

RESEARCH ARTICLE

Nuclear Profilin-1 for DNA Damage Repair Is Involved in Phagocytic Impairment of Senescent Microglia

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Received: 7 January 2025 | **Revised:** 17 April 2025 | **Accepted:** 21 April 2025

Funding: This work was supported by a grant (2023R1A2C1006622 to Min-Soo Kwon) and the K-Brain Project (RS-2023-00265515 to Min-Soo Kwon) of the National Research Foundation (NRF) funded by the Ministry of Science and ICT (MSIT), Republic of Korea. This research was supported by KBRI basic research program through Korea Brain Research Institute funded by Ministry of Science and ICT (24-BR-02-04 to Minyeop Nahm). We would like to thank the CheongNyeong Research Foundation (CNRF) for providing a scholarship to Chan Rim.

Keywords: DNA damage repair | phagocytosis | Profilin-1 | senescent microglia

ABSTRACT

Accumulation of DNA damage is a hallmark of cellular senescence and plays a critical role in brain aging. Although the DNA damage repair mechanisms are crucial in cellular senescence, they are not well understood in microglia. In this study, we found that profilin-1 (PFN1), an actin-binding protein, relocates from the cytoplasm to the nucleus in response to DNA double-strand breaks (DSBs) induced by doxorubicin. This nuclear PFN1 subsequently translocates back to the cytoplasm during the recovery period. In response to DSBs, we detected enhanced expression of genes associated with nonhomologous end joining (NHEJ), but not with homologous recombination (HR), along with increased nuclear F-actin accumulation. However, this repair process is compromised when PFN1 is either knocked down or its nuclear transport is blocked. Notably, in DNA damage-induced senescent microglia, increased nuclear localization of PFN1 and nuclear F-actin formation are associated with phagocytic dysfunction. Both ex vivo aged microglia and publicly available single-cell RNA sequencing data from aged mouse brains recapitulate the in vitro findings described above. Despite cytochalasin D treatment for actin depolymerization, the return of PFN1 to the cytoplasm was not facilitated due to its aggregation. We propose that PFN1 plays an important role in DNA damage repair in microglia. In addition, the dysregulation of the nucleocytoplasmic balance of PFN1 alongside DNA damage accumulation may contribute to the phagocytic impairment of microglia in the aged brain.

1 | Introduction

Microglia are the resident immune cells that also carry out immune and nonimmune functions within the central nervous system (CNS) (Borst et al. 2021; Li and Barres 2018). Despite being a relatively small population among CNS cells, microglia have a great impact on CNS homeostasis and a healthy brain environment (Kettenmann et al. 2011). Microglia exhibit a slow

turnover rate and have a long lifespan in the human brain (Reu et al. 2017), which might lead to the accumulation of senescent microglia in the brain as aging (Borghesan et al. 2020). Recent spatiotemporal RNA-sequencing studies have unveiled accelerated aging in glial cells, particularly in white matter. This study has identified microglia as cells vulnerable to brain aging, as determined through Bulk-seq Common Aging Score (CAS) calculations (Hahn et al. 2023).

Notably, senescent microglia rather than reactive microglia have been observed in postmortem brain tissue with neurodegenerative diseases including Alzheimer's disease (AD) (Streit et al. 2009). Recent studies have reported that senescent microglia have been identified in postmortem brain tissues of AD (Hu et al. 2021; Rachmian et al. 2024). In addition, single-nucleus RNA sequencing of the dorsolateral prefrontal cortex in human AD brains revealed multiple microglial clusters, including dystrophic, senescence-like, and canonical inflammatory microglia (Prater et al. 2023). Not only the research articles but also recent review papers have highlighted the functional relevance of microglial senescence in the pathophysiology of neurodegenerative diseases (Lau et al. 2023; Rim et al. 2024). A recently proposed hypothesis suggests that senescent microglia may contribute to brain aging through the propagation of cellular senescence, mediated by the senescence-associated secretory phenotype (SASP) (Gordleeva et al. 2020). Moreover, as resident phagocytes that fail to eliminate other senescent cells, senescent microglia themselves may accelerate brain aging and its related diseases. The therapeutic potential of senolytics—drugs that selectively eliminate senescent cells (Chaib et al. 2022) in a tauopathy model (Bussian et al. 2018) as well as in patients with AD (Gonzales et al. 2023) might be related to senescent microglia. Thus, understanding the physiology of senescent microglia is necessary to combat brain aging-related neurodegenerative diseases (Rim et al. 2024).

Cellular senescence, which is a hallmark of organismal aging, contributes to neurodegeneration (Angelova and Brown 2019). Stressors that contribute to cellular senescence include replicative stress stemming from repetitive cell culture (Linskens et al. 1995), DNA damage accumulation (Rodier et al. 2009), and nuclear barrier-induced senescence (NBIS) (Park et al. 2021). Especially, the accumulation of DNA damage leads to cell cycle arrest and increases the expression of SASPs (Kang et al. 2015; Rodier et al. 2011). For DNA damage repair, most mammalian cells utilize majorly homologous recombination (HR) and non-homologous end joining (NHEJ) (Ceccaldi et al. 2016). When DNA damage occurs, phosphorylation of the serine 139 residue of histone 2A (γ H2AX) recognizes the damage and initiates repair (Nagelkerke and Span 2016). To allow better access for the repair machinery to the site of the DNA double-strand breaks (DSB), the damaged DNA strand is attached to the nuclear lamina via filamentous-actin (F-actin) and unwound to euchromatin state (Caridi et al. 2018, 2019). In addition, F-actin level is increased in aged fibroblast, while globular-actin (G-actin) expression remains constant (Phillip et al. 2015). Furthermore, F-actin accumulation in neurons drives brain aging via autophagic dysfunction and shortens the healthspan in *Drosophila* (Schmid et al. 2024).

It is understood that PFN1 acts as an actin-binding protein, which is known to regulate actin polymerization (Alkam et al. 2017; Suarez et al. 2015). PFN1 is primarily located in the cytoplasm and transports nuclear actin to the cytoplasm by means of exportin-6 (Xpo6) (Stuven et al. 2003). Regulating the nucleocytoplasmic transport of PFN1 may support DNA damage repair by controlling F-actin polymerization/depolymerization or other mechanisms within the nucleus. Notably, microglia play a key role in phagocytosing extracellular aggregates through their processes, primarily regulated by actin dynamics

involving cytosolic PFN1 in both physiological and pathological conditions (Castellano et al. 2001; Noh et al. 2023).

Considering the possible role of PFN1 in DNA damage repair and phagocytosis, maintaining intact PFN1 function and its nucleocytoplasmic balance is crucial for DNA repair and aging-related neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) (Schmidt et al. 2021; Wu et al. 2012; C. Yang et al. 2016). In this study, we aim to elucidate the potential link between DNA damage accumulation and phagocytic impairment in senescent microglia, with a focus on the critical role of PFN1.

2 | Materials and Methods

2.1 | Animals

Aged C57BL/6 mice (80-week-old) (Janvier, France) for acute isolation of ex vivo aged microglia and subsequent immunofluorescence analysis were used. Timed-pregnant 13-day C57BL/6 mice (TP13, Koatech Inc. Pyeongtaek, Korea) were used for primary microglia culture. The mice were housed in cages with three to five animals each under specific pathogen-free (SPF) conditions at $22^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and an alternating 12-h light–dark cycle at the CHA BIO COMPLEX animal facility (Seongnam, Korea). The animals were provided with food and water ad libitum. The animals were acclimated to the laboratory setting for a minimum of 1 week prior to experiments. To minimize the potential for time-dependent variability, all experiments were conducted during the light phase of the cycle. All experimental animals were handled in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of CHA University (IACUC240084) and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) reporting guidelines.

2.2 | BV2 Cell and Primary Microglia Cultures

BV2 cells were cultured in an uncoated 100 mm² cell culture dish with DMEM (Gibco, #11995–073) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, #16000–044) and 1% penicillin/streptomycin (Gibco, #15140–122). Medium was changed every 3 days. Since BV2 cells cannot fully substitute for mature microglia, we also used primary microglia, which exhibit characteristics more closely resembling those of mature microglia (You et al. 2021). Primary microglial cultures were performed as described in our previous study (You et al. 2021). Briefly, uteri of TP13 mice were dissected and soaked in Hanks' balanced salt solution (HBSS, Invitrogen, USA). The mouse embryo was removed from the uterus using microsurgical instruments under a microscope. The neuroepithelial layer (NEL) of C57BL/6 mice (embryonic day 13.5) was then carefully dissected. The minced tissue was incubated with 1 mL of trypsin–EDTA (Gibco, #15090–046) for 3 min in a 37°C water bath. After centrifugation at $300 \times g$ for 5 min, the neuroepithelial layer cells were plated into 25 T flasks and cultured in DMEM containing 10% FBS, 1x penicillin/streptomycin, and 0.1% GlutaMAX (Gibco, #35–050–061) at 37°C in a 5% CO_2 incubator. The culture medium was replaced with 5 mL fresh growth medium

after 24 h. On day 21, microglia were isolated from NEL cells by magnetic activation cell sorting (MACS) using CD11b microbeads (Miltenyi Biotec, 130-093-634). Primary microglia were plated at appropriate density on poly-D-lysine-coated cell culture plates or dishes. BV2 cells were seeded in 6-well plates at 2×10^6 cells/well for qPCR and Western blot analysis or in 24-well plates at 5×10^4 cells/well for cell viability, phagocytosis, and immunocytochemistry. Primary microglia were seeded in poly-D-lysine-coated 24-well plates at 10^5 cells/well for immunocytochemistry. For molecular analysis, BV2 cells were incubated in DMEM with 1% FBS and 1% penicillin/streptomycin for 24 h to reduce cell cycle and cell activation bias. Primary microglia were seeded in poly-D-lysine-coated 24-well plates at 10^5 cells/well for immunocytochemistry.

2.3 | Acute Isolation of Microglia in Aged Mouse

Brains were isolated from male aged C57BL/6J mice at 90 weeks old. Enzymatic cell dissociation was performed using the Adult Brain Dissociation Kit (Miltenyi Biotec, #130-107-677), according to the manufacturer's instructions. Brain tissue pieces (up to 500 mg) were transferred into a gentleMACS C tube (Miltenyi Biotec, #130-096-334) containing 1950 μ L of enzyme mix 1 (enzyme P and buffer Z). Subsequently, 30 μ L of enzyme mix 2 (enzyme A and buffer Y) was added. The gentleMACS C tube was securely closed and placed in an inverted position on the sleeve of the gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec, #130-096-427). The appropriate gentleMACS program (37C_ABDK_01) was then executed. After dissociation, the sample was briefly centrifuged to gather the samples at the bottom of the tube, then strained through a 70 μ m strainer (Miltenyi Biotec, #130-098-462), washed with DPBS, and centrifuged again. The resulting pellet was then resuspended in cold DPBS. The myelin and cellular debris were eliminated with a debris removal solution, followed by the removal of erythrocytes via a red blood cell removal solution. Finally, microglia were magnetically isolated using microbeads coupled to anti-CD11b mAb, as previously described.

