CD38 & Senescent Cells

LINK BETWEEN SENESCENCE AND AGE-RELATED CELLULAR NAD+ DECLINE

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The NADase CD38 is induced by factors secreted from senescent cells providing a potential link between senescence and age-related cellular NAD$^+$ decline

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ABSTRACT

Tissue nicotinamide adenine dinucleotide (NAD$^+$) decline has been implicated in aging. We have recently identified CD38 as a central regulator involved in tissue NAD$^+$ decline during the aging process. CD38 is an ecto-enzyme highly expressed in endothelial and inflammatory cells. To date, the mechanisms that regulate CD38 expression in aging tissues characterized by the presence of senescent cells is not completely understood. Cellular senescence has been described as a hallmark of the aging process and these cells are known to secrete several factors including cytokines and chemokines through their senescent associated secretory phenotype (SASP). Here we investigated if the cellular senescence phenotype is involved in the regulation of CD38 expression and its NADase activity. We observed that senescent cells do not have high expression of CD38. However, the SASP factors secreted by senescent cells induced CD38 mRNA and protein expression and increased CD38-NADase activity in non-senescent cells such as endothelial cells or bone marrow derived macrophages. Our data suggest a link between cellular senescence and NAD$^+$ decline in which SASP-mediated upregulation of CD38 can disrupt cellular NAD$^+$ homeostasis.

1. Introduction

It has been recently demonstrated that decreases in levels of the intracellular nucleotide nicotinamide adenine dinucleotide (NAD$^+$) and accumulation of senescent cells play important roles in the biology of aging [1–23]. These two mechanisms have emerged as new hallmarks of the aging process, but whether there is a link between these two mechanisms is not known. Cellular senescence is a cell fate that, like replication, differentiation, or apoptosis, can occur at any stage of development, as a consequence of cell damage, or in aging [10–19,23]. In particular, senescence may be initiated by stimuli such as repeated cell division, strong mitogenic signals, oxidative stress, inflammation, and DNA damage [10–19,23]. Senescent cells have been shown to secrete cytokines, growth factors, extracellular matrix modifiers, and other biological compounds that promote chronic "sterile" inflammation and fibrosis through the so-called senescence-associated secretory phenotype (SASP) [13–19,23]. Through these processes senescent cells can contribute to tissue injury [10–19,23]. This concept has been presented in the inflammaging hypothesis [24]. As discussed above, whether there is a link between senescence/SASP and tissue NAD$^+$ decline during aging is not known. We have previously shown that the enzyme CD38 is the main nicotinamide nucleotidase in mammalian tissues [24,25]. Furthermore, we have also demonstrated that CD38 plays a key role in the age-related NAD$^+$ decline [2,9]. However, to date several aspects of the biology of this enzyme remain to be elucidated [26,27]. For instance, what drives CD38 expression in the biology of aging is an open question [27–30]. Surprisingly, the possible link between CD38 expression and the cellular senescence phenotype has not been explored. Here we demonstrated that CD38 expression in cells can be induced by factors associated with the SASP, providing a possible link between these two emerging hallmarks of aging.

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2. Materials and methods

2.1. HUVECs: preparation, induction of senescence, and treatment with conditioned media

Human umbilical venous endothelial cells (HUVECs - ATCC® PCS100013™) were cultured in Vascular Cell Basal Medium supplemented with Endothelial Cell Growth Kit VEGF (ATCC PCS100041). Exposure of HUVECs for 36 h to MEF conditioned media was performed between passage 4 and 6 at 90–100% confluence in serum starved cells (0.5% FBS) amended at time of experiment with 2% FBS.

Senescence was induced in HUVECs which had reached approximately 100% confluence. Two different methods were used: gamma irradiation (γ-IR) or x-ray irradiation. HUVECs were sham-irradiated or irradiated in either a cesium irradiator or an X-Ray irradiator at 10 Gy. The senescence phenotype was observed at d7 post-radiation. HUVEC conditioned media was produced by culturing cells for 24 h in medium containing 2% FBS. Medium was steroid-free.

2.2. Patient-derived preadipocytes (PDP): preparation and induction of senescence

Abdominal subcutaneous adipose tissue for primary pre-adipocyte isolation [16] was obtained during intra-abdominal surgery from 2 consented clinically obese subjects (BMI 46 ± 5.7) undergoing kidney transplant or gastric bypass surgery (female; age 51.75 ± 6.06 [mean ± SEM] years). Preadipocytes are also known as adipose-derived stem cells or fat cell progenitors. Cells were used in experiments between passages 4–8. The protocol was approved by the Mayo Clinic Foundation Institutional Review Board for Human Research.

