Research article

Doxorubicin generates senescent microglia that exhibit altered proteomes, higher levels of cytokine secretion, and a decreased ability to internalize amyloid β

The corrections made in this section will be reviewed and approved by a journal production editor.

Luís Marques^a, Adiv A. Johnson^b, Alexandra Stolzing^{a,*} stolzing@gmail.com

^aSchool of Mechanical, Electrical and Manufacturing Engineering, Loughborough University, Loughborough, United Kingdom

^bNikon Instruments, Melville, NY, USA

*Corresponding author.

Abstract

Cellular senescence is defined by irreversible cell-cycle arrest and is an evolutionarily conserved hallmark of aging. In this study, we generate senescent microglial cells via exposure to the chemotherapy drug doxorubicin. Compared to control cells, doxorubicin-treated microglia exhibited an altered morphology characterized by an enlarged cell size, a flattened appearance, and the development of prominent filaments. Senescent cells harbored elevated levels of senescence associated- β -galactosidase, p16^{Ink4a}, and γ -H2AX. Senescent microglia were also less efficient at internalizing amyloid β and pHrodo bioparticles. A detailed proteomic analysis using SWATH-MS identified 201 proteins that were significantly downregulated and 127 that were significantly upregulated in doxorubicin-treated microglia. Proteins involved in processes such as protein synthesis, RNA damage and repair, and protein degradation were largely downregulated while those compromising the integrity of the cell were predominantly upregulated. Various proteins involved in proteasomal processing were among the most significantly downregulated in senescent cells. Relevant to the deleterious senescence-associated secretory phenotype, senescent cells secreted higher levels of the inflammatory cytokines IL-6, IL-8, TNF- α , and GRO- α . Our data suggest that symptoms of brain aging and age-related neurodegenerative disease may be partially caused by defective phagocytosis, impaired proteasomal processing, and elevated cytokine secretion of senescent microglia.

Keywords: Senescence; Microglia; Alzheimer's disease; Inflammation; Aging; Proteomics

1 Introduction

An ever-growing body of data has demonstrated that cellular senescence, characterized by permanent cell-cycle arrest, is an evolutionarily conserved hallmark of aging [1]. Seminal work from the Jan van Deursen laboratory has shown that drug-inducible clearance of p16^{Ink4a}-positive senescent cells in BubR1 progeroid mice delays the onset of age-related pathologies as well as the progression of established age-related disorders [2]. Follow-up work demonstrated that the clearance of p16^{Ink4a}-positive senescent cells extends median lifespan, delays tumorigenesis, and attenuates age-related organ deterioration in wild-type mice [3]. Analogous research from the laboratory of James Kirkland has found that the clearance of senescent cells either via senolytic agents or the activation of drug-inducible suicide genes enhances insulin sensitivity, improves glucose tolerance, and promotes adipogenesis in obese mice [4]. Strategically removing senescent cells can also prevent age-related bone loss [5] and improve cognitive performance in mice subjected to whole brain irradiation [6]. Conversely, transplanting a small number of senescent cells into older mice shortens both lifespan and healthspan [7]. Moreover, cellular senescence becomes more prevalent with age in human tissues [8,9]. The ability of senescent cells to impact aging has led to the proposal that senescence is a viable target for clinical rejuvenation [10].

While senescent cells can mediate beneficial effects – such as suppressing tumors, maintaining tissue homeostasis, and regulating development [11] – these healthspan and lifespan studies all demonstrate that cellular senescence contributes to aging. Senescent cells generate pro-aging, deleterious effects via the senescence-associated secretory phenotype (SASP) [11]. The SASP is characterized by altered gene expression as well as changes in secreted factors, including the proinflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α). The SASP alters the tissue microenvironment and promotes chronic inflammation [11], which itself occurs pathologically in various regions of the Alzheimer's disease brain [12]. A growing body of data also indicates that the immune system becomes progressively dysregulated with age [13,14]. Senescent cells can additionally secrete various proteins and these secretions are linked to functional effects. For example, senescent fibroblasts secrete proteins related to hemostasis and clearing senescent cells in mice decreases blood clotting [15].

More recently, neurodegeneration has been explicitly linked to senescence in neuronal cells, including glial cells [16]. Bussian et al showed that p16^{Ink4a}-positive senescent astrocytes and microglia accumulate in the MAPT^{P301S}PS19 tau-dependent mouse model of neurodegenerative disease. Clearing these deleterious cells preserves cognitive function by preventing gliosis, tau hyperphosphorylation, and the degeneration of hippocampal neurons. Pharmacological treatment with the senolytic ABT263 (navitoclax) prevented the upregulation of genes associated with senescence and attenuated tau phosphorylation [17]. More broadly, senescent glial cells have been implicated in both Alzheimer's disease and Parkinson's disease [18] and the activity of glial cells can affect organismal stress resistance and longevity [19]. While there are different types of glial cells, microglia are critically important macrophages that play paramount roles in inflammation, neurodegenerative disease, and brain homeostasis [20]. Microglial cells also have a very slow turnover and long lifespans [21], meaning they are theoretically quite susceptible to the effects of senescence.

To better understand senescence, aging, and neurodegeneration, we utilized rat CHME-5 microglial cells to study and characterize microglial senescence. Although initially thought to be of human origin, these cells were recently shown to be derived from rats and are a useful model of neuroinflammation [22,23]. We demonstrate that senescence can be induced in microglia via exposure to the anti-cancer drug doxorubicin (DOX) and that senescent cells exhibit classical markers of permanent cell cycle arrest. The drug DOX was chosen because of its ability to induce senescence in various cell types [15,24,25]. We additionally show that, compared to non-

senescent cells, senescent microglial cells have an enlarged cell body, exhibit an impaired ability to internalize amyloid β , harbor significantly altered proteomes, and secrete higher levels of inflammatory cytokines.