2.4 | Reagents

Cells were treated with 500 nM of Doxorubicin (DOX; Sigma-Aldrich, D1515) for 1 h to induce double-strand break induction (DSB). For actin depolymerization, 5 μ M of Cytochalasin D (CytD; Sigma-Aldrich, C8273) was treated for 30 min. To induce DNA damage-induced senescence (DDIS), primary microglia were treated with 100 nM of DOX for 3 days.

2.5 | siRNA and Plasmid DNA Transfection

BV2 cells were transfected with predesigned siGENOME Mouse Pfn1 siRNA smart pool or nontargeting siRNA for control (Dharmacon, M-062705-01-0005; D-001210-02-05). Cells were transfected with 25 nM total siRNA using RNAiMAX (Invitrogen, #13778030) according to the manufacturer's protocols. To modulate the subcellular localization of PFN1, we inserted the triple repeat nuclear localization signal (NLS; DPKKKRKV DPKKKRKV DPKKKRKV) and nuclear export

signal (NES; MNLVDLQKKLEELDEQQ) with PFN1 into the pEGFP-C1 vector (Clontech, KanR) by polymerase chain reaction. To increase the efficiency of nuclear localization, we used three tandem repeat NLS, as suggested in a previous study (Diamond et al. 2015). Cells were transfected with 500 ng of 3 $\times$ NLS-PFN1-EGFP, NES-PFN1-EGFP, and EGFP-PFN1 DNA using Lipofectamine 3000 (Invitrogen, #13778030) according to the manufacturer's protocols. To record the live cell video, we used the JuLi stage live cell imaging device (NanoEnTek).

2.6 | RNA Extraction and Quantitative PCR

To obtain RNA, cells were digested with 1 mL of TRIzol reagent per 100 mg of tissue (Life Technologies, #15596018). Then, add chloroform to separate the phase-containing RNA and isopropyl alcohol to precipitate the RNA. Air-dry each RNA pellet and dissolve it in DEPC-treated water (Life Technologies, AM9906). Measure the RNA concentration by measuring absorption at 260 nm. Messenger RNA (mRNA) was transcribed in 20 μ L of reaction mixture through reverse transcription utilizing the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, K1622). Power SYBR Green PCR Master Mix (Life Technologies, #4367659) was employed to execute Quantitative PCR. The cyclic conditions comprised an initial enzyme activation at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 20 s, as well as annealing and extension, while detecting SYBR Green that is bound to PCR product at 56°C for 40 s. For normalization purposes, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as an internal control. The comparative CT method was employed to calculate the relative quantities of PCR fragments. The primer sequences are listed in Table S1.

2.7 | Whole Protein Extraction, Nuclear Protein Isolation, and Western Blotting

To collect whole protein from the cells, cells were lysed using RIPA buffer (Sigma #R0278). Also, to isolate nuclear and cytoplasmic proteins, NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo, #78833) were utilized following the manufacturer's instructions. BV2 cells were first treated with trypsin-EDTA and centrifuged at $500 \times g$ for 5 min. Next, ice-cold CER I was added to the cell pellet, which was then thoroughly vortexed for 15 s to completely dissolve the pellet. Subsequently, the tube was placed on ice for 10 min to allow for incubation, after which ice-cold CER II was added to the tubes. The tube was vortexed twice for 5 s and centrifuged for 5 min at $15,000 \times g$. The resulting supernatant was utilized as the cytoplasmic extract. Following this, the insoluble substance was resuspended in ice-cold NER, vortexed for 15 s every 10 min, and incubated a total of 40 min. The tube was then centrifuged at $15,000 \times g$ for 10 min, and the resulting supernatant was utilized as the nuclear extract. Protein concentrations were measured through a detergent-compatible protein assay reagent (Bio-Rad Laboratories, USA) using BSA as the standard. Following the addition of dithiothreitol (5 mM) and bromophenol blue (0.1% w/v), the proteins were boiled and separated by electrophoresis on 10%–15% polyacrylamide gels (Invitrogen). The separated proteins were finally transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad

Laboratories). The membranes were blocked for 1 h on a shaker at room temperature using Tris-buffered saline/0.1% Tween-20 (TBS-T) and 5% nonfat dry milk or 2.5% BSA. Primary antibodies were dissolved in the blocking buffer and then used for immunoblotting of the membranes against specific antibodies. The secondary antibody was carried out at room temperature for 1 h in the blocking buffer. Afterward, the membranes were visualized with an ECL-plus solution from GE Healthcare, USA (RPN2106V1 and V2). Finally, the membranes were exposed to chemiluminescence using a LAS-4000 device (Fujifilm, Japan). The blot was quantified by utilizing ImageJ 1.51 software (the National Institutes of Health in Bethesda, MD, USA). The quantification followed densitometric scanning of films. Antibodies used and their titer in this experiment are below. Rabbit anti-profilin-1 antibody (Cell Signaling Technology; 3237S, 1:1000), Rabbit anti-Lamin B1 antibody (Abcam; ab16048, 1:5000), Rabbit anti-histone H3 antibody (Cell signaling technology; 9715S, 1:5000), Rabbit anti- β -actin antibody (Cell signaling technology; 4970s, 1:5000), Rabbit anti-GAPDH (Cell signaling technology; 2118S, 1:1000), and Goat anti-rabbit IgG-H+I HRP conjugated secondary antibody (Bethyl, A120-101P, 1:10000).

2.8 | Senescence-Associated β -Galactosidase Assay

The primary cultured microglia were seeded onto a 12-mm coverslip in a 24-well cell culture plate. After that, the cells were fixed and stained with the Cellular Senescence Assay Kit (Cell Biolabs, #CBA-230), following the manufacturer's instructions. Random microscopic fields were imaged per sample using bright field microscopy. The percentage of senescent cells was determined by counting the number of SA- β -gal-positive cells stained with turquoise in microglia.

2.9 | Immunocytochemistry

The cells were seeded onto glass coverslips in 24-well plates. The cells were washed using DPBS solution and subsequently treated with a 4% paraformaldehyde fixative. Fixed cells were blocked and permeabilized on a shaker for 1 h at room temperature. The buffer used for permeabilization and blocking was composed of PBS with 5% normal donkey serum, 0.3% Triton X-100, and 1% BSA. To detect aggregated proteins, we used cytoskeletal buffer (CSK buffer) for detergent pre-extraction as described in this article (Zhu et al. 2020). Briefly, cells were incubated with CSK buffer (25 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose, and 0.5% Triton X-100) for 5 min, followed by DPBS rinse and fixation with 4% paraformaldehyde. Indirect immunofluorescence was conducted with the following primary antibodies. Cells were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. After rinsing three times with DPBS containing 1% BSA for 5 min, cells were incubated with secondary fluorescent antibody diluted in 1% BSA in DPBS. Finally, washing 3 times with DPBS, Nuclei were counterstained using ProLong Gold antifade reagent and DAPI (Invitrogen, Carlsbad, California, USA #P36931). Fluorescent images were acquired utilizing a TCSP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany). Antibodies used and their titers are below: rabbit anti-Profilin-1 antibody (Abcam; ab124904, 1:500), goat

anti-IBA-1 antibody (Novus; NB100-1028, 1:500), mouse anti-phospho H2A.X (ser 139) antibody (Millipore; 05-636, 1:1000), rabbit anti-53BP1 antibody (Novus; NB100-904, 1:500), mouse anti-RAD51 antibody (Novus; NB100-148, 1:500), Alexa Fluor 488 donkey anti-goat IgG antibody (Invitrogen; A11055, 1:200), Alexa Fluor 488 donkey anti-rabbit IgG antibody (Invitrogen; A21206, 1:200), Alexa Fluor 594 donkey anti-mouse IgG antibody (Invitrogen; A21203, 1:200), Alexa Fluor 594 donkey anti-rabbit IgG antibody (Invitrogen; A21207, 1:200), Alexa Fluor 647 donkey anti-rabbit IgG antibody (Invitrogen; A31573, 1:200), Alexa Fluor 647 donkey anti-mouse IgG antibody (Invitrogen; A31571, 1:200), Alexa Fluor 488 Phalloidin probe (Invitrogen; A12379, 1:1000).

2.10 | Phagocytosis Assay

To evaluate the phagocytic activity of microglia, they were seeded onto a 12-mm coverslip in 24-well cell culture plates. BV2 cells were seeded at a density of 1×10^5 cells/ml, while primary microglia were seeded at a density of 2×10^5 cells/ml. The cells were exposed to pHrodo Red Zymosan BioParticles conjugate for phagocytosis (Invitrogen, P35364) and were used at 100 μ g/ml for 1 h at 37°C. To inhibit phagocytic activity, ice-cold PBS was added. Cells were washed twice with ice-cold PBS, then fixed and stained with a microglial marker (Iba-1). They were counterstained with DAPI and analyzed using a confocal microscope (TCS SP5, Leica). To quantify the intensity of the pHrodo signal, the cells were fixed with CytoFix buffer (BD, 554655) and utilized the CytoFLEX flow cytometer system (Beckman Coulter) after washing twice with FACS buffer (0.5% BSA in DPBS).

2.11 | Image Analysis

To determine the precise nuclear or cytoplasmic intensity of PFN1, we utilized the intensity ratio of nuclei and cytoplasm tool, which is integrated with Image J software. Initially, we segmented the nucleus by subtracting the nuclei-stained channel (DAPI) from the whole-cell stained channel (PFN1). Then we automatically measured the PFN1 intensity of each nucleus and calculated the nuclear/cytoplasmic ratio (N/C ratio). To assess the extent of DNA double-strand breaks, we merged the DAPI and DSB marker channels (γ H2AX), and subsequently manually counted the co-localized foci per nucleus. We assessed the dystrophic morphology of microglia by counting the loss of fine branches (deramification), as well as the presence of thin, shortened, beaded, tortuous, or fragmented processes and cytoplasm, or any combination of these alterations (Streit et al. 2004). To confirm the fragmented morphology, we utilized FracLac in ImageJ to calculate fractal dimensions and lacunarity (Karperien et al. 2013). Finally, to visualize the fragmented processes of microglia, we performed 3D reconstruction of z-stacked confocal imaging using MATLAB (MathWorks).

