



RESEARCH ARTICLE

Preadipocyte secretory factors differentially modulate murine macrophage functions during aging which are reversed by the application of phytochemical EGCG

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Abstract The present study aimed at evaluating the role of senescent cell microenvironment as an extrinsic causal factor for altered age-associated macrophage functions, and that whether such changes could be ameliorated by the application of tea catechin epigallocatechin gallate (EGCG). To ascertain this, we analyzed the impact of secretory metabolites of proliferating (P) and senescent (S) preadipocyte cells on the induction of phenotypic and functional characteristics associated with aging in macrophages isolated from young (YM) and old (OM) C57BL/6J mice. The role of EGCG as alleviator of preadipocyte media-induced senescence and inflamm-aging was evaluated in OM. Results revealed strong age-related dysregulation in macrophage functions as evident by decreased CD11b expression, enhanced expression of cytokines (IL-6/TNF- α /IL-1 β /IL-10) and cell cycle inhibitors p53/p21^{WAF1}/p16^{Ink4a}, as well as augmentation of M2 phenotype (*Arg1/Msr1/Mrc1*) and SA- β -gal activity. Ex vivo exposure of macrophages (YM and OM) to secretory factors of preadipocytes induced differential effects, and treatment with S culture media largely showed an augmentation of senescent

phenotype, particularly in the YM. Pretreatment with EGCG (10 μ M) to OM caused a dramatic reversal of both age-associated and preadipocyte media-induced changes as evident from upregulation of CD11b and ROS levels, inhibition of inflammatory makers, attenuation of p53/p21^{WAF1}/p16^{Ink4a} expression and SA- β -gal activity. Our results indicate vital role of adipose tissue-mediated extrinsic factors in shaping macrophage phenotype and functions during aging. It is also apparent that EGCG is a promising candidate in developing preventive therapies aimed at alleviating macrophage inflamm-aging and senescence that may help curb incidences of inflammatory disorders in elderly.

Keywords Macrophage · Senescence · Inflammation · EGCG · SA- β -gal · ROS

Abbreviations

YM	Young macrophages
OM	Old macrophages
P	Proliferating preadipocyte cells
S	Senescent preadipocyte cells
EGCG	Epigallocatechin gallate
H ₂ O ₂	Hydrogen peroxide
ROS	Reactive oxygen species

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Introduction

Macrophages are critical regulators of systemic homeostasis involving immunological and inflammatory responses as well as metabolic stability. However, this homeostasis is subject to characteristic age-associated changes and is often held accountable for increased morbidity and mortality in elderly (Sharma et al 2014; Vida et al. 2017). Recent advances in our understanding of molecular mechanisms governing the aging process have highlighted a pivotal role of senescent cells in shaping organismal aging (Bhatia-Dey et al. 2016; Sharma and Padwad 2019). Cellular senescence is a permanent cell cycle arrest characterized by loss of proliferative capacity and presence of certain characteristic cellular markers such as p16^{Ink4a}, p21^{WAF1}, SA- β -gal activity and senescence-associated secretory phenotype (SASP). Emerging evidences have suggested that senescent cells accumulate with age in various tissues and organs and their selective elimination in vivo has been shown to confer anti-aging attributes by delaying tumorigenesis and attenuation of age-related deterioration of several organs including kidney, heart, and fat (Baker et al. 2016). It is thus evident that senescent cells could be vital contributors to the progression of systemic effects of aging. However, it is yet unclear how senescent cells affect resident macrophage phenotype and functions *per se* that could be related to known age-associated dysregulations in macrophages. This is of particular significance to highly plastic and heterogenous cells, such as macrophages, which are in constant interaction with various tissues. In addition to this, direct evidence for the presence of cell senescence in macrophages is rare and unconvincing (Burton and Stolzing 2018), and it has been argued that activation of senescence markers could just represent a sub-class of macrophages (Hall et al. 2017). Moreover, accumulating evidences have established that chronic, sub-cutaneous and low-grade inflammatory activation (inflamm-aging) is amongst the leading causes of age-related morbidity and mortality (Franceschi et al. 2018). However, the exact nature of the source(s) of this inflammatory aggravation remains ambiguous. While it is reasoned that age-associated development of SASP could be an important cause of inflamm-aging; it has also been demonstrated that macrophages develop a pro-inflammatory phenotype during aging that may also contribute to

inflamm-aging (Prattichizzo et al. 2016). Taken together, it is evident that our current knowledge of the causes, characteristics and effects of cellular senescence in macrophages are very limited.

The adipose tissue has been conventionally observed as central to metabolic processes and is involved in the pathogenesis of diabetes and obesity. Emerging studies have also shown that adipose tissue dysfunction plays an essential role in regulating longevity, presumably by affecting inflamm-aging (Palmer and Kirkland 2016; Mau and Yung 2018). The age-associated inflamed adipose tissue is one of the persistent sources of inflammation and has been shown to be heavily infiltrated with macrophages. Further, macrophages have been shown to modulate the differentiation as well function of adipose tissues by paracrine effects (Suganami et al. 2005; Permana et al. 2009). However, studies exploring the role of age-associated changes in adipose tissue on the development of senescence or functional changes in macrophages during aging are very limited. Phytomolecules, such as green tea catechins, have been documented with several beneficial effects both in health and disease. Working on green tea EGCG, we have previously reported its anti-immunosenescence, anti-inflammatory, anti-senescence, senolytic and synbiotic attributes thereby suggesting its multi-faceted potency as an ameliorator of some of the deleterious aspects of aging (Sharma et al. 2017, 2019; Kumar et al. 2019). Notwithstanding this, there is still little knowledge regarding the role of EGCG or other natural bioactive molecules in influencing macrophage senescence and functions during aging, especially when exposed to senescent cell microenvironment. Considering these apparent lacunae in our understanding of macrophage senescence, the present work is aimed at addressing how senescent preadipocytes affect macrophage phenotype and functional changes relating to inflamm-aging and senescence, and whether such deleterious effects could be mitigated by pre-treatment of macrophages to EGCG. To ascertain this, peritoneal macrophages from young and old C57BL/6J mice were cultured in the presence or absence of media of proliferating or senescent 3T3-L1 preadipocytes followed by various cellular, biochemical and molecular analysis. The anti-senescence potential of EGCG in presence or absence of secretory factors of preadipocytes was subsequently evaluated in aged macrophages. 3T3-L1 preadipocytes were

chosen for this work since we had previously observed strong induction of senescence and SASP in this cell line on exposure to hydrogen peroxide (Kumar et al. 2019), which thus, could be utilized as source of senescent factors in the present study.

Methods

Animal husbandry

Young (4 months old) and aged (18 months old) male C57BL/6J mice were procured from the animal house facility of CSIR-IHBT, Palampur. All animals were kept in individual ventilated caging system (Citizen, India) at 25 ± 2 °C temperature, 40–70% relative humidity and were fed on a commercial basal diet and water ad libitum. The experiments were approved and executed as per recommendations of the animal ethics committee of the institute (Approval no. IAEC/IHBTP4/2016).

Isolation and culture of peritoneal macrophages

Murine peritoneal macrophages were isolated as described previously (Sharma et al. 2018). Briefly, macrophages were collected in Dulbecco's Modified Eagle Medium (DMEM) from the peritoneal cavity of mice. The obtained cell suspension was then incubated (10^6 cells/ml) overnight in a CO₂ incubator at 37 °C to allow attachment of the adherent cells. Subsequently, the non-adherent cells were removed after washing with PBS twice, and the remaining adherent macrophages were cultured in complete DMEM media for various cellular analyses. The macrophages from young (YM) and old animals (OM) were cultured separately.

3T3-L1 preadipocytes culture and senescence induction

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were cultured and maintained in DMEM supplemented with 10% FBS and 100 µg/ml of penicillin–streptomycin (15140122; Invitrogen, USA) at 37 °C in a 5% CO₂ incubator. Premature senescence was induced in preadipocytes by repeated exposure to a non-lethal concentration of hydrogen peroxide (150 µM) for 3 h per day and for three consecutive days

followed by additional culturing for three days (in absence of H₂O₂) as reported in our previous work (Kumar et al. 2019). Finally, the supernatant of the senescent preadipocyte cells (S) was collected, centrifuged, aliquoted and stored at – 80 °C till further use. Preadipocytes without H₂O₂ treatment served as proliferative controls and culture supernatant of these proliferative preadipocyte cells (P) was also collected and stored at – 80 °C.

Macrophage culture in media of proliferating and senescent 3T3-L1 cells

After overnight culture of peritoneal exudate followed by removal of non-adherent cells; the adherent peritoneal macrophages from both young and old animals were washed twice and incubated for 48 h in culture supernatants of P or S, while cells cultured in complete DMEM only were kept as control. For assays investigating the effects of EGCG, OM were pretreated with EGCG [@10 µM final concentration] for 24 h before addition of respective 3T3-L1 cell media (P or S) or complete DMEM as control. Cells were then incubated for 48 h in a humidified CO₂ incubator @ 37 °C followed by various cellular analyses.

Flow cytometry

Macrophage activation status was analyzed by flow cytometric determination of the expression of cell surface receptors CD11b and CD80 as reported previously (Lifshitz et al. 2010) using fluorescence-conjugated antibodies: PE-CD11b (Miltenyi Biotech Inc.) and APC-CD80 (Miltenyi Biotech Inc). In brief, peritoneal macrophages were trypsinized and incubated with antibodies for 1 h in dark at 4 °C as per manufacturer's instructions. After centrifugation, the cell pellet was washed twice with PBS followed by resuspension in FACS buffer for data acquisition. AMNIS ImageStream®X Mark II Imaging Flow Cytometer (Merck Millipore, Germany) was utilized for data acquisition while data were analyzed using INSPIRE ImageStream system software. Fluorescence minus one (FMO) controls were used in all staining to accurately identify the specific sub-population sets (Mahnke and Roederer 2007).

