A novel *in vitro* macrophage senescence model to study MacrophAging

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Todos deseamos llegar a viejos, y todos negamos que hayamos llegado.

Quevedo

A mi familia

INDEX

Abstract

Macrophages play a key role in the immune response destroying pathogens directly or releasing mediators which can activate other cells. However, macrophages from aged mice present defects in their functional activities due to the aging process that alter the immune response. Cellular senescence is characterized by a permanent cell cycle arrest and is produced after continuous duplication/reproduction of a cell. The accumulation of senescent cells seems to be involved in and is responsible for the induction of aging. In this regard, due to the difficulty of working with old mice as a source of aged macrophages, in the present study we have considered whether the functions of long-lasting cultures of macrophages from young mice resembles to the functions found in senescent cells responsible for the aging patterns previously described. For this purpose, we have compared a normal macrophage culture (0 days macrophages) and long-period macrophage culture (14 days macrophages) of bone-marrow derived macrophages from 6-weeks old Balb/c mice.

Macrophages from 14 days cultures were positive for senescence markers such as telomere shortening and high β-galactosidase activity. Macrophage senescence was correlated with a reduced proliferation in response to specific growth factors (M-CSF and GM-CSF) due to an increase of cells in G1 phase of the cell cycle. In addition, long-lasting macrophage cultures showed a great number of altered macrophage functions such as antigen presentation and microbicidal reduced capacities and defects in alternative activation, indicating that both pro-inflammatory and anti-inflammatory phenotypes were impaired in these cells which correlate with that observed in macrophages from aged mice, as it was previously demonstrated in our laboratory.

All these data suggest that cellular senescence induced in long-lasting macrophage cultures is a good tool to evaluate the altered macrophage functions and characteristics during the aging process.

Abbreviations

Act.D	Actinomycin D
APS	Ammonium persulfate
Arg-1	Arginase-1
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
AU	arbitrary units
BMDM	Bone marrow-derived macrophages
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Cdk	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor
cDNA	Complementary DNA
CLR	C-type lectin receptor
COPD	Chronic Obstructive pulmonary disease
Cyc.	Cyclin
Cyt.D	Cytochalasin D
DCF-DA	Diclhorfluorescein diacetate
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dymethilsulfoxide
dNTPs	Deoxyribonucleotide triphosphate
dsRNA	Double-stranded RNA
DSS	Dextran sodium sulfate
DTT	D-(L)-dithiotheritol
EDTA	Ethylendiamine tetraacetic acid
EGTA	Ethylen glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fizz1	Found in inflammatory zone
HDACi	Histone deacetylase inhibitor
IBD	Inflammatory bowel disease

IFN-γ	Interferon gamma
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
M1	Classical activated macrophage
M2	Alternatively activated macrophage
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage-colony stimulating factor
Mgl1	Macrophage galactose-type lectin 1
MHC II	Major histocompatibility complex type II
MMP	Metalloproteinase
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NLR	Nucleotide-oligomerization domain(NOD)-like receptor
NOS2	Inducible nitric oxide synthase
OIS	Oncogene-induced senescence
PCR	Polymerase chain reaction
PI	Propidium iodide
PMSF	Phenylmethanesulfonyl fluoride
PPARɣ	Peroxisome proliferator-activated receptor
PRR	Pathogen recognition receptor
qRT-PCR	Quantitative real time PCR
Rb	Retinoblastoma
RLR	Retinoic acid-inducible gene I (RIG)-like receptor
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SA-β-gal	Senescence-associated β galactosidase
SDS	Sodium dodecilsulfate
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
TA-65	Telomerase activator 65

TCA	Tricarboxylic acid
TEMED	N, N, N', N'-tetramethylethylendiamine
TGFβ	Transforming growth factor beta
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet light
Ym1/2	Chitinase-like molecules

Introduction

1. Macrophages in the innate immune system

The innate immune system is an evolutionarily conserved system acting as a first-line of defense against invading microbial pathogens and other potential threats to the host (Rescigno, 2009; Clarke et al., 2010; Hand and Belkaid, 2010). The mucosal surfaces of mammals are in constant interaction with a multitude of commensal and pathogenic microorganisms that include bacteria, viruses, parasites, fungi and protozoa, constituting a diverse microbial ecosystem (Xu and Gordon, 2003). Therefore, the innate immune system has evolved to rapidly detect and respond to harmful stress situations and infectious agents (Kanneganti et al., 2007). This is coordinated by myeloid sentinels, mainly macrophages and dendritic cells, which express a limited number of evolutionarily conserved germline-encoded pattern-recognition receptors (PRRs) (Bauernfeind and Hornung, 2013).

PRRs recognize conserved microbe-specific molecules such as specific nucleic acid structures of microorganisms, microbial cell wall components (lipopolysaccharide, LPS) or lipoproteins and structural proteins for microbial mobility (flagellin) (Franchi et al., 2012). Moreover, PRRs also detects a set of host-derived molecules released from the plasma membrane, nucleus, mitochondria, endoplasmic reticulum and cytosol as ATP or urate crystals (Kobayashi et al., 2010). These molecules are secreted upon “danger” signals, such as cellular stress, damage or non-physiological death and modulate the immune system playing important roles in inflammation and tissue repair processes during infection (Krysko et al., 2011).

PRRs can be classified according to their ligand specificity, function, localization and/or evolutionary relationships. The main families of PRRs include the Toll-like receptors (TLRs), the nucleotide oligomerization domain(NOD)-like receptors (NLRs) and the C-type lectin receptors (CLRs) (Kawai and Akira, 2006). TLRs and CLRs recognize mainly extracellular and endosomal microbial signals and facilitate signal transduction across cell membranes, leading to pro-inflammatory gene expression through the transcription factor NF-kB (Akira and Takeda, 2004; Pizarro-Cerda and Cossart, 2006). If the bacteria invade the cell, cytosolic PRRs mainly those belonging to the NLRs family aid in amplifying pro-inflammatory responses (Strober et al., 2006; Mariathasan and Monack, 2007). Recently, other PRR families that appear to guard the intracellular environment have emerged. These include the RIG-I-like receptor (RLR) and the HIN200 families (Takeuchi and Akira, 2010; Lamkanfi and Dixit, 2012).

Then, microorganisms modulate the innate immune system through the activation of multiple PRRs together or sequentially (Franchi et al., 2012). In the inflamed tissue, the recognition of microbial agents by macrophage PRRs leads to a multitude of transcriptional and posttranslational modifications that result in pro-inflammatory cytokines, chemokines and growth factors being secreted into circulation in a high coordinated manner. These factors induce the migration of polymorphonuclear leukocytes and professional phagocytes to the site of infection or injury, to rapidly eliminate the pathogen and repair the damage (Lamkanfi and Dixit, 2012).

In this sense, alterations in the innate immune system regulation due to disorders in immune responses can lead to chronic inflammation, tumorigenesis, tissue destruction and autoimmune diseases (Coussens and Werb, 2010; Elinav et al., 2013).

1.1 Macrophage origin and differentiation

In this context, the mononuclear phagocyte system which embraces a family of cells that includes committed precursors in the bone marrow, circulating blood monocytes and tissue macrophages and dendritic cells has acquired great importance in the study of different pathologies in the last years (Hume, 2008). Cells of the mononuclear phagocyte lineage progress through a series of defined morphologically distinct stages; a common myeloid progenitor shared with granulocytes giving rise to monoblasts, promonocytes, and thence monocytes, which migrate to the tissues (Takahashi, 2000).

Metchnikoff originally identified and characterized macrophages more than 100 years ago, and his phagocytosis report won him the Nobel Prize for Medicine in 1908. The growth and differentiation of macrophages is produced into tissues and depend on lineage-determining cytokines, such as macrophage colony-stimulating factor (M-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), and is defined by interactions with stroma in haematopoietic organs (Celada and Nathan, 1994). Interleukin-3 (IL-3), KIT, tumour-necrosis factor (TNF)-family proteins and TNF-receptor-related molecules contribute to macrophage determination, as do key transcription factors such as PU.1 (Gordon, 2003; Hume, 2008).

A common progenitor gives rise to tissue macrophages, myeloid dendritic cells (DCs) and osteoclasts, which are distinct, irreversibly differentiated sublineages (Lech et al., 2012). Monocytes are able to leave the bone marrow into the bloodstream and, once

they are constitutively distributed, enter into all tissue compartments of the body. These migration processes (Figure 1) are produced through the interaction between adhesion molecules at the surface of monocytes and vascular endothelial cells (Valledor et al., 1998). Adhesion molecules control cell migration from the blood and through endothelia, the interstitium and epithelia. These include integrins (β1, β2 and others), immunoglobulin-superfamily molecules (such as CD31), selectins and epidermal growth factor seven-transmembrane spanning (EGF-TM7)-type receptors related to the F4/80 (EMR1) antigen (Lech et al., 2012). Cytokines such as transforming growth factor-β, chemokines and growth factors, often bound to local proteoglycans, influence the expression of a range of macrophage genes (Hume, 2008). During monocyte migration into tissues, monocyte maturation ends and then they undergo differentiation to become multifunctional tissue macrophages. In this sense, monocytes are considered to be immature macrophages (Gordon, 2003). Resident macrophage populations (Figure 1) in different organs, such as Kupffer cells (liver), alveolar macrophages (lung) and microglia (central nervous system, CNS), adapt to their local microenvironment since they are required to perform particular functions. The signals that are responsible for tissue-specific phenotypes of macrophages include surface and secretory products of neighbouring cells, and extracellular matrix (Gordon, 2003). Moreover, it is possible that monocyte-derived tissue macrophages can re-enter the bloodstream and differentiate into dendritic cells, depending on local stimuli such as phagocytosis (Gordon, 2003).

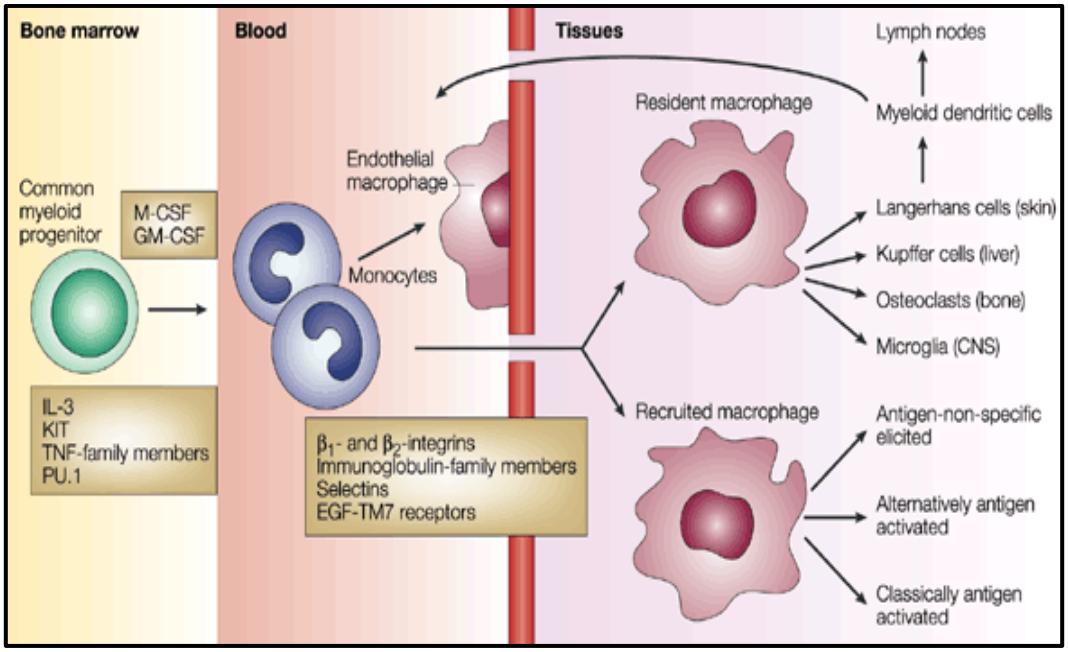


Figure 1. Macrophage differentiation process. IL, interleukin; M-CSF, macrophage colony-stimulating factor from macrophages, GM-CSF, granulocyte-macrophage colony-stimulating factor. Adapted from Gordon, 2003.

Tissue-resident macrophages cease to proliferate, but they undergo local activation in response to various inflammatory and immune stimuli (Hume, 2008). The enhanced recruitment of monocytes and precursors from bone-marrow pools results in the accumulation of tissue macrophages that have enhanced turnover and an altered phenotype. These macrophages are classified as being 'elicited', as in the antigen-non-specific response to a foreign body or sterile inflammatory agent, or as being 'classically activated' or 'alternatively activated' by an antigen-specific immune response (Gordon, 2003) (see point 1.2). Nevertheless, it is difficult to distinguish originally resident macrophages from more recently recruited, elicited or activated macrophages, because macrophages adapt to a particular microenvironment.

Thus, macrophages in a steady state or during an inflammatory process are a crucial part of the host defense fighting pathogens during an infection and, once resolved, in wound healing processes. But they also can be harmful, causing or exacerbating inflammatory diseases such as osteomyelitis, atherosclerosis and multiple sclerosis. All these indicate that these cells have a high phenotypical plasticity according to the environment conditions (Biswas and Mantovani, 2010).

1.2 Macrophage sutypes

Macrophages are a heterogeneous population of innate myeloid cells with diverse functionality due to different transcriptional profiles. Their main function is to recognize pathogens and orchestrate a signaling response in order to modulate the adaptive immune response through antigen processing and presentation. These cells are also key regulators in processes centered on generation and resolution of inflammation, as well as tissue repair (Mosser and Edwards, 2008). In addition, macrophages are required to maintain homeostasis which involves phagocytosis of debris and pathogens, dead cell clearance and matrix turnover processes (Steinman and Idoyaga, 2010).

The multifunctionality of macrophages is associated to the type of macrophage receptor involved or activated and the presence of determined cytokines in the microenviroment (Taylor et al., 2005). Analogous to the T helper type 1 (Th1) and T helper type 2 (Th2) polarization, macrophages have been defined in two different states of polarized activation: the classically activated (M1) macrophage phenotype

and the alternatively activated (M2) macrophage phenotype (Figure 2) (Mantovani et al., 2002; Gordon and Taylor, 2005).

Classical activation provides to macrophages their characterization as effector cells in Th1 cellular immune responses. The recognition of LPS and the signaling pathways induced by IFN-γ, a Th1 type cytokine, polarize macrophages towards the pro-inflammatory phenotype (M1 phenotype) which induces the production of large amounts of pro-inflammatory cytokines such as TNF-α, IL-12 and IL-23 (Figure 2). The secretion of this kind of cytokines supports the induction of adaptive immune responses based on Th1 and Th17 cell inflammatory processes. Furthermore, the antimicrobial functions of M1 macrophages are associated to up-regulation of enzymes, such as inducible nitric oxide synthase (NOS2) that generates nitric oxide (NO) (MacMicking et al., 1997).

In contrast, the alternative activation of macrophages seems to be involved in immunosuppression and tissue repair. The signaling pathway induced by Th2 type cytokines, such as IL-4, produces an anti-inflammatory macrophage phenotype (M2 phenotype) which drives to the secretion of high levels of IL-10 and IL-1RA and low expression of pro-inflammatory cytokines (Hao et al., 2012). M2 macrophages act as immunoregulators exerting diverse functions that favor elimination of parasites, reduction of inflammation, promotion of tissue remodeling and proliferation (Hao et al., 2012).

M2 macrophages can be further divided into different subsets (Figure 2): M2a, M2b, and M2c based on gene expression profiles (Mantovani et al., 2004). The M2a subtype is produced by IL-4 or IL-13. The M2b is elicited by IL-1R ligands or exposure to immune complexes plus LPS. The M2c subtype is generated by IL-10, TGF-β and glucocorticoid hormones (Hao et al., 2012).

In addition, it has also been described a fourth type of M2 macrophage, the M2d macrophage phenotype. This subset of macrophages is characterized by an IL-10^{high} and IL-12^{low}, as normal M2 profiles, but with some features of tumor-associated macrophages (TAMs) (Duluc et al., 2007).

As it has been mentioned before, M-CSF and GM-CSF cytokines are implicated in the differentiation of monocytes to macrophages. However, GM-CSF can also polarize monocytes to M1 macrophage subtype with a pro-inflammatory cytokine profile (e.g. IL-23), and treatment with M-CSF produces an anti-inflammatory cytokine profile (e.g. IL-10) similar to M2 macrophages (Jaguin et al., 2013).

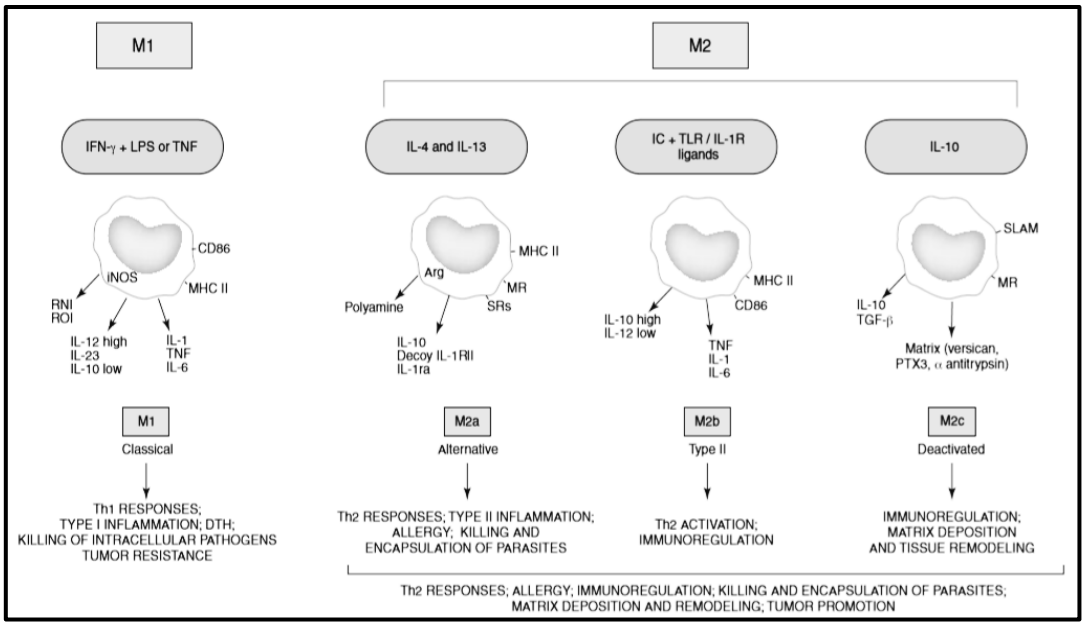


Figure 2. Macrophage subsets. The proposed M1-M2 macrophage model includes M1 phenotype induced by IFN-γ, LPS or TNF-α and M2 phenotype subdivided to define similarities and differences between IL-4 (M2a), immune complex + TLRs ligands (M2b), and IL-10 and glucocorticoids (M2c). Adapted from Martinez and Gordon, 2014.

Interestingly, although both phenotypes exhibit many distinct characteristics, they are involved in arginine metabolism through distinct biochemical pathways to produce their definitive features. Th1-type cytokines or conserved microbial structures induce macrophage NOS2, which oxidized arginine into OH-arginine and then into NO. In contrast, Th2-type cytokines induces the expression and activation of arginase-1 which metabolizes arginine into urea and ornithine, which is then subsequently converted into proline and polyamines (putrescine, spermidine, and spermine). Proline mediates the production of collagen, while polyamines induce cell proliferation. Therefore, macrophage activation requires high concentrations of arginine either for the microbicidal activity required to eliminate pathogens or for the restoration of the tissue and the extracellular matrix (Classen, 2009). In this sense, the involvement of different pathways sharing a common substrate suggests that macrophages present plasticity between these phenotypes.

In the last years, several works have explored the expression of gene markers that could characterize M1 and M2 macrophage populations. In this sense, several markers have been related with M1 phenotype such as TNF-α, IL-1β and IL-6 (Loke et al., 2002; Martinez et al., 2008). In the case of alternative activation, although there are several controversies, it seems clear that at least in mice, besides arginase-1 (*arg1*), other proteins are strongly associated with the M2 phenotype, including mannose receptor

(MR) encoded by *mrc1* gene, galactose receptor (GR) encoded by *mgl1* gene, “resistin-like” (Relm; also referred as “found in inflammatory zone” (*fizz1*)), transforming growth factor β (*tgfβ*), chitinase-like molecules (also called *ym1/2*) and the nuclear associated peroxisome proliferator-activated receptor γ (*pparγ*). The vast majority of these M1 and M2 markers are highly regulated at transcriptional level during macrophage polarization (Mosser and Edwards, 2008; Gordon and Martinez, 2010).

Many works have been reported regarding to the study of M1/M2 regulation. They found different and essential signaling pathways and transcription factors upstream of the expression of M1 and M2 related genes. Although it has been established that STAT1 and STAT6 plays a crucial role in the IFN-γ and IL-4 signaling, respectively (Classen, 2009), several publications have showed the role of certain transcription factors to generate any of both macrophages activation phenotypes. This is the case of NF-κB; NF-κB subunit p50 has been associated with the alternative activation of macrophages (Porta et al., 2009), whereas NF-κB subunit p65 is clearly involved in the signaling pathway required for the classical phenotype (Ray et al., 2010).

This classification of macrophage subsets is based on the establishment of differences and similarities in macrophage responses to diverse stimuli. In this sense, the main limitations of the current view are, first, it ignores the source and context of the stimuli, second, the M1 and M2 stimuli do not exist alone in tissues and third, macrophages may not form clear-cut activation subsets nor expand clonally. In addition, updated knowledge of cytokine signaling, the role of cytokines in the development of the hematopoietic system and transcriptomic and proteomic analysis reveal a far more complex picture (Martinez and Gordon, 2014).

1.3 Inflammatory response

Inflammation is a tightly regulated process initiated after tissue injury or infection. The inflammatory process has as main function restore homeostasis eliminating the aggression and removing the injured tissue (Allen et al., 2010; Dupaul-Chicocine et al., 2010; Zaki et al., 2010). In this sense, the coordinated feat of professional phagocytes (neutrophils, monocytes and macrophages) is critical.

The phagocytic activity and microbicidal capacity of macrophages are crucial to achieve the eradication of the pathogenic agent. In normal physiological conditions, macrophages are resident phagocytic cells involved in tissue homeostasis, through the

clearance of apoptotic cells and the secretion of growth factors (Geissmann et al., 2010). However, in a situation of microbial invasion macrophages activities go far beyond eating and killing, they also regulate innate and adaptive immunity producing several cytokines and chemokines and present antigens to activate and differentiate T cells linking the innate with adaptive immunity (Gordon, 2003).

Once the aggression occurs, during the early stages of the inflammatory response (Figure 3), macrophages are activated classically inducing pro-inflammatory M1 phenotype, as it has been mentioned before (see point 1.2). In this phase, M1 macrophages produce diverse mediators, such as cytokines, NO and ROS that destroy pathogens combined with an increased expression of major histocompatibility complex (MHC) class II and CD86 enhancing their antigen-presenting capacity to T lymphocytes (Gordon, 2003). The persistence of inflammation often results in tissue damage and the immune system permanently is challenged for developing anti-inflammatory mechanisms (Martinez and Gordon, 2014).

When the pro-inflammatory stimulus is removed from the inflamed tissue, macrophages are alternatively activated, M2 phenotype, by Th2-type cytokines (Figure 3), such as IL-4 or IL-13, and initiate anti-inflammatory processes to reconstruct the damaged tissue with the removal of apoptotic cells, production of collagen, etc., (Classen et al., 2009). Moreover, M2 macrophages exhibit enhanced phagocytic activity and increased expression of MHC class II molecules for antigen presentation (Martinez and Gordon, 2014). Therefore, anti-inflammatory macrophages protect organs and surrounding tissues against detrimental immune responses promoting tissue remodeling.

Several *in vitro* studies have shown macrophages adaptation to a changing microenvironment (Stout and Suttles, 2004). It has been shown that macrophages *in vitro* are capable of complete repolarization from M2 to M1, and change again in response to fluctuations in the cytokine environment (Davis et al., 2013). However, there are contradictory opinions about this fact because it is not known what extent these distinct activation states exist *in vivo* and whether macrophage phenotype is determined forever or whether it remains constantly malleable (Gordon and Taylor, 2005). Ma and co-workers demonstrated that M2 macrophages presented within the inflammatory tissue were not recruited from the blood, they were derived from M1 macrophages (Ma et al., 2011). Then, after M1 activation of macrophages they can become to M2 phenotype in the inflammatory locus. This conversion was called “macrophage switch” or “switching” and occurs rapidly at the level of gene expression,

protein, metabolite and microbicidal activity (Kuroda et al., 2009; Davis et al., 2013). In addition, it has been hypothesized that phagocytosis could be involved in the switch from M1 to M2 or from inflammatory to anti-inflammatory macrophages (Arnold et al. 2007). In this sense, phagocytosis drives to the final steps of the pro-inflammatory phase eliminating the remaining microorganisms and also participates in the tissue repair removing apoptotic and senescent cells (Valledor et al., 2010). However, due to the variety and complexity of phagocytosis processes, further studies are required to clarify the role of phagocytosis in the macrophage switch.

