Cytokine 91 (2017) 13–29

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Relationship of inflammatory profile of elderly patients serum and senescence-associated secretory phenotype with human breast cancer cells proliferation: Role of IL6/IL8 ratio

Bertha Alicia Barajas-Gómez [a,1], Oscar Rosas-Carrasco [b], Sandra Lisbeth Morales-Rosales [a,1], Gibrán Pedraza Vázquez [a,1], Viridiana Yazmín González-Puertos [a], Teresa Juárez-Cedillo [c], Jorge Antonio García-Álvarez [d], Norma Edith López-Diazguerrero [a], Pablo Damián-Matsumura [e], Mina Königsberg [a], Armando Luna-López [f,*]

[a] Depto. Ciencias de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, Ciudad de México, Mexico
[b] Depto. Investigación Clínica-Epidemiológica, Instituto Nacional de Geriatría, Ciudad de México, Mexico
[c] Depto. Investigación Epidemiológica, Instituto Nacional del Seguro Social, Ciudad de México, Mexico
[d] Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad de México, Mexico
[e] Depto. Biología de la Reproducción, Universidad Autónoma Metropolitana-Iztapalapa, Ciudad de México, Mexico
[f] Depto. Investigación Básica, Instituto Nacional de Geriatría, Ciudad de México, Mexico

ARTICLE INFO

Article history:
Received 5 July 2016
Received in revised form 15 November 2016
Accepted 2 December 2016

Keywords:
Inflammation
SASP
Proliferation
IL-6
IL-8
Senescence
Aging

ABSTRACT

Aging is considered a systemic, chronic and low-grade inflammatory state, called "inflammaging", which has been contemplated as a risk factor for cancer development and progression in the elderly population. Cellular senescence is a multifactorial phenomenon of growth arrest and distorted function, which has been recognized as a contributor to aging. Senescent cells have an altered secretion pattern called Senescent Associated Secretory Phenotype (SASP), that comprise a complex mix of factors including cytokines, growth factors, chemokines and matrix metalloproteinases among others. The SASP secreted by accumulated senescent cells during old age has been related to local inflammation that leads to cellular transformation and therefore may be supporting the inflammaging process.

Here, we evaluated if the pro-inflammatory profile within the serum obtained from elderly patients (EPS) was able to induce cellular proliferation in the breast cancer transformed cell line (MCF-7), in a similar way to the proliferation stimulated by the SASP obtained from WI-38 primary cells prematurely induced to senescence by oxidative stress (SIPS). At the same time, the participation of IL-6/IL-8 ratio was determined.

Our results showed that not all the EPS increased MCF-7 proliferation. However, there was an interesting relationship between IL-6 and IL-8 concentrations, when the IL-6 was higher than IL-8. Similar results were found with SASP from SIPS-WI-38 on the MCF-7 proliferation. Although it is known that those cytokines are fundamental factors to induce proliferation; the occurrence of other components in the cellular microenvironment is necessary to carry out this effect.

1. Introduction

Aging is a natural, universal and irreversible process that is characterized by the decrease of biochemical, structural and physiological functions in the organisms, that all together, make them hamper their competence to adapt to environmental changes and lose their resilience. Among the most interesting processes that deregulate during aging are DNA repair ability, apoptosis regulation, immune response, oxidative homeostasis and inflammation modulation [1].

This deregulation leads to a progressive deterioration, which has been related to countless diseases [2]. It is known that cancer prevalence exponentially increases with age [3,4].

The inflammation is a physiological response that protects the tissues against noxious stimulus such as pathogens, damaged cells or xenobiotics. This reaction involves immune cells, blood vessels

* Corresponding author at: Depto. Investigación Básica, Instituto Nacional de Geriatría, Blvd Adolfo Ruiz Cortines 2767, San Jerónimo Lídice, La Magdalena Contreras, CP: 10200 México DF, Mexico.
E-mail address: allbioexp@yahoo.com (A. Luna-López).
[1] Posgrado en Biología Experimental de la Universidad Autónoma Metropolitana.

http://dx.doi.org/10.1016/j.cyto.2016.12.001
1043-4666/

and molecular signaling mechanisms [5,6]. Inflammation primary purpose is to eliminate the initial cellular lesion, remove damaged cells and tissues, and create a suitable microenvironment to start the wound healing process. Despite the fact that inflammation is a highly regulated process, chronic inflammation may promote some illnesses such as cardiovascular diseases and cancer [5]. Chronic inflammation has also been recognized as the previous step to the beginning and progression of different cancer types [7]. Inflammation is characterized by the increment of cytokines, such as TNF-α that can stimulate reactive oxygen species (ROS) production [8,9], increasing the probability of tumorigenic transformation.

Recently it has been reported that aging is considered a systemic, chronic and low-grade inflammatory state, called "inflammaging", which has been considered a risk factor for morbidity and mortality in the elderly population [10–12].

Inflammaging etiology and its precise participation in the progressive deterioration observed during aging are still unknown. However, several mechanisms that increase inflammation have been proposed: i) Damaged cells and molecules accumulation, due to an increment in their origination and/or elimination; ii) Noxious products infiltration increase due to oral and gastric microbiota inefficient regulation; iii) Coagulation processes intensification associated with age; iv) Senescent cells accumulation [10]. This last feature is interesting because senescent cells accumulate through age, as a result of a deficient immune system that can no longer eliminate them.

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 [13,14]. Various pathways for senescence induction have been proposed; the most studied is replicative senescence due to telomere attrition called replicative senescence (RS). Senescence can be prematurely attained when cells are exposed to diverse stimuli such as oxidative stress and others (Stress-Induced Premature Senescence, SIPS) [15–17]. Senescent cells have an altered secretion pattern called Senescent Associated Secretory Phenotype (SASP) that comprise a complex mix of factors including cytokines, growth factors, chemokines and matrix metalloproteinases among others [18–20]. The SASP has been related to local inflammation that leads to cellular transformation and other diseases [21–23], and therefore may be supporting inflammaging.

The SASP composition can be modulated depending on the stimulus that induced senescence [24–26], and its effect might be beneficial or deleterious in adjacent cells. Some of them have been related to increased cell duplication [25,27], mesenchymal–epithelial transition (MET) [28] among others. The interleukins 6 and 8 (IL-6 and IL-8) are components of SASP that stimulate or inhibit proliferation depending on the cellular physiological context, by activating WNT signaling [29–31].

IL-6 and IL-8 are components of the inflammatory response associated with cancer development and progression. There are reports that IL-6 increases with age and induces gene expression of proteins related to cell cycle progression, like cyclin D1 [32]; while IL-8 rise during chronic inflammatory processes and stimulate proliferation, MET, invasion, and metastasis [33].

The aim of this work was to evaluate if the presence and participation of the IL6/IL-8 ratio in the pro-inflammatory profile of the serum obtained from elderly patients (EPS) and in the SASP obtained from WI-38 primary cells could be able to induce SIPS and cellular proliferation in the human breast cancer cell line MCF-7.

MCF-7 was chosen as a cellular model to evaluate the proliferation induced by EPS and the SASP from WI38 senescent cells because it is well known that this cell line has a high proliferative profile, but a low metastasis response [34], which allowed us to evaluate this effect directly. MCF-7 cells are also a suitable to study IL-6 involvement in different biological processes in regards to oncogenic transformation, invasion, and metastasis, because the IL-6 basal expression is very low [32,100]. In contrast to other ER-α negative breast cancer lines, which produce higher concentrations of that cytokine [100,101]. Also, MCF-7 cells are known to increase their proliferation when stimulated with exogenous IL-6, by a mechanism involving the activation of the IL-6/pSTAT3 (Tyr-705) signaling pathway, which has been highly related to cell proliferation in ER-α positive breast cancer cells [32,100,102]. Contrariwise, ER-α negative breast cancer cell lines do not proliferate when stimulated with exogenous IL-6 [32,100,103].

Our results showed that not all the EPS increased MCF-7 proliferation, but there was an interesting relationship between IL-6 and IL-8 concentrations in the EPS and the SASP from SIPS-WI-38. In both of them, IL-6 was higher than IL-8, in a particular ratio that induced cellular proliferation. Nevertheless, it is known those cytokines are fundamental factors to induce proliferation, the occurrence of other components in the cellular microenvironment is necessary to carry out this effect.

2. Materials and methods

2.1. Geriatric cohort

Blood samples were obtained from 64 elderly patients at the Mexican Social Security Institute (IMSS), under informed consent. The protocol was approved by the Research Ethics Committee of the National Institute of Geriatrics, with the registration number SIRES: DI-PI-004/12.

2.1.1. Inclusion criteria

A sub-group of patients of 60 years and more that correspond to the Study of Aging and dementia in Mexico City were included. The original study cohort was 3015 participants representative of patients attended Mexican Institute of Social Security (IMSS) in Mexico City were included in the study as long as they were not diagnosed with any contagious disease, dementia or cancer. The samples were initially classified by age and gender [35]. The age ranged from 60 to 83 years, mean 72 ± 5.2. Of whom 36 were men and 28 women.

The blood was obtained from the forearm and was immediately centrifuged at 1500 rpm for 15 min to get the serum, which was aliquoted in Eppendorf tubes (1 mL) and stored at −70 °C until used. This serum will from now on be called EPS: Elderly Patient Serum.

2.1.2. Exclusion criteria

Serums with cytokines values higher than 1000 pg/ml were excluded from the study because those values suggest a clinical state related to sepsis or infection before the sampling.

2.2. Cell culture

MCF-7 cell line (ATCC no. HTB-22) was used in this study. Cells were cultured in DMEM (GIBCO-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS; GIBCO-BRL, Gaithersburg, MD, USA), L-Glutamine (200 mM), sodium pyruvate (100 mM), 1% non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL, Gaithersburg, MD). The medium was replaced every three days. Cells were trypsinized upon reaching confluence and re-plated to continue time in culture. Cells were grown at 37 °C in 60 mm-diameter culture multi-well plates (Corning, Acton MA, USA) in an atmosphere of 95% air and 5% CO_2.

2.3. Cellular viability

MCF-7 cells were incubated for several days with each one of the 64 elderly patient's serums (EPS). To determine any changes in cellular viability due to the addition of EPS to the culture media 2×10^5 cells were seeded into 24-well plates (Corning, Acton MA, USA). Cells were treated with only DMEM, only EPS 10%, or DMEM –SBF 5% plus EPS 10%, for 12, 48, 72 or 96 h, without changing the media.

To determine cellular viability treated cells were trypsinized, and a 20 μl aliquot was stained with an equal volume of a 0.4% trypan blue physiological solution (trypan blue exclusion). The number of living cells in 10 μl of this suspension was scored using five fields of a hemocytometer under a phase-contrast optical microscope as described elsewhere [36].

2.4. Cellular proliferation

MCF-7 cells were seeded at 1×10^4 density into 96-well plates (Corning, Acton MA, USA) using the standard conditions mentioned above plus 10% of each one of the EPS and cellular proliferation was determined at 24, 48, 72 or 96 h using the CCK-8 kit, which measures at the same time cellular functionality and proliferation (Dojindo, Japan) [107,108]. 10 μL of CCK-8 solution were added to each well and were incubated for 1 h at 37 °C, following the manufacturer instructions. After that time, the absorbance at 450 nm was detected in a DTX 880, Multimode Detector (Beckman Coulter). Along with the treated cells a proliferation curve was performed using increasing numbers of cells as suggested in the kit (5, 10, 20, 40, 80, 160×10^3 cells) in order to relate the cell number with the absorbance obtained due to the dehydrogenases activity, and to determine the cellular proliferation during the experimental time points.

2.5. IL-6 and IL-8 receptors evaluation by immunofluorescence

MCF-7 untreated cells (5×10^3) were washed with PBS and fixed with 4% paraformaldehyde for 30 min and incubated in blocking buffer (2% BSA, 0.2% non-fat milk, 0.4% Triton X100 in PBS) for 1 h at room temperature. Cells were then washed and incubated for another h with the primary antibody IL-6-R (sc-661, Santa Cruz Biotechnology, USA) or IL-8-R (sc-30008, Santa Cruz Biotechnology, USA), dilution 1:50. Cells were washed three times with PBS-Tween 1% and incubated for 1 h with the secondary antibody ALEXA FLUOR 488 anti-rabbit dilution 1:1000 (Invitrogen, USA) for IL-6-R or with ALEXA FLUOR 594 anti-rabbit dilution 1:1000 (Invitrogen, USA) for IL-8-R. After three more washes, cells were incubated with DAPI 10 μM for 5 min to stain DNA and mark the nucleus. Cells were washed again twice and mounted with fluorescent mounting medium (Dako Cytomation, Glostrup Denmark). Images were obtained with a confocal microscope (Carl Zeiss).

2.6. Cytokine concentration in the EPS

Eight cytokines: IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, and TNFα were quantified from 10 μL of each EPS using the commercial Bio-Plex Pro™ Human Cytokine 8-plex Assay (Biorad Hercules CA) as described by the manufacturer. The quantification in the kit is based on the quantity of proteins adhered to their specific antibodies as previously reported [110].

2.7. Effect of IL-6 and IL-8 on MCF-7 proliferation

MCF-7 cells were seeded in 96 well plates at a 1×10^4 cellular density. After 24 h culture medium was changed and DMEM/FBS-5% plus 10% EPS was added along with IL-6 antibody (sc-7920, Santa Cruz Biotechnology, USA), IL-8 antibody (sc-7922, Santa Cruz Biotechnology, USA), IL6R (sc-661 Santa Cruz Biotechnology, USA) or IL8R (sc-30008 Santa Cruz Biotechnology, USA). Other experiments were performed using the same conditions: DMEM/FBS-5% plus 10% EPS, but different human recombinant IL-6 or human recombinant IL-8 concentrations were used (2, 4, 6 or 8 pg/mL) (RB Systems, USA), the control was DMEM/FBS-5% plus IL-6 or IL-8 without EPS. Treated cells were incubated at 37 °C, for 24, 48, 72 or 96 h. Cellular proliferation was determined using the CCK-8 kit (Dojindo, Japan) as described above.

2.8. WI-38 cell line stress-induced premature senescence (SIPS)

WI-38 cells were seeded at a cell density of 1×10^3 cells per well on a 6-well multi-chamber (Corning, Acton MA, USA) in (MEM) (GIBCO-BRL, Gaithersburg, MD) supplemented with FBS-10% (GIBCO-BRL, Gaithersburg, MD, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL, Gaithersburg, MD SIPS was induced as described previously [36,37]. After 24 h, cells were incubated with 75 μM H_2O_2, for 2 h at 37 °C and 5% CO_2; and after that, the cells were allowed to recover in fresh culture medium for 48 h before measuring proliferation or SA-β–gal activity.

Cellular proliferation was determined using the CCK-8 kit (Dojindo, Japan) as described above and senescence was evaluated using the SA-β–gal activity [38], on days 2, 4, 6, 8 and 10 after SIPS induction. The conditioned medium (CM) containing the SASP was recovered at the same days. CM of untreated WI-38 cells was used as a control for the experiments.

2.9. Effect of SASP from WI-38-SIPS cells on cell proliferation of MCF-7

MCF-7 cells were seeded at a cell density of 1×10^3 cells per well on a 24-well multi-chamber (Corning, Acton MA, USA). After 24 h, the medium was replaced with DMEM-FBS-5% supplemented with SASP-10% from WI-38-SIPS cells, collected on day 6 after SIPS induction. Day 6 was chosen because 75% of the cells were positive to the SA-β–gal assay. CM from non-senescent WI-38 cells was used as a control.