Analysis of intracellular ROS production

Cells staining positive to intracellular ROS production were determined using H₂DCFDA (D399; Molecular Probes, USA) dye as per manufacturer's protocol. In brief, cells were stained with H₂DCFDA (10 μM) for 30 min and the oxidation of the dye to 2,7-dichlorofluorescein (DCF) was captured in several images by fluorescence microscopy using the EVOS FL Auto2 Imaging System (Thermo Scientific, USA). The cells were manually counted and results are presented as percent ROS positive cells.

Senescence-associated β-galactosidase activity

β-galactosidase activity in macrophages were identified by using a commercially available kit (K802; Biovision, USA) as per manufacturer's protocol. In brief, cells were washed with PBS, fixed and incubated overnight in a staining solution in dark at 37 °C in the absence of CO₂. Next day, cells were observed under EVOS FL Auto 2 Imaging System (Thermo Scientific, USA) for the presence of blue color as a marker of β-galactosidase activity. Multiple images were recorded and percentage senescent cells were manually counted.

Measurement of cytokines in culture supernatants

After respective treatments, the cell supernatants were collected, centrifuged and aliquoted for the estimation of levels of IL-1β, IL-6, TNF-α, and IL-10 using sandwich ELISA kits following the manufacturer's protocol (eBioscience, San Diego, CA, USA). Samples were read by a Multi-Mode microplate reader (BioTek Instruments Inc, USA) as per manufacturer's instructions.

Table 1 List of primer sequences used for qRT-PCR

S. No.	Gene	Forward primer	Reverse primer
1	<i>Arg1</i>	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
2	<i>Mrc1</i>	CTCTGTTTCAGCTATTGGACGC	CGGAATTTCTGGGATTTCAGCTTC
3	<i>Msr1</i>	GCACAATCTGTGATGATCGCT	CCCAGCATCTTCTGAATGTGAA
4	<i>p53</i>	TTCTGTAGCTTCAGTTCATTGG	ATGGCAGTCATCCAGTCTTC
5	<i>p21</i>	TGCATCCGTTTCACCCAACC	CTCATTTCCTCAAAGTGCTATTTCAGG
6	<i>p16</i>	CCCAACGCCCCGAACT	GCAGAAGAGCTGCTACGTGAA
7	<i>GAPDH</i>	TCACTCAAGATTGTCAGCAATGC	TCATGAGCCCTCCACAATG

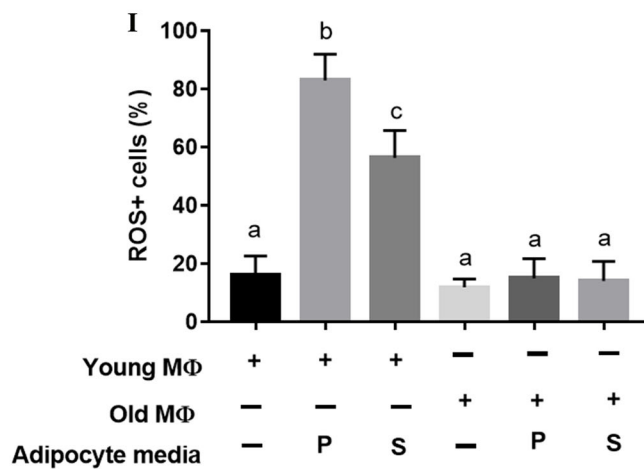
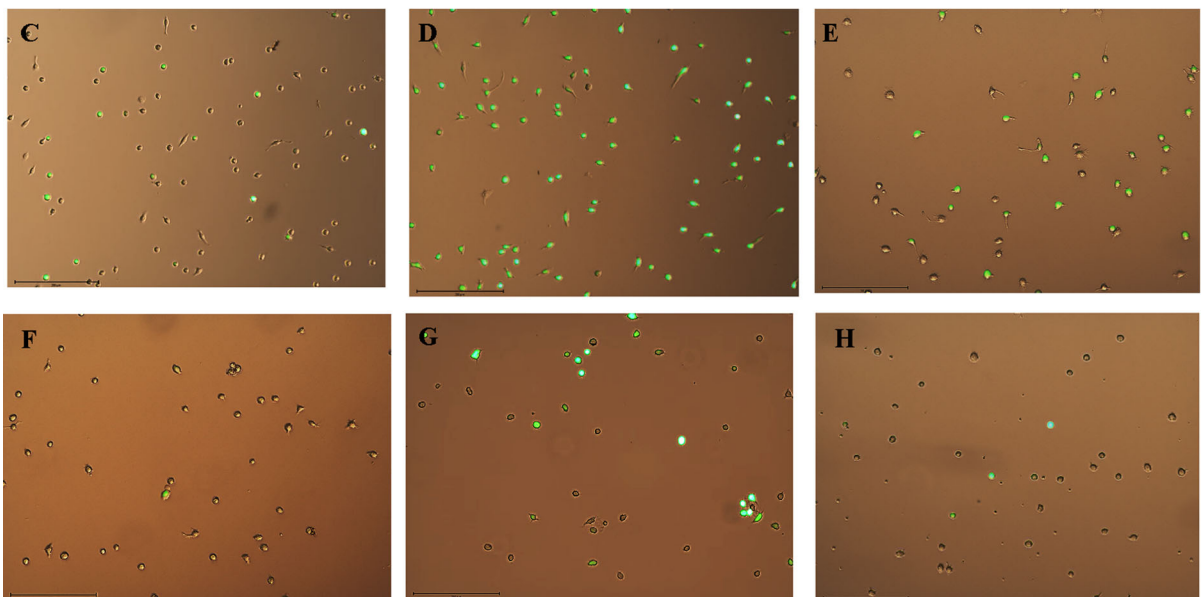
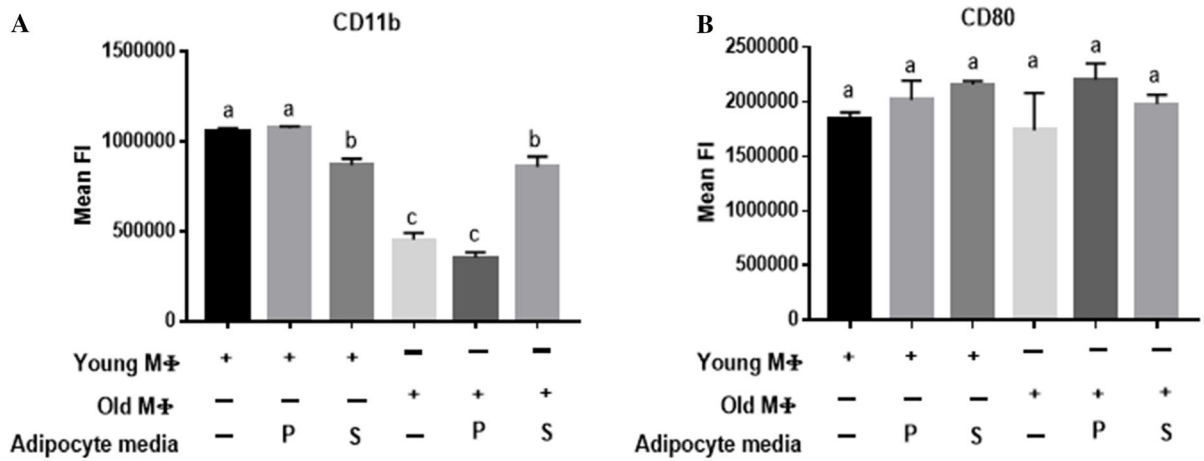
Fig. 1 Effect of proliferating (P) and senescent (S) preadipocyte media on activation of macrophages. Macrophages from young and old animals were treated in presence or absence of P or S preadipocyte media for 48 h and analyzed for **a** CD11b expression, **b** CD80 expression. Intracellular ROS levels were measured in young macrophages. **c** Control. **d** Treated with P preadipocyte media. **e** Treated with S preadipocyte media, and in old macrophages. **f** Control. **g** Treated with P preadipocyte media. **h** Treated with S preadipocyte media. **i** ROS + cells (%). Values are mean ± SEM (n = 3); values with different letters are significantly different at p < 0.05

Total RNA isolation and qRT-PCR

Total cellular RNA was isolated by using commercially available Qiagen RNeasy mini kit (74104, Qiagen, Germany). RNA was quantified and qualitatively assessed using a nanodrop instrument following which one step qRT-PCR was performed using QuantiFast SYBR Green PCR kit (204054; Qiagen, Germany). The primer sequences used for mRNA examination are provided in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to normalize the expression of tested genes. The PCR reactions were performed in 96-well plates using the Step one Plus™ Real-Time PCR system (Applied Biosystems, USA) and ΔΔCt method was used to determine the relative mRNA quantification (Sharma et al. 2017).