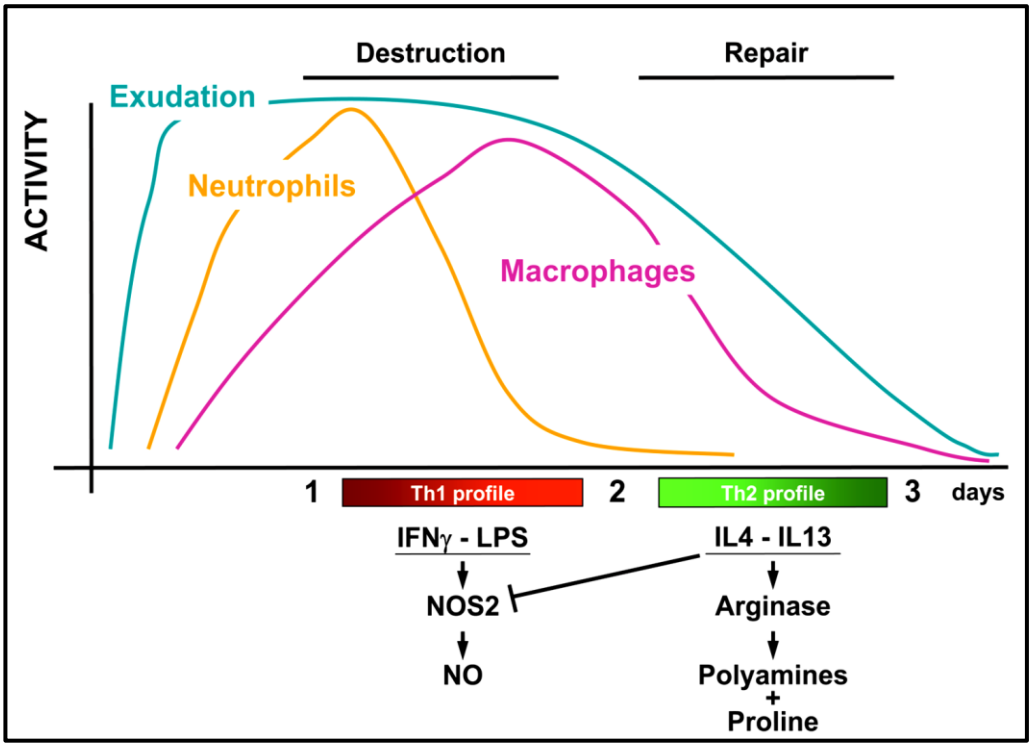


Figure 3. Schematic overview of the inflammatory response process. After the initial homeostasis phase, neutrophils and M1 macrophages dominate the inflammation phase of the inflammatory response destroying pathogens. Once the microbial agent is eliminated, macrophages are polarized to M2 phenotype in order to repair injured tissues.

1.4 Other macrophage functions

Due to the key role played by macrophages in the immune system we generally forget that these cells are also central in many other biological functions. In this sense,

macrophages participate in development, bone resorption and iron and cholesterol metabolisms (Gordon et al., 2014).

The importance of macrophages in development is emphasized by the numerous systems affected by macrophage depletion in the M-CSF-deficient mouse, including somatic growth, development of the pancreas and nervous system, and male and female fertility (Pollard, 2009; Gow et al., 2010). As an example, Langerhans cells (epidermal macrophages) contribute to the control of proliferation and differentiation of keratinocytes (Lau et al., 2008).

Another critical macrophage-related cell is the osteoclast. Osteoclasts are multinucleated giant cells formed in bones by fusion of monocyte-like cells in the presence of M-CSF (Helming and Gordon, 2007). Collaborating with osteoblasts, these cells participate in bone production and remodeling. However, the main function of osteoclasts is to resorb living bone, by integrin-dependent adhesion to the bone surface. Moreover, they are able to regulate the differentiation of osteoblast precursors and the movement of hematopoietic stem cells from the bone marrow to the bloodstream (Takayanagi, 2007).

In addition, stromal macrophages have been implicated in the metabolism of iron (Hanspal and Hanspal, 1994; Sadahira, et al.,, 1995; Spring et al., 2013). These cells express EbR, which promotes adhesion of erythroblasts and myeloblasts (Morris et al., 1991) and CD163, involved in iron recycling from heme, after uptake of erythrocytes (Kristiansen et al., 2001).

Finally, macrophages are also involved in the uptake of modified lipoproteins and cholesterol by scavenger receptors such as CD36 and CD68 (Moore and Freeman, 2006). The internalization of these molecules results in foam cell formation and the polarization to M1 macrophages inducing an inflammatory response. Furthermore, these cells alter the expression of genes involved in lipid metabolism, secretion of pro-coagulants and metalloproteinases (Libby, 2013).

2. Cellular senescence

Cellular senescence is an irreversible program of cell cycle arrest triggered in normal somatic cells in response to a variety of intrinsic and extrinsic factors including telomere attrition, DNA damage, physiological stress and oncogene activation (Kuilman et al., 2008).

Cellular senescence was officially described more than five decades ago when Hayflick and co-workers demonstrated that diploid cells had a restricted capacity to multiply in culture (Hayflick and Moorhead, 1961). This fact was interpreted as an organismal protective mechanism from cancer, since cancer cells proliferate indefinitely *in vitro*, and as the origin of aging at organ and organismal levels, typified by the loss of regenerative capacity and functionality of tissues *in vivo*. In this sense, senescent cells have been observed in mammals in association with aging (Nussey et al., 2013).

These findings suggest that cellular senescence can be by one hand beneficial (tumor suppressive) and by the other hand deleterious (limit tissue restitution). Although some researchers assume that natural selection has favored genes providing advantages to the organism during the reproductive years, at the expenses of deterioration in the distant future (Kuilman et al., 2008), recently Blagosklonny and collaborators understand senescence process as the result of two opposing signals: cell growth and the blockage of cell cycle (Blagosklonny, 2013).

Thus, cellular senescence is a permanent condition in which normal cells are unable to proliferate further (Campisi and D'Adda di Fagagna, 2007). In contrast to cellular quiescence, senescence cannot be reversed by altering the cellular environment, by removing cell contact inhibition or providing abundant nutriments *in vitro* (Serrano, 2010). Furthermore, senescence is distinct from terminal differentiation, as it is not the end of a programmed differentiation process (Shay and Wright, 2005).

Senescent cells, even they stop proliferation and synthesize DNA, are viable almost indefinitely *in vitro*. They typically suffer dramatic morphological and functional changes and present an altered gene and protein expression profile (Serrano and Blasco, 2001; Narita et al., 2003). To characterize and detect senescent cells different biochemical assays have been developed such as senescence associated-β-galactosidase staining (SA-β-Gal) (Dimri et al. 1995; Shelton et al. 1999; Pascal et al. 2005), telomere dysfunction (Sebastián et al., 2009) and activation of DNA damage response (Takai et al., 2003), among others. However, these distinct features are not completely

connected with known senescent signaling pathways and, therefore, senescence mechanism requires further investigations to be entirely defined.

2.1 Morphological and molecular changes in senescence

Upon entering senescence, cells suffer dramatic modifications. Senescent cells acquire large volume, flattened cytoplasm morphology accompanied by changes in nuclear structure, gene expression, protein processing and metabolism (Campisi et al., 1996; Campisi, 1997; Chen, 2000). These alterations are also observed during the process of aging *in vivo* (Dimri et al., 1995; Campisi, 2000; Narita et al., 2003).

Most of the mechanistic studies of senescence have been focused on the irreversible growth arrest (Parajuli et al., 2014; Renna et al., 2014; Zhang et al., 2015). However, the cause of cell enlargement has not been well studied. Some works link senescent morphogenesis with cell cycle arrest since it is believed that both phenomena are connected (Kuilman et al., 2010). In this sense, senescent morphogenesis could be a programmed event involving cell cycle checkpoint proteins which control the morphological changes, such as p53 and Rb whose hyperphosphorylation abrogate the cell cycle and is also linked with senescence morphology (Chen et al., 1998).

A large number of morphological studies focus on cytoskeletal proteins, mainly in actin polymerization (Hou et al., 2013; Biran et al., 2015). In this sense, it has been demonstrated that senescent cells although present similar protein levels of actin they have an increased rate of actin polymerization or decreased rate of actin filament depolymerization (Chen et al., 2000), necessary to maintain the enlarged cells. However, the involvement of cell cycle checkpoint proteins is not clear. The inactivation of Rb is necessary but not sufficient for senescence morphogenesis, supporting that alteration in senescent morphology is not simply a consequence of inhibition of cell proliferation (Chen et al., 2000).

Changes in cell morphology and adhesion are important parameters of cancer metastasis and invasion (Button et al., 1995; Gumbiner, 1996). Tumor cells are often smaller and have less actin stress fibers compared to their normal counterparts (Otto, 1990; Button et al., 1995; Zigmond, 1996). Therefore, it is believed that senescent or senescent-like cells could contribute to tumor suppression at least by cell enlargement and changes in cytoskeletal proteins.

2.2 Factors inducing cellular senescence

The extensive sets of stimuli that can induce senescence indicate the complexity of the senescence process. The growth arrest is due in part to the gradual loss of DNA at the ends of chromosomes (telomeres shortening), produced in each cell division (Nakamura et al., 2008). Short telomeres originate a persistent DNA damage response (DDR), which initiates and maintains the senescence growth arrest (d'Adda di Fagagna, 2003; Takai et al., 2003; Herbig et al., 2004; Rodier et al., 2009; Rodier et al., 2011). In fact, DNA double strand breaks inducers, such as ROS, are particularly effective senescence inducers (DiLeonardo et al., 1994). Moreover, histone deacetylase inhibitors (HDACi), which relax chromatin, activate the DDR response triggering ATM and p53 proteins activation (Bakkenist and Kastan, 2003), and promoting senescence (Ogryzko et al., 1996; Munro et al., 2004), as it has been demonstrated by sodium butyrate, a well-known HDACi (Vargas et al., 2014). Finally, many normal cells become senescent after robust mitogenic signals produced by oncogenes (*ras*, *raf* and *c-myc*) or pro-proliferative genes (*E2F*) (Serrano et al., 1997; Lin et al., 1998; Zhu et al., 1998; Dimri et al., 2000; Davis, 2012) which enhance DDR by failed replication origins and replication fork collapse (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007).

However, senescence can also be caused without DDR signaling through "culture stress" (tissue culture plastic, serum, etc) not detected *in vivo* (Fusenig and Boukamp, 1998; Ramirez et al., 2001; Yaswen and Stampfer, 2002; Parrinello et al., 2003). In these circumstances cells induce pro-proliferative/pro-survival kinases (Alimonti et al., 2010) and increase the expression of the cyclin-dependent kinase inhibitors (CDKi), such as $p21^{WAF1}$ and $p16^{INK4a}$ that normally enforce the senescence growth arrest (Mc-Connell et al., 1998; Rodier et al., 2009).

2.2.1 Cellular senescence induced by telomere shortening

Historically, telomere shortening has been implicated as one of the major mechanisms of replicative senescence (Hayflick's limit) (d'Adda di Fagagna, 2008). Human chromosome telomere ends are special structures composed of stretches of repetitive tandem hexameric units-TTAGGG and associated telomeric proteins that cap the ends of linear chromosomes protecting them from degradation and fusion during DNA repair

process (Reaper et al., 2004) (Figure 4). The length of human telomeres ranges from a few to 10–15 kbs.

The term “end-replication problem” describes the inability of standard DNA polymerases to fully replicate the ends of the chromosomes which are consequently shortened at each mitotic cycle (Campisi and D’Adda di Fagagna, 2007). This causes the estimated loss of around 50 to 200 base pairs during each cycle of replication at the 5′ end of the lagging strand. Therefore, when the length of one or more telomeres gets below a certain threshold (1-4 kbs), the exposed telomeric DNA ends are recognized as double-strand breaks (DSBs) by the DNA damage response (DDR) mechanism (explained in section 2.2.2) driving to senescence.

Although telomere shortening might not be primarily involved in the acute induction of senescence (Shay and Wright, 2005), together with the accumulation of oxidative stress (see section 2.2.2) might increase the possibility of a cell to enter in cellular senescence. It has been reported that replicative telomere attrition with high levels of DNA damage can result in an increase of senescent cells in different tissues and organs ultimately resulting in decreased function and pathology and causing organismal aging (Von Zglinicki et al., 2005).

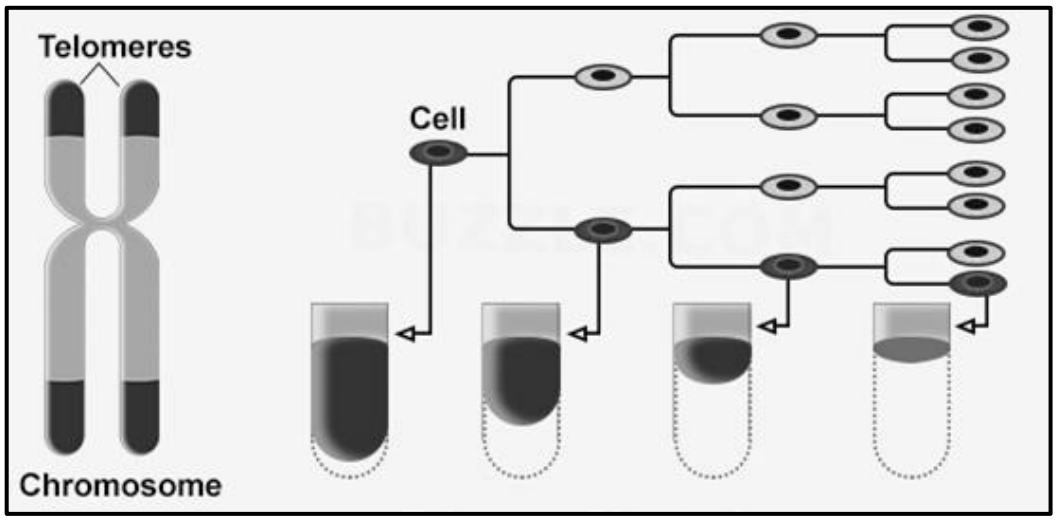


Figure 4. Telomere attrition. Size of telomeres is shortened in each cell division until a critically telomere length that induces senescence.

The discovery of the reverse transcriptase telomerase revolutionized the telomere attrition research. In contrast to DNA-polymerase, telomerase is capable of elongating

the telomeres. In this sense, telomerase deficient mouse has inactive telomerase and consequently reduced telomere length in each following generation causing premature aging (Di Leonardo et al., 1994).

Some works try to link telomere length with lifespan, although this hypothesis is still not clear. It has been estimated that females have longer telomeres than men and in African Americans telomeres generally are longer than in White Americans (Rodier et al., 2011). In addition, mice strains with longer telomeres do not seem to have an increased lifespan compared to mice strains with shorter telomeres. How these variations in telomere size alter lifespan are still unknown, although the most putative premise is that the shortest telomeres are contributing most to the expected lifespan (Robles and Adami, 1998).

2.2.2 Cellular senescence induced by DNA damage

The human genome is constantly exposed to genotoxic stress, such as radiation (X or γ-rays, UV) and DNA-interacting drugs (bleomycin, doxorubicin, mitomycin, etoposide) and oxidative stress (Robles and Adami, 1998; Touissant et al., 2002; Parrinello et al., 2003; Bavik et al., 2006). Oxidative stress results in formation/accumulation of reactive oxygen species (ROS) (Chen et al., 1998; Reina-San-Martin et al., 2003; Blander et al., 2003). The generation of ROS and free radicals is considered as one of the mechanisms of aging, affecting DNA and other cellular constituents and activating the transcription of inflammatory genes (Langen et al., 2003; Ramsey and Sharpless, 2006). According to some data, oxygen radicals can also directly participate in the induction of senescence (Serrano et al., 2007).

These diverse factors can cause DNA double-strand breaks (DSBs), the most dangerous type of DNA damage, with a similar structure to short or dysfunctional telomeres (Von Zglinicki et al., 2005). DSBs are associated with physiological processes as a consequence of metabolism (Takahashi and Ohnishi, 2006).

To guarantee the integrity of the genome, cells activate a refined protective system that stops cell cycle to allow the repair of the damage. It was demonstrated that the Ser139 phosphorylation of histone H2A, called H2AX, is immediately induced by DSBs (Rogakou et al., 1998). The phosphorylated form of H2AX is referred to as γ-H2AX (Rogakou et al., 1998; Helt et al., 2005) which stops cell cycle progression and activates DNA repair (Rogakou et al., 1998; Kastan and Lim, 2000; Bakkenist and

Kastan, 2003). Thus, H2AX phosphorylation is a useful marker for DSBs detection (Rogakou et al., 1999).

The first phase of the repair process is the detection of DNA damage through Mre11/Rad50/NBS1 (MRN) complex whose assembly is induced by H2AX phosphorylation and functions as a DNA damage sensor (Matsuda et al., 2013). This important complex is localized in nuclear foci around the breaks stabilizing the structure and prepares it for potential repair (Zhao et al., 2008). In this sense, H2AX-knockout cells present impaired recruitment in DNA repair foci of NBS1 protein of the MRN complex and 53BP1 and BRCA1 (mediator proteins of DNA damage response) (Celeste et al., 2003) which consequently produces defects in DNA damage repair and chromosome abnormalities (Petersen et al., 2001; Bassing et al., 2002; Reina-San-Martin et al., 2003, Xie et al., 2004; Kao et al., 2006).

One of the most critical kinase-activating cell cycle checkpoints following DNA damage is ATM (ataxia-telangiectasia mutated) (Ostan et al., 2008). ATM kinase is considered as a major physiological mediator of H2AX phosphorylation in response to DSB formation (Kastan and Lim, 2000; Bakkenist and Kastan, 2003). In addition, the MRN complex is also involved in the recruitment and activation of ATM and ATR (ATM and Rad3related) protein kinases, which in turn activate checkpoint-1/2 (CHK1/2) kinases leading to phosphorylation and thereby stabilization of a variety of target genes including p53 (Ou et al., 2005).

When DNA injuries are repairable, cells temporary stop proliferation and repair the damage through the mechanism described above (Rodier et al., 2011). In contrast, severe or irreparable DNA damage, such as complex breaks or uncapped telomeres, causes many cells to senesce or die with persistent DNA damage foci, constitutive DDR signaling and chronic p53 activation. These persistent changes precede establishment of senescence-associated phenotypes, including growth arrest (Tchkonia et al., 2013) and senescence-associated secretoy phenotype (SASP) (Wu et al., 2015), or the well-known programmed cell death, apoptosis (Childs et al., 2014).

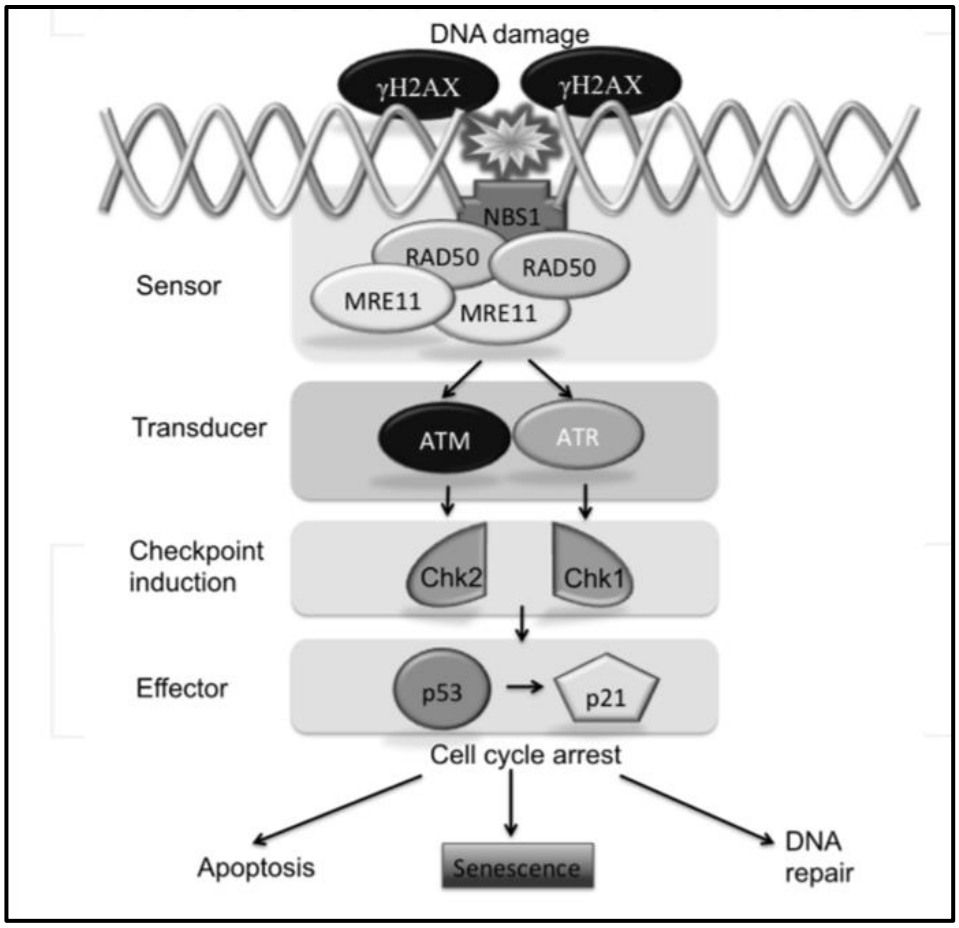


Figure 5. DNA damage response. The activation of H2AX triggers a signaling pathway initiated by the formation of the MRN complex. Then activated ATM and ATR kinases induce the activation of DNA damage response checkpoint driving to the phosphorylation of p53 and abrogating the cell cycle progression. Depending on the damage level and the circumstances of the cell, cells repair the damage, or become apoptotic or senescent. Adapted from Becker and Haferkamp., 2013.

2.2.3 Cellular senescence induced by oncogenes and hyper-proliferative signals

Oncogenes are mutant forms of normal genes that have the ability to transform cells stimulating uncontrolled cell division (Gorgoulis and Halazonetis, 2010). However, induction of senescence or apoptosis is the frequent outcome of oncogene activation in normal cells. In this sense, senescence induction must be directed to neutralize excessive activation of the cell cycle that drives oncogenic transformation (Campisi and D'Adda di Fagagna, 2007). Thus, oncogene-induced senescence (OIS) is a powerful

protective mechanism to elude tumor formation because avoids the proliferation of cells that overexpressed aggressive oncogenes.

The pathways mediating OIS are not totally clarified but, the irreversible cell cycle blockage involves activation of both Rb and p53 pathways (Braig and Schmitt, 2006) accompanied by the upregulation of the CDK inhibitors *p15*^{INK4B}, *p16*^{INK4A} and *p21* (Reddy and Li, 2011).

OIS can be triggered by *E2F1*, *c-myc* and *Ras* (Grandori et al., 2003; Lazzerini et al., 2005; Courtois-Cox et al., 2006). The increase of *Ras* was demonstrated to trigger cell division followed by growth arrest produced by the accumulation of p53 and p16^{INK4A} proteins and the phosphorylation of pRb (Serrano et al., 1997), suggesting resemblances to tumor suppressor mechanisms. The proof of OIS has been demonstrated *in vivo* in human and mouse tumor models (Braig et al. 2005; Chen et al. 2005; Collado et al. 2005; Michaloglou et al. 2005; Courtois-Cox et al. 2006; Dankort et al. 2007). Furthermore, mutations in *Ras* and *Raf* have been observed to induce cellular senescence *in vivo*. Senescence occurs in benign but not in advanced tumors, supporting the first *in vitro* observation that activation of these pathways lead to an initial burst of proliferation before causing cellular senescence (Courtois-Cox et al., 2008).

Additionally, similar to replicative senescence, oncogene-induced senescence has a causal link to reactive oxygen species (ROS) accumulation and can be abolished by culturing cells in the presence of antioxidants (Lee et al., 1999). Therefore, OIS appears to be engaged by a variety of intrinsic and extrinsic signals but is not clearly associated with telomere shortening (Hornsby, 2007). Independent of the mechanism, cells that have undergone OIS and replicative senescence share common features such as flat morphology, SA-β-Galactosidase activity and induction of cell cycle inhibitory proteins such as p53 and p16^{INK14} (Serrano et al., 1997; Ferbeyre et al., 2002).

2.3 The secretory phenotype of senescent cells

Cellular reprogramming carried out during senescence results in stable blockage of cell cycle progression and in different morphology and function, as it has been mentioned before. However, the senescent phenotype is not limited to these alterations since senescent cells are metabolically active. Therefore, it has been reported that culture

medium of senescent cells, with a persistent DNA damage, is enriched with secreted proteins including growth factors (GM-CSF, G-CSF), inflammatory cytokines (IL-1α, IL-1β, IL-6), chemokines (MCP-1, CXCL8) and extracellular proteases (MMP-3, MMP-10) (Shelton et al. 1999; Krtolica and Campisi 2002; Acosta et al. 2008; Coppé et al. 2008; Kuilman et al. 2008; Wajapeyee et al. 2008; Augert et al. 2009; Coppé et al., 2010). The specific secretory pattern of senescent cells, also demonstrated *in vivo*, has been denoted as "senescence associated secretory phenotype" (SASP) and entails changes in the released of >40 factors that exert a broad range of effects on the local tissue microenvironment (Coppé et al., 2008).

Senescence develops gradually from an initiation point (DDR) to entirely senescent phenotype (irreversible growth arrest). In this sense, transitional phases define the establishment of senescence, which are largely unidentified with respect to kinetics and central mechanisms, including the effects produced by SASP factors (Passos et al. 2010). The mechanisms that origin and sustain the SASP are uncertain. Nevertheless, some studies reported that components of the SASP, such as IL-6 and IL-8, can maintain the senescence cell cycle arrest via an auto-regulatory feedback mechanism- (Kuilman et al., 2008; Wajapeyee et al., 2008). Many of SASP factors can contribute to normal tissue repair. For example, it has been demonstrated that IL-6, IL-8, MMP, growth factors and proteases participate in wound healing, attractants for immune cells kill pathogens and stem or progenitor cells, communicating cellular damage/dysfunction to the surrounding tissue and if needed, stimulating repair (Coppé et al., 2008).

The SASP inflammatory cytokines are of particular interest because they can play a role in much age-related pathologies (Campisi et al., 2008). IL-1α and IL-β are minor SASP components which, compared to IL-6 and IL-8, are secreted at low levels (McCarthy et al., 2008). IL-1 (α/β forms) is a multifunctional cytokine that regulates inflammatory and immune responses mainly by originating a signal transduction cascade which lastly induces IL-6 and IL-8 expression (McCarthy et al., 2008).