2.12 | Public Omics Data Analysis

To support our in vitro and in vivo experiments, we utilized one proteomics dataset from the PRIDE database and two distinct single-cell RNA-sequencing datasets from the National Center

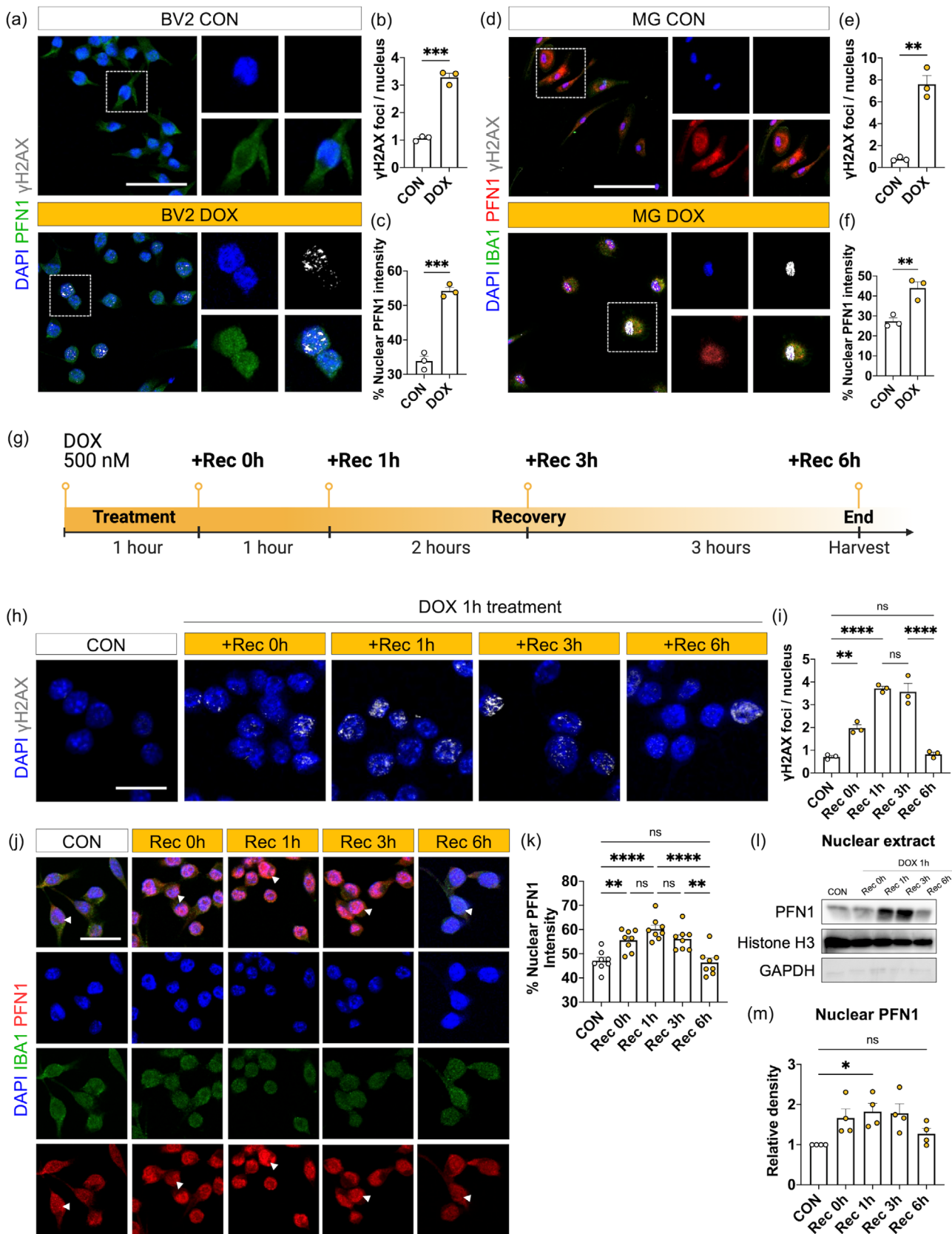


FIGURE 1 | Legend on next page.

for Biotechnology Information Gene Expression Omnibus (NCBI GEO). R (v4.2.0) and Seurat (v5.1.0) were used for downstream analysis following the standard workflow, which was provided

by Satija Lab. Quality control and filtering criteria were applied in accordance with those described in the original publication (Ximerakis et al. 2019; Zhang et al. 2022). Pathway analysis

FIGURE 1 | Profilin-1 is rapidly transported to the nucleus in response to DNA damage in microglia. (a) Representative images of immunostained profilin-1 (PFN1) and phosphorylation of the serine 139 residue of histone 2A (γ H2AX) from control and doxorubicin (DOX)-treated BV2, and (b) its quantification of γ H2AX foci per nucleus of each field in a ($n = 3$). (c) Quantification of percent of PFN1 intensity in nucleus of each field in a ($n = 3$). (d) Representative images of immunostained PFN1 and γ H2AX of DOX-treated microglia, and (e) its quantification of γ H2AX foci per nucleus of each field in d ($n = 3$). (f) Quantification of percent of PFN1 intensity in nucleus of each field in d ($n = 3$). (g) Schematic illustration of the DOX-induced DNA damage and its recovery timepoint utilized in this study. (h) Representative images of immunostained γ H2AX from control and DNA damage recovery (0 h, 1 h, 3 h, 6 h), and (i) its quantification of γ H2AX foci per nucleus of each field ($n = 3$) in e. (j) Representative images of immunostained PFN1 from control and DNA damage recovery (0 h, 1 h, 3 h, 6 h), and (k) its quantification of percent of PFN1 intensity both in cytoplasm and nucleus ($n = 8$) in j. (l) Representative immunoblots of PFN1 and Histone H3 (nuclear marker) and GAPDH (cytoplasmic marker) in BV2 nuclear extract. (m) Relative density is defined as the ratio of respective protein density as a percentage of Histone H3 density normalized to CON density ($n = 4$). Data information: Scale bar is 50 μ m for (a, d, h, and j). Graphs are represented as Mean $\pm$ SEM, (****) $p < 0.0001$, (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$; two-tailed unpaired Student's t -test was used to test for significant differences of (b, c and e, f). One-way ANOVA and a subsequent Tukey *post hoc* test were used to test for significant differences of (i, k, and m). Repair scheme (g) is created with Biorender.com. Three independent experiments were performed throughout the study.

was performed using the web-based Database for annotation, visualization, and integrated discovery (DAVID; NIH) with cut-off criteria for determining statistical significance ($-\log_2$ (fold change > 1), and p -value < 0.05). We reanalyzed proteomics data from doxorubicin-induced senescent-like CHME-5 cells using the STRING database (Szklarczyk et al. 2023) and metascape (Zhou et al. 2019).

2.13 | Statistical Analysis

To provide a more accurate depiction of the statistics, we first detected any statistical outliers and carried out normality testing through normality testing utilizing the ROUT test (with $Q < 0.5\%$). Subsequently, the cleaned dataset was assessed for normality using the Kolmogorov–Smirnov and Shapiro–Wilk tests. We performed unpaired t -tests and paired t -tests to evaluate statistical significance between two groups. Furthermore, we conducted a One-way Analysis of Variance (ANOVA) with Tukey's post hoc test to compare means among more than two groups using GraphPad Prism 10 for Mac software (GraphPad, La Jolla, CA). The statistically significant differences are represented by the following values: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

3 | Results

3.1 | Rapid PFN1 Translocation to the Nucleus Upon DNA Damage

To investigate the subcellular location of PFN1 in response to acute DNA damage, we used doxorubicin (DOX), a chemical known for causing DNA DSBs by topoisomerase II inhibition (Yang et al. 2015). We treated BV2 cells and primary microglia with DOX at a concentration of 500 nM for 1 h. At this concentration and timepoint, γ H2AX foci were increased in BV2 cells (Figure 1A,B), and primary microglia after DOX treatment (Figure 1D,E), without cytotoxicity (Figure S1). We found that the increased nuclear intensity of PFN1 in both BV2 cells (Figure 1A,C) and primary microglia (Figure 1D,F) in response to acute DNA damage. This is consistent with previous findings that show in keratinocytes, PFN1 translocates into the nucleus in response to DOX-induced DSBs (DOX-DSBs) (Lee et al. 2021).

Further experiments with BV2 cells transfected with an EGFP-tagged PFN1 confirmed that PFN1 moves to the nucleus during DOX-DSBs (Figure S2). To better understand the DNA damage repair process of microglia, we designed an experiment to track the DNA repair process (Figure 1G). The DNA damage response peaked at 3 h after DOX washout (3-h recovery: Rec 3 h) and decreased by 6 h (Rec 6 h) (Figure 1H,I). Electrophoresis analysis of DNA fragments confirmed that microglia were able to repair the DOX-DSBs within 6 h in our design (Figure S3). Consistent with the DNA damage response, the intensity of PFN1 in the nucleus gradually increased until Rec 3 h and returned to control levels by Rec 6 h (Figure 1J,K). Western blot analysis of the nuclear extract of BV2 cells showed significantly increased PFN1 expression at Rec 1 h (Figure 1L,M). Together, these findings demonstrate the dynamic response of PFN1 in microglia to DOX-DSBs, highlighting its translocation to the nucleus, which may be involved in DNA damage repair.

3.2 | Nuclear PFN1 for DNA Double-Strand Breaks Repair

We then shifted focus to understanding the DSB repair mechanisms in microglia. The two main pathways for DSB repair are NHEJ and HR (Scully et al. 2019). To identify the primary pathway involved, we used 53BP1 and RAD51 as markers for NHEJ and HR, respectively (Zhao et al. 2020). Following DSB induction, we observed an increase in the co-localization of 53BP1 and γ H2AX, which peaked at Rec 3 h (Figure 2A–C). However, RAD51 foci were not detected at any point during the recovery period (Figure 2D). We also observed an increase in the expression of NHEJ-related genes, including *Xrcc6*, *Xrcc5*, and *Xrcc4*. However, there was no significant change in the expression of HR-related genes like *Rad51* and *Rad54b* (Figure 2E).

