

# Senescence associated secretory phenotype profile from primary lung mice fibroblasts depends on the senescence induction stimuli

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**Abstract** Cellular senescence is a multifactorial phenomenon of growth arrest and distorted function, which has been recognized as an important feature during tumor suppression mechanisms and a contributor to

aging. Senescent cells have an altered secretion pattern called Senescence-Associated Secretory Phenotype (SASP) that comprises a complex mix of factors including cytokines, growth factors, chemokines, and matrix metalloproteinases. SASP has been related with local inflammation that leads to cellular transformation and neurodegenerative diseases. Various pathways for senescence induction have been proposed; the most studied is replicative senescence due to telomere attrition called replicative senescence (RS). However, senescence can be prematurely achieved when cells are exposed to diverse stimuli such as oxidative stress (stress-induced premature senescence, SIPS) or proteasome inhibition (proteasome inhibition-induced premature senescence, PIIPS). SASP has been characterized in RS and SIPS but not in PIIPS. Hence, our aim was to determine SASP components in primary lung fibroblasts obtained from CD-1 mice induced to senescence by PIIPS and compare them to RS and SIPS. Our results showed important variations in the 62 cytokines analyzed, while SIPS and RS showed an increase in the secretion of most cytokines, and in PIIPS only 13 were incremented. Variations in glutathione-redox balance were also observed in SIPS and RS, and not in PIIPS. All senescence types SASP displayed a pro-inflammatory profile and increased proliferation in L929 mice fibroblasts exposed to SASP. However, the behavior observed was not exactly the same, suggesting that the senescence induction pathway might encompass dissimilar responses in adjacent cells and promote different outcomes.

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## Introduction

Aging is an irreversible process characterized by the progressive loss of physiological and structural integrity of cells and organisms (Rajawat et al. 2009; López-Otin et al. 2013). In humans, besides increasing vulnerability to death, it deepens the risk of cancer and cardiovascular and neurodegenerative diseases (López-Otin et al. 2013). Hayflick and Moorhead described senescence for the first time, relating it with the limited number of cellular replications (Hayflick et al. 1961; Hayflick 1965). Although senescence was discovered as an *in vitro* phenomenon, the presence of senescent cells has also been shown *in vivo* during aging (Dimri et al. 1995; Cosme-Blanco et al. 2007) and is more recently associated with development (Storer et al. 2013; Muñoz-Espin et al. 2013).

Cellular senescence is recognized as a tumor suppressor mechanism because damaged cells undergo cell cycle arrest preventing aberrant proliferation (Wagner et al. 2001; Campisi et al. 2007); however, accumulation of senescent cells can also contribute to the aging phenotype due to pleiotropic antagonism (Campisi 2013). The principal pathways described to achieve senescence include p53/p21 and/or pRB/p16<sup>INK4a</sup> (Muller 2009), which are triggered by DNA damage response (DDR) (Rodier et al. 2007). These pathways can be stimulated by telomere erosion during what has been called replicative senescence (RS) (Blazer et al. 2002; Dimri et al. 2005); nevertheless, it has been suggested that senescence *in vivo* might be prematurely attained mainly as a response to different stressors independently of cellular duplications number, *i.e.*, independently of telomere shortening (Rodier and Campisi 2011). Senescence can be induced by diverse stimuli such as stress-induced premature senescence (SIPS) after H<sub>2</sub>O<sub>2</sub> treatment (Chen et al. 2005; López-Diazguerrero et al. 2006), exposition to UV and gamma radiation (Lee et al. 2011), or hyperoxia (Toussaint et al. 2000), along with oncogenic stress (Bartkova et al. 2006), proteasome inhibition (Torres et al. 2006; Bitto et al. 2010), or mitophagy impairment (Kang et al. 2011; Fujii et al. 2012).

Senescent cells secrete diverse molecules to the extracellular environment, including growth factors, metalloproteinases, cytokines, chemokines, and other

molecules with paracrine and autocrine activities (Rodier et al. 2011). This secretion is one of the most interesting characteristics of senescent cells and is called senescence associated secretory phenotype (SASP). Factors that are secreted by senescent cells include interleukins 6, 7, and 8 (IL-6, IL-7, IL-8); Monocyte Chemoattractant Protein 2 (MCP-2); Macrophage Inflammatory Protein 3 $\alpha$  (MIP-3 $\alpha$ ); Growth Regulated Oncogene alpha (GRO $\alpha$ ); Hepatocyte Growth Factor (HGF); Insulin-like Growth Factor Binding Protein (IGFBP); and others (Coppé et al. 2008; Coppé et al. 2010; Laberge et al. 2015). Certain cytokines and chemokines included in SASP can attract and activate cells of the immune system in order to promote its clearance (Krizhanovsky et al. 2008). Nevertheless, in older individuals, where the immune system is compromised (McElhaney et al. 2009), the SASP secreted by senescent cells accumulated in tissues and significantly altered the cellular microenvironment and structure (Campisi et al. 2011). Studies have suggested a link between senescence and various age-related diseases including primarily cancer (Kiecolt-Glaser et al. 2003) and neurodegenerative disorders such as Alzheimer's disease (Bitto et al. 2010; Bhat et al. 2012).

As mentioned, it has been proposed that numerous stimuli are capable to bring on diverse types of senescence; therefore, it is noteworthy to investigate if senescent cells might have a different SASP profile as a function of the stimuli that induced senescence. Hence, the aim of this work was to use three different stimuli to induce senescence and determine the effects on SASP profile. We characterized the secretion of some SASP components in response to oxidative stress (SIPS) and proteasome inhibition induced premature senescence (PIIPS) in primary lung mice fibroblasts and compared them with SASP from replicative senescence (RS). Since the glutathione-redox balance is known to play a significant role in regulating cellular immune responses (Alam et al. 2010; Reynaert 2011), GSH/GSSG ratio was determined. Our results showed that SASP profile in RS and SIPS were very similar, but both were very different from PIIPS profile.