Statistical analyses

Results are presented as mean ± standard error of the mean (SEM) per group. Statistical differences were analyzed using GraphPad Prism (Version 6) software by one-way analysis of variance (ANOVA) followed



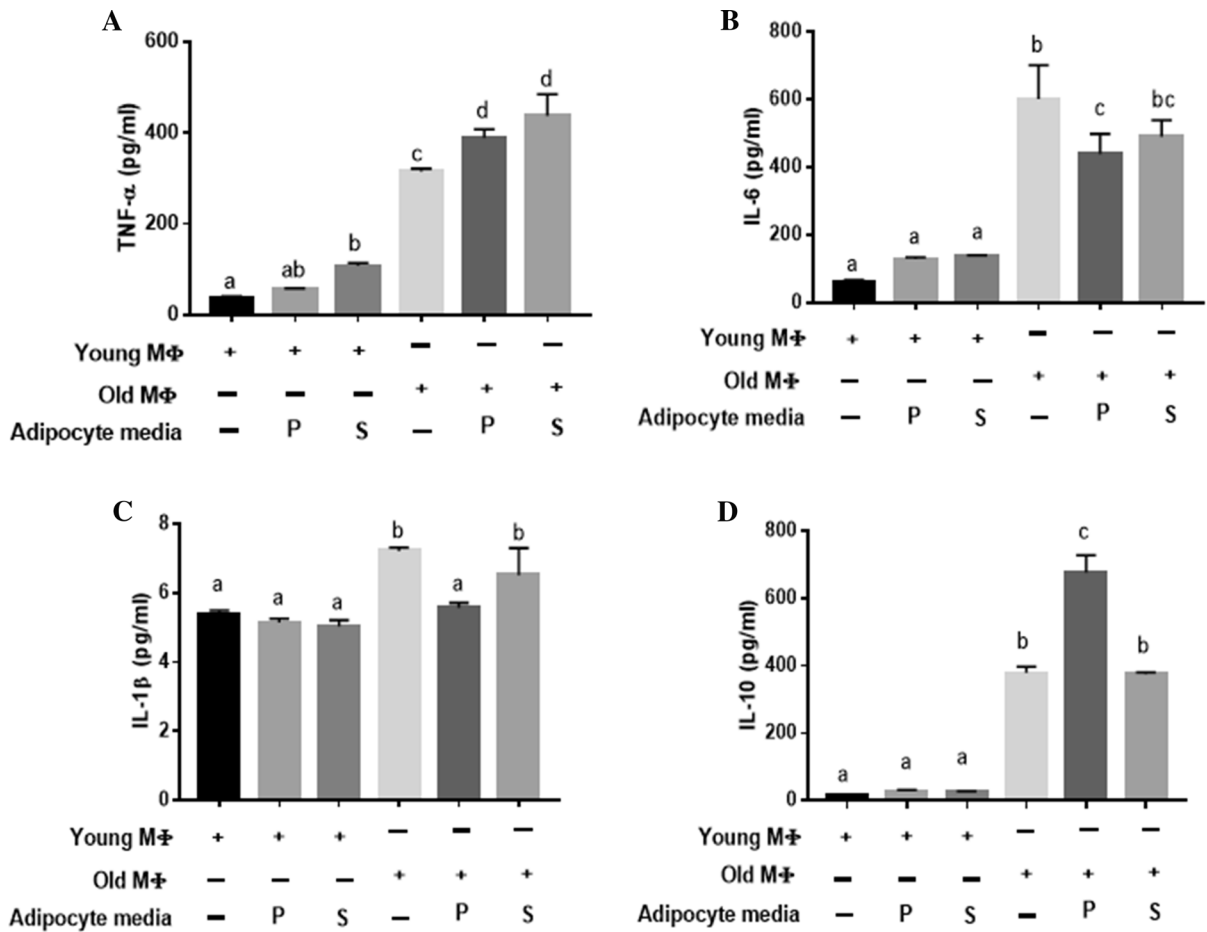


Fig. 2 Effect of proliferating (P) and senescent (S) preadipocyte media on pro-inflammatory attributes of macrophages. Macrophages from young and old animals were treated in presence or absence of P or S preadipocyte media for

48 h and analyzed for levels of cytokines **a** TNF- α , **b** IL-6, **c** IL-1 β , **d** IL-10. Values are mean \pm SEM (n = 3); values with different letters are significantly different at $p < 0.05$

by Tukey test to identify means which were considered significant at $p \leq 0.05$.

Results

Macrophage activation is differentially modulated with age and in response to secretory factors of preadipocyte media

To understand how macrophage functions are influenced by age as well as secretory factors of preadipocytes; YM and OM were cultured in presence or absence of culture media of P or S. Distinct morphological changes due to age as well as due to the presence of preadipocyte media were observed in

macrophages. OM were larger and elongated with irregular morphology while a more consistent and smooth morphology was evident in YM. Macrophages exposed to S media were visibly more heterogeneous and elongated in both YM and OM. Flow cytometric analyses revealed significant downregulation in the expression of CD11b in OM as compared to YM (Fig. 1a). Although no significant changes in macrophage CD11b expression (in either YM or OM) could be observed in presence of P media; however, when exposed to S media, a significant decrease in YM and a significant increase in OM CD11b expression was observed (Fig. 1a). No significant changes of any treatment could be observed for CD80 expression (Fig. 1b). Analyses of intracellular ROS positive cells revealed no significant age-associated changes in

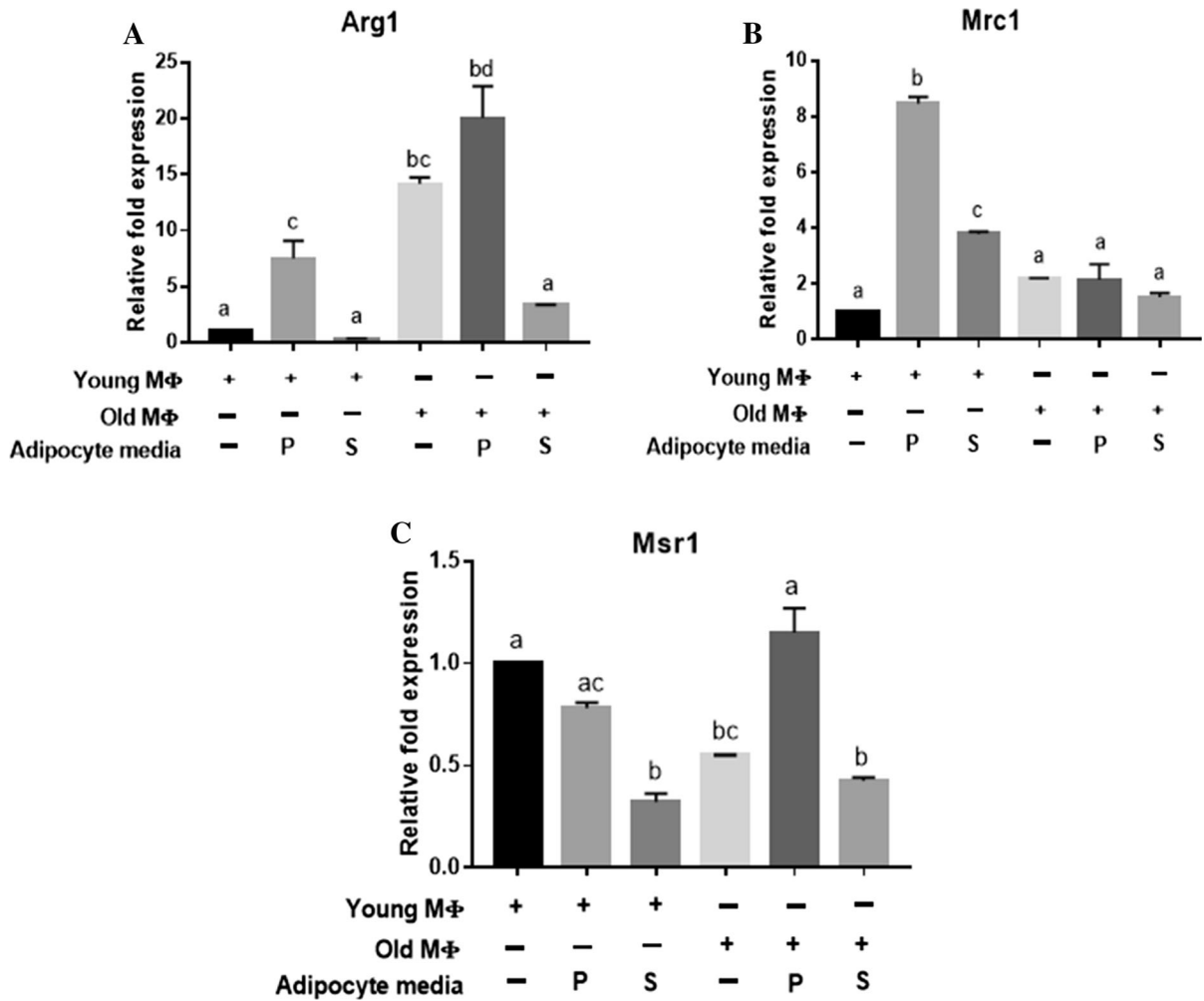


Fig. 3 Effect of proliferating (P) and senescent (S) preadipocyte media on macrophage M2 phenotype. Macrophages from young and old animals were treated in presence or absence of P or S preadipocyte media for 48 h and

relative gene expression of M2 markers were analyzed **a** Arg1, **b** Mrc1, **c** Msr1. Values are mean ± SEM (n = 3); values with different letters are significantly different at p < 0.05

resting YM or OM (Fig. 1c, f, i). However, a strong increase in ROS generating cells was observed in YM when exposed to media of both P and S (Fig. 1d, e, i). On the other hand, the relative ROS generation in OM was rather subdued with no significant change in ROS positive cells in presence of either P or S media (Fig. 1f–i).