PFN1, primarily known for its role in actin polymerization in the cytoplasm (Davey and Moens 2020) can support the repair of DNA damage by facilitating the polymerization of F-actin within the nucleus after DOX treatment. Our hypothesis is supported by the observations that cytoplasmic PFN1 is translocated into the nucleus following DNA damage, resulting in an increase in nuclear F-actin intensity. The intensity peaked at Rec 3 h and returned to control level at Rec 6 h (Figure 2F). We analyzed publicly available proteomics data from DOX-induced

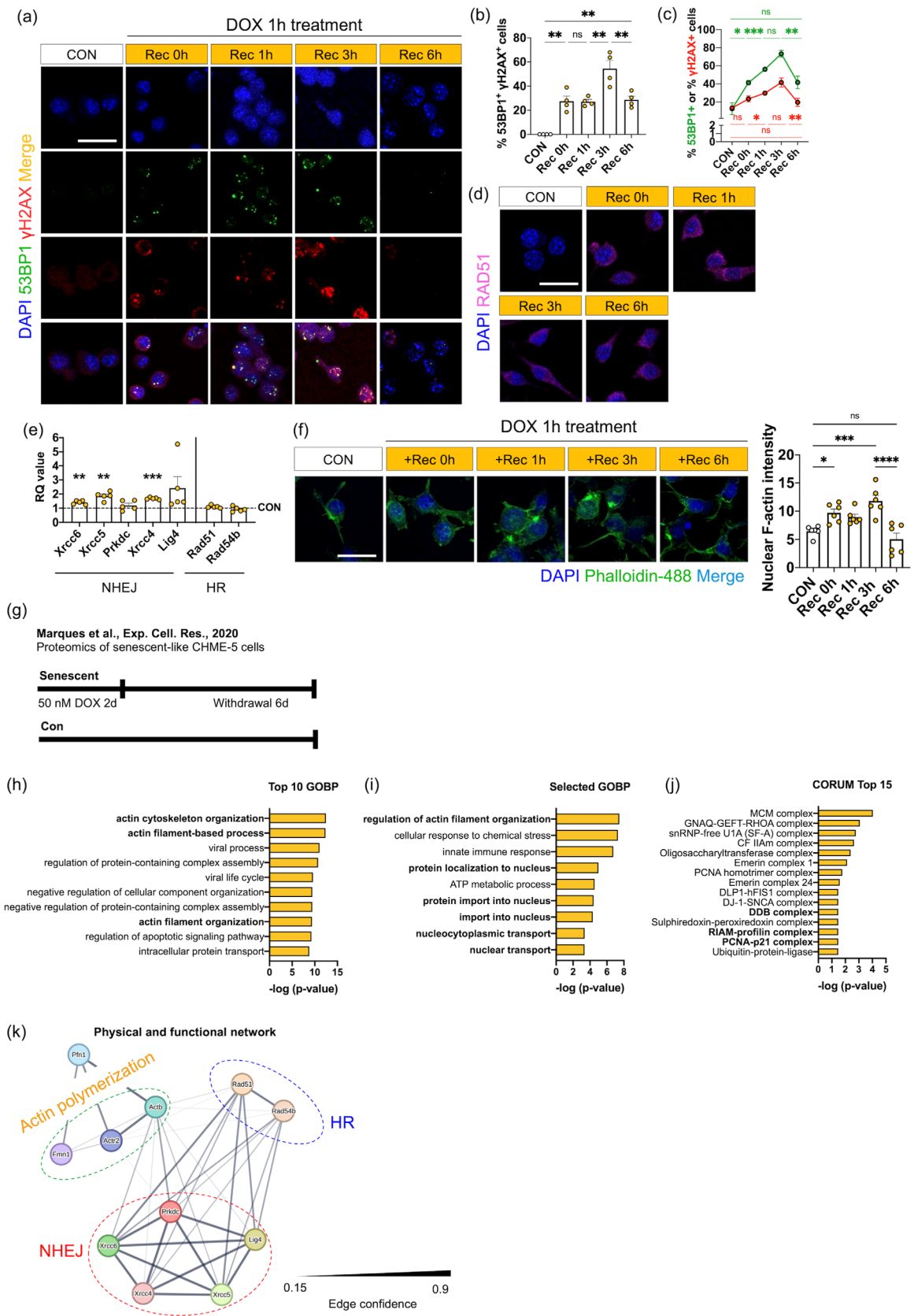


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senescent-like CHME-5 cells (Marques et al. 2020) to support our findings (Figure 2G). The analysis revealed that the senescent-like CHME-5 cells exhibit alterations in actin dynamics and

nucleocytoplasmic transport (Figure 2H,I). Furthermore, we identified that differentially expressed proteins were linked to the DNA damage response complex and profilin complex in

FIGURE 2 | Nuclear profilin-1 supports DNA damage repair through non-homologous end joining in microglia. (a) Representative images of immunostained γ H2AX with p53 binding protein 1 (53BP1) from the DOX-Rec scheme, and (b) its quantification of 53BP1 and γ H2AX double-positive foci of each field in (a) ($n=4$). (c) Quantification of DNA damage foci of each field; green dots and line indicate % 53BP1-positive cells, and red dots and line indicate % γ H2AX-positive cells. (d) Representative images of immunostained γ H2AX with Radiation sensitive protein 51 (RAD51) from the DOX-Rec scheme. (e) NHEJ-/HR-related mRNA level after DOX treatment ($n=5$). The RQ values are the ratios of the respective genes as a percentage of CON. (f) Representative images of immunostained F-actin with phalloidin-488 from the DOX-Rec scheme, and its quantification of nuclear F-actin intensity of each field ($n=3$). (g) Information on publicly available proteomics data. (h) Top 10 ranked GO biological processes (GOBP) in senescent-like CHME-5 cells, and (i) selected statistically significant GOBP and (j) top 15 CORUM protein complexes. (k) Protein–protein interaction analysis using the STRING database. Data information: Scale bar is 50 μ m for (a, d, and f). Graphs are represented as Mean $\pm$ SEM. (****) $p < 0.0001$, (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$, two-tailed unpaired Student's t -test was used to test for significant differences of (e). One-way ANOVA and a subsequent Tukey *post hoc* test were used to test for significant differences of (b, c, and f). Three independent experiments were performed throughout the study.

senescent-like CHME-5 cells (Figure 2J). Protein–protein interaction prediction analysis further indicated that PFN1 may interact with actin polymerization-related proteins, with their neighbors associated with NHEJ and HR (Figure 2K). Based on the STRING protein interaction network, we hypothesized a potential indirect interaction between DNA repair genes and PFN1. Taken together, our findings suggest that DOX-DSBs promote the nuclear translocation of PFN1, accompanied by the up-regulation of several NHEJ genes, but not HR genes in BV2 cells.

3.3 | Impaired Nuclear Shuttling of PFN1 in DNA Damage Repair System

To confirm whether nuclear PFN1 plays a role in DSB repair, we used murine *Pfn1* siRNAs to suppress its expression, which effectively reduced both *Pfn1* mRNA and protein levels (Figure S4A,B). As expected, we observed decreased F-actin expression following *Pfn1* knockdown (Figure S4C). *Pfn1*-silenced BV2 cells showed increased DNA damage and a suppressed DNA damage repair, implying that the absence of PFN1 hinders the DNA repair process (Figure 3A).

Our finding indicates that the reduction of PFN1 in BV2 cells leads to disruption of DSB repair. This highlights the significant role of PFN1 in managing nuclear actin dynamics through its nuclear presence for efficient DSB repair in microglia. To explore this further, we produced PFN1 constructs tagged with either a nuclear localization signal (NLS) for nuclear translocation or a nuclear export signal (NES) for cytoplasmic retention (Figure 3B). We confirmed the precise control over PFN1 localization in BV2 cells transfected with these constructs, which include 3x NLS and NES tags fused to green fluorescent protein (GFP) to directly indicate PFN1's subcellular location (Figure 3C). After transfecting these constructs into BV2 cells for 3 days, DNA damage was induced, followed by a 6 h recovery period (Figure 3D). BV2 cells transfected with either NES-PFN1 or NLS-PFN1 (refer to NES and NLS, respectively) exhibited a mild increase in DNA damage, likely due to the transfection toxicity itself. Notably, BV2 cells expressing cytoplasm-dominant PFN1 (NES-DOX) showed less efficient repair than BV2 cells expressing nucleus-dominant PFN1 (NLS-DOX) after same recover period (6h), while NLS-DOX repaired DNA damage to levels similar to the control (Figure 3E,F). To minimize the confounding effects of endogenous PFN1, we conducted a simultaneous knockdown of mouse *Pfn1* using siRNA while co-transfecting cells with either NLS- or NES-PFN1 constructs. To

prevent potential off-target interactions between the mouse siRNA and the introduced constructs, all constructs were designed to express recombinant human PFN1 with EGFP. The siPFN1-NES transfected group showed more severe DNA damage, whereas the siPFN1-NLS group showed less DNA damage compared to other groups (Figure 3G). Together, these data suggest that subcellular location of PFN1 is important for supporting DNA damage repair in microglia.

3.4 | Nuclear F-Actin Counteracting the Phagocytic Capacity

Building on our findings that PFN1 remains in the nucleus during DOX-DSBs in microglia, and considering its crucial role in actin dynamics, we hypothesized that the nuclear localization of PFN1 could influence nuclear F-actin polymerization. This polymerization, in turn, may affect microglial phagocytosis during DNA damage repair.

The phagocytic capacity of microglia can be assessed using pHrodo-conjugated zymosan bioparticles that emit red fluorescence when internalized into phagolysosomes. We observed that a reduction in phagocytic capacity at Rec 3h with slight recovery at Rec 6h (Figure 4A,B). This was accompanied by increased nuclear F-actin intensity at Rec 3h, which returned to control levels by Rec 6h (Figure 4A,C). At 12h postrecovery (Rec 12h), both phagocytic function and nuclear F-actin levels returned to levels comparable to the CON (Figure 4A–C). Finally, we confirmed temporally decreased phagocytic capacity during DNA damage recovery using live cell imaging (Figure 4D–F). To evaluate whether the subcellular localization of PFN1 can affect the phagocytic function of microglia, we co-transfected the BV2 cells with mouse siPFN1 and recombinant PFN1 with NES/NLS tags, as previously described. The siPFN1+NLS BV2 cells phagocytosed zymosan particles less frequently than the siPFN1+NES BV2 cells (Figure 4G,H). These suggest that nuclear F-actin formation, possibly driven by PFN1 presence in the nucleus, disturbs microglial phagocytic function during the DNA damage repair process (Figure 4I).