SASP is known to have an autocrine as well as a paracrine effect in neighboring cells (Acosta et al. 2013) and to induce cell proliferation in transformed cells (Campisi et al. 2011; Zacarias-Fluck et al. 2015). So, in order to determine if the distinctive SASP, secreted by cells that arrived to senescence by different stimulus, is able to provoke a dissimilar effect on cells predisposed

to immortalization, L929 cell line (originally derived from lung mice fibroblasts) was exposed to the SASP obtained from the different senescence (RS, SIPS, and PIIPS). L929 is a transformed cell line that exhibits an unlimited growth potential and has been used for many years to compare transformed cells with primary cultures (Abken et al. 1993). As expected, the response was not the same, but interestingly cells treated with the different SASP increased their proliferation, suggesting that the pro-inflammatory profile maintained in all senescent types might be sufficient to induce proliferation, but might as well have a different proficiency to do it.

## Materials and methods

### Chemicals

All chemicals and reagents were of the highest analytical grade, and unless otherwise indicated, they were purchased from Sigma (St. Louis, MO).

### Animals

Primary fibroblasts were isolated from the lung of neonatal (3–7 days old) CD-1 mice (*Mus musculus*), provided by the closed breeding colony of the Universidad Autónoma Metropolitana-Iztapalapa (UAM-I). All procedures with animals were strictly carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Mexican Official Ethics Standard 062-ZOO-1999.

### Cell culture

Lung fibroblasts were obtained according to a protocol previously established (López-Diazguerrero et al. 2006); pools of lungs from three animals were used for each cell culture. Cells were grown at 37 °C, 5 % CO<sub>2</sub> in MEM supplemented with 10 % FBS (GIBCO-BRL), 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO-BRL). The medium was replaced every 3 days. Cells were trypsinized upon reaching confluence and replated to continue time in culture.

### Induction of senescence

Six days after isolation, primary lung fibroblasts were trypsinized and seeded at a density of  $3 \times 10^3$  cells per

well on a 24-well multichamber (Corning, Acton MA, USA). After 72 h in culture, cell premature senescence was induced.

### *Stress-induced premature senescence (SIPS)*

Cells were incubated with 75 µM H<sub>2</sub>O<sub>2</sub> dissolved in the culture medium for 2 h at 37 °C and 5 % CO<sub>2</sub>; H<sub>2</sub>O<sub>2</sub> was removed by changing the medium and the cells were allowed to recover in fresh culture medium for 48 h before further measurements.

### *Proteasome inhibition-induced premature senescence (PIIPS)*

Cells were incubated with 5 nM epoxomicin dissolved in the culture medium; the medium was not removed until the normal medium replacement. Epoxomicin at the same concentration was added to the culture medium throughout subsequent medium changes. In order to determine proteasome inhibition in epoxomicin treated cells, chymotrypsin activity was determined with the fluorogenic substrate Suc-LLVY-AMC at different time points, as previously described (Torres et al. 2006) data not shown.

### *Replicative senescence (RS)*

Cells were allowed to proliferate in culture until they reached the Hayflick limit, as previously reported by our group (Königsberg et al. 2007; Triana-Martínez et al. 2014).

### Proliferating rate and senescence determination

Determinations were performed every 3 days by triplicate starting at day 9 after isolation until day 21. All determinations were always performed with subconfluent cultures in three independent cultures from independent donor animals. To prevent quiescence due to contact inhibition, cell cultures that achieved confluence were trypsinized and the entire population was seeded in a larger well (six-well multi-chambers) (Corning, Acton, MA, USA). The entire population of cells in a well was quantified and scored in order to measure the proliferation rate as described elsewhere (López-Diazguerrero et al. 2006).

### [<sup>3</sup>H] Thymidine labeling for DNA synthesis

Determinations were performed every 3 days by triplicate in SIPS, PIIPS, and RS cells, in the same conditions described above for proliferating rate. An aliquot of 1  $\mu$ Ci [<sup>3</sup>H]-thymidine (NEN, USA) was used as previously described (López-Diazguerrero et al. 2006).

### p16 and $\beta$ -galactosidase immunofluorescence experiments

Seven days after isolation, primary lung fibroblasts were trypsinized and seeded on coverslips at a cell density of  $3 \times 10^3$  cells/coverslip. After 48 h, cells were treated to induce SIPS and PIIPS or untreated in the case of RS. Preparations for immunocytochemistry were made at different days for each group: 15, 18, and 21 for RS, and 12 and 15 for SIPS and PIIPS. Cells were fixed in 3 % formaldehyde/PBS for 15 min and washed twice with 2 % PBS-tween (PBS-t). After that, cells were incubated with 200:1 of protein blocker for 10 min, washed again, and incubated for 1 h with anti-p16 (dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed twice with PBS-t and incubated for 1 h with anti- $\beta$ -galactosidase (dilution 1:200; Abcam, Cambridge, MA). After three extra washes, cells were incubated for 1 h with anti-rabbit secondary antibody (1:500; Alexa Fluor<sup>®</sup> 488), washed twice with PBS-t, and incubated for 1 h with anti-mouse secondary antibody (1:500; Alexa Fluor<sup>®</sup> 594). Finally, cells were washed three times and mounted with fluorescent mounting medium (DakoCytomation, Glostrup Denmark) and 0.01 % DAPI (4,6-diamino-2-phenylindol) on glass slides. Single plane images were obtained with a confocal microscope LSM-META-Zeiss Axioplan 2 imaging at  $\times 30$  with the Diodo Laser 405 nm for DAPI and Ar/ML 458/488/514 nm for ALEXA. Cells were considered senescent when they were positive for both p16 (nuclear) and  $\beta$ -galactosidase (cytosolic).

### SASP analysis

The components present in the SASP secreted by the different senescent-induced cells were analyzed and compared at selected time points for each kind of senescence (day 15 for SIPS and PIIPS, day 21 for RS, and day 9 for control non-senescent cells). MEM supplemented medium was changed to MCDB105-free serum (Conditioned Media, CM), and cells were incubated for

48 h. After that time, CM was recovered and frozen at  $-80$  °C for further analysis. When all samples were collected, CM was thawed and concentrated 15-fold (final volume 1 mL). Sixty-two cytokines were analyzed with the kit Mouse Cytokine Array C3 AMM-CYT-3 (RayBiotech Inc., Norcross, GA) following instructions from kit protocol. Briefly, membranes were incubated in blocking buffer for 1 h and 1 mL of each sample was added to the membranes and incubated further for 2 h. Afterwards, membranes were washed with the corresponding washing buffers and incubated for two additional hours with 1 mL of biotin conjugated-antibody mixture. Membranes were washed again and incubated with HPR-streptavidin for 2 h. After a final wash, 1 mL of detection buffer was added to the membrane for 2 min to detect the conjugation signal. Membranes were exposed to x-ray detection films for 10–15 min and then exposed in an image processor (Kodak X-Omat1000A). Images were examined with the software ImageJ v.1.47 to obtain a semi-quantitative analysis and data were normalized to cell number in each sample.

