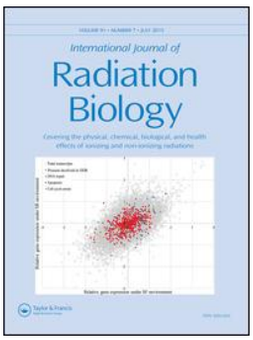

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ORIGINAL ARTICLE

Cytokine profile of breast cell lines after different radiation doses

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ABSTRACT

Purpose: Ionizing radiation (IR) treatment activates inflammatory processes causing the release of a great amount of molecules able to affect the cell survival. The aim of this study was to analyze the cytokine signature of conditioned medium produced by non-tumorigenic mammary epithelial cell line MCF10A, as well as MCF7 and MDA-MB-231 breast cancer cell lines, after single high doses of IR in order to understand their role in high radiation response.

Materials and methods: We performed a cytokine profile of irradiated conditioned media of MCF10A, MCF7 and MDA-MB-231 cell lines treated with 9 or 23 Gy, by Luminex and ELISA analyses.

Results: Overall, our results show that both 9 Gy and 23 Gy of IR induce the release within the first 72 h of cytokines and growth factors potentially able to influence the tumor outcome, with a dose-independent and cell-line dependent signature. Moreover, our results show that the cell-senescence phenomenon does not correlate with the amount of 'senescence-associated secretory phenotype' (SASP) molecules released in media. Thus, additional mechanisms are probably involved in this process.

Conclusions: These data open the possibility to evaluate cytokine profile as useful marker in modulating the personalized radiotherapy in breast cancer care.

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KEYWORDS
Ionizing radiation; breast cancer; cytokines; inflammation

Introduction

Radiation therapy (RT) is a treatment used for many types of cancer: more than 50% of cancer patients receive RT, often used in combination with surgery and chemotherapy (Bernier et al. 2004; Di Maggio et al. 2015).

Besides the direct effects of radiation in reducing cancer cell viability, RT may induce modifications on local microenvironments that can affect tumor development (Formenti and Demaria 2009; Sologuren et al. 2014).

Breast cancer (BC) represents a highly heterogeneous tumor at both clinical and molecular levels. In particular, ionizing radiations (IR), mainly used in conventional external beam RT and in intraoperative electron radiotherapy (IOERT), may be used to destroy any remaining mutated cells in the breast or in the surrounding tumor area after surgery (Bernier et al. 2006; Bravatà 2015; Bravatà et al. 2015; Bravatà et al. 2013a, 2013b; Kraus-Tiefenbacher et al. 2007; Minafra et al. 2012, 2014; Offersen et al. 2009; Williams et al. 2014). According to specific eligibility criteria, IOERT BC treatments may be conducted using two modalities. The first, a single radiation dose of 21–23 Gray (Gy), corresponding to the administration of the entire sequence of a conventional adjuvant RT, could be delivered during the exclusive IOERT treatment. The second, IOERT may be conducted as a boost of 9–12 Gy, followed by conventional external RT treatment (Di Maggio et al. 2015; Minafra and Bravatà 2014; Orecchia and Leonardo 2011; Smith et al. 2009; Veronesi et al. 2010; Wallner et al. 2004).

RT and in particular high IR treatments, activate both pro- and anti-proliferative signal pathways producing an imbalance in cell fate decision regulated by several genes and factors involved in cell cycle progression, survival and/or cell death, DNA repair and inflammation. It has long been recognized that the immune microenvironment surrounding neoplastic cells plays a double-edged sword role in tumor natural history. More precisely, immunological factors can suppress tumor development by killing cancer cells or inhibiting their growth. In addition, immune cells are able to induce an immunosuppressive microenvironment that contributes to promote tumor progression (Balkwill and Mantovani 2001; Di Maggio et al. 2015; Rodemann and Blaese 2007). Inflammatory cytokines, growth factors and proteases can affect cancer cell invasion as well as radiation tissue complications such as fibrosis, genomic instability and thus can greatly influence intrinsic cellular radiosensitivity (Campa et al. 2013; Caruso et al. 2004; Frey et al. 2014; Grivennikov et al. 2010; Hall and Hei 2003; Lumniczky and Sáfrány 2006, Multhoff and Radons 2012; Sologuren et al. 2014).

As recently described by our group, IR could stimulate inflammatory factors to affect cell fate via multiple pathways regulating gene expression, fibrosis and invasive processes

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(Di Maggio et al. 2015). The cytokine profile in the tumor microenvironment is highly dynamic and subjected to multiple changes during tumor development (Desai et al. 2013; Lathers and Young 2004). Moreover, in response to stress such as IR exposure, tumor cells modulate their own cytokine secretion in order to control the cancer therapy outcome (Collins et al. 2000; Kil et al. 2012; Lau et al. 2009; Lev et al. 2004). These secreted factors also interact with surrounding cells and hence, may determine the magnitude of damage to non-targeted tissue via the bystander effect (Desai et al. 2013; Mothersill et al. 2004; Munro 2009; Prise and O'Sullivan 2009).

The aim of this study was to highlight in a descriptive way, for the first time to our knowledge, the cytokine profile secreted in a conditioned medium (CM) by the human MCF10A mammary epithelial cell line, MCF7 and MDA-MB-231 BC cell lines after single high radiation doses. This study has revealed that high IR may modify, independently of the radiation dose used, immunological factor secretion in time-dependent and cell line phenotype-dependent manners. Thus, we could hypothesize that cytokines released following IR treatment, might guide cell fate and trigger, in some conditions, the selection of a surviving cell fraction with a more radioresistant phenotype.

Material and methods

IOERT treatment

The NOVAC7 Intraoperative Electron Radiation Therapy (IOERT) system producing electron beams of 4, 6, 8 and 10 MeV nominal energies was used to perform treatment plans. The beam collimation was performed through a set of polymethyl methacrylate (PMMA) applicators. Cell irradiation setup and the dose distribution were conducted as previously reported (Bravatà et al. 2015; Minafra et al. 2015; Russo et al. 2012). IOERT cell treatments were conducted with two dose values: 9 Gy (in 'boost' scheme) and 23 Gy (according to the 'exclusive' modality) to the 100% isodose at a dose rate of 3.2 cGy/pulse.