2.10. Western blot analysis

Treated and untreated MCF-7 cells were trypsinized and resuspended in lysis buffer M-PER (Pierce Chemical, Rockford, IL, USA) supplemented with proteases inhibitor (Complete; Roche Applied Science, Indianapolis, IN, USA), 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 0.1 mM dithiothreitol (DTT). Cell homogenates were incubated at 4 °C for 5–10 min and centrifuged at 14,000g, 4 °C for 20 min. Protein concentration was determined in the supernatants using a commercial Bradford reagent (BioRad, Hercules CA, USA) [109]. Cell lysates were separated on 13% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Invitrogen), and probed with anti-IL-6Rα (C-20) (sc-661), anti-IL-8RA/B (H-100) (sc-30008), anti-Cyclin D1(M-20) (sc-718), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Phospho-Akt (Ser473) (9271S) (Cell Signaling Technology, Danvers, MA, USA) or anti-STAT3 (phosphoY705) (ab76315) (ABCAM, USA). Membranes were washed 3 times with TBS-Tween and incubated with a horseradish peroxidase-conjugated α-mouse IgG secondary antibody (Pierce, Rockford, IL, USA) for 1 h. After the 3 washes, the blots were developed using a commercial chemiluminescent reagent (Supersignal Pierce, Rockford, IL, USA.

2.11. Data analysis

Data are reported as the means ± SD for at least three independent experiments performed in triplicate. Kruskal–Wallis test followed by Dunn's post hoc test was used to compare the proliferation results. A 0.05 level of probability was used as a minimum criterion of significance.

The ANOVA test followed by the Tukey-Kramer variance analysis was used to compare the SA-β-Gal assay; a 0.05 level of probability was also used as a minimum criterion of significance.

3. Results

3.1. Inflammatory profile from Elderly patient's serum (EPS)

This study consisted of the analysis of serum from a cohort of 64 elderly patients, of which 43.75% were women and 56.25% men, with an age range of 63–83, and an average of 71.46 years (±SD = 5.2).

To determine the relationship of the chronic inflammatory state during aging and its effect on MCF-7 cells proliferation, the inflammatory condition of our study cohort was evaluated. EPS was classified according to the donor's age. Our results showed that there are not a significant difference among the cytokines evaluated by groups of age as observed in Table 1A. However, when the cytokine concentration in the elderly cohort was compared with the reported in healthy young and adult donors (Table 1B), it was evident that the pro-inflammatory cytokines like IL-2, IL-6, IL-8 and GM-CSF increased in the elderly, while the anti-inflammatory cytokines, IL-4 and IL-10 decreased. Interestingly, other pro-inflammatory proteins like INF-γ and TNF-α that have been related to an acute inflammatory response were not different among cohorts. These results indicate that our study cohort displayed a low intensity and chronic inflammatory state during aging.

3.2. Culture media conditions to evaluate the effects of EPS on MCF-7 proliferation

MCF-7 culture conditions include 10% FBS for optimal growth, so to evaluate EPS effect in its proliferation, we attempted to minimize the FBS concentration used. Hence, MCF-7 cells were grown with 2.5, 5 or 10% FBS and cell proliferation was evaluated. The results presented in Fig. 1A show that cells grown with 2.5% FBS for 96 h did not proliferate, and their number was statistically different from cells grown with 10% FBS ($p \leqslant 0.05$ at 24 h and $p \leqslant 0.01$ from 48 h on). Cells grown with 5% FBS presented an 88% decrease in proliferation during the first 24 h in comparison to the cells grown with 10%FBS ($p \leqslant 0.05$), probably due to an adaptation period. At 48 h, their proliferation was not different from the cells cultivated with 10% FBS, and although their proliferation at 72 and 96 h remained 20–35% lower ($p \leqslant 0.05$), they still continued to proliferate. To verify that this decrease in cell number was indeed a decline in proliferation and was not because cells were dying cellular viability was determined at the different time points. Fig. 1B shows a 100% viability in cells grown with 5% FBS, and no differences in viability were observed when 10% EPS was added to the culture medium. However, MCF-7 cells significantly decreased their viability and gradually died when the FBS was completely substituted by 10% EPS. Therefore, 5% FBS + 10% EPS was chosen as the suitable condition to evaluate the effects of EPS on MCF-7 proliferation.

Table 1B
Cytokines concentration reported the serum obtained from healthy young and adults. The table shows the concentration reported in healthy young and adult patient's serum. The values reported for each cytokine are the minimum and maximum reported by different studies performed by other groups, as stated by the references. The values are clearly different from the concentrations determined in Table 1 for EPS.

Cytokine	Concentration in adults and/or young healthy patients (pg/μL)	References
IL-2	1.12–1.6	[68,69,91,92]
IL-4	4.2–12.61	[68,69,93–95]
IL-6	0.98–1.68	[66,67,93,96,97]
IL-8	1.48–3.3	[66–69]
IL-10	6.01–21.2	[68,69,94,96,98]
GM-CSF	4.5–10.82	[68,69,99]
IFN-γ	2.93–4.79	[68,93,97,98,95]
TNF-α	1.30–3.24	[66,67,93,96]

3.3. Effects of EPS on MCF-7 proliferation

Once the culture conditions were established, EPS effect on MCF-7 proliferation was evaluated at different incubation time points (24, 48, 72 and 96 h). Interestingly, when the MCF-7 cells were grown only with 5% FBS, and was compared the average from the 64 sera, no significant differences were found (Fig. 1C). Nevertheless, when was analyzed the effect on proliferation of each one of the 64 EPS on MCF-7 cells, was clear that some EPS increased the proliferation, compared to the cells grown only with FBS 5%. However, some EPS did induce low MCF-7 proliferation or any effect (Fig. 1D).

3.4. Relationship between age and gender of EPS donors with MCF-7 proliferation

To determine a relationship between age of donors, and the proliferation observed with the sera, four age groups were established as follows: (1) donors younger than 65 years old; (2) donors between 66 and 70 years old; (3) donors between 71 and 75 years old; and (4) donors older than 76 years old. Our results showed that the EPS groups 2 and 3 increased the MCF-7 proliferation

Table 1A
Elderly patients serum (EPS) inflammatory profile. The inflammatory condition of a cohort of 64 elderly patients was determined using the BIOPLEX kit as described in materials and methods. EPS were classified according to the donor's age. The represented value is the average ± SD of 17 patients 65 years old; 23 patients between 66 and 70; 13 patients between 71 and 75 years old and 11 patients older than de 76 years. To compare the results, the ANOVA testy was used, and no significant differences (probability column) among the cytokines evaluated in none of the age groups were observed.

Cytokine (pg/mL)	⩽65 (years)	66–70 (years)	71–75 (years)	⩾76 (years)	Probability
IL-2	2.40 ± 1.09	3.01 ± 1.61	3.15 ± 1.52	2.65 ± 1.01	0.508
IL-4	1.83 ± 1.01	1.65 ± 0.53	1.67 ± 0.62	2.35 ± 0.51	0.731
IL-6	4.04 ± 1.97	3.95 ± 1.96	3.90 ± 1.26	4.64 ± 1.98	0.981
IL-8	4.21 ± 1.91	4.44 ± 2.63	4.01 ± 1.98	4.75 ± 1.92	0.909
IL-10	2.76 ± 1.88	2.75 ± 1.11	2.60 ± 1.05	2.66 ± 1.48	0.939
GM-CSF	2.13 ± 1.27	2.42 ± 1.15	2.35 ± 1.14	2.13 ± 1.28	0.764
IFN-γ	2.18 ± 1.46	2.17 ± 1.41	2.22 ± 1.87	2.43 ± 1.46	0.996
TNF-α	1.95 ± 1.23	1.92 ± 1.11	2.09.55 ± 1.31	2.22 ± 1.24	0.919

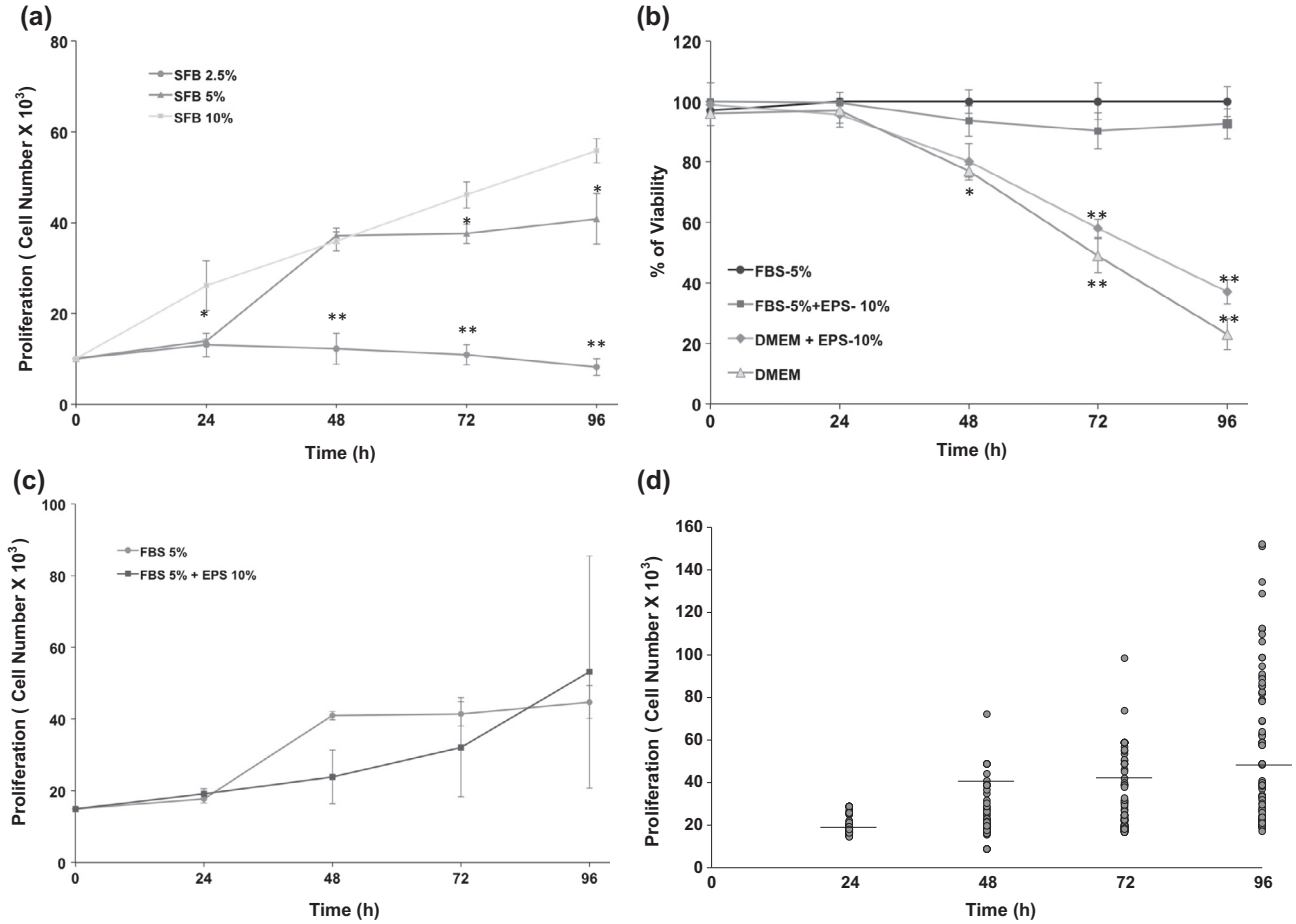

Fig. 1. Cell culture conditions using EPS. (A) MCF-7 cellular proliferation using different FBS concentrations at different time points (24, 48, 72 and 96 h). (B) MCF-7 cellular viability was determined during the different growing conditions as described in materials and methods. (C) MCF-7 cellular proliferation was determined after 24, 48, 72 and 96 h after EPS 10% exposure as described in Materials and Methods. In B and C, the points represent the mean ± SD of 64 EPS evaluated independently. (D) Graphical representation of MCF-7 cells proliferation incubated with each EPS individually. Horizontal lines represent the average of the control MCF-7 cells proliferation incubated only with-5% FBS. Cellular proliferation was determined using the CCK-8 and the viability was determined using the trypan blue exclusion dye as described materials and methods. The data were compared using the Kruskal-Wallis test followed by Dunn multiple comparisons test. Significant differences were considered as (∗) $p < 0.05$; or (∗∗) $p < 0.01$ compared to the control MCF-7 cells grown only in SFB-5%.

when were compared to cells grown only with FBS 5% ($p < 0.05$) (Fig. 2A). However, no significant difference was observed between those groups. Moreover, sera from groups 1 and 4 (younger than 65 and older than 76) did not increase cell proliferation. When the data was analyzed individually, we found that some EPS groups increased MCF-7 proliferation, and some of them decreased it (Fig. 2B). These results suggest that the proliferation induction does not depend on the age of donor EPS.

To determine whether gender was a required condition to increase MCF-7 proliferation, EPS by gender were analyzed. The results presented in Fig. 2C show that there are not differences in the average EPS between from female or male on cellular proliferation. But once more, when were analyzed individually, various EPS from both genders were capable of enhancing MCF-7 proliferation (Fig. 2D).

Since induction of proliferation did not depend on the age or gender, EPS was classified due to their ability to stimulate proliferation after 96 h of treatment, and three groups were formed: (1) EPS that increased proliferation (IP) compared to the control cells grown only with FBS 5% (EPS-IP), (2) EPS whose effects on proliferation were similar (SP) to the control cells (EPS-SP), and (3) EPS that decreased MCF-7 proliferation (DP) when compared to the controls (EPS-DP).

In brief, we determined that 15 of the 64 samples behaved like EPS-IP and they increased 110% the MCF-7 proliferation ($p < 0.05$), while 36 samples were grouped as EPS-SP because they did not have any effect on proliferation. Thirteen samples were classified as EPS-DP and decreased 50% cellular proliferation ($p < 0.05$) (Fig. 3). In this paper, we will focus only on the effects of EPS-IP. However, further studies are required to explore why some EPS diminish cellular proliferation.

3.5. Clinical status of Elderly patients and its relation with proliferation capacity of EPS

One important feature to explore was if the induction of proliferation by EPS was associated with the existence of one or more pathologies. Therefore, a statistical analysis was performed considering the most common diseases and the comorbidity in the elderly donors of EPS.

The prevalent pathologies analyzed were hypertension, diabetes mellitus, hypercholesterolemia, arthritis, osteoporosis, and gastritis.

