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Original Article

Male Monocyte-Like Cells are Prone to a Senescence-Induced Pro-inflammatory State

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ABSTRACT: Chronic systemic inflammation in the elderly is a hallmark of aging and a major contributor to age-related diseases. It both results from and promotes the accumulation of senescent immune cells. While the sex difference in the aging process has been widely studied, the impact of cellular sex on immune cell senescence remains unclear and was the focus of this present study. Senescence was induced in human male (THP-1) and female (HL-60) monocyte-like cells by treatment with D-galactose for 48 h. The expression of metabolic sensors (Sirt1 and pAMPK), mitochondrial biogenesis and respiration, reactive oxygen species formation, as well as pro-inflammatory markers was investigated alongside senescence markers. Treatment with D-galactose resulted in significant reduction of the nuclear proteins HMGB1 and lamin B1, and an upregulation of senescence-associated secretory phenotype factors including *IL-6*, *VEGF*, *TGF-β*, and *MMP-9*, in male and female cells. The expression of the metabolic sensors Sirt1 and pAMPK was reduced, whereas mitochondrial ROS production and mitochondrial gene expression were elevated in both male and female cells to a similar extent. In contrast, D-Galactose-induced senescence was accompanied by a significant elevation of pro-inflammatory markers (NF-κB, *TNF-α*, *IL-1β*, *HLA-DR*, and *MCP-1*) primarily in male monocytes, whereas primary monocytes did not display sex differences. This study suggests that male immune cells are more prone to developing a pro-inflammatory state under senescent stimuli. These findings highlight the potential significance of sex-specific anti-inflammatory therapies.

Keywords: Cellular Senescence, Immunosenescence, Inflammaging, Monocyte-Like Cells, Mitochondrial Homeostasis

INTRODUCTION

Aging is accompanied by chronic low-grade systemic inflammation. This phenomenon is characterized by elevated levels of pro-inflammatory mediators, including IL-1β, IL-6, and TNF-α [1-3]. Inflammation is an important predictor of morbidity and mortality among elderly individuals [1, 4-6]. Although the source of inflammation remains incompletely understood, there is growing evidence highlighting the role of monocytes and macrophages [7]. In addition to aging, biological sex, particularly sex hormones such as estrogen and testosterone, significantly impacts the inflammatory response [8]. Several studies have shown that male peripheral blood mononuclear cells (PBMCs) exhibit heightened susceptibility to lipopolysaccharide (LPS) stimulation, resulting in increased production of pro-inflammatory factors such as TNF-α, compared to female cells [8-10]. Consistent with these findings, our previous research demonstrated that male murine bone marrow

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macrophages are more responsive to LPS treatment and exhibit a more pronounced pro-inflammatory phenotype than female macrophages [11]. Additionally, treatment with 17β-estradiol alleviates the inflammatory phenotype in pro-inflammatory M1 female macrophages, which is accompanied by upregulation of Sirt3 and mitochondrial protein deacetylation [12]. Importantly, these sex-related differences in the inflammatory state persist in elderly individuals, with older men exhibiting higher levels of pro-inflammatory markers, while older women tend to show increased levels of IL-10, which influences the immune response [8].

Chronic systemic inflammation may also affect immune cells during aging, particularly via the induction of immune cell senescence, also known as immunosenescence [13], which impairs their functionality and responsiveness [14]. Generally, cellular senescence is characterized by a state of cell cycle arrest triggered by intracellular or extracellular stress or damage [15, 16]. Although not proliferating, senescent cells remain metabolically active and release numerous factors i.e., pro-inflammatory cytokines, chemokines, growth factors, proteases, bioactive lipids, and extracellular vesicles, known as the senescence-associated secretory phenotype (SASP) [17, 18]. The SASP can promote paracrine senescence and maintain an increasingly senescent and inflammatory milieu within the tissue [19]. In particular, senescent macrophages express elevated levels of pro-inflammatory factors, e.g., TNF-α, IL-6, and IL-1β [20-22], whereas the anti-inflammatory cytokine IL-10 is significantly decreased in senescent macrophages [13, 23]. Furthermore, senescent cells release reactive oxygen species (ROS) due to disturbed mitochondrial function [24].

The potential contribution of cellular sex to the senescent phenotype of immune cells remains unclear and was the focus of the present study. The results reveal a significant upregulation of senescence markers, accompanied by impaired mitochondrial homeostasis in both cell types, whereas specifically male monocyte-like cells developed a pro-inflammatory phenotype.

MATERIAL AND METHODS

In vitro studies with human monocytic-like cells

Human male THP-1 cells (ATCC® TIB-202™) and human female HL-60 (ACC 3, DSMZ, Germany) were cultivated in RPMI 1640 medium (Gibco, Germany), L-glutamine, 10 % Fetal Bovine Serum Advanced (Capricorn Scientific, Germany), and 1 % Penicillin/ Streptomycin (Biochrom, Germany) at 37°C and 5 % CO_2 [25]. Cells were harvested by centrifugation (5 min /300 rpm) and resuspended in fresh medium every 2 days. All experiments were performed in duplicate and the experiments were repeated a minimum of five times to ensure replicability.

Isolation and cultivation of human monocytes

Healthy age-matched men and women (age: 35-45 years, 4 women, 4 men) donated 80 ml of blood for the isolation of peripheral blood mononuclear cells (PBMCs). All participants provided written informed consent. Sample collection and experimental protocols were approved by the Scientific Board of Charité – Universitätsmedizin Berlin (EA1/070/16). All experiments were conducted in accordance with German regulations and ethical standards as outlined in the Declaration of Helsinki. PBMCs were isolated from freshly collected blood by Ficoll density gradient centrifugation (Sigma-Aldrich, Germany) [25]. Monocytes were isolated from PBMCs with human CD14 MicroBeads (Miltenyi Biotec, Germany) and seeded on 6-well plates ($1x10^6$ cells/well) in RPMI 1640 medium (Gibco, USA) supplemented with 4 ng/ml human macrophage colony-stimulating factor (M-CSF) (Peprotech, Hamburg), 10% Fetal Bovine Serum (FBS) Advanced (Biotech, USA) and 1% penicillin/ streptomycin (Biochrom, Germany) at 37°C with 95% humidity and 5% CO_2 for 72 h.

Cellular senescence induction

Human THP-1 cells, human HL-60 cells and human primary monocytes were treated with 250 mM D-galactose (Sigma-Aldrich, USA) for 48 h to induced senescence [26, 27].