The most important cytokine of the SASP is IL-6, a pleiotropic pro-inflammatory cytokine (Coppé et al., 2008). IL-6 secretion has been shown to increase markedly after DNA damage- and OIS senescence of mouse and human keratinocytes, melanocytes, monocytes, fibroblasts and epithelial cells (Kuilman et al., 2008). The senescence response, SASP in particular, may reduce health by stimulating both the inflammation associated with aging as well as the development of specific age-related diseases (Freund et al., 2010).

Interestingly, this secretory phenotype seems to act as a double edged sword regarding tumor initiation and maintenance. It has been shown that the SASP can have pro-tumorigenic effects; for instance, it has been shown that senescent mesenchymal cells increase the tumorigenicity of nearby breast cancer cells (Freund et al., 2010). Likewise, it has been proposed that loss of proliferative competition of senescent cells can allow progression of leukemic cells during aging (Bilousova et al., 2005). Additionally, abnormal secretion of cytokines and growth factors by the SASP can accelerate this process in aged and chronically injured organ systems. However, the SASP also presents antitumor effects. It has been reported that in liver cancer mouse model the activation of p53 induces senescence, an up-regulation of inflammatory cytokines and stimulation of innate immune responses causing tumor cell clearance (Xue et al., 2007).

2.4 Senescent cells biomarkers

Although senescence can be induced by a wide spectrum of factors, senescent cells present specific characteristics that allow their identification in both *in vitro* and *in vivo*. These biomarkers reflect changes in morphology, gene expression, functionality, metabolism and, definitely, cell division (Kuilman et al., 2010). However, in order to correctly identify senescent cells, cells under study have to present several of these biomarkers, being essential an irreversible growth arrest.

2.4.1 Cellular morphology

Cell senescence is commonly accompanied by remarkably morphological alterations (see point 2.1) which depend on the specific senescence-induced factor (Kuilman et al., 2010). These modifications can range from produce multi-nucleation, to become cells large, flat, refractile or even generate extensive vacuolization as a result of endoplasmic reticulum stress caused by the unfolded protein response (Denoyelle et al., 2006). For example, *Raf*-dependent senescence normally originates a more spindle-shaped morphology (Chan et al. 2005; Michaloglou et al. 2005). In contrast, cells senescing due to *Ras* overexpression, persistent DDR or stress become flat (Chen and Ames, 1994; Serrano et al., 1997; Chen et al., 2001; Parrinello et al., 2003; Denoyelle et al., 2006). In this sense, the comparison of cellular shapes between

growing cells with those suspected to be senescent can help to identify them as senescent cells.

2.4.2 Telomeres length

Telomere shortening has been associated with DNA damage-induced senescence process (see section 2.2). Telomere length test is a very easy measurement based on a specific controlled quantitative real time PCR (see point 8.4 from Material and Methods) that allow quantify the shortening of telomeres due to successive cell divisions and compare it with normal cells (Sebastián et al., 2009).

2.4.3 Senescence-associated β-galactosidase (SA-β-gal) activity

SA-β-gal activity is the most exploited marker in identifying senescent cells because of the simplicity of the assay method and its apparent specificity for senescent cells that can distinguish them from quiescent cells (Dimri et al., 1995). The technique is based on the observation that β-galactosidase activity measured at a suboptimal pH (pH 6.0) specifically stains senescent cells, while the same procedure carried on at pH 4.0 uniformly stains all type of culture cells *in vitro*. SA-β-gal activity has been also detected *in vivo* in organs of old individuals, suggesting that cellular senescence is an aging mechanism of organisms where senescent cells accumulate in tissues (Campisi and D'Adda di Fagagna, 2007). Although it is unclear the function of SA-β-gal in senescence, it has been reported that this activity is due to the increase in lysosomal content in senescent cells (Dimri et al., 1995).

2.4.4 Cell cycle progression

Withdraw from the cell cycle is the only indispensable marker for the identification of all types of cellular senescence both *in vitro* and *in vivo*. However, quiescence and terminal differentiations share this particularity. Thus, the irreversible growth arrest cannot be used in isolation to detect senescent cells.

Some studies have demonstrated that exist some conditions that allow escape from this irreversible state, such as dephosphorylation of p53 and the inactivation of some

interleukins (Beausejour et al. 2003; Dirac and Bernards 2003; Coppé et al. 2008; Kuilman et al. 2008). Although it is a rare event in cultured cells, it is very relevant *in vivo* for cancer progression (Vredeveld and Peeper, unpubl.). Additionally, it has been shown that the reversibility depends on the expression of $p16^{INK4A}$ prior to entering senescence (Beausejour et al., 2003).

2.4.5 DNA damage foci

The detection of the MRN complex (localized in DSBs which induces the signaling pathway of the DDR, see section 2.2), has been suggested to be a cellular senescence biomarker (Lee et al., 2006). Similarly, the phosphorylation of H2AX (γ-H2AX), produced by DSBs which promote the assembly of the MRN complex and the subsequent signaling, is also a useful marker for senescence (Ben-Porath and Weinberg, 2004).

2.4.6 Reactive oxygen species (ROS)

Although oxidative stress certainly plays a key role in senescence since senescent cells present high levels of ROS (see point 2.2), how it increases and what the cellular targets are remains unclear (Lu and Finkel 2008). However, ROS production is easily measured and represents an important marker for identifying senescent cells.

2.4.7 Apoptosis resistance

It has been demonstrated that several, but not all, senescent cells show resistance to apoptosis upon apoptotic conditions comparing with young cells (Hampel et al., 2005). However, the underlying mechanism is poorly understood due to the complexity of study this process induced for multiple apoptotic agents in cells of different functionalities.

2.5 Molecular mechanisms inducing cellular senescence

p53 and pRb molecular pathways have been widely studied to investigate senescence mechanisms confirming their relevance in this process (Figure 6).

p53 is a well-known protein important for the correct progression of the cell cycle since it maintains the genomic integrity. It has been demonstrated that mutations in the p53 gene or dysfunctional p53 protein result in an uncontrolled cell division (Vogelstein et al., 2000). Moreover, p53-deficient mice present spontaneous tumors and in practically 50% of all human cancer p53 does not work properly (Donehower et al., 1992; Donehower et al., 1995).

p53 is activated under stress signals and induces cell cycle arrest, apoptosis or senescence. Some evidences suggest that high p53 activity drive to organismal aging regulating genes by transcriptional activation or proteins by direct binding (Murphy, et al., 1999; Guimaraes and Hainaut 2002). In addition, p21 is involved in the p53 tumor suppressor activity. In this sense, p53 activation causes upregulation of p21 which in turns is able to inhibit the activity of CDK-cyclin complexes blocking the cell cycle at different stages and inducing senescence (Gartel et al., 1996; Colman et al., 2000; Taylor and Stark 2001).

pRb protein is ubiquitously expressed in all tissues and is regulated in a cell cycle-dependent manner acting as a general regulator of cell cycle (Cobrinik et al., 1992). Its main function is to bind negatively to the E2F family of transcription factors in G1/S phase of the cell cycle in order to block the cell cycle progression (Morris and Dyson, 2001). However, pRb is also critical for the senescence process. Although it has been difficult to obtain experimental data, it is believed that pRb is involved in the maintenance of the senescent state (Narita et al., 2003). pRb activity is also linked to $p16^{INK4}$ function (Pavel et al., 2003). $p16^{INK4}$ is a tumor suppressor whose main function consists in inactivate cyclin D-CDK complexes resulting in the inhibition of CDK4/CDK6 that then is unable to phosphorylate pRb (Haller et al., 2002). Moreover, $p16^{INK4}$ is considered as a senescence marker since is upregulated upon replicative senescence and is maintained at high level in senescent cells (Stein et al., 1999).

Although p53/p21 and p16/pRB signaling pathways can act synergistically to induce senescence, most senescent cells exhibit just one of these two mechanisms

(Magalhaes, 2004). However, they are not fully understood and downstream effectors are not completely indentified.

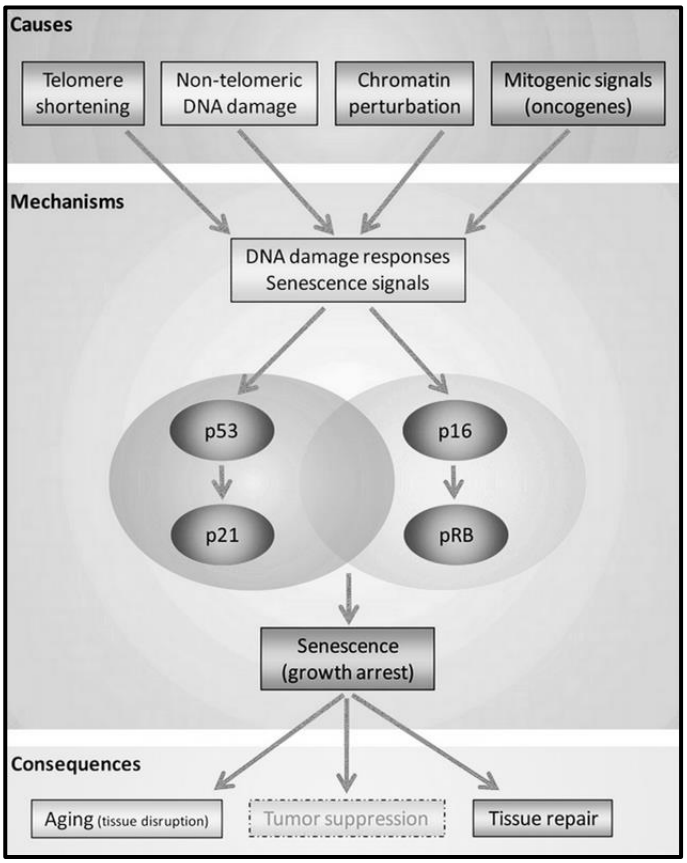


Figure 6. Cellular senescence mechanisms. Although various stressors can undergo cellular senescence, the signaling induced converge mainly to two different pathways: p53 and pRb whose activation provokes aging, tumor suppression and tissue repair. Adapted from Naesens, 2011.

3. Cellular senescence and its role in aging

Aging can be defined as the time-related deterioration of the physiological functions required for survival and fertility. The connection between aging and senescence processes has been studied intensively (Campisi et al., 2011; Schraml and Grillari, 2012). Actually, senescent cells have been observed in mammals in association with aging. In this sense, it has been demonstrated that the accumulation of senescent cells *in vivo* in various tissues, such as skin, liver, kidney or brain, is linked to altered tissue functions resulting in aging (Campisi and D'Adda di Fagagna, 2007).

In fact, it is not clear what happens to senescent cells in the body since they behavior depends on the cell type. For example, it has been demonstrated that senescent fibroblasts from mice are resistant to apoptotic stimuli while senescent endothelial cells are more sensitive (Wang, 1995; Hoffman et al., 2001). Another important point is the impact on the accumulation of senescent cells in the immune system response. It has been observed that senescence is characterized by the alteration in gene expression of some cytokines, chemokines and surface markers involved in inflammation (Yoon et al., 2004; Minamino and Komuro, 2007; Ren et al., 2009). Although these changes could induce senescent cells elimination, mainly by macrophages (Xue et al., 2007), it is required further investigations in different aging models as well as in human studies.

The idea that senescence contributes to the development of the process of aging is rational but is still under debate owing to the controversial results obtained (Lorenzini et al., 2005; Patil et al., 2005). Some studies are in favor of a key role of senescence in the development of organismal aging and age-related pathologies. In this sense, it has been shown that senescent cells and their products (predominantly SASP derived molecules) induce the elderly phenotype. For instance, senescence in breast caused by deterioration of the structure or in altered migration and proliferation of cells caused by changed expression of extracellular matrix macromolecules (Campisi et al., 2011; Noureddine et al., 2011) is similar to skin aging or cognitive impairment (Alzheimer's or Parkinson's disease) produced by senescent cells (Bitto et al., 2010; Salminen et al., 2011; Velarde et al., 2012).

In addition, the role of senescent cells was confirmed in several age-related diseases, such as macular degeneration, COPD (Chronic Obstructive Pulmonary Disease), emphysema and insulin resistance (Campisi et al., 2011). However, due to the complexity of the process, it is unknown whether this increased number of senescent cells in tissues is the cause or the consequence to becoming older and whether it is good or bad for the organism. Therefore, the mechanism involved in the causal senescence-aging process has to be clarified.

3.1 Aging, senescence and cancer

The cellular response prompted by senescence inducing stimuli (short telomeres, DNA damage, overexpression of certain oncogenes and cellular stress, among others) is controlled by p53 and pRb pathways, establishing and maintaining the process of

senescence (Serrano and Blasco, 2001; Ben-Porath and Weinberg, 2004; Colavitti and Finkel, 2005; Passos and Von Zglinicki, 2006), as it has been mentioned in section 2.5 of this introduction.

In the last years, it has become clear that the senescence response is a potent and an efficient cell autonomous tumor suppressive mechanism. In this regard, senescence inducing factors, such as DNA damage or stress, prevent the initiation of tumorigenesis (Dimri, 2005). Consistent with this data, the blockage of cell proliferation presented by senescent cells depends mainly on p53 and pRb pathways (Itahana et al., 2001; Ohtani et al., 2004), two of the most powerful tumor suppressor routes. Thus, genetic mutations or functional defects of the genes or proteins involved in these mechanisms favor the malignant transformation of cells preventing the bypass to the senescent phenotype.

The tumor suppressive character of senescence seems to be clear, however, there are some investigations suggesting that the SASP could promote malignant phenotypes, even *in vivo* (Bavik et al., 2006; Coppé et al., 2006; Coppé et al., 2008). It has been shown that the SASP induces the cellular transition of epithelial to mesenchymal in culture, favoring the progress of invasive and metastatic carcinoma, due to the release of certain SASP components such as IL-6, IL-8 and GRO (growth related oncogene) (Krtolica et al., 2001; Roninson, 2002; Dilley et al., 2003).

In addition, in mouse xenograft studies have been demonstrated that the secretion of MMPs produced by senescent cells induces tumor growth and invasiveness *in vivo* (Liu and Hornsby, 2007). Although these studies did not demonstrate a direct connection between SASP factors and the appearance of tumors, they support that SASP factors, released by senescent cells accumulated with age, can produce the conversion of neighboring premalignant cells to malignant (Hasty et al., 2003).

3.2 Immunosenescence and Inflammaging in the macrophage lineage

One of the most recognized effects of getting old is the dysregulation of the innate and adaptive immune function with advancing age (Figure 7), thus leading to increase susceptibility to viral and bacterial infections, reactivate latent viruses and decrease response to vaccines (Montecino-Rodriguez et al., 2013).

In fact, the aging alteration of the immune system results in defects in the initiation and in the resolution of immune responses, process called immunosenescence, and chronic low-grade inflammation, termed inflammaging. This chronic subclinical condition has been associated with increased mortality and major incidence of immune diseases and cancer in the elderly as well as an increased prevalence of metabolic syndrome, atherosclerosis and neurodegenerative diseases (Franceschi et al., 2007; Montecino-Rodriguez et al., 2013).

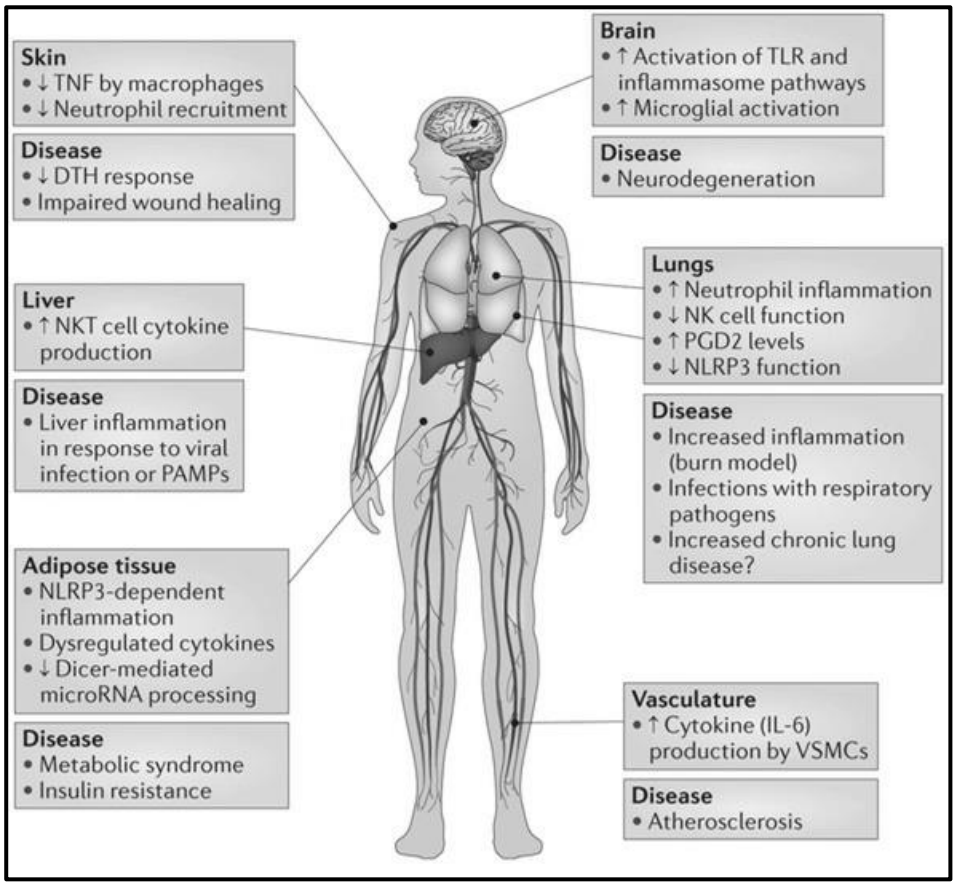


Figure 7. Dysregulation of the innate immune system associated with aging and disease. Diminished innate immune responses are found in some cases such as the skin. However, other tissues present hyperactive innate immune system as the brain. Adapted from Shaw et al., 2013.

Deterioration of the innate immunity seems to be the established mechanism linked with age-related infections (Colonna-Romano et al., 2008), however, since innate immune activity is required to develop an appropriate adaptive immune response, age-related deficits in innate immune functions might therefore change both cell-mediated and humoral adaptive immune reactions (Djukic et al., 2014).

Given the importance of macrophages to orchestrate the progression of the immune response (see section 1.3 of this introduction) deficiencies in macrophage activities associated with age, process known as MacrophAging (Sebastián et al., 2005), contribute significantly to the disorder of the immune responses observed in the elderly.

For instance, some studies in humans and mice have demonstrated that TLR expression and function fail with age (Aspinall et al., 2007). This alteration reduces the production of normal levels of pro-inflammatory cytokines and chemokines causing the no activation of T and B cells and several processes involved in the adaptive immune system (Aspinall et al., 2007). On the contrary, Boehmer and co-workers reported that TLR expression is not affected by advanced age and explained the low levels of pro-inflammatory cytokines by impaired intracellular signaling produced by reduced p38 and JNK MAPKs phosphorylation (Boehmer et al., 2004).

Furthermore, it has been shown that the reduced expression of MHC II of aged macrophages is connected with a declined T cells clonal expansion (Seth et al., 1990; Garg et al., 1996; Solana et al., 2006). In this sense, the elimination of pathogenic microorganisms by phagocytosis is also impaired in aged macrophages (Antonini et al., 2001; Mancuso et al., 2001) as well as the chemotactic activity and the macrophage production of chemokines such as MIP-1α/β and MIP-2 (Ortega et al., 2000; Swift et al., 2001).

All of these deficiencies are not only produced by reduced macrophage functions. The communication between the cells of the immune system and tissue cells seems to be impaired. For example, in aging the vascular endothelium decreased the expression of cell adhesion molecules (Ashcroft et al., 1998) and VEGF and EGF receptors (Ashcroft et al., 1997; Ashcroft et al., 2002).

On the other hand, the progressive pro-inflammatory state shown in healthy-aged individuals, termed inflammaging, mentioned before, consists in a higher basal inflammatory state in the absence of an immune challenge. The elevated pro-inflammatory circulating cytokines includes IL-6, TNF-α and IL-1β which can affect the phenotypes and functions of cells and cause susceptibility to a poor prognosis after systemic insults (Franceschi et al., 2000).

There are many processes that can induce or contribute to inflammaging. Immunosenescence is one of them. Defective regulation of the complement pathway drives to local inflammation that in age can cause degenerative diseases (Gallenga et al., 2014). In addition, as adaptive immunity is declined, the alteration of the innate

immunity, mainly macrophage functions, could result in hyperactivity to undertake the burden (Franceschi et al., 2000; McElhaney, 2009; Shaw et al., 2011).

Persistent senescent cells can also induced inflammaging. Through the SASP, senescent cells can modify the tissue microenvironment and then the function of neighboring normal or premalignant cells (Campisi et al., 2007; Baker et al., 2011). Senescent cells are localized at sites of many age-related pathologies and it has been shown that their elimination in prematurely aged mice prevents several age-related pathologies (Coppé et al., 2010).

Several molecules can be accumulated with age and act as endogenous damage, such as ATP, urate crystals and free radicals from oxidative stress, among others (Dall'Olio et al., 2013). These compounds can be recognized by the innate immunity through a network of sensors called inflammasomes that once activated can initiate an immune reaction required for physiological repair. As damage accumulates, this response can become chronic (Franceschi et al., 2000).

Other source of inflammaging is represented by microbial constituents of the organism such as gut microbiota (Biagi et al., 2011). As gut presents an impaired physical function with aging and gut microbiota changes with aged, these "new" microbes or their products can escape from the gut and initiate an inflammatory response (Wikby et al., 2006; Larbi et al., 2008).

However, it is still under study whether this low-grade of inflammation is pathogenic or adaptive. Chronic inflammation leads to tissue deterioration but is also part of normal tissue restoration. Centenarian healthy-individuals present systemic inflammation with high levels of IL-6, TNF-α and IL-8 in plasma but also a delay in the development of chronic aged-related diseases such as type II diabetes, cardiovascular disease and cancer (Varadhan et al., 2014). This fact suggests that inflammaging is compatible with health and longevity and proposes that there are others factors, not only high levels of pro-inflammatory cytokines, determinant for health or disease in the elderly.

Given the easiness with which macrophages adapt to changes in their microenvironment, the evidence that aging modifies tissue milieu suggests that targeting aged-related factors might reestablish the correct macrophage function in the elderly. Nevertheless, these factors are unknown and could be very abundant. It has been postulated that oxidative stress alters several transcription factors involved in the inflammatory response, such as NFκB or PPARɣ (Lavrovsky et al., 2000). In this

sense, the research into the effect of aging on macrophages is clearly needed in order to control infectious disease in the elderly.

Objectives

Aging has been associated with defects in immune function characterized mainly by decreased immune response to bacterial and viral infections, altered wound healing processes and dysregulated cytokines and chemokines secretion. The essential role of macrophages to coordinate innate and adaptive immune response to pathogenic insults and to maintain homeostasis led us to explore the effects of aging on senescent macrophages. In this sense, deficits in macrophage activity participate considerably to the altered immune responses observed in the elderly.

The effects of aging on macrophages were addressed in two specific objectives:

1. Characterization of a novel macrophage senescent model based on long-period cultures of BMDM.
2. Determination of impaired functional activities in senescent macrophages.

Materials and Methods

1. Buffers and solutions

Laboratory stock solutions and common buffers were prepared as described in weblink:http://onlinelibrary.wiley.com/doi/10.1002/0471143030.cba02as00/abstract., (Current Protocols in Cell Biology, 2001). Buffers used in this thesis were specified below.

Protein lysis buffer for Western Blot

1 M HEPES-NaOH (7.5 pH), 0.5 M EGTA, 180 mM β-glycerophosphate, 1 M $MgCl_2$, 1% NP-40, 10 µg ml^{-1} Aprotinine,10 µg ml^{-1} Leupeptine, 1 mM DTT,1 mM PMSF,1 mM Orthovanadate and 30 µg ml^{-1} Iodoacetamide.

Senescence-associated β galactosidase staining solution

1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM $K_4(FeCN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 150 mM NaCl and citrate/phosphate buffer pH6 (0.1 M citric acid, 0.2 M Na_2HPO_4).

FACS buffer

PBS 1X, 2% Fetal Bovine Serum (FBS) and 0.1% NaN_3.

FACS fixed buffer

PBS 1X, 2% Fetal Bovine Serum (FBS), 0.1% NaN_3 and 2% paraformaldehyde.

Griess reagent

1% sulfanilamide, 0.1% N-(1-napthyl)ethyl-enediamine dihydrochloride (NED) and 2.5% phosphoric acid.

Arginase activity stop solution

Acid solution mixture of H_2SO_4:H_3PO_4:H_2O 1:3:7.

Electrophoresis buffer (10X)

30 g of Tris base

10 g of SDS

144 g of Glycine

Dissolved in distilled water and bring volume up to 1 L.

Transfer buffer (10X)

30.3 g of Tris base

1 g of SDS

144 g of Glycine

Dissolved in distilled water and bring volume up to 1 L.

20% methanol was added to 1X transfer buffer before use.

Laemmli-SDS-PAGE gels

Resolving gels (for 20ml)

V (ml)	**8%**	**12%**	**15%**
H_2O	9.3	6.6	4.6
30% Acrylamide solution	5.3	8	10
1.5 M Tris (pH 8.8)	5	5	5
10% SDS	0.2	0.2	0.2
10% APS	0.2	0.2	0.2
TEMED	0.008	0.008	0.008
Proteins resolved	NOS2, STAT3, STAT5	p38, JNK, ERK	IL-1β, p53, ɣ-H2A, H1

Stacking gels (for 5 ml)

V (ml)	**4%**
H_2O	3.07
30% Acrylamide solution	0.67
1.5 M Tris (pH 8.8)	1.25
10% SDS	0.05
10% APS	0.05
TEMED	0.005

Laemmli sample loading buffer (5X)

For 20 ml:

4.5 ml of 1 M Tris pH 6.8

11.5 ml of 87% glycerol

1 g of SDS

1.5 ml of 1% Bromophenol blue

0.8 g of DTT (154.25 g mol^{-1})

Add up to 20 ml water and rotate on a wheel for 30 min. Store in aliquots at -20ºC.