No significant differences were found when comparing each of the pathologies with each of the EPS according to the classification defined by its ability to induce proliferation. As shown in Table 2, the comorbidity was analyzed using Charlson index adapted to Mexican population [39]. The three EPS groups classified due to their ability to stimulate proliferation after 96 h of treatment, obtained a value less than 1, indicating that the individuals in

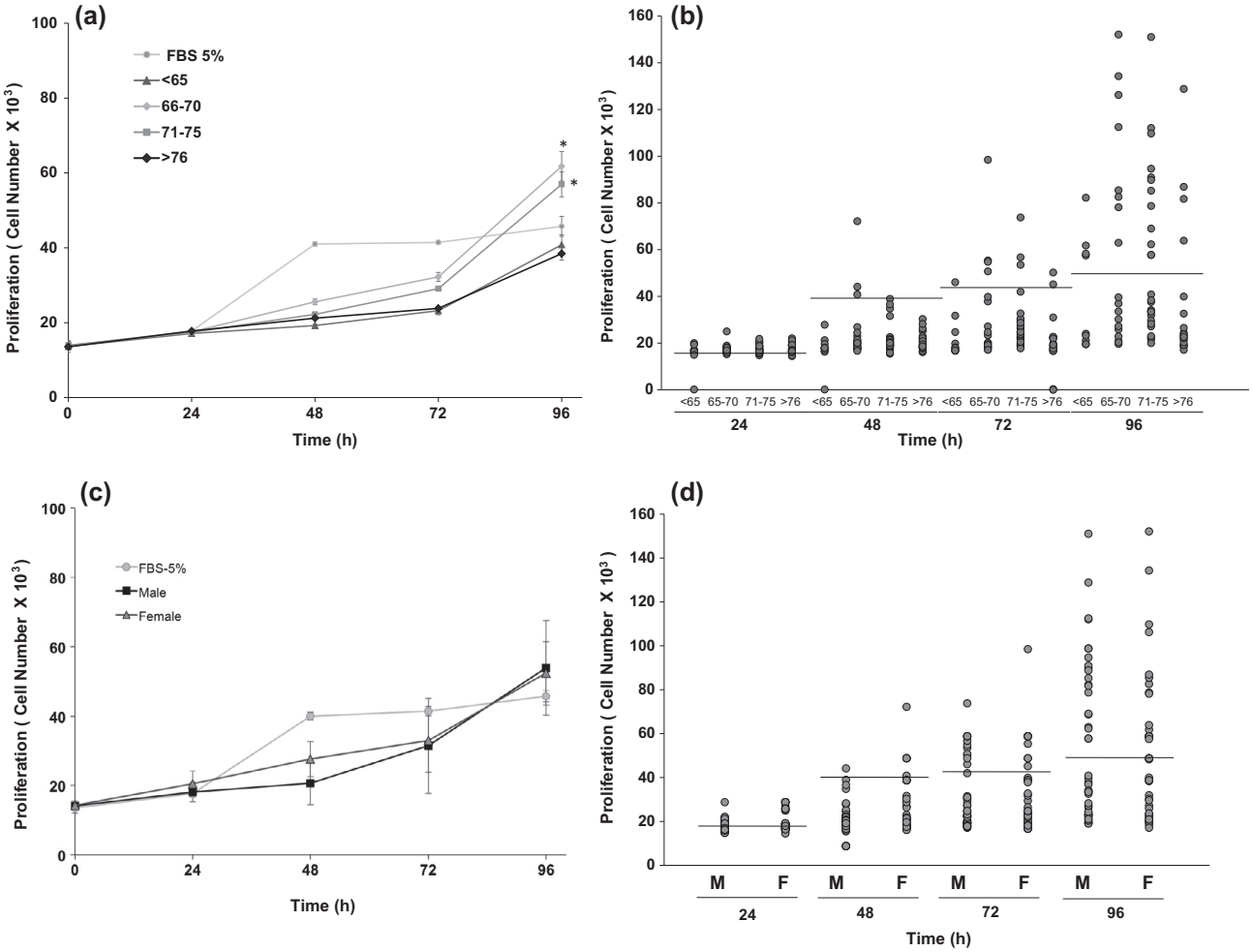

Fig. 2. Relationship between age and gender of EPS donors with MCF-7 proliferation. (A) MCF-7 cells proliferation after incubation with EPS classified according to EPS donor age determined at different time-points as described in Materials and Methods. Each point represents the mean ± SD of 8 individual determinations for EPS obtained from donors under 65; 18 EPS obtained from donors in the range from 66 to 70 years old; 23 EPS obtained from donors in the range from 71 to 75, and 15 EPS obtained from donors over 76. (B) Graphical representation MCF-7 cells proliferation incubated with each EPS individually separated by age range. Horizontal lines represent the average of the control MCF-7 cells proliferation incubated only with-5% FBS. (C) MCF-7 cells proliferation after incubation with EPS classified according to EPS donor gender determined at different time points as described in Materials and Methods. Each point represents the mean ± SD of individual determinations performed for each of the 28 EPS obtained from women donors and 36 EPS from men donors. (D) Graphical representation of MCF-7 cells proliferation incubated with each EPS individually separated by gender. Horizontal lines represent the average of the control MCF-7 cells proliferation incubated only with-5% FBS. Significant differences were considered as $^{*}p < 0.05$ compared to the control MCF-7 cells grown only in SFB-5%.

our cohort presented a state of mild comorbidity, thus discarding that the effect on proliferation was due to a particular disease or to a set of them.

3.6. Cytokine concentration in the EPS

To understand the increment of the proliferation of MCF-7 by some EPS, we determined the concentration of eight of the most representative cytokines reported in SASP. The results presented in Fig. 4 demonstrate that IL-2, IL-4, and TNF-α did not show any significant differences among the three EPS groups. Moreover, IL-6 concentration in EPS-IP was found to be 29 and 21% higher than EPS-SP and EPS-DP respectively ($p < 0.05$), while no differences were found between EPS-DP and EPS-SP. IL-10 concentration displayed a similar behavior than IL-6, where IL-10 was 45 and 57% higher in EPS-IP than in EPS-DP and EPS-SP respectively ($p < 0.05$), and no differences were found between them. Interferon-gamma (IFN-γ) and granulocyte and macrophage colony-stimulating factor (GM-CSF) concentrations in EPS-IP were equal to EPS-SP, and both of them were higher than EPS-DP ($p < 0.05$). Interestingly, IL-8 showed an opposite behavior than IL-6, since IL-8 concentration was higher in EPS-SP and EPS-DP than in EPS-IP (68 and 43% respectively; $p < 0.01$), whereas EPS-SP had an additional 25% higher concentration than EPS-DP ($p < 0.05$).

3.7. Participation of IL-6 in MCF-7 proliferation induced by EPS-IP

Before evaluating the participation of IL-6 and IL-8 in the induction of proliferation of MCF-7, the IL-6 receptor (IL-6R) and IL-8 receptor (IL-8R) in MCF-7 were evaluated in the control cells by immunofluorescence as demonstrated in Fig. 5.

We evaluate if the single addition of exogenous IL-6 was sufficient to induce proliferation (Fig. 6A). The MCF-7 cells were incubated with IL-6 (6 and 8 pg/mL), and the proliferation was evaluated at 96 h. Cellular proliferation increased 170 and 150% with each concentration used ($p < 0.05$). The addition of 6 pg/mL of IL-6 to MCF-7 cells treated with EPS-IP showed an additional 60% increment on proliferation and 40% with the 8 pg/mL treatment. Both treatments were significantly different from the control cells grown only with FBS 5% and from cells grown with FBS 5% plus EPS-IP 10% ($p < 0.05$). No differences were observed between

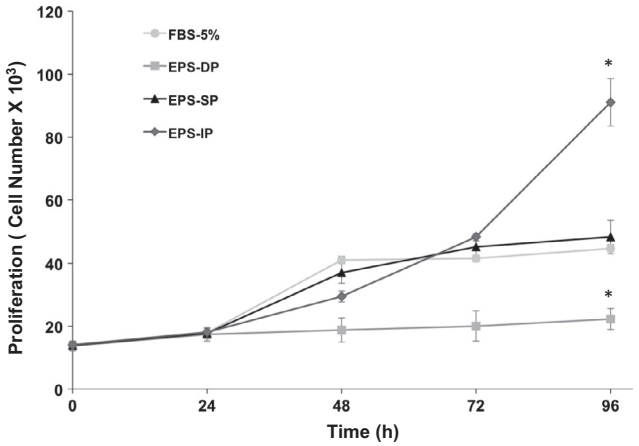

Fig. 3. EPS according to their ability to induce MCF-7 cells proliferation. EPS were classified due to their ability to stimulate proliferation after 96 h of treatment, and three groups were formed: (1) **EPS-IP**: EPS that increased proliferation compared to the control cells grown only with FBS 5% (15 samples); (2) **EPS-SP**: EPS whose effects on proliferation were similar to the control cells (36 samples); and (3) **EPS-DP**: EPS that decreased MCF-7 proliferation when compared to the controls (13 samples). Each point represents the mean ± SD of individual determinations EPS in each group. Significant differences were considered as $^{*}p < 0.05$ compared to the control MCF-7 cells grown only in SFB-5%.

the two IL-6 treatments, suggesting that the effect of IL-6 probably has a saturation value between 6 and 8 pg/mL and that IL-6R expression levels might mediate this response.

To further confirm the involvement of IL-6 present in the EPS-IP, a specific IL-6 antibody was added to MCF-7 cells before to EPS-IP treatment to inhibit IL-6 attachment to IL-6R. Our results showed that when IL-6 was compromised or trapped, EPS-IP-induced proliferation was abrogated; and proliferation returned to the basal levels observed in the control group. To assure that this abrogation was not due to some unspecific antibody effect, the same experiment was performed using two unspecific immunoglobulins, one from mouse origin and the other from rabbit. No significant differences were found when we compared to EPS-IP proliferation effect. Finally, to demonstrate that IL-6R was functional, we added a specific IL-6R antibody to MCF-7 cells before the EPS-IP treatment to inhibit IL-6 attachment to its receptor. MCF-7 proliferation significantly decreased, almost to control levels, thus confirming IL-6R functionality. The results presented in Fig. 6A corroborate that IL-6 contained in EPS has an essential role in the induction of MCF-7 proliferation. This is highly relevant because although the previous experiments showed that increasing or decreasing IL-6 concentration affects cellular proliferation, other components contained in the EPS-IP are participating.

Table 2
EPS donor's clinical status according to their ability to induce MCF-7 cells proliferation. Clinical analysis of the different pathologies presented by the elderly patients donors classified by their ability to induce proliferation. The comorbidity state (Charlson Index), gender and age were also analyzed.

Variable	EPS-IP	EPS-SP	EPS-DP	Probability
Age mean ± SD	71.4 ± 1.37	71.7 ± 0.88	71.3 1.47)	0.959
Sex (Women) n (%)	8 [53]	15 [41]	5 [39]	0.690
Arterial hypertension n (%)	11 [73]	20 [56]	10 [77]	0.278
Diabetes mellitus n (%)	4 [27]	17 [47]	6 [46]	0.391
Osteoarthritis n (%)	2 [13]	2 [6]	1 [8]	0.652
Gastritis n (%)	2 [13]	5 [14]	1 [8]	0.846
Osteoporosis n (%)	1 [7]	1 [3]	1 [8]	0.719
Hypercholesterolemia n (%)	1 [7]	3 [8]	1 [8]	0.578
Charlson index mean ± SD	0.466 ± 0.216	0.805 ± 0.140	0.846 ± 0.233	0.371

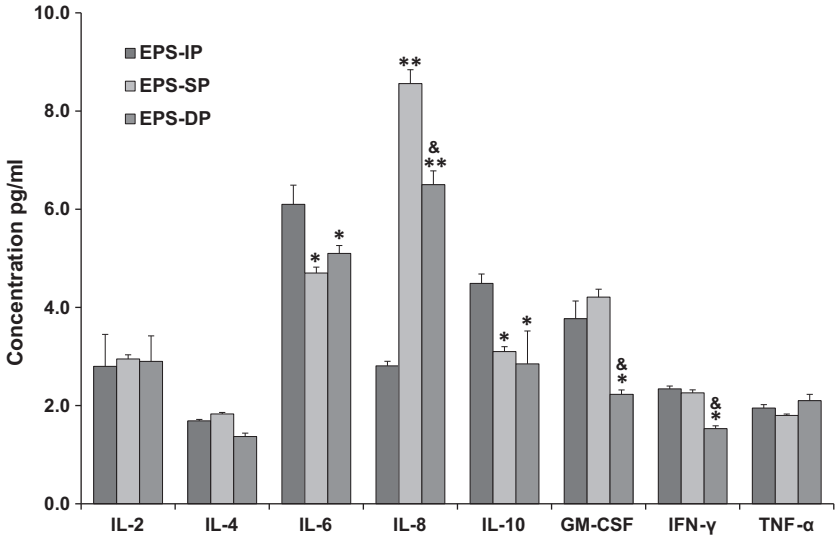

Fig. 4. Cytokines inflammatory profile. The concentration of eight cytokines in each one of the 64 EPS samples was quantified as described in materials and methods. EPS were classified according to their ability to induce MCF-7 cells proliferation. The values represented are mean ± SD of 15 EPS-IP samples, 36 EPS-SP samples, and 15 EPS-DP samples. Significant differences were considered as $^{*}p < 0.05$; or $^{**}p < 0.01$ for EPS-IP; while the differences for EPS-SP were labeled as & $p < 0.05$.

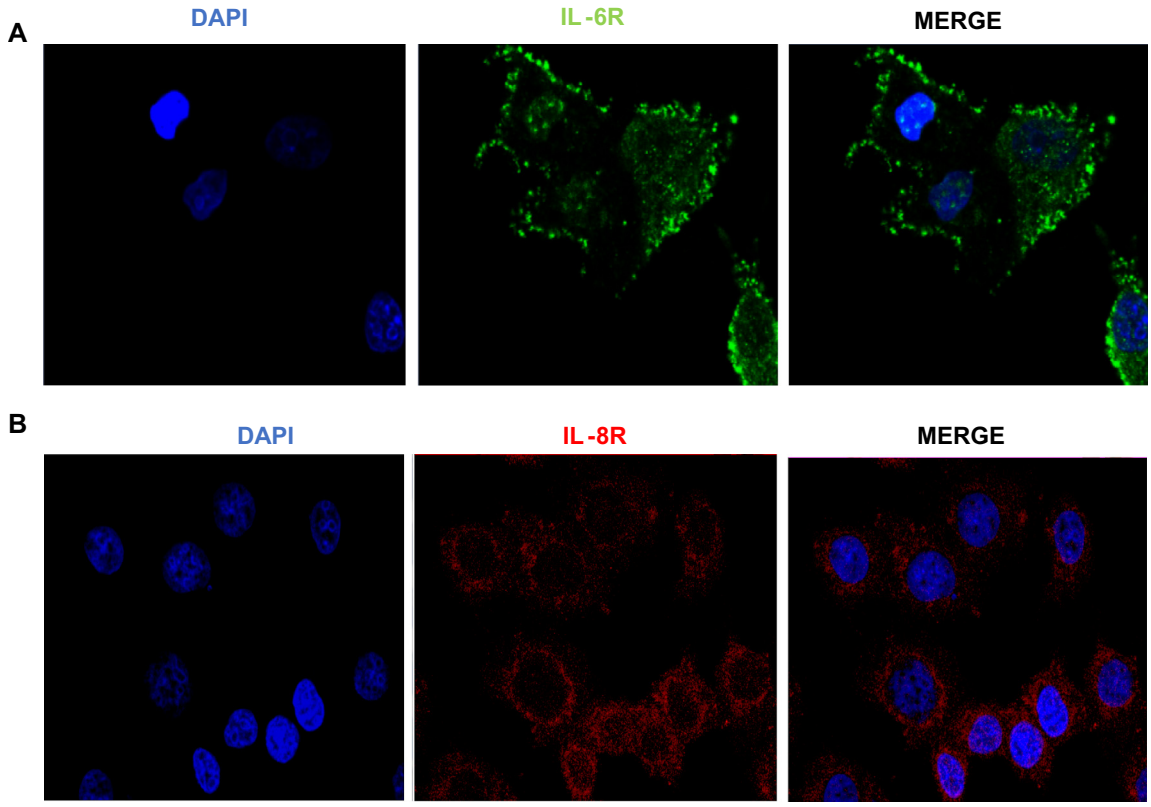

Fig. 5. IL-6 and IL-8 receptors in MCF-7 cells. IL-6R and IL8R representative image in MCF-7 cells. The receptors were determined by immunohistochemistry using confocal microscopy. Alexa-Fluor 488 secondary antibody was used to determine IL-6R, Alexa-594 for IL-8 and DAPI was used to stain the nucleus.