Activation of ERs and ARs

D-galactose stimulated THP-1 and HL-60 cells were starved in phenol-free RPMI 1640 medium and 2.5% charcoal-stripped FCS (Biochrom, Germany) for 48 h prior to estradiol (E2) or testosterone treatment. After starvation, cells were treated with 10 nmol/l water soluble E2 (Sigma-Aldrich, Germany) or its vehicle (10 nmol/l dextrin; Sigma-Aldrich, Germany) for 24 h [11]. In addition, cells were also treated with 10 nmol/l testosterone or ethanol as the vehicle control for 24 h.

RNA isolation and quantitative real-time (RT)-PCR

Total RNA from monocyte-like cells was isolated using RNA-Bee (Amsbio, UK), and a quantitative real-time PCR was performed with Brilliant SYBR Green qPCR master mix (Applied Biosystems, USA), as described [28]. The relative amount of target mRNA was determined using the comparative threshold (Ct) method

as previously described [28]. The mRNA contents of target genes were normalized to the expression of hypoxanthine phosphoribosyl transferase (HPRT) and ribosomal protein lateral stalk subunit P0 (RPLP0) (Supplementary table 1). The results are expressed as fold change relative to the untreated group after normalization.

Protein extraction and immunoblotting

THP-1 and HL-60 cells were homogenized in Laemmli buffer, as described [11]. Proteins were quantified using the BCA assay (Thermo Scientific Pierce Protein Biology, Germany). Equal amounts of total proteins were separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were immunoblotted overnight with the following primary antibodies: PGC-1α (1:1000, #54481, Abcam, UK), HMGB1 (1:1000, #6893, Cell Signaling, USA), Lamin B1 (1:1000, #13435, Cell Signaling, USA), Sirt1 (1:1000, #8469, Cell Signaling, USA), pAMPK (1:2000, Thr172, #2535L, Cell Signaling, USA), AMPK (1:2000, #2532L, Cell Signaling, USA) and NFκBp50 (1:500, #8414, Santa Cruz, USA). Equal sample loading was confirmed by tubulin (1:15000, #3873, Cell Signaling, USA) or GAPDH analysis (1:5000, #97166, Cell Signaling, USA). Immunoreactive proteins were detected using ECL Plus (GE Healthcare, UK) and quantified with ImageLab (Bio-Rad Laboratories, USA). All samples were run on the same gel.

Mitochondrial mass analysis

The amounts of nuclear and mitochondrial DNA (mt-DNA) were analyzed in monocyte-like cells via quantitative real-time PCR. Briefly, the measurement of mitochondrial content was conducted with the ratio of mt-DNA (*RNR2*) to nuclear DNA (*β-globulin)* [29].

Mitochondrial respiration analysis

THP-1 and HL-60 cells were seeded in an Agilent XFe96 Seahorse cell culture plate at a density of 7×10^4 cells/well for THP-1 cells and a density of 1 x 10^5 cells/well for the HL60 cells, with one well per corner of the plate containing supplemented media without cells to serve as a background control. Cells were treated with 125 mM and 250 mM D-galactose for 24 h. A Seahorse utility plate (Seahorse Bioscience, USA) containing 200 µl of water per well, along with a Seahorse injector port and probe plate, was incubated overnight at 37°C without CO_2. On the following day, the media in the Seahorse cell culture plate was replaced with Seahorse XF assay buffer (supplemented with 10 mmol/l glucose and 2 mmol/l glutamine) and incubated in a CO_2-free incubator at 37°C for one hour. The designated injector ports were filled with the following MitoStress Test inhibitors: 1 µmol/l oligomycin (an ATP synthase inhibitor used to calculate mitochondrial O_2 consumption rate); 2 µmol/l carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), an ionophore that uncouples ATP synthesis from the electron transport chain to assess maximal mitochondrial oxidative phosphorylation (OXPHOS); and 0.5 µmol/l antimycin and 0.5 µmol/l rotenone (inhibitors of Complex I and III, respectively, used to determine mitochondrial and non-mitochondrial O_2 consumption) [12]. The MitoStress Test Assay was performed for 135 minutes on a Seahorse XF-e96 Bioanalyzer (Agilent) following the calibration of the utility plate with the injector port plate according to the manufacturer's instructions.

Measurement of mitochondrial ROS

Monocyte-like cells were incubated with 10 µmol/l MitoSox™ Red (Invitrogen™, Germany) for mitochondrial ROS measurement for 30 min at 37°C as described previously [30]. Then cells were washed twice with PBS containing calcium-chloride (1 mmol/l) and lysed with Triton X-100 buffer. The fluorescence intensity was analyzed by excitation at 490 ± 10 nm and emission at 595 ± 20 nm using a VICTOR X multilabel plate reader and subsequently normalized to protein content.

ELISA

The supernatants of untreated and D-galactose treated cells were collected and ELISA assays for TNF-α (1:200, BioLegend, Germany), IL-6 (1:200; BioLegend, Germany), IL-1β (1:200, BioLegend, Germany), and VEGF (1:200, BioLegend, Germany) were performed according to the manufacturer's instructions.

Flow cytometry

The purity of the $CD14^+$-isolated human monocytes was assessed by flow cytometry with human anti-CD45-VioBlue (Cat#130-110-637, Miltenyi Biotech, Germany), human anti-CD14-APC-Vio770 (Cat#130-113-706, Miltenyi Biotec, Germany), and human anti-CD3 FITC (Cat#130-113-128, Miltenyi Biotec, Germany) as previously described (data not shown) [25]. Flow cytometry was performed on an LSRII instrument (BD, Germany) and data were analyzed using FlowJo software.

Statistical analysis

Data was evaluated using the Mann-Whitney test (non-parametric test). Statistical significance was defined as p

< 0.05. Results with p< 0.05 were considered statistically significant; p< 0.01 highly significant and p< 0.001 very highly significant. All statistical analyses were performed using Prism 10 for Windows (GraphPad Software, San Diego, USA).