3.5 | Nuclear PFN1 in Low Phagocytic Capacity of Senescent Microglia

Given that DNA damage is a hallmark in senescent cells, we hypothesized that senescent microglia also exhibit increased

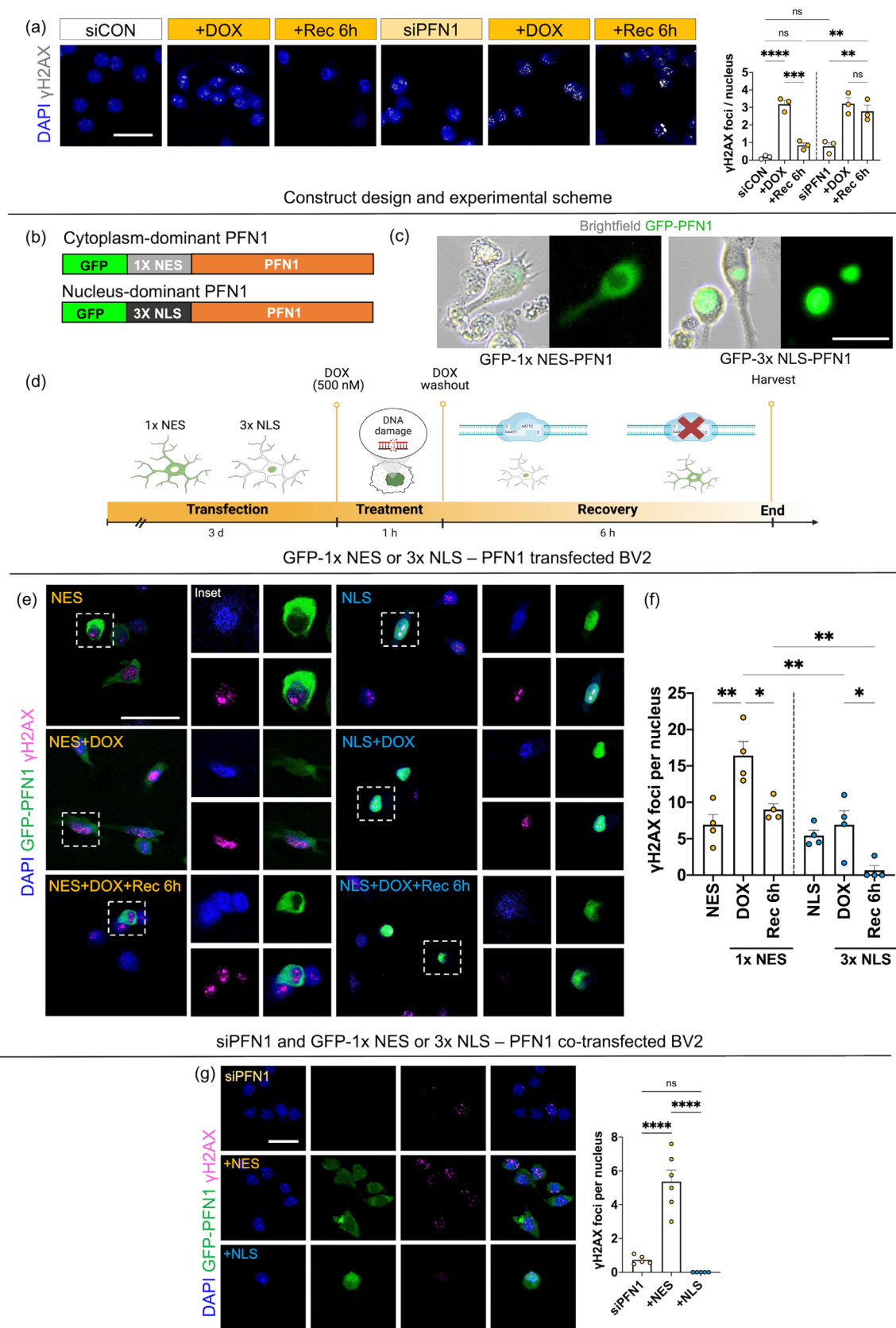


FIGURE 3 | Legend on next page.

retention of PFN1, along with accumulation of persistent DNA damage. To test this hypothesis, we induced DDIS with mouse primary microglia. Briefly, we treated a low dose of DOX

(100 nM) for 3 days (Figure 5A). We confirmed the induction of DDIS phenotype through senescence-associated β -galactosidase (SA β -gal) staining (Figure 5B), along with increased expression

FIGURE 3 | Blocking PFN1 nuclear shuttling impairs DNA damage repair of microglia. (a) Representative images of immunostained γ H2AX from siCON, siPFN1 following DNA damage recovery (6h) and its quantification of γ H2AX foci per nucleus of each field in D ($n=3$). (b) Schematic depicting the construct design utilized in this study. (c) Representative images of green fluorescence protein (GFP) - nuclear export signal (1 $\times$ NES) - PFN1, GFP-nuclear localization signal (3 $\times$ NLS) - PFN1 transfection. (d) Schematic depicting the transfection, DNA damage recovery scheme utilized in this study. (e) Representative images of immunostained γ H2AX from NES-/NLS-PFN1 transfection. White dotted line indicates cropped and magnified cell, and (f) its quantification of γ H2AX foci per nucleus of each field in e ($n=4$). (g) Representative images of immunostained γ H2AX from simultaneously NES-/NLS-PFN1 overexpression and endogenous PFN1 knockdown in BV2, and its quantification of γ H2AX foci per nucleus of each field in F ($n=5$). Data information: Scale bar is 50 μ m for (a, c, e, and f). Graphs are represented as Mean $\pm$ SEM. (****) $p < 0.0001$, (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$, One-way ANOVA and a subsequent Tukey *post hoc* test were used to test for significant differences of (a, f, and g). Schematic illustration (b, d) is created with [Biorender.com](https://biorender.com). Three independent experiments were performed throughout the study.

of p21 and p53, which are markers elevated in response to persistent DNA damage (Figure 5C). However, p16 or proinflammatory cytokines were not increased (Figure 5C). Finally, we observed elevated γ H2AX foci in DDIS-MG (Figure 5D). DDIS-MG with senescent characteristics exhibited nuclear retention of PFN1 (Figure 5E,F) and showed decreased phagocytosis (Figure 5G), consistent with the findings in previous results using BV2 cells (Figures 1–4). The accumulation of DNA damage and the increase in SA β -gal activity remained unchanged even after a 3-day DOX washout following DDIS (Figure 5S,A,B). Consistent with these findings, nuclear PFN1 accumulation in DNA-damaged microglia also persisted after the 3-day DOX washout following DDIS (Figure 5S,C).

We postulated that the DNA damage accumulation in microglia triggers the translocation of PFN1 into the nucleus, where it may facilitate F-actin polymerization to support the DNA repair process, as suggested by our data and previous reports. This process could hinder the return of PFN1-actin complexes to the cytoplasm (Stuven et al. 2003; Wang et al. 2021). Firstly, we investigated whether the nuclear retention of PFN1 in DDIS-MG was associated with alterations in the expression of exportin-6 (Xpo6), the primary transporter of PFN1-actin complexes. Our findings revealed that Xpo6 expression was reduced in DDIS-MG (Figure 5H). Collectively, senescent microglia with DNA damage accumulation showed impaired phagocytosis, which was associated with the nuclear retention of PFN1.

To compare our functional analysis of DNA damage-induced senescent microglia and the transcriptome of in vivo senescent microglia, we reanalyzed publicly available single-cell RNA sequencing dataset from (Zhang et al. 2022), with the Seurat V5 standard pipeline. This dataset showed stable expression of microglia-specific markers in the myeloid cell population. Using this dataset, we identified microglia within the myeloid clusters using the microglia-specific marker Tmem119 (Figure 5S6A,B). Microglia from aged mice showed alterations in DNA damage response, lipid metabolism pathway, innate immune response, and regulation of cellular senescence (Figure 5S6C,D). To investigate the role of senescent microglia in the aged brain, we identified microglia sub-clusters and discovered three distinct microglial populations (Figure 5S6E). In the aged brain, p16ink4a/CDKN2A expressing microglia are more prevalent compared to the young brain (Figure 5S6F), despite the difficulty in detecting the sequencing read. To identify the senescence phenotype microglia cluster, we examined the expression patterns of SASP-related genes. Cluster 0 microglia were identified as the

senescent phenotype, showing elevated expression of p16ink4a/CDKN2A and increased SASPs compared to clusters 1 and 2 (Figure 5S6G). Further pathway analysis revealed that actin cytoskeleton, phagosome, and DNA repair-related pathways were altered in the senescence phenotype microglia cluster compared to nonsenescent microglia (Figure 5S7A–C). Finally, we analyzed phagocytosis-related gene expression patterns using gene sets from the GO ontology, including GO:0006911 (phagocytosis, engulfment) and GO:0030041 (actin filament polymerization). Our analysis revealed significantly altered expression of actin polymerization and phagocytosis-related genes in microglia cluster 0, which we identified as the senescence phenotype microglia (Figure 5S7D,E). Together, the publicly available scRNA-seq dataset of the aged mouse brain confirms that senescent phenotype microglia exhibit impaired phagocytosis, altered actin dynamics, and DNA damage accumulation.

3.6 | Recapitulation in In Vivo and Ex Vivo Aged Microglia

A recently published paper suggested that the percentage of senescent microglia is increased in aged mouse brain and AD mouse models, with γ H2AX expression (Rachmian et al. 2024). These microglia showed increased γ H2AX expression along with Cdkn2a/p16^{Ink4a} expression in the Cy-TOF experiment (Figure 5S8). This published data suggests that DNA damage-accumulated senescent microglia in the aged brain may contribute to brain aging or pathological mechanisms. First, we investigated the DNA damage accumulation in the aged mouse brain and found an increase in γ H2AX-incorporating microglia compared to the young mouse brain (Figure 6A,B). Dystrophic morphology and fragmentation of microglial processes have been proposed as in vivo hallmarks of microglial senescence (Streit 2006; Streit et al. 2004; Tischer et al. 2016). Therefore, we examined microglial morphology in the brains of young (10-week-old) and aged (>80-week-old) mice. An increased number of dystrophic microglia was observed in aged mice (Figure 6C,D). Subsequent FracLac analysis revealed reduced fractal dimensions and increased lacunarity, indicating fragmented microglial processes (Figure 6E).

Next, we isolated microglia from aged mice (90-week-old; Aged microglia) using MACS to assess the DNA damage accumulation and the PFN1 distribution ex vivo (Figure 6F). Finally, we confirmed that aged microglia indeed exhibited DNA damage accumulation and nuclear retention of PFN1 (Figure 6G,H).