Senescence was induced in PDPs which had reached approximately 60% confluence using an X-Ray irradiator at 10 Gy. Media was subsequently changed twice weekly post-irradiation. Conditioned media from treated and untreated cells was harvested at d20 post-IR following incubation of cells for 24 h with serum-free media. It was centrifuged prior to use.

2.3. MEFs: preparation and induction of senescence

Primary mouse embryonic fibroblasts (MEFs) were generated from C57BL/6 as previously described [2,9]. Briefly, embryos were harvested at d13.5 post-conception, mechanically disaggregated and digested in trypsin/EDTA (0.25%) for 1 h. Cells were cultivated in DMEM containing FBS, sodium pyruvate, glutamine, nonessential amino acids, and antibiotics in a humidified incubator at 37 °C with 5% CO_2.

Senescence was induced in MEFs which had reached approximately 75% confluence by administering 10 Gy in a cesium irradiator. Media was subsequently changed at d4 and d7 post-irradiation. Conditioned media from treated and untreated cells was harvested at d10 following incubation of cells for 24 h with serum-free DMEM. MEF CM was centrifuged prior to use. Senescent cells expressed increased *p16-INK4a* or *p21Cip1* by 10 days post-irradiation.

2.4. Preparation of bone marrow-derived macrophages (BMDMs)

Mouse BMDMs were isolated as described before in Matalonga et al. [31] from aged mice (18–20 months). BMDM cells were seeded at 5 million cells/60 mm^2 dish and cultured for 24 h with conditioned media collected from senescent or non-senescent cells and amended with 2% FBS.

2.5. Western blot analysis

Western immunoblotting was performed as previously described in Tarrago et al. [9] using the following antibodies: mouse CD38 (R&D Systems AF4947) and β-Tubulin (Abcam Ab 15568).

2.6. Enzymatic activity

Measurement of CD38 hydrolase activity with nicotinamide 1,N6-ethenoadenine dinucleotide (Et-NAD) as substrate was performed as described previously [24,25].

2.7. Quantification of mRNA

Total RNA was isolated from cells and mouse tissues using TRIzol or Qiagen RNeasy kits. cDNA was synthesized using the Qiagen QuantiTect or ABI High Capacity cDNA Reverse Transcription kit. Quantitative real-time PCR was performed using commercially available TaqMan gene expression probes (Applied Biosystems, see table below), according to the manufacturer's instructions, on a BioRad CFX384 thermal cycler. The relative mRNA abundance of target genes was calculated by the $2^{(-ddCq)}$ method. The expression changes were calculated relative to control.

2.8. Statistical analysis

TaqMan Gene Expression Assays(Mouse)	
Gene Symbol	Probe ID
Cd38	Mm01220906_m1
Cdkn2a	Mm0049449_m1
Cdkn1a	Mm04205640_g1
Il1b	Mm00434228_m1
Il6	Mm00446190_m1
Nos2	Mm00440502_m1
Hprt	Mm01545399_m1
Tbp	Mm00446971_m1
TaqMan Gene Expression Assays (Human)	
Gene Symbol	Probe ID
CD38	Hs01120071_m1 _m1
CDKN1A	Hs00355782_m1 _m1
CDKN2A	Hs00923894_m1 _g1
CDKN2D	Hs00176481_m1 _m1
CXCL8	Hs00174103_m1 _m1
GAPDH	Hs02758991_g1 _m1
IL6	Hs00985639_m1 _m1
TP53	Hs01034249

Data were analyzed by a two-tailed Student's *t*-test and a one-way ANOVA with a Bonferroni's post hoc test. Analyses were performed using GraphPad Prism 7.