Cell cultures, collection of irradiated conditioned media

The human non-tumorigenic breast epithelial MCF10A cell line and human breast adenocarcinoma MCF7 and MDA-MB-231 cell lines, characterized by different tumorigenic aggressive phenotypes (Lacroix and Leclercq 2004), were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to ATCC instructions. Cells were maintained in an exponentially growing culture condition at 37 °C in a 5% CO_2 incubator and were irradiated at subconfluence under sterile conditions. Forty-eight hours before irradiation, cells (1×10^6) were seeded in 100-mm Petri dishes, one for each collection time post-treatments (30 min, 1, 3, 6, 24, 48 and 72 h). Twenty-four hours before irradiation, the medium was replaced with a fresh one. At the defined times after exposure to 9 and 23 Gy IR doses, irradiated conditioned media (ICM) were collected and stored at −80 °C until use. In addition, in the case of radioresistant cell fractions (RCF) of MCF10A 9 Gy and MDA-MB-231 9 Gy, at 7, 14 and 21 days post-irradiation the media was collected as described above. For each cell line, one Petri dish with the same cell number compared to treated cells, were seeded and grown under the same experimental conditions. Thus, their complete CM was collected and used as control (basal, i.e. untreated). Moreover, complete media without cells were incubated under the same experimental conditions and used as the blank controls. Schedule of irradiation and collection of ICM and CM are shown in Figure 1.

Clonogenic survival assay

The clonogenic survival assay was performed as previously described (Bravatà et al. 2015; Minafra et al. 2015). Colonies were allowed to grow under normal cell culture conditions for 2–3 weeks and then fixed and stained for 30 min with 6% glutaraldehyde and 0.5% crystal violet (both from Sigma-Aldrich, St. Louis, MO). Colonies with more than 50 cells were counted manually under a Zeiss Axiovert phase-contrast microscope (Carl Zeiss, Germany). Moreover, to detect the cell radiation effect, cells throughout the course of the assays were monitored for cell morphology and growth pattern by taking photographs in random fields for each treatment.

Senescence detection assay

Senescence detection assays, performed by senescence associated β-galactosidase (SA-β-gal) activity, were also conducted as previously described (Bravatà et al. 2015; Minafra et al. 2015). Twenty-four hours after irradiation, MCF10, MCF7 and MDA-MB-231 cells were seeded in triplicate at a density of 100 cells per well in two-well chamber slides. At 3 and 5 days after irradiation, senescent cells were identified by a senescence associated β-galactosidase (SA-β-gal) assay using a Senescence Cell Staining kit following the manufacturer's instructions (Sigma-Aldrich). Senescent cells were evaluated using a Zeiss Axioskop microscope (Carl Zeiss, Göttingen, Germany) under a ×20 lens. Five random fields of cells were photographed for each treatment and the percentage of SA-β-gal-positive cells was calculated.

Cytokine, chemokine and growth factor analysis

Media were collected after the following time points post-exposure to 9 and 23 Gy IR doses: 30 min, 1, 3, 6, 24, 48 and 72 h. In addition, for MCF10A and MDA-MB-231 RCF fractions, media were collected using the following time points: 7, 14 and 21 days after 9 Gy IR exposure. CM and ICM were stored at −80 °C until use. Immediately before the cytokine assay, thawed samples were centrifuged at 12,000 rpm for 5–10 min to allow precipitation of any lipid excess that may interfere with subsequent analysis. CM of untreated MCF10A, MCF7 and MDA-MB-23 cells, seeded with the same cell number compared to treated cells, were collected in the same time points and used for data normalization. The samples were analyzed using Luminex and ELISA technologies, described as follows.

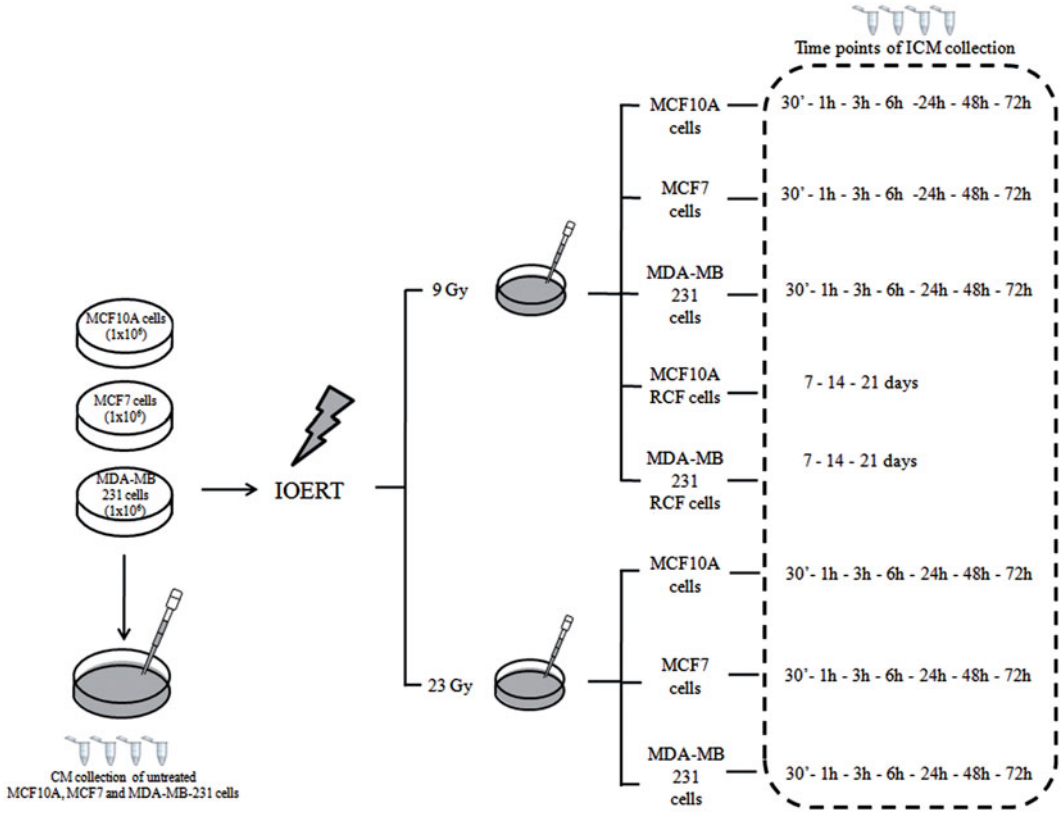

Figure 1. Schedule of irradiation and collection of CM and ICM.