2. Reagents

All products used are summarized in Table 1 and and kits used in Table 2.

Table 1. Detailed reagents used.

Reagent	Short name	Function	Company	Reference
^{3}H-thymidine	-	Radioactive nucleoside	Amersham	TRK300
Actinomycin D	Act.D	Transcription inhibitor	Sigma-Aldrich	A-1410
Adenosine triphosphate	ATP	P2x7R agonist	Sigma-Aldrich	A2383
Bromodeoxyuridine	BrdU	Nucleoside	Sigma-Aldrich	B5002
Chloride acid	HCl	Inorganic acid	Sigma-Aldrich	320331
Citric acid	-	Organic acid	Merck	244
Cytochalasin D	Cyt.D	Phagocytosis inhibitor	Sigma-Aldrich	C8273
Dibasic sodium phosphate	Na_2HPO_4	Phosphate based buffer	Innogenetics	30186
Dichlorofluorescin diacetate	DCF-DA	Quantification of reactive oxygen species	Sigma-Aldrich	D6883
Dimethylsulfoxide	DMSO	Solvent	Sigma-Aldrich	D8418
Etoposide	Etop.	DNA damage inducer	Tocris	1226
Hydrogen Peroxide	H_2O_2	ROS inducer	Sigma-Aldrich	H3410
Interferon-γ	IFN-γ	Macrophage activator	Thermo Scientific	RM200120

Interleukin-4	IL-4	Macrophage activator	RD SYSTEMS	404-ML-010
L-arginine	-	aminoacid	Sigma-Aldrich	A5131
Lipopolysaccharide	LPS	TLR4 agonist	Sigma-Aldrich	L3129
Magnesium chloride	$MgCl_2$	Inorganic salt	Sigma-Aldrich	M-9272
N-(1-napthyl)ethyl-enediamine dihydrochloride	NED	Coupling agent for spectrophotometric determination	Sigma-Aldrich	222488
N-acetyl-L-cysteine	NAC	General antioxidant agent	Sigma-Aldrich	A7250
Paraformaldehyde	-	Preparation of fixed samples	Sigma-Aldrich	P-6148
Phosphoric acid	H_3PO_4	Inorganic acid	Fluka-Sigma	40779
Potassium ferricyanide	$K_4(FeCN)_6$	Inorganic salt	Sigma-Aldrich	P9387
Potassium ferrocyanide	$K_3Fe(CN)_6$	Inorganic salt	Sigma-Aldrich	B4252
Propidium iodide	PI	Fluorescent stain for nucleic acids	Sigma-Aldrich	P4170
rGranulocyte macrophage-colony stimulating Factor	rGM-CSF	Recombinant growth factor	Preprotech	250-05
rMacrophage-colony stimulating factor	rM-CSF	Recombinat growth factor	Merck	234378
Sodium azide	NaN_3	Inorganic salt	Sigma-Aldrich	S2002
Sodium chloride	NaCl	Inorganic salt	Fluka-Sigma	71381
Sodium Nitrite	$NaNO_2$	Inorganic salt	Sigma-Aldrich	S2252
Sulfanilamide	-	Blockage of dihydrofolic acid synthesis	Sigma-Aldrich	S9251
Sulfuric acid	H_2SO_4	Inorganic acid	Fluka-Sigma	84720
Telomerase activator 65	TA-65	Telomere length elongation	-	-
Urea	-	Solubilization protein agent	Fluka-Sigma	51458

α-isonitrosopropiophenone	α-ISPP	Colorimetric determination of urea	Sigma-Aldrich	13502

Table 2. Detailed kits used.

Kit	Company	Reference
Annexin V kit	BD Transduction	556547
Genomic blood DNA purification kit	GE Healthcare	27-9603-01
IL-1β ELISA kit	R&D Systems	MLB00C
Mouse Inflammation kit	BD Biosciences	552364
Purelink Dnase kit	Invitrogen	12185-010
Purelink RNA minikit	Ambion	12183018A
SOD activity kit	Sigma-Aldrich	19160
Super script II Reverse Transcriptase	Invitrogen	18064-014

3. Animal strains

Bone Marrow-Derived Macrophages (BMDM) were obtained from different 8 week-old BALB/c mice which were purchased from Charles River Laboratories (Wilmington, MA). All animals were healthy, housed in a barrier system (temperature: 20-26ºC; relative humidity: 40-70%) with a 12 h light-dark cycle. Water and standard diet were available add libitum. Mice were killed by cervical dislocation and the animal use was approved by the Animal Research Committee from the University of Barcelona (number 2523).

4. Generation of Bone Marrow-Derived Macrophages (BMDM)

To obtain BMDM, mice were killed by cervical dislocation and bone marrow was flushed out of the femurs and tibias of adult mice in DMEM medium under aseptic conditions as was previously described (Celada et al., 1984). Cells were suspended by vigorous pipetting and cultured in plastic dishes of 150 cm^2 (Miles Laboratories, Inc.,

Naperville, IL) in complete medium consisting on DMEM rich in glucose containing 20% of FBS, 30% of L-cell conditioned media as a source of M-CSF (see point 6), using 4 plastic dishes and obtaining around 100.10^6 cells per mouse. Media was supplemented with 50 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin. Cells were incubated at 37 ºC in a humidified 5% CO_2 atmosphere. Cells were grown to reach a state of subconfluence and after 7 days a homogeneous population was observed (0 days BMDM), consisting of attached cells, positive for surface macrophage markers such as Cd11b and F4/80 (>90%).

To obtain long-lasting cultures of macrophages (14 days BMDM), a normal mature culture of BMDM (0 days BMDM) was replated in 150 cm^2 plastic dishes ($2x10^6$ cells) in complete medium for 7 days. After this period of time, the macrophages obtained were replated again ($5x10^6$ cells) re-establishing the complete medium for 7 other days to get $25x10^6$ of senescent macrophages per plastic dish.

4.1 Macrophage cultures

After 7 days of macrophage differentiation, BMDM were replated in plastic dishes ($1x10^6$ cells/24-well plates, $3x10^6$ cells/ 60 mm) in order to perform different experiments. After adherence, BMDM were stimulated with different treatments. Treatments were always performed with rM-CSF (10 ng ml^{-1}), rGM-CSF (5 ng ml^{-1}), LPS (10 ng ml^{-1}), IFN-γ (20 µg ml^{-1}), IL-4 (10 ng ml^{-1}), H_2O_2 (5 µg ml^{-1}) and Etoposide (1 µg ml^{-1}). ATP (1 mM) treatment was performed for the last 20 min of the indicated stimuli in order to prevent cell toxicity. DMSO was used as vehicle control when inhibitors used for treatments were dissolved in it. For RNA extraction, proliferation, cell cycle analysis and detection of phosphorylated proteins, BMDM were cultured in quiescent conditions (DMEM and 10% FBS) for 16 h before treatments in order to stop cell cycle.

5. Cell line cultures

5.1 L929 cell line

L929 fibroblast cell line (ATCC CCL-1) was derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse. NCTC clone 929

Clone of strain L was one of the first cell strains to be established in continuous culture, and clone 929 (also known as L929) was the first cloned strain developed. Clone 929 was established (by the capillary technique for single cell isolation) from the 95th subculture generation of the parent strain. These cells were cultured in tissue culture plates with high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C and 5% CO_2.

5.2 J558L cell line

J558L cell line (ATCC TIB-6) was derived from a BALB/c mouse plasmacytoma B lymphocyte. These cells were cultured in tissue culture plates with high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C and 5% CO_2.

5.3 MDA-MB-231 cell line

The MDA-MB-231 breast cancer cell line (ATCC HTB-129) was obtained from a patient. With epithelial-like morphology, the MDA-MB-231 breast cancer cells appear phenotypically as spindle shaped cells. In vitro, the MDA-MB-231 cell line has an invasive phenotype. This MDA-MB-231/GFP cell line stably expresses GFP; the gene was introduced using lentivirus. These cells were cultured in tissue culture plates with high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C and 5% CO_2.

6. Obtention of Macrophage-Colony Stimulating Factor (M-CSF) supernatant

Conditioned medium (L-cell) to generate macrophages from bone marrow was obtained from conditioned medium of the mouse fibroblast cell line L929 (see point 5.1), which produces large quantities of M-CSF during proliferation. This is the only growth factor produced by these fibroblasts affecting macrophages. The addition of monoclonal antibodies against M-CSF to the medium blocks production of macrophages in culture (Lokeshwar and Lin 1988). $7x10^5$ L929 cells were cultured in high glucose DMEM supplemented with 10% FBS at 37°C and 5% CO_2. Cells were

growth in 150 mm flasks up to confluence and after 7 days the supernatant was collected, centrifuged to remove the cells in suspension and kept in aliquots at -80 ºC until the moment of use. Once thawed, the aliquots were stored at 4 ºC to prevent degradation of M-CSF resulting from freezing and thawing cycles. The content of M-CSF was determined by a test of proliferation (^{3}H-thymidine incorporation) in macrophages from mouse bone marrow. In our studies we use the concentration of 30% of supernatant to differentiate bone marrow to macrophages which is equivalent to 1200 U ml^{-1} of recombinant M-CSF (eBioscience, San Diego, CA), since this dose is able to saturate the M-CSF receptors on the surface of macrophages (Stanley, 1985).

7. Obtention of Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) supernatant

GM-CSF supernatant was obtained from conditioned medium of the mouse B myeloma cell line J558L (see point 5.2), which produces large quantities of GM-CSF during proliferation. This is the only growth factor produced by these cells affecting macrophages. $1x10^6$ J558L cells were cultured in high glucose DMEM supplemented with 10% FBS at 37°C and 5% CO_2. Cells were growth in 150 mm flasks up to confluence and after 5-7 days the supernatant was collected, centrifuged to remove the cells in suspension and kept in aliquots at -80 ºC until the moment of use. Once thawed, the aliquots were stored at 4 ºC to prevent degradation of GM-CSF resulting from freezing and thawing cycles. The content of GM-CSF was determined by a test of proliferation (^{3}H-thymidine incorporation) in macrophages from mouse bone marrow. The concentration of 10% of supernatant is equivalent to 20 ng ml^{-1}.

8. Experimental analysis

8.1 RNA extraction from cultured cells

$3x10^6$ quiescent BMDM (60 mm plastic dishes), were stimulated with different treatments. Total RNA from macrophage cultures were extracted using PureLink™ RNA miniKit following manufacturer's instructions (Ambion, Paisley, UK).

8.2 cDNA synthesis

For cDNA synthesis, 1 µg RNA of each experimental sample was retrotranscribed with Superscript II Retrotranscriptase kit from Invitrogen, as described by the manufacturer (Carlsbad, CA).

8.3 Quantitative real-time PCR (qRT-PCR) analysis

For qRT-PCR analysis SYBR Green PCR Core Reagents and the ABI Prism 7900 Detection System were used from Applied Biosystems (Arlington Heights, IL). The threshold cycle (CT) was defined as the cycle number at which the fluorescence corresponding to the amplified PCR product is detected. PCR arbitrary units (AU) were defined as the mRNA levels normalized to the housekeeping gene HPRT1 expression in each sample. The primer sequences are detailed in Table 3.

Table 3. Primers used in qRT-PCR analysis.

Gene	Forward Sequence 5'------- 3'	Reverse Sequence 5'------- 3'
36B4	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC
Arg-1	TTGCGAGACGTAGACCCTGG	CAAAGCTCAGGTGAATCGGC
b-actin	ACTATTGGCAACGAGCGGTTC	AAGGAAGGCTGGAAAAGAGCC
Bcl-2	CGATGGTGTGGTTGCCTTATG	GTCTACTTCCTCCGCAATGCTG
Catalase	AGCATATTGGAAAGAGGACCCC	ATTTCACTGCAAACCCCCG
Cdk2	CCATTCTCACCGTGTCCTTCA	CCAAAGGCTCTTGCTAGTCCAA
Cdk4	TGGAGCGTTGGCTGTATCTTT	TGGAGGCAATCCAATGAGATC
c-Myc	AACAGCTTCGAAACTCTGGTGC	CGCATCAGTTCTGTCAGAAGGA
cycD1	TGCTGCAAATGGAACTGCTTC	CATCCGCCTCTGGCATTTT
cycE	GTTCACAGGAGGTTTGGCAAAA	TGACCTCATCTGTGGTTCCAGG

E2F1	ATTGGTAAGCGGCTTGAAGG	TTGCAGCTGTGTGGTACAGATG
Fizz1	CCTTCTCATCTGCATCTCCCTG	GCTGGATTGGCAAGAAGTTCC
Hprt1	ATCATTATGCCGAGGATTTGG	GCAAAGAACTTATAGCCCCC
IA-b	ACCCAGCCAAGATCAAAGTGC	TGCTCCACGTGACAGGTGTAG
il-10	TCCTTAATGCAGGACTTTAAGGG	GGTCTTGGAGCTTATTAAAAT
il-1β	TGGGCCTCAAAGGAAAGAAT	CAGGCTTGTGCTCTGCTTGT
Mgl-1	TGAGAAAGGCTTTAAGAACTGGG	GACCACCTGTAGTGATGTGGG
Mrc-1	AGATAATGGCCTCCGTTTGGTT	TGCTGAGCTCTCCACAGGTG
Nos2	GCCACCAACAATGGCAACA	CGTACCGGATGAGCTGTGAATT
p16	TGAATCTCCGCGAGGAAAGCGAAC	CTGCTACGTGAACGTTGCCCATCA
p21	CTGCTACGTGAACGTTGCCCATC	CTGTGGGGTGAGGAGGAGCATGA
p27	GGCCTTCGACGCCAGACGTAA	CTTCACCCCCATGCTGACTCCTCA
Raf	AGGTGGTTGACCCAACTCCA	TGCCGTGTTTTGCGC
Sod1	AATGTGACTGCTGGAAAGGACG	GCCAATGATGGAATGCTCTCC
Sod2	TCTCTTTAGATCAGCGAAGCCC	GGTCATCTCTGCCAAACTTGC
Tgf-β	TCGCCAGTCCCCCAAGCCAG	CAGCAATGGGGGTTCGGGCA
Tnf-α	CCAGACCCTCACACTCAGATC	CACTTGGTGGTTTGCTACGAC

8.4 Telomere length quantification by real-time PCR

$3x10^6$ unstimulated BMDM were replated in 60 mm plastic dishes. Telomere length was analyzed as described (Sebastián et al., 2009). Briefly, genomic DNA was isolated using the GFX genomic blood DNA purification kit from Amershan Biosciences (Buckinghamshire, UK), and real time PCR was performed as in RNA expression analysis using 35 ng of DNA/well. Telomere length was calculated as the ratio of telomere repeat copy number to single copy gene (36B4) copy number (T/S ratio). This ratio should be proportional to the average telomere length. To confirm that the number of copies of the single copy gene per cell that was effectively PCR-amplified was the

same in all samples being studied, we quantified the relative ratio of 36B4 copies to b-actin copies. This ratio was = 1.0, indicating that equal copy numbers of 36B4 per cell were amplified in all DNA samples. Primer sequences (Table 3) and real-time conditions for telomere amplification were as described (Sebastián et al., 2009): 95ºC for 5 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min.

8.5 Determination of protein concentration

Total protein was quantified using Bradford assay according to manufacturer instructions (Sigma-Aldrich). Protein concentrations were measured with a spectrophotometer at an absorbance of 570 nm. For the calculation of protein concentrations a BSA-standard curve was used.

8.6 Western blotting

Proteins from treated macrophages were extracted using lysis buffer for western blot analysis (see point 1). Samples were centrifuged at 13.000 rpm for 20 min at 4ºC and the supernatant protein concentrations were measured by Bradford assay. In the case of IL-1β and NOS2, 20 µg of protein was used and resolved by SDS-PAGE. For P-p53 P-p38, P-JNK, P-ERK1/2, P-STAT3, P-STAT5 and ɣ-H2AX western blots, macrophages were cultured in quiescent conditions (90% DMEM and 10% FBS) for 16-18 h. Then, cells were treated as indicated and 40 µg of total protein from cell lysates were resolved by SDS-PAGE. After transference using a semi-dry transfer system, nitrocellulose membranes were blocked with 5% milk in TBS-0.1%Tween and incubated with primary antibodies overnight at 4ºC. After three washes, membranes were incubated for 1 h with fluorescent dye-labeled or peroxidase-conjugated anti-rabbit or anti-mouse IgG. Enhanced fluorescence detection was then performed using Odyssey Classic System (Li-cor Biosciences, Lincoln, NE) or using ECL from Thermo Scientific (Rockford, IL) with x-ray films. β-actin and H1 antibodies were used as loading control.

In Table 4 the information of all antibodies used for western blotting in this Thesis is summarized.

Table 4. Primary and secondary antibodies used for western blotting.

Primary antibody	Company	Reference
ɣ-H2AX	Millipore	05-636
H1	Santa Cruz	sc-10806
IL-1β	Abcam	ab9722
NOS2	Santa Cruz	sc-8017
P-ERK	Cell Signal	9101
P-JNK	Cell Signal	9912
P-p38	Cell Signal	9211S
P-p53	Cell Signal	9284
P-STAT3	Cell Signal	4904P
P-STAT5	Cell Signal	9351S
STAT5	R&D Systems	PA-ST5A
β-actin	Sigma	A5441

Secondary antibody	Company	Reference
Mouse IgG (Alexa Fluor 680)	Invitrogen	A21057
Mouse IgG (Alexa Fluor 800)	Rockland	610-731-124
Rabbit IgG (Alexa Fluor 680)	Invitrogen	A21076
Rabbit IgG (Alexa Fluor 800)	Rockland	611-131-122
Rabbit IgG peroxidase-conjugated	Jackson	111-035-003

8.7 Protein surface expression

Surface expression of Mac1 (Cd11b), MHC II (I-Aβ) and TLRs were analyzed with monoclonal anti-mouse antibodies, as described previously (Goñalons et al., 1998). $1x10^6$ BMDM were activated with IFN-γ (20 μg ml^{-1}) for 48 h or LPS (10 ng ml^{-1}) for 24 h at 37ºC. Then, macrophages were collected and washed in ice-cold PBS 1X. Macrophages were resuspended in 100 μl FACS Buffer (see point 1) and then incubated on ice for 30 minutes with anti-CD16/CD32 mAb (1 μg ml^{-1}) to block Fc receptors. They were then incubated for 30 minutes on ice with the specific FITC-conjugated or PE-conjugated primary antibody (see Table 5) and washed by centrifugation through PBS 1X at 4°C, avoiding light. Finally, samples were fixing with FACS fixed buffer (see point 1). Stained cell suspensions were analyzed using an FC500 flow cytometer (Coulter Corp., Hialeah, FL). The parameters used to select cell populations for analysis were forward and side light scatter. As a control for specificity, we used an irrelevant antibody of the same isotype.

Table 5. List of antibodies used for surface protein expression.

Primary antibody	Company	Reference
Cd11b-PE	BD Pharmingen	557397
CD16/CD32	BD Pharmingen	553142
I-Aβ-FITC	BD Pharmingen	553623
TLR2-FITC	Invivogen	mab-mtlr2f
TLR4-FITC	Invivogen	mab-mtlr4md2f
TLR5-FITC	Invivogen	mab-mtlr5md2f
TLR9-FITC	Invivogen	mab-mtlr9md2f

8.8 Cytokine determination

Supernatants from BMDM were analyzed for IL-1β production by ELISA and for IL-12, IL-10 and TNF-α production by Mouse Inflammation kit, a cytometric bead array. Macrophages were treated with the indicated stimuli in 24 micro-well plates (10^6 cells $well^{-1}$) for 24 h. Supernatants from these experiments were obtained and centrifuged at 7000 rpm. The supernatants were frozen at -80ºC until cytokine determination. The user's manual instructions were followed for the IL-1β immunoassay kit (R&D Systems, Minneapolis, MN) and for the Mouse Inflammation kit (BD biosciences). Cytometric bead array samples were analyzed using an FC500 flow cytometer (Coulter Corp., Hialeah, FL) and FCAP array software.

8.9 Cellular counting of proliferating cells

Macrophage number was analyzed by cellular counting. BMDM were deprived of M-CSF for 16-18 h. Then, macrophages ($3x10^2$ cells μl^{-1}) were replated in 60 mm plastic dishes and incubated at 37ºC in the presence of different percentages of specific growth factors supernatants (M-CSF or GM-CSF) for 24 h. Stimulated macrophages were washed with PBS 1X and incubated with trypsin for 5 min at 37ºC, centrifuged at 1500 rpm for 5 min and resuspended in 500 µl of PBS 1X. Number of cells was determined using a Neubauer chamber.

8.10 Analysis of DNA content with propidium iodide (PI)

Cell cycle was analyzed as described (Xaus et al., 1999). $5x10^5$ of quiescent BMDM were stimulated for 24 h with rM-CSF (10 ng ml^{-1}) or rGM-CSF (5 ng ml^{-1}). Then, macrophages were collected and fixed with EtOH 95% and incubated with propidium iodide (0.2 µg ml^{-1}). Immediately, samples were analyzed by XL-Coulter flow cytometer and cell cycle distributions were determined with the Multicycle program (Watson, Flow Jo).

8.11 Proliferation assay

Macrophage proliferation was measured by ^{3}H-thymidine incorporation as described (Sebastián et al., 2009). BMDM were deprived of M-CSF for 16-18 h. Then, macrophages (10^5 cells well^{-1}) were replated in 24 micro-well plates and incubated for 24 h in the presence of different percentages of specific growth factors supernatants (M-CSF or GM-CSF). After this period of time, the medium was removed and replaced with media containing ^{3}H-thymidine (1 µCi ml^{-1}). After two additional h of incubation at 37°C, the medium was removed and the cells were fixed in ice-cold 70% methanol. After three washes in ice-cold 10% TCA, the cells were solubilized in 1% SDS, 0.3 N NaOH. Radioactivity was counted by liquid scintillation using a 1500 Packard Tri-Carb scintillation counter (Meriden, CT) and expressed in cpm (counts per minute).

8.12 BrdU labeling of proliferating cells

Macrophage proliferation was also measured by BrdU incorporation. BMDM were deprived of M-CSF for 16-18 h. Then, macrophages (10^5 cells well^{-1}) were replated in 60 mm plastic dishes and incubated for 24 h in the presence of rM-CSF (10 ng ml^{-1}) or rGM-CSF (5 ng ml^{-1}). After this period of time, BrdU (10 µg ml^{-1}) was added to the medium for 2 h at 37ºC. Then, macrophages were washed with PBS 1X, resuspended with FACS buffer (see point 1) and blocked with anti-CD16/CD32 mAb (1 µg ml^{-1}) for 30 min on ice. After 3 washes with FACS buffer, macrophages were incubated with 10 µl of BrdU-FITC antibody (BD Biosciences, 556028) for 1 h on ice. Then, the stained was fixed with FACS fixed buffer (see point 1) and samples were analyzed by flow cytometry using FC500 cytometer (Beckman Coulter, Inc., Pasadena, CA). FlowJo software was used to analyze data that was represented as BrdU fluorescence intensity.

8.13 Apoptosis assay

Apoptosis was measured using a fluorescent Annexin-V kit (Santa Cruz, CA). Macrophages (3-5x10^5 cells well^{-1}) were stimulated with rM-CSF (10 ng ml^{-1}), rGM-CSF (5 ng ml^{-1}) for 24 h, Etoposide (1 µg ml^{-1}) for 1 h, H_2O_2 (5 µg ml^{-1}) for 10 min or

actinomycin D (5 µg ml^{-1}) for 12 h. Stimulated macrophages were washed with PBS 1X and incubated with trypsin for 5 min at 37ºC, centrifuged at 1500 rpm for 5 min and resuspended in 500 µl annexin-V binding buffer. Just before measurement, Annexin-V (25 µM) and propidium iodide (0.2 µg ml^{-1}) were added for 10 min. Samples were analyzed by flow cytometry using FC500 cytometer (Beckman Coulter, Inc., Pasadena, CA). FlowJo software was used to analyze data that was represented as % of Annexin-V and PI positive cells.

8.14 Senescence-associated β-galactosidase activity staining (SA-βgal staining)

SA-βgal staining is a cytochemical assay based on production of a blue-dyed precipitate that results from the cleavage of the chromogenic substrate X-Gal. Thus, it measures the enzymatic activity to hydralyze β-galactosides into monosaccharides carried out in a suboptimal pH (pH6) in which only senescent cells develop staining. 10^6 unstimulated BMDM were replated on slideflasks. After adherence, macrophages were washed with PBS 1X; fixed in paraformaldehid 3% for 5 minutes and incubated with SA-β-gal staining solution (see point 1) for 18 h at 37ºC. Then, staining solution was eliminated with 3 washes of PBS 1X and 20% glycerol was added to the cells. Images were obtained through an inverted microscope (Nikon TE200+Olympus DP72 PSA1).

8.15 Analysis of macrophage ROS production

Reactive oxygen species (ROS) levels were determined by FACS analysis. Quiescent BMDM (5.10^5 $well^{-1}$) were replated in 60 mm plastic dishes. After adherence, macrophages were stimulated in the presence or absence of NAC (2 mM) with rM-CSF (10 ng ml^{-1}), rGM-CSF (5 ng ml^{-1}), LPS (10 ng ml^{-1}) for 24 h or H_2O_2 (5 µg ml^{-1}) for 10 min. After this period of time, BMDM were treated with the probe DCF-DA (25 µM) for 20 minutes at 37ºC, avoiding light. Then, macrophages were collected and washed with PBS 1X. Immediately, fluorescence intensity was analyzed using FC-500 flow cytometer (Coulter) and represented as arbitrary units (AU) as previously described (Sebastian et al., 2009).

8.16 Superoxide dismutase (SOD) activity measurement

SOD, which catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. The determination of the enzymatic activity was measured using a specific indirect process based on colorimetric method. Macrophages were treated with the indicated stimuli in 24 micro-well plates (10^6 cells $well^{-1}$) for 24 h. Supernatants from BMDM were obtained and centrifuged at 7000 rpm. The user's manual instructions were followed for SOD activity assay kit (Sigma-Aldrich) and represented as SOD activity percentage.