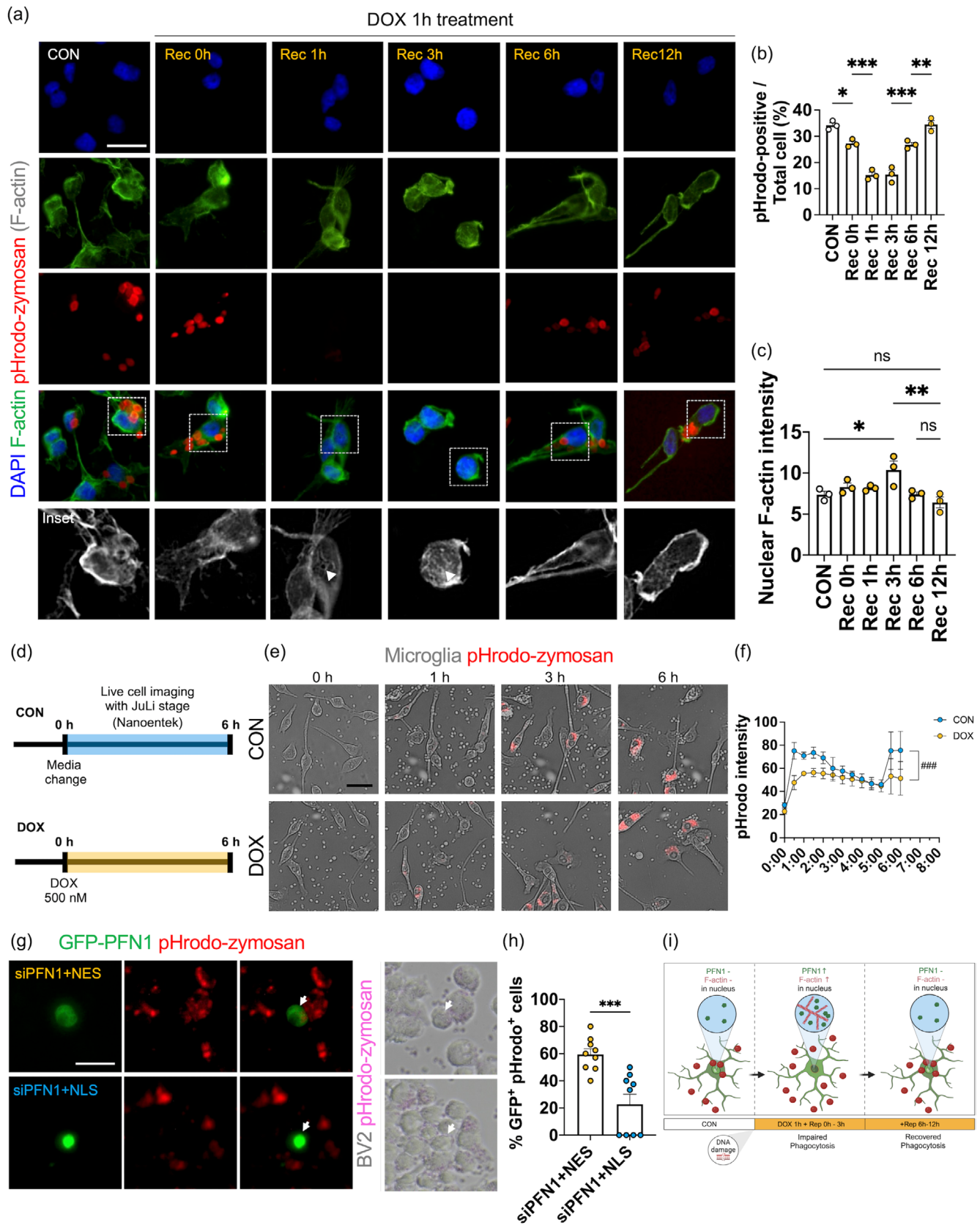


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To investigate the link between impaired phagocytosis and actin polymerization in aged brain microglia, we used a published scRNA-seq dataset [GSE129788], which sequenced whole brain

cells of 8 young mice and 8 aged mice. We identified 38 cell clusters as authors already reported (Figure S9A). Sequentially, we compared microglia-specific marker expression through Cx3cr1,

FIGURE 4 | Nuclear F-Actin in phagocytic dysfunction in acute DNA damage. (a) Representative images of microglial phagocytosis during DNA damage recovery (Rec 0 h, 1 h, 3 h, 6 h, 12 h). (b) Flow cytometric analysis of microglia phagocytosis. Intensity of pHrodo-zymosan indicates the relative amounts of beads, which are engulfed and transported to the lysosome (low pH). (c) Quantification of nuclear F-actin intensity of each field in a ($n = 3$). (d) The schematic illustration of live cell imaging of DOX-REP phagocytosis. (e) The representative time course image of microglia phagocytosis, and (f) its pHrodo-intensity quantification. (g) Representative images of phagocytosis of siPFN1 and NES-/NLS-PFN1-EGFP co-transfected BV2 cells, and (h) its quantification of the percentage of GFP⁺ and pHrodo-zymosan⁺ cells of each field in F (9 fields, three independent experiments). (i) The schematic illustration for phagocytosis regulation through PFN1 and F-actin during acute DNA damage. Data information: Scale bar is 50 μ m for (a, e, and g). Graphs are represented as Mean $\pm$ SEM, (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$ for One-way ANOVA (b, c) with Tukey's test and unpaired t -test (h), and (###) $p < 0.001$ for two-way ANOVA (f). Schematic illustration (d, i) is created with [Biorender.com](https://biorender.com).

C1qa, Tmem119, and P2ry12 among the clusters (Figure S9B). Clusters 3 and 8 exhibited high expression of microglia-specific markers (Figure S9C). We also found an increase in p16Ink4a/CDKN2A-expressing microglia in aged mice compared to young mice (Figure S9D). Furthermore, the expression of SASPs was increased in aged microglia (Figure S9E). As a result, pathway analysis revealed significant alterations in innate immune-related pathways, cholesterol metabolism, actin cytoskeleton, and phagosome pathways in aged microglia (Figure S10A–C). In parallel with pathway analysis, gene expression related to actin and phagocytosis was examined using gene sets from Gene ontology. Most of the annotated genes associated with phagocytosis and actin filament polymerization were significantly altered in aged microglia, but not in young microglia as shown in Figure S10D,E. In summary, our analysis of publicly available scRNA-seq datasets revealed that aged and senescent microglia undergo significant alterations in pathways related to phagocytosis and actin polymerization. These findings suggest that the accumulation of DNA damage in microglia within the aging brain may impair phagocytic function with senescent characteristics, likely due to the dysregulation of nuclear actin polymerization and the retention of PFN1 in the nucleus.

3.7 | Actin Depolymerization Is Not Enough to Rescue Nuclear Retention of PFN1

Given that the primary function of PFN1 is to facilitate F-actin formation, its actin binding affinity is essential for nucleocytoplasmic transport of both PFN1 and actin. This suggests that impaired actin dynamics may contribute to the retention of PFN1 in the nucleus. To investigate this hypothesis, we utilized cytochalasin D (CytD), a drug known to induce actin depolymerization.

We found that DDIS-MG showed increased nuclear F-actin intensity, which was recovered to control levels following CytD treatment (Figure 7A). Unexpectedly, PFN1 does not respond to CytD, which means that actin depolymerization is not enough to rescue nuclear retention of PFN1 (Figure 7B). Next, we hypothesized that PFN1 may co-localize with DNA damage foci to facilitate the repair of long-term DNA damage. To test this, we performed detergent pre-extraction using CSK buffer (Figure 7C). Notably, we observed the nuclear insoluble PFN1 in DDIS-MG following detergent pre-extraction. This was confirmed through orthogonal views of z-stacked images (Figure 7D) and maximum intensity projections of z-stacked images (Figure 7E). Our data demonstrate that, in the context of long-term DNA damage, microglia exhibit nuclear PFN1 foci; however, these foci do not co-localize with DNA damage foci (Figure 7D,E). Additionally, the number of

nuclear insoluble PFN1-positive microglia was significantly increased in DDIS-MG compared to CON-MG (Figure 7E). These findings suggest that nuclear insoluble PFN1 in DNA damage-induced senescent microglia and this aggregation is independent of F-actin polymerization and depolymerization.

4 | Discussion

In this study, we report that nuclear PFN1 supports DNA damage repair; however, its failure to return to the cytoplasm is associated with phagocytic dysfunction in DDIS-MG. In addition, we identified nuclear PFN1 retention along with DNA damage accumulation in DDIS-MG, which appears recovered due to its aggregation. These results were recapitulated in in vivo aged brain, ex vivo aged microglia, and publicly available single-cell RNA sequencing dataset.

Microglia, as dynamic innate immune cells, maintain CNS homeostasis through immune surveillance, migration, and phagocytosis, all of which are commonly regulated by actin dynamics (Abd-el-Basset and Fedoroff 1994; Hickman et al. 2018; Uhlemann et al. 2016). Microglial phagocytic dysfunction in clearing toxic protein aggregates is associated with neurodegenerative diseases such as Alzheimer's disease (AD), ALS, and Parkinson's disease (PD) (Lepiarz-Raba et al. 2023; Noh et al. 2023; Tremblay et al. 2019). Moreover, it has been reported that the phagocytic capacity of microglia decreases with brain aging (Deczkowska et al. 2018). Recently FDA-approved therapeutics for targeting amyloid- β in AD, such as aducanumab and lecanemab, have the mechanism to support microglial phagocytosis (Sevigny et al. 2016). Senescent microglia are a common feature in aging-related neurodegenerative diseases and their prevalence increases with brain aging (Angelova and Brown 2019; Shahidehpour et al. 2021; Streit et al. 2009). It has been well known that DNA damage and subsequent accumulation of senescent cells can lead to low-level chronic neuroinflammation in the brain (Admasu et al. 2021). However, the effects of DNA damage accumulation on microglial phagocytosis remain unknown.

Our study aimed to address three important questions regarding the characteristics of DNA-damaged microglia in brain aging and neurodegeneration:

1. How do microglia respond to DNA damage? A previous report suggests that DNA damage-induced senescence-like CHME-5 cell lines exhibited alterations in PFN1 protein complex, actin regulation at the proteome level (Marques et al. 2020). It supports our results that nuclear

