

Efferocytosis by Macrophages Attenuates Inflammatory Responses Following Ultraviolet B-Induced Apoptosis in Corneal Stromal Cells

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PURPOSE. To investigate the role of efferocytosis in maintaining corneal immune homeostasis after ultraviolet B (UVB)-induced keratocyte apoptosis and its impact on inflammatory responses in both in vitro and in vivo settings.

METHODS. Human corneal stromal fibroblasts (HCFs) were exposed to UVB radiation (150 mJ/cm²) to induce apoptosis and co-cultured with M1 macrophages using a transwell system. In this in vitro efferocytosis model, UVB-irradiated HCFs (BHCFs) were evaluated for efferocytosis-related markers, including milk fat globule epidermal growth factor 8 (MFG-E8) and MER proto-oncogene, tyrosine kinase (MERTK), as well as inflammatory cytokines such as interleukin (IL)-1 β and IL-6. Paracrine effects on nearby M1 macrophages were assessed by analyzing changes in cytokine profiles and expression of myeloid/macrophage markers. To validate the physiological relevance of these findings, an in vivo mouse model was established by subconjunctival injection of clodronate liposomes in UVB-exposed mice to reduce corneal macrophages.

RESULTS. BHCFs showed higher TUNEL positivity and significantly more efferocytosis when co-cultured with M1 macrophages. This was accompanied by upregulation of MFG-E8 and MERTK and downregulation of IL-1 β and IL-6. Microenvironmental M1 macrophages exhibited reduced IL-1 β , increased transforming growth factor beta 1, and downregulation of CD14, CD68, CD80, and CD11c. In vivo macrophage reduction impaired MERTK activation and failed to suppress IL-1 β upregulation in the cornea.

CONCLUSIONS. Efferocytosis contributes to corneal immune homeostasis after UVB-induced apoptosis by resolving inflammation and modulating macrophage phenotype. These findings support the existence of an efferocytic mechanism in the corneal stroma.

Keywords: corneal stromal fibroblasts, efferocytosis, inflammation, macrophage, UVB

Ultraviolet (UV) radiation is categorized by wavelength into UVC (100 to 290 nm), UVB (290 to 320 nm), and UVA (320 to 400 nm) depending on its wavelength.¹ UVC is absorbed by the earth's atmospheric ozone layer.² In contrast to UVA, which can penetrate the full thickness of the cornea,^{1,3} shorter wavelengths like UVB are more readily absorbed by the cornea, making it highly susceptible to UVB damage.¹ For instance, the acute excessive exposure to UVB naturally or by welder's flash leads to corneal epithelial damage and loss, called photokeratitis.¹

UVB exposure may activate cell membrane K⁺ channels in a dose-dependent manner, leading to the loss of intracellular K⁺ and the initiation of apoptotic signaling.⁴⁻⁶ Although the apoptotic signals including caspases-9, -8, and -3 are rapidly activated even on exposure to the ambient level of UVB in corneal limbal epithelial cells in vitro, the

corneal epithelium is generally resistant to UVB-induced apoptosis during ordinary outdoor activity. Such discordance between in vitro and in vivo conditions was supported by a hypothesis that high concentration of K⁺ in tears prevented the loss of intracellular K⁺ in corneal epithelial cells after UVB-exposure.^{5,7} Although the corneal epithelium has a much higher absorption coefficient for UVB than the stroma, the corneal stroma absorbs a greater total amount of UVB because of its considerably greater thickness.⁸ In fact, after in vivo exposure to UV radiation at 300 nm (within UVB range), apoptosis was observed by TUNEL staining in keratocytes, as well as corneal epithelial cells.⁹ However, the keratocytes in the corneal stroma are not directly exposed to tears because of the tight junction barrier of the corneal epithelium and are therefore not protected by tear-mediated self-defense mechanisms against

UVB. Based on these previous findings, we questioned why we do not see the corneal stromal inflammation frequently in clinical settings despite the presence of apoptosis of keratocytes under UVB-exposure.

Efferocytosis is a critical physiological process by which phagocytes recognize, engulf, and clear apoptotic cells, playing a vital role in tissue homeostasis, inflammation resolution, and immune tolerance.^{10,11} This highly regulated mechanism involves several steps, including the release of "find-me" signals by dying cells, recognition of "eat-me" signals on apoptotic cell surfaces, and the subsequent engulfment and processing of cellular debris.¹² However, comprehensive investigations into the specific mechanisms and implications of efferocytosis in corneal health and disease are still lacking. Recent studies have begun to shed light on the importance of efferocytosis in maintaining corneal homeostasis. Notably, it was demonstrated that adjunctive thymosin β -4 treatment enhances macrophage effector cell functions, including efferocytotic activity and phagocytosis, in a murine model of *Pseudomonas aeruginosa*-induced keratitis, thereby improving disease outcome.¹³ However, comprehensive investigations into the specific mechanisms and implications of efferocytosis in corneal health and disease are still lacking. This gap in knowledge presents an opportunity to elucidate the potential role of efferocytosis in corneal wound healing, inflammation resolution, and maintenance of corneal transparency.¹³

The purpose of this study is to investigate the role of efferocytosis in the corneal stroma in response to UVB-induced keratocyte apoptosis. By examining this mechanism, we aim to understand how the cornea responds to UVB exposure and maintains tissue homeostasis.

METHODS

Ethics Approval

Approval was obtained from the Institutional Review Board of Chung-Ang University Hospital (Approval No. 2106-006-465) for the use of cadaveric ocular tissues in primary corneal cell culture, adhering to the principles outlined in the Declaration of Helsinki.

Isolation and Culture of Human Corneal Stromal Fibroblasts

The isolation and culture of human corneal stromal fibroblasts (HCFs) were performed following the protocol outlined in our previous study.¹⁴ Briefly, corneal tissues free of ocular disease were obtained, with the central 8-mm section removed for transplantation. The peripheral corneal tissues were washed with PBS containing a $5 \times$ antibiotic solution and then placed in six-well plates for HCF culture. After a 10-minute adhesion, minimum essential medium with 10% fetal bovine serum and antibiotics was added. Cells were cultured at 37°C in 5% CO₂ until reaching 80% to 90% confluence and then subcultured with TrypLE Express Enzyme. Third to fifth-passage cells were used for experiments.

Polarization of Macrophages From THP-1 Monocytes

M1 macrophages were derived from the THP-1 cell line (TIB-202; ATCC, Manassas, VA, USA) following established

methods.^{15–17} THP-1 cells were cultured in RPMI 1640 medium (WELGENE, Gyeongsan, Korea) with supplements and seeded in six-well plates. Cells were first differentiated into M0 macrophages with 50 nM phorbol 12-myristate 13-acetate (PMA; #P8139, Sigma-Aldrich, St. Louis, MO, USA) for 24 hours, then polarized to M1 macrophages with 100 ng/mL of lipopolysaccharide (LPS; #L2630, Sigma-Aldrich) and 20 ng/mL of interferon-gamma (IFN- γ ; no. 570216, BioLegend, San Diego, CA, USA) for another 24 hours.

UVB Irradiation of HCFs

HCFs were seeded in six-well cell culture plates at a density of 1.2×10^5 cells per well and were incubated for 24 hours before irradiation. For UVB irradiation, the culture medium was removed, and cells were covered with 750 μ L of Gibco Dulbecco's phosphate-buffered saline solution (Thermo Fisher Scientific, Waltham, MA, USA) per well. Cells were then irradiated with 0–150 mJ/cm² UVB using a UV irradiation system (Bio-Link 312; Vilber, Marne-la-Vallée, France) and then were cultured for 24 hours in fresh culture medium.

In Vitro Co-Culture System for Efferocytosis

Following our previous study's protocol,¹⁸ HCFs or UVB-irradiated HCFs (BHCFs) were seeded in six-well plates at a density of 1.2×10^5 cells per well and cultured for 24 hours. The co-culture system was set up with transwell inserts (8.0 μ m pore size; no. 37506; SPL Life Sciences, Pocheon, Korea) placed in the six-well plates. The 8.0 μ m pore size was selected based on a previous study that has used this pore size for macrophage migration and invasion assay using a Transwell in vitro system.¹⁹ M1 macrophages were resuspended in RPMI 1640 at 3×10^5 cells per insert. The co-culture of M1 macrophages and HCFs was then incubated at 37°C in a humidified incubator with 5% CO₂ for 24 hours.