3. Results and discussion

3.1. Senescent cells in vitro do not express CD38

We have postulated that increased CD38 expression and accumulation of $CD38^+$ cells in tissues with aging could be linked to the senescent cell phenotype [20]. Thus, we first investigated whether

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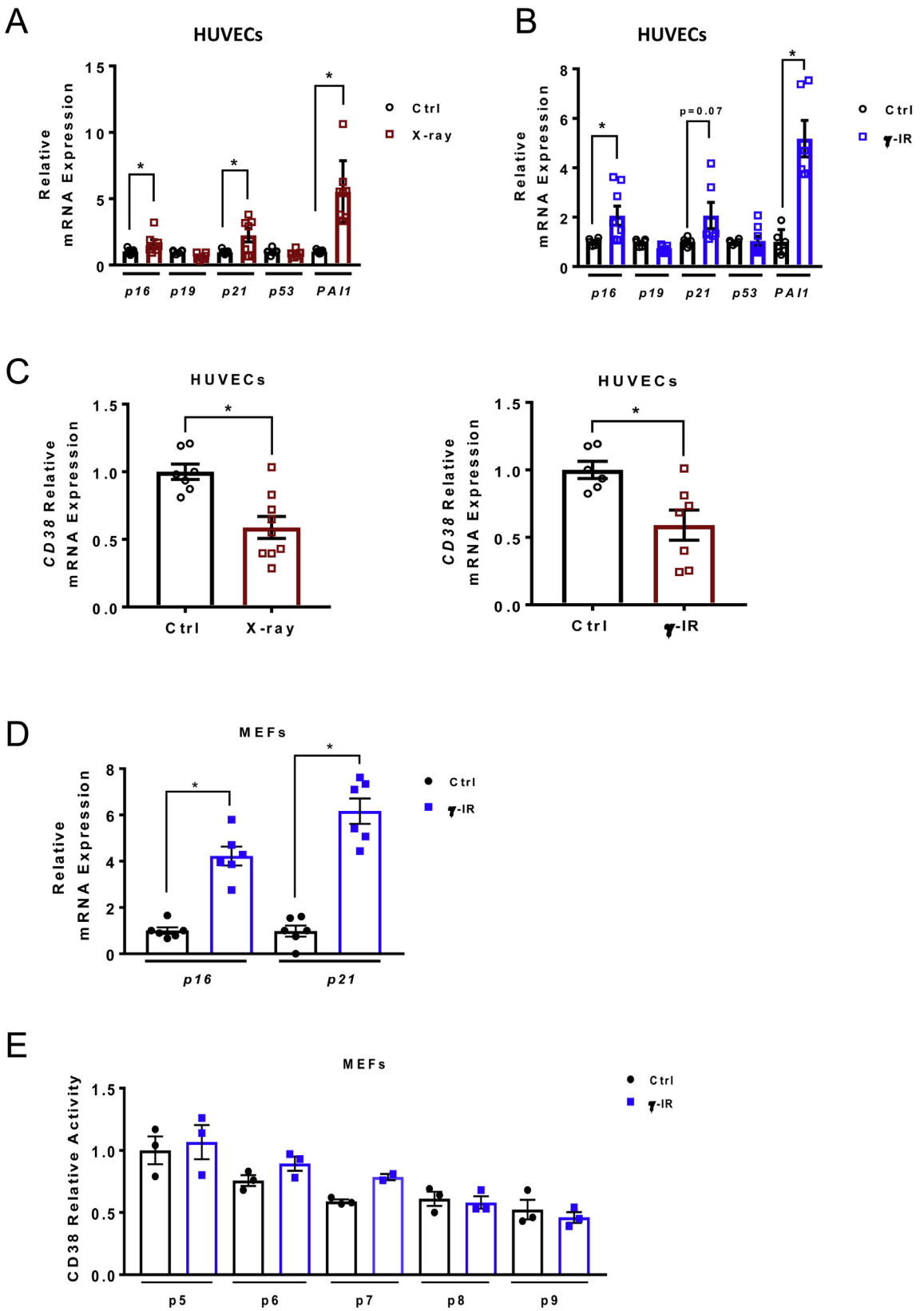

Fig. 1. CD38 expression is not increased in senescent cells. (A–B) Relative mRNA levels of senescence-related genes in HUVECs treated with (A) X-ray irradiation (IR) (n = 7–9) or (B) γ-IR (n = 4–8). (C) Relative mRNA levels of *CD38* in HUVECs treated with X-ray (n = 7–9) or -IR (n = 6–7). (D) Relative mRNA levels of senescence-related genes in irradiated MEFs (n = 6). Relative mRNA levels (A–D) were determined by qRT-PCR. (E) CD38 relative enzymatic activity in multiple independent irradiation-induced senescent MEFs (passage 5–9) (n = 2–3). Data are mean ± SE, *p < 0.05 vs control.