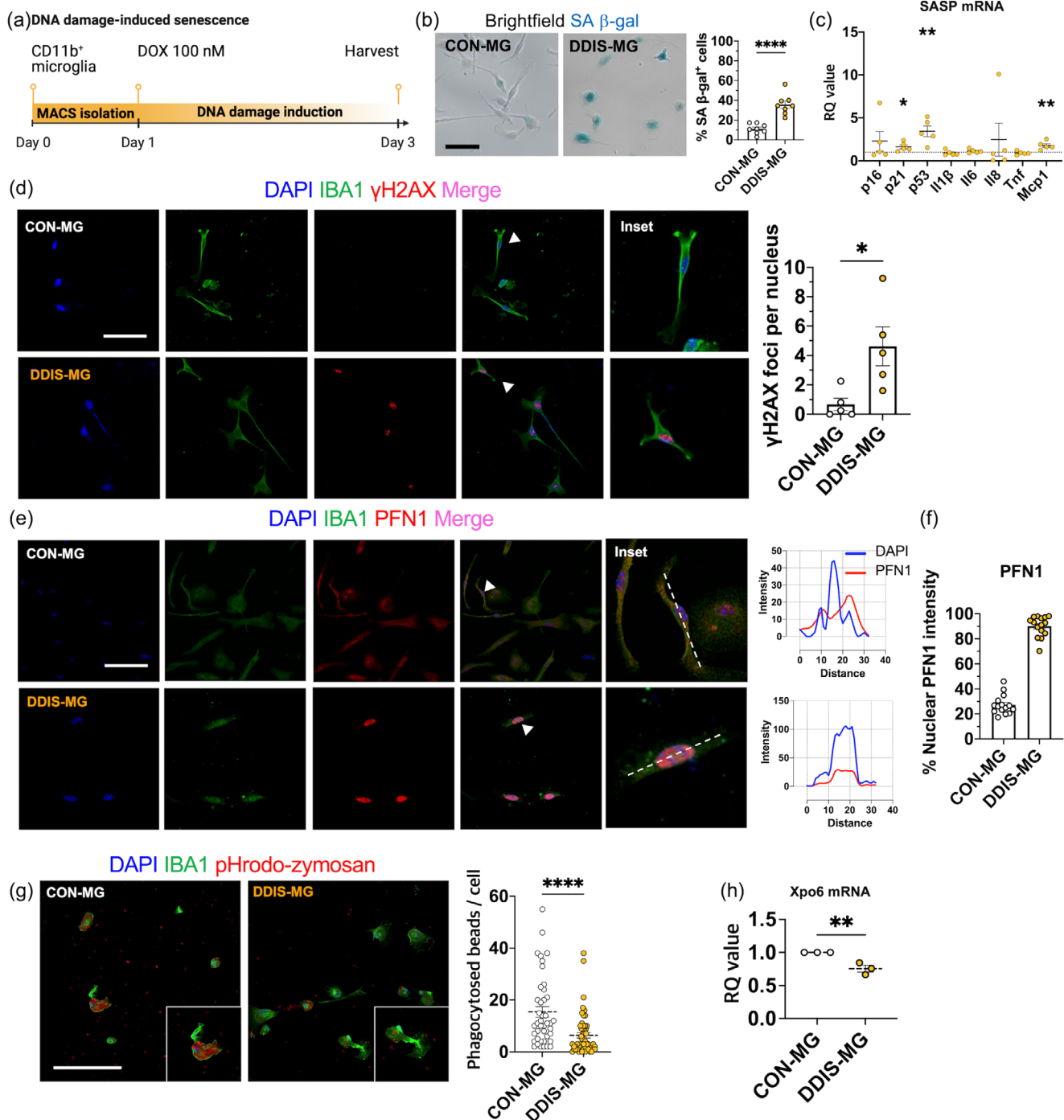


FIGURE 5 | Profilin-1 nuclear retention and impaired phagocytosis of DNA damage-induced senescent microglia. (a) Schematic illustration of DNA damage-induced senescent microglia experiment. (b) Representative images of SA β-gal staining from control microglia (DIV3; CON-MG) and DNA damage-induced senescent microglia (DDIS-MG). Quantification of SA β-gal positive cells of each field in B. CON-MG and DDIS-MG ($n=8$). (c) the senescence-associated secretory phenotype (SASP) mRNA expression of DDIS-MG. The RQ values are the ratio of the respective genes as a percentage of CON-MG. (d) Representative images of immunostained γH2AX from CON-MG and DDIS-MG, and its quantification of the percentage of γH2AX foci of each field in D ($n=5$). (e) Representative images of immunostained PFN1 from DDIS-MG, and intensity plot of the representative cell in the field. (f) The quantification of nuclear PFN1 intensity in DDIS-MG compared to CON-MG ($n=15$). (g) Representative images of phagocytosis of CON-MG and DDIS-MG, and its quantification of phagocytosed beads per cell. (h) The exportin-6 mRNA expression of DDIS-MG. Data information: Scale bar is 50 μm for (b, d, e, and g). Graphs are represented as Mean ± SEM, (****) $p < 0.0001$, (**) $p < 0.01$, (*) $p < 0.05$; a two-tailed unpaired t -test was used to test for significant differences of (b–d, f–h). Schematic illustration (A) is created with [Biorender.com](https://biorender.com). Three independent experiments were performed throughout the study.

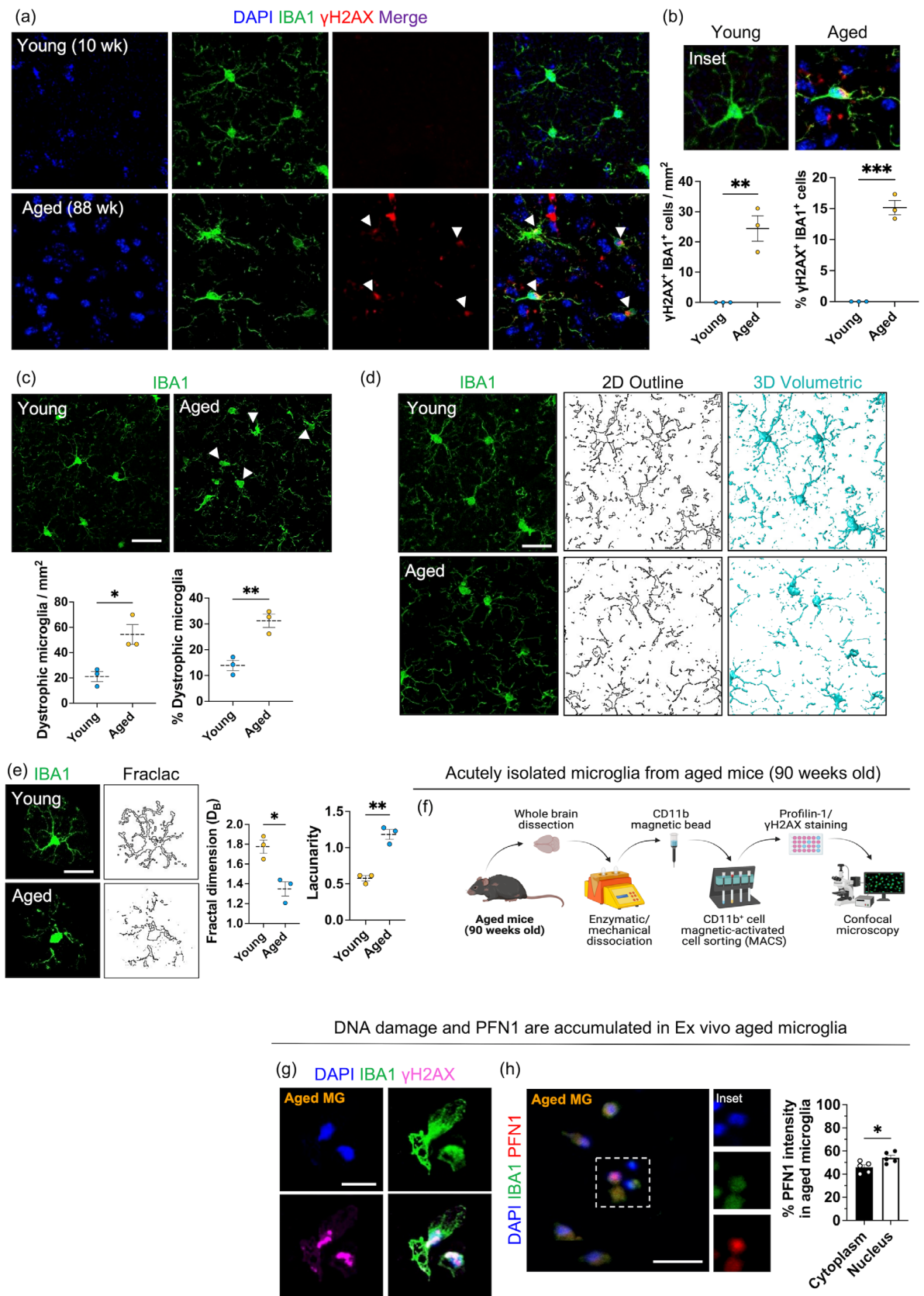


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translocation of PFN1 in the microglia, particularly in the DNA damage-induced senescence manner. We also found that nuclear PFN1 facilitates the repair of DOX-induced

DNA damage via NHEJ. F-actin polymerization in the nucleus is implicated in both HR and NHEJ (Peric-Hupkes et al. 2010). Within the nucleus, actin monomers polymerize

FIGURE 6 | DNA-damaged microglia with fragmented processes in vivo and ex vivo. (a) Representative images of γ H2AX-positive microglia in vivo, and (b) its magnified inset and quantification (young and aged, $n = 3$, respectively). (c) The representative images of IBA1-positive microglia and quantification of the percentage of dystrophic microglia (5 field of view were acquired across 3 mice of each group). (d) Image J and MATLAB processed images of microglia of young and aged mice. 2D outlines were acquired with Image J, and 3D volumetric reconstruction was performed with z-stacked images using MATLAB code. (e) Representative images of FracLac analysis of young and aged microglia and its quantifications. (f) Schematic illustration of the method for obtaining Ex vivo microglia from aged mice. (g) Representative images of immunostained γ H2AX from acutely isolated microglia from aged mice (90-week-old: Aged MG). (h) Representative images of immunostained PFN1 from acutely isolated microglia from 90-week-old mice, counterstained for DAPI. The white dotted line indicates cropped and magnified cells. Quantification of the percentage of nuclear PFN1 intensity. Data information: Scale bar is $50\mu\text{m}$ for (a–d, g–h), $25\mu\text{m}$ for (e). Graphs are represented as Mean $\pm$ SEM, (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$; two-tailed unpaired t -test was used to test for significant differences.

into F-actin, anchoring chromatin with DNA lesions and unwinding it to provide sufficient space for DNA damage repair machinery to bind, thereby facilitating the repair of DSBs (Caridi et al. 2019; Kyheroinen and Vartiainen 2020). Despite the limitation of lacking direct evidence that PFN1 directly regulates NHEJ machinery, our results demonstrate that NHEJ is the primary mechanism for early DNA damage repair in microglia, particularly in the context of DOX-DSBs. It is reasonable to propose that microglia utilize NHEJ to repair DNA damage, as this pathway functions throughout all phases of the cell cycle, whereas HR is dependent on cell cycle progression (Lieber 2008).

2. Is DNA damage relevant to impaired phagocytosis in senescent microglia? In our investigation, nuclear PFN1 translocation during acute DNA damage was associated with temporary phagocytic dysfunction. Given the critical role of the DNA damage repair mechanism in the normal immune response of innate immune cells, such as macrophages (Pereira-Lopes et al. 2015), the transient reduction in phagocytic function during DNA damage repair in microglia can be considered a momentary compromise, crucial for the restoration of homeostasis and functional activity. In DDIS-MG, nuclear PFN1 appears incapable of returning to the cytoplasm with its aggregation and increased nuclear F-actin. Considering the critical roles of PFN1 and F-actin dynamics in regulating phagocytosis (Funes et al. 2024), we propose that subcellular imbalances in PFN1 and F-actin contribute to decreased phagocytic capacity observed during DNA damage repair and in the context of chronic DNA damage accumulation. Another possible explanation for impaired phagocytosis in DDIS-MG involves the role of actin dynamics. Genome-wide CRISPR screening has demonstrated that actin polymerization and filopodia formation are crucial for regulating phagocytosis (Haney et al. 2018). Since PFN1 is the primary regulator of actin polymerization, its mislocalized distribution may disrupt phagocytosis by altering the balance of the actin cytoskeleton. In addition, a recent study reported elevated F-actin levels in the brains of aged *drosophila* (Schmid et al. 2024), which potentially leads to autophagy and mitochondrial metabolism dysregulation. Given that both actin polymerization, DNA repair, and nucleocytoplasmic transport are all energy-intensive processes (Kitabatake et al. 2020; McCullagh et al. 2014), it is plausible that PFN1 nuclear translocation and subsequent increase in nuclear F-actin following DNA damage

may impair phagocytic function in an energy-dependent manner in the aged brain.