Cell Viability (MTT) Assay

HCFs were seeded in 96-well plates and cultured for 16 hours before performing the MTT assay to assess cell viability. The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; no. M2128, Sigma-Aldrich) was prepared in PBS at 5 mg/mL. MTT solution 10 μ L was added to each well containing 100 μ L of medium, and the plates were incubated at 37°C in the dark for two hours. After incubation, the medium was replaced with 100 μ L of dimethyl sulfoxide to dissolve the formazan crystals, and absorbance was measured at 570 nm using a Spectramax i3x microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Purification of Total RNA and Real-Time qRT-PCR

Total RNA was extracted from 1.2×10^5 cells of HCFs or BHCFs per well and 3×10^5 of polarized M1 macrophages per co-culturing insert. The experimental protocol followed our previous study.¹⁸ Cells were lysed using NucleoZOL (no. 740404.200; Macherey-Nagel, Düren, Germany), and 200 μ L of RNase-free water was added to each lysate. After thorough mixing by inversion, the samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. From the resulting supernatant, 500 μ L was carefully transferred and mixed

TABLE. Sequences of PCR Primers

Gene		Primer Sequence	Product Size (bp)	Accession Number
<i>BCL2</i> (Human)	Sense	GCCCTGTGGATGACTGAGTA	117	NM_000633.3
	Antisense	GAAATCAAACAGAGGCCGCA		
<i>Bcl2</i> (Mus musculus)	Sense	CTGAGTACCTGAACCGGCAT	70	NM_009741.5
	Antisense	AGTTCCACAAAGGCATCCCA		
<i>BAX</i> (Human)	Sense	CGCCCTTTTCTACTTTGCCA	72	NM_001291428.2
	Antisense	GTTCTGATCAGTTCCGGCAC		
<i>Bax</i> (Mus musculus)	Sense	GGAAGGCCTCCTCTCCTACT	72	NM_001411994.1
	Antisense	GTGAGGACTCCAGCCACAAA		
Active <i>APAF1</i> (Human)	Sense	GGACCCCTCAAGAGGATATG	179	NM_013229.3
	Antisense	GTGGGGAGAAGTCACAGTAC		
<i>CASP9</i> (Human)	Sense	GTTTGAGGACCTTCGACCAGCT	129	NM_001229
	Antisense	CAACGTACCAGGAGCCACTCTT		
<i>MFGE8</i> (Human)	Sense	TTCAGCTGGAACCCCTCCTA	100	NM_001114614.3
	Antisense	GGAAGATCTGCAGCCACTGA		
<i>Mfge8</i> (Mus musculus)	Sense	CACTGTGAAACCGGTTGTCT	78	NM_001045489.1
	Antisense	AGACGAGGCGGAAATCTGTG		
<i>Pros1</i> (Mus musculus)	Sense	TGGCAAGGAGACAGGTGTCAGT	110	NM_011173
	Antisense	GAGCAGTGGTAACTTCCAGGAG		
<i>MERTK</i> (Human)	Sense	GTACCAGCCTGCCTTGATGT	74	NM_006343.3
	Antisense	CCTGGGGAAATGGCTACGTT		
<i>Mertk</i> (Mus musculus)	Sense	TTGGGCTGTGGAAGTAGGG	71	NM_001420413.1
	Antisense	AGCACCTGCCATCAATCGAC		
<i>IL1B</i> (Human)	Sense	CCACAGACCTTCCAGGAGAATG	131	NM_000576.3
	Antisense	GTGCAGTTCAGTGATCGTACAGG		
<i>Il1b</i> (Mus musculus)	Sense	CAGGCAGGCAGTATCACTCA	86	NM_008361.4
	Antisense	TGTCCTCATCCTGGAAAGGTC		
<i>IL6</i> (Human)	Sense	AATAACCACCCCTGACCCAAC	168	NM_000600.4
	Antisense	AATCTGAGGTGCCATGCTAC		
<i>Il6</i> (Mus musculus)	Sense	CCGGAGAGGAGACTTCACAG	102	NM_031168.2
	Antisense	TCCACGATTTCCCAGAGAAC		
<i>TGFB1</i> (Human)	Sense	CTCGCCAGAGTGGTTATCTT	123	NM_000660.5
	Antisense	AGTGTGTTATCCCTGCTGCA		
<i>Il10</i> (Mus musculus)	Sense	AAGTCCAAGACCAAGGTGTC	182	NM_010548.2
	Antisense	TCCGTTAGCTAAGATCCCTGG		
<i>CD14</i> (Human)	Sense	AGCCTAGACCTCAGCCACAA	138	NM_000591.4
	Antisense	CTTGGCTGGCAGTCCCTTAG		
<i>ITGAM</i> (i.e., <i>CD11B</i>) (Human)	Sense	GCTTTGGTGGCTTCCTTTGTG	80	NM_001145808.2
	Antisense	CTGGAAGGAGCCAGAACCTG		
<i>Itgam</i> (i.e., <i>Cd11b</i>) (Mus musculus)	Sense	AGTGCTGGGAGACGTGAATG	120	NM_001082960.1
	Antisense	GCACTGAGGCTGGCTATTGA		
<i>ITGAX</i> (i.e., <i>CD11C</i>) (Human)	Sense	CGTTCGACACATCCGTGTA	217	NM_001286375.1
	Antisense	TTTGCTCTCCATCAITTC		
<i>CD68</i> (Human)	Sense	GCTACATGGCGGTGGAGTACAA	262	NM_001251.2
	Antisense	ATGATGAGAGGCAGCAAGATGG		
<i>CD80</i> (Human)	Sense	CTCTTGGTGCTGGCTGGTCTTT	136	NM_005191
	Antisense	GCCAGTAGATGCGAGTTTGTGC		
<i>Adgre1</i> (i.e., <i>F4/80</i>) (Mus musculus)	Sense	CTGTAACCGGATGGCAAACCT	92	NM_010130
	Antisense	CTGTACCCACATGGCTGATG		
<i>GAPDH</i> (Human)	Sense	CACCAGGGCTGCTTTAACTCTGGT	131	NM_002046.5
	Antisense	CCTTGACGGTGCCATGGAATTTGC		

with an equal volume of isopropanol. The mixture was incubated at room temperature (RT) for 15 minutes and centrifuged again at 13,000 rpm for 15 minutes to collect RNA pellets. The pellets were then washed and resuspended in RNase-free water. Subsequently, 1 µg of purified RNA was reverse-transcribed into cDNA using a cDNA synthesis kit (no. K1622; Thermo Fisher Scientific). Real-time qRT-PCR was performed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and relative gene expression was calculated using the comparative Ct method, normalized to the reference gene GAPDH. Primer sequences used are shown in the [Table](#).

Protein Isolation and Western Blot Analysis

Proteins were extracted from 1.2×10^5 cells of HCFs or BHCFs per well and 3×10^5 of polarized M1 macrophages per co-culturing insert. Following the protocol from our previous study,¹⁴ total cellular protein was extracted from cultured cells with 70 µL of RIPA buffer (no. R0278; Sigma-Aldrich) containing a protease inhibitor cocktail (no. 78441; Thermo Fisher Scientific). Protein concentration was measured with the BCA assay (no. 23227; Thermo Fisher Scientific). A 30 µg protein sample was separated on a 12.5% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Merck Millipore, Billerica, MA, USA) at 100 V

in transfer buffer containing 0.3% Tris, 1.4% glycine, and 20% methanol using a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk in TBS-T for one hour at RT.

The membrane was then incubated overnight at 4°C with primary antibodies against Bax (no. 60267-1-Ig; Proteintech, Rosemont, IL, USA), B-cell lymphoma 2 (Bcl-2; no. sc-7382; Santa Cruz Biotechnology, Dallas, TX, USA), caspase 9 (no. 9502S; Cell Signaling Technology, Danvers, MA, USA), Apoptotic protease activating factor 1 (APAF1; no. 29022-1-AP; Proteintech), CD80 (no. 66406-1-Ig, Proteintech), MER proto-oncogene, tyrosine kinase (MERTK; no. PA5-15028; Thermo Fisher Scientific), milk fat globule-EGF Factor 8 (MFG-E8; no. 67797-1-Ig; Proteintech), and β -actin (no. sc-47778; Santa Cruz Biotechnology), all diluted 1:1000 in TBS-T with 5% BSA. Secondary antibodies diluted 1:2000 in TBS-T with 5% skim milk were applied and incubated for one hour at RT. Signals were visualized with the ECL Prime Detection Reagent (no. RPN2232; Merck, Rahway, NJ, USA), and band intensities were normalized to β -actin.

