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Age-dependent effects of the recombinant spike protein/SARS-CoV-2 on the M−CSF− and IL-34-differentiated macrophages *in vitro*

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ABSTRACT

The SARS-CoV-2 virus causes elevated production of senescence-associated secretory phenotype (SASP) markers by macrophages. SARS-CoV-2 enters macrophages through its Spike-protein aided by cathepsin (Cat) B and L, which also mediate SASP production. Since M-CSF and IL-34 control macrophage differentiation, we investigated the age-dependent effects of the Spike-protein on SASP-related pro-inflammatory-cytokines and nuclear-senescence-regulatory-factors, and CatB, L and K, in mouse M−CSF− and IL-34-differentiated macrophages. The Spike-protein upregulated SASP expression in young and aged male M−CSF−macrophages. In contrast, only young and aged male IL-34-macrophages demonstrated significantly reduced pro-inflammatory cytokine expression in response to the Spike-protein *in vitro*. Furthermore, the S-protein elevated *CatB* expression in young male M−CSF−macrophages and young female IL-34-macrophages, whereas *CatL* was overexpressed in young male IL-34- and old male M−CSF −macrophages. Surprisingly, the S-protein increased CatK activity in young and aged male M−CSF −macrophages, indicating that CatK may be also involved in the COVID-19 pathology. Altogether, we demonstrated the age- and sex-dependent effects of the Spike-protein on M-CSF and IL-34-macrophages using a novel *in vitro* mouse model of SARS-CoV-2/COVID-19.

1. Introduction

The current COVID-19 pandemic, caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is characterized by an overwhelming macrophage (Mϕ)-induced cytokine storm, which leads to the mild-to-severe respiratory disease and death caused by pneumonia [1]. Severe symptomatology is more prevalent in males than in females, and both morbidity and mortality are aggravated by age [1]. These differences have been adjudicated to age- and gender-dependent expression of receptors that facilitate the entry of SARS-CoV-2 virus to the cell, along with the age-related co-morbidities [2].

As a characteristic structural component of the virion membrane of SARS and MERS coronaviruses, the Spike protein (S-protein) is responsible for the viral entry into Mϕ, by activation of cysteine protease cathepsins [3]. The activated Mϕ produces a distinct panel of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and IL-6, which are collectively termed senescence-associated secretory phenotype (SASP). This Mϕ polarization and activation is triggered by the ligation of Macrophage colony stimulating factor (M-CSF) and IL-34, with the CSF-1 receptor [4]. The M−SCF−induced Mϕ are highly immunostimulatory, whereas the IL-34 activated Mϕ possess immunosuppressive characteristics [5]. Furthermore, M-CSF differentiated Mϕ are more susceptible to viral infection and correlate with higher mortality due to viral disease, whereas Il-34 differentiated Mϕ is shown to possess elevated resistance to viral infection [6]. Incidentally, the SARS-CoV-2 S-protein binds to the surface proteins on peripheral blood mononuclear cells and induces the production of M-CSF, which then mediates the pro-inflammatory SASP cytokine storm that characterizes severe COVID-19 symptomatology [7].