2 Materials and methods

2.1 CHME-5 cell culture

Immortalized rat microglial CHME-5 cells (procured from Dr. Yarua Jaimes) were cultured in DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific) in a humidified atmosphere at 37°C and in air containing 5% CO₂. When cells reached 70–80% confluence, they were trypsinized by incubation with TripLE (Thermo Fisher Scientific) for 5 min and then inactivated by the addition of fresh growth medium equivalent to three times the volume of the trypsin solution used for cell detachment. After centrifuging the cell suspension at 200×g's for 5 min at room temperature, the supernatant was discarded, and the remaining pellet resuspended in an appropriate volume of culture medium. Cell concentration and viable cells were counted using a NucleoCounter NC-3000 automated mammalian cell counter (Chemometec, Allerød, Denmark) and an appropriate number of cells were then reseeded into a fresh T-flask. Cells were utilized at passage 8 for experimental analysis.

2.2 Determination of cell viability and cell number

After trypsinization, the cell suspension was taken up into a Via-1 Cassette (Sartorius Stedim, Göttingen, Germany) and loaded into the NucleoCounter NC-3000 automated mammalian cell counter to determine total cell number and viability. This was accomplished via an automated image analysis based on acridine orange uptake and DAPI exclusion.

2.3 DOX treatment

In pilot experiments, cells were treated with various concentrations of the drug DOX to identify the optimal concentration that would lead to senescence. Tested DOX concentrations included: 25 nM, 50 nM, 75 nM, 100 nM, 125 nM, 150 nM, 175 nM, 200 nM, 225 nM, 250 nM, 275 nM and 300 nM. 50 nM DOX was chosen because it was the lowest concentration capable of inducing senescence without causing cell death. Stock solutions of DOX (1 mM, Sigma-Aldrich, Saint Louis, Missouri, United States of America) were prepared and stored in the dark at −20 °C. Upon use, these aliquots were diluted in DMEM medium supplemented with 10% FBS immediately before treating cells. Cells were exposed to DOX for 48 h followed by medium removal, washed with PBS twice, and further cultured for 6 days with a fresh media change every 72 h. Cell morphology was also monitored through culture time under a phase contrast inverted microscope (Leica Microsystems, Wetzlar, Germany).

2.4 SA- β -gal staining

SA- β -gal staining was performed according to a protocol published by Dimri et al [26]. Cells were incubated at 37 °C (without CO₂) with the following freshly prepared SA- β -gal (Thermo Fisher Scientific) stain solution: 1 mg of 5-bromo4-chloro-3-indolyl P3-D-galactoside - X-Gal (Thermo Fisher Scientific) per ml (stock = 20 mg of dimethylformamide per ml), 40 mM citric acid, sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂. Staining of cells was evident after 6–8 h and reached peak levels after 12–16 h. To detect lysosomal β -Gal as a positive control, the citric acid/sodium phosphate solution was used at a pH of 4.0. To selectively detect senescent cells, the citric acid/sodium

phosphate solution was used at a pH of 6.0. This resulted in only cells with residual lysosomal activity being stained in blue.

2.5 Immunocytochemistry

Cells were washed twice with PBS after culture and incubated with 4% paraformaldehyde (PFA) in PBS (Thermo Fisher Scientific) for 15 min at room temperature followed by two washes with PBS. For intracellular protein targets, cells were incubated for 10 min with 0.1% Triton X-100 followed by 2 washes with PBS before blocking with 1% bovine serum albumin (BSA) (SERVA, Heidelberg, Germany). The primary antibody was incubated for 1 h at room temperature and washed with PBS twice followed by incubation with a secondary antibody for 1 h at room temperature. The following primary antibodies used were: p16^{ink4a} (1:500, ab209579, Abcam), γ -H2AX (1:20, #564718, BD Biosciences), and Arg1 (1:100, #610708, BD Biosciences). The following secondary antibodies were used: NL-557 anti-Rabbit IgG (1:200, R&D Systems, Minneapolis, Minnesota, United States of America) and NL-557 anti-Mouse IgG (1:200, R&D Systems). Cells were washed with PBS twice, incubated with DAPI (0.1 μ g/mL) for 10 min for nuclear staining, and washed twice more with PBS.

2.6 Flow cytometry

Detached cells were resuspended in growth medium and loaded onto a 96 well plate (5×10^5 cells per well). The plate was centrifuged for 5 min at 200 $\times$ g's. The supernatant was removed and the cells resuspended and washed with cell staining solution (BioLegend, San Diego, California, United States of America). Cells were resuspended in 100 μ l of FVS450 viability dye (diluted 1:1000, BD Biosciences, Franklin Lakes, New Jersey, United States of America), incubated for 10 min at room temperature protected from light. Cells were then washed twice with cell staining buffer, fixed and made permeable with 200 μ l of BD Cytfix/Cytoperm Buffer (BD Biosciences) for 30 min at room temperature, washed with 1x BD Perm/wash buffer (BD Biosciences), centrifuged at 300 $\times$ g's for 5 min, and resuspended in cell staining buffer. 100 μ l of antibody dilutions or isotype controls were added to the cells and incubated at room temperature for 30 min in the dark. Anti-CDKN2A/p16^{INK4a} (1:500, Abcam, Cambridge, United Kingdom) and anti- γ H2AX (1:20, BD Biosciences) antibodies were used. Cells were washed twice with cell staining buffer, centrifuged at 300 $\times$ g's for 5 min, and then filtered via cell strainers (Thermo Fisher Scientific) into the final tube.

For SA- β -Gal staining experiments, 1 μ M bafilomycin A1 was added to the culture medium to neutralize the pH and incubated for 30 min at 37°C (without CO₂), followed by the addition of 10 μ g/mL of DDAOG (1,3-Dichloro-9,9-Dimethylacridin-2-One-7-yl, β -D-Galacto-pyranoside, #D6488, Thermo Fisher Scientific) and incubated for 1 h at 37 °C. Cells were washed twice with PBS and analyzed in a BD FACSCanto™ II flow cytometer (BD Biosciences). A minimum of 10,000 events (forward scatter/side scatter) were recorded for each sample.