ELISA

To quantify the levels of IL-6, culture supernatants were collected from HCFs BHCs co-cultured with M1 macrophages. Human IL-6 ELISA kits (no. EH2IL6; Thermo Fisher Scientific) were used according to the manufacturer's protocols. The supernatants were first clarified by centrifugation at 13,000 rpm for 30 minutes at 4°C. Subsequently, 100 μ L of each sample was subjected to ELISA. Absorbance was measured at 450 nm using a microplate reader (SpectraMax i3x; Molecular Devices), and cytokine concentrations were determined based on standard curves.

TUNEL Assay Staining

HCFs were seeded on cover glasses in four-well plates at 4×10^4 cells per well and incubated for 24 hours before UVB irradiation to induce apoptosis. Apoptotic cells were detected using the In Situ Cell Death Detection Kit (no. 11684795910; Roche, Basel, Switzerland) according to the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde (no. BPP-9004, Tech&Innovation, Chuncheon, Korea) for 20 minutes at RT, followed by three 10-minute PBS washes.

The cells were then permeabilized on ice with 50 μ L of 0.1% Triton X-100 in 0.1% sodium chloride for three minutes. TUNEL staining solution (50 μ L) was applied, and the cells were incubated for one hour at RT and then washed three times with PBS. Cover glasses were mounted with VECTASHIELD Antifade Mounting Medium containing DAPI (no. H-1200-10; Vector Laboratories, Novato, CA, USA) and analyzed under an inverted fluorescence microscope (DMI8; Leica, Wetzlar, Germany).

Fluorescence Labeling of M1 Macrophages

M1 macrophages were resuspended in 100 μ L of Diluent C containing 1 μ L of PKH26 red dye (no. PKH26GL; Sigma-Aldrich) to label the cells for tracking. After 10min of incubation at RT, the reaction was stopped by the addition of 200 μ L of PBS containing 10% BSA. These fluorescently labeled M1 macrophages were subsequently used in efferocytosis assays for visualization purposes.

In Vivo Reduction of Macrophages in Murine Corneas

Animal experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Balb/c mice (7 weeks old; Orient Bio Inc., Seongnam, Korea) were used to investigate the role of macrophage-mediated efferocytosis in UVB-induced corneal inflammation. Macrophage depletion in the cornea was performed as previously described.^{20,21} Briefly, 5 μ L of clodronate-containing liposomes (5 mg/mL; Clodrosome Liposomal Clodronate Suspension; no. CLD-8901; Encapsula Nanosciences LLC, Brentwood, TN, USA) or control PBS-containing liposomes (5 mg/mL; Encapsome Control Liposome Suspension, no. CLD-8901; Encapsula Nanosciences LLC) were injected subconjunctivally using a 10 μ L NanoFil syringe (World Precision Instruments, Sarasota, FL, USA) fitted with a 35-gauge blunt metal needle (no. NF35BL; World Precision Instruments) on days -5 and -2.5 before UVB exposure. Macrophage reduction in the cornea was confirmed by flow cytometry 2.5 days after the second injection. All procedures, including treatment and sacrifice, were performed under anesthesia induced by intramuscular injection into the thigh muscle with 10 μ L/g body weight of a 1:10 PBS-diluted mixture of tiletamine-zolazepam (Zoletil; Virbac, Carros, France) and xylazine (Rompun; Bayer, Leverkusen, Germany) in a 6:4 ratio.

In Vivo Corneal UVB Irradiation

After anesthesia, each mouse was placed in a brown opaque container that shielded the body from UVB exposure while allowing only the head to remain exposed. While lying on its side, one eye was irradiated with UVB for three minutes and 20 seconds, corresponding to a total fluence of 1 J/cm², using a UV irradiation system (Bio-Link 312, Vilber). Immediately afterward, the mouse was repositioned onto the opposite side, and the contralateral eye was irradiated under identical conditions.

Flow Cytometry Analysis

To isolate cells from the murine cornea, circular incisions were made at the limbus parallel to the iris using ophthalmic corneal scissors. Under a dissection microscope, the iris was carefully removed from the posterior surface of the cornea. For enzymatic digestion, 0.002 g of DNase I (no. 10104159001; Roche) and 0.004 g of collagenase type I (no. 17018029; Invitrogen-Gibco) were added to 1 mL of RPMI-1640 medium (WelGENE) in a 1.5 mL microtube, with four corneas processed per tube to generate one analysis sample. The mixture was incubated at 37°C in a water bath for 60 minutes, with gentle inversion every 10 minutes. Enzymatic activity was stopped by adding 200 μ L of fetal bovine serum. The digested cell suspension was filtered through a 70 μ m cell strainer (no. 93070, SPL Life Sciences) with 5 mL of RPMI-1640. The filtrate was spun in a centrifuge at 300g for seven minutes, the supernatant was discarded, and the pellet was resuspended in PBS.

Cells were washed with PBS and stained with Fixable Viability Stain 510 (no. 564406; BD Biosciences, San Jose, CA, USA) to discriminate between live and dead cells. After viability staining, cells were washed with FACS buffer (1% BSA and 0.01% sodium azide in PBS) and spun in a centrifuge at 300g for seven minutes. Fc receptors

were blocked using TruStain FcX (no. 101320; BioLegend). Cells were then incubated with fluorescently conjugated antibodies targeting surface markers. The following fluorochrome-conjugated antibodies were used: APC-Cy7 anti-CD45 (no. 557659; BD Biosciences), RY703 anti-Ly6C (no. 571456; BD Biosciences), BB515 anti-CD11b (no. 565586; BD Biosciences), and RB705 anti-F4/80 (no. 570288; BD Biosciences). Antibody staining solutions were prepared using Brilliant Stain Buffer (no. 563794; BD Biosciences) following the manufacturer's instructions. Samples were incubated on ice in the dark for 30 minutes. After staining, cells were washed with 1 mL of FACS buffer, spun in a centrifuge at 300g for seven minutes at 4°C, and resuspended in 300 μ L of FACS buffer.

Single-stained controls were prepared using UltraComp eBeads Plus Compensation Beads (no. 01-3333-42; Thermo Fisher Scientific) for spectral compensation. Unstained samples were included to define negative populations. Flow cytometric analysis was performed using a BD FACSymphony A1 cytometer (BD Biosciences), and data were analyzed using FlowJo software (BD Biosciences).

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software (version 10; GraphPad Software Inc., La Jolla, CA, USA). Initially, if the data satisfied the assumption of normality in all groups, a parametric test was used; otherwise, a nonparametric test was applied. For comparisons among three or more groups, either a parametric one-way ANOVA followed by Tukey's post-hoc test or a nonparametric Kruskal-Wallis test followed by Dunn's post-hoc test was performed. For comparisons between two groups, either a parametric Student *t*-test or a nonparametric Mann-Whitney U test was used. Graphical data are presented as mean $\pm$ standard error, with statistical significance set at $P < 0.05$.

RESULTS

Alteration of Cell Viability and Apoptotic Signals in HCFs by UVB Irradiation

First, an experiment was conducted to establish the conditions for UVB-induced apoptosis of HCFs. UVB fluence in the range of 0 to 150 mJ/cm^2 was applied to HCFs to produce BHCFs, resulting in a dose-dependent decrease in cell viability. Specifically, under the condition of 150 mJ/cm^2 UVB exposure, an average reduction in cell viability was most prominent. As a result, in subsequent experiments, UVB with a fluence of 150 mJ/cm^2 was applied (Fig. 1).