3.8. IL-8 involvement in MCF-7 proliferation induced by EPS-IP

To demonstrate other components in the EPS-IP and particularly the participation of IL-8 the MCF-7 cells were incubated with EPS-IP supplemented with recombinant IL-8 (6 pg/mL) and cell proliferation was determined after 96 h. The results showed (Fig. 6B) that hyperproliferative capability of EPS-IP fell 50% ($p \leqslant 0.05$). However, proliferation was 55% higher than cells with 5% FBS, suggesting that elevated IL-8 concentrations might also induce MCF-7 proliferation.

To understand if IL-8 by itself was able to induce MCF-7 proliferation, cells were incubated with recombinant IL-8 (6 pg/mL) in the absence of EPS, and the cells did not increase their proliferation. When IL-8 antibody was added to MCF-7 cells grown with EPS-IP, cellular proliferation decreased 57%. However, it was still 48% above to the proliferation observed only with FBS-5% ($p \leqslant 0.05$). We also explored IL-8 R functionality and participation in MCF-7 proliferation, by incubating MCF-7 cells with IL-8R specific antibody before to their incubation with EPS-IP. The results show a decrease in proliferation at 96 h, similar to that observed in inhibition with the anti-IL-8 antibody. This demonstrates IL-8R functionality and participation within MCF-7 cellular proliferation. These results, along with the results in Fig. 6A, suggests that the participation of IL-8 is required to induce cell proliferation, but its effect must be combined with other serum components.

3.9. IL-6/IL-8 ratio

To verify if the relation and their relative proportions between IL-6 and IL-8 are an important factor to induce MCF-7 proliferation, cells were incubated with different ratios of recombinant IL-6 and IL-8, in the absence of EPS-IP. Different proportions of IL-6/IL-8 ratio were used (0, 0.33, 1 and 3), and proliferation was quantified after 96 h.

The results in Fig. 6C show that when the ratio IL-6/IL-8 was 3 (first bar) the MCF-7 proliferation was induced (27%; $p \leqslant 0.05$). This observation confirms that to induce proliferation, IL-6 must be in a higher proportion compared to IL-8. Moreover, the proliferation induction (27%) It was not as high as what was observed with EPS-IP. This difference might be related to the other inflammatory component participation.

3.10. Effect of the SASP from WI-38-SIPS cells on MCF-7 proliferation

To understand the differences in the inflammatory profile composition in the EPS groups, we decided to evaluate and compare the effects of EPS-IP on MCF-7 proliferation with the SASP from WI-38 senescent.

WI-38 cells were induced to SIPS when incubated with 75 μM H_2O_2 for 2 h. The results show a decrease in proliferation in WI-38 SIPS cells of 1.77 times at 2 days ($p < 0.05$); 6.85 times at 4 days ($p < 0.05$), 19.86 times at 6 days ($p < 0.01$) and 20 times less at 8 days ($p < 0.01$) with respect to control (Fig. 7A), with an increment of positive cells to SA-β-Gal ($p < 0.01$). After treatment at day 4, the SIPS cells reached 75% of cell proliferation (Fig. 7B). Fig. 7C and D are representative images of control WI-38 cells (7C) and senescent WI-38 (7D) on day 6 after SIPS induction.

MCF-7 cells were incubated for 96 h with the SASP from WI-38-SIPS of 6 days, and the proliferation increased significantly ($p < 0.05$) compared to the control MCF-7 cells (20% at 48 h, 23% at 72 h and 34% at 96 h) (Fig. 8). The MCF-7 cells incubated with the conditioned medium (CM) of WI-38 non-senescent cells did not exhibit significant differences with control cells.

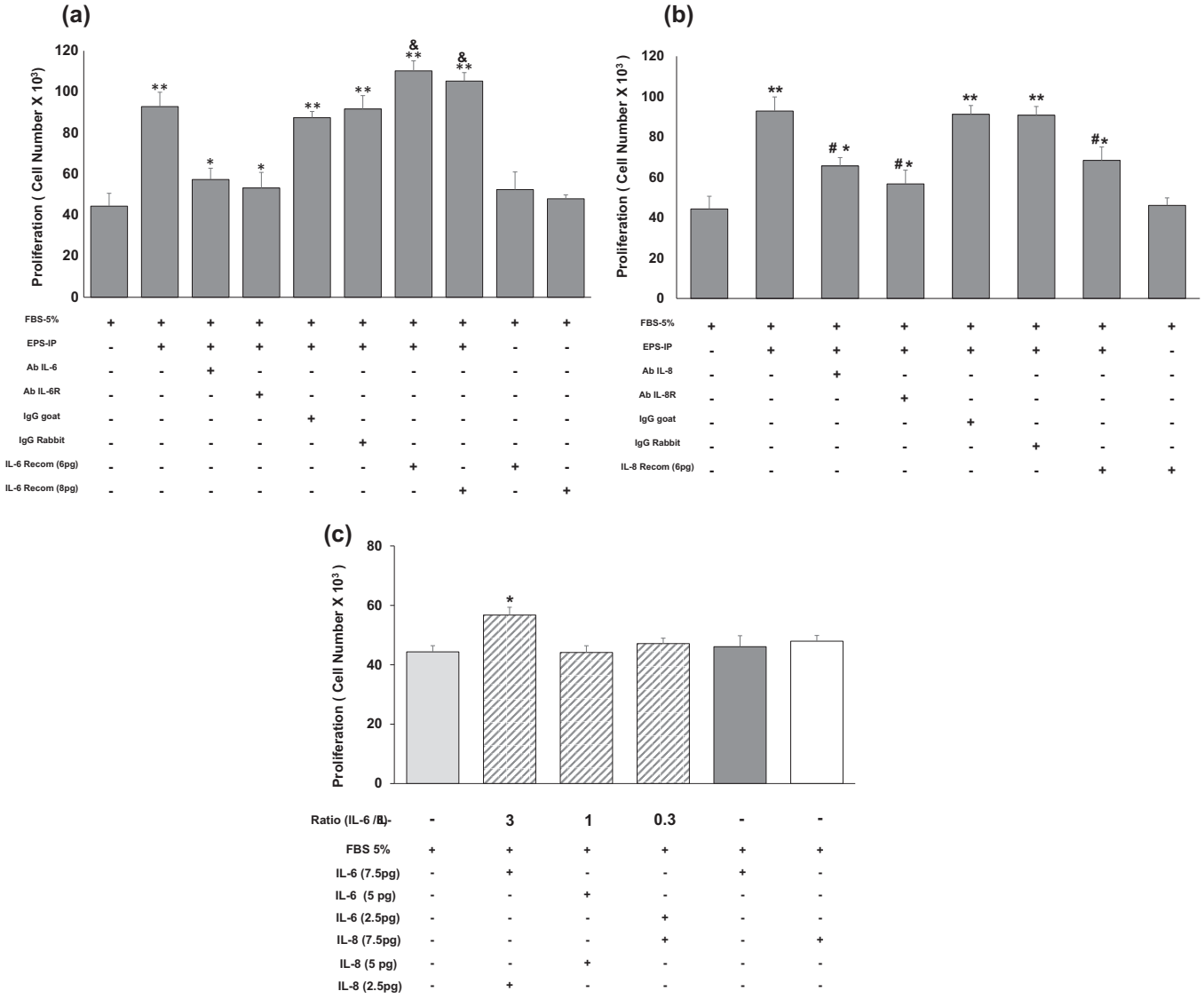

Fig. 6. IL-6 and IL-8 involvement in MCF-7 cellular proliferation incubated with EPS-IP. (A) Recombinant IL-6 (6 and 8 pg/mL) was added along with the EPS-IP and proliferation was determined after 96 h. IL-6 effect was abrogated by adding anti-IL-6 (6 pg/mL) or anti-IL-6R (6 pg/mL). The specificity of that inhibition was evaluated by adding unspecific IgG (mouse and rabbit). (B) Recombinant IL-8 (6 pg/mL) was added along with the EPS-IP and proliferation was determined after 96 h. IL-8 effect was abrogated by adding anti-IL-8 (6 pg/mL) or anti-IL-8R (6 pg/mL). The specificity of that inhibition was evaluated by adding unspecific IgG (mouse and rabbit). In A and B each point represents the mean ± SD of 15 EPS-IP samples. The ANOVA test was used followed by the Tukey-Kramer multiple comparisons test. Significant differences were considered as $^{*}p < 0.05$; or $^{**}p < 0.01$ with respect to cells incubated only with FBS 5% and & $p < 0.05$ with respect to cells incubated with EPS-IP. (C) MCF-7 cells were incubated recombinant IL-6 and IL-8 at with different ratios in the absence of EPS-IP, as described in materials and methods. The data shown are the mean ± SD of 4 independent experiments. The ANOVA test was used followed by the Tukey-Kramer multiple comparisons test. Significant differences were considered as $^{*}p < 0.05$ for cells incubated only with FBS 5%.

3.11. Inflammatory profile in SASP from WI-38-SIPS cells and CM from non-senescent WI-38 cells

To determine the expression of the cytokines contained in the SASP, eight of them were evaluated in SASP obtained from WI-38-SIPS cells and in the CM obtained from non-senescent WI-38 cells. The analysis shown in Fig. 9, revealed that only IL-6 and IL-8 showed significant differences in the SASP obtained from WI-38-SIPS compared to the CM from non-senescent WI-38 cells. IL-6 increased 95%, while IL-8 decreased 47% ($p < 0.05$). The remaining cytokines did not present significant differences.

3.12. Induction of proliferation by IL-6 and IL-8 from SASP

Since the SASP from WI-38-SIPS increased MCF-7 cell proliferation (34%) at 96 h, recombinant IL-6 (6 pg/mL) was added to the SASP of treated cells, and again an additional increase in proliferation was observed (87% compared to control group; $p < 0.01$) (Fig. 10A). When IL-6 antibody was added before to SASP treatment, the increase in proliferation was abrogated. Additionally, MCF-7 cells were also incubated with CM obtained from WI-38 non-senescent cells and no significant proliferation was observed. Neither significant change was observed when the MCF-7 cells were incubated with recombinant IL-6 at different concentrations (Fig. 10A).

Similar results were obtained when MCF-7 cells were incubated with the SASP obtained from WI-38-SIPS and the recombinant IL-8 (6 pg/mL). The IL-8 recombinant decreased the cellular proliferation in a similar way to the control group levels, and the same was observed when IL-8 antibody was added (Fig. 10B). It is important to notice that EPS-IP increased 54% the proliferation of MCF-7 when it was compared with the proliferation induced by the SASP

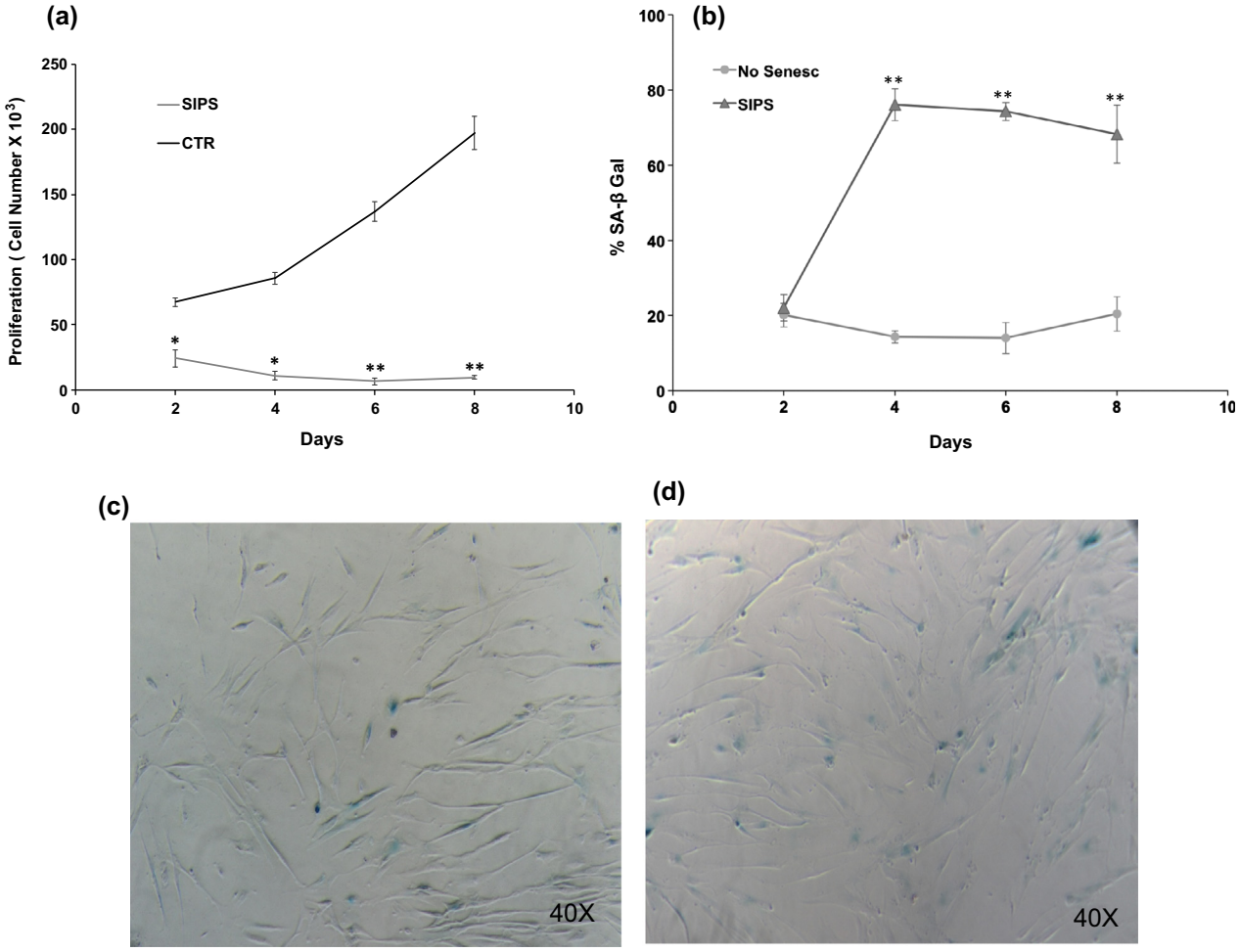

Fig. 7. Stress-induced premature senescence (SIPS) in WI-38 cells. (A) WI-38 cellular proliferation after H_2O_2 treatment to induce SIPS, as described in materials and methods. (B) SA-β-gal positive WI-38 cells at different days after H_2O_2 treatment. (C) Representative image of not senescent WI-38 cells. (D) Representative image of senescent WI-38 cells after 6 days of H_2O_2 treatment. Data represent the mean ± SD of three independent experiments by triplicate. Significant differences were considered as $^{*}p < 0.01$ for non-senescent cells.