8.17 Analysis of macrophage NO production

NO production was measured by a colorimetric method based on the reaction of the Griess reagent. This system detects nitrite (NO_2^-) which is a stable and nonvolatile breakdown product of nitric oxide (NO). BMDM were replated in 96 micro-well plates (10^4 cells $well^{-1}$) in complete medium and stimulated with IFN-γ (20 µg ml^{-1}) and/or LPS (10 ng ml^{-1}) for 24 h at 37ºC. After this period of time, 100 µl of supernatant sample was mixed with 100 µl of Griess reagent (see point 1), avoiding light. After waiting 10 min, optical density at 540 nm was measured and expressed as production of NO µM. Standard curve was used with different concentrations of $NaNO_2$ (µM).

8.18 Analysis of macrophage arginase activity

Arginase activity was measured by a colorimetric method, as previously described (Classen et al., 2009). In this assay, arginase reacts with L-arginine and undergoes a series of reactions to form an intermediate that generates the colored product. BMDM were replated in 96 micro-well plates (10^4 cells $well^{-1}$) in complete medium and stimulated with IL-4 (10 ng ml^{-1}) for 24 h at 37ºC. After this period of time, macrophages were washed with PBS 1X and 100 µl of Triton x-100 (0.1%), 100 µl of Tris-HCl (50 mM, pH7.5) and 10 µl of $MnCl_2$ (10 mM) were added. 100 µl of this mixture was incubated at 56ºC for 7 min and 100 µl of L-arginine (0.5 M, pH9.7) was

added. Then, samples were incubated at 37ºC for 1 h. After that, the reaction was stopped with 800 µl of arginase activity stop solution (see point 1). Then, 40 µl of α-ISPP (6% in EtOH) was added in each sample. Finally, samples were incubated first at 95ºC for 30 min and at 4ºC for also 30 min. Standard curve was used with different concentrations of urea and arginase activity was measured at 540 nm and expressed as mU.

8.19 Phagocytosis of apoptotic cells

The phagocytic activity of macrophages was measured by flow cytometry and by fluorescent microscopy. MDA-MB-231/GFP cells (see point 5.3) were treated with actinomycin D (5 µg ml^{-1}) for 6 h to induce apoptosis. After this period of time, these cells were washed 3 times with PBS 1X and added to 10^5 BMDM, which were replated in 60 mm of plastic dishes (for cytometric analysis) or in slideflasks (for microscopy analysis), in a proportion of 10:1 (10 apoptotic cells per one macrophage) with the presence or absence of a pre-stimulation of cytochalasin D (2 µg ml^{-1}). After 1 h of incubation at 37ºC, BMDM were washed with PBS 1X. For flow cytometry measurement BMDM were resuspended in FACS buffer and samples were analyzed using a FC500 flow cytometer (Coulter Corp., Hialeah, FL). The parameters used to select cell populations for analysis were forward and side light scatter. For phagocytic images, after washed BMDM with PBS 1X, 20% glycerol was added to the cells. All pictures were acquired with a fluorescent inverted microscope (Leica Nikon TE200+Olympus DP72 PSA1).

9. Statistical analysis

The results shown correspond to at least 3 independent experiments, each experiment performed in triplicate, unless otherwise indicated in the figure legend. Data are expressed as the average ±SD (n≥9). The two-tailed t-test of the indicated groups was applied and significance of each particular group comparison was represented as "*" when $p \leq 0.05$ or "**" when $p \leq 0.01$.

Results

Characterization of a novel macrophage senescent model based on long-period cultures of BMDM

Macrophages are key cells in innate and adaptive immune functions. These cells may act directly, by destroying bacteria, parasites, viruses and tumor cells, or indirectly, by releasing mediators such as IL-1, TNF-α, etc, which can regulate other cells. Macrophages are also responsible for processing antigens and presenting digested peptides to T lymphocytes, and for tissue damage repair.

Macrophage functions are altered in old age in humans, mice and rats, thereby contributing to the immunosenescence of adaptive and innate immunity. For instance, these cells acquire increased adhesion to the extracellular matrix and a flattened and much enlarged phenotype with a vacuolated morphology (Chang, Broude et al., 1999; Serrano and Blasco, 2001; Narita et al., 2003). Phagocytic activity, cytokine and chemokine secretion, antibacterial defenses such as the production of reactive oxygen and nitrogen intermediates, infiltration and wound repair functions in the late phase of inflammatory response, and antigen presentation are also altered in aged macrophages (Herrero et al., 2002; Sebastián et al., 2009).

Bone-marrow derived macrophages (BMDM) cellular model is a homogeneous primary culture able to respond physiologically to differentiation, proliferation and activation. In addition, the easy way of isolate these cells makes it the most used model to study particular activities of macrophage biology, including aging (Herrero et al., 2002; Sebastián et al., 2009; Shaik-Dasthagirisaheb et al., 2010; Barrett et al., 2015). For these reasons, BMDM were selected as the cellular model to be used in our studies. Since cellular senescence has been associated with aging, we have compared characteristics of normal macrophage cultures (0 days macrophages) and long-period macrophage cultures (14 days macrophages) of BMDM to study the aging process of macrophages avoiding use aged mice.

Long-term cultures of BMDM display a senescent phenotype

Macrophages require the presence of growth factors, such as M-CSF or GM-CSF to proliferate and survive (Lacey et al., 2012). The stimulation of starved macrophages (macrophages deprived of M-CSF for 16 h) with different percentages of conditioned media of both growth factors induced the incorporation of radioactive ^{3}H-thymidine in the cellular DNA. M-CSF supernatant produced high levels of macrophage proliferation from 10% of M-CSF concentration (Figure 8A). Large quantities of M-CSF were not

able to increase macrophage proliferation probably because M-CSF receptors were saturated.

In the case of GM-CSF, the proliferation rate induced by this growth factor was not so elevated in comparison with radioactive levels produced by M-CSF stimulation (Figure 8A and B). However, GM-CSF was able to induce macrophage proliferation at very low concentrations (from 0.25%, being maximal at 0.5% of GM-CSF supernatant) and decreased at higher doses (5%) (Figure 8B). Consistently, recombinant M-CSF and GM-CSF were able to produce cell cycle progression from G1 phase observed in control macrophages (starved BMDM) to G2/M phase of the cell cycle (Figure 8C).

As we expected, these facts indicate that macrophages obtained from mice bone-marrow respond to conditioned media and recombinant M-CSF and GM-CSF which induced the activation of cell cycle and proliferation.

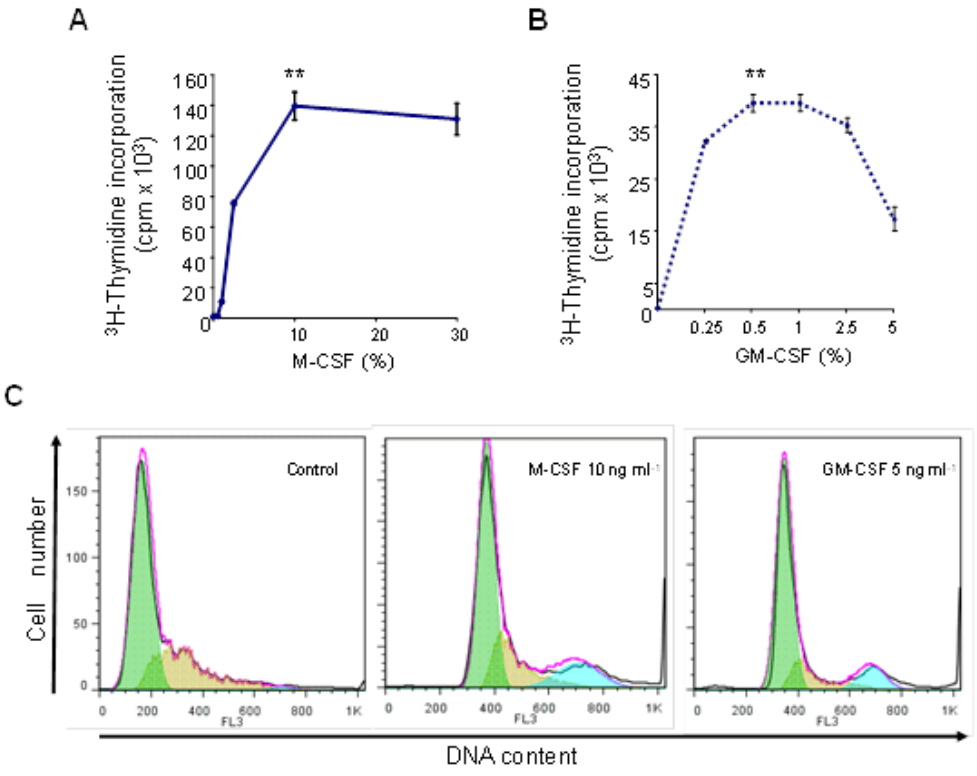


Figure 8. BMDM proliferate in response to specific growth factors, M-CSF and GM-CSF. (A and B) Starved BMDM were stimulated with different percentages of M-CSF (%) and GM-CSF (%) conditioned medium for 24 h. Proliferation induced by these growth factors was determined by ^{3}H-thymidine incorporation. Radioactivity was expressed in cpm (counts per minute). (C) Starved BMDM were treated with recombinant M-CSF (10 ng ml^{-1}) or GM-CSF (5 ng ml^{-1}) for 24 h. After this period of time, DNA content was determined by flow cytometry using propidium iodide (PI) staining. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. **p<0.01 corresponds to the statistical significance between indicated pair comparisons.

In order to investigate macrophage aging through cellular senescence process, we designed a culture procedure to achieve senescent macrophages (long-period BMDM) from a mature culture of young cells.

For this purpose, once bone-marrow differentiated to BMDM (0 days BMDM, see section 4 of Material and Methods), 2.10^6 macrophages were replated in renewal complete medium (30% M-CSF and 20% FBS) for 7 days, obtaining a primary culture of 7 days BMDM. After this period of time, 5.10^6 of 7 days BMDM were replated again re-establishing the complete medium for 7 days more to get long-lasting cultures of macrophages termed 14 days BMDM (Figure 9A). To compare biological functions and cellular characteristics related to senescence and aging, we elaborate this method considering getting these different macrophage cultures simultaneously to perform the experimental analysis properly, planning the sacrifice of the mice every 7 days consecutively.

Once these long-term cultures of macrophages were obtained, we decided to investigate if they presented senescent features. It is well known that senescence is associated with a permanent reduction of the proliferative capacity of cells (Chen, 2007). Therefore, we decided to measure it in response to M-CSF and GM-CSF after starvation conditions. As we expected, while 0 days BMDM presented normal levels of thymidine incorporation, macrophages from long-term cultures showed a reduced proliferative capacity in both M-CSF- and GM-CSF-induced proliferation (Figure 9B).

Although, GM-CSF-dependent macrophage proliferation was similar between 7 and 14 days BMDM cultures, the reduction of the M-CSF proliferative capacity in 14 days BMDM was more important than in 7 days BMDM (Figure 9B). Thus, we decided to compare normal macrophages (0 days BMDM) with 14 days BMDM, using these cells as long-period cultures of macrophages for experimental analysis.

In addition, to discard that this culture method modified phenotypical features of macrophages, we demonstrated that these long-period cultures are ordinary BMDM, but with a restricted capacity of multiplying, through forward and side scatter analysis as well as the measurement of surface expression of Cd11b, a well-known macrophage marker (Xu and Gordon, 2003). In this sense, cellular size and complexity of 14 days BMDM population were exactly the same as normal macrophages (Figure 10A). Similarly, the fluorescence intensity of Cd11b staining in both cultures was high at analogous levels (Figure 10B).

These results indicate that, although long-lasting BMDM cultures present an impaired proliferative capacity in response to two specific macrophage growth factors, they still have phenotypical macrophage characteristics.

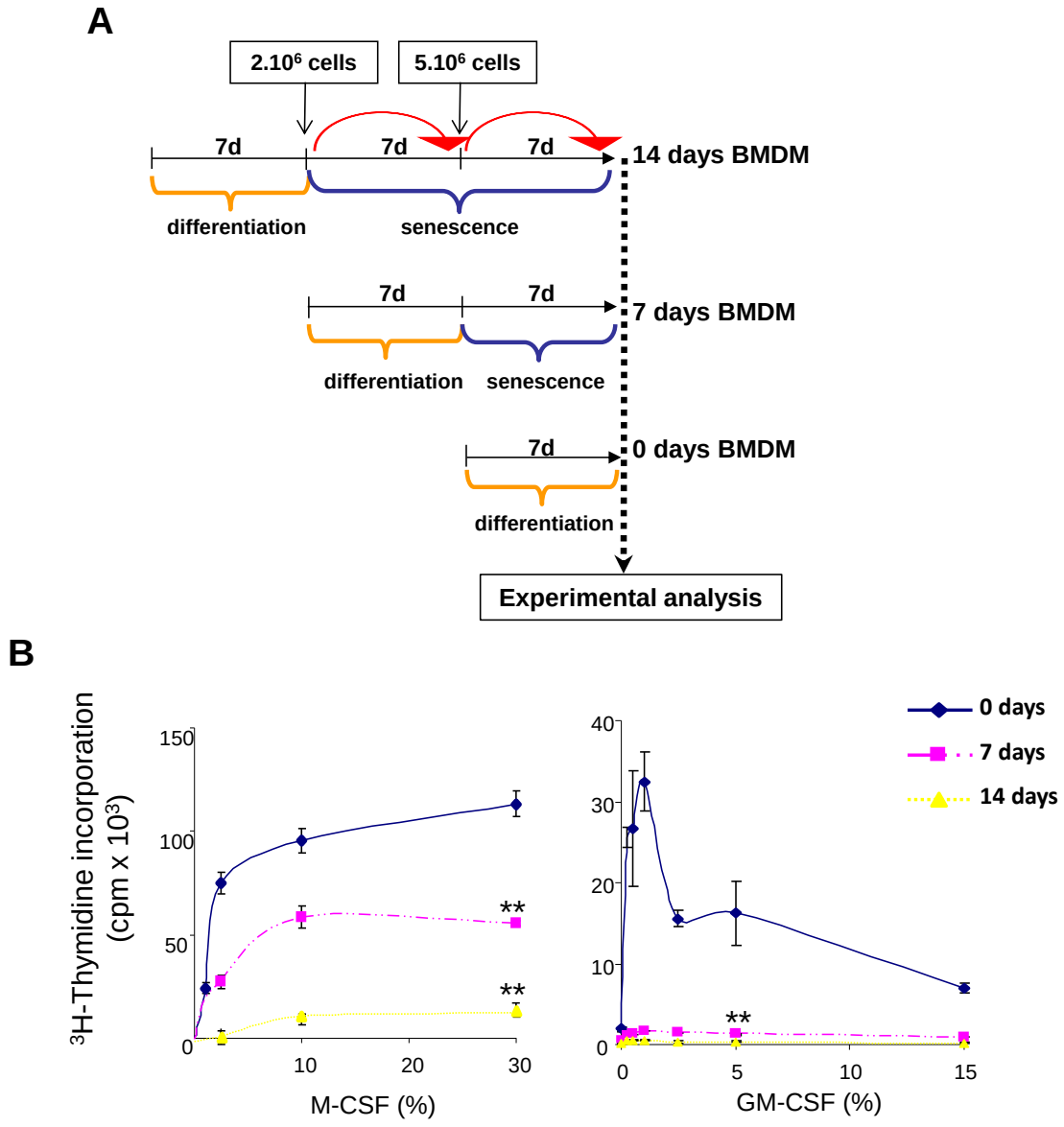


Figure 9. Long-term cultures of BMDM present a reduced proliferative capacity in response to M-CSF and GM-CSF. (A) Schematic representation of normal and long-period BMDM cultures obtention to perform experimental analysis. (B) Starved BMDM were stimulated with different percentages of M-CSF (%) and GM-CSF (%) conditioned medium for 24 h. Proliferation induced by these growth factors was determined by ^{3}H-thymidine incorporation. Radioactivity was expressed in cpm (counts per minute). Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. **p<0.01 corresponds to the statistical significance between indicated pair comparisons.

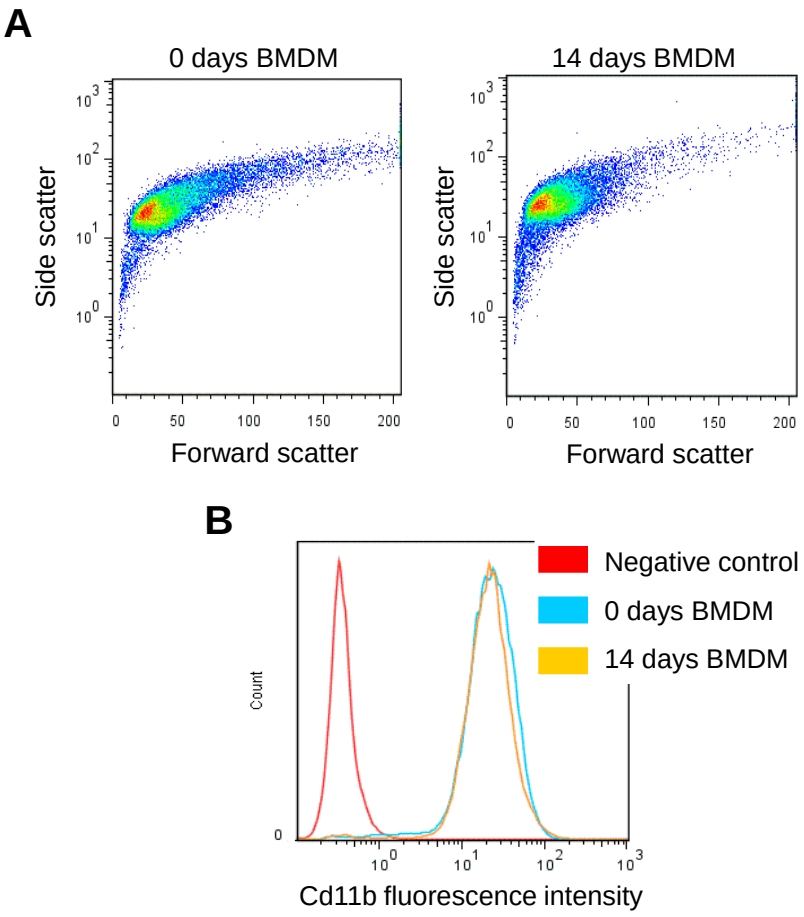


Figure 10. Characterization of normal and long-term BMDM obtained from *in vitro* differentiation of bone marrow cells in mice. (A and B) 10^5 BMDM from normal and long-lasting cultures were collected and analyzed by flow cytometry. (A) Granularity and cell surface were detected by side scatter and forward scatter measurements. (B) Cd11b surface expression was analyzed by anti-Cd11b staining, as previously described in material and methods. All assays are representative for at least 3 independent experiments.

While cellular senescence is induced by a wide variety of conditions, senescent cells display a number of characteristics that allow their identification (Kuilman et al., 2008). Some of these biomarkers reflect the activation of mechanisms that contribute to the senescence program, but others are the consequence of this process induction (Campisi and D'Adda di Fagagna, 2007).

Cellular senescence is generally accompanied by morphological changes, which may be quite striking. Depending on the cell type, cells can become large, flat, multinucleated and even refractile (Hou et al., 2013; Biran et al., 2015). In the case of macrophages we observed that un-stimulated 14 days BMDM seemed to be bigger and rounder comparing with cells from normal cultures (Figure 11, above images).

In addition, long-term cultures of macrophages were positive for the senescence-associated β-galactosidase (SA-β-gal) activity specific staining (Figure 11, bottom images). This enzymatic activity increases in senescent cells due to an expansion of the lysosomal compartment, giving rise an increase in its activity that can be detected at a suboptimal pH 6 (Kuilman et al., 2008).

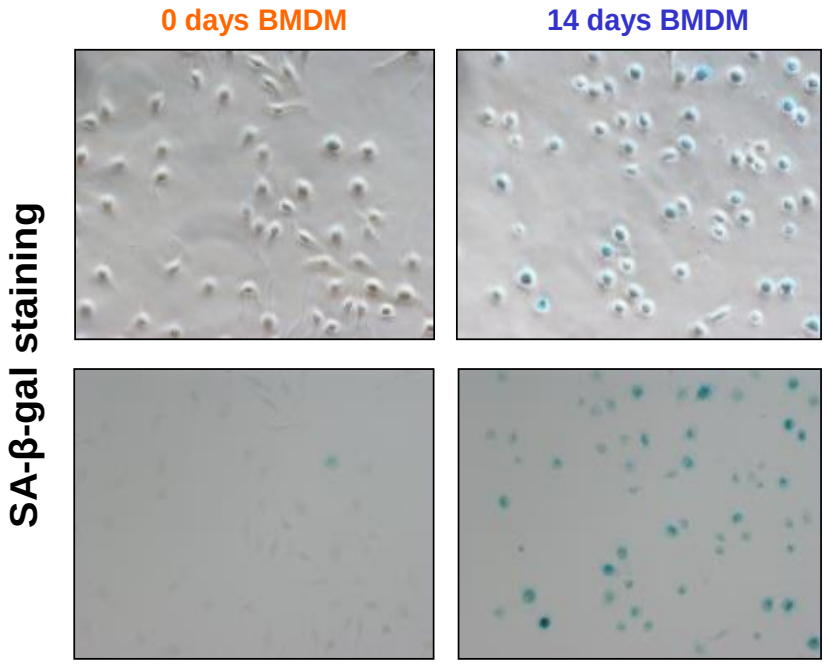


Figure 11. Long-lasting cultures of macrophages show an increased β-galactosidase activity. 10^6 BMDM from normal and long-lasting cultures were incubated in the presence of the SA-β-gal staining solution for 18 h at 37 ºC, as it was indicated in Material and Methods section. Specific staining for senescent cells were performed based on the activity of β-galactosidase in a suboptimal pH. Bottom pictures correspond to the same image but with contrast phase. An inverted microscope was used to obtain images. This assay is representative for at least 3 independent experiments.

Another important senescent biomarker is the telomere shortening (Rodier et al., 2011). Telomeres are chromatin structures that cap and protect the end of chromosomes. Telomere loss has been shown in several cell types during aging but in particular, cells of the immune system are highly susceptible to telomere attrition because of their high proliferative potential (Sebastián et al., 2009). During aging *in vitro*, the telomeres shorten at each subcultivation and for this reason we purposed to analyze the telomere length in long-term macrophage cultures.

In this sense, telomeres length was measured by real-time PCR and 14 days BMDM presented shorter telomeres than those from normal macrophage cultures (Figure 12A).

When telomeres reach a critical minimal length, their protective structure is disrupted and triggers a DNA damage response (DDR), which is associated with the activation of γ-H2AX, a phosphorylated form of the histone variant H2AX (d'Adda di Fagagna, 2003; Takai et al., 2003; Herbig et al., 2004; Rodier et al., 2009). 14 days BMDM stimulated with M-CSF and GM-CSF for 24 h increased γ-H2AX levels, indicating that these cells presented high levels of DNA damage comparing with normal BMDM (Figure 12B).

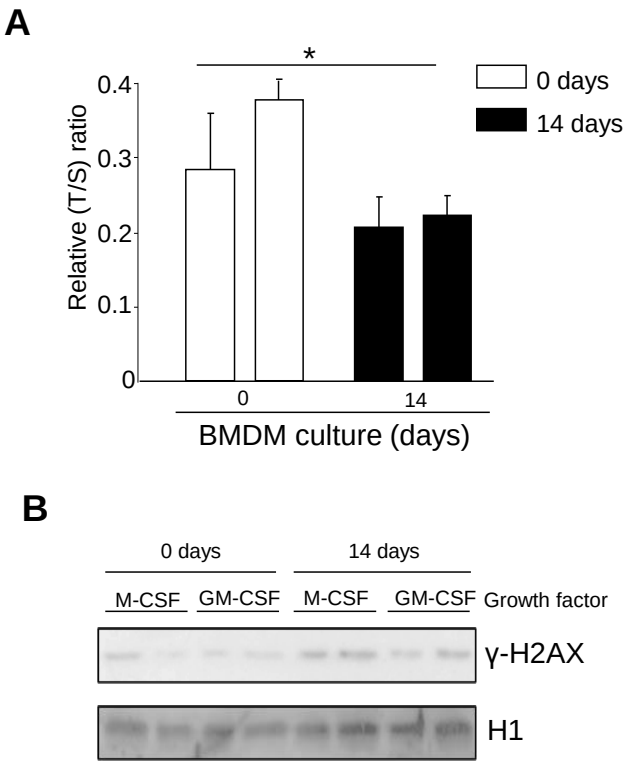


Figure 12. Long-lasting cultures of BMDM show shorter telomeres and higher levels of DNA damage. (A) Telomere length was measured by real-time PCR in normal and 14 days macrophages. The ratio of relative telomere to single copy gene (36B4) amplification (T/S) was shown by two independent experiments. (B) Starved BMDM were treated with recombinant M-CSF (10 ng ml^{-1}) or GM-CSF (5 ng ml^{-1}) for 24 h and DNA damage was determined by γ-H2AX immunoblotting. H1 image was used as loading control for western blots. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05 corresponds to the statistical significance between indicated pair comparisons.

An important mechanism that leads to a wide spectrum of intracellular damage during aging is extended exposure to reactive oxygen species (ROS) generated by cellular metabolism (Kregel and Zhang, 2007). The formation and accumulation of ROS and free radicals affect DNA stability and other cellular elements (Blander et al., 2003; Langen et al., 2003; Ramsey and Sharpless, 2006). Therefore, ROS contributes to the senescence process inducing a DDR (Serrano et al., 2007).