Given that the Bax-to-Bcl-2 ratio and Apaf1 activation are established indicators of apoptosis progression, we examined the expression changes of these markers in UVB-irradiated HCFs. The Bax-to-Bcl-2 ratio is known to determine the susceptibility of a cell to apoptosis,²² whereas Apaf1 is a central component of the apoptosome, playing a crucial role in the intrinsic pathway of apoptosis.²³ UVB irradiation at 150 mJ/cm^2 fluence suppressed the expression of Bcl-2 and activated the expression of Bax in HCFs, resulting in an increased Bax-to-Bcl-2 ratio (Figs. 2A–C, 2F). In addition, both the expression of the active form of *APAF1* and the Apaf-1 protein expression were upregulated after UVB exposure (Figs. 2D, 2F). The *CASP9* mRNA level and

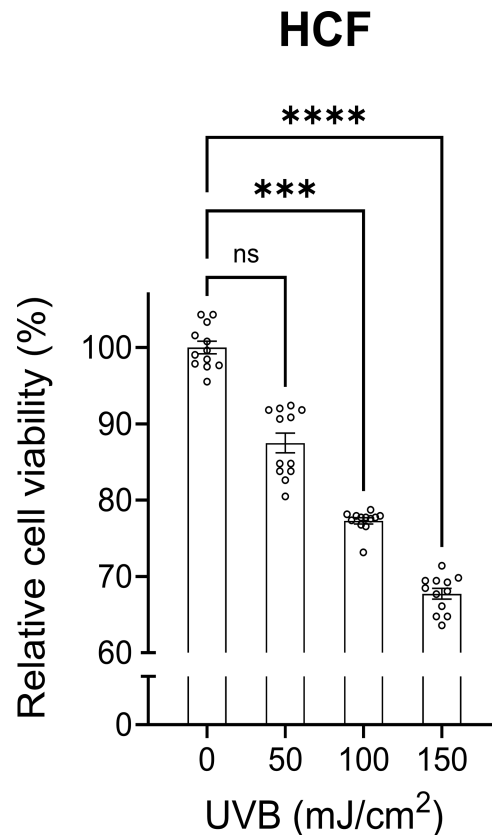


FIGURE 1. Cell viability (MTT assay) of HCFs irradiated with various fluences of UVB, ranging from 0 to 150 mJ/cm^2 . The cell viability of HCFs decreased in an inverse manner depending on the UVB dose. $n = 12$. Data are presented as mean $\pm$ SEM. **** $P < 0.0001$ and *** $P < 0.001$. ns, not significant. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post-hoc test.

the ratio of cleaved (Cl) to full-length cysteine-aspartic acid protease (caspase) 9 (Cl-Cas9/Cas9) were upregulated in BHCFs compared to HCFs (Figs. 2E, 2F).

Efferocytosis of UVB-Irradiated HCFs by M1 Macrophages in an In Vitro Efferocytosis System

After confirming that apoptosis signals are activated in HCFs by UVB irradiation, we identified "eat-me" signals that play an important role in efferocytosis. MFG-E8 is a crucial bridging molecule in this process, with MFG-E8 binding phosphatidylserine on apoptotic cells and engaging $\alpha\text{v}\beta 3/5$ integrins on phagocytes.²⁴ Consistent with these roles, *MFG8* was upregulated in BHCFs compared to HCFs (Fig. 3A).

To investigate efferocytosis between BHCFs and M1 macrophages, we used a transwell co-culture system (Fig. 3B). The transwell insert was equipped with 8 μ m pores, allowing M1 macrophages seeded in the upper chamber to migrate naturally toward BHCFs located in the lower chamber. This pore size was selected to permit controlled macrophage migration while maintaining a physical separation between the two cell populations prior to efferocytic interaction. Before co-culture, apoptosis was induced in HCFs by UVB irradiation. The apoptotic cells were labeled with a TUNEL assay (green), and M1 macrophages were stained with PKH26 (red). PKH26-labeled M1 macrophages

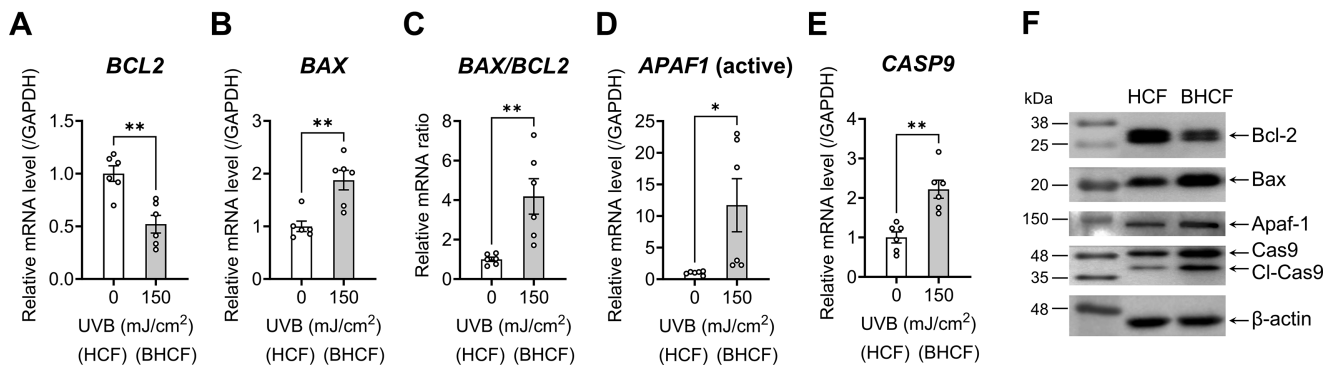


FIGURE 2. Induction of apoptotic signals in UVB-irradiated HCFs. (A–E) Real-time RT-PCR analysis for *BCL2*, *BAX*, the *BAX*-to-*BCL2* expression ratio, the active form of *APAF1*, and *CASP9* in control HCFs and HCFs irradiated with 150 mJ/cm² of UVB (BHCFs). ***P* < 0.01 and **P* < 0.05. *n* = 6. Statistical analyses were performed using Student's *t*-test (A, C, D, and E) or Mann-Whitney U test (B). Data are presented as mean ± SEM. (F) Representative Western blot images of Bax, Bcl-2, Apaf-1, the full length of Cas 9, and the Cl-Cas 9 in control HCFs and BHCFs.