Luminex assays. The samples were tested for a panel of 17 cytokines and chemokines [IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, IFN-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1b (MIP-1b), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF)] using Bio-plex kit (BioRad, Milan, Italy) and following the manufacturer's instructions. The assay was carried out using the Luminex system (BioRad, Munchen, Germany), based on the measurement of fluorescent signals released by a suspension of microspheres with immobilized cytokine specific antibodies, in 96-well plates. The combination of the fluorimetric signal of the microspheres with that released by a secondary antibody allows the measurement of cytokine concentration-related signals converted by a processor. The assay was performed using an eight-point standard curve for every cytokine. Samples were analyzed on a Luminex 100 device (BioRad), and the data were evaluated using the Bio-Plex Manager software (BioRad). Standards, internal controls, and samples are reported as means of duplicate measurements.

ELISA assays. Secreted TGFβ2 and VEGFA were determined by the human TGF-β2 ELISA assay (RayBiotech, Norcross, GA) and human VEGF ELISA Kit (Boster Biological Technology Co., Pleasanton, CA), respectively, following the manufacturer's instructions. The assays were performed using an eight-point standard curve for each assay. Standards and samples were loaded into the 96-well plate and TGF-β2 and VEGFA molecules in the samples were bound to the wells by immobilized specific antibodies coated on the plate bottom. Standards and samples were reported as means of triplicate measurements. At the end of each assay, the intensity of the solution color changes (from blue to yellow) were measured at 450 nm using VICTOR3 multilabel counter instrument (Perkin Elmer, Alameda, CA). Data analyses were performed comparing MCF10A, MCF7 and MDA-MB-231 ICMs at different collection time points, versus CMs of untreated cells used as reference samples.

Statistics

Concentration data were collected as mean values ± standard deviations from three replicated dosages and expressed as pg/ml of three independent experiments. Then, data in the Tables 1–3 were reported as expression normalized values of each cell line with respect to untreated cells. The Mann–Whitney non-parametric test was used to evaluate the differences in cytokine levels between treated and untreated samples. Differences were considered significant when a p value $<.05$ was obtained.

PubMatrix

All proteins assayed in this work were analyzed using the PubMatrix tool (Becker et al. 2003). In this way, lists of terms, such as protein names, can be assigned to a genetic, biological, or clinical relevance in a flexible systematic fashion in order to confirm our assumptions. Thus, bibliographic relationships between proteins and some selected queries such as IR, radiation, cancer, BC, inflammation, apoptosis, NF-Kb, STAT-3, MCF10A, MCF7 and MDA-MB-231 were analyzed in

Table 1. ICM cytokine profile of MCF10A mammary epithelial cell line exposed to different IR doses.

	9 Gy MCF10A			23 Gy MCF10A			9 Gy MCF10A-RCF[a]		
Analytes	24 h	48 h	72 h	24 h	48 h	72 h	7 days	14 days	21 days
IL-1b	1.01	1.42	2.02	1.41	1.81	2.12	0.51	1.32	1.44
IL-6	0.91	***2.64***	***2.09***	1.33	***1.84***	***1.86***	0.39	1.48	1.07
TNFα	1.02	1.18	1.56	1.17	1.34	1.45	0.40	1.14	1.30
IL-12	0.00	0.00	0.69	0.00	0.00	0.00	0.00	0.00	0.69
INF-γ	1.06	1.37	1.37	1.17	1.34	1.40	0.52	1.25	1.42
IL-4	1.01	1.40	1.37	1.34	1.40	1.32	0.50	1.32	1.48
IL-13	0.82	0.93	1.02	0.89	1.06	1.04	1.48	1.48	1.48
IL-8	0.97	***2.57***	***5.04***	1.79	***3.35***	***5.58***	0.22	0.86	***2.60***
MCP-1	1.10	***1.94***	***9.40***	1.31	1.71	***4.22***	0.64	***4.09***	***25.81***
MIP-1b	0.89	***2.70***	***3.00***	***1.97***	***2.70***	***3.64***	0.00	***5.13***	***8.00***
IL-2	1.07	1.21	1.48	1.24	1.35	1.45	0.73	1.31	1.47
IL-7	1.02	1.67	***2.46***	1.43	***1.95***	***2.52***	0.10	1.06	***3.06***
G-CSF	1.03	***3.48***	0.00	***2.04***	0.00	0.00	0.31	0.00	0.00
IL-17	0.89	1.67	***2.33***	1.67	***2.11***	***2.18***	0.00	***2.14***	***2.74***
TGFβ2[b]	0.65	1.05	1.16	0.56	1.07	1.44	0.08	1.07	***1.95***
VEGFA[b]	0.87	1.03	1.04	0.71	0.96	0.93	0.10	1.03	1.16

Values (pg/ml) were normalized using CM of untreated MCF10A cells. Significantly modified cytokine concentrations respect to untreated cells ($p < .05$, Mann–Whitney non-parametric test), were marked in italic bold in the table. IL-5, IL-10 and GM-CSF were undetectable. [a]MCF10A-RCF: radio-resistant cell fraction. [b]ELISA tests.

Table 2. ICM cytokine profile of MCF7 BC cell line exposed to different IR doses.

	9 Gy MCF7			23 Gy MCF7		
Analytes	24 h	48 h	72 h	24 h	48 h	72 h
IL-6	1.24	1.26	***2.69***	1.39	***1.88***	***3.18***
IL-12	0.00	0.96	0.00	0.73	1.40	1.29
INF-γ	1.09	1.00	1.09	0.91	0.65	0.48
IL-8	0.84	0.69	1.00	0.84	0.54	1.00
MCP-1	***5.36***	***7.48***	***4.42***	***5.79***	***4.42***	***7.22***
IL-2	0.98	0.91	0.83	0.76	0.99	0.89
GM-CSF	1.31	1.12	1.26	1.20	0.00	0.00
IL-17	1.52	0.96	1.44	1.15	1.26	1.04
TGFβ2[a]	0.88	1.26	1.38	0.99	1.20	1.39
VEGFA[a]	0.89	1.80	2.36	0.72	1.31	2.04

Values (pg/ml) were normalized using CM of untreated MCF7 cells. Significantly modified cytokine concentrations respect to untreated cells ($p < .05$, Mann–Whitney non-parametric test), were marked in italic bold in the table. IL-1B, IL-4, Il-5, IL-7, IL-10 IL-13, G-CSF, MIP-1b and TNFα values were under detection limits. [a]ELISA tests.

order to understand data and to draw useful conclusions (Figure 2).