### GSH and GSSG determination

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined in RS, SIPS, and PIIPS cells at the same days used for the previous experiments using the fluorometric assay previously described by Galván-Arzate (2005). The final results were expressed as micrograms of GSH or GSSG per milligram of protein.

### L929 mice fibroblast treatment with SASP medium

Seven days after the isolation, primary fibroblasts were seeded in 24-well plates at a cellular density of  $2.5 \times 10^4$  per well. Senescence was induced at day 9 as described before. At the same time points used before (day 15 for SIPS and PIIPS, and day 21 for RS), cells were washed with PBS, and medium was replaced with fresh MEM-10 % FBS. After 48 h, medium (*SASP-medium*) was collected and frozen at  $-80$  °C until use. At the same time, L929 mice fibroblasts were seeded on coverslips at a cellular density of  $2.5 \times 10^4/\text{cm}^2$  and maintained in MEM + 10 % FBS for 48 h. After that time, the medium was replaced with MEM supplemented with *SASP-medium* from SIPS, PIIPS, and RS at 1:1 rate. Importantly, in order to compare the results, the number of cells

treated with each conditioned medium was always the same as that of the cells from which the CM was taken.

L929 mice fibroblasts were maintained in this condition for 72 h. Control cells were cultured with the conditioned media from non-senescent cells (C-NS). Cell number was determined every 24 h, as described above, to determine proliferation.

### Statistical analysis

Data are reported as the means  $\pm$  SD for at least three independent experiments using cells from different donor animals. The ANOVA test followed by the Tukey-Kramer variance analysis and paired Student *t* test were used to compare data. A 0.05 level of probability was used as a minimum criterion for significance.

## Results

### Oxidative stress and proteasome inhibition induce premature senescence in mice lung fibroblasts

To compare the effects of different stressors on senescence, cellular proliferation rate and DNA synthesis were evaluated in untreated and H<sub>2</sub>O<sub>2</sub> or epoxomicin treated cells. As shown in Fig. 1a, proliferation rate for RS logarithmically increased during the first days in culture until day 15 when cells ceased their proliferation as expected. When oxidative stress-induced senescence and proteasome inhibition-induced senescence were compared with RS, a significant difference was observed ( $p < 0.05$ ). Figure 1b illustrates that [<sup>3</sup>H]-thymidine incorporation has a complementary behavior to the proliferation rate. There was a rapid increase in DNA synthesis for RS cells, with a significant decrease after day 15 ( $p < 0.05$ ) and stable [<sup>3</sup>H]-thymidine incorporation in stress-induced cells, which was significantly different from RS during the whole experiment ( $p < 0.05$ ). Altogether, these experiments show that oxidative stress and proteasome inhibition stopped proliferation of lung mice fibroblasts.

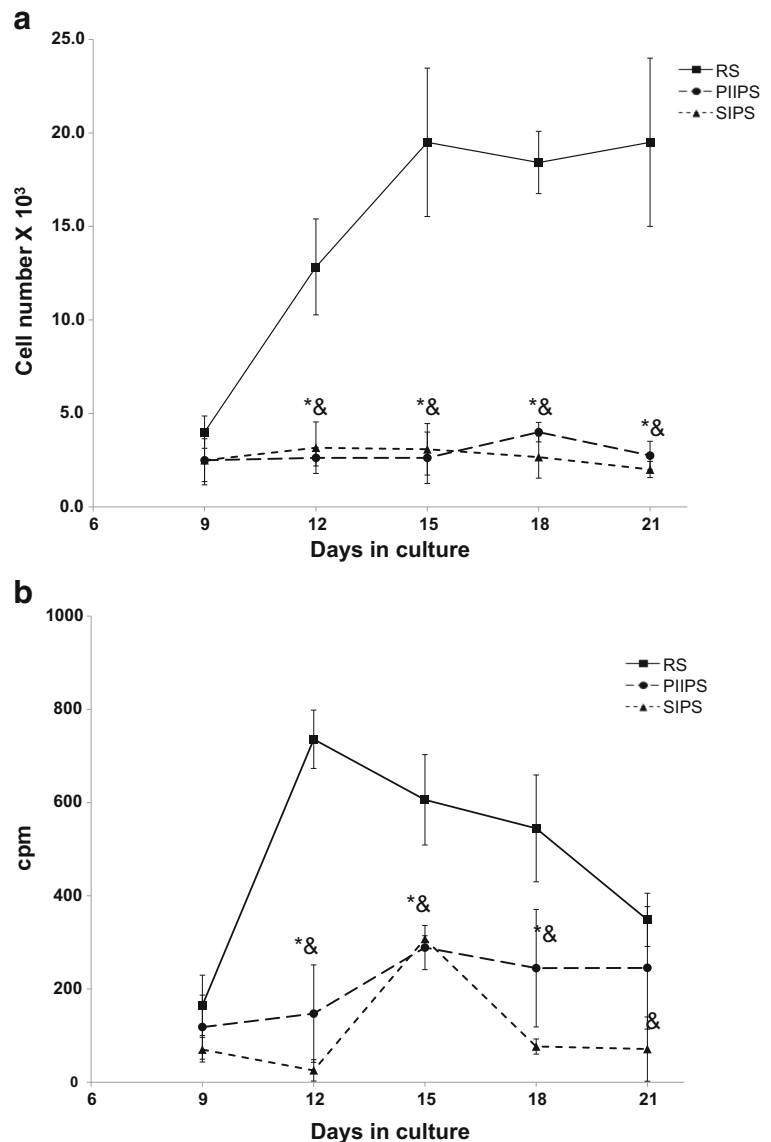
In order to verify if the proliferation arrest observed was indeed associated to senescence, we evaluated two molecular markers associated to this phenomenon: the cell cycle inhibitor p16 and the enzyme  $\beta$ -galactosidase ( $\beta$ -gal). Figure 2a shows that non-treated cells gradually increased both markers' signal, starting from unclear signals at day 9 and reaching intense signals at day 21.