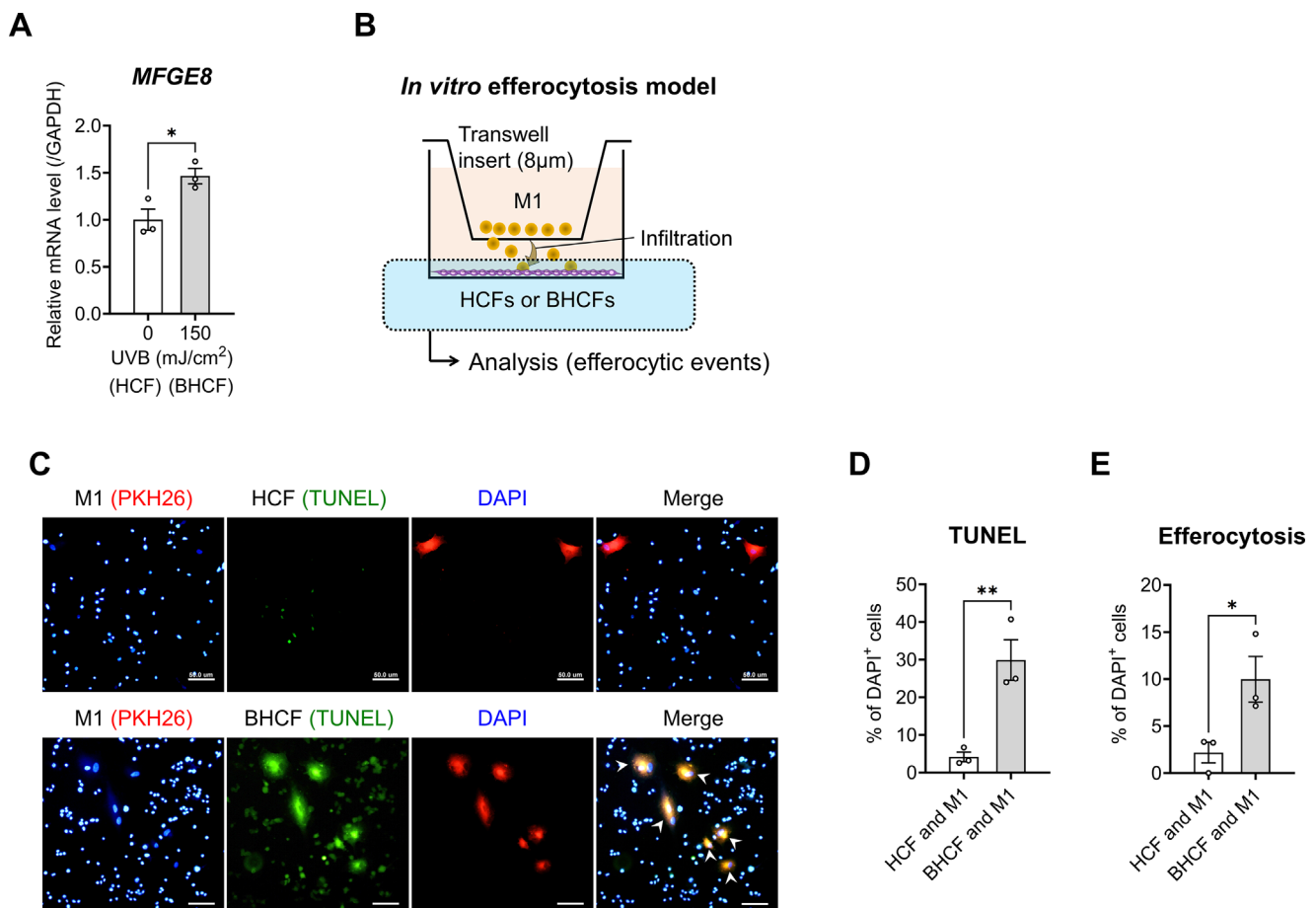


FIGURE 3. Induction of efferocytosis and associated signaling in UVB-irradiated HCFs. (A) Real-time RT-PCR analysis for the efferocytotic ligand *MFGE8* in control HCFs and HCFs irradiated with 150 mJ/cm² of UVB (BHCFs). *n* = 3. (B) Schematic diagram of the transwell-based co-culture system involving M1 macrophages and either control HCFs or UVB (150 mJ/cm²)-irradiated HCFs (BHCFs). The transwell insert with 8 μm pores allowed M1 macrophages seeded in the upper chamber to migrate to the lower chamber and interact with apoptotic BHCFs. (C) Immunofluorescence analysis of in vitro efferocytosis in a co-culture of HCFs or BHCFs with TUNEL staining and PKH26 red-labeled M1 macrophages. Co-localization of apoptotic BHCFs and M1 macrophages (white arrowheads) was interpreted as evidence of efferocytosis. Scale bars: 50 μm. (D, E) Quantification of TUNEL-positive cells and efferocytic events as a proportion of total DAPI-positive cells in HCF or BHCF co-cultures. *n* = 3. Statistical analyses were performed using Student's *t*-test. ***P* < 0.01 and **P* < 0.05. Data are presented as mean ± SEM.

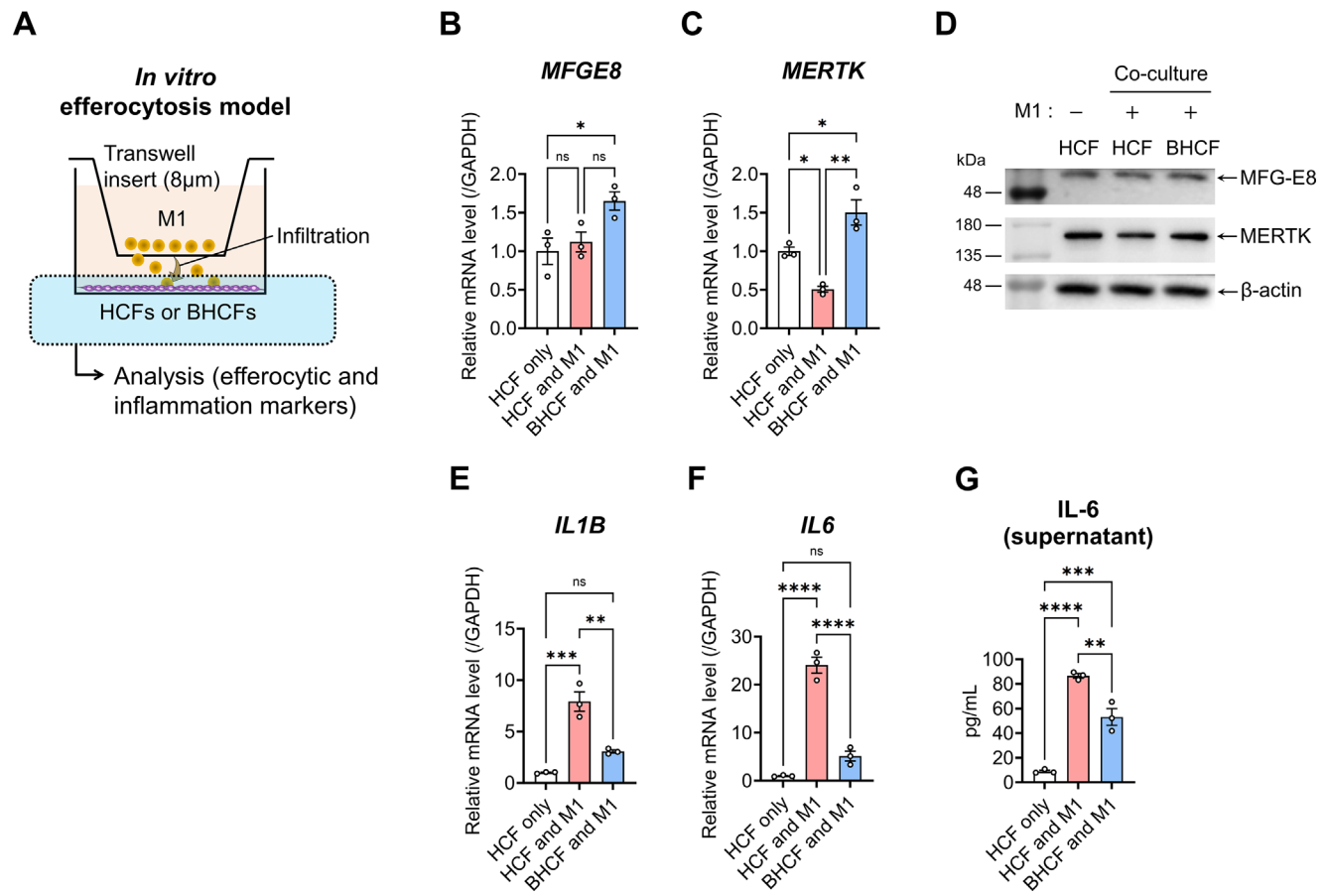


FIGURE 4. Efferocytosis and inflammation-related signals in UVB-irradiated HCFs co-cultured with M1 macrophages within an in vitro efferocytosis model. **(A)** Schematic diagram of the transwell-based co-culture system involving M1 macrophages and either control HCFs or UVB (150 mJ/cm²)-irradiated HCFs (BHCFs). **(B, C)** Real-time RT-PCR analysis of the efferocytosis-related ligand *MFGE8* and the receptor *MERTK* in three groups: non-irradiated HCFs without co-culture with M1 macrophages (i.e., HCF only), non-irradiated HCFs co-cultured with M1 macrophages (i.e., HCF and M1), and UVB-irradiated HCFs co-cultured with M1 macrophages (i.e., BHCF and M1). The cells used for analysis were confined to the bottom area of the well (inside a dotted rounded rectangle). *n* = 3. **(D)** Representative Western blot images of MFG-E8 and MERTK in three groups. *n* = 3. **(E, F)** Real-time RT-PCR analysis of the innate immune-related inflammatory cytokines *IL1B* and *IL6* in three groups. *n* = 3. **(G)** ELISA analysis of IL-6 protein levels in the culture supernatant from three groups. *n* = 3. Statistical analyses were performed using one-way ANOVA followed by Tukey's post-hoc test. *****P* < 0.0001, ****P* < 0.001, ***P* < 0.01, and **P* < 0.05; ns, not significant. Data are presented as mean ± SEM.

were then seeded onto the transwell insert above either HCFs or BHCFs. The proportion of TUNEL-positive cells was significantly higher in the BHCF group, confirming successful induction of apoptosis (Figs. 3C, 3D). In addition, the frequency of orange co-localized signals, which indicate efferocytosis between M1 macrophages and apoptotic cells, was significantly increased in the BHCF co-culture group compared to the HCF co-culture group (Figs. 3C, 3E). These findings suggest that M1 macrophages located near UVB-induced apoptotic HCFs are actively involved in the efferocytosis process.