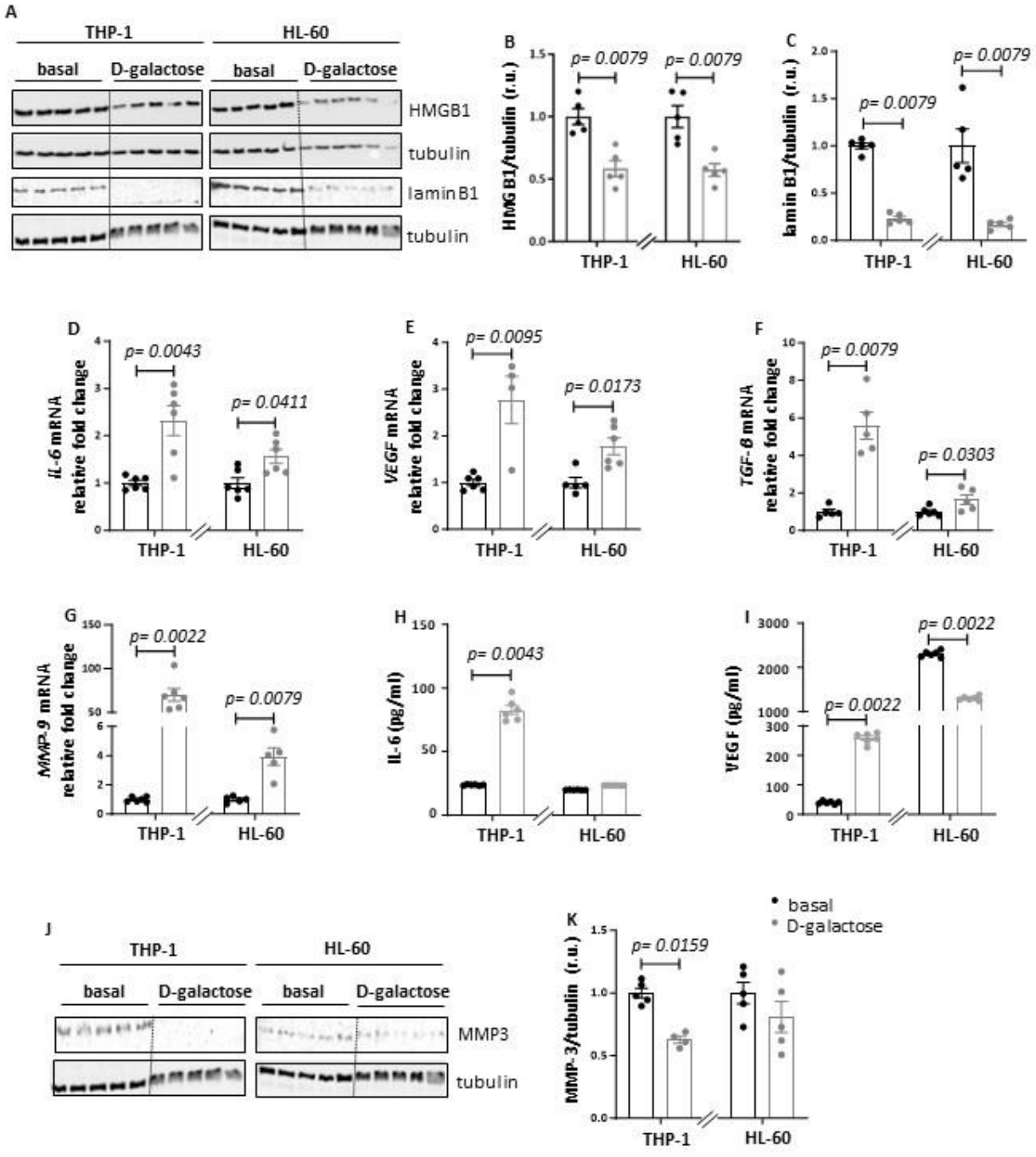

Figure 1. D-galactose induces cellular senescence in male and female monocyte-like cells. Western blot analyses and statistics of **(A and B)** HMGB1, **(A and C)** lamin B1, (J and K) MMP-3. Real-time PCR analyses of **(D)** *IL-6*, **(E)** *VEGF*, **(F)** *TGF-β*, and **(G)** *MMP-9* mRNA expression. ELISA analyses of **(H)** IL-6 and **(I)** VEGF were performed with supernatants of THP-1 and HL-60 cells untreated or treated with D-galactose (250 mM for 48 h). Data are shown as the means ± SEM (n = 4-6/group). All data, except for H and I, were normalized to the corresponding control, and Western Blot data are expressed in relative units (r.u.). Statistical analysis was conducted using the Mann-Whitney test. p value between p< 0.05 and p< 0.005.

RESULTS

D-galactose induces cellular senescence in monocyte-like cells

To characterize the model of cellular senescence, the expression of typical senescence markers was examined in male THP-1 and female HL-60 monocyte-like cells. A 48-h treatment with D-galactose reduced the expression of the nuclear proteins HMGB1 and lamin B1 equally in both cell types (p = 0.079) (Fig. 1 A-C). Furthermore, the expression of several SASP-related markers, i.e., *IL-6*, *VEGF*, *TGF-β*, and *MMP-9*, was significantly elevated at mRNA level following D-galactose treatment in both male and female monocyte-like cells (p < 0.05) (Fig. 1D-G). In addition, the IL-6 and VEGF concentration in supernatant was significantly elevated following D-galactose treatment in male monocyte-like cells (p= 0.0043 and p= 0.0022, respectively), whereas in HL-60 cells, D-galactose treatment significantly decreased VEGF levels (p= 0.0022) (Fig. 1H and I). In contrast, in primary human monocytes, D-galactose significantly increased IL-6 and VEGF concentration in supernatant in monocytes from male and female donors (p= 0.0286) (Supplementary Fig. 1A and B). The MMP-3 protein levels were significantly decreased in male monocyte-like cells following D-galactose treatment (p= 0.0159), whereas in HL-60 cells, MMP-3 expression remained unchanged (p> 0.05) (Fig. 1J and K).