Moreover, from day 18 on, p16 co-localized with DAPI staining in the nucleus (arrows in Fig. 2a), suggesting that this cell cycle regulator was performing its function inhibiting the CDK4/6–cycline D complexes and, therefore, cell cycle inhibition. Cells treated with H<sub>2</sub>O<sub>2</sub> or epoxomicin (Fig. 2b) augmented both markers' signal since day 12 (3 days after the treatment). This is consistent with the early arrest on cell proliferation observed in these groups. Only cells that were positive for p16 staining in the nucleus and  $\beta$ -gal in the cytosol were considered as senescent. Figure 2c shows a close-up of the merged images for RS, SIPS, PIIPS, and control cells. Supplementary Figure 1 shows the unmerged images for each marker (p16, DAPI,  $\beta$ -gal). Figure 2d represents the percentage of positive senescent cells. As observed, non-treated cells became senescent gradually obtaining almost 30 % of positive cells at day 15 and more than 70 % at day 21, implying RS. H<sub>2</sub>O<sub>2</sub>-treated cells quickly reached almost 80 % positive cells from day 12, and epoxomicin-treated cells showed 40 % positive cells at day 12 and 85 % at day 15. These results confirm the idea that oxidative stress and proteasome inhibition are stimuli capable to induce premature senescence in mice lung fibroblasts and coincide with previous reports of our laboratory (López-Diazguerrero et al. 2006; Triana-Martínez et al. 2014) and others (Torres et al. 2006). To simplify terminology, untreated primary fibroblasts, which were led to senesce in culture, will be abbreviated as RS (replicative senescence), while H<sub>2</sub>O<sub>2</sub>-treated primary fibroblasts will be termed as SIPS (stress-induced premature senescence) and epoxomicin-treated cells will be called as PIIPS (proteasome inhibition-induced premature senescence cells).

### Redox status during RS and stress-induced senescence

Redox status changes have been recognized as promoters of senescence; therefore, to compare what is happening during senescence induction by different stimuli, GSH/GSSG ratio was quantified (Fig. 3). As expected, and in concordance with previous data (Triana-Martínez et al. 2014), there was a significant decrease in GSH/GSSG ratio ( $p < 0.05$ ) during senescence in RS and SIPS when compared with control non-senescent cells, but no difference was observed between them, pointing towards a more oxidized environment during these senescence. Moreover, no differences in redox state were observed in PIIPS compared to non-senescent cells, but a significant difference was obtained

**Fig. 1** Proliferation rate during RS, PIIPS, and SIPS. **a** Cellular proliferation. Senescence was induced as described in “Materials and methods” section and determinations were obtained in RS (*squares*), PIIPS (*circles*), and SIPS (*triangles*) every 3 days starting 9 days after the cell isolation. **b** DNA synthesis assayed by [<sup>3</sup>H]-thymidine incorporation was determined in RS, SIPS, and PIIPS cells every 3 days starting 9 days after the cell isolation as described in “Materials and methods” section. Each point represents the mean  $\pm$  SD of three independent experiments performed by triplicate. (\*) SIPS statistical significance against RS ( $p < 0.05$ ). (&) PIIPS statistical significance against RS ( $p < 0.05$ )

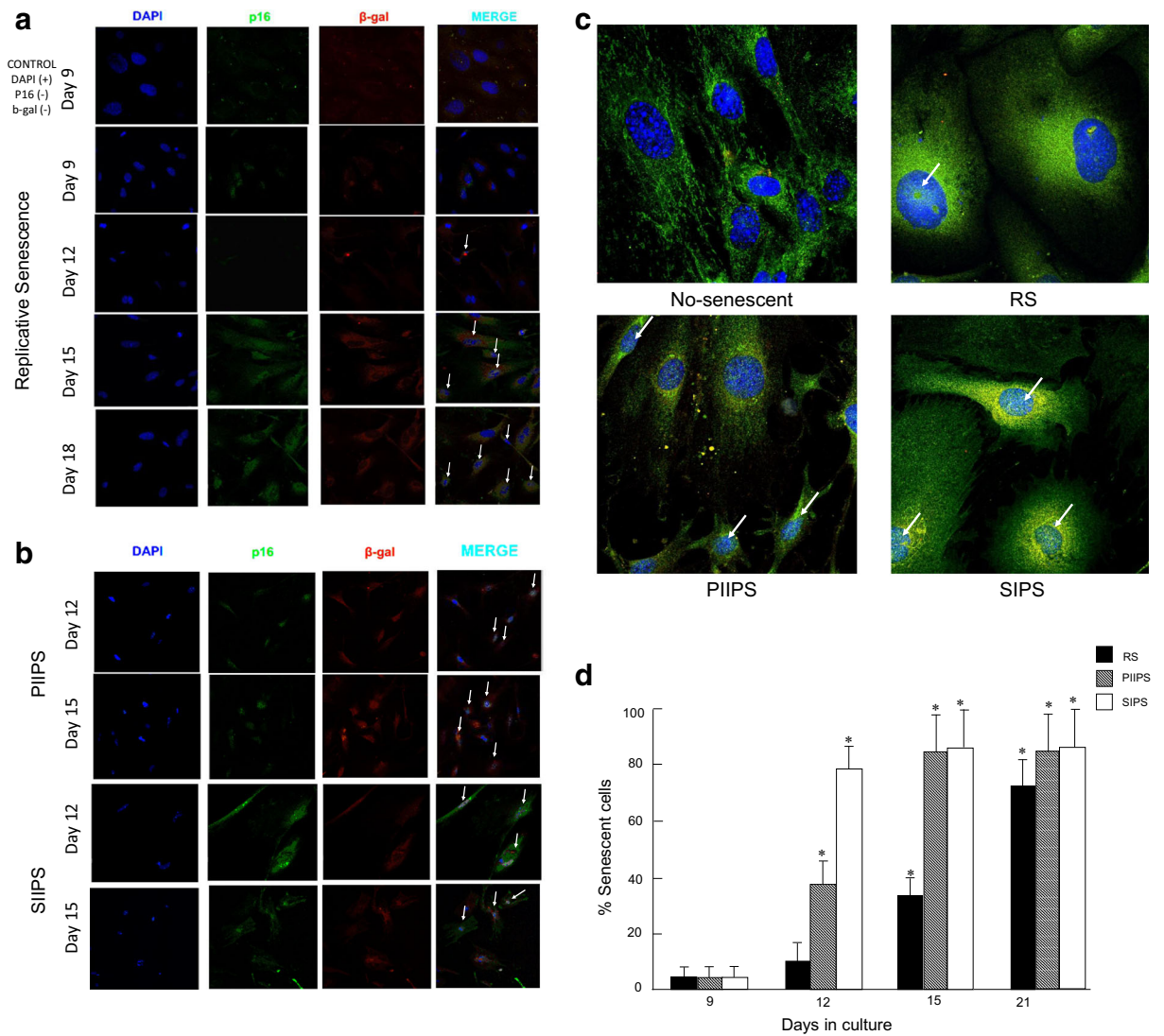


against SIPS, suggesting that probably redox modifications play a different role during PIIPS induction.