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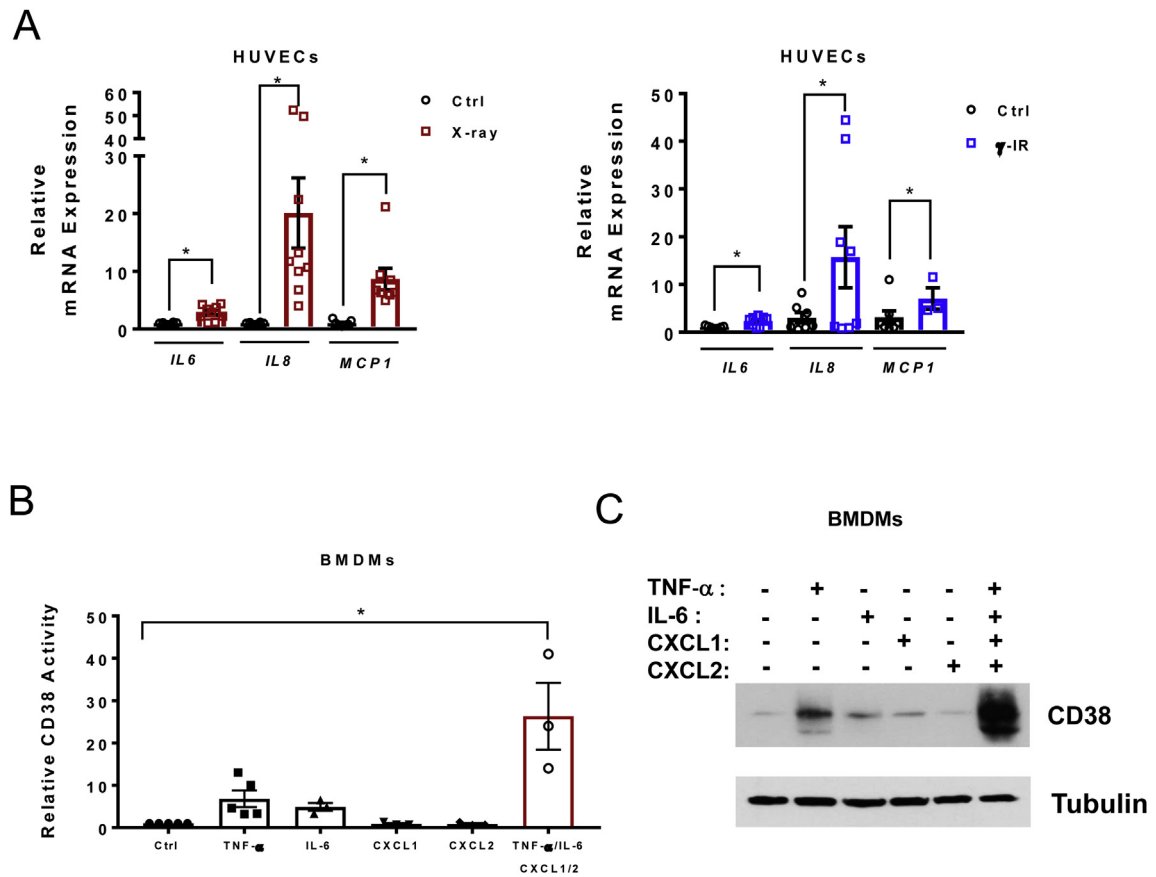

Fig. 2. Expression of SASP factors in senescent HUVECs and induction of CD38 expression by a combination of potential SASP factors. (A) mRNA expression of selected SASP factors (*IL-6, IL-8, MCP-1*) in senescent HUVECs, determined by qRT-PCR (n = 7–9). (B) CD38 activity (n = 3–5) and (C) a representative immunoblot showing CD38 levels in BMDMs treated with various factors found in the SASP. Data are mean ± SE, *p < 0.05 vs control.

or not induction of the cellular senescence phenotype *in vitro* could lead to expression of CD38 in cells. We induced senescence in human umbilical vein endothelial cells (HUVECs) by DNA damage through exposure to x-ray irradiation (x-ray IR) (Fig. 1A) or gamma irradiation (γ-IR) (Fig. 1B), as described before by our group (12). Markers of the senescent phenotype include p21, $p16^{Ink4a}$, and PAI1 (Fig. 1A and B), and are induced by these treatments [10–19,23]. We observed that, although our cells presented several markers of senescence after induction of the senescence phenotype, they showed no induction of CD38 mRNA compared to non-senescent cells (Fig. 1C). Very similar results were obtained when MEFs were induced to become senescent (Fig. 1D and E). In fact, although these cells expressed markers of cellular senescence such as p16 and p21, no changes in CD38 activity were observed even when several independent cell passages were induced to become senescent (Fig. 1D and E).