Aged macrophages showed perturbed inflammatory homeostasis which was strongly influenced by exposure to P & S media

Cytokine analyses in macrophages was performed to assess modulation of inflammatory response with age and in presence of secretory factors of P and S culture media. A categoric and robust increase in all measured cytokines (IL-1β/IL-6/TNF-α/IL-10) was observed in OM as compared to YM (Fig. 2a–d). Culture of YM in S media significantly enhanced TNF-α levels while no significant effects of P media on cytokine production could be observed (Fig. 2a–d). In contrast, OM

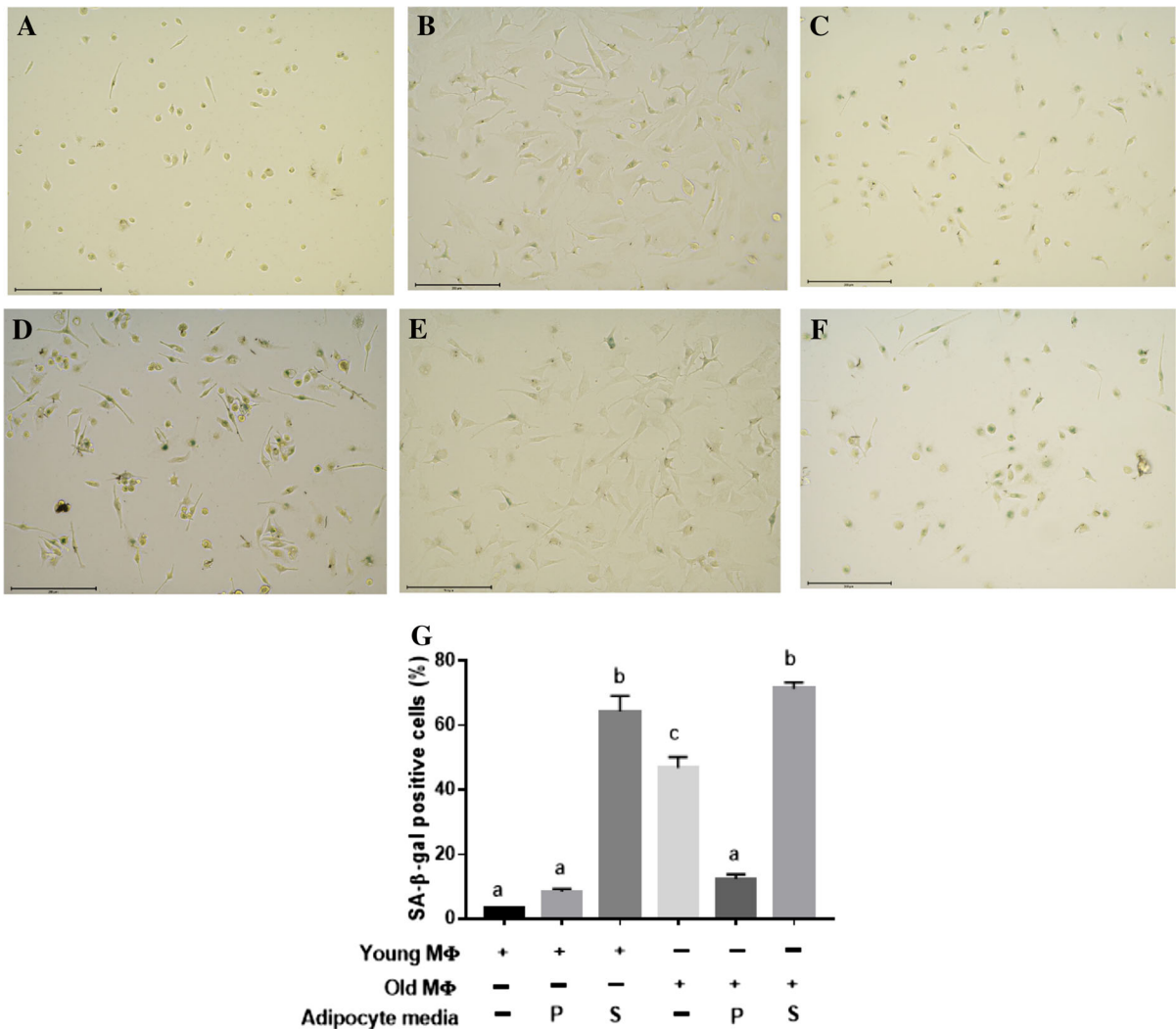


Fig. 4 Exposure to proliferating (P) and senescent (S) preadipocyte media modulates SA-β-gal activity in macrophages. Macrophages from young and old animals were treated in presence or absence of P or S preadipocyte media for 48 h and SA-β-gal activity was analyzed in young macrophages. **a** Control. **b** Treated with P preadipocyte media. **c** Treated with

S preadipocyte media, and in old macrophages. **d** Control. **e** Treated with P preadipocyte media. **f** Treated with S preadipocyte media. **g** % SA-β-gal positive cells. Values are mean ± SEM (n = 3); values with different letters are significantly different at p < 0.05

cultured in presence P and S media showed significant differential response to measured cytokines with no distinct discernible pattern (Fig. 2a–d). Levels of IL-10 appeared to be strongly modulated in OM with a significant increase in presence of P culture medium while a significant decrease in presence of S culture medium was observed (Fig. 2d).

M2 polarization in macrophages is influenced by both age and exposure to preadipocyte media

To assess the extent of M2 polarization in macrophages, gene expression levels of *Arg1*, *Mrc1* and *Msr1* were determined. Two genes associated with M2 phenotype, *Arg1* and *Mrc1* appeared upregulated in OM as compared to YM (Fig. 3a, b). Presence of secretory factors of proliferating preadipocytes in both YM and OM appeared to result in activation of M2

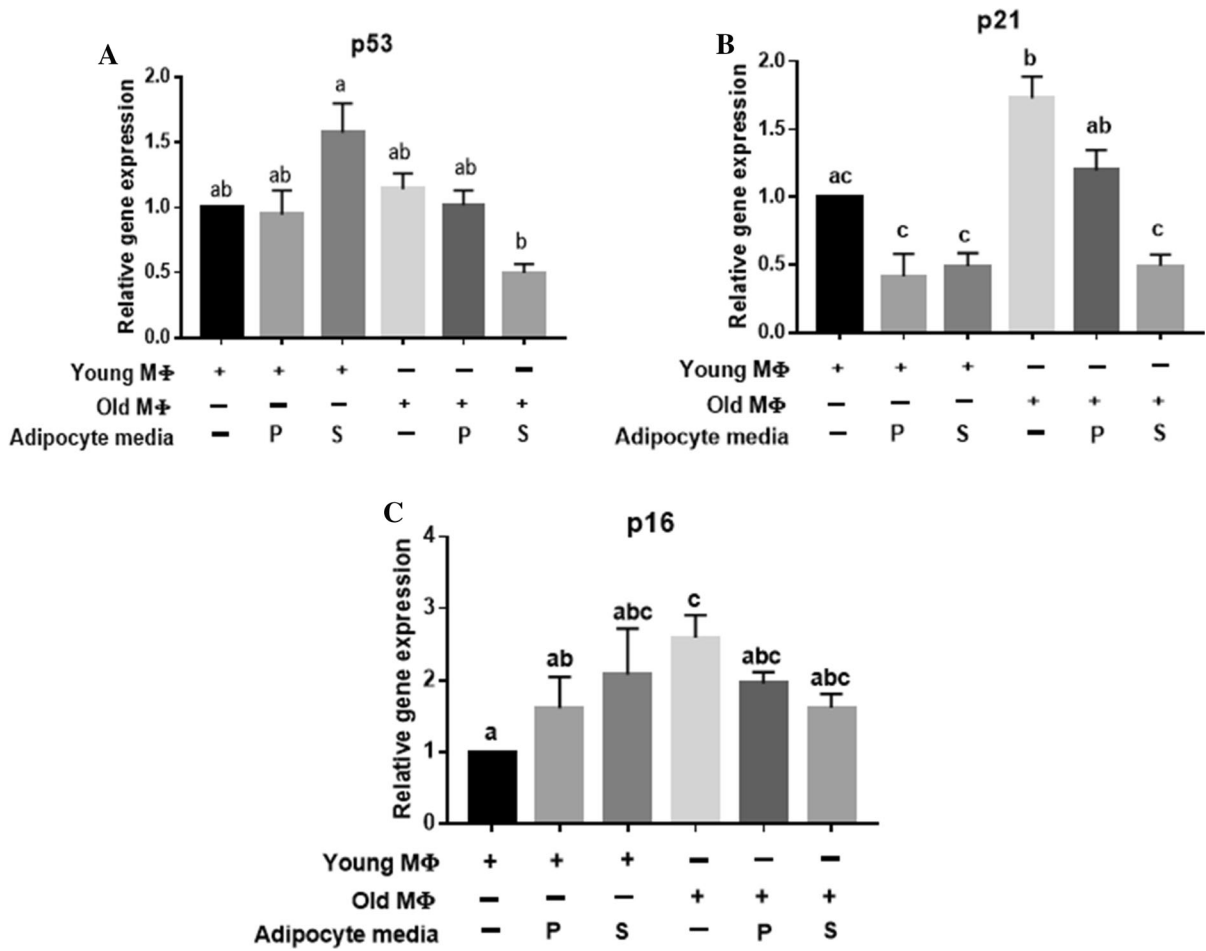


Fig. 5 Effect of proliferating (P) and senescent (S) preadipocyte media on gene expression of cell cycle inhibitors in macrophages. Macrophages from young and old animals were treated in presence or absence of P or S

preadipocyte media for 48 h and relative gene expression of **a** p53, **b** p21, **c** p16 was analyzed. Values are mean ± SEM (n = 3); values with different letters are significantly different at p < 0.05

phenotype (Fig. 3a–c). On the other hand, an invariable suppression in M2 phenotype, as evident by diminished gene expression of *Arg1*, *Mrc1* and *Msr1*, was observed when YM and OM were cultured in presence of secretory metabolites of senescent preadipocytes (Fig. 3a–c).

