression of iNOS, CD86, Arg-1, and CD206 in macrophages was detected by RT-qPCR. (F) CD86 and CD206 positive macrophage counts analyzed by flow cytometry. (G) The protein expression of IRF3 and pIRF3 in macrophages was detected by WB.

All experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

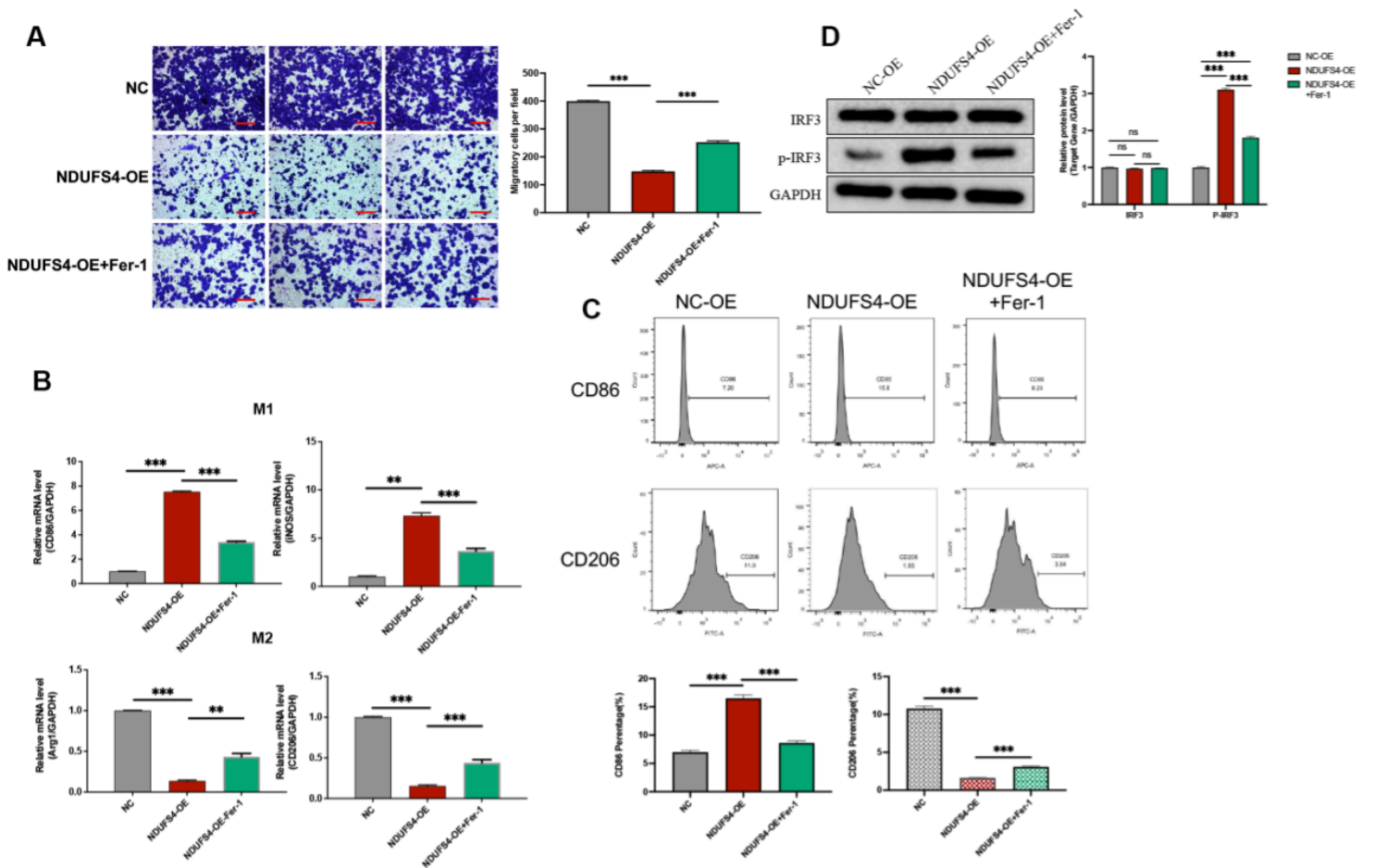


Figure 3

NDUFS4-overexpressed skin fibroblasts induced macrophage transition to M1 phenotype.

BJ cells were transfected to overexpress NDUFS4 or treated with Fer-1 (10 μ M) and then exposed to UVB irradiation. Subsequently, these BJ cells were co-cultured with THP-1 macrophages using Transwell chambers. (A) The migratory capacity of macrophages was probed using the Transwell assay. Representative images are shown at a total magnification of $\times 40$ (scale bar: 100 μ m). (B) The mRNA expression of iNOS, CD86, Arg-1, and CD206 in macrophages was detected by RT-qPCR. (C) CD86 and CD206 positive macrophage counts analyzed by flow cytometry. (D) The protein expression of IRF3 and pIRF3 in macrophages was detected by WB.

All experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

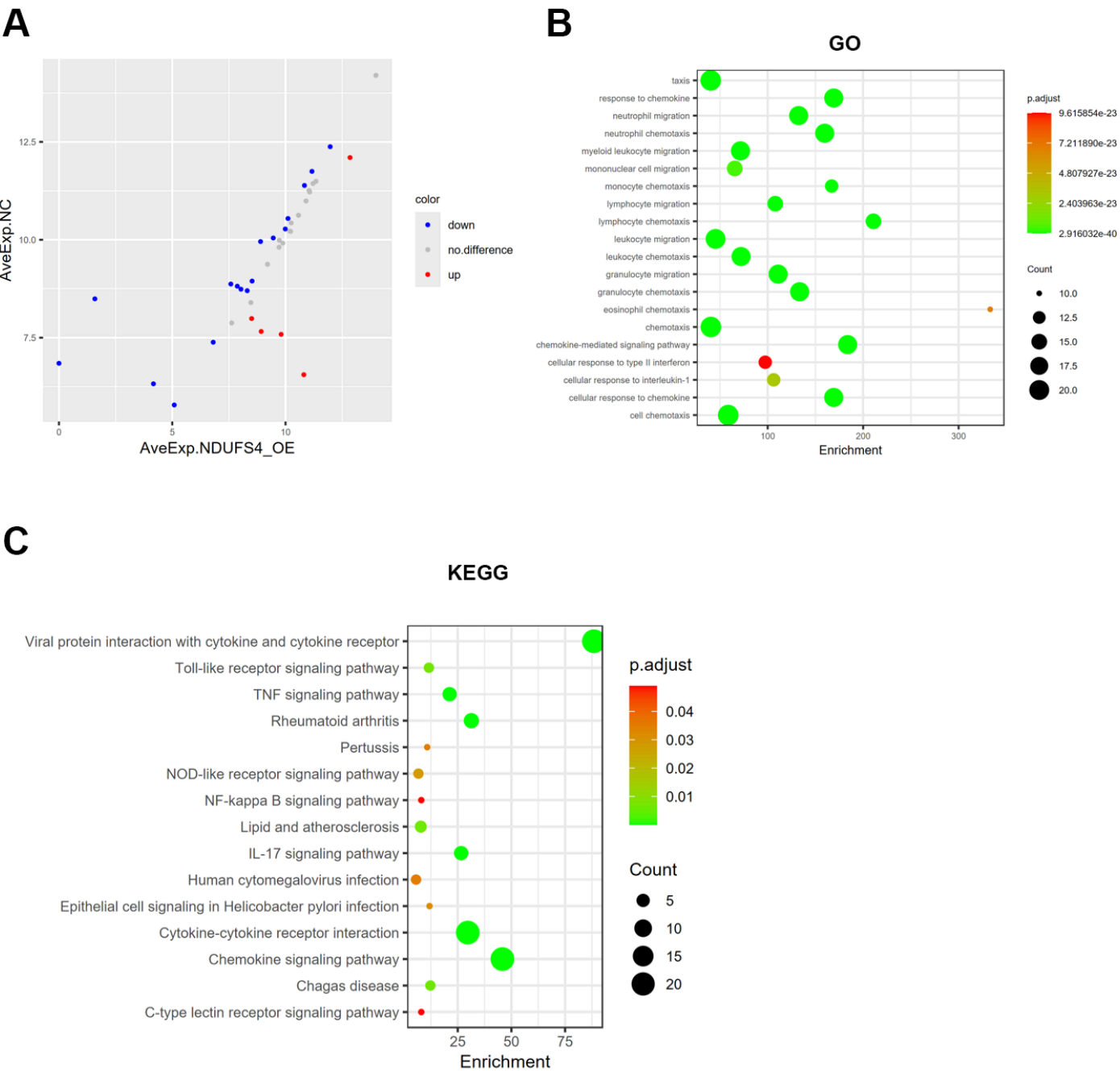


Figure 4

Proteomics-based screening of downstream proteins regulated by NDUFS4.

BJ cells were transfected with NDUFS4 overexpressing lentivirus or control lentiviral vectors and then exposed to UVB irradiation. Subsequently, supernatants from BJ cells were used for proteomics techniques. (A) Volcanoplot was performed to identify differentially expressed proteins (DEPs). (B) GO enrichment analysis of DEPs. (C) KEGG enrichment analysis of DEPs.