Senescence associated secretory phenotype displays a different profile depending on the stimuli that induced senescence

Since different stimuli were able to induce premature senescence in our model, we inquired if the cells could send different signals through the SASP components during each type of premature senescence (SIPS vs. PIIPS) compared to SASP

observed during RS. In order to make an easier analysis of the 62 cytokines analyzed using a semi-quantitate kit (RayBiotech), changes in cytokines are reported as relative optical density (ROD) of each spot, normalized to the cytokine secretion in non-senescent cells. Representative membranes used for SASP analysis are shown in Supplementary Figure 2. Cytokines were classified in four groups as follows: (a) Chemokines (Fig. 4a), (b) Inflammatory cytokines (Fig. 4b), (c) Growth factors (Fig. 4c), and (d) Other cytokines and soluble receptors (Fig. 4d).



**Fig. 2** Senescence determination: p16 and  $\beta$ -galactosidase expression. Fixed cells were incubated with anti-p16 and anti- $\beta$ -galactosidase antibodies as described in “Materials and methods” section. Representative immunocytochemistry images are shown for RS (a) and PIIPS and SIPS (b). Cells where p16 (green) colocalized with DAPI (blue), along with  $\beta$ -galactosidase high intensity in the cytosol (red), were considered senescent. c Close-up for merged images for control (day 9), RS (day 21), PIIPS

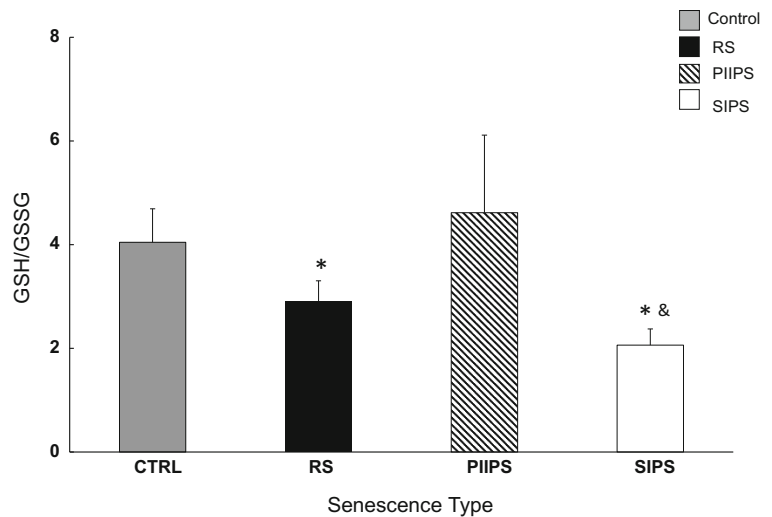
(day 15), and SIPS (day 15) cells. The unmerged images for these pictures are shown in supplementary Figure 1. d Percentage of senescent cells scored. Each bar represents the mean  $\pm$  SD from five random fields of each preparation that were analyzed. Preparations were obtained from three independent experiments by triplicate. \*Statistical significance compared against pre-senescent cells (day 9) ( $p < 0.05$ )

A general overview of the analysis revealed a noteworthy difference in the secretion profile between PIIPS and the other two types of senescence; while most of the cytokines augmented their secretion in SIPS and RS, this secretion was diminished in PIIPS. Remarkably, the most reported and studied SASP components (e.g., IL-6, IL-12, IL-10, MIP-2, and IFN- $\gamma$ ) increased their secretion in the three types of senescence. Despite the

differences among them, these results point towards the existence of a well-conserved SASP phenotype. Nevertheless, it is important to recall that the amount of secreted cytokines and their combination might differently affect neighboring cells.

In RS, practically all the cytokines analyzed, with exception of Fas L, IL-4, and IL-9, increased their secretion. However, in PIIPS, the only cytokines that

**Fig. 3** Redox state. GSH/GSSG ratio was measured as described in Galván-Arzate (2005). Bars represent the mean  $\pm$  SD of three independent determinations accomplished by triplicate. \*Statistical significance against control ( $p < 0.05$ ). &Statistical significance against PIIPS ( $p < 0.05$ )



increased were CD30 L, CD40, ORG-2, IFN- $\gamma$ , IL-3, IL-6, IL-10, IL-12/p40/p70, MCP-1, MIP1- $\gamma$ , MIP2, MIP3- $\alpha$ , and RANTES. All the other cytokines decreased their secretion in this kind of induced senescence. The two most notable changes in the secretion were IL-10 and IL12/p40/p70 with almost 256-fold ( $\log_2$  scale) in RS and 16- to 32-fold in the other two groups. Also, TNF- $\alpha$  increased its secretion almost 32-fold in SIPS and 8-fold in RS. It is important to mention that the average of the increase for the other cytokines was around 8-fold.

In PIIPS, the most notable decrease in the secretion was observed for SDF1- $\alpha$ , almost 0.015625-fold, followed by IL-13, L-selectin, TARC, and TPO with almost 0.625-fold. On average, the other cytokines decreased their secretion almost 0.125-fold. So, even though there might be a representative or typical SASP phenotype, our data strongly suggest the existence of a differential secretion of some components depending on the stimuli that induced senescence.