Expression of SA-β-gal is increased in aged macrophages and in presence of secretory metabolites of senescent preadipocytes

A distinct and significant increase in SA-β-gal positive cells was observed in OM as compared to YM (Fig. 4g). Interestingly, numbers of cells positive for

SA-β-gal remarkably increased in presence of media of senescent preadipocytes in both YM and OM (Fig. 4g). On the other hand, a significant decrease in SA-β-gal + cells, as compared to control, was observed in OM cultured in presence of media of proliferative preadipocytes, while no such effects on YM could be observed (Fig. 4g). Representative images of SA-β-gal staining are presented in (Fig. 4a–f).

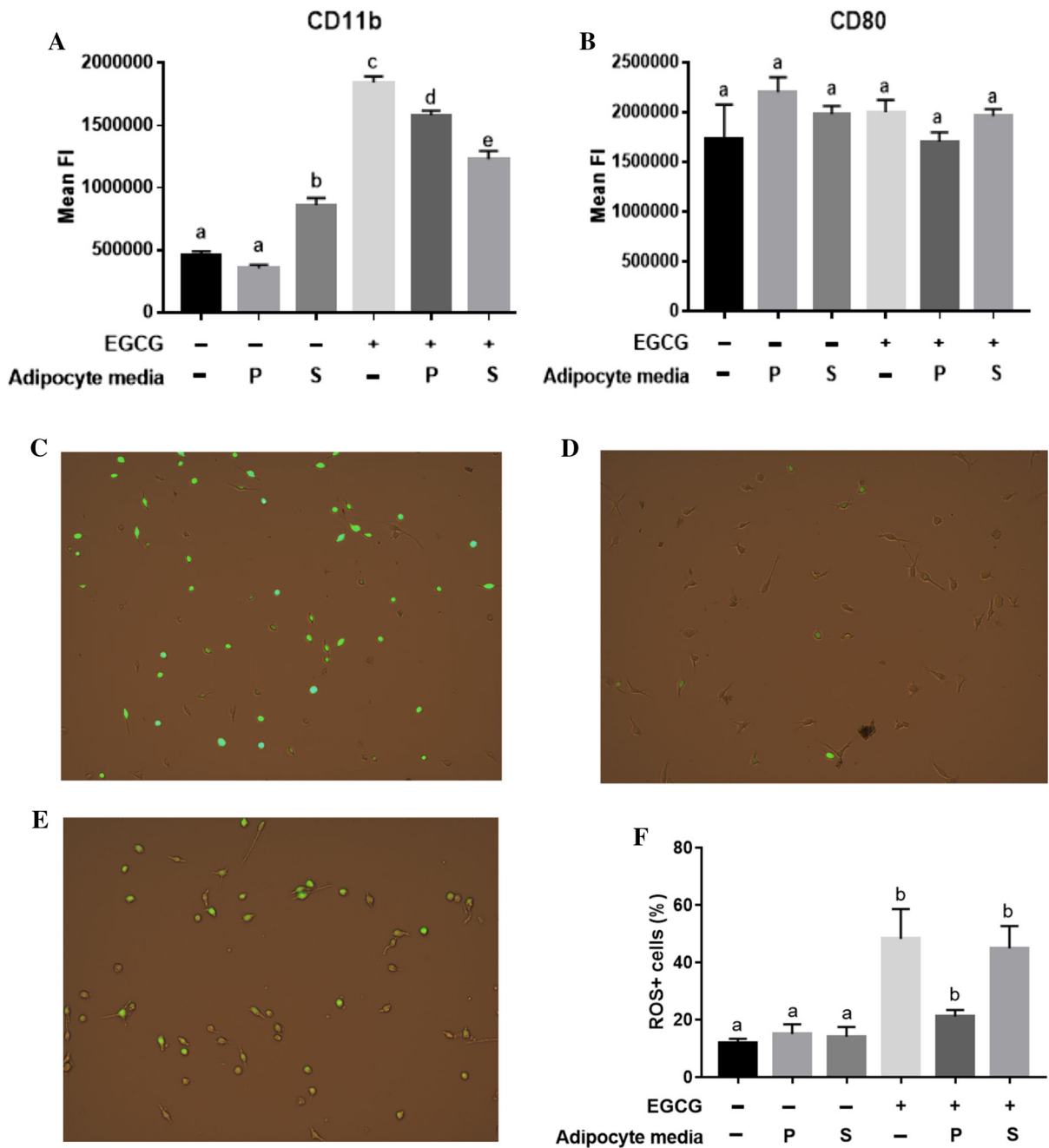


Fig. 6 Effect of EGCG on proliferating (P) and senescent (S) preadipocyte media-induced changes in old macrophages. Macrophages from aged animals were treated in presence or absence of EGCG (10 μ M) for 24 h prior to treatment with P or S preadipocyte media for 48 h and analyzed by flow cytometry for **a** CD11b expression. **b** CD80 expression. Intracellular ROS

levels were measured in old macrophages. **c** Treated with EGCG alone. **d** Treated with EGCG and P preadipocyte media. **e** Treated with EGCG and S preadipocyte media. **f** ROS + cells (%) Values are mean \pm SEM (n = 3); values with different letters are significantly different at $p < 0.05$

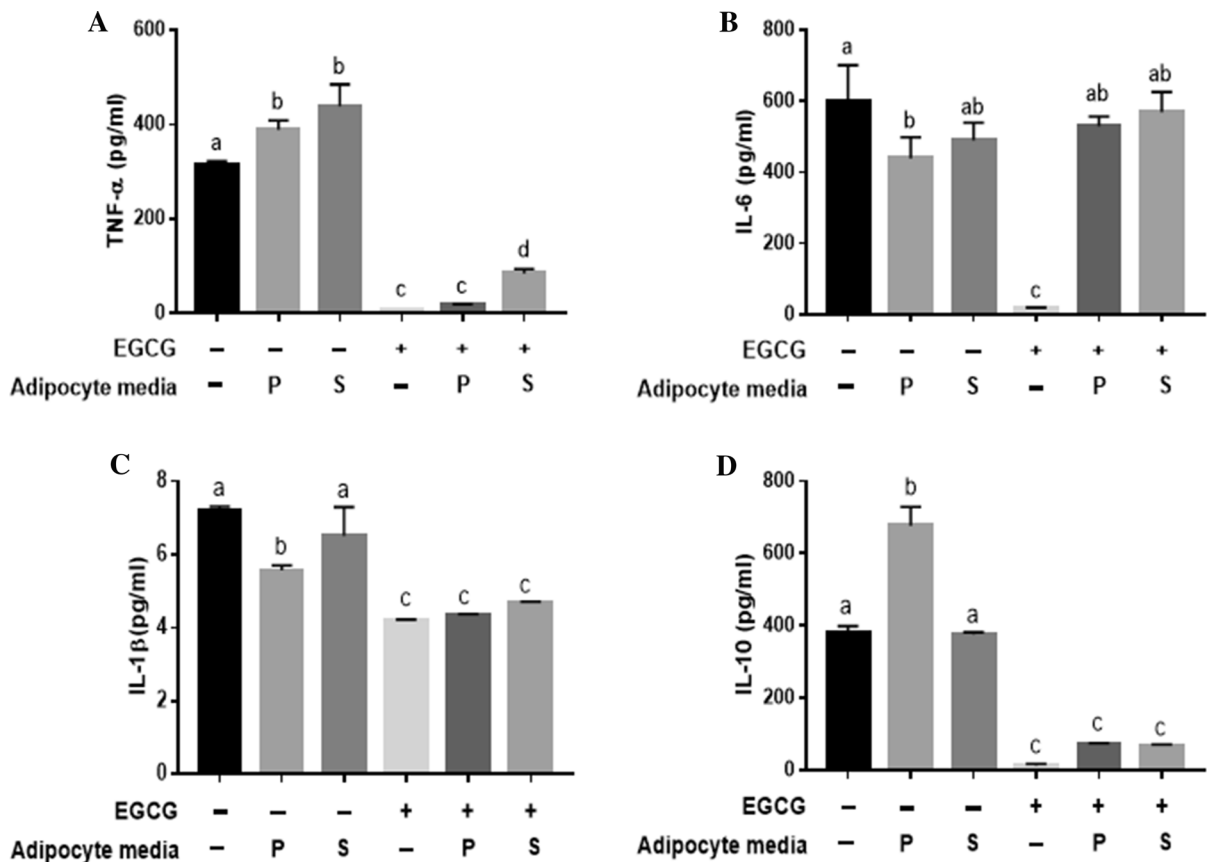


Fig. 7 Effect of EGCG on proliferating (P) and senescent (S) preadipocyte media-induced changes in inflammation status of old macrophages. Macrophages from aged animals were treated in presence of EGCG (10 μ M) for 24 h prior to treatment

with P or S preadipocyte media for 48 h and analyzed for levels of **a** TNF- α , **b** IL-6, **c** IL-1 β , **d** IL-10. Values are mean \pm SEM ($n = 3$); values with different letters are significantly different at $p < 0.05$

Differential effects of age and secretory factors of preadipocytes on cell cycle inhibitors in macrophages

Relative gene expression of p53 showed slight increase (non-significant) in YM exposed to S media while a decrease (non-significant) was evident in OM treated with S media as compared to YM (Fig. 5a). No significant change in p21^{WAF1} expression could be observed in YM cells irrespective of the treatment while OM showed significant increase in p21^{WAF1} expression as compared to control YM group. However, exposure of OM to S media showed gradual decrease in p21^{WAF1} expression as compared to OM control which was at par with YM control (Fig. 5b). Similarly, p16^{Ink4a} expression showed a significant increase in OM as compared to YM with non-

significant variations in the remaining groups (Fig. 5c).