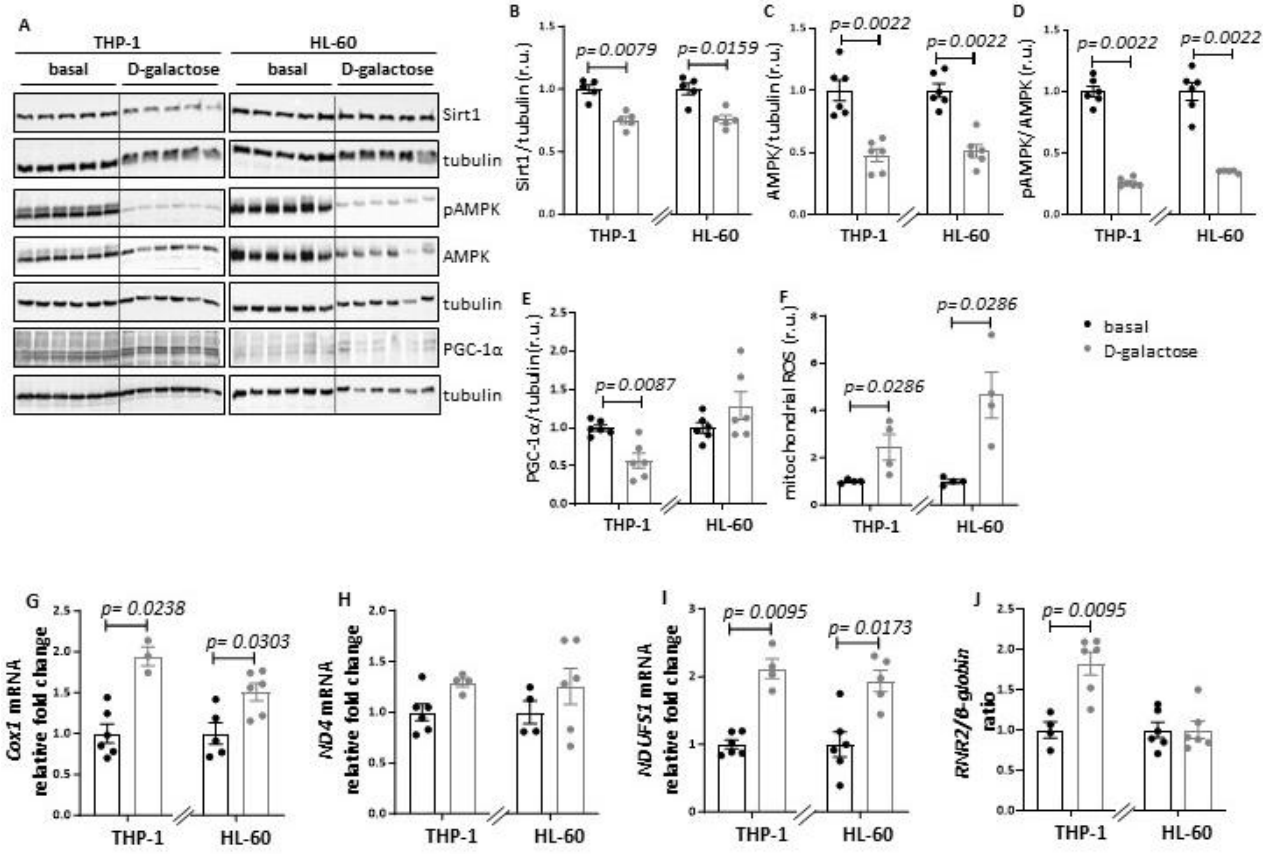

Figure 2. Effects of D-galactose on the expression of metabolic sensors and mitochondrial homeostasis in male and female monocyte-like cells. Western blot analyses and statistics of **(A and B)** Sirt1, **(A and C)** total AMPK, **(A and D)** phosphorylated AMPK (Thr172), and **(A and E)** PGC-1α were performed with lysates of THP-1 and HL-60 cells untreated or treated with D-galactose (250 mM for 48 h). **(F)** Analysis of mitochondrial ROS (MitoSox measurement) in untreated or treated with D-galactose (250 mM for 48 h) THP-1 and HL-60 cells. **(G-I)** Relative mRNA expression of mitochondria- and nucleus-encoded genes in THP-1 and HL-60 cells untreated or treated with D-galactose (250 mM for 48 h). **(J)** Mitochondrial mass presented as *RNR2/β-globin* ratio. Data are shown as the means ± SEM (n = 3-6/group). All data were normalized to the corresponding control, and Western Blot data are expressed in relative units (r.u.). Statistical analysis was conducted using the Mann-Whitney test. p value between p< 0.05 and p< 0.005.

To assess whether sex hormones influence the senescence phenotype of monocyte-like cells, THP-1 and HL-60 were treated with E2 and testosterone following D-galactose treatment. E2 treatment had no significant effect

on *TGF-β* or *MMP9* expression in THP-1 cells regardless of D-galactose treatment (p> 0.05) (Supplmentary Fig. 2A and B). In HL-60 cells, however, E2 treatment significantly increased *TGF-β* expression in untreated cells (p= 0.0136) (Supplmentary Fig. 2A). In contrast, testosterone treatment decreased TGF-β expression in untreated THP-1 and HL-60 cells (p= 0.0848 and p= 0.0664, respectively), while the MMP9 expression remained unchanged (p> 0.05) (Supplementary Fig. 3A and B).