3.2. Cytokines and chemokines expressed by senescent cells induce CD38 expression in macrophages

Induction of cellular senescence in cultured cells did not lead to an increase in the expression of *CD38*; therefore, we next tested the hypothesis that factors secreted by senescent cells could increase CD38 expression and activity in non-senescent cells. It has been previously demonstrated that senescent cells have a secretory phenotype that includes TNF-α, IL-6, IL-8, IFN-γ, MCP-1 and many other chemokines and cytokines (12). Thus, first we established the expression of some of these inflammatory genes considered SASP factors (*IL-6, IL-8, MCP-1*) in our HUVECs exposed to x-ray- or γ-IR (Fig. 2A).

Next, we investigated whether components of the SASP were able to regulate the expression of CD38 in non-senescent cells. M1 macrophages are the prototype CD38-expressing cells, and it has been demonstrated that induction of the M1 phenotype by LPS, TNF-α, and activators of the LXR receptor leads to increased CD38 expression in these cells [20,27–31]. In fact, CD38 is proposed to be one of the main markers of induction of the M1 phenotype in macrophages [32]. Here we show that representative SASP factors (TNFα, IL-6, CXCL1, CXCL2) had small or no significant effects on CD38 activity or protein expression when given individually, but when combined promoted a synergistic effect that led to a strong increase in CD38 activity (Fig. 2B) and CD38 protein levels (Fig. 2C) in murine bone marrow-derived macrophages (BMDM).

3.3. Conditioned media from senescent cells induces CD38 expression in macrophages and endothelial cells.

We next tested whether or not conditioned media derived from senescent cells could induce CD38 expression in both inflammatory (BMDM) and non-inflammatory (HUVEC) cells (scheme Fig. 3A). We focused on BMDM and HUVECs because previous reports demonstrate that CD38 *in vivo* is preferentially expressed in inflammatory and endothelial cells [9,33]. In support of our hypothesis, we observed that exposure to conditioned media from x-ray- or γ-IR-induced senescent HUVECs leads to increases in CD38 mRNA expression and NADase activity in mouse BMDMs compared