EGCG treatment reversed age-dependent and preadipocyte media-induced changes in macrophage activation and inflammation

Pretreatment of EGCG strikingly reversed the loss of CD11b expression in OM with a significant increase of over 3 folds in observed fluorescence intensity (Fig. 6a). Similarly, EGCG treated cells showed robust response to P and S culture media and a significant increase in the expression of CD11b was observed in OM (Fig. 6a). However, no significant difference in CD80 expression could be observed (Fig. 6b). Analyses of cells positive for resting intracellular ROS levels also showed a significant

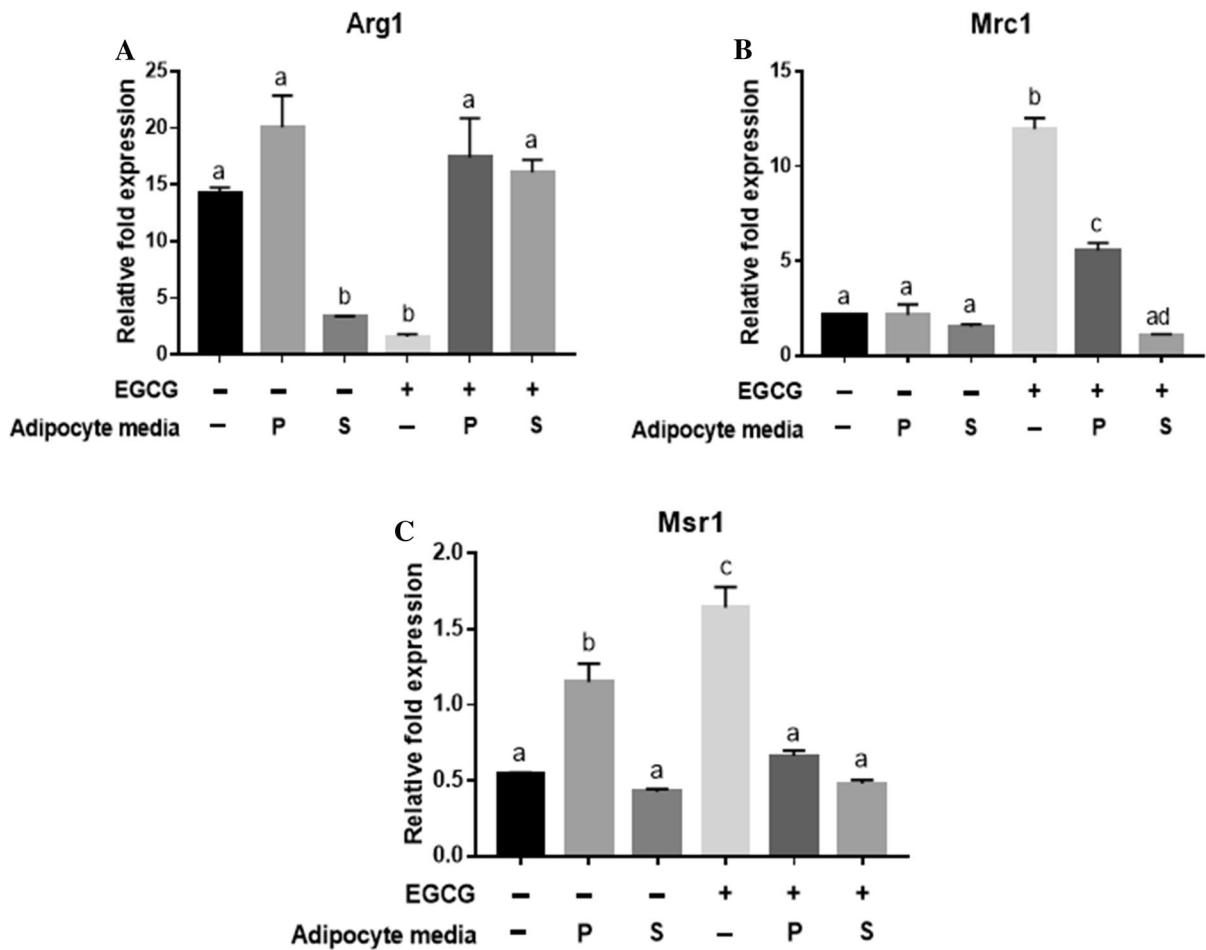


Fig. 8 Effect of EGCG on proliferating (P) and senescent (S) preadipocyte media-induced changes on markers of M2 polarization in old macrophages. Macrophages from aged animals were treated in presence of EGCG (10 μ M) for 24 h

prior to treatment with P or S preadipocyte media for 48 h and analyzed for relative gene expression of **a** Arg1, **b** Mrc1, **c** Msr1. Values are mean \pm SEM (n = 3); values with different letters are significantly different at p < 0.05

increase in EGCG treated OM as well as in EGCG treated OM exposed to P or S media OM (Fig. 6c–f). Further, application of EGCG indicated a strong inhibitory influence on all analyzed cytokine levels in OM. EGCG treatment categorically inhibited the apparent increase in cytokines (IL-1 β /IL-6/TNF- α /IL-10) levels in aged OM as compared to YM suggesting strong anti-inflamm-aging attributes (Fig. 7a–d). Except for IL-6, concentration of all measured cytokines decreased in EGCG treated OM when exposed to either P or S media (Fig. 7a–d).

Treatment with EGCG modulated M2 phenotype in old macrophages

Analyses of EGCG treatment on markers of macrophage M2 phenotype with age and on culture with P & S media suggested evidence of significant modulation. Expression of *Arg1* with age and upon culture in S media was effectively and dramatically reversed by EGCG treatment, while *Mrc1* expression appeared to significantly increase in EGCG treated cells except when stimulated with S media (Fig. 8a, b). *Msr1* expression, on the other hand, was only positively influenced by EGCG treatment in absence of P or S media (Fig. 8c).

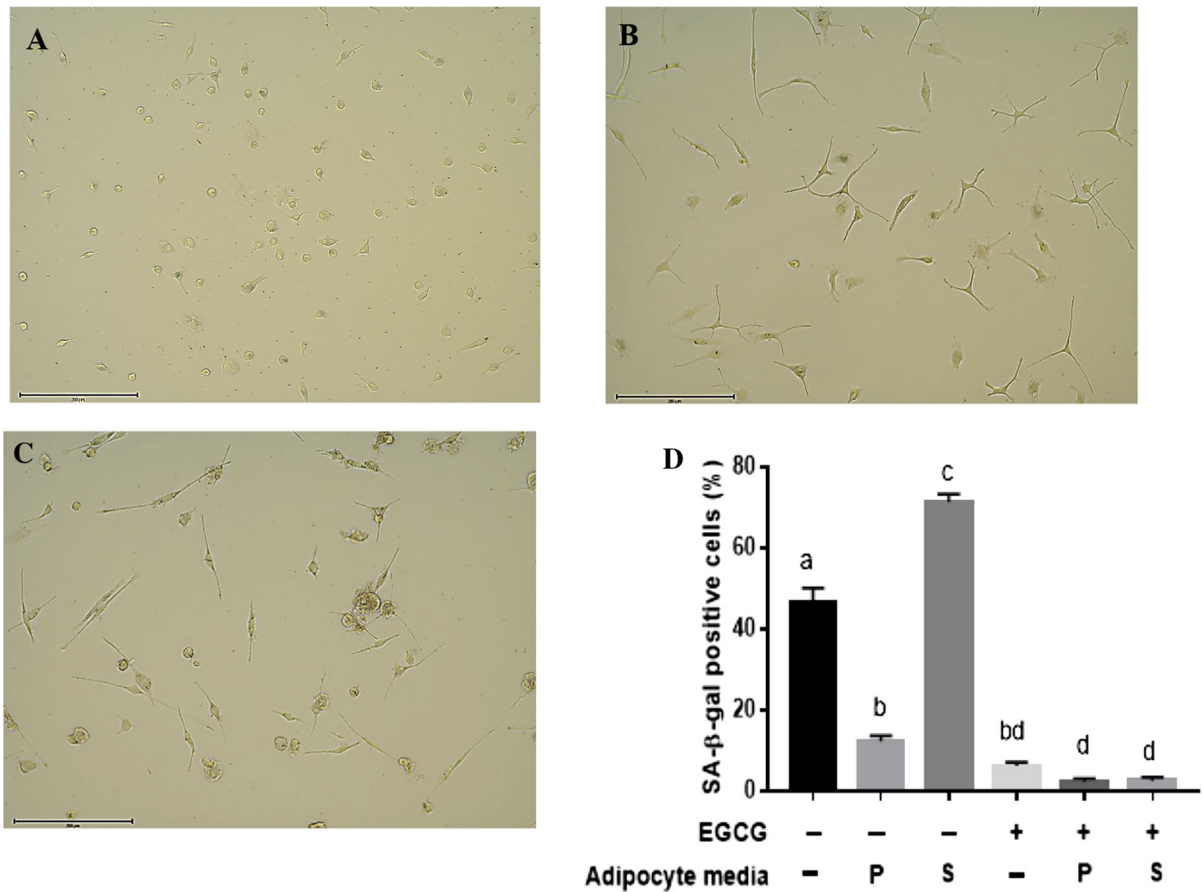


Fig. 9 Effect of EGCG on proliferating (P) and senescent (S) preadipocyte media-induced changes in SA-β-gal activity of old macrophages. Macrophages from aged animals were treated in presence of EGCG (10 μM) for 24 hours prior to treatment with P or S preadipocyte media for 48 h and analyzed for SA-β-

gal activity. **a** Cells treated with EGCG alone. **b** Cells treated with EGCG and P preadipocyte media. **c** Cells treated with EGCG and S preadipocyte media. **d** % SA-β-gal positive cells. Values are mean ± SEM (n = 3); values with different letters are significantly different at p < 0.05

EGCG treatment abolished SA-β-gal activity and suppressed the expression of cell cycle inhibitors in macrophages

Observations of SA-β-gal assay in OM treated with EGCG revealed a remarkable abrogation of SA-β-gal expression as compared to untreated control and a significant decrease in numbers of SA-β-gal + cells (Fig. 9a–d). Similar observations were recorded for cells stimulated with P and S media wherein a robust inhibition of SA-β-gal activity on account of EGCG pretreatment was observed (Fig. 9a–d). Treatment with EGCG significantly downregulated the relative gene expression of p53, p21^{WAF1} and p16^{Ink4a} in OM as compared to untreated control (Fig. 10a–c). The

suppression appeared more pronounced in p21^{WAF1} and p16^{Ink4a} suggesting robust effects of EGCG in modulating macrophage proliferation during aging.