Changes in Inflammatory Signals in Efferocytic HCFs in an In Vitro Efferocytosis System

Next, in an in vitro efferocytosis system, we evaluated the changes in inflammatory signals alongside efferocytosis-related markers in UVB-irradiated (150 mJ/cm²) HCFs co-cultured with M1 macrophages (Fig. 4A). When non-irradiated HCFs were co-cultured with M1 macrophages,

there was no significant increase in the expression of *MFGE8*, an efferocytosis ligand, or *MERTK*, a representative receptor mediating efferocytosis at the synapse between phagocyte and apoptotic cells (Fig. 4B, 4C).²⁵ In contrast, co-culture with UVB-irradiated apoptotic HCFs led to a significant increase in the expression of both *MFGE8* and *MERTK*, indicating efferocytosis activation (Figs. 4B, 4C). At the protein level, MFG-E8 and MERTK showed a modest increase in BHCF co-cultures compared to HCF co-cultures; however, these changes were less pronounced than those observed at the mRNA level (Fig. 4D).

Expression levels of *IL1B* and *IL6*, known as innate immune-related inflammatory cytokines,²⁶ were markedly increased when M1 macrophages were co-cultured with non-irradiated HCFs (Figs. 4E, 4F). In contrast, both *IL1B* and *IL6* were significantly suppressed in M1 macrophages co-cultured with UVB-irradiated HCFs (Figs. 4E, 4F). This trend was further supported by ELISA analysis of the co-culture supernatant, which showed significantly lower IL-6 protein levels in BHCF-M1 co-cultures compared to HCF-M1 co-cultures (Fig. 4G). This inverse relationship between

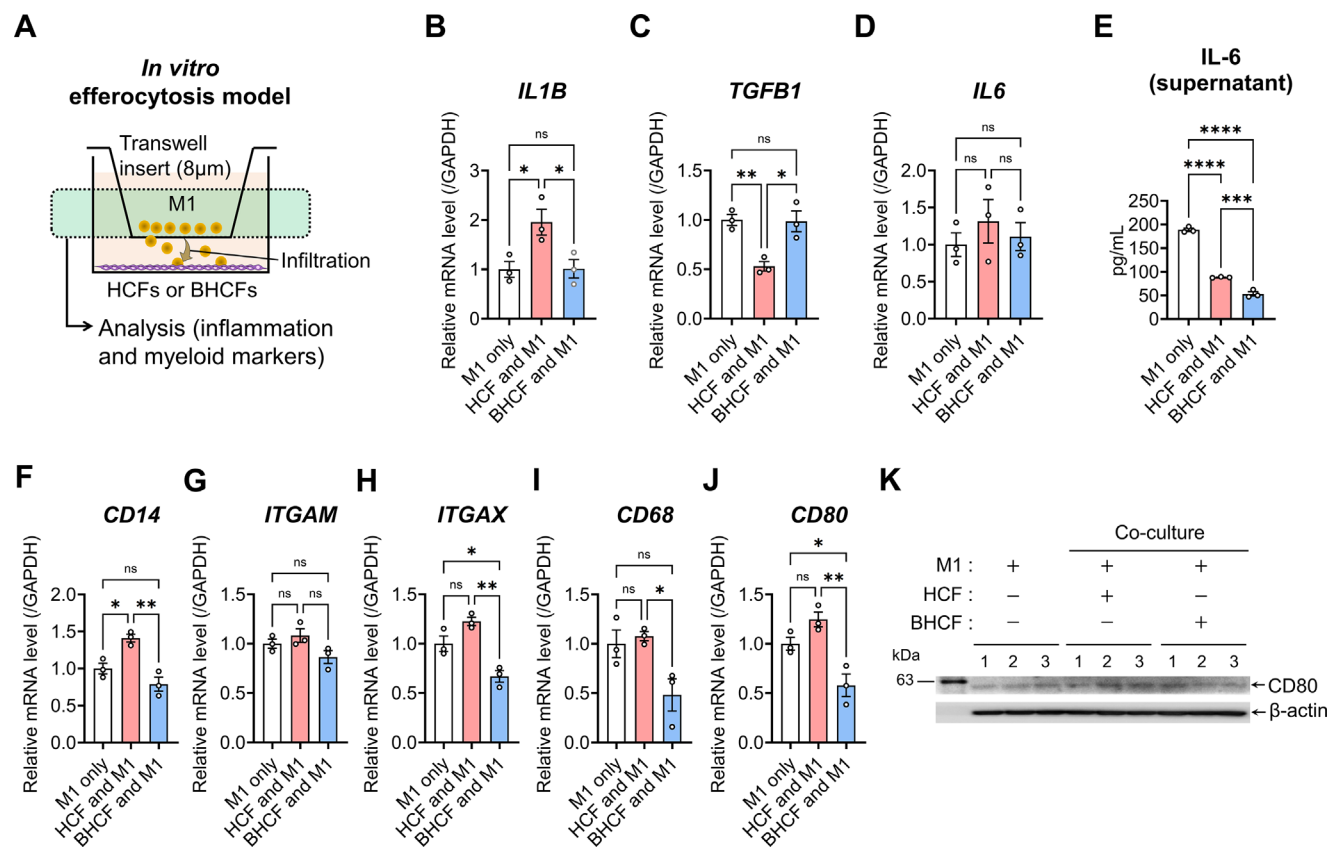


FIGURE 5. Paracrine regulation of microenvironmental M1 macrophages in response to the efferocytosis of UVB-irradiated HCFs. **(A)** Schematic diagram of the transwell-based co-culture system involving M1 macrophages and either control HCFs or UVB (150 mJ/cm²)-irradiated HCFs (BHCFs). **(B–D)** Real-time RT-PCR analysis of the pro-inflammatory cytokines *IL1B*, *IL6*, and the anti-inflammatory cytokine *TGFB1*, in microenvironmental M1 macrophages under three co-culture conditions: M1 macrophages without co-culture with HCFs or BHCFs (i.e., M1 only), non-irradiated HCFs co-cultured with M1 macrophages (i.e., HCF and M1), and BHCFs co-cultured with M1 macrophages (i.e., BHCF and M1). The cells used for analysis were limited to the microenvironmental M1 macrophages on the transwell insert (inside a dotted rounded rectangle). $n = 3$. **(E)** ELISA analysis of IL-6 protein levels in the supernatants from three groups. $n = 3$. **(F–J)** Real-time RT-PCR analysis of the monocyte/myeloid/M1 macrophage-related markers, including *CD14*, *ITGAM* (i.e., *CD11B*), *ITGAX* (i.e., *CD11C*), *CD68*, and *CD80* in three groups. $n = 3$. **(K)** Representative Western blot images of CD80 in three groups. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$; ns, not significant. Data are presented as mean $\pm$ SEM.

efferocytosis activation and inflammatory signal suppression supports the known role of efferocytosis in promoting the resolution of inflammation.^{27,28}

Paracrine Feedback of Efferocytosis to Microenvironmental M1 Macrophages

After confirming that efferocytosis of BHCFs by M1 macrophages reduces inflammation within the efferocytic cell population (Fig. 4), we next examined how this efferocytosis influences nearby macrophages (Fig. 5A). This experiment aimed to simulate whether the polarization of newly recruited macrophages participating in efferocytosis is affected by the surrounding microenvironment.

Compared to the phagocytosis of non-UVB-irradiated HCFs, the efferocytosis of UVB-irradiated (150 mJ/cm²) HCFs led to decreased *IL1B* expression and increased the expression of the anti-inflammatory transforming growth factor beta 1 (*TGFB1*) in the transwell-separated M1 macrophages (i.e., in vitro microenvironmental M1 macrophages) (Figs. 5B, 5C). However, *IL6* mRNA levels

showed only a nonsignificant trend toward change among groups (Fig. 5D). In contrast, analysis of the co-culture supernatant revealed that IL-6 protein levels in the supernatants were most strongly reduced in BHCF-M1 co-cultures, suggesting that efferocytosis suppresses IL-6 secretion from M1 macrophages (Fig. 5E).