2.7 Amyloid beta internalization assay

An amyloid beta (A β) internalization assay was performed using A β (1–42), HiLyte™ Fluor 488 – labelled, human (488-labelled A β) (AnaSpec, Fremont, California USA). 488-labelled A β was resuspended in 1% NH₄OH solution and aggregated overnight at 37 °C according to the manufacturer's protocol and previously described methods [27]. 488-labelled A β was then added to the cell and cultured to a final concentration of 0.5 μ M followed by incubation overnight at 37 °C with 5% CO₂. Cells were then washed with PBS, trypsinized, resuspended in medium to inactivate the trypsin, washed twice with PBS, and detected via flow

cytometry using the BD FACSCanto™ II flow cytometer (BD Biosciences). Images of the cells were acquired using a Nikon Eclipse TS100 fluorescence microscope (Nikon Instruments, Europe BV, Amsterdam, Netherlands) before preparing cells for flow cytometry.

2.8 Internalization assay using pHrodo™ zymosan Bioparticles™

An additional internalization assay was done to assess the lysosomal internalization of pH rodo™ red Zymosan bioparticles (Thermo Fisher Scientific). Particles are conjugated to pHrodo™ dye, a fluorogenic dye that dramatically increases in fluorescence as the pH of its surroundings becomes more acidic (e.g., lysosomal internalization). The protocol was followed according to manufacturer's instructions. Briefly, pH rodo™ red Zymosan bioparticles were thoroughly vortexed, added to the cell culture at 0.5 mg/mL, incubated overnight with 5% CO₂ at 37°C, washed with PBS, trypsinized, and detected using the BD FACSCanto™ II flow cytometer (BD Biosciences). Images of the cells were taken using a Nikon Eclipse TS100 fluorescence microscope (Nikon Instruments Europe BV) before preparing cells for flow cytometry.

2.9 Proteome analysis

Cells were trypsinized, washed twice with DPBS, and centrifuged at 200×g's for 5 min. Supernatants were discarded and cell pellets were instantly frozen in N₂ and kept at –80 °C until further analysis. After thawing, proteins were extracted by resuspending the pellets in 100 µL of lysis buffer [50 mM Tris (pH 7.4); 5 mM EDTA; 150 mM NaCl; 1% (v/v) Triton X-100] supplemented with protease inhibitors (Roche, Basel, Switzerland), and incubated at 4 °C for 30 min. Cells were then passed 30 times through a 25 gauge needle at 4 °C. Samples were centrifuged at 15,000×g's for 15 min at 4°C. The supernatant was collected and quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific).

25 µg of each sample were run on a 4–12% Bis-Tris gel under denaturing conditions for 5 min at 200 V with MES Running Buffer (Thermo Fisher Scientific). For each sample the whole lane was cut, reduced and alkylated with 10 mM of DTT (45 min at 56°C) and 55 mM iodoacetamide (IAA, 30 min at 20°C in the dark), respectively, and digested with trypsin (10 ng/µl) overnight at 37 °C (Promega, Madison, Wisconsin, United States of America). The tryptic peptides were extracted from the gel with acetonitrile (ACN), desalted using C18 microcolumns (3 M, Maplewood, Minnesota, United States of America), and concentrated on a SpeedVac (Thermo Fisher Scientific). Samples were resuspended in water with 0.1% (v/v) formic acid before being analyzed by LC-MS.

2.10 Generation of the spectral reference library

3 µg of each sample was used for information-dependent acquisition (IDA) analysis by NanoLC-MS using a TripleTOF 6600 (AB Sciex LLC, Framingham, MA, USA). A reversed phase nanoLC with a trap and elution configuration was performed using a Nano cHiPLC Trap column (200 µm × 0.5 mm ChromXP C18-CL 3 µm 120 Å) and a nano column (75 µm × 15 cm ChromXP C18-CL 3 µm 120 Å). Water with 0.1% (v/v) formic acid (solvent A) and acetonitrile with 0.1% (v/v) formic acid (solvent B) were used. Sample was loaded in the trap column at a flow rate of 2 µL.min⁻¹ for 10 min using 100% (v/v) solvent A. Peptide separation was performed in the nano column at a flow rate of 300 µL.min⁻¹, applying a 90 min linear gradient of 5%–30% (v/v) of solvent B. Protein samples were subjected to two IDA runs. The mass spectrometer was set for IDA scanning full spectra (400–2000 *m/z*) for 250 ms. The top 50 ions were selected for subsequent MS/MS scans (150–1800 *m/z* for 40 ms each) using a total cycle time of 2.3 s. The selection criteria for parent ions included a charge state between +2 and +5 and counts above a minimum threshold of 125 counts per second. Ions were excluded from

further MS/MS analysis for 12 s. Fragmentation was performed using rolling collision energy with a collision energy spread of 5. The spectral library was created by combining all IDA raw files (4 runs in total) using ProteinPilot™ software (v5.0 AB Sciex LLC) with the Paragon algorithm and with the following search parameters: search against *Homo sapiens* from Uniprot/SwissProt database (release 2018_03); trypsin digestion; iodoacetamide cysteine alkylation; through identification efforts.

After a false discovery rate (FDR) analysis, only those with a FDR <1% were considered (514 proteins). The output of these searches, in the form of a group file, was used as the reference spectral library. To identify differently expressed proteins between non-senescent and senescent cells, a *t*-test analysis was conducted: all proteins with a fold change higher than 1.5 in relative abundance and a $p < 0.05$ were considered to be differentially expressed. For each sample, 3 technical replicates and 2 biological replicates were considered.

2.11 SWATH-MS analysis and targeted data extraction

For quantitative analysis, 3 µg of each sample were subjected to SWATH – Sequential Windowed data independent Acquisitions of the Total High-resolution Mass Spectra. Similar chromatographic conditions to the previously described IDA run were used. The mass spectrometer was operated in a cyclic product ion data independent acquisition (DIA). A variable windows calculator (AB Sciex LLC) and a SWATH acquisition method editor (AB Sciex LLC) were used to set up the SWATH acquisition. A set of 32 overlapping windows (containing 1 *m/z* for the window overlap) was constructed, covering the precursor mass range of 400–1250 *m/z*. A 200 ms survey scan was acquired at the beginning of each cycle and SWATH MS/MS spectra were collected for 96 ms, resulting in a cycle time of 3.322 s. Rolling collision energy with a collision energy spread of 15 was used. The spectral alignment and targeted data extraction of DIA samples were performed using PeakView v.2.2 (AB Sciex LLC) with the reference spectral library. For data extraction, the following parameters were used: six peptides/protein, six transitions/peptide, peptide confidence level of >97%, FDR threshold of 1%, excluded shared peptides, and an extracted ion chromatogram (XIC) window of 6 min with a width set at 20 ppm. A total of 514 proteins were quantified under these conditions.