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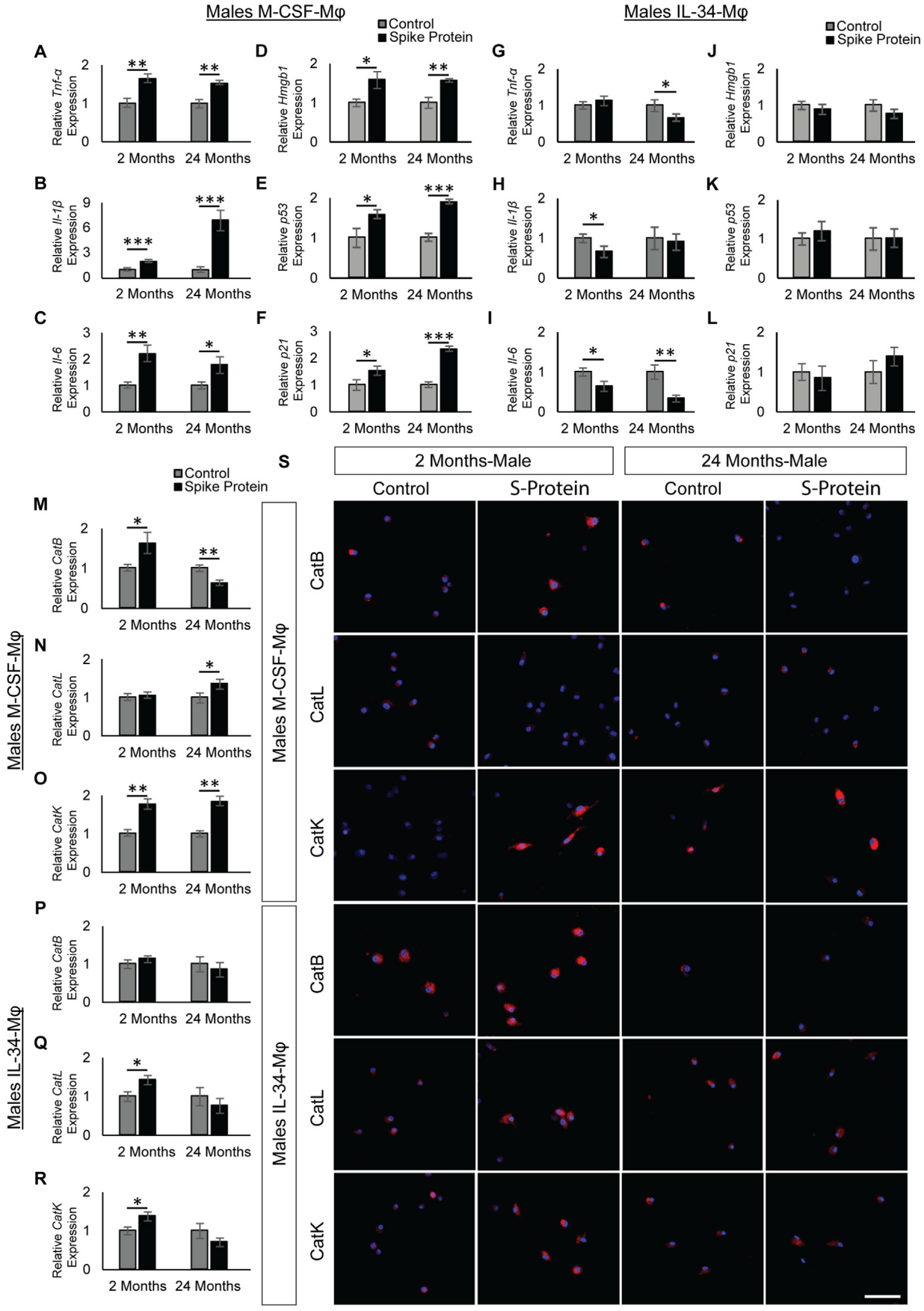
Males M-CSF-Mφ
Males IL-34-Mφ
Control
Spike Protein
A
B
C
D
E
F
G
H
I
J
K
L
Relative Tnf-α Expression
Relative Il-1β Expression
Relative Il-6 Expression
Relative Hmgb1 Expression
Relative p53 Expression
Relative p21 Expression
2 Months
24 Months
S
2 Months-Male
24 Months-Male
Control
S-Protein
M
N
O
P
Q
R
Relative CatB Expression
Relative CatL Expression
Relative CatK Expression
Males M-CSF-Mφ
Males IL-34-Mφ
CatB
CatL
CatK

Although the production of SASPs, which includes pro-inflammatory cytokines and chemokines, senescence-regulatory proteins and lysosomal cathepsins (Cat), by Mϕ is well established in various infectious and sterile experimental models of age-related inflammation [8], the differential effect of the SARS-CoV-2 S-protein on M−CSF− and IL-34Mϕ remains elusive in the context of age and gender. Thus, this study aimed to evaluate the age- and sex-dependent effects of the SARS-CoV-2 recombinant S-protein on the expression of pro-inflammatory cytokines (*TNF-α*, *IL-1β*, *IL-6,* macrophage migration inhibitory factor *(MIF)*), nuclear senescence-regulatory proteins (*Hmgb1*, *p53, p21*), and cathepsins (*CatB*, *CatL* and *CatK)* using M-CSF and IL-34 primed bone marrow derived macrophages, as a potential *in vitro* experimental model of COVID-19/SARS-CoV-2.