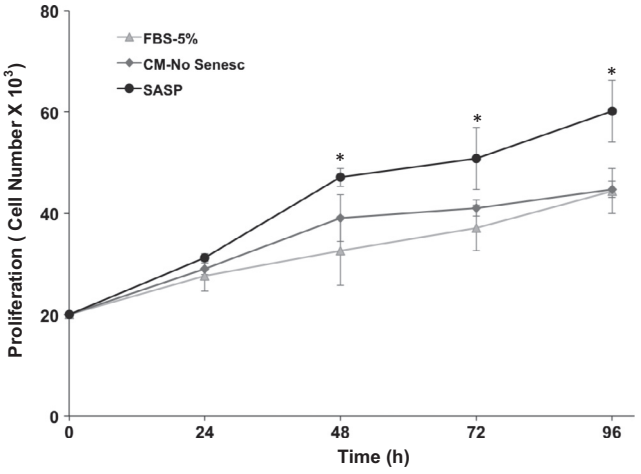

Fig. 8. SASP obtained from Stress-Induced Premature Senescence (SIPS) WI-38 cells, and its effects on MCF-7 proliferation. MCF-7 cells proliferation was determined at 24, 48 72 and 96 h after the incubation with SASP obtained from SIPS WI-38 cells on day 6 after senescence induction or with conditionate media obtained from control non-treated Wi-38 cells (CM-No senesc), as described in materials and methods. Data represent the mean ± SD of three independent experiments by triplicate. Significant differences were considered as $^{*}p < 0.05$ compared to control MCF-7 cells incubated only with-5% FBS.

from WI-38-SIPS. The cytokines concentration was also higher in EPS-IP than in the SASP. These results suggest that the differences in cell proliferation might be related to the combination and proportion of EPS-IP components.

3.13. IL-6/IL-8 ratio in EPS, SASP and CM

Finally, to explain a possible relationship between IL-6 and IL-8 during the effect of cellular proliferation induction, the ratio IL-6/IL-8 was determined and correlated with the proliferative competency. The cytokines ratio obtained from the SASP of WI-38-SIPS and the EPS-IP was 3.55 and 2.18 respectively, while the ratio for the CM of WI-38 cells non-senescent, EPS-SP and EPS-DP was lower than 1(0.83, 0.55 and 0.78 respectively). This suggests that to induce MCF-7 proliferation, IL-6 must be in a higher proportion than IL-8 (Table 3).

3.14. IL-6/IL-6R/Akt (Ser 473)/STAT3/Cyclin D1 pathway

Since IL-6/IL-6R/Akt (Ser 473)/STAT3/Cyclin D1 has been reported as one of the main signaling pathways related with cellular proliferation after IL-6 incubation in MCF-7 cells [100,102], its components were evaluated by Western blot (Fig. 11A). Our results showed a significant increase in IL-6R in all the treatments in comparison to 5% FBS-only: 50% increase in EPS-IP ($p < 0.05$), 30% after

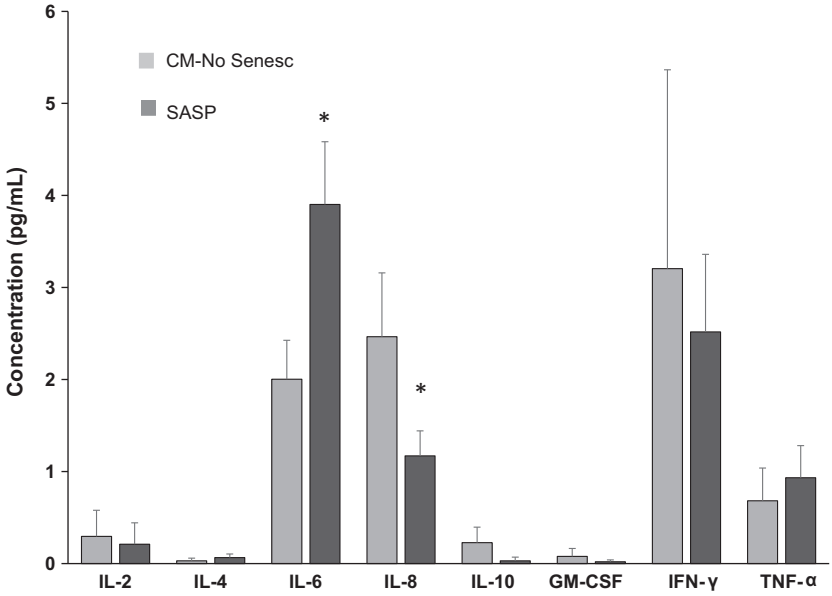

Fig. 9. Cytokines concentration in the SASP of SIPS WI-38 cells. Cytokines concentration was determined as described in materials and methods. Each bar represents the mean ± SD of 3 independent experiments by triplicate. Statistically significant differences were considered as $^{*}p < 0.05$ for non-senescent conditioned media (Non-Senescent CM).

IL-6/IL-8 ratio 0.3 ($p < 0.05$), 56% in IL6/IL-8 ratio 1.0 ($p < 0.05$) and 40% in IL6/IL-8 ratio 3.0 ($p < 0.05$) (Fig. 11B). Those results suggest a positive feedback mechanism for IL-6 stimulus and confirm IL-6R functionality. The next component evaluated was pSTAT3 (Tyr-/705) (Fig. 11A and C), its levels also increased in all the treatments in comparison to 5% FBS-only. EPS-IP treatment increased pSTAT3 (Tyr-/705) in 36.6% ($p < 0.05$), while the different IL-6/IL-8 ratios showed 33.9% increase in ratio 0.3 ($p < 0.05$), 33.8% in ratio 1.0 ($p < 0.05$) and 28.6% in ratio 3.0 ($p < 0.05$), suggesting an important MCF-7 proliferative response induced by recombinant IL-6.

Interestingly, Cyclin D1 expression only showed significant differences against FBS 5% in the cells treated with EPS-IP (48%; $p < 0.05$) and when the IL-6/IL-8 ratio 3.0 was used (42%; $p < 0.05$) (Fig. 11D). The increment in Cyclin D1 expression correlates with the proliferation increment observed during those treatments (Fig. 6A and B).

In order to confirm the participation of another important protein during pSTAT3 activation, pAkt was evaluated, because this protein has been reported to phosphorylate STAT3 [100,102]. Our data showed a 29% increase in pAkt (Ser 473) expression after EPS-IP treatment in comparison to 5% FBS-only ($p < 0.05$). Increments were also observed after the treatments with the different IL-6/IL8 ratios, 44.4% in ratio 0.3, 44% in ratio 1.0 and 40% in ratio 3.0, all of them $p < 0.05$ (Fig. 11E).

Finally, to understand the mechanism that is involved in the regulation of Cyclin D1 and MCF-7 cells proliferation, we decided to evaluate IL-8R expression levels in the different treatments. The results showed an increment in IL-8R expression after the treatment with IL-6/IL/8 ratio 1.0, 96% ($p < 0.05$) and an increment of 145% after the treatment with IL-6/IL-8 ratio 3.0 ($p < 0.05$). These results were also different from EPS-IP treatments (Fig. 11F). The previous results point towards a mechanism that induces IL-8R expression when IL-8 concentrations are lower than IL-6 concentrations, and this mechanism might be activating different cellular processes. Interestingly, this mechanism is not observed in the treatment with EPS-IP where the inflammatory microenvironment could be regulating IL-8R activity.

The above is interesting because most likely Akt (Ser 473) activation could be related to IL-8 presence and activity to induce different cellular processes, other than cell proliferation, such as migration, survival or metastasis.

4. Discussion

Aging has been considered a state of chronic low-grade inflammation, known as "inflammaging". The association of this inflammatory state with cancer progression has a great interest in aging studies [11,40,41]. The inflammatory profile observed in our elderly cohort agrees with the characteristics described for "inflammaging" [42–44], such as a decrease in anti-inflammatory cytokines (IL-4 and IL-10), an increment in pro-inflammatory cytokines related with chronic states (IL-6 and IL-8) and mild increases in pro-inflammatory cytokines associated with acute states (TNF-α, INF-γ, IL-2).

Interestingly our results showed that only 23.4% of EPS was able to induce the MCF-7 cellular proliferation. Nevertheless, all the EPS presented a chronic low-grade inflammatory profile. All groups (EPS-IP, EPS-SP and EPS-DP) contained different cytokines concentrations and proportions, and the fact that EPS-IP was the only group that induced proliferation points toward the importance of the composition of the inflammatory microenvironment and its relationship with hyperproliferation and cancer development. Zeng et al. [45] reported that the inflammatory profile in three different groups of elderly patients: i) healthy, ii) multiple myeloma (MM) and iii) monoclonal gammopathy of undetermined significance (MGUS), showed similar, chronic low-grade inflammatory profiles. However, only the cytokines composition in MM profile was able to stimulate cancer development and progression. Another study, using cytokines secreted by T lymphocytes from old mice and from young knockout mice for GPAT (a protein that regulates cellular proliferation and cytokines production), were able to induce cellular proliferation in PC-3 and LNCaP prostate cancer cell lines. Interestingly, even though both groups presented a chronic low-grade inflammatory profile, the cytokine composition was different [46], suggesting that the inflammatory profile or inflammaging is not enough to induce proliferation, but it is important to consider the proportion of the constituent within the microenvironment.

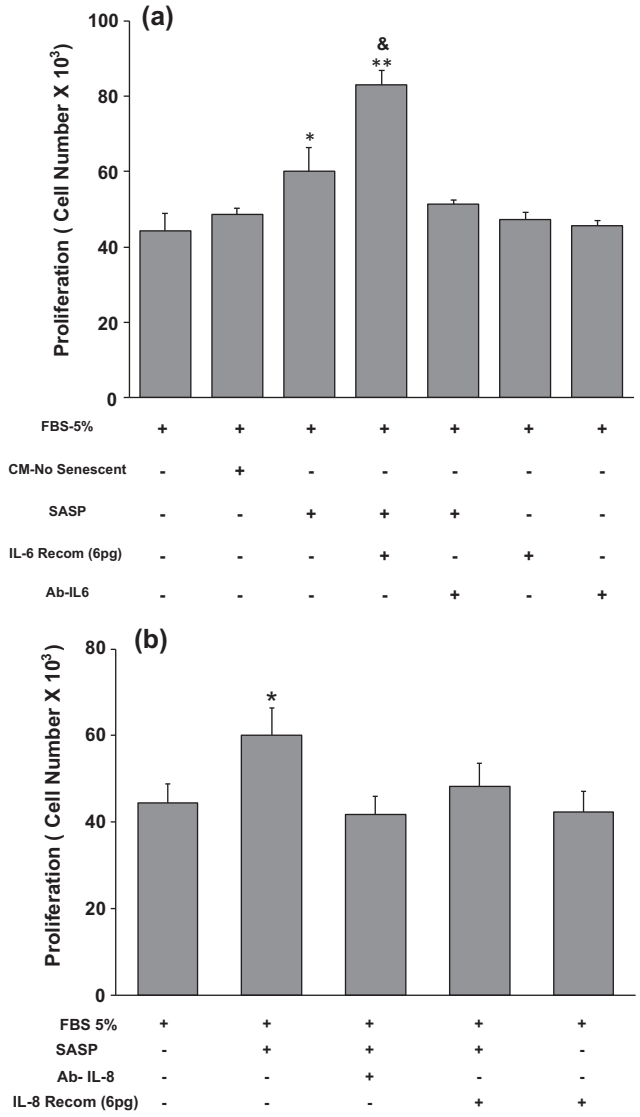

Fig. 10. IL-6 and IL-8 involvement in MCF-7 cellular proliferation incubated with the SASP form SIPS-WI-38 cells. (A) MCF-7 cells were grown with the SASP obtained from SIPS-WI-38 cells, supplemented with recombinant IL-6 (6 pg/mL), and proliferation was quantified at 96 h. Additionally, some cells were incubated with anti-IL-6 to abrogate the effect. (B) MCF-7 cells were grown with the SASP obtained form SIPS-WI-38 cells, supplemented with recombinant IL-8 (6 pg/mL), and proliferation was quantified at 96 h. Additionally, some cells were incubated with anti-IL-8 to abrogate the effect. The data shown are the mean ± SD of 4 independent experiments. The ANOVA test was used followed by the Tukey-Kramer multiple comparisons test. Significant differences were considered as $^{*}p < 0.05$, $^{**}p < 0.01$ with respect to cells incubated only with FBS 5%, and & $p < 0.05$ for cells incubated only with the SASP.

Table 3
IL-6/IL-8 ratio in the different culture conditions. The table show the ratio obtained for the two cytokines contained in the SASP from WI-38-SIPS, the conditioned medium from non-senescent WI-38 cells and the different EPS obtained from the 64 elderly patients classified as EPS-IP, EPS-SP, and EPS-DP. The cytokines concentrations were obtained from the results in Figs. 4 and 9.

	IL-6 (pg/mL)	IL-8 (pg/mL)	Ratio IL-6/IL-8
CM-NO SENESC	2 ± 0.42	2.4 ± 0.69	0.83 ± 0.06
SASP	3.9 ± 0.68	1.1 ± 0.27	**3.55 ± 0.58**
EPS-IP	6.1 ± 0.37	2.8 ± 0.65	**2.18 ± 0.46**
EPS-SP	4.7 ± 0.55	8.6 ± 0.43	0.55 ± 0.17
EPS-DP	5.1 ± 0.23	6.5 ± 0.34	0.78 ± 0.11

Statistically significant differences were considered as bold values as $p < 0.05$ with respect non-senescent conditioned media (Non-Senescent CM) or EPS-SP and EPS-DP.

Here we evaluated two main components IL-6 and IL8 that have been reported in "inflammaging" during cancer progression. IL-6 is a potent pleiotropic inflammatory cytokine, associated with proliferation and *anti*-apoptotic regulation. IL-6 also participates in immune response, hematopoiesis, and ontogenesis [47–49].

IL-8 has been related with cancer progression due to angiogenic promotion, neutrophil recruitment, along with proliferation, survival, and migration of tumor cells [50,51]. High levels of IL-8 have been reported in mammary gland, colon, cervical, gastric, lung, ovarian and others human carcinomas [50].

In our results, IL-6 concentration in EPS-IP was higher than in EPS-SP and EPS-DP, concurring with some other studies where IL-6 has been shown to increase with age and organismal deterioration [12,52–54]. Nevertheless, the analysis of the age of EPS-IP donors compared with EPS-SP and EPS-DP donors did not show significant differences. Surprisingly no differences were either found in EPS groups in relationship with the pathologies where IL-6 is reported high during aging [55–60], nor in association with Charlson comorbidity index, suggesting that the IL-6 concentration contained in the EPS-IP is not related to the donor functional impairment and neither to age. However, IL-6 concentration might be related to the damage repair mechanisms present in EPS-IP donors that lead to damage accumulation within the cells.

High concentrations of IL-6 have also been correlated with increased cellular proliferation in different types of cancer like breast cancer [61,62], cervical [63], prostate [64,65], etc.