Results

Clonogenic survival assay, morphological and senescence analysis of IOERT-treated cells

To evaluate MCF10A, MCF7 and MDA-MB-231 cell viability in terms of reproductive capacity, we performed a clonogenic survival assay according to the method of Franken et al. (2006), as previously described (Bravatà et al. 2015; Minafra et al. 2015). The results of this assay showed that 23 Gy exposure inhibited colony-forming ability of MCF10A cells. On the contrary, following 9 Gy treatment, a surviving RCF (9.6%) with reproductive capacity, was found and maintained in culture up to 3-weeks post-treatment (Figure 3(A,B)). Moreover, 9 and 23 Gy exposure inhibited completely the colony-forming ability of MCF7 (Figure 4(A,B)). In the case of MDA-MB-231 BC cells, 23 Gy treatment inhibited cell growth and proliferation. While, 3-weeks after the 9 Gy exposure an RCF was observed (6.4%) (Figure 5(A,B)).

Cell morphology modifications IR-induced by using 9 and 23 Gy of IR, was similar for all cell lines assayed. Overall, cell damage at both the membranous and cytoplasmic levels was observed starting from 72 h post-treatments and increased within 1 week (data not shown). In addition, irradiated MCF7 and MDA-MB-231 cells displayed a large flat cell shape with evident macroscopic plasma membrane and nucleus alterations (Figures 4(A) and 5(A)). These observations indicated a typical senescent phenotype (the well-known 'fried egg'), which we confirmed by SA-β-Gal activity as shown in Figure 6. On the contrary, MCF10A cells did not reveal a radiation-induced senescent phenotype, as displayed in Figures 3(A), 5(A) and 6(A).

CM inflammatory profile of human MCF10A after RT

MCF10A ICMs collected after 30 min, 1, 3, 6, 24, 48 and 72 h post-IR treatments with 9 and 23 Gy, were analyzed in order to quantify and evaluate cytokine, chemokine and growth factor profiles. Cell media, exposed to 9 and 23 Gy doses, did not show relevant amounts of these factors during the early time points after treatments (30 min, 1, 3, 6 h), with respect to untreated cells, as their quantities were below the range detected by the instrument (data not shown). Thus, here we have reported experimental data obtained at 24, 48 and 72 h time points after RT. The MCF10A cytokine signature was very similar for the two doses used in the post-irradiation time windows analyzed (24–72 h), as shown by a similar trend (Table 1). Among all the molecules investigated, relative expression values significantly higher compared to untreated cells, were marked in bold Italics in the Table 1. In summary, our data suggest that treated cells normalized to untreated ones, react to irradiation with a progressive increased production of inflammatory molecules, sustained by a mild rise of IL-6 release. In addition, a gradually strong increase of chemokine release (IL8, MCP-1 and MIP-1b), known as an attractant of immune cells to the inflammation site, was observed together with an increased secretion of the growth factors IL-7. IL-17, involved in vivo by enhancing

Table 3. ICM cytokine profile of MDA-MB-231 BC cell line exposed to different IR doses.

	9 Gy MDA-MB-231			23 Gy MDA-MB-231			9 Gy MDA-MB-231-RCF[a]		
Analytes	24 h	48 h	72 h	24 h	48 h	72 h	7 days	14 days	21 days
IL-1b	0.00	0.55	***2.75***	0.00	1.02	1.28	***21.63***	***45.91***	***67.53***
IL-6	***4.54***	***2.42***	***4.38***	***3.97***	***16.98***	***27.9***	***53.30***	***143.70***	***248.58***
TNFα	0.82	1.30	***5.30***	0.58	1.99	***3.26***	***3.26***	***4.79***	***5.45***
IL-10	1.45	0.92	***1.94***	***2.08***	***2.44***	***2.35***	1.37	1.81	***2.36***
IL-12	***11.22***	***2.68***	1.34	***3.81***	***4.61***	1.91	0.03	0.04	0.05
INF-γ	***2.04***	***3.15***	***11.57***	***2.37***	***4.75***	***7.72***	***3.30***	***4.66***	***4.80***
IL-4	***4.50***	***10.25***	***27.75***	***5.00***	***12.75***	***20.25***	***2.06***	***3.49***	***4.19***
IL-13	0.88	0.71	1.79	1.00	1.00	1.29	0.13	0.16	0.21
IL-8	***5.69***	***12.92***	***52.3***	***6.09***	***26.6***	***21.01***	0.00	0.00	0.00
MCP-1	***2.04***	***3.35***	***6.32***	1.51	***3.91***	***4.54***	0.75	***2.97***	***9.28***
MIP-1b	1.35	***2.52***	***6.20***	1.57	***4.71***	***4.09***	1.55	***2.51***	***2.93***
IL-2	***1.87***	***1.87***	***3.07***	1.32	***2.27***	***2.16***	0.52	0.68	0.72
IL-7	***2.83***	***3.19***	***3.73***	***2.18***	***4.11***	***3.40***	0.78	0.92	1.51
G-CSF	***5.15***	***9.47***	***36.6***	***8.65***	***14.90***	***18.40***	***8.12***	***51.43***	***87.84***
G-CSF	***2.19***	***2.81***	***7.01***	***1.98***	***5.54***	***3.68***	***15.32***	***25.29***	***23.59***
IL-17	***7.48***	***9.55***	***18.98***	***7.48***	***12.37***	***12.77***	1.23	1.85	***1.99***
TGFβ2[b]	***4.9***	***5.56***	***4.62***	***3.08***	***5.66***	***3.69***	1.46	***2.78***	***3.19***
VEGFA[b]	***5.04***	***7.55***	***6.00***	***3.04***	***4.30***	***3.88***	1.18	***2.04***	***2.38***

Values (pg/ml) were normalized using CM of untreated MDA-MB-231 cells. Significantly modified cytokine concentrations respect to untreated cells ($p < .05$, Mann–Whitney non-parametric test), were marked in italic bold in the table. IL-5 was undetectable. No detectable concentrations of cytokines were evaluable in MDA-MB-231 cell cultures 7 days after 23 Gy irradiation. [a]MDA-MB-231-RCF: radio-resistant cell fraction. [b]ELISA tests.