2. Materials and methods

2.1. Cells

Bone marrow derived macrophages (BMDMs) were isolated from the femurs and tibias of young (two-month old) and aged (twenty-four-month old) male and female C57BL/6 mice (NIA Aged Rodent Colony located at Charles River) and plated at a density of 1×10^5 cells per well, in alpha-MEM (Life Sciences) supplemented with 10% FBS (Atlanta Biologicals), 1% Anti-Anti, 1% L-glutamine, 1% MEM-NEAA (Life Sciences), and 30 ng/ml recombinant mouse M-CSF or IL-34 proteins (Biolegend) for 3 days. On day 4, the culture media was replaced and supplemented with 10 ng/ml of COVID 19 recombinant Spike Protein (MyBiosource). The samples were collected, and images were taken, after 24 h.

2.2. RNA extraction and quantitative PCR

Total RNA was extracted using the PureLinkTM RNA Mini Kit (Ambion, Life Technologies), according to the manufacturer's instructions, and reverse transcription of 1 μg of total RNA was performed using the Verso cDNA Synthesis Kit (Thermo Scientific), following the manufacturer's recommendations. The gene expressions were measured using PowerUp™ Sybr™ Green Master Mix (Applied Biosystems Diagnostics) or TaqMan™ Universal PCR Master Mix (Applied Biosystems, Life Technologies), in the AriaMx Real-time PCR System (Agilent) and quantified using the AriaMX Software Version 1.3. The data was analyzed using the ΔΔCq method normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences are available upon request.

2.3. Cathepsins activity assay

The intracellular activities of cathepsin B, L and K were evaluated using the Magic Red™ Cathepsin (Immunochemistry Technologies, Bio-Rad) kits, following the manufacturer's recommended protocol. Images were acquired with a Zeiss LSM780 confocal microscope.

2.4. Statistical analysis

The collected data were analyzed using the Student's *t*-test for the comparison between two groups, or a one-way ANOVA with post hoc Tukey's test for the comparisons among different groups. A $p < 0.05$ was considered statistically significant. Data were analyzed using Sigma Plot v.14 software.

3. Results and discussion

To the best of our knowledge, the S-protein significantly elevated expression of *Tnf-α*, *Il-1β*, *Il-6* and *Mif* RNAs in aged male M−CSF−Mϕ (Fig. 1A–C, Suppl. Fig. 1), whereas only *Tnf-α*, *Il-1β* and *Il-6* were overexpressed in young male Mϕ (Fig. 1A–C). In contrast, IL34-Mϕ from old male mice showed an anti-inflammatory effect, with inhibition of *Tnf-α* and *Il-6* (Fig. 1G, I), while inhibition of *Il-1β* and *Il-6* were observed in IL-34-Mϕ from young male mice (Fig. 1H–I). These data indicate that the S-protein increased the expression of SASP cytokines in M−CSF−Mϕ while decreasing it in IL-34-Mϕ, from male mice. For young and aged female MCSF-Mϕ exposed to the S-Protein, no significant fluctuations in the expression of the *Tnf-α*, *Il-1β*, *Il-6* and *Mif* genes were observed (Fig. 2; Suppl. Fig. 1). Furthermore, only a significant increase in the expression of *Il-1β* (Fig. 2I) was detected in young female IL-34 Mϕ, whereas no changes were observed in any of the reported cytokines in aged female IL34-Mϕ. These data corresponded to a recently published observation showing that high serum levels of TNF-α and IL-6 have been identified in the COVID-19 patients, with IL-6 being significantly higher in critically ill patients [9]; however, the gender or age distribution of these data have not been reported. Additionally, in COVID-19 patients, M-CSF, but not IL-34 expression, has been assessed, and found to be elevated [10]. Although, M-CSF and IL-34 have been identified in chronic inflammation, their specific contribution to the inflammatory process has not yet been detailed [11]. Our sex and age specific observations in mouse Mϕ correlated with statistical findings of high prevalence and severity of COVID-19 symptoms in senior male patients [2,12]. Additionally, our data clearly demonstrated the expected inflammatory phenotype induced by the SARS-CoV-2 in M-CSF, but not in IL-34, differentiated Mϕ.