Mass spectrometry (MS) data was obtained using an UniMS – Mass Spectrometry Unit (ITQB NOVA/iBET, Oeiras, Portugal).

2.12 Ingenuity® pathway analysis (IPA®)

Comparative bioinformatics analysis was performed using IPA software from Qiagen (Hilden, Germany) [28]. SWATH-MS data were uploaded to IPA in the format of an excel file with three columns (“protein ID”/“fold-change” (senescent/non-senescent)/“p-value”) and a Core analysis was performed. From the Core analysis, information was extracted regarding biological functions, canonical pathways, individual proteins, proteins networks, and related diseases.

2.13 Transwell co-culture of DOX-treated and control CHME-5 cells

Co-culturing was done in 6 well plates transwells (0.4 µm/24 mm inserts polyester membrane, Thermo Fisher Scientific) in the absence of cell-cell contact. After senescent cells were generated (50 nM DOX for 48 h followed by 6 days of normal culture media), they were washed twice with PBS and the inserts placed in a 6 well plate where non-senescent cells were cultivated in the bottom for 24 h beforehand (at 3500 cells/cm²). Both cell populations were then co-cultured in the transwell plate for 72 h at 37°C, 5% CO₂ in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS. At the end of the experiment, supernatant medium from the bottom and insert wells was collected and stored at –80 °C. The cells were then trypsinized for 5 min at 37°C

and cell number and viability were counted. The cells were subsequently washed two times with PBS, centrifuged at 200×g's, and cell pellets stored at −80°C. The non-senescent control condition was generated using cells cultured at the bottom of the plate with an empty transwell.

2.14 Elisa

IL-6 and TNF- α were quantified in cell culture media using commercial enzyme-linked immunosorbent assays (ELISAs) (Thermo Fisher Scientific), per the manufacturer's instructions. Absorbance was read at 450 nm in a Labtech FLUOstar® Omega microplate reader (BMG LABTECH, Ortenberg, Germany).

2.15 Luminex immunoassay

Cytokine levels in cell culture media were determined using a multiplexed bead immunoassay and measured with a Luminex MAGPIX instrument (Luminex Corporation, Austin, Texas, United States of America). Interleukin-8 (IL-8) and GRO- α were measured using the Premixed Multi-Analyte Kit (R&D Systems) following the manufacturer's instructions. The cytokine magnetic bead kit provides a minimum detectable concentration of CXCL8/IL-8 (1060 pg/mL) and GRO- α (11,320 pg/mL). 96-well plates were analyzed on the Luminex® MAGPIX® CCD Imager instrument to measure cytokine concentrations followed by a 5-parameter logistic curve-fitting method from a standard curve of each respective analyte and normalized to cell number.

2.16 Statistical analyses

All statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad Software, San Diego, California, United States of America). Statistical comparisons of two groups were performed using an unpaired student's *t*-test. For statistical comparisons involving more than two groups, a one-way analysis of variance (ANOVA) was performed to determine differences between groups. For MS data analysis, a *t*-test was conducted to identify differently expressed proteins between non-senescent and senescent cells. All proteins with a fold change higher than 1.5 in relative abundance and a *p* < 0.05 were considered to be differentially expressed. Presented error bars represent standard deviation.

3 Results

3.1 Treatment with DOX alters cellular morphology and elevates SA- β -gal levels

DOX is a commonly used drug to induce senescence in different cell types [15,24,25]. After testing a variety of DOX concentrations (25 nM, 50 nM, 75 nM, 100 nM, 125 nM, 150 nM, 175 nM, 200 nM, 225 nM, 250 nM, 275 nM, and 300 nM), we determined that 50 nM was the lowest concentration that could induce characteristics of senescence without causing cell death and was therefore the concentration we chose to use. Cells were exposed to DOX for 48 h followed by six days of culture in standard medium. Compared to control cells (Fig. 1 A), DOX-treated cells acquired a distinct morphology easily visualized by transmitted light microscopy (Fig. 1 B). As is typical of other cells that have undergone irreversible cell cycle arrest [29], DOX-treated CHME-5 cells acquired a unique phenotype characterized by an enlarged cell size, a flattened morphology, and the development of prominent filaments (Fig. 1B). At a pH of 6, the SA- β -gal assay also distinguishes DOX-treated cells (positive staining) from control (negative staining) cells (Fig. 1C). Visually, it is clear that DOX-treated cells express substantially higher levels of β -gal compared to controls (Fig. 1C). To demonstrate that the assay works, a positive control experiment was done at a pH of 4 to show β -gal staining in lysosomes of control cells (Fig. 1 C). While 16.8% $\pm$ 3.12% of control cells contained β -gal, 87.6% $\pm$ 4.27% of DOX-treated cells contained β -gal (*p* < 0.0001) (Fig. 1D).

Fig. 1

Senescent microglia exhibit an altered morphology and increased staining of SA-β-gal. Transmitted light microscopy shows that untreated cells (A) and senescent DOX-treated cells (B) have different morphologies. Senescent cells exhibited an altered appearance characterized by an enlarged cell size, a flattened morphology, and the development of prominent filaments. Scale bars = 100 μm. C) Although non-senescent cells showed minimal staining at a pH of 6.0 (upper right panel), senescent cells exhibited increased staining of SA-β-gal at a pH of 6.0 (left panel). As a positive control, non-senescent cells displayed SA-β-gal at a pH of 4.0 (bottom right panel). Scale bars = 100 μm. D) Quantitative summary of the expression of SA-β-gal in either non-senescent or senescent cells. While 16.8% ± 3.12% of non-senescent cells contained β-gal, 87.6% ± 4.27% of senescent cells contained β-gal ($p < 0.0001$). Values represent the mean value of independent experiments done in triplicate. Statistical differences were determined by a t -test and error bars represent standard deviation. DOX = doxorubicin.