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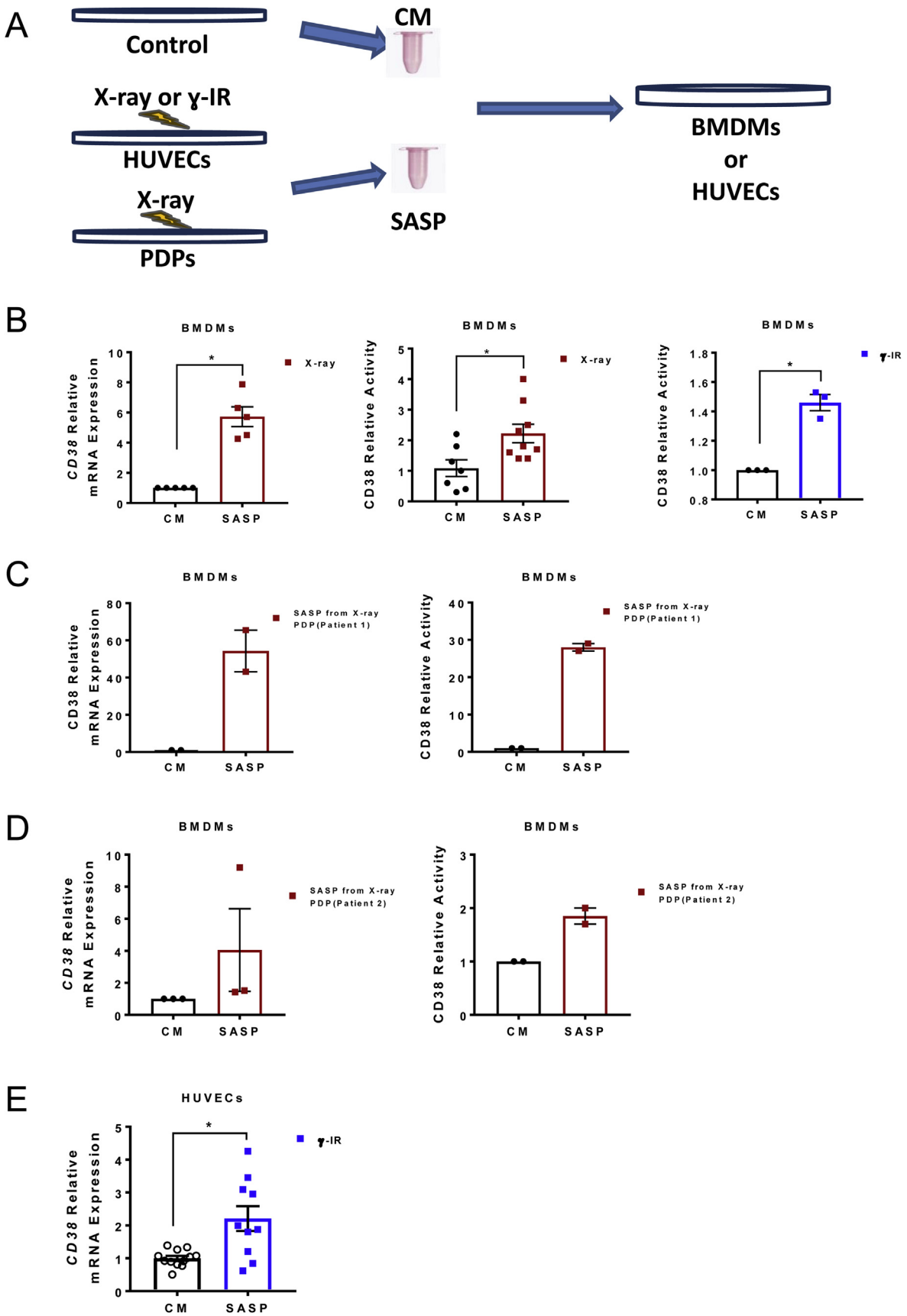

Fig. 3. CD38 is induced in non-senescent cells by secreted factors (SASP) from senescent cells. (A) Scheme showing production of control conditioned media (CM) and SASP (senescent cell conditioned media) from HUVECs or PDPs for treatment of non-senescent cells. (B) Relative CD38 mRNA expression and activity in BMDMs treated with CM or SASP from x-ray- (n = 7–9) or γ-IR-treated (n = 3) HUVECs. (C and D) CD38 mRNA expression and activity in BMDMs treated with CM or SASP from PDP originating from (patient 1 in C) or (patient 2 in D) (n = 2–3). (E) Relative *CD38* expression (n = 4–6) in HUVECs treated with CM or SASP from γ-IR MEFs. Data are mean ± SE, *p < 0.05 vs control.