Consequently, we analyzed intracellular levels of ROS in starvation (basal), growing (M-CSF or GM-CSF) and activating conditions (LPS). Normal BMDM increased ROS levels after hydrogen peroxide (H_2O_2, positive control) and LPS treatments (Figure 13A), while M-CSF and GM-CSF stimulations did not modify ROS quantities from basal conditions (Figure 13A). In addition, the pre-treatment with a general antioxidant, N-acetylcisteine (NAC), reduced ROS, indicating the specificity of the technique. After treatment, 14 days BMDM showed higher ROS levels even in basal and growing conditions in comparison with normal macrophages (Figure 13B), suggesting an increased oxidative stress in long-lasting BMDM cultures.

Cells protect themselves from ROS metabolizing H_2O_2 and oxygen free radicals through different mechanisms. One of them is to convert these dangerous molecules into less-reactive substances through the enzymatic activity of catalase, superoxide dismutase (SOD1 and SOD2) and glutathione peroxidase (Zhao et al., 2008). Catalase reacts with the hydrogen peroxide to catalyze the formation of water and oxygen; SOD1 and SOD2 catalyze the dismutation of the superoxide ion into oxygen and hydrogen peroxide and glutathione peroxidase reduces hydrogen peroxide by transferring the energy of the reactive peroxides to a very small sulfur-containing protein called glutathione (Rampsey and Sharpless, 2006).

As 14 days BMDM showed high levels of ROS, we evaluated the gene expression of some senescence markers involved in oxidative stress (Kuilman et al., 2008). It has been observed that antioxidant enzymes gene expression such as *catalase*, *SOD1* and *SOD2* is elevated in senescent cells although their activity is impaired (Haan et al., 1996).

In this sense, starved macrophages from normal and long-term cultures were stimulated with M-CSF and GM-CSF for the times indicated and mRNA levels of these enzymes were measured through qRT-PCR. M-CSF and GM-CSF treatments induced the overexpression of *catalase* and *SOD2*, but not *SOD1*, in macrophages from long-period cultures (Figure 14A, B and C). However, consistently with the published data,

SOD activity was impaired in these macrophages at growing and activating conditions (Figure 14D) preventing the elimination of free superoxide radicals.

This data suggests that the reduction of SOD activity presented in 14 days BMDM probably contributes to the accumulation of ROS in macrophages which produduces DNA damage and, therefore, the induction of cellular senescence.

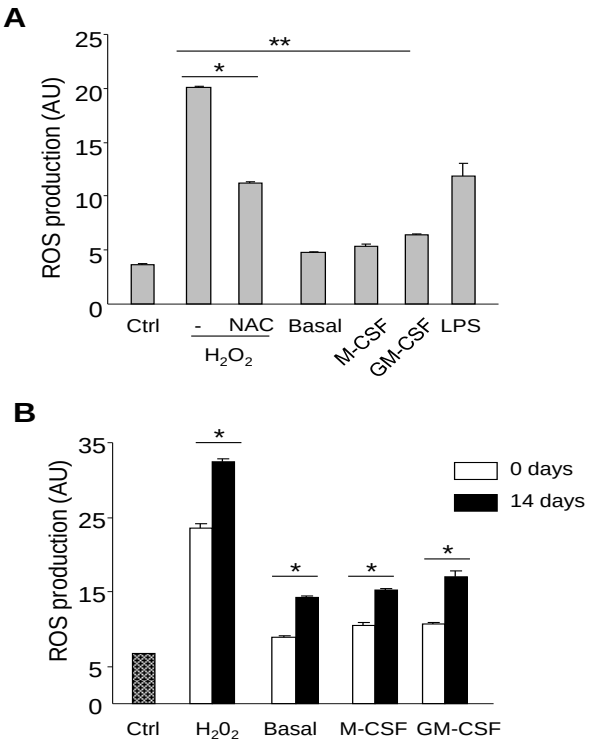


Figure 13. Long-lasting cultures of BMDM present an increased oxidative stress. (A and B) Intracellular ROS levels were analyzed by FACS using DCF-DA staining in macrophages stimulated in the presence or absence of NAC (2 mM) with recombinant M-CSF (10 ng ml^{-1}), GM-CSF (5 ng ml^{-1}), LPS (10 ng ml^{-1}) for 24 h or H_2O_2 (5 µg ml^{-1}) for 10 min. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

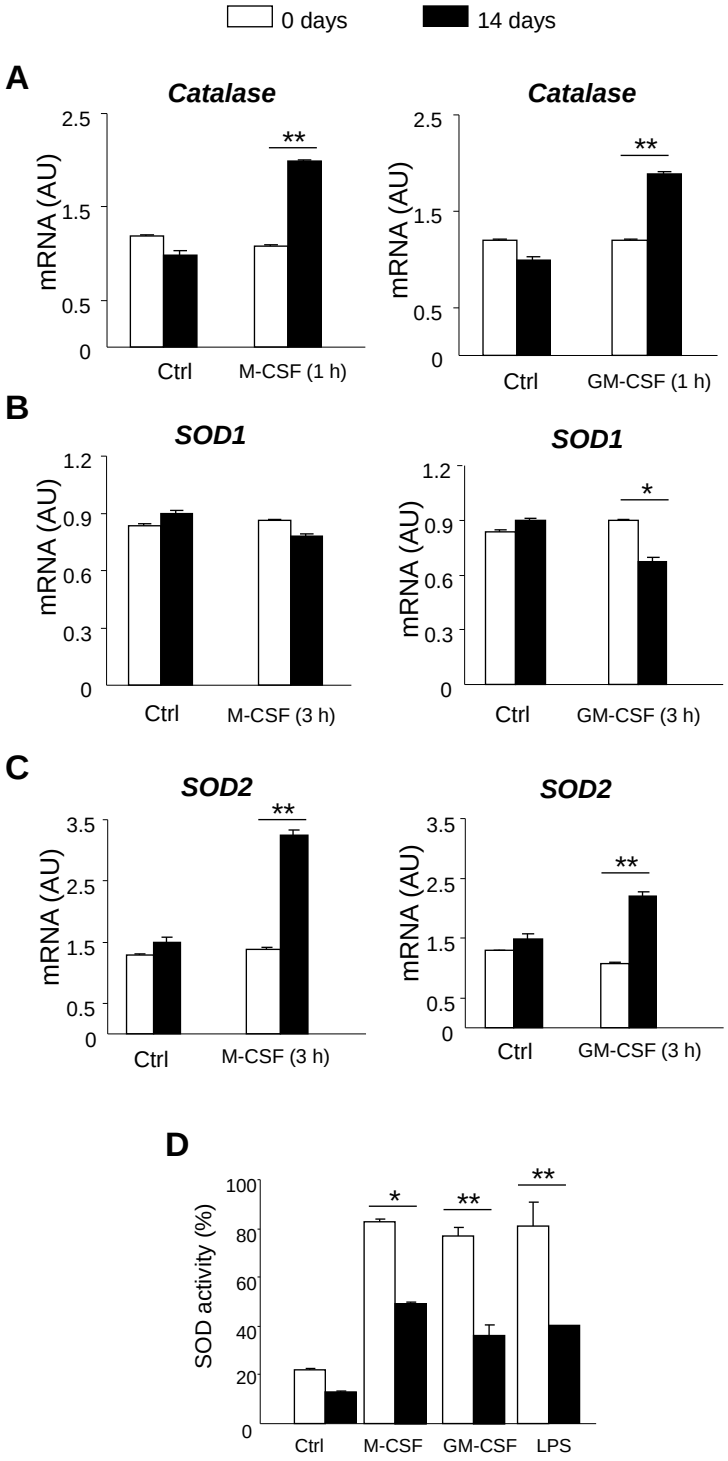


Figure 14. Some senescence markers involved in oxidative stress are overexpressed in long-period BMDM cultures. (A to C) The levels of *Catalase*, *SOD1* and *SOD2* were measured by qRT-PCR in starved BMDM treated with recombinant M-CSF (10 ng ml^{-1}) and GM-CSF (5 ng ml^{-1}) for the indicated times. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. (D) The determination of SOD enzymatic activity was performed by a specific colorimetric method. 10^6 macrophages well $^{-1}$ were treated with M-CSF (30%), GM-CSF (1%) or LPS (10 ng ml^{-1}) for 24 h. Supernatants were collected and analyzed. The enzymatic activity was expressed as percentage of activity. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are

representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Taken together, these results indicate that long-lasting macrophage cultures present a senescent phenotype but not quiescent or terminal differentiated, since most of these characteristics are absent in quiescent cells (Sharpless, 2004).

DNA damage recovery is not affected in long-term cultures of BMDM

The accumulation of DNA damage can induce the altered gene expression produced in long-lasting macrophage cultures that modifies macrophage functional activities (Di Micco et al., 2006). Our results indicated that 14 days BMDM presented high levels of DNA damage (as it was shown in Figure 13).This damage can be generated by ROS production and/or by a decrease in damage repair capacities. In this sense, we designed an experiment to determine the repair capacity after oxidative stress induction (Figure 15A). For this purpose, we used a stimulus that can induce oxidative stress and, therefore, DNA damage upon growing conditions: H_2O_2 treatment. After stimulation with H_2O_2 for just 1 h, complete medium with M-CSF or GM-CSF was reestablished to promote cell recovery. After 24 h of recovery, γ-H2AX levels were measured to detect DNA damage.

In the case of M-CSF-dependent DNA repair, H_2O_2 treatment in 14 days BMDM caused an increase in the levels of γ-H2AX, as it has been reported in macrophages from old mice (Sebastián et al., 2009). This high levels could be reduced after 24 h of recovery (Figure 15B), indicating that the capacity of DNA repair after oxidative stress induction was not impaired in these cells. Similarly, damaged 14 days BMDM showed reduced levels of γ-H2AX after GM-CSF-dependent recovery, indicating that DNA repair process induced by GM-CSF was not affected (Figure 15C).

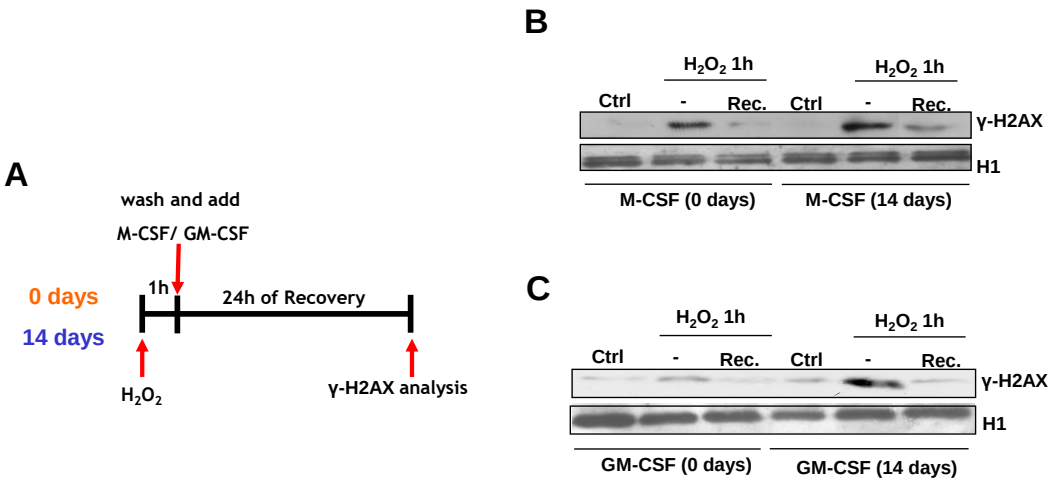


Figure 15. The DNA repair capacity of senescent cells is reduced after the induction of oxidative stress in M-CSF-dependent recovery conditions. (A) Schematic representation of BMDM stimulation to assay macrophage DNA repair capacity. (B and C) M-CSF (30%)- and GM-CSF (1%)-stimulated macrophages (24 h) were treated with H_2O_2 (5 µg ml^{-1}) for 1 h; the cells were washed and led to recover (Rec.) for 24 h. DNA damage was determined by γ-H2AX immunoblotting. H1 image was used as loading control for western blots. All assays are representative for at least 3 independent experiments.

It has been previously described that treatment of BMDM with Actinomycin D (Act.D), Etoposide (Eto.) and H_2O_2 induces apoptosis (Kozmar et al., 2010; Jog et al., 2013). These pro-apoptotic stimuli induced elevated number of annexin-V/PI positive cells in young macrophages (Figure 16A and B). However, although DNA damage was increased in long-lasting BMDM cultures, it was not translated to a high percentage of apoptotic cells, quite the contrary, 14 days BMDM reduced sensitively the number of apoptotic cells after treatment with apoptotic stimuli (Figure 16B), being this apoptosis resistance a senescent characteristic extensively studied (Hampel et al., 2005).

In addition, we explored whether the altered gene expression associated with senescent cells was involved in this protection to die. Thus, we evaluated the mRNA expression of *Bcl-2*, a well-known anti-apoptotic gene (Lin et al., 2001). 14 days BMDM showed high levels of *Bcl-2* mRNA after stimulation with M-CSF and GM-CSF (Figure 16C), suggesting that *Bcl-2* high expression presented by long-lasting BMDM cultures contributed to the protection to apoptosis.

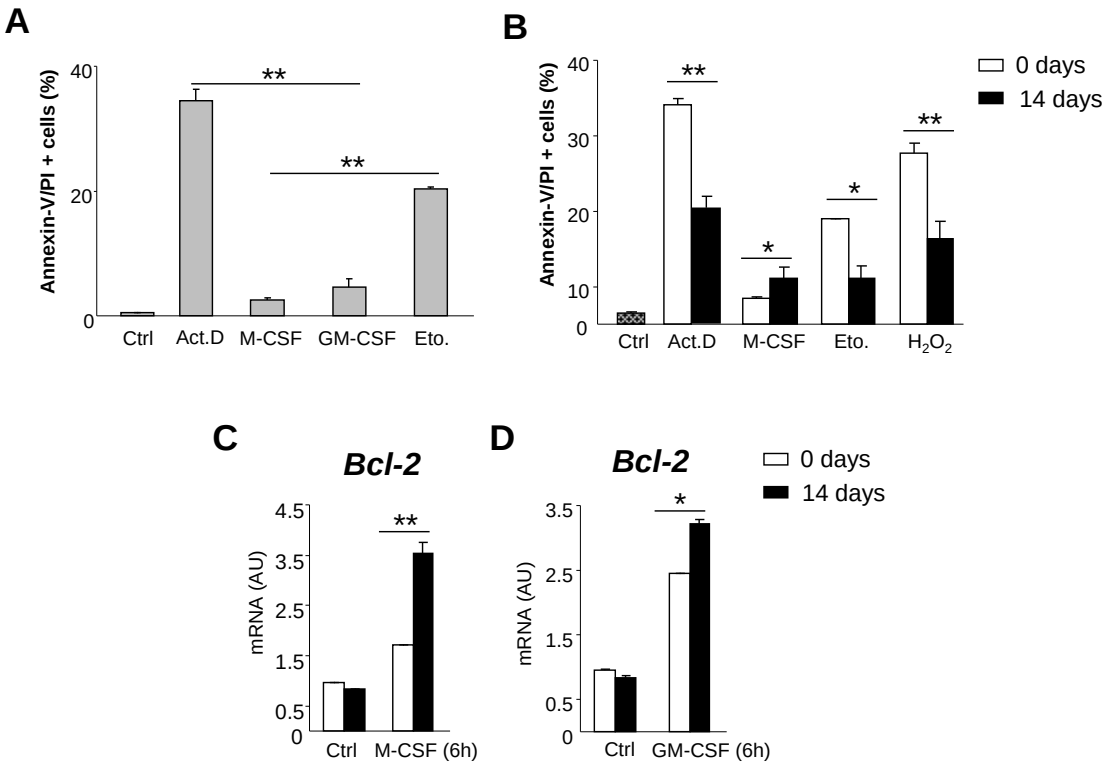


Figure 16. Long-period BMDM cultures are resistant to apoptosis. (A and B) Apoptosis of macrophages cultures from normal and long-term cultures was measured quantifying the annexinV/PI positive cells by flow cytometry. Macrophages were treated with actinomycinD (ActD, 5 µg ml^{-1}) for 12 h, M-CSF (10 ng ml^{-1}), GM-CSF (5 ng ml^{-1}) for 24 h, Etoposide (1 µg ml^{-1} , Eto.) for 1 h and H_2O_2 (5 µg ml^{-1}) for 10 min. (C and D)The levels of *Bcl-2* was measured by qRT-PCR in starved BMDM treated with recombinant M-CSF (10 ng ml^{-1}) and GM-CSF (5 ng ml^{-1}) for the indicated times. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Molecular mechanism involved in cell cycle arrest observed in long-period cultures of BMDM

Cellular senescence is characterized by cessation of cell growth which could be produced after continuous duplication/reproduction of a cell (Campisi et al., 2007). Moreover, with the possible exception of embryonic stem cells (Miura et al., 2004), most division-competent cells, including some tumor cells, can undergo senescence

when appropriately stimulated (Shay and Roninson, 2004; Campisi and d'Adda di Fagagna, 2007).

As permanent cell cycle arrest is the essential feature which describes senescent cells (Kuilman et al., 2008), we decided to study the molecular mechanism involved in the reduced proliferative capacity observed in 14 days BMDM.

As it was demonstrated before (Figure 9B), macrophage proliferation in response to M-CSF and GM-CSF was impaired in 14 days BMDM, measured biochemically and by cellular counting (Figure 17A to C).

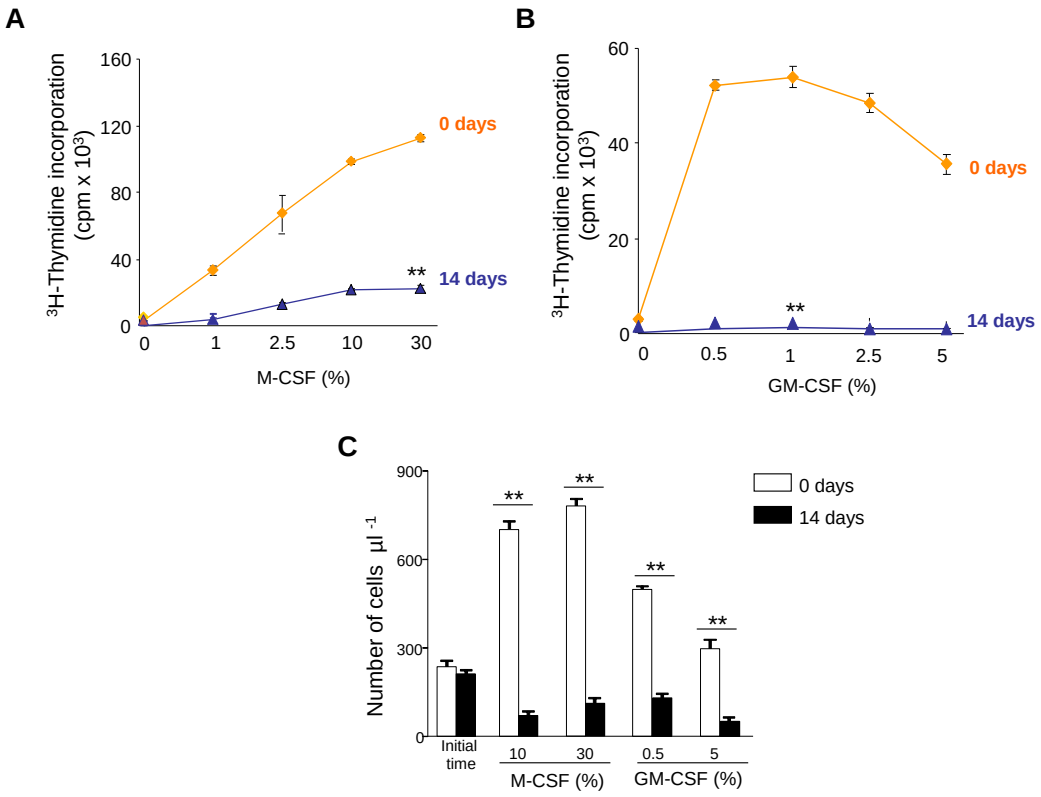


Figure 17. Proliferation is reduced in long-period BMDM cultures. Starved BMDM from normal and long-lasting cultures of macrophages were stimulated with different percentages of M-CSF (%) and GM-CSF (%) conditioned medium for 24 h. (A) Proliferation induced by these growth factors was determined by ^{3}H-thymidine incorporation. Radioactivity was expressed in cpm (counts per minute). (B) Cell number was analyzed by cellular counting using Neubauer chamber. Macrophage concentration was expressed as number of cells µl^{-1}. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. **p<0.01 corresponds to the statistical significance between indicated pair comparisons.

This was also supported by a proliferation assay based on BrdU incorporation. As we expected, after 24 h of M-CSF and GM-CSF treatments, starved 14 days BMDM presented low levels of fluorescence intensity associated with BrdU cellular incorporation (Figure 18A and B).

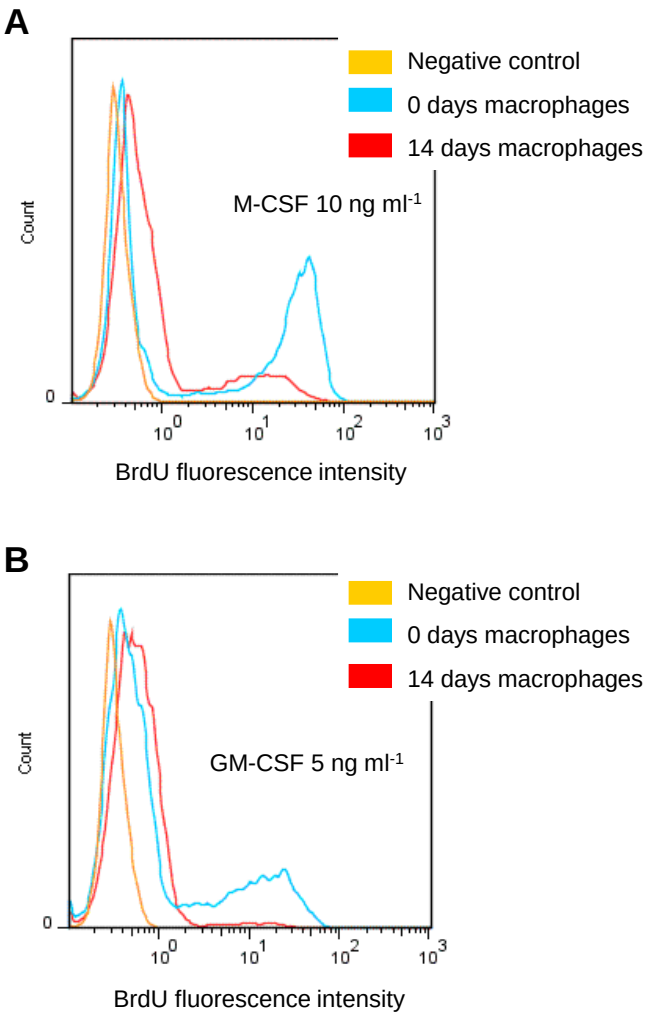
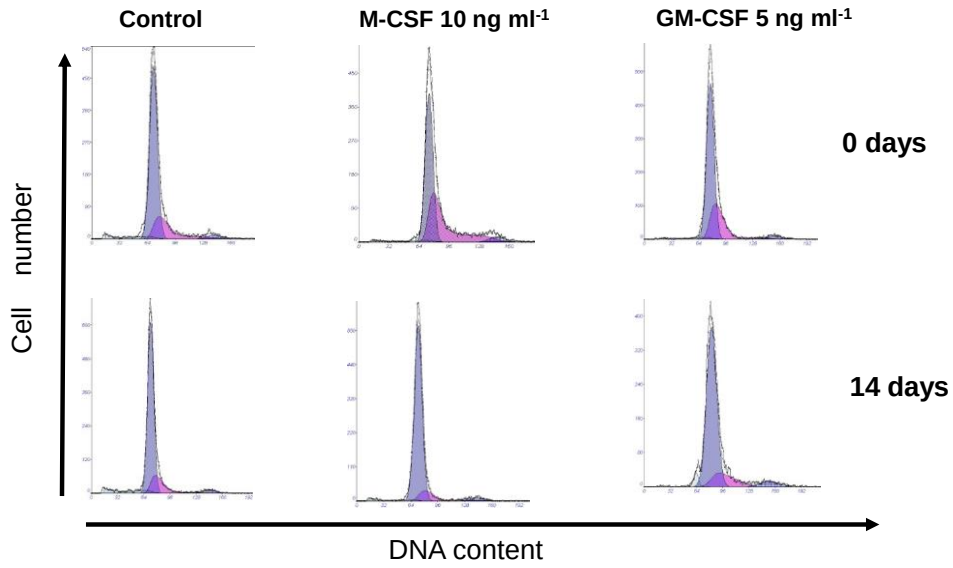


Figure 18. Long-term cultures of BMDM present a decreased BrdU incorporation in response to M-CSF and GM-CSF. (A and B) Starved BMDM were stimulated with recombinant M-CSF (10 ng ml^{-1}) or GM-CSF (5 ng ml^{-1}) for 24 h. Proliferation induced by these growth factors was determined by BrdU incorporation. Proliferation was represented by cell cycle graph and expressed as fluorescence intensity. All assays are representative for at least 3 independent experiments.

Furthermore, this reduced proliferative capacity was also assayed with specific flow cytometry program (Multicycle Program) through the analysis of DNA content with PI. This qualitative and quantitative experiment showed that 14 days BMDM, in response

to recombinant M-CSF and GM-CSF treatments, presented high number of macrophages at G1 phase and very low amount of cells at G2/M phase of the cell cycle comparing with normal BMDM (Figure 19A and B), indicating that 14 days BMDM seem to stop cell cycle at G1 phase.