SASP from RS, SIPS, and PIIPS induced proliferation in L929 mice fibroblasts

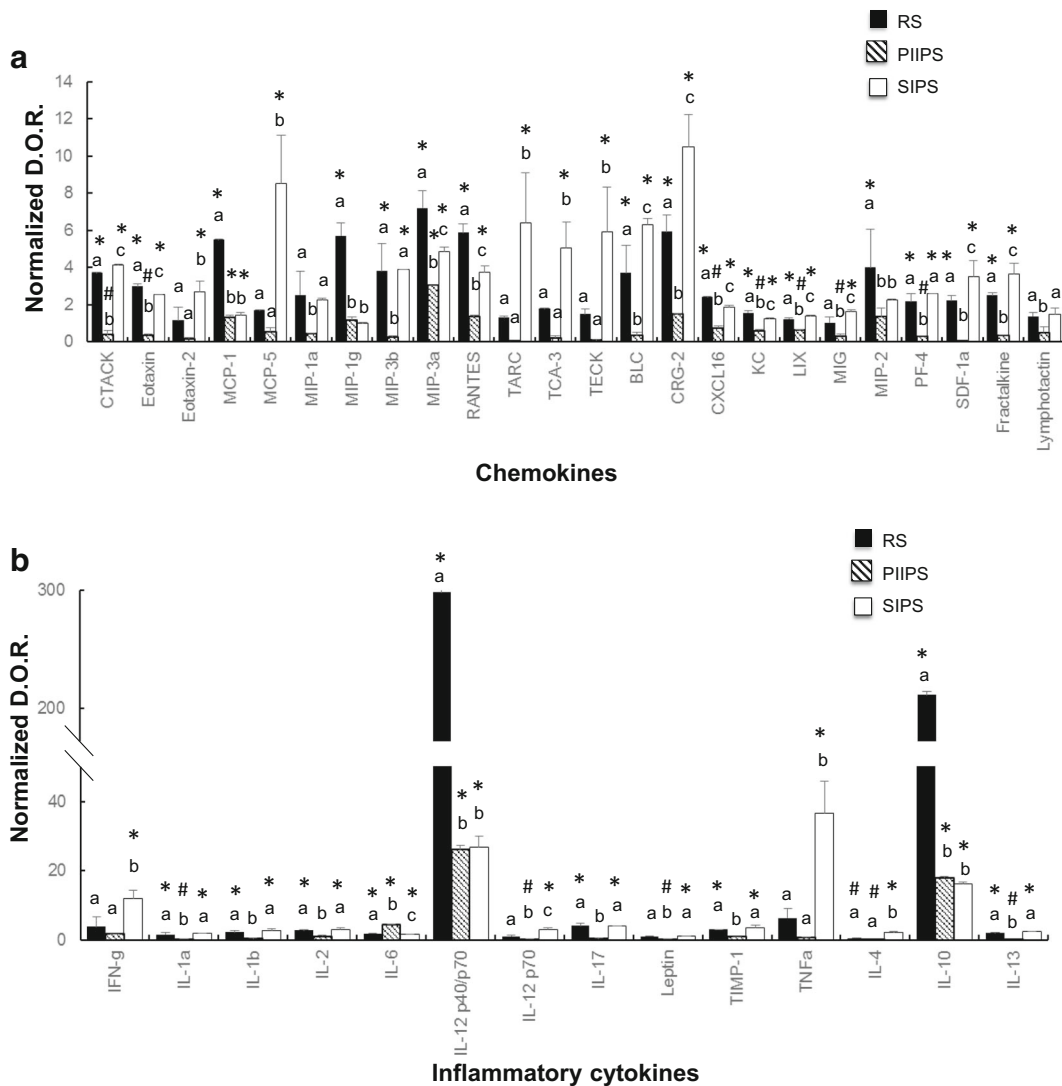
Since SASP profile was different depending on the senescence induction stimuli and in view of the fact that one of SASP effects is proliferation induction in pre-malignant adjacent cells, we evaluated if the SASP obtained from RS, SIPS, and PIIPS could have different effects on proliferation induction in the mice fibroblast cell line L929. Figure 5 shows L929 cellular

proliferation after treated with MEM supplemented with SASP collected from RS, PIIPS, or SIPS cells for 24, 48, and 72 h (SASP-Medium 1:1) (Fig. 5). All SASP types induced L929 proliferation, which was significantly different ( $p < 0.05$ ) from the untreated cells after 48 h, implying that the pro-inflammatory profile displayed by these senescent cells was enough to induce proliferation despite the differences observed in other cytokines. Interestingly, cells treated with PIIPS-SASP took longer to induce proliferation, suggesting that different SASP might have dissimilar effects on adjacent cells or at least different competency to modify them.

## Discussion

Cellular senescence is an intricate and multifactorial phenomenon of growth arrest and distorted function (Vijg and Campisi 2008), which has been recognized as an important feature during tumor suppression and a contributor to aging (Rodier and Campisi 2011). Various pathways have been proposed to explain senescence induction; the most studied is replicative senescence due to telomere shortening (Cristofalo et al. 2004; Rodier et al. 2009, 2010), along with other premature-induced senescence provoked by diverse stimuli such as oxidative stress and radiation exposure (Toussaint et al. 2000; López-Diazguerrero et al. 2006; Lee et al. 2011), autophagy impairment (Kang et al. 2011; Fujii et al. 2012), proteasome inhibition (Torres et al. 2006; Bitto et al. 2010), and oncogenic stress (Bartkova et al. 2006).





**Fig. 4** SASP profile depending on the inducer of senescence. Conditioned media from each type of senescence was collected and processed as described in “Materials and methods” section. Sixty-two cytokines were analyzed and distributed as follows: chemokines (a), inflammatory cytokines (b), growth factors (c), and other cytokines and soluble receptors (d). Relative Optical Density (R.O.D.) was measured using the software ImageJ v.1.47

and normalized with the control and cell number of each sample. The statistical analysis was independently done for each single cytokine. Bars having the same letter are statistically equal, while different letters mean a statistical difference. The symbol \* indicates statistically significant increase ( $p < 0.05$ ), and the # symbol indicates statistically significant decrease ( $p < 0.05$ )

It has been suggested that in vivo, senescence is mainly attained as a response to the different stressors more than due to telomere attrition (Rodier and Campisi 2011); hence, numerous transduction pathways might overlap and entail a constant crosstalk among them. It has also been suggested that a common feature to all described types of senescence is a shift to a pro-oxidant redox state (Dilley et al. 2003; Jones et al. 2002; Muller 2006). A

previous result from our laboratory (Triana-Martínez et al. 2014) showed that while SIPS induction was delayed by almost 50 % when cells were treated with the antioxidant trolox immediately after H<sub>2</sub>O<sub>2</sub> treatment, only a mild difference in senescence establishment was observed for the RS, suggesting that whereas the milieu becomes more oxidant, other pathways activated by the diverse senescence inducers might be modified as well,