3.2 DOX-treated microglial cells express both p16^{Ink4a} and γ-H2AX

We next measured the expression levels of p16^{Ink4a}, which is the most commonly utilized cell cycle regulator marker of senescence in human tissues [9]. We found that 83.4% ± 4.23% and 15.3% ± 3.51% of DOX-treated and control cells, respectively, express this marker ($p < 0.0001$) (Fig. 2A). Similarly, the histone γ-H2AX is yet another protein associated with senescence that is a marker of double-stranded DNA breaks and telomere shortening [30]. While 1.99% ± 0.75% of control microglia expressed γ-H2AX, 93.1% ± 3.75% of DOX-treated cells expressed γ-H2AX ($p < 0.0001$) (Fig. 2B). Representative fluorescence images of cells labelled with p16^{Ink4a} (Fig. 2A) or γ-H2AX (Fig. 2B) are provided.

Fig. 2

Microglial cells that have undergone senescence show increased expression of p16^{Ink4a} and γ-H2AX. A) Flow cytometry measurements revealed that 15.3% ± 3.51% and 83.4% ± 4.23% of non-senescent and senescent cells, respectively, express p16^{Ink4a} ($p < 0.0001$). Representative immunostaining of p16^{Ink4a} (red) and DAPI (blue) of non-senescent (left) and senescent cells (right) are shown. Scale bars represent 25 μm. B) 1.99% ± 0.75% of non-senescent microglia expressed γ-H2AX and 93.1% ± 3.75% of senescent-cells expressed γ-H2AX ($p < 0.0001$). Representative immunostaining of γ-H2AX (red) and DAPI (blue) of non-senescent (left) and senescent cells (right) are shown. Scale bars represent 25 μm. Expression levels were quantified via flow cytometry. These collective results represent the mean value of independent experiments done in triplicate. Statistical differences were determined by a *t*-test and error bars represent standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3 Microglia that have been treated with DOX exhibit an impaired ability to internalize Aβ and pHrodo particles

Phagocytic clearance is performed by microglia in the brain and is highly relevant to Alzheimer's disease [31]. Thus, we sought to investigate whether or not DOX-treated cells could properly internalize Aβ. Using a fluorescence assay that we quantified via flow cytometry, we found that DOX-treated cells were significantly worse at internalizing Aβ compared to untreated cells (Fig. 3A). Internalization was measured by identifying cells with intracellular FITC fluorescence. 95.1% ± 2.74% of untreated microglia could internalize Aβ while 69.9% ± 9.05% of DOX-treated microglial cells could internalize Aβ (Fig. 3A). This 25.2% drop was statistically significant ($p < 0.05$).

alt-text: Fig. 3

Fig. 3

Senescence decreases internalization efficiency in microglia. A) Senescent cells had reduced intracellular FITC fluorescence associated with amyloid beta (A β) internalization. 95.1% $\pm$ 2.74% of non-senescent microglia could internalize A β while 69.9% $\pm$ 9.05% of senescent microglial cells could internalize A β ($p < 0.05$). B) Senescent cells less efficiently internalize pHrodo bioparticles, which emit fluorescence once internalized into lysosomes. Approximately 82% $\pm$ 3.84% or 60% $\pm$ 2.90% of non-senescent or senescent cells, respectively, were capable of internalizing these particles ($p < 0.01$). Values represent the mean value of independent experiments done in triplicate for both the A β (A) and pHrodo bioparticle (B) experiments. Statistical differences were determined by a t -test and error bars represent standard deviation.

We were also interested in assessing how efficiently microglia could degrade engulfed particles. We therefore utilized pH-dependent zymosan bioparticles conjugated to a pHrodo dye. This dye is sensitive to pH and begins to emit fluorescence only after being internalized inside lysosomes. The assay revealed that approximately 82% $\pm$ 3.84% and 60% $\pm$ 2.90% of control and DOX-treated cells, respectively, were capable of internalizing these particles inside lysosomes (Fig. 3B). This 22% disparity was statistically significant ($p < 0.01$).

3.4 The proteome of DOX-treated microglia

Proteomic analysis is an effective tool for identifying proteins and pathways that are unique to specific conditions, diseases, or drug responses [13,32]. To better understand how DOX treatment impacts cellular functions in microglia, we performed a proteomic analysis using SWATH-MS in both DOX-treated and control cells. SWATH-MS allows for both the identification and the relative quantification of proteins while comparing between two different conditions. This technique has been previously utilized to provide insights into various

biological processes, cell types, and diseases relevant to aging, including the insulin signaling/insulin-like growth factor 1 pathway [33], Alzheimer's disease [34], and mesenchymal stem cells [35].

Overall, our SWATH-MS analysis unveiled a total of 514 proteins (Fig. 4A + Table S1). Of these, 342 proteins were significantly different ($p < 0.05$) between untreated and treated CHME-5 cells (Fig. 4A). Of these 342 proteins, 201 were downregulated and 127 were upregulated (Fig. 4A). If the p -value was less than 0.05, the fold change threshold for a protein to be considered upregulated or downregulated was >1.5 and <0.7 , respectively (Fig. 4A). This is analogous to guidelines that have been used previously in the literature [36]. Having identified these 342 differentially upregulated or downregulated proteins, we analyzed this dataset using IPA® Software from Qiagen [28]. This allowed us to determine which cellular functions and networks were altered in DOX-treated microglia. This analysis revealed that the following five biological functions were the most different between untreated and treated microglial cells: protein synthesis, RNA damage and repair, cell death and survival, protein degradation, and cellular compromise (Fig. 4B). The top 20 upregulated and top 20 downregulated proteins are presented in Tables 1 and 2, respectively. The five most upregulated proteins (Table 1) were transforming protein RhoA (40.7 fold change), prosaposin (12.5 fold change), cathepsin B (11.6 fold change), mammalian ependymin-related protein 1 (10.7 fold change), and ATP-dependent 6-phosphofructokinase (10.4-fold change). The five most downregulated proteins (Table 2) were microtubule-associated protein 4 (32.1 fold change), mitochondrial dicarboxylate carrier (29.8 fold change), 40S ribosomal protein S26 (26.3 fold change), eukaryotic translation initiation factor 6 (21.5 fold change), and transgelin-2 (19.2 fold change). Various genes relevant to translation, cytoskeleton integrity, proteasome activity, mitochondrial function, and other processes are also included in these top 20 lists (Tables 1 and 2).