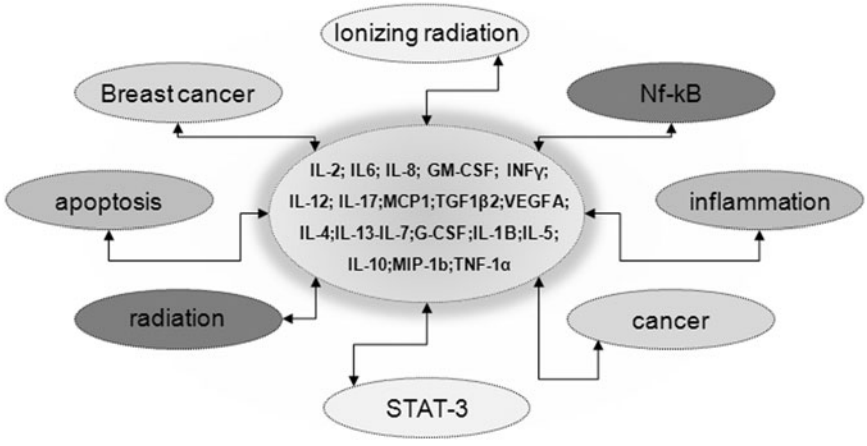

Figure 2. The figure displays the documented interaction between immunological factors and some relevant processes investigated in this work.

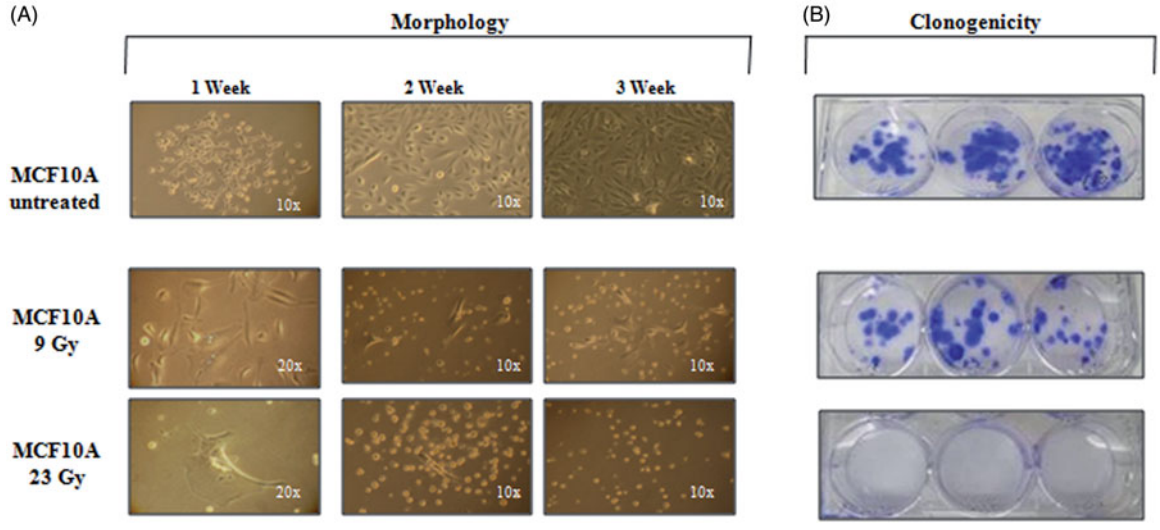

Figure 3. (A) Morphological evaluation of MCF10A cells. (B) MCF10A clonogenic survival analysis.

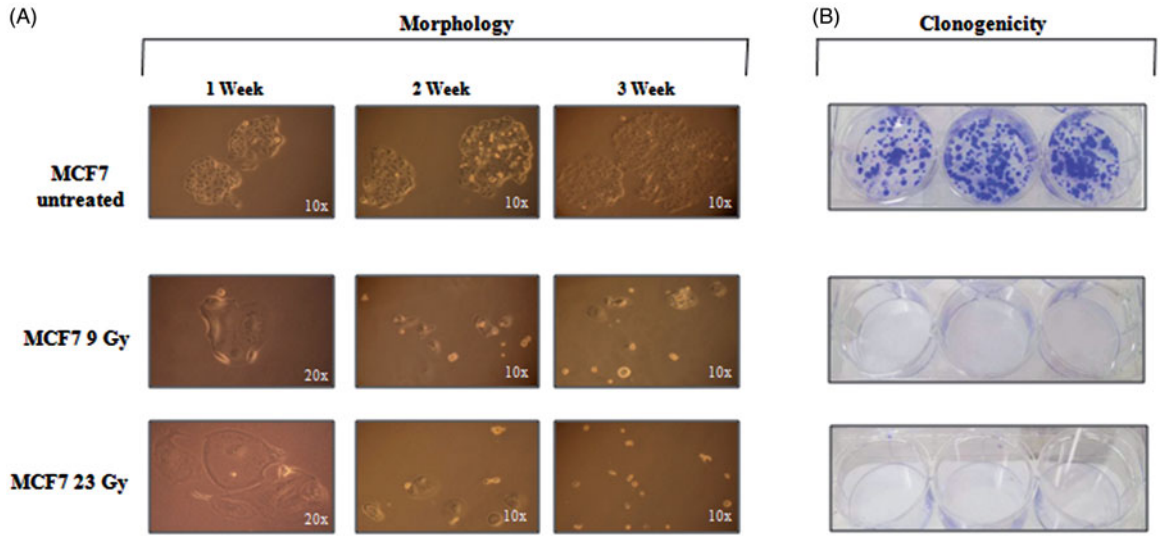

Figure 4. (A) Morphological evaluation of MCF7 cells. (B) MCF7 clonogenic survival analysis.