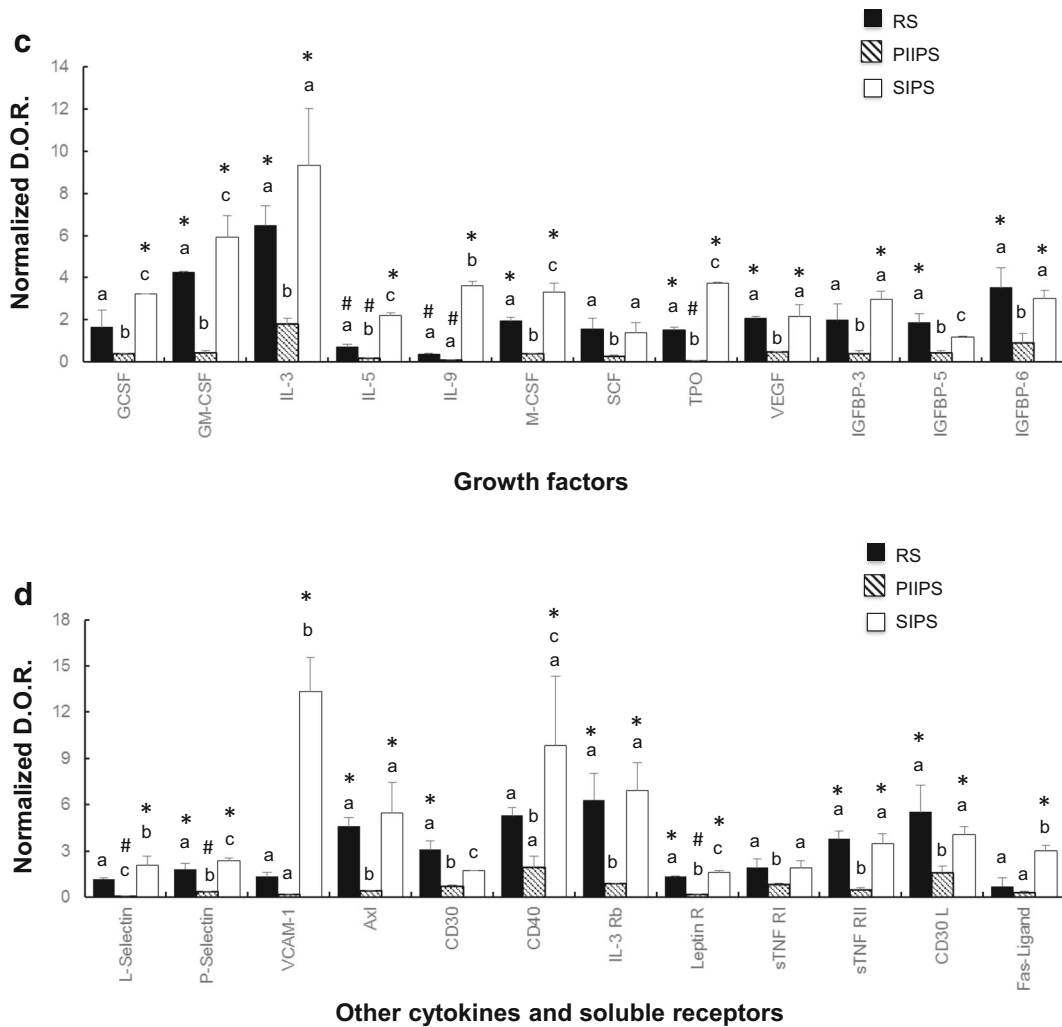


Fig. 4 continued.

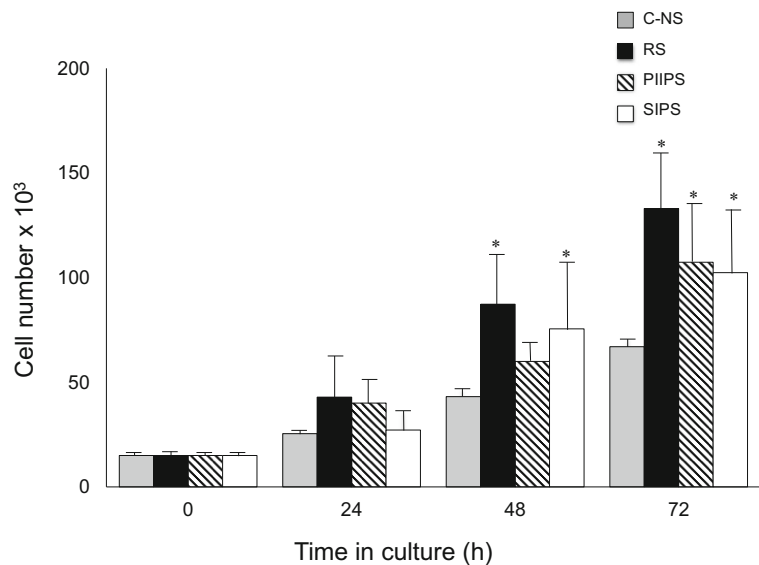
leading to diverse and characteristic outcomes in each senescence. An important consequence might be the secretion of distinctive SASP components, depending on the senescence pathway, which ultimately could differently affect neighbor cells and might induce diverse diseases including cancer and aging.

In this work, we characterized oxidative stress and proteasome inhibition-induced premature senescence (SIPS and PIIPS) in primary lung mice fibroblasts and compared them with replicative senescence (RS). The results showed here are consistent with our previous reports of SIPS induction in this model (López-Diazguerrero et al. 2006; Triana-Martinez et al. 2014) and with published work by Torres and coworkers (2006) where senescence was induced in

human fibroblasts WI-38 and rat astrocytes using epoxomicin or lactacystin. Accordingly, similarities and consistencies were observed among RS, SIPS, and PIIPS regarding the classical parameters used to evaluate cellular senescence, such as proliferation arrest and p16 and β-galactosidase overexpression. Hence, based on all the above, we wanted to determine if senescent cells secrete a different SASP profile as a function of the stimuli that caused senescence induction.