The expression of monocyte, myeloid and M1 macrophage-related markers, including *CD14*, *ITGAX* (i.e., *CD11C*), *CD68*, or *CD80*, was not suppressed, and in some cases even increased, when M1 macrophages were co-cultured with non-irradiated HCFs. However, their expression was significantly downregulated in M1 macrophages co-cultured with apoptotic BHCFs (Figs. 5F–J). Although the protein expression of M1 macrophage marker CD80 did not show a strong change, possibly because of its low abundance as a membrane protein, two out of three M1 samples co-cultured with BHCFs exhibited a marked reduction in CD80 expression according to Western blot analysis (Fig. 5K). This suggests that in a simple phagocytic environment, M1 macrophages are exposed to signals that promote an M1-like phenotype, whereas under efferocytosis-inducing conditions, paracrine feedback

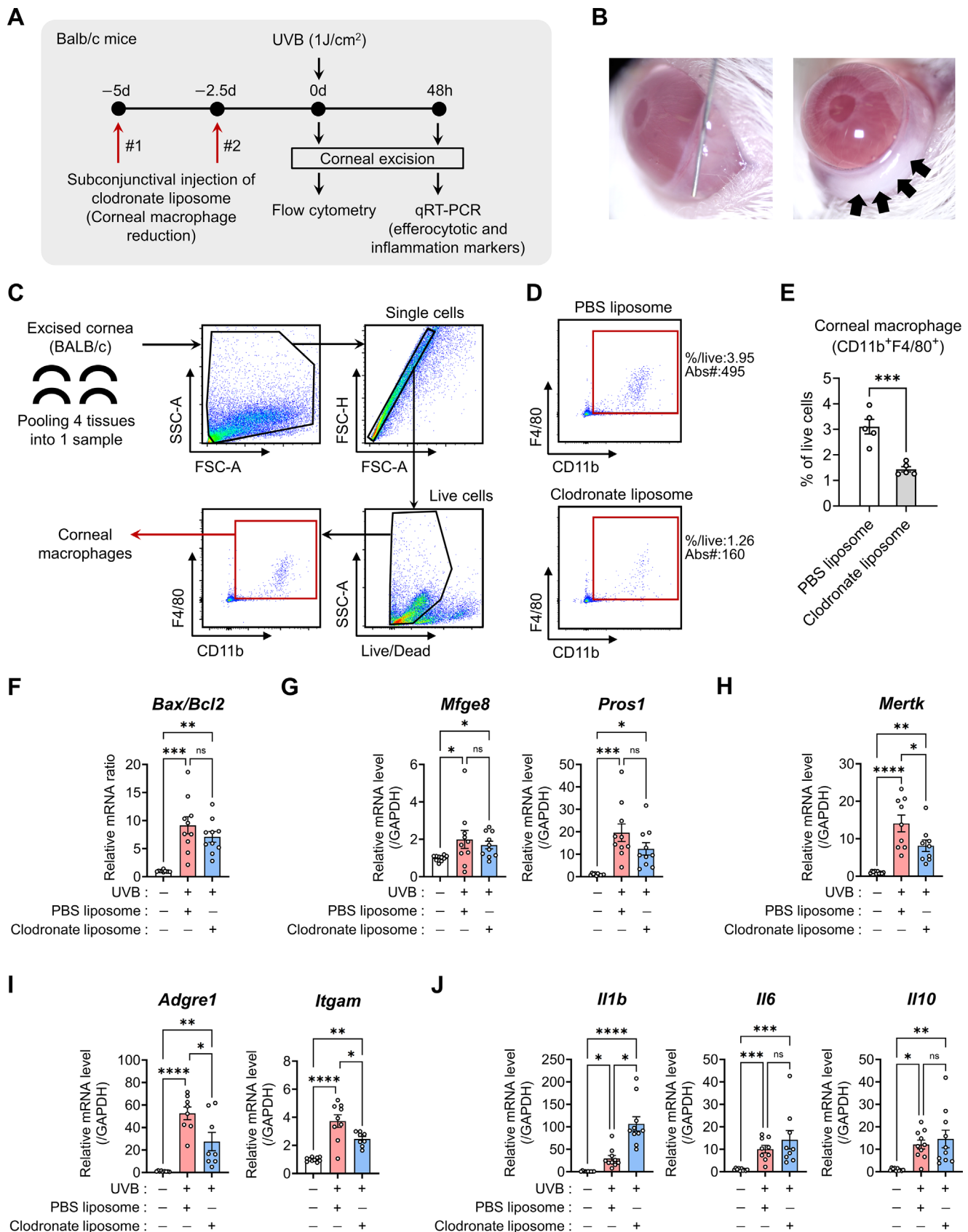


FIGURE 6. In vivo validation of efferocytosis-mediated anti-inflammatory effects using a corneal macrophage reduction mouse model. **(A)** Experimental timeline showing subconjunctival injection of clodronate liposomes five and 2.5 days before UVB exposure (1 J/cm²) in Balb/c mice, followed by corneal excision at 48 hours for flow cytometry and gene expression analysis. **(B)** Representative images of mouse eyes before and after subconjunctival clodronate liposome injection. Arrows indicate the injected liposomes at the subconjunctival

area. (C) Gating strategy for the flow cytometric identification of CD11b⁺F4/80⁺ macrophages in mouse corneas. (D, E) Quantification of the frequency of CD11b⁺F4/80⁺ cells in PBS liposome-injected and clodronate liposome-injected groups. $n = 5$ samples from 20 eyes. (F–H) Real-time RT-PCR analysis of apoptotic (*Bax*-to-*Bcl2* ratio), efferocytosis-related ligands (*Mfge8*, *Pros1*), and efferocytosis-related receptor *Mertk* in the cornea in three different groups: UVB-unirradiated mouse, UVB-irradiated mice injected with either PBS liposomes or clodronate liposomes subconjunctivally. $n = 9$ to 10 eyes per group. (I) Real-time RT-PCR analysis of macrophage marker *Adgre1* (i.e., *F4/80*) and myeloid marker *Itgam* (i.e., *Cd11b*) in the cornea in three different groups. $n = 8$ to 10 eyes per group. (J) Real-time RT-PCR analysis of inflammatory *Il1b*, *Il6*, and anti-inflammatory *Il10* in the cornea in three different groups. $n = 9$ to 10 eyes per group. Statistical analysis was performed using Student's *t*-test (E) and one-way ANOVA followed by Tukey's post-hoc test (F–J). **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$; ns, not significant. Data are presented as mean $\pm$ SEM.

mechanisms may suppress M1 polarization in surrounding macrophages.

In Vivo Validation of Efferocytosis-Mediated Anti-Inflammatory Effects Using a Corneal Macrophage Reduction Model

To further validate the immunological mechanism of efferocytosis in the resolution of inflammation under UVB-stimulated conditions, an in vivo experiment was conducted using a corneal macrophage reduction mouse model. To induce a reduction in corneal macrophages, Balb/c mice were subconjunctivally injected with clodronate liposomes five days and 2.5 days before UVB exposure to the cornea. At the time of UVB exposure (day 0), a decrease in the number and proportion of corneal macrophages was confirmed via flow cytometry (Figs. 6A–C). Specifically, the population of CD11b⁺F4/80⁺ macrophages in the cornea was significantly reduced in the clodronate liposome group compared to the PBS liposome control group (Figs. 6D, 6E). Furthermore, when corneal macrophages were categorized into Ly6C-positive monocyte-derived macrophages and Ly6C-negative resident macrophages, both subsets were significantly reduced following clodronate liposome injection (Supplementary Fig. S1).