Discussion

Although direct evidence linking macrophages to longevity and inflamm-aging are scarce; it has been demonstrated that selective depletion of macrophages from aged mice can inhibit peripheral nerve degeneration (Yuan et al. 2018), diminish increased pro-inflammatory cytokine production (Bouchlaka et al. 2013) as well as improve anti-tumor T cell activity (Duong et al. 2018) relative to young macrophages.

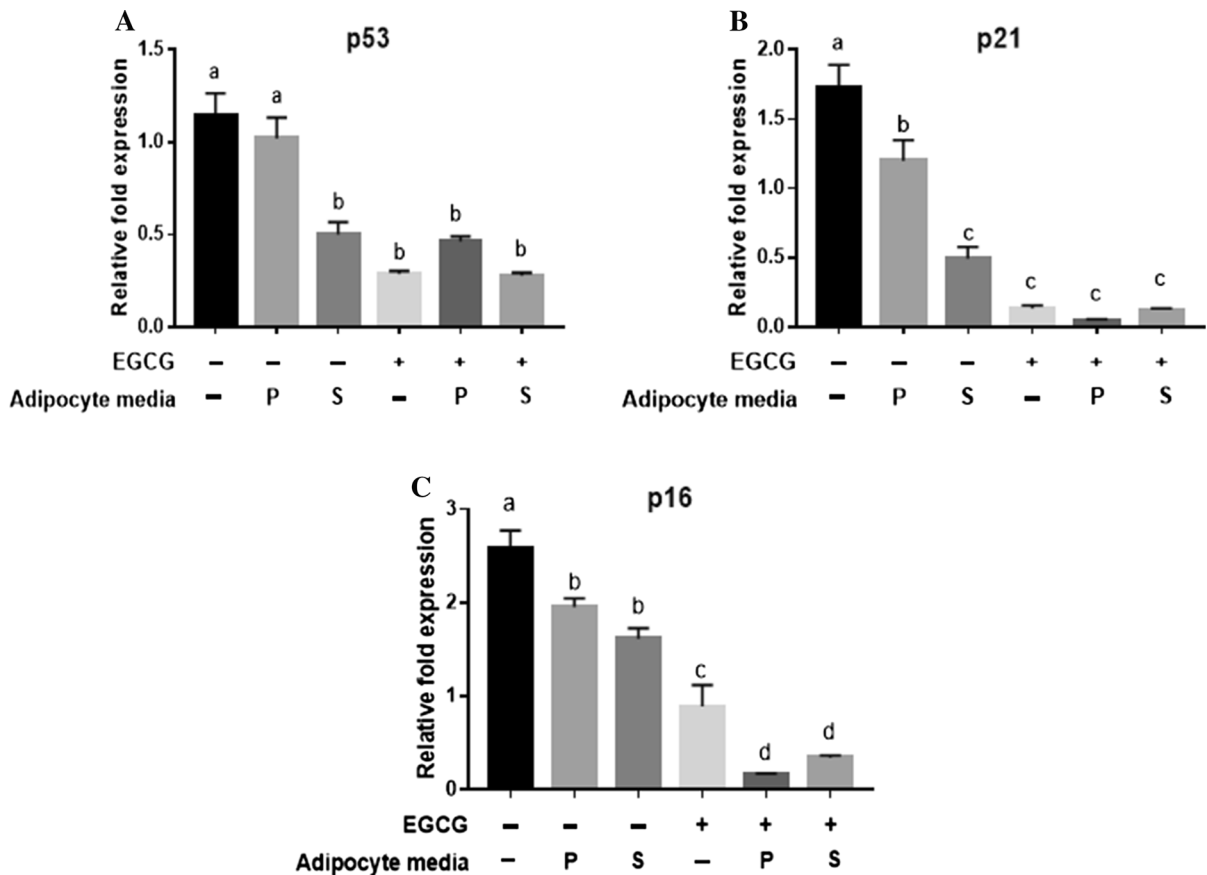


Fig. 10 Effect of EGCG on proliferating (P) and senescent (S) preadipocyte media-induced changes in the expression of cell cycle inhibitors of old macrophages. Macrophages from aged animals were treated in presence of EGCG (10 μ M) for 24

hours prior to treatment with P or S preadipocyte media for 48 h and relative gene expression of **a** p53, **b** p21, **c** p16 was analyzed. Values are mean \pm SEM ($n = 3$); values with different letters are significantly different at $p < 0.05$

The age-associated intrinsic changes in macrophage functions are well known, however, studies identifying the role of extrinsic factors, particularly those related to the senescent cell microenvironment, in shaping macrophage aging are rare (van Beek et al. 2019). The present work thus attempted to understand how aged adipose tissue contributes to macrophage dysfunctions pertaining to senescence, and whether these changes could be mitigated by the treatment of tea catechin EGCG to macrophages. Integrin CD11b is essential for inflammatory cell activation and migration while ROS production capability is directly correlated with respiratory burst-potential of macrophages. In the present study, expression of CD11b was greatly suppressed in OM, however, in the presence of S media, OM showed a significant increase in CD11b expression while a corresponding decrease was

observed in YM. This differential behavior indicates that in the presence of SASP microenvironment (such as in S media), the potency to mount inflammatory response may be compromised in YM while OM may become more responsive and are thus likely to contribute to inflammatory aggravation (inflammaging) as often observed in elderly. This assertion is also substantiated by the observed age-dependent increase in pro-inflammatory cytokines expression in OM which appeared to be preserved even when exposed to P or S media, in contrast to YM which did not show any appreciable change. It was interesting to note that the Th2 cytokine IL-10 was also upregulated in OM which may be indicative of counterbalancing attempts of aging macrophages to prevent the apparent inflammatory aggravation, but nonetheless represents perturbed functional capacity in OM as compared to

YM. However, a cautious approach is required while interpreting these results as unlike majority of other studies, macrophages analyzed in present work were *not* stimulated with LPS or any other external agent and thus represent modulation of the native functional capacity of macrophages. Although there are several reports describing effects of macrophages on adipose tissue differentiation using co-culture systems (Suganami et al. 2005; Permana et al. 2009; Oliver et al. 2009); however, studies detailing reverse stimulation of macrophages and its subsequent physiological effects using preadipocytes are rare. It has been previously reported that when J774 macrophages are exposed to unstimulated and proliferating preadipocyte medium, there is no increase in IL-6 or TNF- α production compared with untreated cells (Berg et al. 2004). Our results in YM appeared to corroborate this, however our observations with OM indicated that aged macrophages are more sensitive even to the secretory factors of P media, thereby suggesting age-specific effects of proliferating preadipocytes. Further, YM showed slightly enhanced TNF- α /IL-6 levels in presence of S media but with little effect on anti-inflammatory cytokine IL-10, thereby implying evidence for the notion that secretory factors of senescent preadipocytes *in vivo* could act as extrinsic modulators of macrophages that may drive the known pro-inflammatory behavior of aged macrophages (inflamm-aging).

Macrophage polarization is another indicator of altered phenotype and functions during aging (Mahub et al. 2012). Disruption of the normal balance of M1/M2 macrophages appears to be an important factor in disease pathogenesis including cancer, atherosclerosis, autoimmune disease, osteoporosis and neurodegeneration (Becker et al. 2018). However, the exact nature of age-associated polarizing behavior of macrophages remains controversial. There are reports suggesting that aging in macrophages is accompanied by a shift towards M1 phenotype and is characterized by an increased pro-inflammatory behavior (Barrett et al. 2015; Thornton et al. 2015). However, it has also been proposed that alternatively activated M2 like macrophages, which are yet pro-inflammatory, can accumulate in tissues and promote inflamm-aging (Hall et al. 2017; van Beek et al. 2019). These conflicting observations could be attributed to the oversimplification of the general concept of M1 versus M2 polarization, as well as the nature of

experimental design (*ex vivo* or LPS stimulated macrophages). In the present study, based on observations of pro-inflammatory cytokines and genes associated with M2 phenotype (*Arg1/Mrc1/Msr1*), it is plausible to assert that OM showed a latency towards M2 phenotype accompanied by perturbed inflammatory homeostasis. An interesting observation was apparent when YM macrophages were exposed to P media wherein a strong increase in *Arg-1* and *Mrc-1* expression was observed. This behavior was similar to a report by Lujambio et al. (2013), wherein it was detected that macrophages exposed to secretory factors of proliferating hepatic stellate cells expressed M2 phenotype as evident from upregulation of genes *Mrc1* and *Msr1*. Thus, it appears that even in presence of ‘healthy’ proliferating cells, macrophage polarization can be altered. Interestingly though, except for *Mrc1*, YM exposed to S media did not show any discrete increase in M2 phenotype, while a corresponding increase in pro-inflammatory phenotype was still evident. However, OM exposed to S media appeared to show a gradual decrease in M2 phenotype but with still perturbed pro-inflammatory response as evident from inflammatory cytokines measurement. Together, it is reasonable to conclude that the exposure of macrophages to P media enhanced M2 polarization in both YM and OM, but without significantly influencing pro-inflammatory behavior. However, exposure of macrophages to S media decreased the M2 phenotype while still maintaining the pro-inflammatory phenotype in YM and OM. This signifies that in an environment of aging adipose tissue, a shift towards heightened pro-inflammatory response with a concomitant decrease in M2 phenotype is plausible regardless of the intrinsic state of macrophage (*i.e.* young or old).