A



B

	G1	G2/M
0 days		
Ctrl	82.1±2.9	18.9±2.2
M-CSF	53.8±0.7	46.2±1.4
GM-CSF	64.5±1.7	35.5±1.5
14 days		
Ctrl	86.6±0.2	14.4±0.5
M-CSF	78.8±3.6*	21.2±2.2*
GM-CSF	77.5±2.3*	22.5±1.5*

Figure 19. Long-term cultures of BMDM seem to stop cell cycle at G1 phase. (A and B) Starved BMDM from normal and long-term cultures were stimulated with recombinant M-CSF (10 ng ml^{-1}) or GM-CSF (5 ng ml^{-1}) for 24 h. (A) Cell cycle assay was performed with PI and analyzed by flow cytometry. (B) Representative table of quantitative measurement of the above cell cycle analysis. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *$p<0.05$ corresponds to the statistical significance between indicated pair comparisons.

The molecular machinery involved in the cell cycle mechanism is dependent on the activation of a cyclin-dependent kinases (CDKs) protein family (Bringold and Serrano, 2000). When quiescent cells are induced to proliferate, CDK4 and CDK6 are firstly activated by association with cyclin D. Activation of CDK4-6/cyclin D kinase complex leads to subsequent activation of the CDK2/cyclin E and CDK2/cyclin A complexes, which in turns mediate DNA replication and cell cycle progression (Bringold and Serrano, 2000).

Moreover, there is a group of proteins known as Cyclin-dependent kinase inhibitors (CDKi), which reversely regulates cell cycle progression, mainly p16, p19, p21 and p27. p16 and p19 specifically interact and block the CDK4 and CDK6 kinases, inhibiting cell cycle initiation, whereas p21 and p27 inhibit CDK2/cyclin E and A kinases (Canepa et al., 2007).

It has been demonstrated that senescence suppresses the molecular machinery of cell cycle progression inducing the up-regulation of specific cell cycle inhibitors, such as CDKi (mainly p16, p19 or p21) which leads to Rb hyperphosphorylation and cell cycle arrest (Itahana et al., 2004). However, senescence also promotes the reduction of cell cycle positive regulators expression, such as cyclins (mainly cyclin D and cyclin E) and CDKs, such as CDK2 and CDK4 (Taylor and Stark, 2001).

In this sense, in order to investigate the cell cycle blockage presented in 14 days BMDM, we evaluated the mRNA expression of important senescence-related mediators for the cell cycle progression. Normal BMDM showed high levels of *cyclin D1* (*cycD1*) and *cyclin E* (*cycE*) after M-CSF treatment. However, consistently with the published data, 14 days BMDM displayed reduced levels in the same conditions, being more significant the decrease of *cyclin E* mRNA levels than *cyclin D1* (Figure 20A). Similarly, GM-CSF-dependent cell cycle progression induced the expression of *cyclin D1* and *E* in normal BMDM, but mRNA levels of both cyclins were reduced in 14 days BMDM (Figure 20B).

Furthermore, the mRNA expression of other cell cycle positive regulators, *Cdk2* and *Cdk4*, were also increased after M-CSF and GM-CSF stimulation in normal BMDM (Figure 21A and B). Nevertheless, the expression levels of these two kinases were reduced in 14 days BMDM (Figure 21A and B).

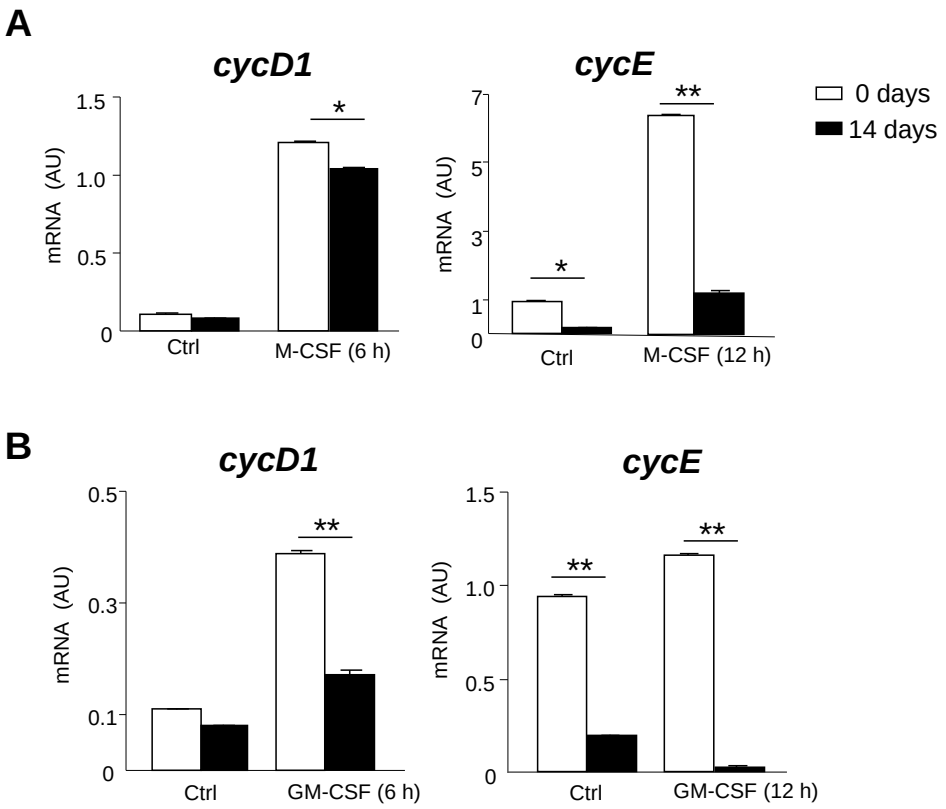


Figure 20. Cell cycle positive regulators are down-regulated in long-lasting cultures of BMDM. (A and B) *CyclineD1 (cycD1) and CyclineE (cycE)* levels were measured qRT-PCR in starved BMDM treated with recombinant M-CSF (10 ng ml^{-1}) and GM-CSF (5 ng ml^{-1}) for the indicated times. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

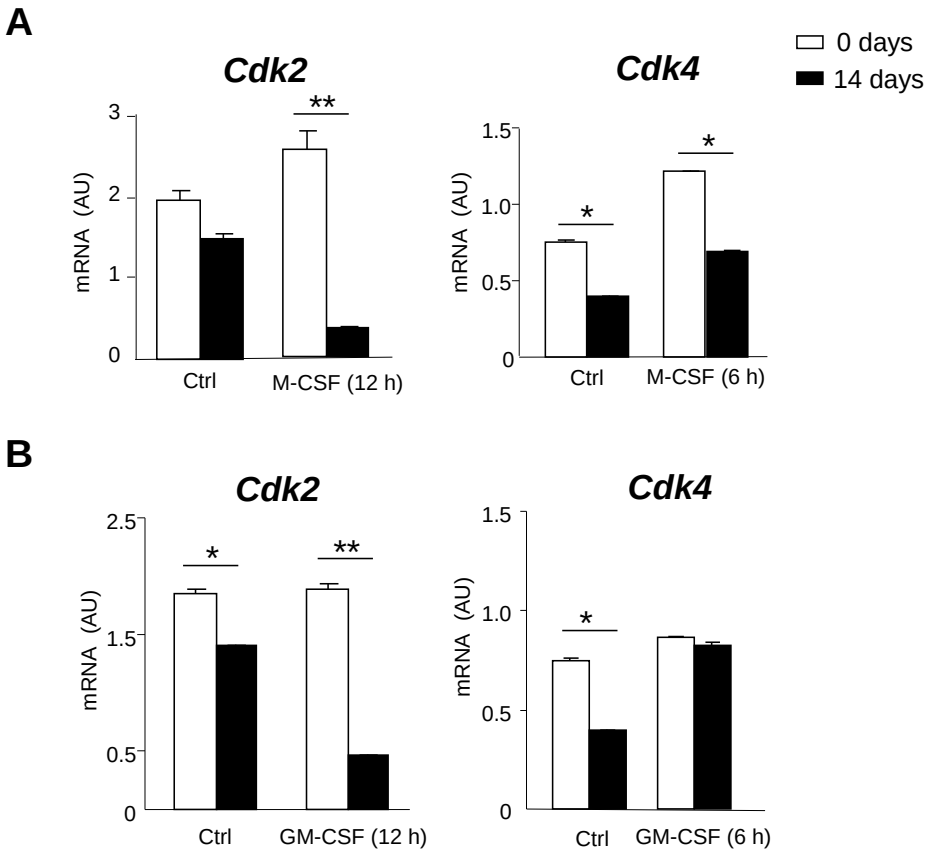


Figure 21. Cell cycle positive regulators are down-regulated in long-lasting cultures of BMDM. (A and B) The levels of *Cycline-dependent kinase2 (Cdk2) and Cycline-dependent kinase4 (Cdk4)* were measured by qRT-PCR in starved BMDM treated with recombinant M-CSF (10 ng ml^{-1}) and GM-CSF (5 ng ml^{-1}) for the indicated times. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

We also explored the potential role of cell cycle inhibitors in macrophage senescence-dependent proliferation blockage. In this sense, we stimulated normal and long-lasting cultures of BMDM with M-CSF or GM-CSF growth factors. Whereas we could not detect differences in *p21* mRNA levels between normal and 14 days BMDM, the reduced proliferative capacity presented in long-period cultures of BMDM seems to be affected by extraordinary levels of *p16* and high levels of *p27* mRNA expression in 14 days BMDM (Figure 22A and B).

Taken together, these data suggest that 14 days BMDM are unable to progress in the cell cycle mechanism due to the impaired cell cycle regulation. On the one hand,

positive regulators of cell cycle, such as *cyclin E* and *Cdk2*, are down-regulated, obtaining a reduced amount of cyclin-kinase complexes and, therefore, damaging the advancement of cell cycle. Additionally, this is also accompanied and sustained by the overexpression of some CDKi, such as *p27* and *p16*.

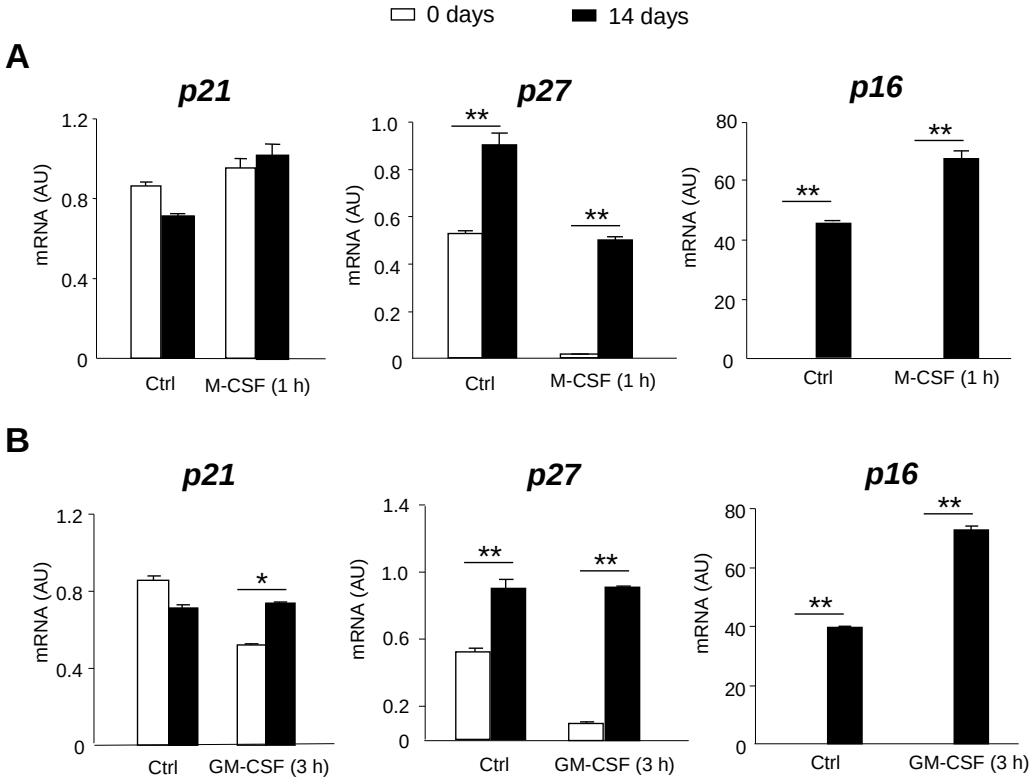


Figure 22. *p27* and *p16*, mainly, are involved in the cell cycle blockage of long-lasting cultures of BMDM. (A and B) The levels of *p21, p27* and *p16* were measured by real-time PCR in starved BMDM treated with recombinant M-CSF (10 ng ml^{-1}) and GM-CSF (5 ng ml^{-1}) for the indicated times. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Next, to further evaluate the involvement of senescence-related oncogenes (OIS) in the impaired cell cycle regulation observed in long-lasting cultures of BMDM, we measured the mRNA levels of some oncogenes, such as *c-Myc*, *Raf* and *E2F1*, that are also important senescence markers (Grandori et al., 2003; Courtois-Cox et al., 2006; Coutois-Cox et al., 2008).

c-Myc is an oncogenic transcription factor that is frequently upregulated in human malignancies (Nesbit et al., 1999), such as melanoma (Ross and Wilson, 1998; Greulich et al., 2000; Kraehn et al., 2001). In normal human cells, overexpression *of c-Myc* promotes the acquisition of a transformed phenotype in cooperation with other cellular oncogenes (Boehm et al., 2005). Moreover, in mice *c-Myc* upregulation has been shown to induce tumorigenesis in numerous transgenic models (Lutz et al., 2002). However, it is also demonstrated that the overexpression of *c-Myc* is correlated with the progression and maintenance of cellular senescence (Zhuang et al., 2008).

In the case of *Raf* oncogene its overexpression can promote a robust induction of senescence produced by two different senescence pathways that work in parallel: the accumulation of CDKi, such as p16, p21 and p27 and the activation of DNA-damage mechanisms with the subsequent phosphorylation of ɣ-H2AX (Jeanblanc et al., 2012).

Similarly, the transcription factor *E2F1* has been proved to be a cellular senescence modulator whose overexpression suppresses the progression of cancer and induce cellular senescence, providing the possibility to use *E2F1* as a therapeutic target in the treatment of cancer (Park et al., 2006).

As we expected, 14 days BMDM showed increased mRNA expression of *c-Myc* after M-CSF and GM-CSF treatment comparing with the amounts produced by normal BMDM (Figure 23A and B). Similarly, the stimulation of 14 days BMDM with both growth factors induced high levels of *Raf* and *E2F1* mRNA expression (Figure 23A and B), indicating that cellular senescence induced in long-period cultures of BMDM is also supported by oncogenes overexpression.

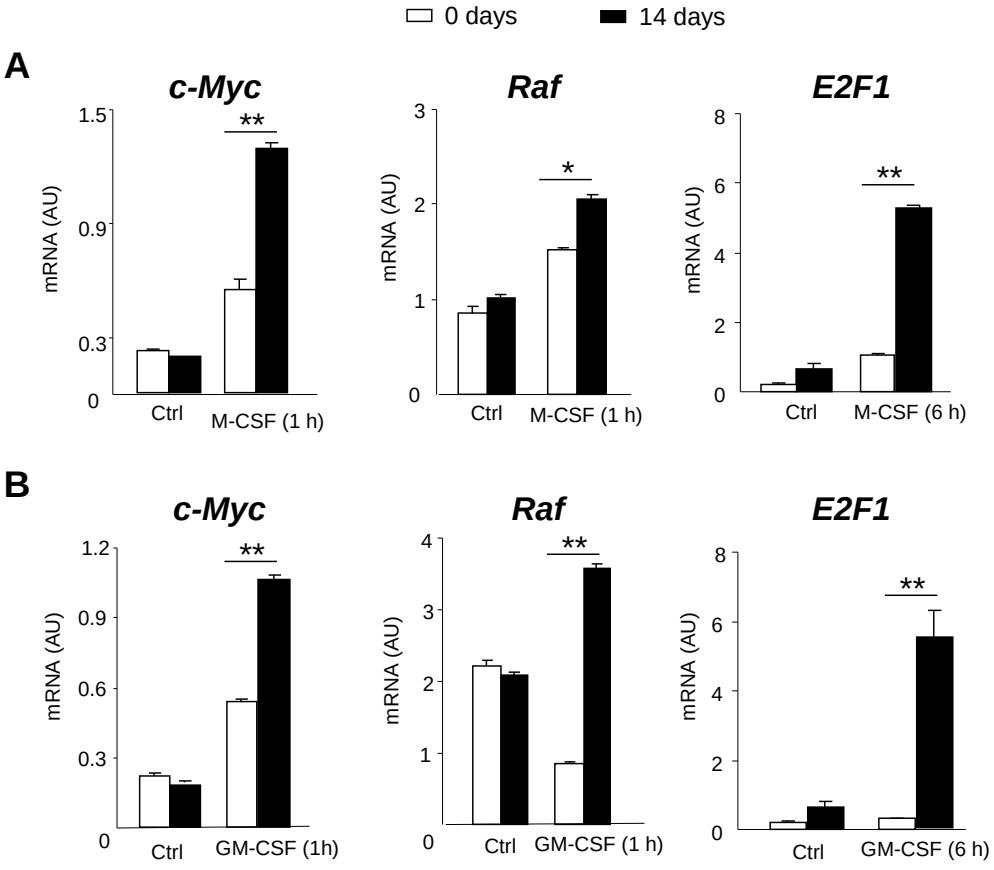


Figure 23. Senescence markers involved in cell cycle regulation are overexpressed in long-lasting cultures of BMDM. (A and B) The levels of *c-Myc, Raf and E2F1* were measured by qRT-PCR in starved BMDM treated with recombinant M-CSF (10 ng ml^{-1}) and GM-CSF (5 ng ml^{-1}) for the indicated times. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Furthermore, in a previous work from our laboratory, it was demonstrated that GM-CSF growing pathway was altered in macrophages from old mice due to Janus kinase 2 (Jak2)/STAT5 impaired signaling pathway (Sebastián et al., 2009). STAT5 is involved in the proliferation of many cell types. Once phosphorylated, STAT5 dimerizes and translocates to the nucleus where it binds to DNA-specific sites and activates the transcription of target genes (Hou et al., 1995; de Groot et al., 1998). In this sense, the cell cycle arrest shown by 14 days BMDM was correlated with a decrease in the phosphorylation of STAT5 (Figure 24A and B) in response to GM-CSF.

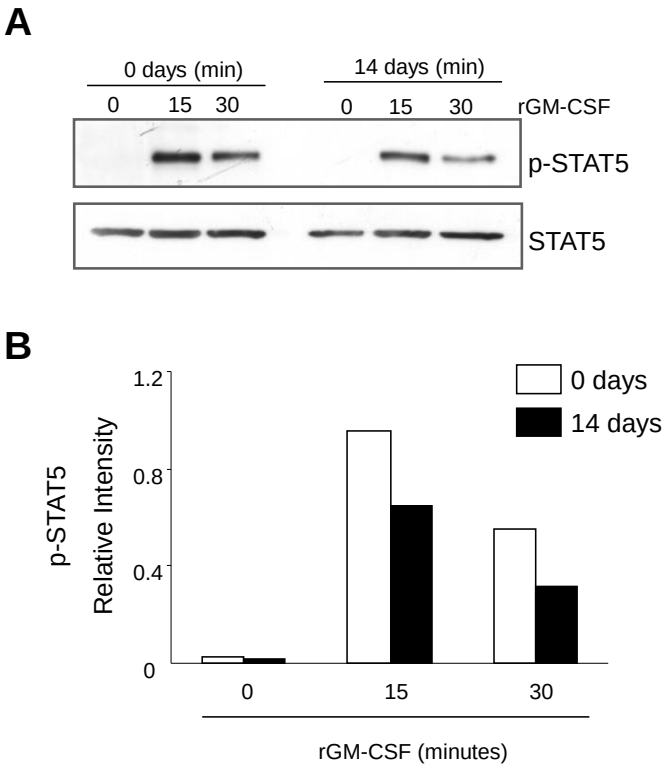


Figure 24. The phosphorylation of STAT5 is reduced in long-period BMDM cultures. Starved BMDM were stimulated with recombinant GM-CSF (5 ng ml^{-1}) for the times indicated. (A) STAT5 phosphorylation was determined by p-STAT5 immunoblotting. (B) Representative graph of the quantitative measurement of the immunoblotting above. STAT5 image was used as loading control for western blots. All assays are representative for at least 3 independent experiments.

Next, in order to investigate the signaling pathway involved in the blockage of cell cycle progression presented by long-lasting cultures of BMDM, we also evaluate the activation of crucial MAPKs implicated in macrophage proliferation, such as extracellular signal-related kinase (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK (Valledor et al., 2008). MAPKs are evolutionarily conserved serine/threonine kinases involved in the transduction of externally derived signals that regulate cell growth, differentiation and apoptosis (Seger et al., 1995).

These kinases directly modulate downstream targets by phosphorylation, including additional protein kinases, components of the cytoskeleton and transcriptional regulators which in turn promote immediate early gene expression (Valledor et al., 2008).

In a previous study, we observed that activation of the ERK cascade is specifically required for macrophages to proliferate in response to M-CSF and GM-CSF (Valledor

et al., 1999). The proliferation of normal BMDM induced by GM-CSF produced the phosphorylation at 15 min of p38 MAPK, JNK and ERK1/2 (Figure 25A and B). However, GM-CSF induced an overactivation and more sustained ERK and p38 MAPK phosphorylation in 14 days BMDM, whereas JNK activity was not affected, as determined by the prolonged phosphorylation kinetics (up to 2 h) presented by ERK and p38 MAPK activity in response to GM-CSF (Figure 25B).

Previous studies of our research group have demonstrated that alteration in the kinetics of ERK1/2 phosphorylation have profound effects on macrophage proliferation and activation (Valledor et al., 2008), but no previous data on the role of p38 MAPK was described in those studies.

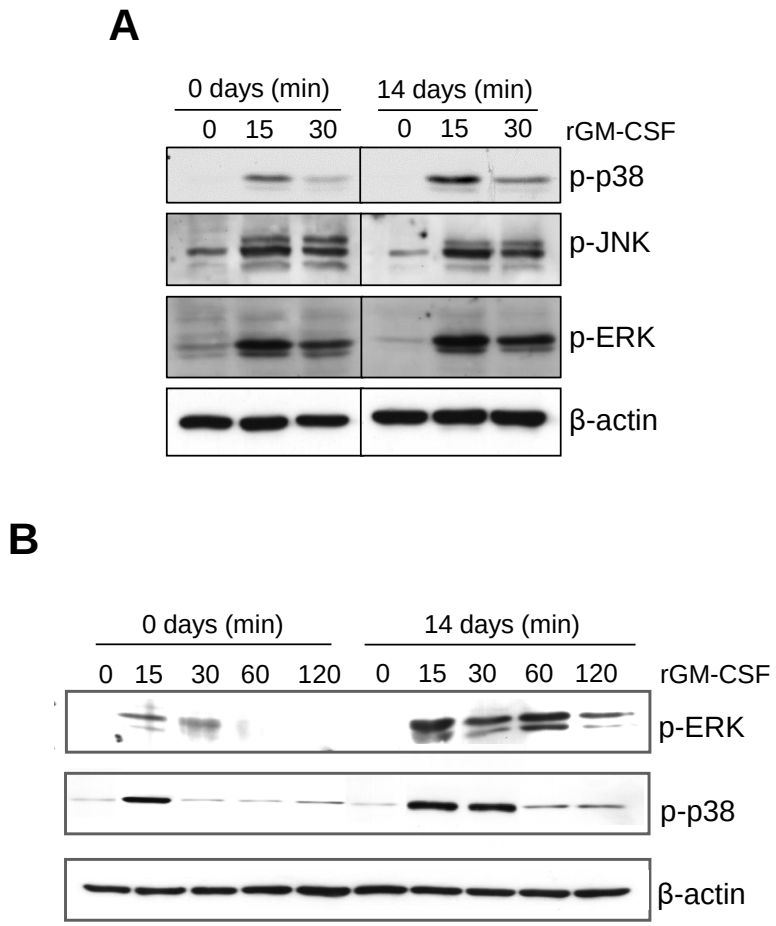


Figure 25. GM-CSF treatment induces prolongation of p38 MAPK and ERK phosphorylation in long-period BMDM cultures. Starved BMDM were stimulated with recombinant GM-CSF (5 ng ml^{-1}) for the times indicated. (A and B) ERK, JNK and p38 phosphorylation was determined by p-ERK, p-JNK and p-p38 immunoblotting. β-actin image was used as loading control for western blots. All assays are representative for at least 3 independent experiments.

Interestingly, this also occurred when these cells were stimulated with M-CSF (Figure 26A and B) but this behavior is distinct to macrophages from old mice since they did not alter MAPK activity (Sebastián et al., 2009). Nevertheless, some data implicate MAPK overactivation with senescence (Sebastián, Lloberas and Celada, 2009). These observations suggest that STAT5, ERK and p38 activity play an important role in the arrest of cell cycle progression induced by GM-CSF and M-CSF in long-term cultures of BMDM.

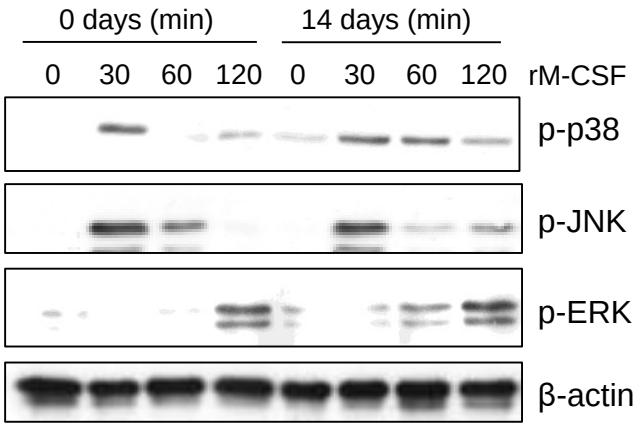


Figure 26. M-CSF treatment induces prolongation of p38 MAPK phosphorylation in long-period BMDM cultures. Starved BMDM were stimulated with recombinant M-CSF (10 ng ml^{-1}) for the times indicated. (A and B) ERK, JNK and p38 phosphorylation was determined by p-ERK, p-JNK and p-p38 immunoblotting. β-actin image was used as loading control for western blots. All assays are representative for at least 3 independent experiments.

p16 **and telomeres shortening are involved in the cellular senescence molecular mechanisms of long-period BMDM cultures**

At molecular level, replicative senescence is controlled by two different pathways which comprise the tumor suppressor proteins pRb and p53 and their corresponding effectors p16 and p21 (Dimri and Campisi, 1995; Campisi, 2001). Consistently, inactivation of these proteins results in bypass of senescence.