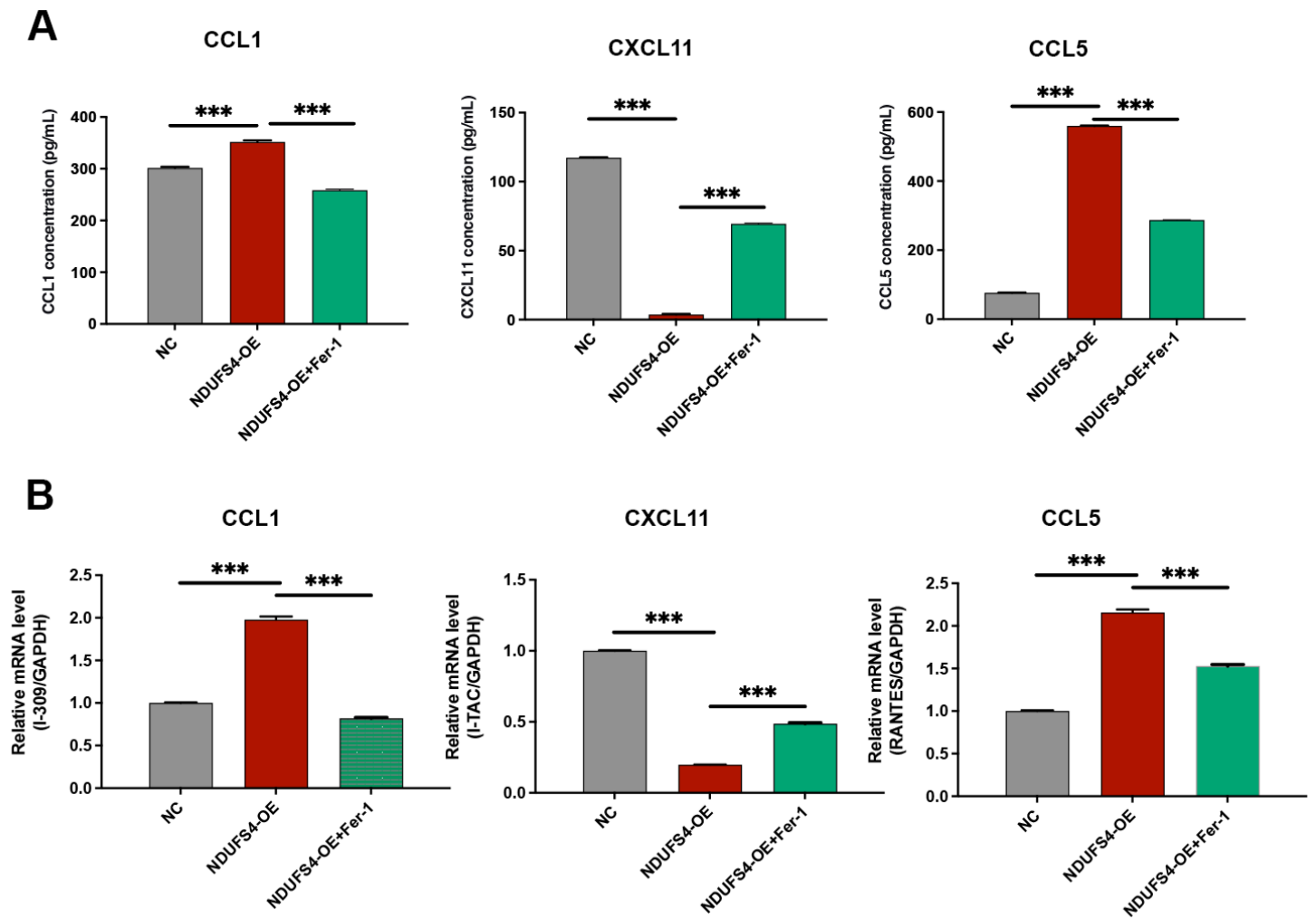


Figure 5

NDUFS4 regulated the expression of chemokines.

BJ cells were transfected to overexpress NDUFS4 or treated with Fer-1 (10 μ M) and then exposed to UVB irradiation. (A) The release levels of CCL1, CXCL11, and CCL5 in BJ cell supernatants were analyzed by ELISA. (B) The mRNA expression of CCL1, CXCL11, and CCL5 in BJ cell supernatants was detected by RT-qPCR.

All experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

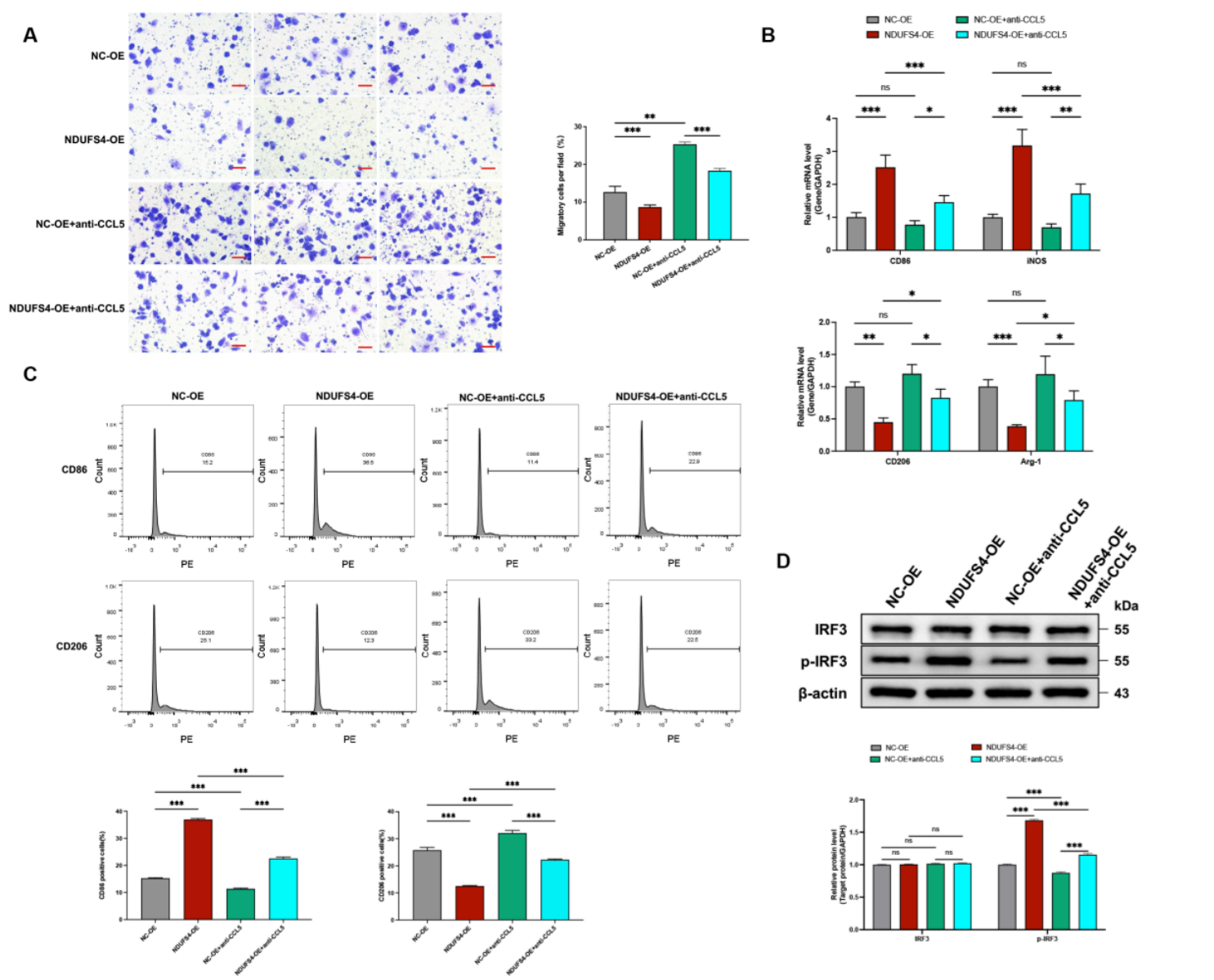


Figure 6

Overexpression of NDUFS4 in skin fibroblasts induced macrophage M1 polarization by promoting CCL5 release.

BJ cells were transfected to overexpress NDUFS4 or treated with anti-CCL5 and then exposed to UVB irradiation. The culture supernatants from these BJ cells were collected and used to co-culture with THP-1-derived macrophages. (A) The migratory capacity of macrophages was probed using the Transwell assay. Representative images are shown at a total magnification of $\times 40$ (scale bar: 100 μm). (B) The mRNA expression of iNOS, CD86, Arg-1, and CD206 in macrophages was detected by RT-qPCR.

(C) CD86 and CD206 positive macrophage counts analyzed by flow cytometry. (D) The protein expression of IRF3 and pIRF3 in macrophages was detected by WB.

All experiments were performed in triplicate. Data are presented as mean $\pm$ SD. *P<0.05, **P<0.01, ***P<0.001.

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