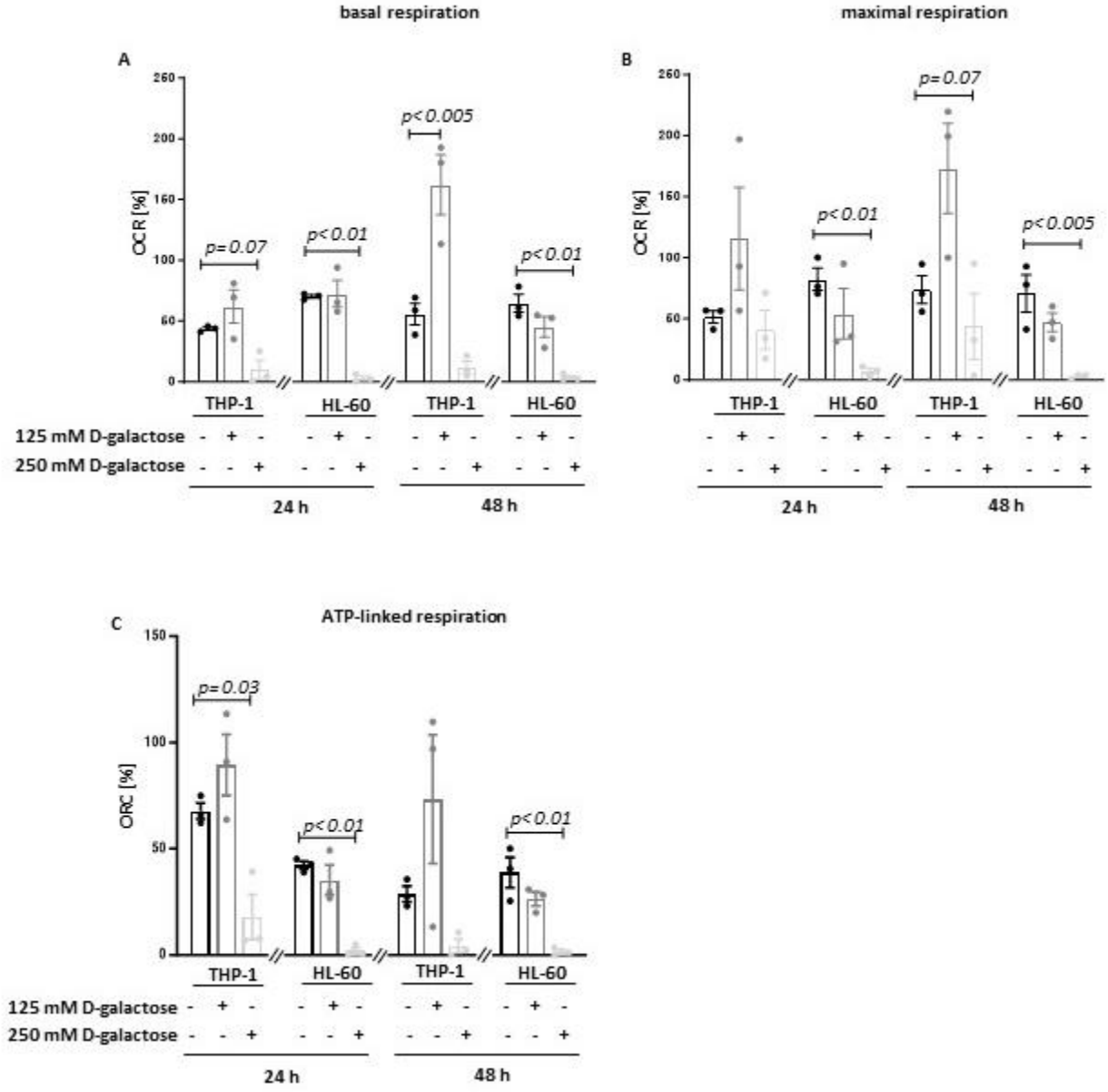

Figure 3. Effects of D-galactose on the mitochondrial respiration in male and female monocyte-like cells. Statistics of **(A)** basal respiration, **(B)** maximal respiration, and **(C)** ATP-linked respiration in THP-1 and HL-60 cells untreated or treated with D-galactose (125 mM or 250 mM for 24 or 48 h). Data are shown as the means ± SEM (n = 3/group with 5 technical replicates). Statistical analysis was conducted using the Mann-Whitney test. p value between p< 0.05 and p< 0.005.

Effect of D-galactose on metabolic sensors and mitochondrial homeostasis

Sirt1 and AMPK play a key role in aging processes, and the expression and activity of both metabolic sensors are reduced in elderly individuals [31]. In the present study, we found that in senescent monocyte-like cells, the total protein level of Sirt1 and AMPK, as well as the phosphorylated form of AMPK, were significantly downregulated in both cell types (p < 0.05) (Fig. 2A-D). Further analysis of mitochondrial biogenesis revealed that

PGC-1α was significantly decreased in male senescent monocyte-like cells ($p = 0.087$) (Fig. 2E).

Since downregulation of Sirt1 and AMPK may promote mitochondrial dysfunction and oxidative stress, mitochondrial homeostasis was analyzed. Mitochondria in D-gatalctose-treated cells were characterized by elevated ROS formation ($p = 0.0286$) (Fig. 2F), suggesting dysfunctional mitochondria. In addition, the expression of mitochondria-encoded gene *Cox1* and the nucleus-encoded gene *NDUFS1* was significantly elevated in male and female cells ($p < 0.05$) (Fig. 2G and I), whereas mitochondrial mass was elevated only in male cells ($p = 0.095$) (Fig. 2J).

To further investigate the effects of D-galactose on mitochondrial homeostasis, we analyzed the mitochondrial respiration in our immunosenescence model. Treatment with 250 mM D-galactose for 24 or 48 h profoundly decreased the basal and maximal respiration of THP-1 and HL-60 cells. (Fig. 3A and B). In contrast, administration of 125 mM D-galactose for 48 h caused significant increase of basal and maximal respiration in THP-1 cells ($p < 0.005$ and $p = 0.07$, respectively), while the mitochondrial respiration in HL-60 cells was not affected (Fig. 3A and B). In addition, a significant reduction in ATP-linked respiration was observed in both cell types exclusively at high concentrations of D-galactose (250 mM) following 24 h or 48 h exposure ($p< 0.01$) (Fig. 3C).