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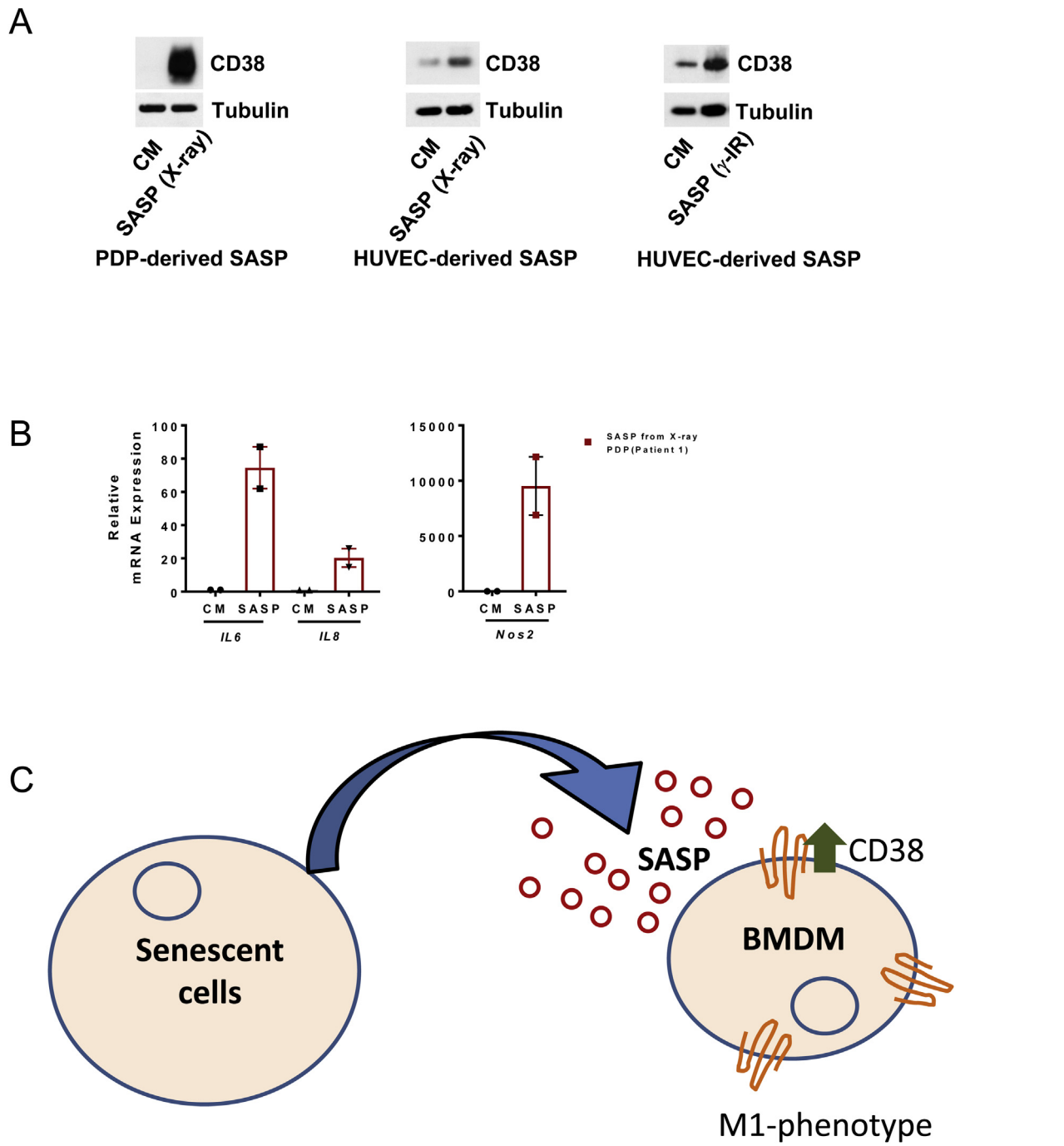

Fig. 4. CD38 and other M1 markers in macrophages treated with SASP. (A) Representative immunoblots demonstrating levels of CD38 in BMDMs treated with CM (control conditional media) or SASP (x-ray- or γ-IR-induced senescent cell conditioned media) from PDPs or HUVECs. (B) mRNA expression of other M1 markers, namely *IL-6*, *IL-8*, and *Nos2* (inducible nitric oxide synthase), in BMDMs treated with CM or SASP from PDPs. (C) Model of SASP-induced CD38 expression in macrophages. Data are mean ± SE, $^{*}p < 0.05$ vs control.

to control condioned media (Fig. 3B).

It has been previously demonstrated that pre-adipocytes are one of the main cells that become senescent *in vivo* [10–12]. Thus, we next tested the possibility that conditioned medium derived from senescent human preadipocytes (PDPs) induces the expression and activity of CD38 in mouse BMDMs. In support of our hypothesis we observed that conditioned media from senescent preadipocytes derived from two independent patients induced CD38 expression and CD38 NADase activity in BMDM, although media from one of the patients caused a much stronger induction of CD38 than the other (Fig. 3C and D). We also observed that the SASP from senescent cells also induced CD38 expression in endothelial cells (Fig. 3E).

We further investigated if SASP increased protein expression of CD38 in BMDMs. Indeed, we found that SASP from different senescent cells induced a robust increase in CD38 protein levels in macrophages (Fig. 4A). Compatible with the idea that the SASP could induce the M1 macrophage phenotype, we also observed that the SASP from senescent cells increased expression of other cellular markers of the M1 phenotype such as IL-6, IL-8, and iNOS (Fig. 4B

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and C). These data together demonstrate that SASP from different cell types can induce CD38 expression in both macrophages and endothelial cells.