In one of the first SASP comparative studies, Coppé and coworkers (2010) reported that SASP profile in mice fibroblasts depends on the environmental oxygen concentration, while in a posterior work, they demonstrated that non-expressing p16<sup>INK4a</sup> senescent cells did

**Fig. 5** SASP effect on L929 proliferation. L929 mice fibroblasts were exposed up to 72 h to SASP-MEM media (1:1) from primary fibroblast. Proliferation was determined as described in “Materials and methods” section. Control cells were cultured with the conditioned media from non-senescent cells (C-NS). Each bar represents the mean  $\pm$  SE from three independent determinations in triplicate. \*Statistical significance against untreated cells at the same day ( $p < 0.05$ )



not secrete SASP even when they were positive for other markers of senescence, suggesting that SASP is only present in those senescent cells where genomic or/and epigenomic changes have occurred (Coppé et al. 2010, 2011; Campisi 2013). Here, we induced senescence with three different stimuli and then we utilized antibody arrays to detect multiple proteins simultaneously to compare the SASP profile. This approach provides an overview on the complexity of the immune response; thus, it is worthy to notice that significant changes in cytokines/chemokines profiles were found concerning to the secretion of some SASP components. In particular, we found that SIPS and RS have a similar SASP profile, which is very different from PIIPS profile that showed a remarkable decrement in the secretion of most of the cytokines analyzed, but preserved the increased secretion of the classical pro-inflammatory cytokines. This difference between the types of senescence concerning SASP is consistent with the difference concerning redox state; importantly, we found that RS and SIPS have more common attributes than PIIPS and these two characteristics suggest that RS and SIPS are modulated by common molecular pathways different than PIIPS. Hence, the precise mechanisms governing these effects are still not known. This could be partially explained if we assume that SIPS and RS have a very important shift to a pro-oxidant redox state (Dilley et al. 2003; Jones et al. 2002; Muller 2006; Triana-Martínez et al. 2014), which is apparently different in PIIPS (Fig. 3).

It is well known that complete abrogation of proteasome activity induces apoptotic cell death (Lopes et al. 1997; Pandit et al. 2011; Cheng et al. 2011); therefore, cellular senescence induced by proteasome impairment must only be attained as a partial inhibition, and some activity should be preserved (Torres et al. 2006; Bitto et al. 2010). Proteasome partial inhibition promotes a sub-lethal accumulation of ubiquitinated proteins that cannot be degraded, inducing cellular senescence instead of apoptosis (Cheng et al. 2011). It has been reported (Torres and Perez 2008) that proteasome inhibitors induce ROS and mitochondrial dysfunction, generating an important effect on protein ubiquitination and further degradation as discussed before. However, depending on the proteasome inhibitor and the concentrations used, it might be possible that PIIPS cells would still be able to buffer redox state and maintain a “healthy” glutathione-redox balance. In this context, one proposed hypothesis is that primary fibroblasts induced to PIIPS might modulate protein synthesis rate in order to avoid a greater accumulation of ubiquitinated proteins, which might be lethal. This decrease in protein synthesis rate could have an impact in the general profile of SASP components, reducing their production at the synthesis level, but maintaining the synthesis and secretion only for some SASP components, which might have some important (but still not well-defined) functions, such as IL-6, IL-10, and IL-12, among others.

It is interesting to note that IFN- $\gamma$ , one of the main regulators of Th1 response, was increased in the three

types of senescence. Consequently, it was expected to find a lower level of cytokines IL-4 and IL-10. However, only IL-4 decreased in PIIPS, while IL-10 secretion increased in the three SASP profiles. On the other hand, IL-12 (p40/p70) that is considered to be critical for Th1 differentiation was high in SR, PIIPS, and SIPS despite the IL-10 increment. Interestingly, the IL-12 (p70) secretion only decreased in PIIPS. It is known that IL-10 has a particular and singular behavior, but usually inhibits IL-1, IL-6, TNF- $\alpha$ , and GM-CSF, but our results showed that all cytokines, except IL-6, decreased only in PIIPS. This finding suggests that the interaction and control of cytokines production and the classical Th1/Th2 cross-talking profiles are deregulated in SR and SIPS, but are partially conserved in PIIPS.

Remarkably, another function of IL-10 is to inhibit nitric oxide synthesis in monocytes and macrophages, which in this case could be related with a global redox status modulation (Li et al. 2014), an aspect that is consistent with our previous results of GSH status. Besides, it is known that interleukin IL-12 (p70) promotes the differentiation of the Th1 cells and the production of IL-2 and IFN- $\gamma$  thereby supporting cellular immunity, while IL-12 (p40) acts as an antagonist of IL-12 p70 (Suzuki et al. 2003; Alam et al. 2010). Previous studies have reported that oxidative stress, in particular GSH status, suppresses the IL-12 (p40/p70) production resulting in a Th2 polarized immune response (Murata et al. 2002a, 2002b; Kamide et al. 2011). This condition might explain why SIPS and RS exhibit a more defined pro-inflammatory profile than PIIPS. In this sense, it would be interesting to determine changes in SASP profile throughout time. However, a deeper analysis of the data will help to comprehend and integrate the entire phenomenon.

Since the inflammatory components of SASP are supposed to promote cellular proliferation in adjacent cells (Campisi et al. 2011; Zacarias-Fluck et al. 2015), L929 were subjected to the SASP obtained from RS, SIPS, and PIIPS. Remarkably, our results showed that all treated cells increased their proliferation when compared to untreated cells, even though this effect was achieved more slowly when L929 were exposed to PIIPS-SASP. Interestingly, albeit most of the SASP components were different in PIIPS in comparison to RS and SIPS, SASP pro-inflammatory profile, which was conserved in the three senescence types, might be sufficient to induce proliferation in some kind of transformed cells. This is because, as mentioned before,

some of the most reported SASP components such as IL-6, IL-12, MIP-2, and IFN- $\gamma$  increased their secretion in all senescence studied. Hence, it is still necessary to study other SASP effects such as migration or wound healing response, as well as immune system activation, in order to determine the diverse outcomes induced by the different SASP. Clearly, more experiments are needed to understand the physiological implications of these results; however, it is noteworthy that SASP obtained from different premature-induced senescence has different components and the effects adjacent cells might be distinct.

Lastly, the more exciting assumption may be that different conditions or stimuli of senescent induction might arouse various transcription pathways that could produce and secrete SASP with distinctive profiles. This idea is challenging because this would mean that SASP effect on neighbor cells might not only depend on their kind and accumulation but also on the SASP components secreted and the pathway by which the cells achieved senescence, and this could have a different impact in diseases and aging establishment.

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