Since the S-protein induced production of pro-inflammatory SASP cytokines from Mϕ (Figs. 1 and 2) and the increased susceptibility of senescent cells to viral infection [13], we next evaluated the effect of the S-protein on the expression of key nuclear senescence-regulatory proteins, including *Hmgb1*, *p53* and *p21*, in MCSF- and IL34-Mϕ. Exposure to the S-protein induced the overexpression of *Hmgb1*, *p53* and *p21*, by MCSF-Mϕ (Fig. 1D–F), but not in IL34-Mϕ, isolated from old and young male mice (Fig. 1J-L). In contrast, *Hmgb1* and *p21* were significantly overexpressed in young female IL34-Mϕ (Fig. 2D,F), but not in MCSF-Mϕ. Interestingly, no changes were observed in M−CSF− and IL-34-Mϕ from old female mice, which may potentially be affected by the SARS-CoV-2 through different signaling pathways. HMGB1 is a damage associated molecular pattern (DAMP) alarmin, which amplifies senescence-associated inflammation [14]. Furthermore, HMGB1 is a mediator of the inflammatory cell infiltration to the lungs and contributes to the reprograming of Mϕ towards a pro-inflammatory phenotype, which is upregulated in the aging lungs and kidneys [15]. Similarly, p53 and its downstream gene p21 are upregulated in fibrotic lung disease and are known to be activated in response to

Fig. 1. The effects of SARS-CoV-2 Spike Protein on the expression of Senescence-associated secretory phenotype (SASP) markers in M−CSF− and IL-34-primed macrophages (Mϕ) **isolated from bone marrow of young and aged male C57BL/6 mice*isolated from bone marrow of young and aged male C57BL.*** The changes in the mRNA genes expression of pro-inflammatory cytokines (A-C, G-I), nuclear senescence-regulatory proteins (D-F, J-L) and cathepsin B, L and K (M–R), as well as the intracellular cathepsins activity (S), were assessed in M−CSF− or IL34-primed bone marrow derived macrophages (M−CSF−Mϕ, IL-34-Mϕ) after 24-h exposure to 10 ng/ml SARS-CoV-2 S-protein (n = 5 samples/condition). Macrophages were isolated from 2-month-old and 24-month-old male and female C57BL/6 mice. Magic Red and Hoechst dyes were used to label cathepsins activity (Red) and nuclei (Blue), respectively. Scale bar: 50 μm. A one-way ANOVA with post hoc Tukey's test for the comparisons among different groups was employed (n = 5 samples/condition); *p < 0.05, **p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