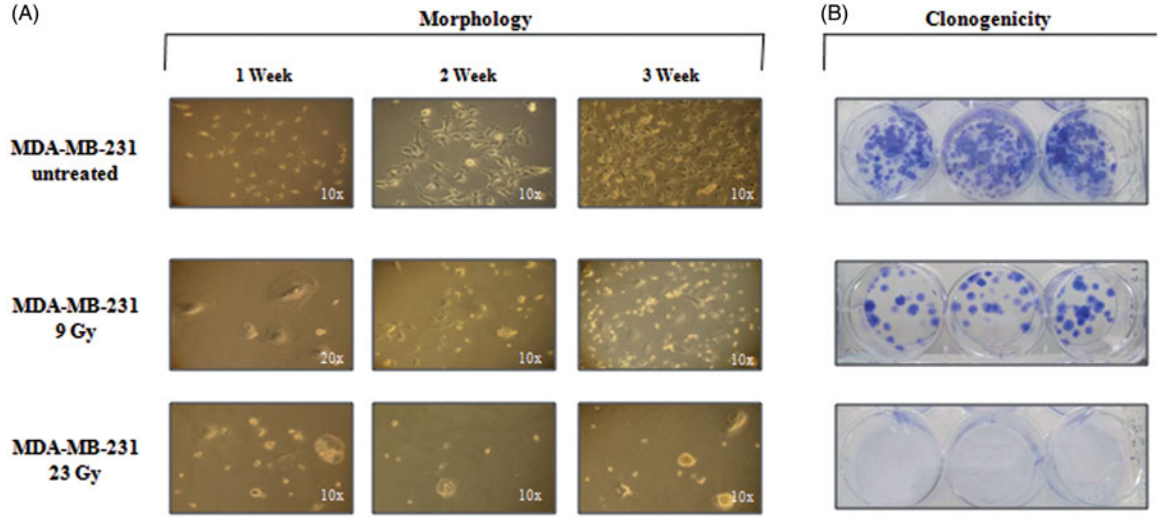

Figure 5. (A) Morphological evaluation of MDA-MB-231 cells. (B) MDA-MB-231 clonogenic survival analysis.

chemokine release and in the generation of T-regulatory cell response (O'Sullivan et al. 2014), is also increased in the late cultures. No long-surving fraction was recovered after 23 Gy treatment. The inflammatory profile of MCF10A radio-resistant surviving RCF was studied at 7, 14 and 21 days post 9 Gy-irradiation (Table 1). Overall, the ICM of MCF10A RCF, is characterized by moderate pro-inflammatory signals maintaining a local cell mediated-type immune response. Chemokine concentration levels, and in particular MCP-1 and MIP-1b secretion, remained high and reached a maximum peak at 21 days. Finally, a slight increase of IL-7 and IL-17 levels was observed, in particular after 21 days post-treatment in ICM of MCF10A RCF compared to the control.

CM inflammatory profile of human MCF7 BC after RT treatment

Table 2 shows the relative expression of immunological molecules, secreted in the medium by MCF7 cells after high radiation treatment. Even in this case, early time points were characterized by very low molecule release, thus, again we only considered the 24, 48 and 72 h post-treatment time points. Overall, the cytokine production of MCF7 cells post-irradiation was quite low: the numerous molecules analyzed (IL-1b, TNF-α, IL-5, IL-4, IL-13, IL-10, MIP-1b, IL-7 and G-CSF) were undetectable in cell cultures exposed both at 9 and 23 Gy.

With respect to untreated cells, the relative production of the other factors was generally reduced and molecule release trends were very similar between the 9 and 23 Gy treatments. Notably, in comparison with the non-tumorigenic MCF10A cell line, these BC cells showed a completely different cytokine signature, in which only some pro-inflammatory cytokines (IL-6 and MCP-1) increased.

CM inflammatory profile of human MDA-MB-231 BC cell lines after RT

As described above for all the cell lines analyzed in this work, during the early time points after RT, a very low