Our results show that the high concentrations of IL-6 in EPS-IP a required condition to induce MCF-7 cellular proliferation because adding IL-6R and IL-6 antibodies blocked IL-6 interaction with its receptor resulted in a proliferation diminished, while adding recombinant IL-6 increased the proliferation. However, the incubation with recombinant IL-6 without EPS-IP was not enough to induce cellular proliferation, implying that more components are needed. On the other hand, IL-8 in EPS-IP is lower than EPS-SP and IPS-DP, but it is present in the same levels as those reported in healthy young and adults [66–69]. The situation is controversial because many studies have shown that IL-8 is increased in different cancer types [70–72]. Here we found that adding recombinant IL-8 along with EPS-IP abrogates the MCF-7 proliferation. Similar results were obtained when the IL-8 antibody was added in excess, to annul IL-8 participation, proposing that the IL-8 from EPS-IP is required to induce MCF-7 proliferation. This was corroborated when MCF-7 cells were incubated with IL-6 and IL-8 recombinant at different ratios in the absence of EPS-IP. The only effective ratio of IL-6/IL-8 that induced cellular proliferation was 3:1. A possible explanation is that the elevated IL-8 concentration reported in different cancer types has been related with processes such as migration, angiogenesis, and tumor microenvironment [73,74]. However, since our experiments were performed in the MCF-7 cell line, which is more tumorigenic that metastasic, IL-8 within the EPS-IP might have been able to induce proliferation in that cell line, because IL-8 induce Cyclin D1 and B1 levels to activate PI3 K/Akt and Raf/MEK/ERK signaling pathways, both related to cellular proliferation [75]. Interestingly, when we evaluated the signaling pathway IL-6R/IL-6/Akt (Ser 473)/pSTAT3 (Tyr 705)/Cyclin D1, we observed that it activates only after EPS-IP or IL-6/IL-8 ratio 3.0 treatments, which are the treatments that showed the highest proliferation rates. However, other participants in that pathway such as IL-6R, pSTAT3 (Tyr 705) and Akt (Ser-473), are also active in the other treatments. This result illustrates the complexity in the regulation mechanism that controls MCF-7 cellular proliferation after EPS-IP treatment. One plausible explanation is that tumor microenvironments, where IL-6 are in higher proportions than IL-8, might preferably use the proliferation pathway IL-6R/IL-6/Akt (Ser 473)/pSTAT3 (Tyr 705)/Cyclin D1; this suggestion coincides

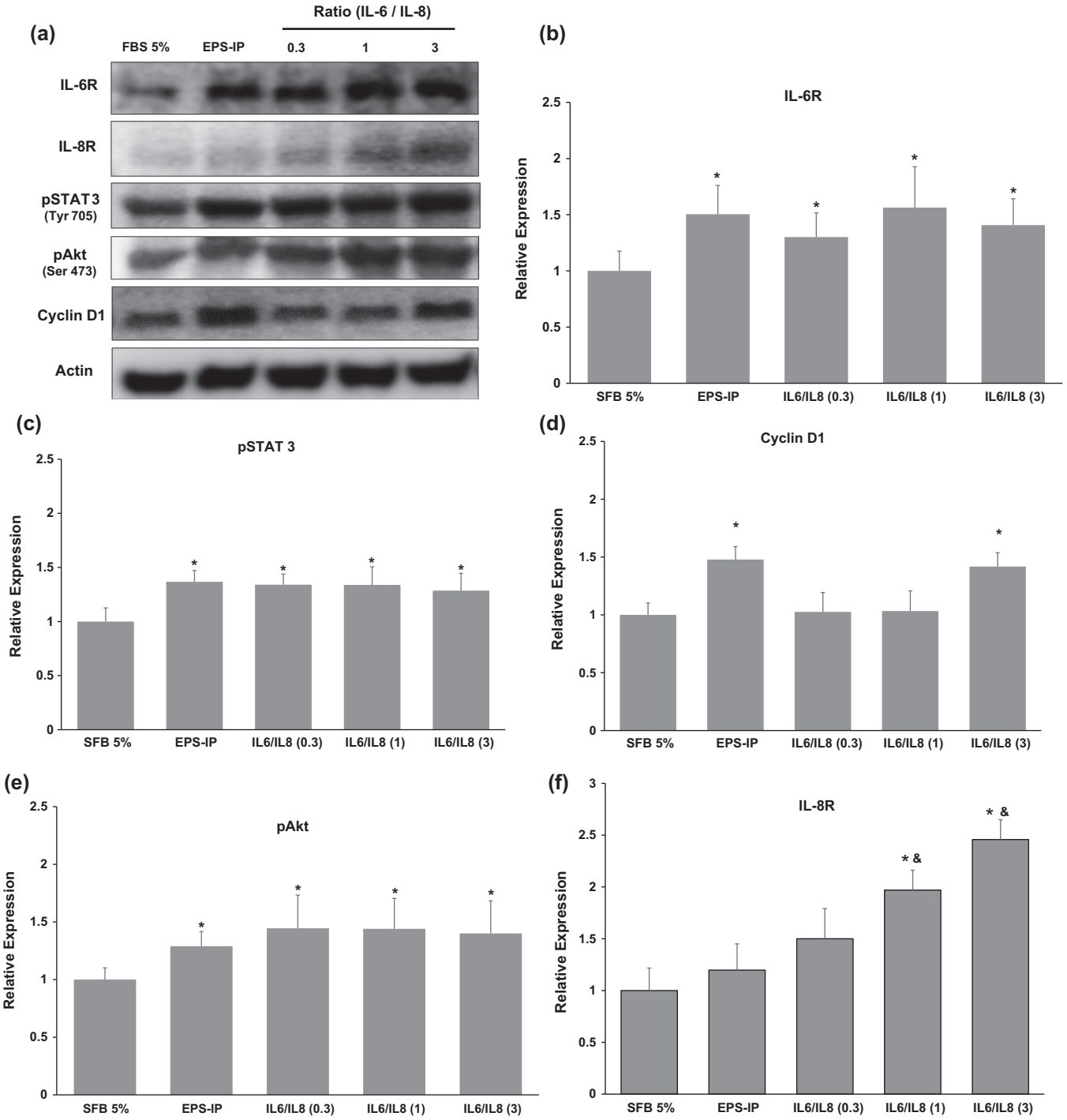

Fig. 11. IL-6/IL-6R/Akt (Ser 473)/STAT3/Cyclin D1 Pathway. Protein levels of several components in the signaling pathway were evaluated in MCF-7 cells subjected to all the treatments as described in materials and methods. (A) Representative blot for IL-6/IL-6R/Akt (Ser 473)/STAT3/Cyclin D1 after 96 h treatment. (B–F) Densitometric analysis for each protein. Each analysis was normalized against the actin control. Each point represents the mean ± SD of three determinations performed in independent experiments. (∗) Statistical significance for controls was considered. (&) Statistical significance for EPS-IP.

with the results obtained by other research groups and in which the participation of IL-6 in the processes of oncogenic transformation such as MET, migration and metastasis has been confirmed [32,100,102,104]. Regarding the explanation of Akt and STAT3 activation when IL-8 concentration is equal or greater than that of IL-6, it is possible that the cells might be performing other cellular processes related to MET, invasion or cellular survival. This idea is supported by other studies that have reported that those processes have been related to the activation of signaling pathways where STAT3 and Akt are involved when the IL-8 is present in elevated concentrations [105,106]. Still, more experiments are needed to explain these differences, as well as IL-6 and IL-8 along with IL-2, 4, 10, TNF-α, IFN-γ, and GM-SCF participation in oncogenic transformation.

It is known that after tissue injury, the inflammatory process is activated in a strictly regulated manner in order to induce wound healing. Conversely, during chronic inflammation (“inflammaging”) the process has been related to the stimulus persistency or/

and a failure in molecular mechanisms that regulate the inflammatory process and wound healing events.

The cytokine inflammatory profile contained within the EPS-IP could be related to a deficiency of the sera donors in repairing tissue damage, and those conditions might also be connected with other events present in the aging process such as accumulation of senescent cells [76,77,38].

Recently it has been reported that senescent cells can modify the tissue microenvironment by secreting of molecules known as senescent associated secretory phenotype (SASP), which is able to stimulate cellular proliferation, metastasis, MET and favors cancer establishment [78–80]. Interestingly, the proportions of the concentration of IL-6 and IL-8 from SASP of WI-38-SIPS cells, which also induced MCF-7 cellular proliferation, was similar to the EPS-IP. This result concurs with other studies where IL-6 and IL-8 have been reported as SASP components [30,31] related to cell proliferation, tumorogenesis and metastasis in vivo and in vitro [81–83].

Another important contribution of our results is the validation that an IL6/IL-8 ratio more than 2.0 can induce cellular proliferation of MCF-7, and that values less than 1.0 do not induce such effect. Previous reports consistent with our results showed an IL-6/IL-8 ratio higher that 3.0 observed in patients with prostate cancer [84]. The same ratio was reported for the induction of proliferation of Glioma Stem Cell (GSC) [85]. Recently, a study showed that incubation of conditioned media from mesenchymal stem cells (MSC) with an IL-6/IL-8 ratio of 1.8, induced the hyperproliferation of a cell line of colorectal cancer [86].

Importantly that is not the increment of cytokines which gives the effect of inducing proliferation, but is rather the ratio of IL-6/IL-8 as mentioned before. It has been observed in MM patients, that even though they present higher IL-6 and IL-8 levels in comparison with healthy patients, the IL-6/IL-8 ratio was lower than 0.5 [45]. All the above suggest that IL-6/IL-8 ratio may vary depending on the cell type and the origin of conditioned media used to induce cellular proliferation.

A clear aspect of our results, besides the description of the ratio of IL-6/IL-8 on the induction of proliferation, is that we found components of SASP or inflammaging that can participate in this effect. Our data showed an increase in other cytokines such as IL-10, IFN-γ, and GM-CSF in EPS-IP. It has been reported that IL-10 is present in tumor environments and activates the Jak-STAT signaling pathway, which has been related to proliferation processes [87]. IFN-γ has also been related to hyperproliferation in dermic psoriasis [88] and in oval cells proliferation during liver regeneration in chronic and acute models [89]; this is interesting because EPS-IP might be related with the cellular response to damage. GM-CSF has been found in benign and malign tumor microenvironments in relation with proliferation, differentiation, and migration of endothelial cells, and also has an aberrant expression in solid tumors like osteosarcomas, gliomas and lung adenocarcinomas [90]. The involvement of IL-10, IFN-γ and GM-CSF on the proliferation of MCF-7 cells, may be related to the establishment of the microenvironment necessary so that it can induce proliferation, and probably its effect could be related to culture cell establishment which is observed between 24 and 72 h incubation with EPS (Figs. 1C, 2A, C and 3).

In summary, we can conclude that an important factor during induction of cell proliferation is the composition of the inflammatory profile in the microenvironment, which along with SASP secreted by senescent cells have been related to the high cancer incidence during old age. The development of new therapies should, therefore, be related to understanding the composition and relationship of the inflammatory components contained in the "inflammaging" and the SASP, to improve treatments that would have the ability to modulate their inflammatory character.

Acknowledgements

The authors would like to thank the CBS-UAMI Confocal Core for confocal images acquisition and analysis. This work was supported by INGER DI-PI004/2012 and CONACyT's grant CB 2012-1-178349. As well as the "Red Temática de Investigación en Salud y Desarrollo Social" from CONACyT. Barajas-Gómez, Morales-Rosales and Pedraza-Vázquez are CONACyT scholarship holders.

References

[1] P.L. Minciullo, A. Inferrera, M. Navarra, G. Calapai, C. Magno, S. Gangemi, Oxidative stress in benign prostatic hyperplasia: a systematic review, Urol. Int. 94 (3) (2015) 249–254, http://dx.doi.org/10.1159/000366210.
[2] A.M. Miniño, M.P. Heron, S.L. Murphy, K.D. Kochanek, Centers for disease control and prevention national center for health statistics national vital statistics system. Deaths: final data for 2004, Natl. Vital Stat. Rep. 55 (19) (2007) 1–119.
[3] L. Balducci, W.B. Ershler, Cancer and ageing: a nexus at several levels, Nat. Rev. Cancer 5 (2005) 655–662, http://dx.doi.org/10.1038/nrc1675.
[4] J. Campisi, Cancer and ageing: rival demons, Nat. Rev. Cancer 3 (2003) 339–349, http://dx.doi.org/10.1038/nrc1073.
[5] C. Ricordi, M. Garcia-Contreras, S. Farnetti, Diet and inflammation: possible effects on immunity, chronic diseases, and life span, J. Am. Coll. Nutr. 34 (Suppl 1) (2015) 10–13, http://dx.doi.org/10.1080/07315724.2015.108010.
[6] C.H. Lee, J.S. Chang, S.H. Syu, T.S. Wong, J.Y. Chan, Y.C. Tang, Z.P. Yang, W.C. Yang, C.T. Chen, S.C. Lu, P.H. Tang, T.C. Yang, P.Y. Chu, J.R. Hsiao, K.J. Liu, IL-1β promotes malignant transformation and tumor aggressiveness in oral cancer, J. Cell Physiol. 230 (4) (2015) 875–884, http://dx.doi.org/10.1002/jcp.24816.
[7] S. Maeda, M. Omata, Inflammation and cancer: role of nuclear factor-kappaB activation, Cancer Sci. 99 (5) (2008) 836–842, http://dx.doi.org/10.1111/j.1349-7006.2008.00763.x.
[8] K. Kashfi, Anti-inflammatory agents as cancer therapeutics, Adv. Pharmacol. 57 (2009) 31–89, http://dx.doi.org/10.1016/S1054-3589(08)57002-5.
[9] S.I. Grivennikov, M. Karin, Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage, Ann. Rheum. Dis. 70 (Suppl 1) (2011) i104–i108, http://dx.doi.org/10.1136/ard.2010.14014.
[10] C. Franceschi, J. Campisi, Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases, J. Gerontol. A Biol. Sci. Med. Sci. 69 (Suppl 1) (2014) S4–S9, http://dx.doi.org/10.1093/gerona/glu05.
[11] T. Fülöp, G. Dupuis, J.M. Witkowski, A. Larbi, The role of immunosenescence in the development of age-related diseases, Rev. Invest. Clin. 68 (2) (2016) 84–91.
[12] L. Ferrucci, A. Corsi, F. Lauretani, S. Bandinelli, B. Bartali, D.D. Taub, J.M. Guralnik, D.L. Longo, The origins of age-related proinflammatory state, Blood 105 (2005) 2294–2299, http://dx.doi.org/10.1182/blood-2004-07-2599.
[13] L. Hayflick, The limited in vitro lifetime of human diploid cell strains, Exp. Cell Res. 37 (1965) 614–636.
[14] J.W. Shay, W.E. Wright, Hayflick, his limit, and cellular ageing, Nat. Rev. Mol. Cell Biol. 1 (1) (2000) 72–76, http://dx.doi.org/10.1038/35036093.
[15] J. Campisi, F. d'Adda di Fagagna, Cellular senescence: when bad things happen to good cells, Nat. Rev. Mol. Cell Biol. 8 (9) (2007) 729–740, http://dx.doi.org/10.1038/nrm2233.
[16] M. Collado, M.A. Blasco, M. Serrano, Cellular senescence in cancer and aging, Cell 130 (2) (2007) 223–233, http://dx.doi.org/10.1016/j.cell.2007.07.003.
[17] T. Kuilman, C. Michaloglou, W.J. Mooi, D.S. Peeper, The essence of senescence, Genes Dev. 24 (22) (2010) 2463–2479, http://dx.doi.org/10.1101/gad.1971610.
[18] R.M. Laberge, Y. Sun, A.V. Orjalo, C.K. Patil, A. Freund, L. Zhou, S.C. Curran, A.R. Davalos, K.A. Wilson-Edell, S. Liu, C. Limbad, M. Demaria, P. Li, G.B. Hubbard, Y. Ikeno, M. Javors, P.Y. Desprez, C.C. Benz, P. Kapahi, P.S. Nelson, J. Campisi, MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation, Nat. Cell Biol. 17 (8) (2015) 1049–1061, http://dx.doi.org/10.1038/ncb3195.
[19] J.M. van Deursen, The role of senescent cells in ageing, Nature 509 (7501) (2014) 439–446, http://dx.doi.org/10.1038/nature13193.
[20] T. Tchkonia, Y. Zhu, J. van Deursen, J. Campisi, J.L. Kirkland, Cellular senescence and the senescent secretory phenotype: therapeutic opportunities, J. Clin. Invest. 123 (3) (2013) 966–972, http://dx.doi.org/10.1172/JCI64098.
[21] N. Loaiza, M. Demaria, Cellular senescence and tumor promotion: is aging the key?, Biochim Biophys. Acta 1865 (2) (2016) 155–167, http://dx.doi.org/10.1016/j.bbcan.2016.01.007.
[22] V.Y. González-Puertos, L.Á. Maciel-Barón, B.A. Barajas-Gómez, N.E. López-Diazguerrero, M. Königsberg, Involvement of phenotype secretor of senescent cells in the development of cancer, aging and the diseases associated with age, Gac. Med. Mex. 151 (4) (2015) 491–500.
[23] R. Pare, T. Yang, J.S. Shin, C.S. Lee, The significance of the senescence pathway in breast cancer progression, J. Clin. Pathol. 66 (6) (2013) 491–495, http://dx.doi.org/10.1136/jclinpath-2012-201081.