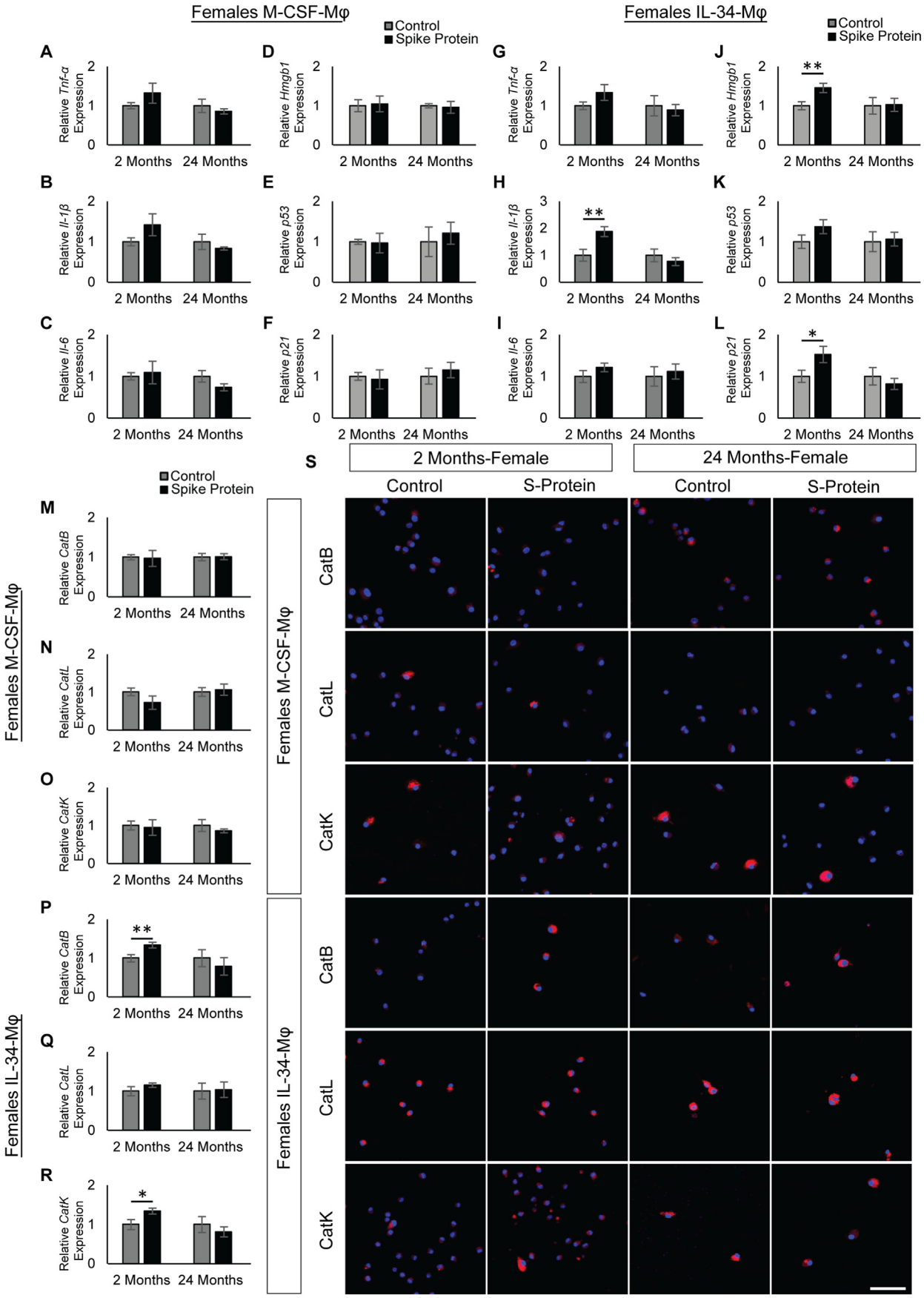

Fig. 2. The effects of SARS-CoV-2 Spike Protein on the expression of Senescence-associated secretory phenotype (SASP) markers in M−CSF− and IL-34-primed macrophages (Mϕ) isolated from bone marrow of young and aged female C57*BL*/6 mice. The changes in the gene expression of inflammatory cytokines (A-C, G-I), senescence markers (D-F, J-I) and cathepsins (M−R), as well as the intracellular cathepsin activity (S), were assessed in M−CSF− or IL-34-Mϕ after 24-h exposure to 10 ng/ml SARS-CoV-2 S-protein (n = 5 samples/condition). Macrophages were isolated from bone marrow of 2-month-old and 24-month-old male and female C57BL/6 mice. Magic Red and Hoechst dyes were used to label cathepsins activity (Red) and nuclei (Blue), respectively. Scale bar: 50 μm. A one-way ANOVA with post hoc Tukey's test for the comparisons among different groups was employed (n = 5 samples/condition); *p < 0.05, **p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DNA damage [16,17]. Furthermore, p53 intervenes in viral activation and has been identified in the bronchial lavage fluid of COVID-19 patients [18,19].

Numerous studies show cathepsins may facilitate the cellular senescence and aging-associated diseases, including osteoporosis and Alzheimer's disease [20]. Furthermore, CatB and L are activated by the S-proteins from the SARS and MERS coronaviruses to mediate membrane fusion and subsequent release of viral RNA into the host cell [3]. Importantly, CatK is not known to be directly associated with viral infection or replication; however, it does induce production of SASPs, including pro-inflammatory TNF-α, IL-6 and IL-1β cytokines, from Mϕ and Mϕ-like osteoclast precursors [21–24]. Thus, we finally assessed the age- and sex-dependent mRNA expression of CatB, L and K, along with their intracellular activity in M–CSF– and IL-34-Mϕ exposed to the S-protein using real time PCR and confocal microscopy assays, respectively.