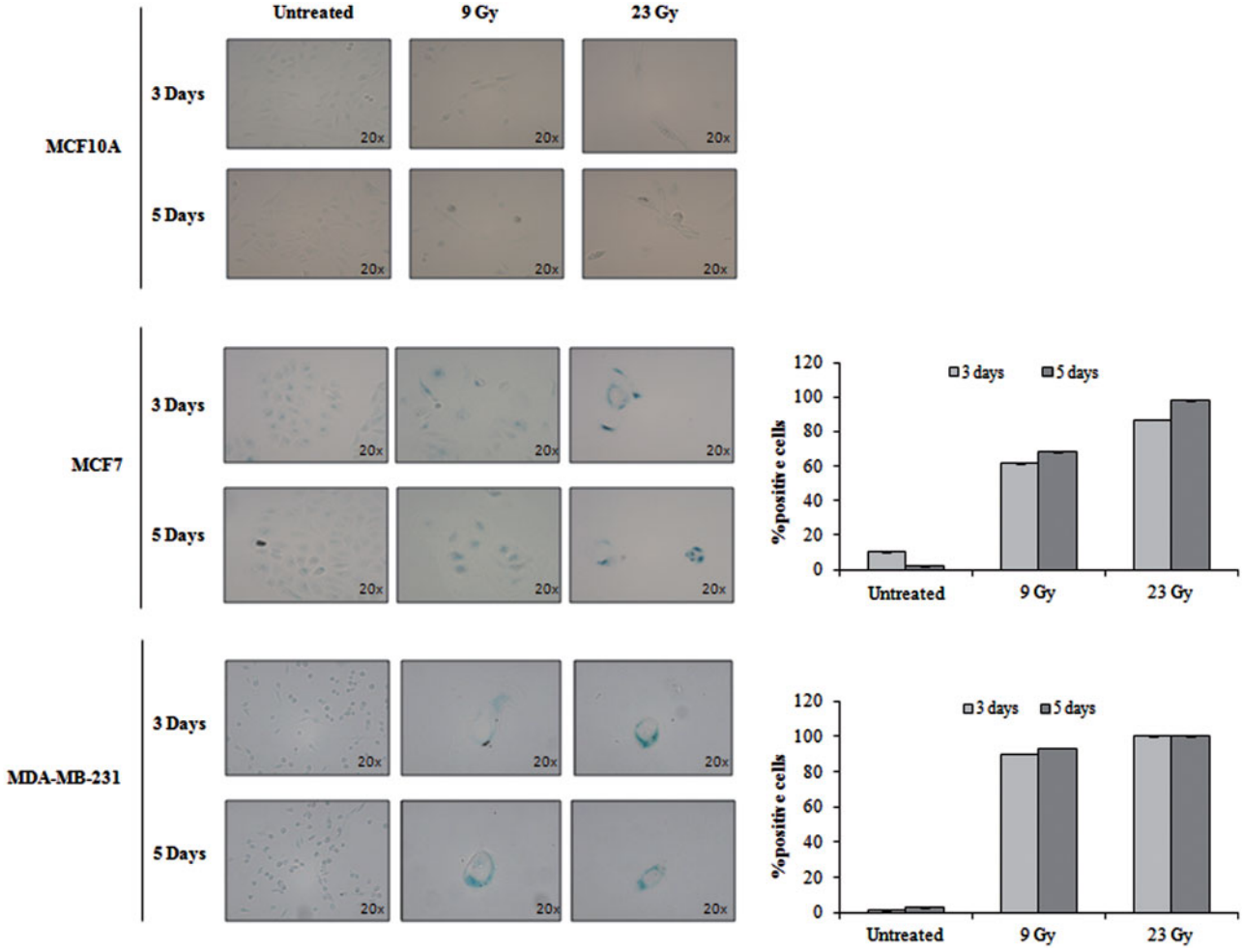

Figure 6. Cell morphology and senescence-associated β-galactosidase (SA-β-Gal) activity in MCF10A, MCF7 and MDA-MB-231 cells treated with 9 Gy and 23 Gy, 3 and 5 days post-IOERT. The graph displays the percentages of SA-β-Gal-positive cells (basal = untreated cells).

amount of molecules were secreted in the medium. Thus, Table 3 shows the relative expression of the immunological factors released by MDA-MB-231 cells at 24, 48 and 72 h post-irradiation. Overall, an over production of virtually all the factors investigated was measured in ICM of MDA-MB-231 cell line with a similar cytokine profile following the 9 and 23 Gy treatment. The cytokine signature was characterized by the progressive increase of TNFα and significantly, of the IL-6 levels. Moreover, an increase of INF-γ, as well as IL-4 with respect to untreated cells at both IR doses, and a strong increase of IL-17 and chemokines (IL8, MCP-1 and MIP-1b) was observed. Growth factors as IL-7, G-CSF and GM-CSF production was progressively enhanced at 24–72 h after the 9 and 23 Gy treatment. Both TGF-β2 and VEGFA levels were higher than untreated cells following the treatments with 9 and 23 Gy. Overall, compared to the non-tumorigenic MCF10A cell line, the immune response profile of MDA-MB-231 cells to irradiation was characterized by an activation of almost all the immunological factors analyzed in this work. As described for the MCF10A RCF cells, no MDA-MB-231 long-surving fraction was recovered after 23 Gy treatment, whereas MDA-MB-231 9 Gy, RCF was observed. The cytokine signature was tested at 7, 14 and 21 days post-irradiation. Table 3 shows that the MDA-MB-231 RCF cells produced a greater amount of nearly all the molecules analyzed compared to the other cell lines studied with exception of IL-8 and IL-12 productions. In particular, increased levels of IL-6, IL-1b, TNF-α, G-CSF, GM-CSF, TGF-β2 and VEGFA were observed. Overall, MDA-MB-231 RCF cells showed the strongest potentially pro-inflammatory secretion profile compared to the other cell fractions analyzed.

Discussion

IR induces in vivo and in experimental models significant effects on immune system modulation through the release of cytokines, chemokines and growth factors (Schaue et al. 2012), which in turn, affect the balance between survival and cell death, as well as metastatic ability and tissue remodeling (Minafra et al. 2014). So, the analysis of the cancer cell cytokine signature is a topic of interest in order to better understand the modification of tumor microenvironment and the cytokine role in cancer progression and radioresistance mechanisms (Bravatà et al. 2013a; Scola et al. 2006, 2014).

Here, we provided a descriptive portrait of radiation-induced cytokine profiles of three different cell lines: the MCF10A non-tumorigenic mammary epithelial cells and the two MCF7 and MDA-MB-231 tumorigenic BC cell lines. The 19 molecules assayed in this work were chosen

according to their involvement in cell radiation response, as described by several authors (Figure 2) (Desai et al. 2013; Li et al. 2012). The literature data report that cytokine production is time-dependent, with an expression peak usually observed at 4–24 h after irradiation with a subsequent decrease to basal levels from 24 h to a few days (Desai et al. 2013; Hong et al. 1995; Li et al. 2012). Here, we explored an early and a late cytokine production profile, analyzing the following time window: from 30 min to 72 h post-IR treatment.

Furthermore, we tested the effects of two doses: 9 Gy (representative of the IOERT BC 'boost' scheme irradiation) and 23 Gy (representative of the BC 'elective' treatment modality), in order to evaluate the differences in the immune response profiles in terms of dose-effects.

Cell viability, evaluated in terms of clonogenic survival, showed that 9 Gy treatment inhibited growth and proliferation of MCF7 BC cells (Bravatà et al. 2015), but not those of MCF10A (Minafra et al. 2015) and MDA-MB-231 BC cell lines, which generated RCFs. RT with 23 Gy inhibited clonogenic activity for all cell lines used. Thereafter, we also studied the cytokine secretion profiles in ICM of MCF10A RCF and MDA-MB-231 RCF, during a very late time window: from 7 to 21 days post-irradiation.

In summary, we investigated the time, cell line, and dose-dependence effects of IR-related cytokine production. To our knowledge, this is the first study evaluating, in such descriptive and extensive way, the immunological signature of one mammary non-tumorigenic and two tumorigenic BC cell lines produced in response to high doses of IR.

The most relevant results were obtained analyzing late time points of ICM collection after irradiation rather than the early one. Overall, we observed a time-dependent and a cell-line dependent cytokine signature post-irradiation in 9 Gy treated cell lines as in 23 Gy dose. In addition, 23 Gy IR had a fundamental lethal effect in all the experiments.