[24] F. Rodier, J.P. Coppé, C.K. Patil, W.A. Hoeijmakers, D.P. Muñoz, S.R. Raza, A. Freund, E. Campeau, A.R. Davalos, J. Campisi, Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion, Nat. Cell Biol. 11 (8) (2009) 973–979, http://dx.doi.org/10.1038/ncb1909.

[25] J.P. Coppé, P.Y. Desprez, A. Krtolica, J. Campisi, The senescence-associated secretory phenotype: the dark side of tumor suppression, Annu. Rev. Pathol. 5 (2010) 99–118, http://dx.doi.org/10.1146/annurev-pathol-121808-102144.

[26] L.A. Maciel-Barón, S.L. Morales-Rosales, A.A. Aquino-Cruz, F. Triana-Martínez, S. Galván-Arzate, A. Luna-López, V.Y. González-Puertos, N.E. López-Díazguerrero, C. Torres, M. Königsberg, Senescence associated secretory phenotype profile from primary lung mice fibroblasts depends on the senescence induction stimuli, Age (Dordr) 38 (1) (2016) 26, http://dx.doi.org/10.1007/s11357-016-9886-1.

[27] G. Yang, D.G. Rosen, Z. Zhang, R.C. Bast Jr., G.B. Mills, J.A. Colacino, I. Mercado-Uribe, J. Liu, The chemokine growth-regulated oncogene 1 (Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis, Proc. Natl. Acad. Sci. USA 103 (44) (2006) 16472–16477, http://dx.doi.org/10.1073/pnas.0605752103.

[28] A. Krtolica, N. Larocque, O. Genbacev, D. Ilic, J.P. Coppe, C.K. Patil, T. Zdravkovic, M. McMaster, J. Campisi, S.J. Fisher, GROα regulates human embryonic stem cell self-renewal or adoption of a neuronal fate, Differentiation 81 (4) (2011) 222–232, http://dx.doi.org/10.1016/j.diff.2011.01.001.

[29] J.P. Coppé, C.K. Patil, F. Rodier, Y. Sun, D.P. Muñoz, J. Goldstein, P.S. Nelson, P.Y. Desprez, J. Campisi, Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor, PLoS Biol. 6 (12) (2008) 2853–2868, http://dx.doi.org/10.1371/journal.pbio.0060301.

[30] J.C. Acosta, A. O'Loghlen, A. Banito, M.V. Guijarro, A. Augert, S. Raguz, M. Fumagalli, M. Da Costa, C. Brown, N. Popov, Y. Takatsu, J. Melamed, F. d'Adda di Fagagna, D. Bernard, E. Hernando, J. Gil, Chemokine signaling via the CXCR2 receptor reinforces senescence, Cell 133 (6) (2008) 1006–1018, http://dx.doi.org/10.1016/j.cell.2008.03.038.

[31] T. Kuilman, C. Michaloglou, L.C. Vredeveld, S. Douma, R. van Doorn, C.J. Desmet, L.A. Aarden, W.J. Mooi, D.S. Peeper, Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network, Cell 133 (6) (2008) 1019–1031, http://dx.doi.org/10.1016/j.cell.2008.03.039.

[32] A.K. Sasser, N.J. Sullivan, A.W. Studebaker, L.F. Hendey, A.E. Axel, B.M. Hall, Interleukin-6 is a potent growth factor for ER-alpha-positive human breast cancer, FASEB J.: Off Publ. Fed. Am. Soc. Exp. Biol. 21 (2007) 3763–3770, http://dx.doi.org/10.1096/fj.07-8832com.

[33] C. Palena, D.H. Hamilton, R.I. Fernando, Influence of IL-8 on the epithelial-mesenchymal transition and the tumor microenvironment, Future Oncol. 8 (6) (2012) 713–722, http://dx.doi.org/10.2217/fon.12.59.

[34] Su. Liu, H. Uppal, M. Demaria, P.Y. Desprez, J. Campisi, P. Kapahi, Simvastatin suppresses breast cancer cell proliferation induced by senescent cells, Sci. Rep. 5 (2015) 17895, http://dx.doi.org/10.1038/srep17895.

[35] S. Sánchez-García, R. Sánchez-Arenas, C. García-Peña, O. Rosas-Carrasco, J.A. Avila-Funes, L. Ruiz-Arregui, T. Juárez-Cedillo, Frailty among community-dwelling elderly Mexican people: prevalence and association with sociodemographic characteristics, health state and the use of health services, Geriatr. Gerontol. Int. 14 (2) (2014) 395–402, http://dx.doi.org/10.1111/ggi.12114.

[36] N.E. López-Diazguerrero, H. López-Araiza, J.C. Conde-Perezprina, L. Bucio, M. C. Cárdenas-Aguayo, J.L. Ventura, L. Covarrubias, M.C. Gutiérrez-Ruíz, A. Zentella, M. Königsberg, Bcl-2 protects against oxidative stress while inducing premature senescence, Free Radic. Biol. Med. 40 (7) (2006) 1161–1169, http://dx.doi.org/10.1016/j.freeradbiomed.2005.11.002.

[37] F. Triana-Martínez, N.E. López-Diazguerrero, L.A. Maciel-Barón, S.L. Morales-Rosales, S. Galván-Arzate, F.J. Fernandez-Perrino, A. Zentella, V.I. Pérez, L.E. Gomez-Quiroz, M. Königsberg, Cell proliferation arrest and redox state status as part of different stages during senescence establishment in mouse fibroblasts, Biogerontology 15 (2) (2014) 165–176, http://dx.doi.org/10.1007/s10522-013-9488-6.

[38] G.P. Dimri, X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, M. Peacocket, J. Campisi, A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Proc. Natl. Acad. Sci. USA 92 (20) (1995) 9363–9367.

[39] O. Rosas-Carrasco, E. González-Flores, A.M. Brito-Carrera, O.E. Vázquez-Valdez, E. Peschard-Sáenz, L.M. Gutiérrez-Robledo, E.J. García-Mayo, Assessment of comorbidity in elderly, Rev. Med. Inst. Mex. Seguro Soc. 49 (2) (2011) 153–162.

[40] M.N. Conley, C.P. Wong, K.M. Duyck, N. Hord, E. Ho, T.J. Sharpton, Aging and serum MCP-1 are associated with gut microbiome composition in a murine model, PeerJ 4 (e1854) (2016) 2016, http://dx.doi.org/10.7717/peerj.1854.eCollection.

[41] C.S. Catana, G.A. Calin, I. Berindan-Neagoe, Inflamma-miRs in aging and breast cancer: are they reliable players?, Front Med. (Lausanne) 2 (2015) 85, http://dx.doi.org/10.3389/fmed.2015.00085. eCollectio.

[42] C. Franceschi, M. Capri, D. Monti, S. Giunta, F. Olivieri, F. Sevini, M.P. Panourgia, L. Invidia, L. Celani, M. Scurti, E. Cevenini, G.C. Castellani, S. Salvioli, Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans, Mech. Ageing Dev. 128 (1) (2007) 92–105, http://dx.doi.org/10.1016/j.mad.2006.11.016.

[43] J.A. Riancho, M.T. Zarrabeitia, J.A. Amado, J.M. Olmos, J. González-Macías, Age-related differences in cytokine secretion, Gerontology 40 (1) (1994) 8–12, http://dx.doi.org/10.1159/000213568.

[44] E. Sartono, Y.C. Kruize, A. Kurniawan, R.M. Maizels, M. Yazdanbakhsh, Depression of antigen-specific interleukin-5 and interferon-gamma responses in human lymphatic filariasis as a function of clinical status and age, J. Infect. Dis. 175 (5) (1997) 1276–1280, http://dx.doi.org/10.1086/593701.

[45] M.M. Zheng, Z. Zhang, K. Bemis, A.R. Belch, L.M. Pilarski, J.E. Shively, J. Kirshner, The systemic cytokine environment is permanently altered in multiple myeloma, PLoS One 8 (3) (2013) e58504, http://dx.doi.org/10.1371/journal.pone.0058504.

[46] A. De Angulo, R. Faris, D. Cavazos, C. Jolly, B. Daniel, L. DeGraffenried, Age-related alterations in T-lymphocytes modulate key pathways in prostate tumorigenesis, Prostate 73 (8) (2013) 855–864, http://dx.doi.org/10.1002/pros.22631.

[47] K. Ishihara, T. Hirano, IL-6 in autoimmune disease and chronic inflammatory proliferative disease, Cytokine Growth Factor Rev. 13 (2002) 357–368, http://dx.doi.org/10.1016/S1359-6101(02)00027-8.

[48] W.W. Lin, M. Karin, A cytokine-mediated link between innate immunity, inflammation, and cancer, J. Clin. Invest. 117 (2007) 1175–1183, http://dx.doi.org/10.1172/JCI31537.

[49] K.T. Chang, C.Y. Huang, C.M. Tsai, C.H. Chiu, Y.Y. Lok, Role of IL-6 in neuroendocrine differentiation and chemosensitivity of non-small cell lung cancer, Am. J. Physiol. Lung Cell Mol. Physiol. 289 (2005) L438–L445, http://dx.doi.org/10.1152/ajplung.00033.2005.

[50] D.J. Waugh, C. Wilson, The interleukin-8 pathway in cancer, Clin. Cancer Res. 14 (21) (2008) 6735–6741, http://dx.doi.org/10.1158/1078-0432.CCR-07-4843.

[51] C. Gabellini, D. Trisciuoglio, M. Desideri, A. Candiloro, Y. Ragazzoni, A. Orlandi, G. Zupi, D. Del Bufalo, Functional activity of CXCL8 receptors, CXCR1 and CXCR2, on human malignant melanoma progression, Eur. J. Cancer 45 (14) (2009) 2618–2627, http://dx.doi.org/10.1016/j.ejca.2009.07.007.

[52] J. Wei, H. Xu, J.L. Davies, G.P. Hemmings, Increase of plasma IL-6 concentration with age in healthy subjects, Life Sci. 51 (1992) 1953–1956, http://dx.doi.org/10.1016/0024-3205(92)90112-3.

[53] W.B. Ershler, W.H. Sun, N. Binkley, S. Gravenstein, M.J. Volk, G. Kamoske, R.G. Klopp, E.B. Roecker, R.A. Daynes, R. Weindruch, Interleukin-6 and aging: blood levels and mononuclear cell production increase with advancing age and in vitro production is modifiable by dietary restriction, Lymphokine Cytokine Res. 12 (1993) 225–230.

[54] J. Walston, D.E. Arking, D. Fallin, T. Li, B. Beamer, Q. Xue, L. Ferrucci, L.P. Fried, A. Chakravarti, IL-6 gene variation is not associated with increased serum levels of IL-6, muscle, weakness, or frailty in older women, Exp. Gerontol. 40 (2005) 344–352, http://dx.doi.org/10.1016/j.exger.2005.01.012.

[55] T. Singh, A.B. Newman, Inflammatory markers in population studies of aging, Ageing Res. Rev. 10 (3) (2011) 319–329, http://dx.doi.org/10.1016/j.arr.2010.11.002.

[56] P. Toth, A. Csiszar, D. Sosnowska, Z. Tucsek, P. Cseplo, Z. Springo, S. Tarantini, W.E. Sonntag, Z. Ungvari, A. Koller, Treatment with the cytochrome P450 ω-hydroxylase inhibitor HET0016 attenuates cerebrovascular inflammation, oxidative stress and improves vasomotor function in spontaneously hypertensive rats, Br. J. Pharmacol. 168 (2013) 1878–1888, http://dx.doi.org/10.1111/bph.12079.

[57] G.R. Mundy, Osteoporosis and inflammation, Nutr. Rev. 65 (2007) S147–S151, http://dx.doi.org/10.1111/j.1753-4887.2007.tb00353.x.

[58] K. Ikewaki, Y. Terao, H. Ozasa, Y. Nakada, J. Tohyama, Y. Inoue, M. Yoshimura, Effects of atorvastatin on nuclear magnetic resonance-defined lipoprotein subclasses and inflammatory markers in patients with hypercholesterolemia, J. Atheroscler. Thromb. 16 (2009) 51–56, http://dx.doi.org/10.5551/jat.E563.

[59] V. Clérigues, M.I. Guillén, M.A. Castejón, F. Gomar, V. Mirabet, M.J. Alcaraz, Heme oxygenase-1 mediates protective effects on inflammatory, catabolic and senescence responses induced by interleukin-1β in osteoarthritic osteoblasts, Biochem. Pharmacol. 83 (2012) 395–405, http://dx.doi.org/10.1016/j.bcp.2011.11.024.

[60] G.H. Kang, H.J. Lee, K.S. Hwang, S. Lee, J.H. Kim, J.S. Kim, Aberrant CpG island hypermethylation of chronic gastritis, in relation to aging, gender, intestinal metaplasia, and chronic inflammation, Am. J. Pathol. 163 (2003) 1551–1556, http://dx.doi.org/10.1016/S0002-9440(10)63511-0.

[61] L. Kozlowski, I. Zakrzewska, P. Tokajuk, M.Z. Wojtukiewicz, Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients, Rocz Akad. Med. Bialymst. 48 (2003) 82–84.

[62] X.P. Jiang, D.C. Yang, R.L. Elliott, J.F. Head, Reduction in serum IL-6 after vaccination of breast cancer patients with tumour-associated antigens is related to estrogen receptor status, Cytokine 12 (2000) 458–465, http://dx.doi.org/10.1006/cyto.1999.0591.

[63] R. Srivani, B. Nagarajan, A prognostic insight on in vivo expression of interleukin-6 in uterine cervical cancer, Int. J. Gynecol. Cancer: Off J. Int. Gynecol. Cancer Soc. 13 (2003) 331–339.

[64] V. Michalaki, K. Syrigos, P. Charles, J. Waxman, Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer, Br. J. Cancer 90 (2004) 2312–2316, http://dx.doi.org/10.1038/sj.bjc.6601814.

[65] A. Azevedo, V. Cunha, A.L. Teixeira, R. Medeiros, IL-6/IL-6R as a potential key signaling pathway in prostate cancer development, World J. Clin. Oncol. 2 (2011) 384–396, http://dx.doi.org/10.5306/wjco.v2.i12.384.