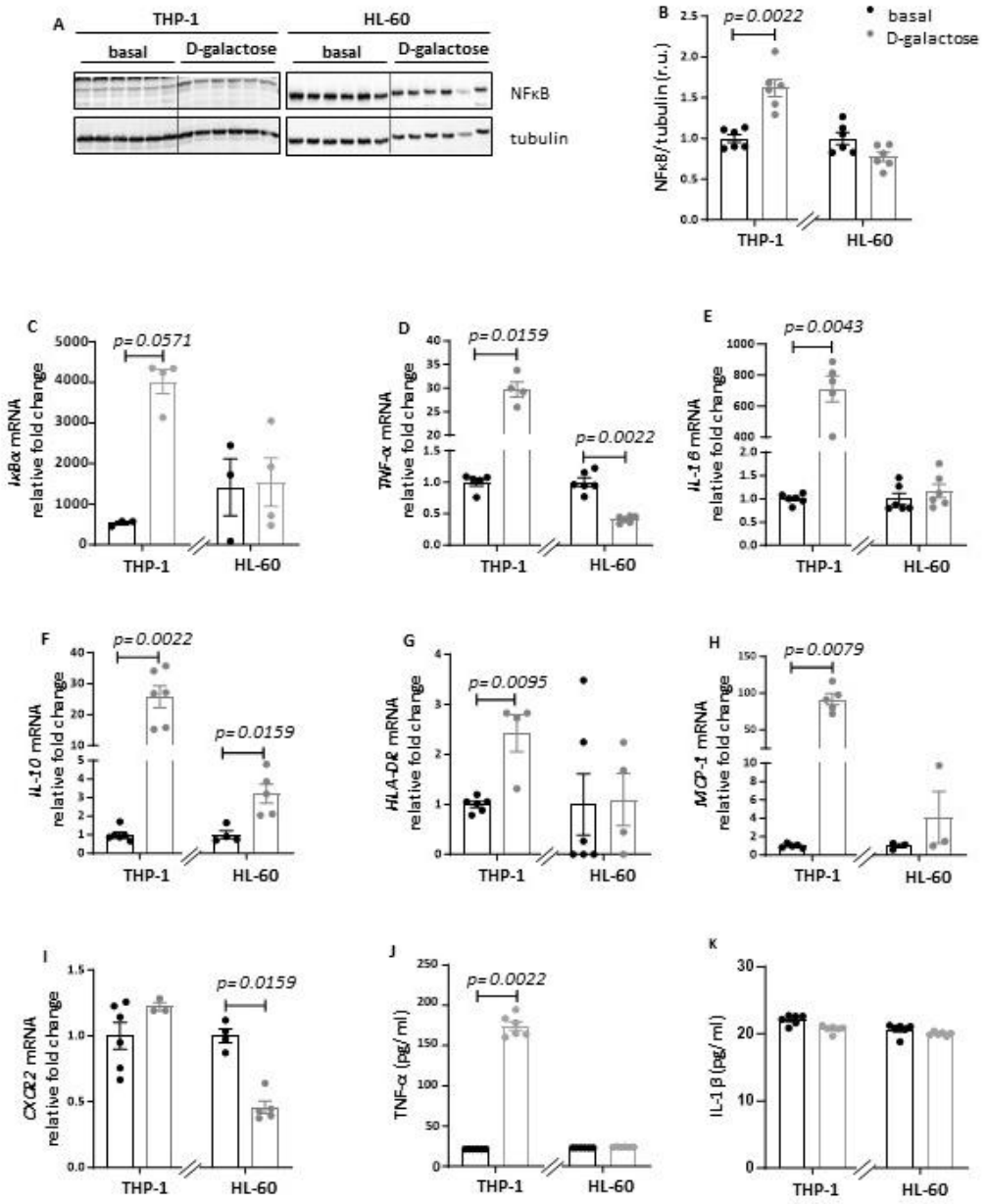

Figure 4. D-galactose induces a pro-inflammatory phenotype in THP-1 cells. (A-B) Western blot analysis and statistics of NF-κB. Real-time PCR analyses of **(C)** *IκBα*, **(D)** *TNF-α*, (E) *IL-1β*, (F) *IL-10*, **(G)** *HLA-DR*, **(H)** *MCP-1* and **(I)** *CXCR2* mRNA expression, and ELISA analyses of **(J)** TNF-α and **(K)** IL-1β were performed with lysates of THP-1 and HL-60 cells, untreated or treated with D-galactose (250 mM for 48 h). Data are shown as the means ± SEM (n = 3-6/group). All data, except for C, J, and H, were normalized to the corresponding control and Western Blot data are expressed in relative units (r.u.). Statistical analysis was conducted using the Mann-Whitney test. p value between $p< 0.05$ and $p< 0.005$.

Sex differences in the inflammatory response of senescent monocyte-like cells

We have previously reported that male macrophages are more susceptible to LPS and exhibit a more pronounced pro-inflammatory phenotype than female cells [11]. In the current study, we investigated whether the induction of cellular senescence affects the expression of pro-inflammatory mediators in monocyte-like cells. NF-κB, a prominent pro-inflammatory factor and key regulator of cytokine expression, was significantly upregulated in male THP-1 cells following treatment with D-galactose, while its expression remained unchanged in female HL-60 cells ($p= 0.0022$ and $p> 0.05$, respectively) (Fig. 4A and B). The mRNA expression of *IκBα* was increased in THP-1 cells following treatment with D-galactose ($p=0.0571$) (Fig. 4C). Similar to NF-κB, the mRNA expression of the pro-inflammatory mediators *TNF-α* and *IL-1β*, was significantly elevated only in male senescent monocyte-like cells (Fig. 4D and E). In THP-1 cells treated with D-galactose, TNF-α concentration in supernatant was significantly elevated, whereas IL-1β concentration was not affected ($p= 0.0022$ and $p> 0.05$, respectively) (Fig. 4J and K). In accordance, D-galactose treatment significantly increased the TNF-α concentration in both male and female primary monocytes ($p= 0.0286$), while IL-1β levels remained unchanged ($p> 0.05$) (Suppl. Figure 1C and D). The mRNA level of the anti-inflammatory cytokine *IL-10* was significantly upregulated in both cell lines following D-galactose treatment; however, male senescent monocyte-like cells showed a markedly higher elevation ($p= 0.022$ and $p= 0.0159$, respectively) (Fig. 4F). D-galactose increased the expression of *HLA-DR* and *MCP-1* solely in male THP-1 cells ($p= 0.0095$ and $p= 0.0079$, respectively) (Fig. 4G and H), while *CXCR2* expression was downregulated only in female HL-60 cells ($p= 0.0159$) (Fig. 4I).

E2 treatment had no effect on the expression of the pro-inflammatory markers *TNF-α*, *IL-1β,* and *MCP-1* in either THP-1 cells or in HL-60, independent of D-galactose treatment ($p> 0.05$) (Suppl. Figure 2C-E). In contrast, testosterone treatment significantly increased MCP-1 expression in senescent HL-60 cells ($p= 0.0448$) (Supplementary Fig. 3E).

Non-senescent THP-1 cells treated with either E2 or testosterone exhibited significantly high expression of *IL-1β*, *MCP-1*, *MMP9* and *TGF-β* compared to non-senescent HL-60 cells ($p< 0.0001$). Conversely, *TNF-α* expression was significantly increased in non-senescent HL-60 cells following E2 or testosterone treatment ($p< 0.01$ and $p< 0.001$, respectively) (Supplemenatry Fig. 4A and B).

DISCUSSION

The primary aim of this study was to explore the sex-specific differences in the impact of cellular senescence on mitochondrial homeostasis and the inflammatory state of monocytes. The main findings are as follows: (i) In both male and female monocyte-like cells D-galactose treatment resulted in cellular senescence indicated by several senescence markers, including SASP-related mediators. (ii) Cellular senescence led to comparable downregulation of metabolic sensors (Sirt1 and AMPK), suppression of mitochondrial respiration, and an elevation of mitochondrial ROS formation in male and female cells. (iii) Cellular senescence elicited a significant pro-inflammatory response specifically in male monocyte-like cells.

There are various methods to induce cellular senescence *in vitro*: radiation (ionizing and ultraviolet), activation of oncogenes, oxidative stress, and mitochondrial dysfunction [32]. One widely-used approach in *in vivo* and *in vitro* studies is treatment with D-galactose, which is naturally found in the body and various foods [33].