Despite extensive research on macrophage biology, the defining evidence for the existence of senescent macrophages remains elusive (Burton and Stolzing 2018). Development of p16^{Ink4a}, p21^{WAF1} expression and SA- β -gal activity are amongst the prevalent markers of cell senescence. Fuentes et al. (2011) previously reported the expression of senescence marker p16^{Ink4a} in human adipose tissue macrophages that contributed to the risk of type 2 diabetes and pro-inflammatory behavior. Another report identified inverse correlation between Ki67 proliferation expression and SA- β -gal activity thereby claiming that SA- β -gal actually labels macrophages with decreasing

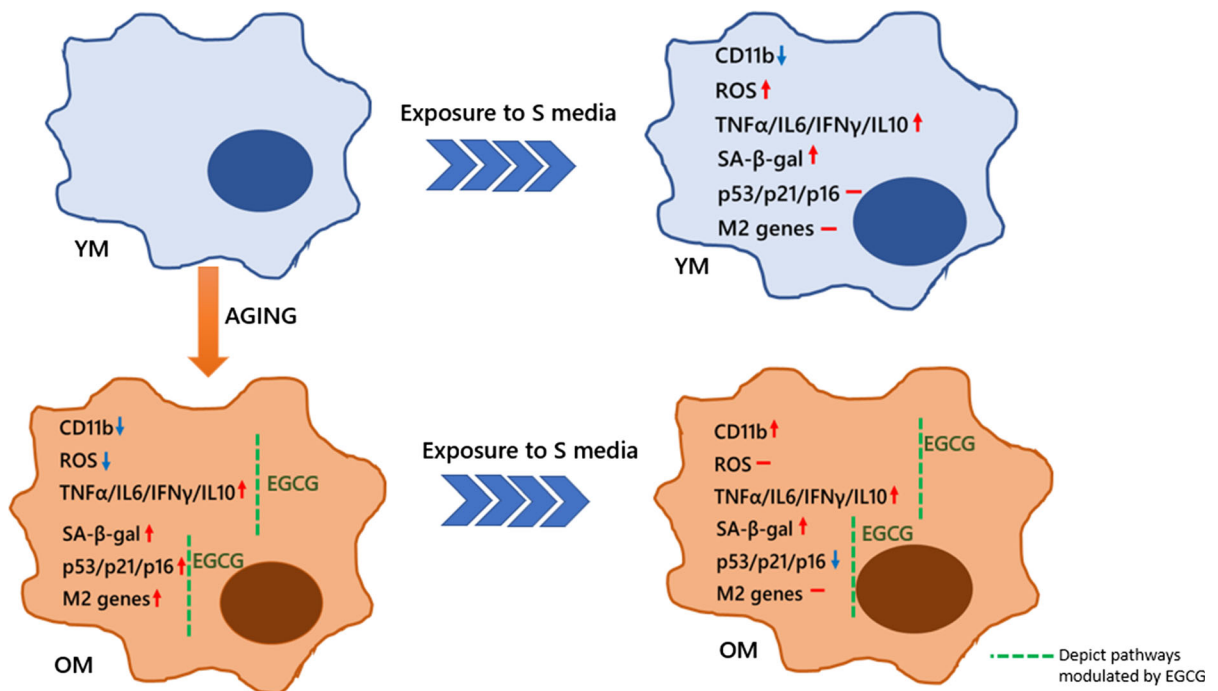


Fig. 11 Schematic description of the observed effects of age, senescent preadipocytes media and EGCG application on phenotype and functions of young (YM) and old (OM) macrophages

proliferation tendency (Holt and Grainger 2012). However, a recent report has argued that presence of a common marker of senescence- SA- β -gal, could only be a physiological response of macrophages and not indicative of senescence *per se* (Hall et al. 2017). There is thus considerable uncertainty and lack of enough studies aimed at defining the senescence program in macrophages. In the present work, we observed very high levels of SA- β -gal activity and a significant increase in p16^{Ink4a} and p21^{WAF1} gene expression in OM as compared to YM suggesting an age-related association of various senescence markers. However, the response of YM and OM to secretory factors of preadipocytes, particularly to the S media, were curious. YM exposed to S media appeared to enhance p53/p16^{Ink4a} gene expression in accordance with increased SA- β -gal activity. However, in OM, the effects of S media on p53/p21^{WAF1}/p16^{Ink4a} gene expression was not so discrete and in fact a general decreasing trend was observed despite a very high SA- β -gal expression. While the exact reasons for this observation are not clear but it certainly suggests that OM respond differently to senescent preadipocytes which may directly influence their proliferation

capacity. Moreover, in the presence of P media, SA- β -gal activity in OM significantly decreased but when exposed to S media, both YM and OM exhibited even a higher percentage of SA- β -gal activity. These observations suggest that SA- β -gal activity is dynamic in macrophages and is responsive to the prevalent microenvironment as proposed by Hall et al. (2017). Taken together, our results suggest that in macrophages, SA- β -gal expression vis-à-vis activation of cell cycle inhibitors is correlated with respect to age only but not regarding exposure to SASP factors. This is an interesting observation that warrants further specific studies to ascertain the underlying mechanism and influence on macrophage-preadipocytes biology vis-à-vis cellular senescence.

Application of EGCG dramatically changed the effects of age as well as exposure to P and S media on the phenotype and functions of OM. It was observed that EGCG enhanced the activation status of resting OM as evident by increased CD11b expression and ROS production. However, it was also noted that EGCG treated OM when co-exposed to P or S did not show any unwarranted aggravation of CD11b expression as otherwise observed in OM treated with S alone.

This was further corroborated by robust attenuation of pro-inflammatory markers despite the observed increase in activation of OM in presence of EGCG. In fact, EGCG treatment strongly abrogated age-associated changes in cytokines expression in OM which appeared to be very similar in profile to YM. In this regard, the present study suggests that EGCG could enhance the reactivity and functional response of OM by reversing age as well as preadipocyte media-induced decline in the activation status but without any exaggerated pro-inflammatory effect which may beneficially influence elderly health by improving response to infections and curbing inflamm-aging. EGCG supplementation also modulated inflammatory behavior (TNF- α /IL-1 β) of OM when exposed to P or S media. Curiously though, while EGCG appeared to suppress IL-6 levels in resting OM; no such effect was apparent in OM when exposed to P or S media suggesting differential and limited effects of EGCG in presence of adipocyte secretory factors. We have previously reported that EGCG supplementation enhances innate immune responses during aging in vivo (Sharma et al. 2017) while several other studies have also identified anti-inflammatory effects of EGCG in the wake of an external stimulation or inflammatory challenge (Ohishi et al. 2016; Chu et al. 2017). EGCG application appeared to further enhance M2 polarization in OM but did not seem to affect it when exposed to P or S media. Therefore, it can be concluded that EGCG ameliorates pro-inflammatory and compromised activation of macrophages but differentially affects M1/M2 polarization. EGCG is known for its anti-inflammatory attributes and there is evidence linking M2 phenotype promoting activities of EGCG (Chu et al. 2018) which is corroborated in present study. Analysis of senescence markers in OM also suggested modulatory attributes of EGCG. SA- β -gal positive cells were remarkably reduced in EGCG treated OM, regardless of the exposure to P or S media. EGCG application also strongly decreased gene expression of p53, p21^{WAF1} and p16^{Ink4a} as compared to control or in presence of P/S media. Previously, we have observed that EGCG can suppress age-associated expression of p53/p21^{WAF1}/p16^{Ink4A} as well as SA- β -gal activity in senescent preadipocytes ultimately enhancing cell proliferation capacity and abrogating senescence (Kumar et al. 2019). However, given the lack of correlation in SA- β -gal activity and expression of cell cycle markers as observed in OM exposed to S

media; the impact and relevance of EGCG in inhibiting both SA- β -gal activity and cell cycle inhibitors in this particular co-culture system requires further exploration.

Conclusion

The present study attempted to ascertain the effects of two-way interactions involving different cell types and prevailing microenvironment on macrophage functions relating to aging. Our study provides evidence that secretory factors of proliferating and senescent preadipocytes, strongly albeit differentially, influence macrophage biology that could be correlated with macrophage aging phenotype (Fig. 11). In particular, the exposure of YM to senescent preadipocyte media strongly induced changes very similar to OM suggesting the contribution of extrinsic factors in augmenting macrophage aging. It is also implied that SASP-associated S media strongly contributes to some of the observed age-related changes in macrophage functions, especially in reference to inflamm-aging and cell cycle inhibitors (Fig. 11). EGCG strongly attenuated both age-associated as well as preadipocytes media-induced changes in macrophage functions and thus appears as a promising candidate for nutrition-induced modulation of macrophage senescence. Further research exploring different aspects of this experiment settings are required to fully determine the effects and mechanisms of accumulating senescent cells on macrophage aging.

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