In young males, S-protein was found to significantly elevate CatB and CatK genes expression and intracellular activities in M–CSF–Mϕ (Fig. 1 M, O), whereas CatL and CatK expressions and activities were increased in IL-34-Mϕ (Fig. 1Q–R). Conversely, the expression and activity of CatB was inhibited while those for CatL and CatK were increased in old males M–CSF–Mϕ (Fig. 1 M–O). No effects of S-protein were observed on IL-34-Mϕ isolated from aged mice. Our data also demonstrated that the expressions and activities of CatB and CatK were significantly increased in young female IL-34-Mϕ (Fig 2P,R); however, the expression and intracellular activities of CatB, CatL and CatK in response to S-protein were neither affected in young and old female M–CSF–Mϕ nor in old female IL-34-Mϕ (Fig. 2). While the importance of CatB and CatL in the SARS coronavirus-induced inflammation has been reported in previous studies [25], this study detected, for the first time, the increased expression and intracellular activity of CatK in M–CSF–Mϕ and IL-34-Mϕ exposed to the S-protein of SARS-CoV-2. Therefore, further investigations are warranted to elucidate the role of CatK in the age and sex associated severity of COVID-19 symptoms.

4. Conclusion

The mechanisms by which Spike-protein/SARS-CoV-2 induces the expression of inflammatory SASP cytokines, which characterize the severe COVID-19 clinical profile, are still to be determined. These inflammatory cytokines are largely produced by cells of the innate immune response, including Mϕ. Hereby, we demonstrate the manner in which the specific age- and sex-dependent proliferation of Mϕ induced by M-CSF or IL-34 leads to the distinct gene expression of various SASP markers in response to stimulus by the S-protein of SARS-CoV-2. Significantly higher inflammatory and senescent phenotypes were observed in male M–CSF–Mϕ, but not female MCSF-Mϕ, which correlate with clinical data suggesting the male prevalence of COVID-19 [12]. Conversely, an anti-inflammatory effect was observed in IL-34 proliferated male but not female Mϕ *in vitro*. IL-34 has been identified as a target of pro-inflammatory microRNA, miR-31-3p, which mediates inflammation in human lung fibroblasts, by inhibiting IL-34, which, in turn, causes flaring of IL-6 and IL-8 production [26]. Interestingly, a mild inflammatory effect and distinct gene expression phenotype was observed in the S-protein stimulated young female IL34-Mϕ, which was not the case with old female Mϕ. The expression of IL-34 in mild and severe COVID-19 cases should be assessed, with sex and age distinctions, to determine whether IL-34 correlates with SARS-CoV-2 susceptibility. Moreover, IL-34 may be considered a potential therapeutic target for COVID-19 in the male population. Sex and gender related variations in the immune response have been previously reported in SARS-CoV-2 positive patients [27]. In this context, the current study demonstrates that sex- and age-related variations to stimulus by the SARS-CoV-2 S-protein can also be obtained in mouse models of the disease.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.01.104.