To investigate the effects of cellular senescence on the functionality of monocytes, we treated male and female monocyte-like cells with D-Galactose, a compound commonly used as a model for inducing cellular senescence [33-35]. In the majority of *in vitro* studies, including the present one, high concentrations of D-galactose (100-300 mM) have been used to induce cellular senescence, which may raise the question of indirect effects of hyperosmotic environment. Indeed, several reports demonstrated the induction of senescence in various types of cells by culturing in a medium with elevated osmolarity, i.e., 400-450 mOsm [36, 37]. To overcome this problem some studies using D-galactose to induce senescence applied an osmotic control by treating cells with an equal concentration of mannitol and found no effect on cell viability or proliferation [38, 39], although the effects of mannitol on senescence markers have not been investigated. Thus, D-galactose appears to

inhibit cell proliferation via mechanisms other than hyperosmolarity.

Recent studies have shown that a 48-hour exposure to D-galactose can effectively induce key senescence markers, including cell cycle arrest, increased senescence-associated β-galactosidase activity, and upregulation of senescence-related genes such as p16 and p21 [40]. For instance, Saiyasilp et al. (2025) demonstrated robust aging phenotypes and gene expression changes after human dental pulp cells were treated with 10 g/L D-galactose for 48 hours [40]. Similarly, Xu et al. observed premature senescence features in lens epithelial cells following a 48-hour exposure to 125 mM D-galactose [38]. Hou et al. reported elevated senescence markers and SA-β-gal activity in astrocytic cells with D-galactose treatments exceeding 48 hours, indicating that early senescence features initiate within this timeframe [41]. Moreover, D-galactose has been widely used in both in vitro and in vivo models to mimic aging-related cellular dysfunction and senescence, with early molecular and phenotypic changes evident within 48 hours in multiple cell types [42]. However, longer treatment (3–7 days or more) are generally required to observe more extensive morphological alterations and sustained SASP development [43].

Consistent with our experimental approach, Hsieh et al. induced cellular senescence by exposing THP-1 cells to 250 mM D-galactose for 48 h [26]. They observed no cytotoxic effect, as determined by the LDH assay; however, senescence was evident through multiple markers, including G1 phase cell cycle arrest, elevated senescence-associated β-galactosidase activity, and upregulated expression of p16 and p21 [26].

In this study, we used both cell lines (THP-1 and HL-60) from the Leibniz Institute DSMZ, which are of male and female origin, respectively. The karyotype of those THP-1 cells shows an XY chromosomal pattern, while those HL-60 cells show an XX pattern. For analysis, we used THP-1 cells as a model for male monocytes. Adati et al. reported that THP-1 cells possess a diploid 46, XY karyotype with multiple chromosomal aberrations. The authors also emphasized that the male origin of these cells should be taken into account when investigating sex differences [44]. Conversely, HL-60 cells, commonly used as a model for female monocyte-like cells, have been shown by karyotype analysis to contain only a single X chromosome with no Y chromosome, consistent with a female origin [45]. Although both cell lines exhibit chromosomal abnormalities typical for immortalized lines, their distinct sex chromosome complements support their use as models representing male and female monocyte-like cells. The finding of the present study, obtained by treating male THP-1 and female HL-60 monocyte-like cells with 250 mM D-galactose for 48 h, revealed a cellular senescence phenotype in a sex-independent manner. In particular, a significant downregulation of the nuclear proteins HMGB1 and lamin B1, accompanied by an upregulation of key SASP-related mediators, was observed in both male and female monocyte-like cells, even though the effects were substantially more pronounced in male cells. In line with this, multiple studies have reported a stronger phenotypic display of cellular senescence in male compared to female cells [46-48]. In this study, we demonstrated decreased expression of MMP3 at protein level following the induction of cellular senescence in male monocyte-like cells. Consistent with our findings, several studies have reported downregulation of MMP-3 in senescent cells, which may contribute to alterations in extracellular matrix remodeling during aging [49-51].

We also assessed other aging-related parameters, such as the expression of metabolic sensors (e.g., Sirt1 and AMPK) and production of mitochondrial ROS. As expected, the expression of Sirt1 and the activity of AMPK (measured as the pAMPK/AMPK ratio) were significantly reduced in senescent monocytes in a sex-independent manner. In line with our findings, a previous study demonstrated the sex-independent dephosphorylation of AMPK in cardiac aging [31]. Moreover, Zarei et al. found that the long-term treatment with D-galactose significantly downregulated the expression of Sirt1 in the rat heart [52]. Similarly, Xu et al., applying an *in vitro* model of lens epithelial cells treated with 125 mM D-galactose for 24-48 h, revealed a significant accumulation of mitochondrial ROS, reduction of cellular ATP, and Sirt1 downregulation [38].

Mounting evidence suggests that aging and cellular senescence are associated with mitochondrial dysfunction [38]. To determine whether cellular senescence is accompanied by mitochondrial malfunction, oxygen consumption was analyzed in the present study. We found a significant reduction of mitochondrial respiration in senescent cells in a sex-independent manner. In agreement with our data, several studies have demonstrated that D-galactose profoundly affects mitochondrial respiration and ATP production [53-55].

Enhanced ROS formation is a hallmark of cellular senescence and is also induced by D-galactose [38, 56, 57]. In the present study, we observed a significant elevation of mitochondrial ROS after the D-galactose treatment in both cell types, confirming the accumulation of dysfunctional mitochondria. Several reports suggest that aging is associated with an imbalance between ROS generation and the cellular antioxidant defense system [58-60], leading to exacerbated ROS production. Regarding sex-specific differences in ROS production, Gaignard et al. demonstrated increased oxidative stress in aged male mice [61]. However, another study revealed no

sex-related differences in oxidative stress and mitochondrial bioenergetic functions between aged male and female mice [62].

In the present study, reduced Sirt1 expression and AMPK phosphorylation, as well as the mitochondrial respiration, were accompanied by reduced expression of key regulators of mitochondrial function and biogenesis, i.e., PGC-1α in male cells. Surprisingly, our findings showed an elevated expression of OXPHOS-related mitochondrial genes in both male and female senescent monocyte-like cells and an elevated mitochondrial DNA content in male senescent cells. This phenomenon may be due to the upregulation of other pathways leading to OXPHOS gene upregulation and is probably of a compensatory nature.