Aging is characterized by the development of metabolic dysfunction and increased risk for age-related diseases including diabetes, loss of muscle function, and cancers. As the world population ages, the incidence of these conditions will further increase. It is not completely understood how molecular changes during aging lead to metabolic dysfunction and age-related diseases. NAD^+ is a critical nucleotide involved in both catabolic and anabolic reactions, serving as an acceptor or donor of electrons, and also as a substrate for enzymatic processes involved in the control of metabolism, epigenetics, DNA repair, and cell signaling [20,21,26]. Recently, it has been described that cellular NAD^+ levels decline during chronological aging and progeroid states [1–9,34]. Interestingly, this NAD^+ decline appears to play a direct role in the development of age-related metabolic dysfunction, age-related diseases, and a decline in tissue resilience after injury in aging [1–9,33–35]. In fact, cellular NAD^+ decline is emerging as one of the hallmarks of aging [1–9,20,21,26]. Until recently, the prevailing hypothesis had been that activation of DNA-repair enzymes such as PARPs during the aging process would consume and deplete NAD^+ [1–9,20,21,26]. In a recent manuscript, we challenged this paradigm and demonstrated that levels and activity of the NADase CD38 increase during aging, and that this enzyme is the main NAD-consuming enzyme responsible for the aging-related NAD^+ decline [2,9]. We showed that the activity of CD38, but not PARP, increases in several tissues during the aging process, and that genetic ablation of CD38 protects against age-related NAD^+ decline and, mitochondrial dysfunction [2].

Our studies present an extremely novel connection between the NAD^+ catabolizing enzyme CD38 and the senescence/SASP phenotype. Very interestingly, we observed that secreted biological compounds from senescent cells are responsible for the increased expression of CD38, at least in macrophages and endothelial cells. Although all these data indicate that senescent cells via their SASPs can lead to CD38 expression in cells, it does not exclude the possibility that other mechanisms mediated by senescent cells could also lead to the increased expression of CD38 during the aging process. It is also possible that induction of CD38 expression via senescent cells could be mediated by a different mechanism. For example, it has been previously demonstrated that senescent cells can express Major histocompatibility complex II (MHC-II) [36]. Direct interaction of immune cells with the MHC-II in senescent cells could also lead to increased CD38 expression in T cells in the liver.

CD38 expression in several cell types is induced by inflammatory cytokines such as TNF-α and endotoxins via activation of a NF-κB [28–31]. Interestingly, these pathways appear to be involved in the “sterile” inflammation observed during the aging process [17,23], and several cytokines are secreted by senescent cells as part of the SASP [17,23]. Furthermore, it has also been observed that clearance of senescent cells *in vivo* can decrease the expression of several components of the SASP including TNF-α, IFNγ, IL-1b, IL-8 and IL-6 [10–19]. This may indicate that senescent cells *in vivo* may modulate CD38 expression via changes in tissue cytokines and chemokines (Fig. 4C). This hypothesis will be further tested in our laboratory using senolytic agents. Although at this moment we cannot exclude that senescent cells express CD38 *in vivo*, induction of cellular senescence by several different stimuli in mouse and human cells *in vitro* did not increase expression of CD38. These data support the hypothesis that senescent cells regulate CD38 expression in non-senescent cells via their SASPs.

In conclusion, we demonstrate that the factors related to the SASP induce CD38 expression and activity in inflammatory and endothelial cells. Interestingly, we have previously demonstrated that in mammalian tissues the majority of $CD38^+$ cells are comprised of inflammatory and tissue endothelial cells, with low or undetectable expression in tissue parenchymal cells [2,9,33]. Thus, it is possible that SASP factors are one of the main drivers of CD38 expression and NAD^+ decline mediated by $CD38^+$ cells during age-related NAD^+ decline and metabolic dysfunction (Fig. 4C).

Author contributions

E.N.C. and C.C.S.C generated the hypothesis of the manuscript. E.N.C. generated the concept of the manuscript. E.N.C., and C.C.S.C. were involved in design of experiments. C.C.S.C., K.A.H., G.M.W., M.G.T., and T.R.P. performed experiments and analyzed data. All authors contributed to the writing and editing of the manuscript.

Declaration of interests

Dr. Chini holds a patent on the use of CD38 inhibitors for metabolic diseases.

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