alt-text: Fig. 4

Fig. 4

Senescence drastically alters the microglial proteome. A) SWATH-MS analysis identified 342 proteins that were significantly different ($p < 0.05$) between senescent and non-senescent CHME-5 cells. Of these 342 proteins, 201 were downregulated and 127 were upregulated. If the p -value was less than 0.05, the fold change threshold for a protein to be considered upregulated or downregulated was >1.5 and <0.7 , respectively. B) Analysis of this dataset using IPA® Software from Qiagen revealed that the following five biological functions were the most different between non-senescent and senescent microglial cells: protein synthesis, RNA damage and repair, cell death and repair, protein degradation, and cellular compromise.

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Table 1

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Top 20 upregulated proteins in senescent microglial cells.

Entry Name (SwissProt)	Fold Change (Senescent/Non-senescent)	<i>p</i> -value	Protein Name
Q9QUI0	40.7	5E-02	Transforming protein RhoA
P10960	12.5	4E-05	Prosaposin
P10605	11.6	1E-03	Cathepsin B
Q99M71	10.7	1E-06	Mammalian ependymin-related protein 1
P47858	10.4	3E-02	ATP-dependent 6-phosphofructokinase, muscle type
P17164	10.1	1E-09	Tissue alpha-L-fucosidase
Q07009	10.0	4E-02	Calpain-2 catalytic subunit
Q9WTT6	9.6	1E-08	Guanine deaminase
P13233	7.3	1E-09	2',3'-cyclic-nucleotide 3'-phosphodiesterase
Q62952	7.1	2E-04	Dihydropyrimidinase-related protein 3
P49817	6.8	3E-03	Caveolin-1
Q6PFD5	5.7	3E-02	Disks large-associated protein 3
Q7TMM9	5.4	2E-07	Tubulin beta-2A chain
Q5XIP9	5.4	3E-02	Transmembrane protein 43
Q3MIE4	5.2	7E-08	Synaptic vesicle membrane protein VAT-1 homolog
Q6PDM2	4.9	1E-02	Serine/arginine-rich splicing factor 1
Q60930	4.8	2E-06	Voltage-dependent anion-selective channel protein 2
P26051	4.8	1E-04	CD44 antigen
P05942	4.4	2E-04	Protein S100-A4
P52631	4.3	2E-03	Signal transducer and activator of transcription 3

alt-text: Table 2

Table 2

The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Top 20 downregulated proteins in senescent microglial cells.

Entry Name (SwissProt)	Fold Change (Senescent/Non-senescent)	<i>p</i> -value	Protein Name
Q5M7W5	32.1	4E-05	Microtubule-associated protein 4
Q9QZD8	29.8	2E-06	Mitochondrial dicarboxylate carrier
P62856	26.3	7E-11	40S ribosomal protein S26
Q3KRD8	21.5	4E-07	Eukaryotic translation initiation factor 6
Q5XFX0	19.2	3E-05	Transgelin-2
Q8BKC5	18.1	2E-05	Importin-5
Q8BH88	17.7	3E-08	DEP domain-containing protein 1B
Q9R1P3	17.1	5E-07	Proteasome subunit beta type-2
Q8CGC7	15.7	3E-02	Bifunctional glutamate/proline--tRNA ligase
Q6NYB7	15.7	3E-06	Ras-related protein Rab-1A
Q3UV71	14.9	7E-10	Protein O-mannosyl-transferase TMTC1
Q91VR2	14.1	6E-07	ATP synthase subunit gamma, mitochondrial
P09895	13.9	8E-07	60S ribosomal protein L5
Q9QZE5	13.4	2E-02	Coatomer subunit gamma-1
P52293	11.6	6E-03	Importin subunit alpha-1
Q8BK67	11.6	1E-09	Protein RCC2
Q99L47	11.1	2E-07	Hsc70-interacting protein
Q9JM76	11.1	3E-07	Actin-related protein 2/3 complex subunit 3
P62832	11.0	5E-09	60S ribosomal protein L23
P62335	10.7	4E-11	26S proteasome regulatory subunit 10B

Fig. 5 summarizes the major changes within these five biological function categories that occur in DOX-treated microglial cells. Broadly, downregulation was more common than upregulation (Fig. 5). Protein synthesis pathways like protein synthesis, translation, and expression were overwhelmingly downregulated (Fig. 5A). The same was true for RNA metabolism and decay (Fig. 5B). Processes involved in cell death and survival were more mixed (Fig. 5C), though those involved in cancer cell death were largely downregulated (Fig. 5C). Protein degradation pathways involving aggresome formation, protein catabolism, and protein stabilization were mostly downregulated (Fig. 5D) while those involved in cellular compromise were predominantly upregulated (Fig. 5E). The latter involved the upregulation of various processes pertinent to cytoskeletal disruption, multinucleation, and cellular degeneration (Fig. 5E). A more detailed look reveals that several signaling pathways and processes

were impacted ([Supplementary Fig. 1](#)). For example, EIF2 and mTOR signaling were mostly downregulated while glycolysis I, glucogenesis I, and remodeling of epithelial adherens were mostly upregulated ([Supplementary Fig. 1](#)). Our SWATH-MS also showed that, overall, there is a downregulation of the protein ubiquitination pathway ([Supplementary Fig. 2](#)). [Supplementary Figure 2](#) highlights proteasomal components that are downregulated compared to control cells. For example, proteasome subunit beta type-2 ($p = 4.75\text{E-}07$), proteasome subunit beta type-3 ($p = 8.09\text{E-}08$), proteasome subunit alpha type-2 ($p = 9.86\text{E-}11$), ubiquitin-like modifier-activating enzyme 1 ($p = 2.18\text{E-}11$) and 26S proteasome non-ATPase regulatory subunit 11 ($p = 5.69\text{E-}03$) were all downregulated ([Supplementary Table 1](#)).