Finally, we analyzed sex-related differences in the inflammatory state of senescent cells. Our study revealed an upregulation of prominent pro-inflammatory markers (e.g., NF-κB, *TNF-α*, *IL-1β*, *HLA-DR,* and *MCP-1*) exclusively in male senescent monocyte-like cells following D-galactose treatment. In contrast, D-galactose induced a significant inflammatory response in primary human monocytes from both male and female donors. These findings suggest that the inflammatory response associated with D-galactose-induced cellular senescence exhibits sex-specific differences dependent on cell type or origen. Interestingly, mRNA expression of *IκBα* was elevated in THP-1 cells following D-galactose treatment. Typically, upregulation of IκBα suppresses NF-κB expression and activity [63]. However, simultaneous upregulation of both IκBα and NF-κB suggests chronic activation of the NF-κB pathway, which is commonly observed during chronic inflammation and tumor progression [64-66]. MCP-1 and CXCR2 are critical mediators of the SASP and central to the propagation and maintenance of senescence and inflammation [67, 68]. MCP-1 is a dominant SASP chemokine secreted by senescent cells, reinforcing senescence in healthy cells through activation of its receptor CCR2. This axis triggers the ROS-p38-MAPK-p53/p21 pathway in both an autocrine and paracrine manner, amplifying senescence and stabilizing the growth-arrested phenotype. CXCR2, a receptor for several pro-inflammatory chemokines, similarly reinforces the senescent state. Its activation triggers p53-dependent cell cycle arrest and enhances the production of other SASP cytokines, thus amplifying local and systemic inflammatory responses [67, 69]. By measuring MCP-1 and CXCR2 in D-galactose-induced senescent THP-1 and HL-60 cells, we tracked key SASP mediators, enabling detailed analysis of the mechanisms governing senescence-associated inflammation and its propagation. Several studies have reported increased expression of pro-inflammatory mediators in the aged female brain and heart tissue in mice and humans [31, 70]. However, these studies focused on normal aging processes rather than on stress-induced cellular senescence. Consistent with our study, Hsieh et al., observed significant upregulation of IL-10 following treatment with 250 mM D-galactose in a similar model of senescence in THP-1 monocytes [26]. This suggests that D-galactose-induced cellular senescence triggers the inflammatory response in monocytes.

Furthermore, we investigated the role of sex hormones on the senescence and proinflammatory phenotype of THP-1 and HL-60 cells. The observed effects align with established mechanisms: estrogen exerts anti-inflammatory [71] and senescence-suppressing effects by modulating the NF-κB pathway and restricting inflammatory gene expression [71]. In a previous work with male and female bone marrow macrophages, we demonstrated that E2 treatment significantly increased the expression of pro-inflammatory mediators in male but not in female macrophages [11]. Conversely, E2 decreased the TNF-α expression in supernatants from pro-inflammatory RAW264.7 cells [12]. In this study, we observed a reduction of TGF-β in HL-60 cells following E2 treatment, suggesting anti-inflammatory effects in female-derived cells. The effects of estrogen seem highly cell type- and tissue-dependent, influenced by the differential expression of estrogen receptors (ERα and ERβ), coregulators, and interacting transcription factors across diverse cellular contexts. In contrast to estrogen, testosterone promotes pro-inflammatory signaling and exacerbates senescence-associated inflammation [72]. It is known to upregulate MCP-1 and related cytokines in myeloid cells by enhancing inflammatory cascades and can potentiate senescence phenotypes, particularly in promyelocytic lineages such as HL-60 [73]. This mechanism is consistent with the increased MCP-1 observed in senescent HL-60 cells following testosterone treatment [74]).

Strengths and limitations

A key strength of this study lies in the use of well-established male and female monocyte-like cell lines, which allowed us to investigate sex-specific differences in immune cell senescence under standardized in vitro conditions. These immortalized cell models are widely used in immunological research and provide reproducible experimental results and mechanistic insights. Nevertheless, important limitations must be acknowledged. Both cell lines harbor chromosomal abnormalities and differ from primary cells in gene expression and functional responsiveness, which may influence senescence pathways and hormone responsiveness. To address these limitations and increase

the translational relevance of our findings, we validated key results, i.e. IL-6, VEGF, TNF-α, and IL-1β levels in age-matched, primary human monocytes from male and female donors, which confirmed the observed pro-inflammatory senescence-associated phenotype. Future studies are required to integrate in vitro and in vivo models for comprehensive elucidation of sex-dependent mechanisms in cellular senescence and inflammaging.

In addition, THP-1 (monocytic) and HL-60 (promyelocytic) cell lines represent distinct myeloid lineages. Upon differentiation, THP-1 cells closely resemble the phenotype of macrophages [75], whereas HL-60 cells are primarily used to model neutrophil differentiation, though under certain protocols they may acquire monocyte-like traits [76]. In the present study, both cell lines were treated with D-galactose to induce cellular senescence, enabling the assessment of sex-specific differences in senescence and inflammatory signaling. Nonetheless, variations in cytokine responses and inflammatory marker expression are expected, reflecting their differing developmental origins and signaling characteristics. Another limitation of the present study is that we were unable to directly assess the activation of NF-κB. Although total NF-κB protein levels were measured, these alone do not fully capture the activation status of NF-κB signaling. This limitation restricts the conclusions that can be drawn regarding the effects of D-galactose on NF-κB activity.

Conclusion

In conclusion, the present study revealed that although D-galactose leads to comparable cellular senescence in male and female monocyte-like cells, specifically male cells developed the pro-inflammatory state.

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Ethical approval

We obtained informed consent from all study participants. Sample collection and experimental protocols were approved by the Scientific Board of Charité – Universitätsmedizin Berlin (EA4/154/16). All experiments were performed in accordance with the German regulations and the ethical standards outlined in the Declaration of Helsinki.

Author contribution

L.H.S. performed the cell culture experiments, real-time PCR experiments, Western blot experiments, mitochondrial mass measurements, analyzed the data and wrote the main manuscript text. J.T. wrote the main manuscript text. I.A.F. conducted seahorse experiments and analyzed the data. O.V. performed cell cultures from human monocytes and FACS analyses. Y.L. analyzed the data and wrote the main part of the manuscript, and M.B. conceived the project, analyzed the data, prepared the figures, and wrote the main manuscript text. All authors contributed to the manuscript by providing comments and revisions.

Conflict of interest

The authors declare that they have no conflict of interest related to this manuscript.

Supplementary Materials

The supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2025.0871.

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