Concerning the cell line differences observed, three different cytokines signatures can be described for the three cell lines analyzed. Our data seem to suggest that the cytokine production profile of the MCF10A cell line, often used as a model of normal breast epithelial cells (Minafra et al. 2015), could represent how normal breast tissue cells react to high doses of radiation. Here, we show that the MCF10A profile is characterized by signals related to a local inflammation and cell mediated response, while only a mild increase of IL-6 release might be considered to mimic a systemic response. In turn, in respect of the non-tumorigenic MCF10A cells, MCF7 BC cells showed a low and high irradiation dose sensitivity in the production of a reduced number of immunomodulatory molecules. The noteworthy exception is represented by a small but significant increase of IL-6 and MCP-1. Our data are in line with those recently described by Desai et al. (2013), regarding the minimum magnitude secretion of immunological factors secreted in the ICM of MCF7 cells compared to other human cancer cell lines analyzed after radiation exposure. On the other hand, the more aggressive MDA-MB-231 BC cell line was characterized by a secretion of almost all the cytokines assayed in this work. These cells produce much higher levels of IL-6, INF-γ, IL-8, MCP-1 and GM-CSF, factors able, in vivo, to mediate stronger systemic and local cell responses (Fujisaki et al. 2015), whereas anti-inflammatory IL-10 release is relatively limited. Thus, the stronger IR related inflammatory signature of MDA-MB-231 BC cells with respect to MCF7, might reflect their particular aggressive phenotype, as described by several authors (Lacroix and Leclercq 2004). In particular, our data allow us to speculate that these differences, might modify the peri-tumoral microenvironment which could influence the natural history and outcome of the disease, affecting the survival/cell death equilibrium, or modulating invasiveness or fibrosis.

Interestingly, overall the cell line signatures studied revealed that the cytokine secretion by cell lines to high IR doses was characterized by an unbalanced inflammatory response. Notably, IL-10, the major anti-inflammatory cytokine, was undetectable in the conditioned medium of MCF10A, MCF10A RCF and MCF7, or relatively poorly expressed in MDA-MB231 and MDA-MB231 RCF.

Moreover, concerning the behavior of non-tumorigenic and tumorigenic RCFs, monitored at a very late time window (7–21 days), consistent differences were found between the two cell lines investigated. Indeed, for the MCF10A RCF cells we observed moderate pro-inflammatory signal release. Moreover, MDA-MB-231 cells showed signs of an uncontrolled high release of mediators. The long term observation of these RCFs might be considered a useful model showing the differences of cytokine signature between high doses of IR resistant non-tumorigenic and tumorigenic cell lines.

Among the molecules detected, IL-6, IL-8, MCP-1 and their related transcription factors, NF-KB and C/EBPβ, are known to be considered among the in vitro cell senescence molecule patterns (Young and Narita 2009). Using the PubMatrix tool (Becker et al. 2003) their relevance for the 'senescence-associated secretory phenotype (SASP)' was confirmed. The induction of cell senescence phenotype represents a permanent exit from the cell cycle, even if it is not a passive phenomenon, but instead it consists of metabolic changes in protein expression and secretion such as interleukins, chemokines, growth factors and proteases. SASP molecules can induce senescence in many of the surrounding tumor cells in an autocrine and/or paracrine manner. On the other hand, they induce transformation processes in cells predisposed to proliferation (Young and Narita 2009).

In our study, we observed a senescent phenotype only in MCF7 and MDA-MB-231 BC cells following high dose irradiation (Bravatà et al. 2015; Minafra et al. 2015). The literature data report that under in vitro conditions, cells develop a full SASP phenotype at least 5 days after senescence induction and also that cell growth arrest occurs within 24 h (Coppé et al. 2010; Raghuram and Mishra 2014). Interestingly, the amount of SASP molecules secreted by the breast cell lines used in our experiments does not correlate with the induction of the SASP phenotype. Indeed, despite the low levels of IL-6, IL-8 and MCP-1, the MCF7 cells displayed a senescent phenotype (Figure 4). In contrast, 9 Gy treated MCF10A cells, able to secrete significantly higher levels of these cytokines compared with untreated control cells, do not become senescent. In addition, despite the high levels of SASP molecules produced by MCF10A and MDA-MB-231 RCF cells, we did not observe senescence traits from 7 to 21 days post-irradiation.

These observations suggest that, despite the high amounts of SASP molecules that could be released in the tumor microenvironment, additional mechanisms are probably needed to induce the senescent process.

A final remarkable consideration on our results concerns the observation that the dose-effect affects the cell killing efficiency rather than the cytokine signature. These findings, as a whole may broaden our current understanding of BC high radiation response after IR dose treatments, with significant implications on the clinical application of high radiation doses.

Conclusion

IR activates complex cross-linked intracellular pathways able to regulate inflammation, DNA repair, and cell fate, as also recently described by our group (Bravatà et al. 2015; Di Maggio et al. 2015; Minafra et al. 2014, 2015; Pucci et al. 2015). Here, we described the cytokine signatures released by human MCF10A mammary epithelial cell lines, MCF7 and MDA-MB-231 BC cell lines after single high radiation doses of 9 Gy and 23 Gy. Our results reveal that cytokine production is time-dependent, high dose-independent and cell line-dependent. To our knowledge, this is the first study evaluating, in such a descriptive way, the cell specific cytokine profile of phenotypically different breast cell lines in response to high IR doses. The results here described open the possibility to modulate the anticancer IR personalized therapy on the basis of cytokine signature analysis of candidate BC patients. Further studies are necessary for a better comprehension of detrimental or beneficial effects of peri-tumoral microenvironment modifications induced by different modalities of IR treatments.

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Disclosure statement

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Notes on contributors

All authors participated in the conception, design, interpretation, and elaboration of the findings of the study, as well as in drafting and revising the final version. In particular, V.B. designed the study. G.R. studied the irradiation setup, simulations and dose distribution of radiation treatments. G.R., V.B. and L.M. performed IR cell treatments. L.M. maintained cell cultures and carried out clonogenic survival assay, morphological evaluation and irradiated conditioned media collection. V.B. performed cytokine, chemokine and growth factor analysis and helped by F.M.M. performed ELISA tests. V.B. wrote the article and helped by L.M., G.I.F. and F.P.C., conducted data interpretation. M.C. performed senescence assays. D.L. was involved in the interpretation of the findings, drafting and revising of the manuscript final version. M.C.G. participated in the elaboration of the findings of the study, financial support, drafting and revising the final version. All authors read and approved the final content of the manuscript. V.B. was a PhD student of the Pathobiology PhD course at Palermo University, Italy, and this work was submitted in partial fulfillment of the requirement for her PhD degree.

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