[66] S.L. Navarro, Y. Schwarz, X. Song, C.Y. Wang, C. Chen, S.P. Trudo, A.R. Kristal, M. Kratz, D.L. Eaton, J.W. Lampe, Cruciferous vegetables have variable effects on biomarkers of systemic inflammation in a randomized controlled trial in healthy young adults, J. Nutr. 144 (11) (2014) 1850–1857, http://dx.doi.org/10.3945/jn.114.197434.
[67] S.L. Navarro, T.M. Brasky, Y. Schwarz, X. Song, C.Y. Wang, A.R. Kristal, M. Kratz, E. White, J.W. Lampe, Reliability of serum biomarkers of inflammation from repeated measures in healthy individuals, Cancer Epidemiol. Biomarkers Prev. 21 (7) (2012) 1167–1170, http://dx.doi.org/10.1158/1055-9965.EPI-12-0110.
[68] C. Nobles, E.R. Bertone-Johnson, A.G. Ronnenberg, J.M. Faraj, S. Zagarins, B.B. Takashima-Uebelhoer, B.W. Whitcomb, Correlation of urine and plasma cytokine levels among reproductive-aged women, Eur. J. Clin. Invest. 45 (5) (2015) 460–465, http://dx.doi.org/10.1111/eci.12428.
[69] E.R. Bertone-Johnson, A.G. Ronnenberg, S.C. Houghton, C. Nobles, S.E. Zagarins, B.B. Takashima-Uebelhoer, J.L. Faraj, B.W. Whitcomb, Association of inflammation markers with menstrual symptom severity and premenstrual syndrome in young women, Hum. Reprod. 29 (9) (2014) 1987–1994, http://dx.doi.org/10.1093/humrep/deu170.
[70] A. Crucitti, M. Corbi, P.M. Tomaiuolo, C. Fanali, A. Mazzari, D. Lucchetti, M. Migaldi, A. Sgambato, Laparoscopic surgery for colorectal cancer is not associated with an increase in the circulating levels of several inflammation-related factors, Cancer Biol. Ther. 16 (5) (2015) 671–677, http://dx.doi.org/10.1080/15384047.2015.1026476.
[71] S.C. Stone, R.A. Rossetti, A.M. Lima, A.P. Lepique, HPV associated tumor cells control tumor microenvironment and leukocytosis in experimental models, Immun. Inflamm. Dis. 2 (2) (2014) 63–75, http://dx.doi.org/10.1002/iid3.21.
[72] J.S. Lee, S.N. Bukhari, N.M. Fauzi, Effects of chalcone derivatives on players of the immune system, Drug Des. Devel Ther. 9 (2015) 4761–4778, http://dx.doi.org/10.2147/DDDT.S86242. eCollectio.
[73] D.W. Infanger, Y. Cho, B.S. Lopez, S. Mohanan, S.C. Liu, D. Gursel, J.A. Boockvar, C. Fischbach, Glioblastoma stem cells are regulated by interleukin-8 signaling in a tumoral perivascular niche, Cancer Res. 73 (2013) 7079–7089.
[74] P. Zarogoulidis, F. Katsikogianni, T. Tsiouda, A. Sakkas, N. Katsikogiannis, K. Zarogoulidis, Interleukin-8 and interleukin-17 for cancer, Cancer Invest. 32 (5) (2014) 197–205, http://dx.doi.org/10.3109/07357907.2014.898156.
[75] Z. Li, S.X. Lin, Y.J. Liang, Y.S. Zong, Effect of macrophage migration inhibitory factor (MIF) on expression of MMP-2, MMP-9, and IL-8 in nasopharyngeal carcinoma cell strains, Ai Zheng 23 (2) (2004) 130–135.
[76] J. Campisi, Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors, Cell 120 (2005) 513–522, http://dx.doi.org/10.1016/j.cell.2005.02.003.
[77] J.C. Jeyapalan, M. Ferreira, J.M. Sedivy, U. Herbig, Accumulation of senescent cells in mitotic tissue of aging primates, Mech. Ageing Dev. 128 (2007) 36–44, http://dx.doi.org/10.1016/j.mad.2006.11.008.
[78] A. Krtolica, S. Parrinello, S. Lockett, P.Y. Desprez, J. Campisi, Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging, Proc. Natl. Acad. Sci. USA 98 (21) (2001) 12072–12077, http://dx.doi.org/10.1073/pnas.211053698.
[79] J.P. Coppé, K. Kauser, J. Campisi, C.M. Beauséjour, Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence, J. Biol. Chem. 281 (2006) 29568–29574, http://dx.doi.org/10.1074/jbc.M603307200.
[80] D. Liu, P.J. Hornsby, Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion, Cancer Res. 67 (2007) 3117–3126, http://dx.doi.org/10.1158/0008-5472.CAN-06-345.
[81] C. Bavik, I. Coleman, J.P. Dean, B. Knudsen, S. Plymate, P.S. Nelson, The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms, Can. Res. 66 (2006) 794–802, http://dx.doi.org/10.1158/0008-5472.CAN-05-1716.
[82] J.P. Coppe, M. Boysen, C.H. Sun, B.J. Wong, M.K. Kang, N.H. Park, P.Y. Desprez, J. Campisi, A. Krtolica, A role for fibroblasts in mediating the effects of tobacco-induced epithelial cell growth and invasion, Mol. Cancer Res. 6 (7) (2008) 1085–1098, http://dx.doi.org/10.1158/1541-7786.MCR-08-0062.
[83] A. Krtolica, J. Campisi, Cancer and aging: a model for the cancer promoting effects of the aging stroma, Int. J. Biochem. Cell Biol. 34 (11) (2002) 1401–1414, http://dx.doi.org/10.1016/S1357-2725(02)00053-5.
[84] N. Chi, Z. Tan, K. Ma, L. Bao, Z. Yun, Increased circulating myeloid-derived suppressor cells correlate with cancer stages, interleukin-8 and -6 in prostate cancer, Int. J. Clin. Exp. Med. 7 (10) (2014) 3181–3192. eCollection 2014.
[85] P. Parajuli, R. Anand, C. Mandalaparty, R. Suryadevara, P.U. Sriranga, S.K. Michelhaugh, S. Cazacu, S. Finniss, A. Thakur, L.G. Lum, D. Schalk, C. Brodie, S. Mittal, Preferential expression of functional IL-17R in glioma stem cells: potential role in self-renewal, Oncotarget 7 (5) (2016) 6121–6135, http://dx.doi.org/10.18632/oncotarget.6847.
[86] X.B. Wu, Y. Liu, G.H. Wang, X. Xu, Y. Cai, H.Y. Wang, Y.Q. Li, H.F. Meng, F. Dai, J. D. Jin, Mesenchymal stem cells promote colorectal cancer progression through AMPK/mTOR-mediated NF-κB activation, Sci. Rep. 6 (2016) 21420, http://dx.doi.org/10.1038/srep21420.
[87] M. Jung, A. Weigert, M. Tausendschön, J. Mora, B. Ören, A. Sola, G. Hotter, T. Muta, B. Brüne, Interleukin-10-induced neutrophil gelatinase-associated lipocalin production in macrophages with consequences for tumor growth, Mol. Cell Biol. 32 (19) (2012) 3938–3948, http://dx.doi.org/10.1128/MCB.00413-12.
[88] M. Honma, M. Minami-Hori, H. Takahashi, H. Iizuka, Podoplanin expression in wound and hyperproliferative psoriatic epidermis: regulation by TGF-β and STAT-3 activating cytokines, IFN-γ, IL-6, and IL-22, J. Dermatol. Sci. 65 (2) (2012) 134–140, http://dx.doi.org/10.1016/j.jdermsci.2011.11.011.
[89] B. Akhurst, V. Matthews, K. Husk, M.J. Smyth, L.J. Abraham, G.C. Yeoh, Differential lymphotoxin-beta and interferon gamma signaling during mouse liver regeneration induced by chronic and acute injury, Hepatology 41 (2) (2005) 327–335, http://dx.doi.org/10.1002/hep.20520.
[90] E. Obermueller, S. Vosseler, N.E. Fusenig, M.M. Mueller, Cooperative autocrine and paracrine functions of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor in the progression of skin carcinoma cells, Cancer Res. 64 (21) (2004) 7801–7812, http://dx.doi.org/10.1158/0008-5472.CAN-03-3301.
[91] R.T. Davey Jr., R. Lynfield, D.E. Dwyer, M.H. Losso, A. Cozzi-Lepri, D. Wentworth, H.C. Lane, R. Dewar, A. Rupert, J.A. Metcalf, S.L. Pett, T.M. Uyeki, J.M. Bruguera, B. Angus, N. Cummins, J. Lundgren, J.D. Neaton, The association between serum biomarkers and disease outcome in influenza AH1N1 pdm09 virus infection: results of two international observational cohort studies, PLoS One 8 (2) (2013) e57121, http://dx.doi.org/10.1371/journal.pone.0057121.
[92] Q. Qian, P. Li, T. Wang, J. Zhang, S. Yu, T. Chen, L. Yan, Y. Song, X. Liu, Y. Gu, Y. Wang, G. Jia, Alteration of Th1/Th2/Th17 cytokine profile and humoral immune responses associated with chromate exposure, Occup. Environ. Med. 70 (10) (2013) 697–702, http://dx.doi.org/10.1136/oemed-2013-101421.
[93] G.M. Bodienkova, R. Alekseev, E. Boklazhenko, S. Kurchevenko, Inflammation mediators in employees in chronic exposure to neurotoxicants, Int. J. Occup. Med. Environ. Health 27 (4) (2014) 619–626, http://dx.doi.org/10.2478/s13382-014-0284-6.
[94] B. Fang, L. Mai, N. Li, Y. Song, Favorable response of chronic refractory immune thrombocytopenic purpura to mesenchymal stem cells, Stem Cells Dev. 21 (3) (2012) 497–502, http://dx.doi.org/10.1089/scd.2011.0231.
[95] A.S. Tirelli, C. Colombo, E. Torresani, F. Fortunato, A. Biffi, L. Cariani, V. Daccò, A. Carbone, A. Edefonti, F. Paglialonga, M. Conese, Effects of treatment in the levels of circulating cytokines and growth factors in cystic fibrosis and dialyzed patients by multi-analytical determination with a biochip array platform, Cytokine 62 (3) (2013) 413–420, http://dx.doi.org/10.1016/j.cyto.2013.03.029.
[96] M. Postal, K.O. Peliçari, N.A. Sinicato, R. Marini, L.T. Costallat, S. Appenzeller, Th1/Th2 cytokine profile in childhood-onset systemic lupus erythematosus, Cytokine 61 (3) (2013) 785–791, http://dx.doi.org/10.1016/j.cyto.2012.11.023.
[97] A. Díaz, N. Santucci, B. Bongiovanni, L. D'Attilio, C. Massoni, S. Lioi, S. Radcliffe, G. Dídoli, O. Bottasso, M.L. Bay, Increased frequency of CD4+ CD25+ FoxP3+ T regulatory cells in pulmonary tuberculosis patients undergoing specific treatment and its relationship with their immune-endocrine profile, J. Immunol. Res. 2015 (2015) 985302, http://dx.doi.org/10.1155/2015/985302.
[98] M. Bogavac, S. Brkić, D. Celić, N. Simin, J. Matijasević, T. Ilić, Interferon gamma, interleukin 8 and interleukin 10 in serum of patients with the cervical infection and symptoms of the imminent preterm delivery, Srp. Arh. Celok Lek. 141 (9–10) (2013) 623–628.
[99] J. Ramos-Cejudo, C. Oreja-Guevara, L. Stark Aroeira, L. Rodriguez de Antonio, B. Chamorro, E. Diez-Tejedor, Treatment with natalizumab in relapsing-remitting multiple sclerosis patients induces changes in inflammatory mechanism, J. Clin. Immunol. 31 (4) (2011) 623–631, http://dx.doi.org/10.1007/s10875-011-9522-x.
[100] T. Casneuf, A.E. Axel, P. King, J.D. Alvarez, J.L. Werbeck, T. Verhulst, K. Verstraeten, B.M. Hall, A.K. Sasser, Interleukin-6 is a potential therapeutic target in interleukin-6 dependent, estrogen receptor-α-positive breast cancer, Breast Cancer 8 (2016) 13–27, http://dx.doi.org/10.2147/BCTT.S92414.
[101] C. Chavey, F. Bibeau, S. Gourgou-Bourgade, S. Burlinchon, F. Boissière, D. Laune, S. Roques, G. Lazennec, Oestrogen receptor negative breast cancers exhibit high cytokine content, Breast Cancer Res. 9 (1) (2007) R15, http://dx.doi.org/10.1186/bcr1648.
[102] A.W. Studebaker, G. Storci, J.L. Werbeck, P. Sansone, A.K. Sasser, S. Tavolari, T. Huang, M.W. Chan, F.C. Marini, T.J. Rosol, M. Bonafé, B.M. Hall, Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner, Cancer Res. 68 (21) (2008) 9087–9095, http://dx.doi.org/10.1158/0008-5472.CAN-08-0400.
[103] H. Hassan, B. Greve, M.S. Pavao, L. Kiesel, S.A. Ibrahim, M. Götte, Syndecan-1 modulates β-integrin-dependent and interleukin-6-dependent functions in breast cancer cell adhesion, migration, and resistance to irradiation, FEBS J. 280 (10) (2013) 2216–2227, http://dx.doi.org/10.1111/febs.12111.
[104] R.V. Schillace, A.M. Skinner, R.F. Pommier, S. O'Neill, P.J. Muller, A.M. Naik, J.E. Hansen, S.J. Pommier, Estrogen receptor, progesterone receptor, interleukin-6 and interleukin-8 are variable in breast cancer and benign stem/progenitor cell populations, BMC Cancer 14 (2014) 733, http://dx.doi.org/10.1186/1471-2407-14-733.
[105] L. Wang, C. Tang, H. Cao, K. Li, X. Pang, L. Zhong, W. Dang, H. Tang, Y. Huang, L. Wei, M. Su, T. Chen, Activation of IL-8 via PI3K/Akt-dependent pathway is involved in leptin-mediated epithelial-mesenchymal transition in human breast cancer cells, Cancer Biol. Ther. 16 (8) (2015) 1220–1230, http://dx.doi.org/10.1080/15384047.2015.1056409.
[106] D.J. Waugh, C. Wilson, The interleukin-8 pathway in cancer, Clin. Cancer Res. 14 (21) (2008) 6735–6741, http://dx.doi.org/10.1158/1078-0432.CCR-07-4843.

[107] J. Gao, Q. Meng, Y. Zhao, X. Chen, L. Cai, EHD1 confers resistance to cisplatin in non-small cell lung cancer by regulating intracellular cisplatin concentrations, BMC Cancer 16 (2016) 470, http://dx.doi.org/10.1186/s12885-016-2527-3.
[108] Y. Zhu, B. Dai, H. Zhang, G. Shi, Y. Shen, D. Ye, Long non-coding RNA LOC572558 inhibits bladder cancer cell proliferation and tumor growth by regulating the AKT-MDM2-p53 signaling axis, Cancer Lett. 380 (2) (2016) 369–374, http://dx.doi.org/10.1016/j.canlet.2016.04.030.
[109] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[110] A.E. Karnoub, A.B. Dash, A.P. Vo, A. Sullivan, M.W. Brooks, G.W. Bell, A.L. Richardson, K. Polyak, R. Tubo, R.A. Weinberg, Mesenchymal stem cells within tumour stroma promote breast cancer metastasis, Nature 449 (7162) (2007) 557–563, http://dx.doi.org/10.1038/nature0618.