Upon UVB irradiation of the cornea in both clodronate- and PBS-liposome-injected groups, the apoptotic *Bax*-to-*Bcl2* ratio increased, and the expression of efferocytosis ligands, including *Mfge8* and *Pros1*, was upregulated (Figs. 6F, 6G). In contrast, corneal macrophage reduction via clodronate liposome injection led to a significant decrease in the expression of the efferocytic receptor *Mertk* compared to PBS liposome-injected mice (Fig. 6H). Additionally, the expression of *Adgre1*, the gene encoding macrophage marker F4/80, and *Itgam*, a myeloid cell marker, was also reduced after clodronate liposome administration (Fig. 6I). Corresponding with the reduction in corneal macrophages and downregulation of efferocytosis-related signals, *Il1b* expression was significantly elevated in the clodronate liposome-treated corneas, whereas *Il6* and the anti-inflammatory cytokine *Il10* did not show statistically significant differences between the two groups (Fig. 6J).

DISCUSSION

UVB radiation, a potent environmental stressor, inflicts substantial damage to the cornea, potentially triggering various ocular pathologies.¹ While previous research has predominantly focused on UVB's direct effects on corneal cells, our study offers novel insights into efferocytosis' role in maintaining corneal homeostasis after UVB-induced keratocyte apoptosis. Recent advancements in our understand-

ing of corneal wound healing have underscored the corneal epithelium's importance not only as a physical barrier but also as an active participant in immune response and tissue repair processes.^{29,30}

Our experiments established that UVB irradiation induces apoptosis in HCFs in a dose-dependent manner, with the most pronounced effects observed at 150 mJ/cm². This finding aligns with previous studies demonstrating corneal cell susceptibility to UVB-induced damage.⁸ The observed changes in apoptotic markers, including the increased *Bax*-to-*Bcl-2* ratio and upregulation of Cas 9 and APAF1, confirm the activation of the intrinsic apoptotic pathway. This is consistent with established UV radiation responses in various cell types.^{31,32}

A key finding of our investigation is the differential regulation of inflammatory signals during efferocytosis of UVB-irradiated HCFs compared to phagocytosis of non-irradiated HCFs. The increased expression of efferocytosis markers (*MERTK* and *MFGE8*), accompanied by suppression of pro-inflammatory cytokines (*IL1B* and *IL6*) during efferocytosis of UVB-irradiated HCFs (BHCfs), suggests a potential mechanism for preventing excessive inflammation in the cornea following UVB exposure. This anti-inflammatory effect of efferocytosis may be crucial for maintaining corneal clarity and function, a finding that aligns with recent research on efferocytosis in other tissues.^{33,34}

Our observations regarding the paracrine effects of efferocytosis on surrounding macrophages are particularly intriguing. The decrease in *IL1B* levels and increase in *TGFB1* expression in microenvironmental M1 macrophages during efferocytosis of BHCfs, coupled with the suppression of markers for myeloid cells and M1 macrophages, suggest a shift towards a less inflammatory or potentially anti-inflammatory phenotype. This paracrine effect may play a significant role in limiting the extent and duration of inflammation after UVB-induced damage, potentially preventing corneal opacity or scarring. Similar paracrine effects have been observed in other tissues,^{35,36} but our study is the first to demonstrate this phenomenon in the context of corneal wound healing.

The upregulation of *TGFB1* in microenvironmental macrophages during efferocytosis merits particular attention. Although TGF-B1 possesses potent anti-inflammatory properties, it can also promote fibrosis under certain conditions. In the context of corneal wound healing, the balance between its anti-inflammatory and pro-fibrotic effects may be critical. Our findings suggest that the efferocytosis-induced increase in *TGFB1* expression may contribute to the resolution of inflammation in the corneal stroma after UVB exposure. However, further research is imperative to fully elucidate the long-term effects of this increased TGF-B1 expression on corneal fibrosis and transparency.

The suppression of M1 macrophage markers in microenvironmental macrophages during efferocytosis suggests a

potential alteration in macrophage phenotype. However, it is important to note that we did not observe a significant increase in M2 markers using Cd163 and Cd206 (data not shown). This lack of clear M2 polarization could be due to several factors. First, the use of cell lines like THP-1 may not fully recapitulate the complex phenotypic changes observed in primary macrophages.³⁷ Additionally, the decrease in M1 markers does not necessarily equate to M2 polarization, as macrophage phenotypes exist on a spectrum rather than as distinct categories.³⁸ Recent studies have shown that macrophages can adopt various phenotypes beyond the traditional M1/M2 dichotomy, particularly in response to complex stimuli like efferocytosis.³⁹ The functional implications of these phenotypic changes in the context of corneal wound healing require further investigation, preferably using primary cells or *in vivo* models. Future studies should focus on a broader range of markers and functional assays to better characterize the macrophage phenotype after efferocytosis in corneal tissues.

Although qRT-PCR analysis demonstrated clear upregulation of efferocytosis-related genes such as *MFGE8* and *MERTK* in BHCfs and co-cultured M1 macrophages, corresponding changes at the protein level were not clearly observed by Western blotting. Similarly, CD80 protein expression in M1 macrophages co-cultured with BHCfs showed no consistent reduction, despite a significant decrease in its transcript level. This discrepancy may reflect the inherent difficulty of detecting quantitative changes in low-abundance membrane proteins or secreted ligands by Western blot analysis. Additionally, receptor dynamics, post-translational regulation, and rapid ligand turnover may contribute to the limited detectability of these proteins under our experimental conditions.

Although *Il1b* expression was consistently suppressed under efferocytotic conditions and increased after corneal macrophage reduction *in vivo*, IL-6 showed only non-significant changes in M1 macrophages co-cultured with BHCfs, as well as in the *in vivo* models. This divergence may be attributed to the distinct regulatory mechanisms of these cytokines. IL-1 β is tightly regulated by inflammasome activation, which is known to be modulated by efferocytosis.⁴⁰

Our study highlights the potential therapeutic implications of targeting efferocytosis in corneal diseases. Enhancing efferocytosis or modulating its downstream effects could promote faster resolution of inflammation and more efficient wound healing in conditions such as corneal ulcers or after corneal surgery. Understanding the mechanisms by which efferocytosis modulates the inflammatory microenvironment could lead to new strategies for preventing corneal scarring and maintaining transparency. While our findings support the functional importance of efferocytosis, the detailed signaling pathways involved remain to be fully elucidated and warrant further investigation. Similar approaches targeting efferocytosis have shown promise in other fields. For instance, in cardiovascular disease, enhancing efferocytosis has been shown to reduce atherosclerotic plaque progression.⁴¹ In the context of lung inflammation, promoting efferocytosis helped resolve inflammation in models of acute lung injury.⁴² Additionally, in neurodegenerative diseases, stimulating efferocytosis of apoptotic neurons showed neuroprotective effects.⁴³ These studies in other organ systems provide a strong rationale for exploring efferocytosis-targeted ther-

apies in corneal diseases, with the potential to improve outcomes in inflammatory and wound healing conditions of the eye.

A significant limitation of this study is the use of the THP-1 human monocyte cell line to verify phenotypical alterations into macrophages,^{44,45} which may not fully reflect the conditions of macrophages in the cornea. Although THP-1 cells offer advantages such as consistency and ease of use, they do not entirely emulate the responses of primary human macrophages to certain stimuli.^{15,37} In addition, the polarization protocols using THP-1 cells can vary across laboratories.⁴⁶ This variability may affect the reproducibility and generalizability of our findings in other experimental settings. Therefore standardization of differentiation conditions should be considered in future studies. Despite these limitations, THP-1 cells remain a valuable tool for initial investigations into macrophage function, particularly in such as efferocytosis studies. The use of cell lines like THP-1 is often necessary for modeling complex cellular interactions such as efferocytosis *in vitro*, as primary culture of macrophages can be challenging and may not provide the consistency required for such experiments. Furthermore, although most of our findings were derived from *in vitro* experiments, the anti-inflammatory role of efferocytosis and its reliance on corneal macrophages were validated *in vivo* using a clodronate-induced macrophage reduction model, thereby enhancing the physiological relevance of our results.

In conclusion, our study demonstrates that efferocytosis plays a crucial role in maintaining corneal homeostasis after UVB-induced keratocyte apoptosis by facilitating the clearance of apoptotic cells and modulating the inflammatory environment. These findings have important implications for our understanding of corneal wound healing and may inform the development of new therapeutic strategies for UV-induced corneal damage and other inflammatory corneal conditions.

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