3. Why nuclear PFN1 fails to return to cytoplasm in the DNA damage-induced senescence and aged microglia? First, the reduction of exportin-6 (XPO6), a key player in the nucleocytoplasmic transport (NCT) of PFN1 (Stuven et al. 2003), may impede its translocation to the cytoplasm. However, although it is conceivable that low-molecular-weight PFN1 might translocate independently of XPO6 (Witke 2004), the reduction in mRNA levels alone does not fully account for the accumulation of PFN1 in DDIS-MG. Second, we propose that persistent DNA damage may increase F-actin formation in the nucleus and hinder NCT, as evidenced by the inability of actin-depolymerizing agents to facilitate the re-translocation of nuclear PFN1 to the cytoplasm. This suggests that the failure of PFN1 re-localization may be linked not only to nuclear F-actin polymerization but also to additional underlying mechanisms. Lastly, we investigated the potential role of aberrant nuclear PFN1 aggregate formation. Our data confirmed the presence of PFN1 aggregates in DDIS-MG, consistent with a previous study conducted in DNA-damaged HeLa cells (Zhu et al. 2020). Since the detergent extraction removes soluble components, these findings suggest that nuclear PFN1 aggregate formation represents a significant barrier to its trafficking back to the cytoplasm. However, further investigations are necessary to validate our hypothesis, potentially exploring interventions that either prevent or reverse PFN1 aggregate formation.

To extend our hypothesis to the aged brain, we observed increased DNA-damaged microglia in aged mice (90-week-old). Additionally, aged microglia isolated from these aged mice demonstrated nuclear retention of PFN1, accompanied by an accumulation of DNA damage. Therefore, the persistent nuclear localization of PFN1 may be attributed to irreparable DNA damage, consistent with observations in DDIS-MG. Furthermore, we identified that aged microglia and Cdkn2a/P16^{Ink4a}-positive senescent microglia exhibited altered phagosome, actin polymerization, and DNA repair-related transcriptome alterations through analyzing publicly available scRNA seq datasets.

Our findings suggest that mutant PFN1 not only disrupts the axonal transport system but also interferes with DNA damage repair in the pathophysiology of ALS (Hardiman et al. 2017). Several PFN1 mutations, such as G118V, M114T, and C71G, have been

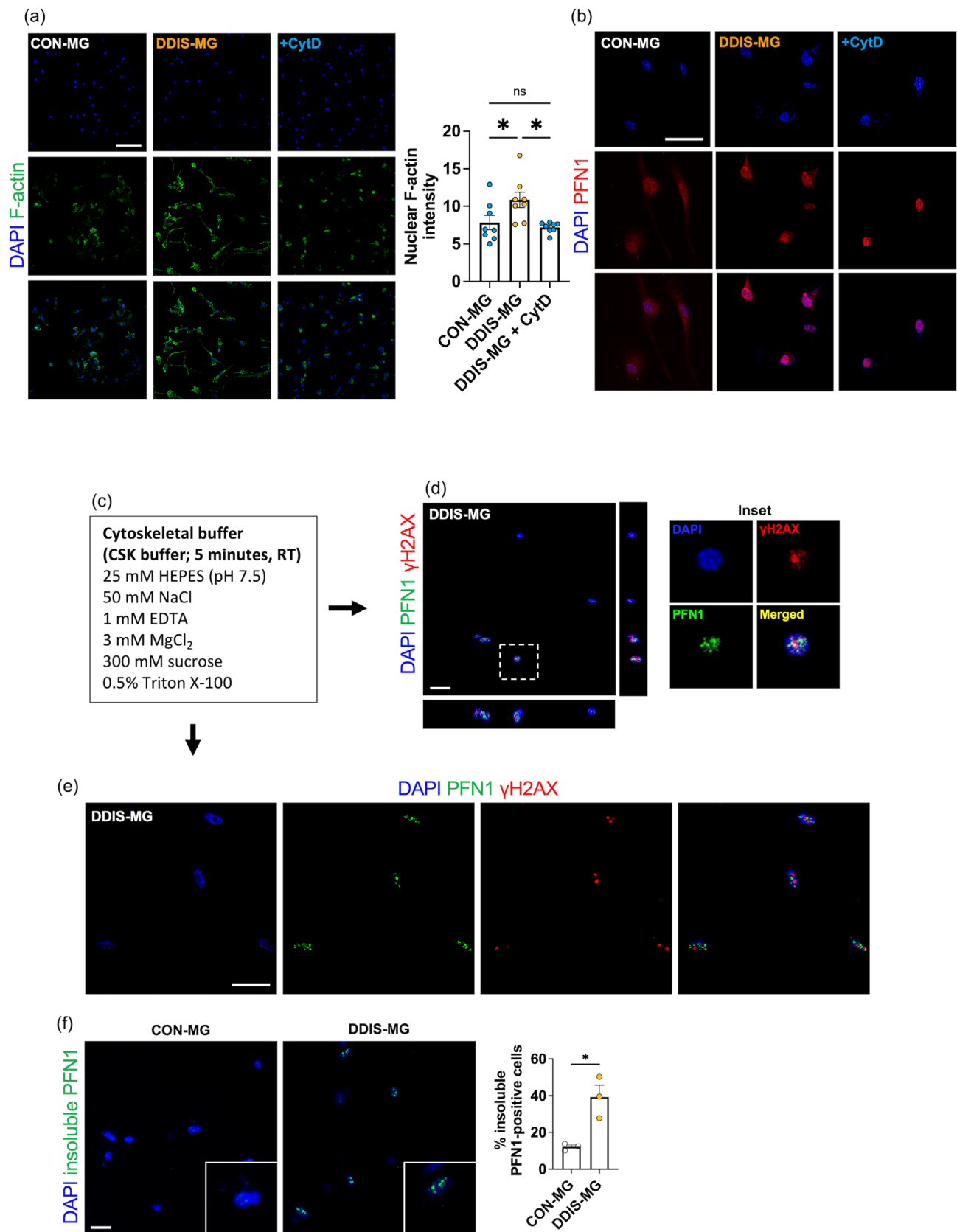


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reported to contribute to ALS pathogenesis, leading to the disruption of the axonal transport system (Wu et al. 2012). The ALS-linked PFN1 disrupts actin dynamics, and the mechanism behind

this perturbation appears to be influenced by the thermodynamic stability of the mutant protein (Schmidt et al. 2021). Moreover, the ALS-associated mutant PFN1 forms cytoplasmic aggregates

FIGURE 7 | Actin depolymerization does not recover PFN1 localization due to PFN1 aggregate formation. (a) Representative images of F-actin staining of CON-MG, DDIS-MG, and DDIS-MG with CytD treatment and its quantification of nuclear F-actin intensity. ($n = 8$). (b) Representative images of PFN1 staining of CON-MG, DDIS-MG, and DDIS-MG with CytD treatment. (c) Composition and treatment condition of detergent pre-extraction. (d) Representative images of detergent-insoluble PFN1 foci with DNA damage foci after detergent pre-extraction with orthogonal view. (e) Maximum intensity projected images of nuclear insoluble PFN1 in DDIS-MG. (f) Representative images of detergent-insoluble PFN1 of CON-MG and DDIS-MG, and its quantification. Data information: Scale bar is 25 μm for (a), 50 μm for (b, d–f). Graphs are represented as Mean $\pm$ SEM, (*) $p < 0.05$, One-way ANOVA with Tukey's test for (a), and two-tailed unpaired t-test was used to test for significant differences in (f).

positive for ubiquitin and p62, and these aggregates sequester endogenous TDP-43 (Tanaka et al. 2016). These cytosolic aggregates of PFN1 can impede DNA damage repair by hindering nuclear localization as well as nuclear actin polymerization. Several researchers reported that induced microglia-like cells from ALS patients derived from peripheral blood mononuclear cells showed DNA damage accumulation with phagocytic dysfunction (Quek et al. 2022) and actin-related phagocytic dysfunction (Noh et al. 2023). Thus, our results support that microglia with mutant PFN1 in ALS might have accumulation of DNA damage and phagocytic dysfunction, showing a senescent phenotype.

This study has limitations. First, BV2 cells have been reported to exhibit a transcriptomic profile distinct from that of mature microglia (You et al. 2021), which highlights their limitations as a model for studying microglial biology. However, BV2 cells have their practical advantages. Their robust proliferative capacity allows for the generation of multiple experimental groups and technical replicates, and they tolerate procedures such as transfection and repeated media changes well (Flury et al. 2025). Additionally, BV2 cells retain key microglial functions, including phagocytosis, migration, and immune responsiveness. Recent studies have also shown that senescent BV2 cells exhibit transcriptomic profiles similar to those of aged primary microglia from mouse and human brains (Skandik et al. 2025). Nevertheless, to address the advantages and limitations of BV2 cells, we validated the reproducibility of our findings through both in vivo and ex vivo experiments. Second, due to the lack of direct evidence linking the pathophysiology of senescent microglia in human brains with neurodegenerative diseases such as ALS and AD, the role of DNA-damaged microglia in the aged human brain requires further investigation. This is particularly important given that our study was conducted using mouse microglia. Despite these limitations, we conclude by reporting the potential role of nuclear PFN1 in DSB repair in microglia and how its failure to return to the cytoplasm impairs phagocytic function in DDIS-MG. Following persistent DNA damage accumulation, PFN1 undergoes nuclear retention and forms detergent-insoluble aggregates. Thus, our findings contribute to the understanding of phagocytic impairment in senescent microglia, with a focus on the DNA damage response involving PFN1, highlighting its role in PFN1-associated pathophysiology.

Author Contributions

Chan Rim: writing – concept, molecular work, original draft. **Soyoung Sung, Hui-Ju Kim:** molecular work, cell analysis, blind image data analysis; **Seung Hyun Kim:** conceptualization, supervision; **Minyeop Nahm** and **Min-Soo Kwon:** conceptualization, supervision, writing – review and editing. All authors reviewed and/or edited the manuscript before submission.

Acknowledgments

The authors have nothing to report.

Ethics Statement

The authors have nothing to report.

Conflicts of Interest

Min-Soo Kwon is a founder of Brainimmunex Inc. This role is outside the present work. All other authors declare no conflicts of interest.

Data Availability Statement

Two different young and old mouse brain scRNA-seq data (Ximerakis et al. 2019; Zhang et al. 2022) are publicly available at GEO under record GSE129788 and GSE178957. As specified in the method section, the Seurat pipeline (v5.1.0) (Stuart et al. 2019) was used for scRNA-seq analysis. The used code included in this article will be provided by the corresponding author upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.