alt-text: Fig. 5

Fig. 5

Key biological processes are differentially downregulated (blue) and upregulated (red) in senescent microglial cells. A) Proteins involved in protein synthesis were predominantly downregulated. The one exception was “stabilization of protein”, which was slightly more upregulated in senescent cells. B) Proteins involved in RNA damage and repair were largely downregulated. C) Proteins involved in cell death and survival were disparately upregulated and downregulated depending on the specific process. D). Protein degradation was mostly downregulated in senescent microglia. E) An upregulation of proteins associated with compromised cellular integrity was observed in senescent cells compared to non-senescent cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5 DOX-treated cells secrete inflammatory cytokines

Given that the SASP is highly relevant to aging and disease [37,38], we sought to investigate whether or not DOX-treated microglia could impact an environment shared with control microglial cells. To do this, we co-cultured both DOX-treated and control cells in a transwell system that featured DOX-treated cells atop the transwell insert and control cells beneath the transwell insert ([Supplementary Fig. 3A](#)). A 0.4 μm pore allowed for indirect interaction and soluble factor exchange between these treated and untreated CHME-5 cells ([Supplementary Fig. 3A](#)). For the control condition, untreated cells were cultured at the bottom of the plate with an empty transwell above them.

We then analyzed mean levels (pg/mL/10⁶ cells) of the cytokines IL-6, IL-8, TNF-α, and GRO-α in control, DOX-treated, and co-cultured cells ([Supplementary Fig. 3B](#)). DOX-treated cells showed significantly higher levels of IL-6 (~4-fold increase, $p < 0.0001$) and IL-8 (~3.5-fold increase, $p < 0.0001$), TNF-α (~5-fold

increase, $p < 0.001$), and GRO- α (~ 2.7 -fold increase, $p < 0.01$) compared to control cells (Supplementary Fig. 3B). In co-cultured cells, the levels of TNF- α and GRO- α were comparable to those observed in DOX-treated cells (Supplementary Fig. 3B). Conversely, a significant decrease in both IL-6 ($p < 0.01$) and IL-8 ($p < 0.001$) was observed in co-cultured cells compared to DOX-treated cells (Supplementary Fig. 3B). For both interleukins, the measured level in co-cultured cells was in between what was observed in DOX-treated and control cells (Supplementary Fig. 3B). Transmitted light microscopy images revealed that, in this unique co-culture environment, untreated cells underwent a morphological shift and became more rounded (Supplementary Fig. 3C). Unlike the DOX-treated cells visualized in Figs. 1 and 2, however, these cells were not enlarged compared to untreated control cells. This suggests that, although these cells show a unique morphology, they are not becoming senescent. While these proof-of-concept co-culture data are intriguing, additional research efforts are warranted to more comprehensively test how DOX-treated and control microglia co-exist together in a shared environment.

4 Discussion

The goal of the study was to establish a model to study microglial senescence. Microglia are an important support macrophage cell type in the brain that are responsible for the phagocytic clearance of pathogens, unwanted molecules, and dying cells [20]. We found that the drug DOX induces several different characteristics of senescence in CHME-5 cells. Compared to control cells, DOX-treated microglia exhibited an altered morphology, increased staining of SA- β -gal, and higher levels of p16^{Ink4a} and γ -H2AX. DOX treated microglia were also less effective at internalizing A β and pHrodo bioparticles. A detailed proteomic analysis revealed that DOX treatment substantially alters the microglial proteome. Broadly, there was a trend towards protein downregulation in these cells. Lastly, we discovered that DOX-treated microglia secrete more of the cytokines IL-6, IL-8, TNF- α , and GRO- α , which are part of the SASP. Taken together, these findings argue that senescent microglial CHME-5 cells can be generated via DOX treatment.

Elevated levels of the senescent markers SA- β -gal, p16^{Ink4a}, and γ -H2AX have all been previously utilized as markers of cellular senescence [18]. Moreover, TNF- α , GRO- α , IL-6, and IL-8 have been discussed as part of the harmful SASP [11]. It is helpful to know that senescent microglia also exhibit these classical markers. Not only does it further validate their use in yet another cell type, but it lends credence to the idea that many features of senescence are conserved across distinctly different cell types. Given that p16^{Ink4a} is a tumor suppressor protein with antiproliferative activity [39], it makes intuitive sense that this would be elevated in cells that have undergone permanent cell cycle arrest. The histone variant γ -H2AX is formed as an early cellular response to double-strand breaks in DNA [40]. Senescence has been proposed as a defensive response to DNA damage [41] and, given that DOX can affect DNA and chromatin dynamics [42], this may be the mechanism by which DOX generates senescence. Although the levels of β -gal and p16^{Ink4a} were much lower in control cells, it was interesting to find that 16.8% of control cells expressed β -gal and that 15.3% of control cells expressed p16^{Ink4a}. This suggests the possibility that at least a subset of cells in our control population became senescent despite not being treated with DOX. Further studies are warranted to determine if this is indeed the case and if background levels of senescence vary between different microglial cell lines.

Because of its known ability to generate senescence in other cell types [15,24,25], we chose to test the ability of DOX to induce senescence in microglia. Given that DOX is an anti-cancer, chemotherapy pharmaceutical [43], our finding that DOX can generate senescent microglia has interesting implications for cancer patients undergoing DOX treatment. Specifically, it raises the concern that DOX treatment may have deleterious effects on microglial cells and therefore on brain health. Indeed, DOX is known to induce cardiotoxicity [44] and has

also been linked to toxicity of the brain, kidney, and liver [45]. A growing body of evidence additionally suggests that DOX treatment may exacerbate cognitive impairment [46]. Thus, it would be intriguing to determine whether or not patients treated with DOX are at a higher risk for brain disorders, including Alzheimer's disease. An argument against this being a concern is that DOX does not efficiently cross the blood-brain barrier [47]. However, certain pharmaceuticals can increase the ability of DOX to cross over into the brain [47]. Whether or not DOX can induce senescence to an extent that is deleterious *in vivo* should be explored in future studies.