References

[1] J. Kopel, et al., Racial and gender-based differences in COVID-19, Front Public Health 8 (2020) 418.
[2] K. Romero Starke, et al., The age-related risk of severe outcomes due to COVID-19 infection: a rapid review, meta-analysis, and meta-regression, Int. J. Environ. Res. Publ. Health 17 (16) (2020).
[3] G. Simmons, et al., Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research, Antivir. Res. 100 (3) (2013) 605–614.
[4] A.I. Segaliny, et al., IL-34 and M-CSF form a novel heteromeric cytokine and regulate the M-CSF receptor activation and localization, Cytokine 76 (2) (2015) 170–181.
[5] E.D. Foucher, et al., IL-34 induces the differentiation of human monocytes into immunosuppressive macrophages. antagonistic effects of GM-CSF and IFN-gamma, PloS One 8 (2) (2013), e56045.
[6] D. Paquin-Proulx, et al., Human interleukin-34-derived macrophages have increased resistance to HIV-1 infection, Cytokine 111 (2018) 272–277.
[7] X. Qiang, et al., Monoclonal Antibodies Capable of Binding SARS-CoV-2 Spike Protein Receptor Binding Motif Specifically Prevent GM-CSF Induction. bioRxiv, 2020.
[8] J. Akkaoui, et al., Contribution of Porphyromonas Gingivalis Lipopolysaccharide to Experimental Periodontitis in Relation to Aging, Geroscience, 2020.
[9] H. Han, et al., Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors, Emerg. Microb. Infect. 9 (1) (2020) 1123–1130.
[10] Y. Chi, et al., Serum cytokine and chemokine profile in relation to the severity of coronavirus disease 2019 in China, J. Infect. Dis. 222 (5) (2020) 746–754.
[11] S. Garcia, et al., Colony-stimulating factor (CSF) 1 receptor blockade reduces inflammation in human and murine models of rheumatoid arthritis, Arthritis Res. Ther. 18 (2016) 75.
[12] C. Penna, et al., Sex-related differences in COVID-19 lethality, Br. J. Pharmacol. 177 (19) (2020) 4375–4385, https://doi.org/10.1111/bph.15207.
[13] M. Malavolta, et al., Exploring the relevance of senotherapeutics for the current SARS-CoV-2 emergency and similar future global health threats, Cells 9 (4) (2020).
[14] A.R. Davalos, et al., p53-dependent release of Alarmin HMGB1 is a central mediator of senescent phenotypes, J. Cell Biol. 201 (4) (2013) 613–629.
[15] Z. Su, et al., HMGB1 facilitated macrophage reprogramming towards a proinflammatory M1-like phenotype in experimental autoimmune myocarditis development, Sci. Rep. 6 (2016) 21884.
[16] H.L. Ou, B. Schumacher, DNA damage responses and p53 in the aging process, Blood 131 (5) (2018) 488–495.
[17] C. Jiang, et al., Serpine 1 induces alveolar type II cell senescence through activating p53-p21-Rb pathway in fibrotic lung disease, Aging Cell 16 (5) (2017) 1114–1124.
[18] R.J. Kraus, et al., Reactivation of epstein-barr virus by HIF-1alpha requires p53, J. Virol. 94 (18) (2020).
[19] Y. Xiong, et al., Transcriptomic characteristics of bronchoalveolar lavage fluid and peripheral blood mononuclear cells in COVID-19 patients, Emerg. Microb. Infect. 9 (1) (2020) 761–770.
[20] A. Wyczalkowska-Tomasik, L. Paczek, Cathepsin B and L activity in the serum during the human aging process: cathepsin B and L in aging, Arch. Gerontol. Geriatr. 55 (3) (2012) 735–738.
[21] L. Hao, et al., Odanacatib, A cathepsin K-specific inhibitor, inhibits inflammation and bone loss caused by periodontal diseases, J. Periodontol. 86 (8) (2015) 972–983.
[22] L. Hao, et al., Deficiency of cathepsin K prevents inflammation and bone

erosion in rheumatoid arthritis and periodontitis and reveals its shared osteoimmune role, FEBS Lett. 589 (12) (2015) 1331–1339.
[23] M.P. Lee, Essential mixed cryoglobulinemia as a cause of gastrointestinal hemorrhage, Am. J. Gastroenterol. 86 (1) (1991) 120–121.
[24] F. Pedica, et al., Cathepsin-k as a diagnostic marker in the identification of micro-granulomas in Crohn's disease, Pathologica 101 (3) (2009) 109–111.
[25] N. Iwata-Yoshikawa, et al., TMPRSS2 contributes to virus spread and immunopathology in the airways of murine models after coronavirus infection, J. Virol. 93 (6) (2019).
[26] R. Guo, L. Zhang, J. Meng, Circular RNA ANKRD36 attends to lipopolysaccharide-aroused MRC-5 cell injury via regulating microRNA-31-3p, Biofactors 46 (3) (2020) 391–401.
[27] N.A.P. Lieberman, et al., In Vivo Antiviral Host Response to SARS-CoV-2 by Viral Load, Sex, and Age, bioRxiv, 2020.