Macrophages can either be activated and primed to kill (M1) or alternatively activated and primed to heal (M2) [48]. Since activated microglia assume a pro-inflammatory phenotype, it is not atypical for non-senescent microglial cells to secrete inflammatory cytokines like IL-6 and TNF- α [49]. While it is not yet clear whether or not there are consistent morphological differences between M1 and M2 macrophages [49], our co-culture experiment revealed that morphology of non-senescent cells changed when placed in an environment with adjacent senescent cells. This suggests the possibility that the altered appearance of these cells could be due to their activation. Since microglia can become activated and secrete various cytokines, it is important to utilize other markers besides cytokines (e.g., SA- β -gal, p16^{Ink4a}, and γ -H2AX) when characterizing senescence in macrophages.

It was interesting to observe that senescent microglia were less efficient at internalizing A β and pHrodo particles. These data suggest that senescent microglial cells are less efficient at phagocytosis than non-senescent cells. Phagocytosis is an integral cellular process that has been implicated in various human diseases, such as inherited retinal disease [50]. Patients with Alzheimer's disease exhibit an abnormal accumulation of A β peptides and, in the brain, microglia are responsible for the phagocytic clearance of A β [31]. In *Drosophila*, phagocytic activity is associated with neurodegeneration and the regulation of lifespan [51]. Lipid-droplet-accumulating microglia buildup with age in mouse and human brains and these cells phagocytosis poorly, secrete proinflammatory cytokines, and generate high levels of reactive oxygen species [52]. The association with lipid droplets is intriguing as accumulating evidence suggests that lipid metabolism strongly regulates lifespan and healthspan [53]. Thus, the accrual of senescent microglia with age may hinder phagocytosis and pave the way for the accumulation of neural plaques and other hazardous materials. Furthermore, the findings from our pH-dependent bioparticle phagocytosis assay suggest that lysosomal degradation is impaired in senescent cells. Lysosomes are highly relevant to aging and disease, as exemplified by the fact that the overexpression or knockdown of lysosomal lipase can impact lifespan and health in model organisms [54]. More generally, a reduced ability of microglia to properly phagocytose would be predicted to hinder the brain's ability to handle pathogens, clear dead or dying cells, and perform important immune functions. Keeping this potential for such broad effects in mind, it is unsurprising that defective microglial phagocytosis has been theorized to contribute to the pathogenesis of Parkinson's disease [55].

Our proteomic comparison between non-senescent and senescent cells revealed a global trend towards downregulation in senescent microglia. The data also helped illuminate what mechanisms underlie our observational changes. For example, we observed that senescent cells had a distinct morphology compared to non-senescent cells. Such morphological changes are typical of cellular senescence [29] and, as would be predicted given such observable changes, protein alterations associated with cytoskeleton disruption were upregulated. Moreover, the actin-associated [56] transforming protein RhoA was the most upregulated protein while microtubule-associated protein 4 was the most downregulated protein. Several proteins were also directly linked to known hallmarks of aging [1] and were among the most upregulated or downregulated proteins. For example, mitochondrial health is known to decline with age and strategies targeting mitochondrial function can

either extend or reduce lifespan in model organisms [1]. Moreover, animal longevity is associated with improved mitochondrial function [57]. Relevantly, mitochondrial dicarboxylate carrier and ATP synthase subunit gamma, mitochondrial were the 2nd and 12th most downregulated proteins, respectively.

Our proteomic analysis revealed that several different proteasomal proteins were downregulated, indicating that proteasomal function may be compromised in senescent cells. A recent proteomic study reported that the epithelial, soluble SASP is depleted in proteins pertinent to proteasomal degradation [58]. The downregulation of proteasomal components may also reflect the decrease in protein synthesis, which is likely a response to cell-cycle arrest. Indeed, the eIF2 signaling pathway – which plays an important role in the initiation of translation – was largely downregulated. Inhibition of the GTPase eIF2 has been shown to prolong life in nematodes [59] and, relevant to human disease, mutations in eIF2B cause the neurological disorder vanishing white matter disease. It was recently shown that long-term treatment with the small molecule eIF2B activator 2BAct can prevent pathology in mice with this disease [60]. It would be intriguing to know if the drug 2BAct or if targeting eIF2 signaling can offset any negative effects induced by senescent cells.

Future studies are warranted to assess how the specific clearance of senescent microglial cells can improve health and ameliorate neurodegenerative disease. A substantial body of work has demonstrated that the removal of senescent cells can elongate healthspan and lifespan in mice [2–5,7,17,61]. It would also be worthwhile to optimize strategies to enhance the phagocytic ability of microglia to yield desirable clinical effects. Pluvinage et al have shown, for example, that the canonical B cell receptor CD22 is a negative regulator of phagocytosis that is upregulated in older microglial cells. Long-term treatment with an antibody that blocks the function of CD22 is sufficient to reprogram microglia and enhance cognitive function in elderly mice [62]. Given the importance of senescent cells in aging and the relevance of microglia to neurodegenerative diseases, it seems feasible that therapies that remove senescent microglia or enhance the function of microglia have the potential to improve patient outcomes.

Author contributions

Luís Marques collected the data and assisted with writing. Adiv A. Johnson helped with writing and data presentation. Alexandra Stolzing is responsible for project design and assisted with writing.

Data availability statement

The proteomics data generated in this study are available online in the Mendeley Data database (<https://doi.org/10.17632/rcb56r2rvh.2>). Any additional data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yexcr.2020.112203>.

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The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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- Microglial cells can be made senescent via treatment with doxorubicin.
 - Senescent microglia express β -galactosidase, p16^{Ink4a}, and γ -H2AX.
 - Senescent microglial cells less efficiently internalize amyloid β .
 - Doxorubicin-treated microglia secreted higher levels of inflammatory cytokines.
 - The proteomes of non-senescent and senescent microglia are significantly different.
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Appendix A Supplementary data

The following are the Supplementary data to this article:

[Multimedia Component 1](#)

Multimedia component 1

alt-text: Multimedia component 1

[Multimedia Component 2](#)

Multimedia component 2

alt-text: Multimedia component 2

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