In this regard, we evaluated the possibility that p53 pathway was involved in the senescence mechanism induced in long-period cultures of BMDM. Normal and 14 days BMDM were stimulated with recombinant M-CSF (growing conditions) and with etoposide (Eto.) and H_2O_2 treatments, as DNA-damage inducers (p53 positive activators) and p53 phosphorylation was measured by western blotting. In both, normal and 14 days BMDM, p53 was not activated by M-CSF treatment, whereas etoposide and H_2O_2 stimulation induced the phosphorylation of p53 at similar levels (Figure 27A). Additionally, as it was demonstrated before (Figure 22), *p21* mRNA expression was similar in both types of macrophage cultures, indicating that p53-p21 senescence pathway is not involved in the molecular mechanism induced in long-term cultures of BMDM (Figure 27B).

In contrast, *p16* mRNA levels were extraordinary high in 14 days BMDM comparing with normal macrophages after stimulation with recombinant M-CSF and GM-CSF growing factors (Figure 27C), suggesting that pRb-p16 pathway seems to be the molecular mechanism implicated in the senescence process produced in 14 days BMDM.

The relative contribution of pRb and p53 cascades to senescence depends on the cell type but both mechanisms ultimately undergo telomeric crisis, resulting in chromosomal instability (Shay and Wright, 2005). Therefore, the dependence of replicative senescence on telomere shortening is evident from its bypass by the ectopic expression of the catalytic subunit of the telomerase holoenzyme (hTERT), which elongates telomeres, thereby abrogating the effect of the end replication problem (Bodnar et al. 1998; Vaziri and Benchimol 1998). In contrast to those cells that need to divide regularly (stem cells and immune cells, among others), many somatic cells are not able to express telomerase, thus, they are incapable to maintain telomeres at a sufficient length to prevent DDR (Masutomi et al., 2003). In this sense, we wanted to determine the importance of telomere attrition observed for the macrophage senescence-related impaired proliferative capacity induced in long-period cultures of BMDM.

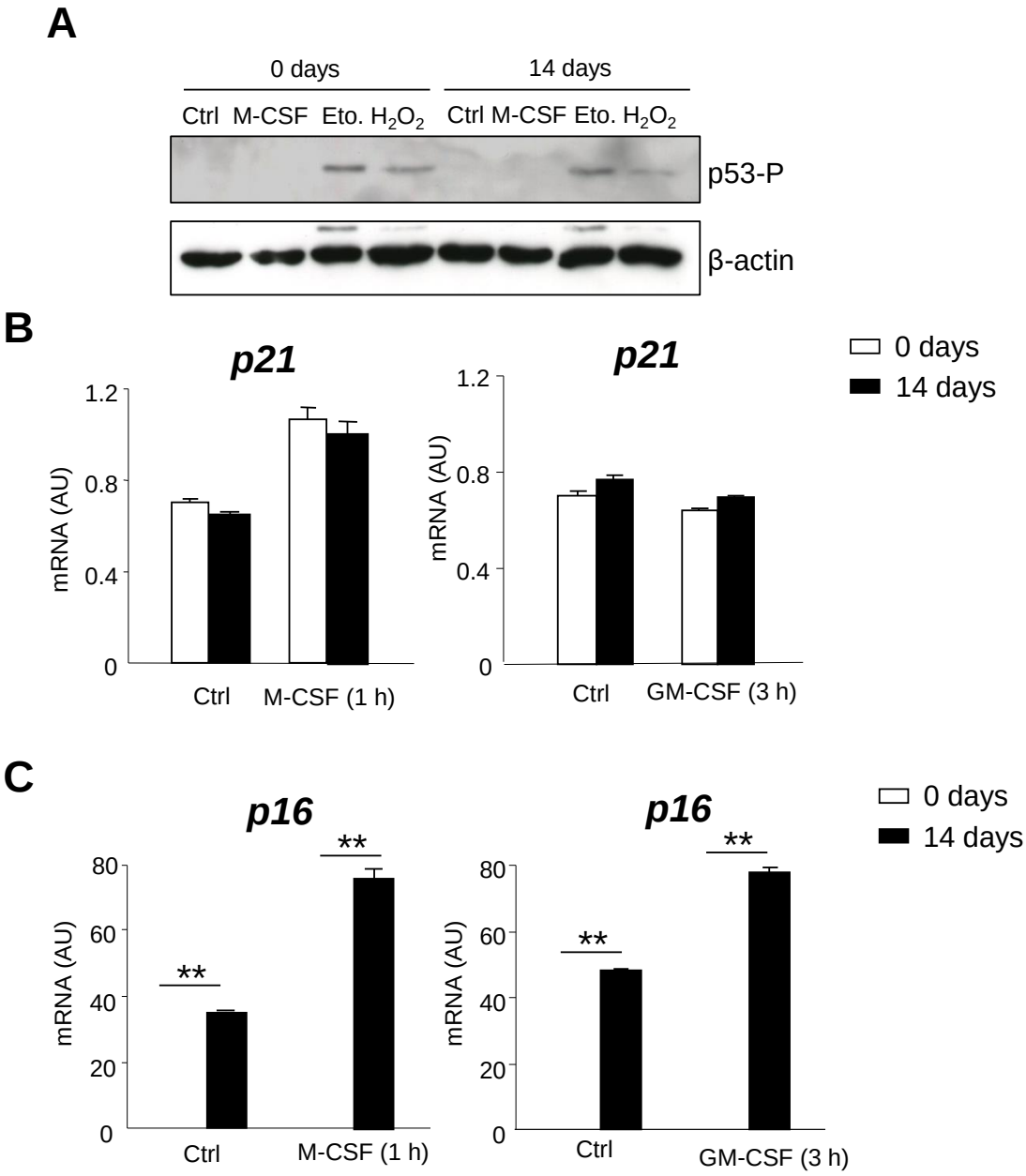


Figure 27. The mechanism involved in the senescence process of long-lasting BMDM cultures seems to be driven by p16. (A) Starved BMDM were stimulated with recombinant M-CSF (10 ng ml^{-1}), Etoposide (1 µg ml^{-1} , Eto.) and H_2O_2 (5 µg ml^{-1}) for 30 min. p53 phosphorylation was measured by p-p53 immunoblotting. β-actin image was used as loading control for western blots. (B and C) The levels of *p21* and *p16* were measured by qRT-PCR in starved BMDM treated with recombinant M-CSF (10 ng ml^{-1}) and GM-CSF (5 ng ml^{-1}) for the indicated times. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. **p<0.01 correspond to the statistical significance between indicated pair comparisons.

For this purpose, we used a weak telomerase activator, termed TA-65 that promotes the elongation of telomere caps on chromosomes (Harley et al., 2011). During long-

period cultures of BMDM generation, TA-65 was added into the complete medium every 48 h up to get 14 days BMDM, and adding DMSO in normal and control 14 days macrophages to adjust the assay. To determine the optimal concentration of TA-65 pre-treatment that induces the elongation of telomeres, first we evaluated the telomere length of normal and 14 days BMDM in the presence or absence of different concentrations of TA-65 through qRT-PCR, being 10 µM the concentration of TA-65 that enhances the highest level of T/S ratio in 14 days BMDM (Figure 28A). Moreover, the increase in the telomere length observed in 14 days BMDM induced by TA-65 (Figure 28B) was correlated with an incomplete increase in the proliferative capacity of 14 days BMDM in response to M-CSF and GM-CSF (Figure 28C), suggesting that telomeres attrition plays a partial role in the impaired proliferative capacity of long-period cultures of BMDM.

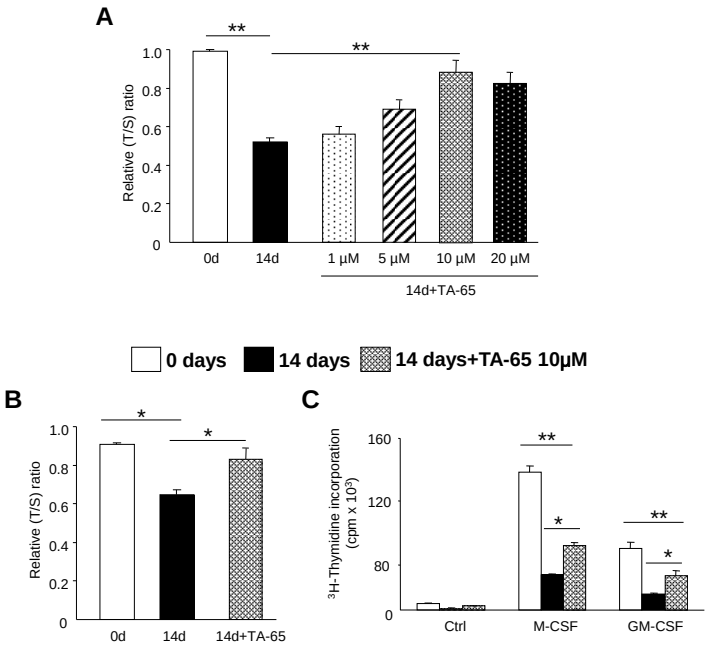


Figure 28. Telomerase activation partially rescues the impaired proliferative capacity of long-period BMDM cultures. (A and B) Telomere length was measured by qRT-PCR in normal and 14 days macrophages with or without TA-65 at the concentrations indicated (see Materials and Methods section). The ratio of relative telomere to single copy gene (36B4) amplification (T/S) was shown by two independent experiments. (C) Starved BMDM were stimulated with M-CSF (30 %) and GM-CSF (1 %) conditioned medium for 24 h with or without TA-65 (10 µM). Proliferation induced was determined by ^{3}H-thymidine incorporation. Radioactivity was expressed in cpm (counts per minute). Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Determination of functional activities in senescent macrophages

Classical and alternative activation phenotypes are impaired in long-term BMDM cultures

The idea of macrophage activation appeared as a result of the observation that macrophages treated with bacterial constituents, such as LPS or flagellin, and IFN-γ developed an enhanced capacity to destroy a wide range of ingested pathogens. Classically activated macrophages (M1 macrophages) have an increased ability to present antigen since they increase the expression of MHC II and CD80/CD86 (Herrero et al., 2002). In addition, M1 macrophages mediate diverse inflammatory effects secreting a variety of cytokines and destroying intracellular pathogens through increasing the production of NO and other effector molecules (Xu and Gordon, 2003).

In contrast, alternative activated macrophages (M2 macrophages), produced by IL-4 or IL-13 treatments, do not display an increased oxidative burst and are inefficient in killing intracellular pathogens. These macrophages are important in clearance of parasitic and extracellular pathogens and promote cell growth and tissue remodeling through the induction of arginase (Gratchev et al., 2001). Moreover, unlike classical macrophage phenotype, M2 macrophages increase the expression of *mannose receptor 1* (*mrc1*) and *macrophage galactose-type lectin 1* (*mgl1*) and specific transcription factors such as *resistin-like molecule alpha* (*Fizz1*) (Stein et al., 1992; Iniesta et al., 2001; Gratchev et al., 2001; Gordon, 2003).

Due to the importance of macrophage activities in the steady state or during an inflammatory process, we next decided to study important macrophage functions based on phenotypic characteristics. Regarding to the macrophage classical activation, we analyzed MHCII mRNA and protein expression induced by IFN-γ in 14 days BMDM. As it was reported in macrophages from old mice (Herrero et al., 2002), we observed a reduction of *I-Ab* expression after 48 h of IFN-γ treatment (Figure 29A). Consistent with the decreased mRNA levels, the protein surface expression of I-Ab was also reduced in 14 days BMDM samples (Figure 29B and C), indicating that antigen presentation capacity is declined in long-period cultures of BMDM.

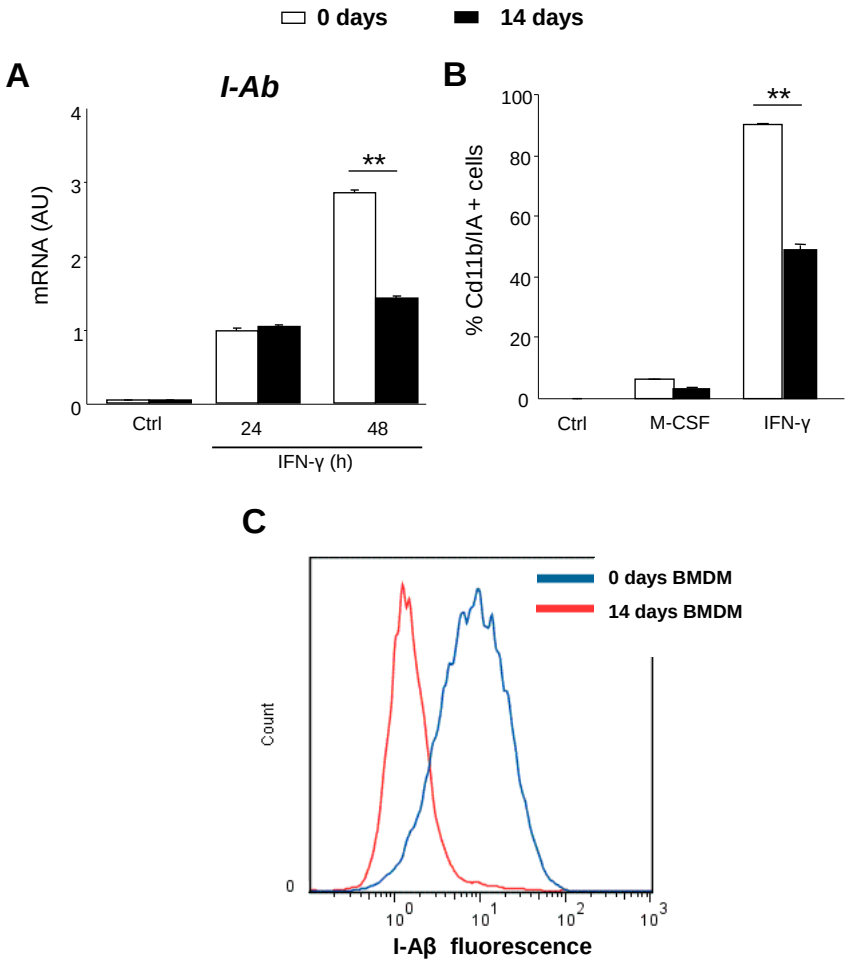


Figure 29. Antigen presentation capacity is impaired in long-period BMDM cultures. (A) The levels of *I-Ab* were measured by qRT-PCR in starved BMDM treated with recombinant IFN-γ (20 µg ml^{-1}) for the times indicated. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. (B and C) MHCII surface expression was measured by flow cytometry. BMDM were stimulated with recombinant IFN-γ (20 µg ml^{-1}) for 48 h and stained with I-Aβ-FITC and Cd11b-PE antibodies. Data were expressed as percentage of positive cells (B) or fluorescence intensity (C). Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. **p<0.01 correspond to the statistical significance between indicated pair comparisons.

In addition, we proposed determine other essential M1 functions such as the ability to destroy intracellular pathogens through the oxidative burst. In this sense, the stimulation with LPS induced the mRNA and protein expression of NOS2 at high levels in normal BMDM (Figure 30A and B). However, 14 days BMDM presented a significant decreased of NOS2 expression at mRNA and protein levels in response to LPS (Figure 30A and B). Correlating with these data, we observed a diminished NO production

induced by LPS or by LPS plus IFN-γ in 14 days BMDM, suggesting that the microbicidal activity was highly reduced in these macrophages (Figure 30C).

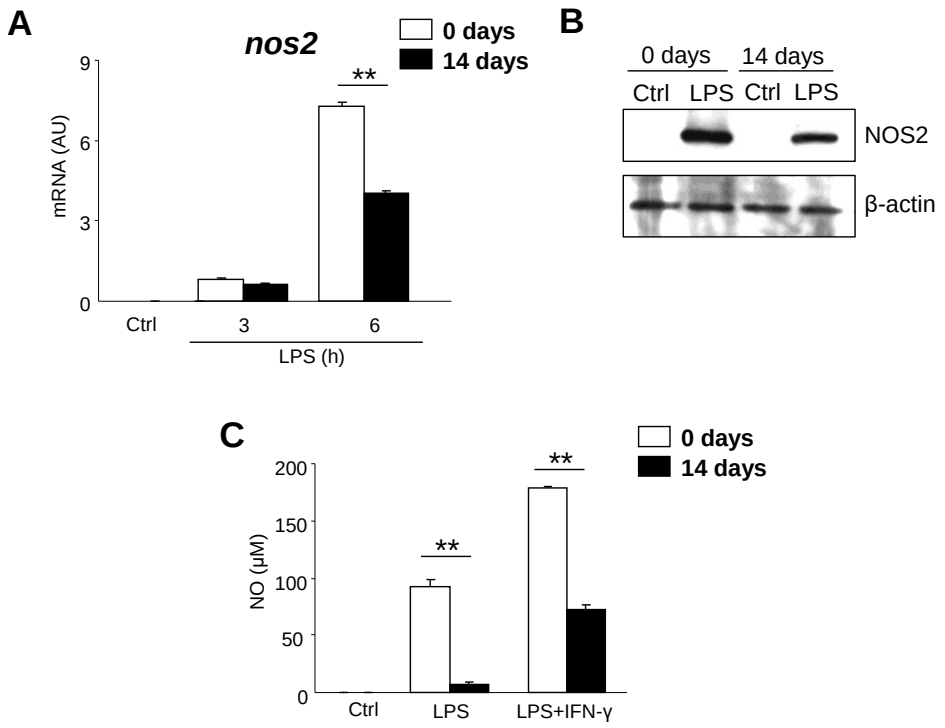


Figure 30. Microbicidal activity is reduced in long-period BMDM cultures. (A) The levels of *nos2* was measured by qRT-PCR in starved BMDM treated with LPS (10 ng ml^{-1}) for the times indicated. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. (B) BMDM were stimulated with LPS (10 ng ml^{-1}) for 24 h. NOS2 protein expression was measured by western blot. β-actin image was used as loading control for western blots. (C) NO production was analyzed by absorbance. BMDM were stimulated with LPS (10 ng ml^{-1}) or with LPS (10 ng ml^{-1}) plus recombinant IFN-γ (20 μg ml^{-1}) for 24 h. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Next, we also evaluated the expression and secretion of pro-inflammatory cytokines in response to LPS. Thus, normal and 14 days BMDM were treated with LPS at different time points and mRNA and protein levels of IL-1β and TNF-α were measured. IL-1β mRNA and protein expression was increased in 14 days BMDM in response to LPS (Figure 31A and B). Consistently, the treatment with ATP, required to induce the release of IL-1β in LPS-primed macrophages through inflammasome activation (Schroder and Tschopp, 2010; Elinav et al., 2013), produced a rise in IL-1β production

in 14 days BMDM (Figure 31C). Similarly, the mRNA levels (Figure 31D) and the secretion of TNF-α (Figure 31E) were also augmented in 14 days BMDM.

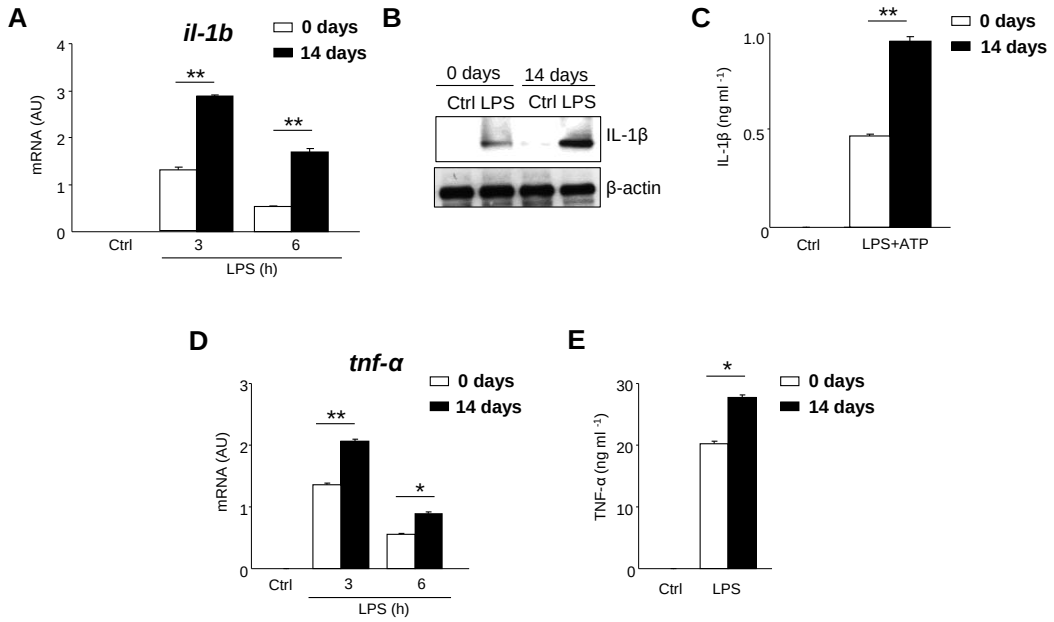


Figure 31. Pro-inflammatory cytokines secretion is increased in long-period BMDM cultures. (A and D) The levels of *il-1b* and *tnf-α* were measured by qRT-PCR in starved BMDM treated with LPS (10 ng ml^{-1}) for the times indicated. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. (B) BMDM were stimulated with LPS (10 ng ml^{-1}) for 24 h. IL-1β protein expression was measured by western blot. β-actin image was used as loading control for western blots. (C and E) Cytokine production was analyzed by ELISA. BMDM were stimulated with LPS (10 ng ml^{-1}) or with LPS (10 ng ml^{-1}) plus ATP (1 mM) for 24 h. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Since it was clear that pro-inflammatory cytokines were overexpressed in long-period cultures of BMDM, we also decided to determine the levels of IL-10, an anti-inflammatory cytokine crucial for limiting the innate immune response to pathogens (Saraiva and O'Garra, 2010). IL-10 is transcriptionally induced by LPS stimulation via STAT-3 activation (Carl et al., 2004) and has an inhibitory effect on the production of several inflammatory cytokines including TNF-α (Armstrong et al., 1996). *Il-10* mRNA and secretion levels were higher in 14 days BMDM comparing with normal BMDM in response to LPS (Figure 32A and B). Additionally, LPS treatment induced a peak of STAT-3 phosphorylation at 15 min in normal BMDM (Figure 32C). However, as we

expected, 14 days BMDM presented an increase STAT-3 activity whose phosphorylation was also sustained at 30 min (Figure 32C).

Taken together, these results indicate that macrophage M1 activation by IFN-γ and LPS stimulation were deficient in long-period BMDM cultures.

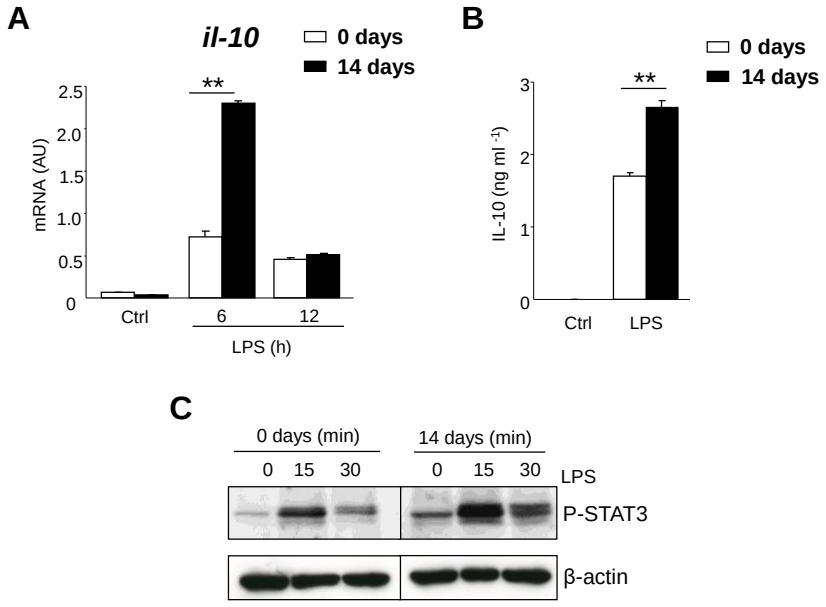


Figure 32. IL-10 secretion is increased in long-period BMDM cultures. (A) The levels of *il-10* were measured by qRT-PCR in starved BMDM treated with LPS (10 ng ml^{-1}) for the times indicated. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. (B) BMDM were stimulated with LPS (10 ng ml^{-1}) for 24 h and IL-10 production was analyzed by ELISA. (C) BMDM were stimulated with LPS (10 ng ml^{-1}) for the times indicated and STAT-3 phosphorylation was measured by western blot. β-actin image was used as loading control for western blots. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. **p<0.01 correspond to the statistical significance between indicated pair comparisons.

An innovative hypothesis of cellular senescence contribution to the age-related changes of the immune system explains that immunosenescence and inflammaging can also be derived from a dysfunction of TLRs expression and TLRs signaling since TLRs activation results in pro-inflammatory mediators induction (Olivieri et al., 2013).

The age-related expression of the TLRs has been extensively studied in cells of the innate immunity, mainly neutrophils and macrophages obtaining complex results. In this sense, we decided to evaluate the surface (TLR-2, -4 and -5) or intracellular (TLR-9) protein levels of several TLRs in our cellular model in response to LPS stimulation.

Whereas we did not observe any difference in TLRs expression at basal levels between normal and 14 days BMDM, TLRs levels after LPS treatment showed alterations (Figure 33). In the case of TLR-2 expression, 14 days BMDM presented lower percentage of positive cells than normal macrophages (Figure 33A). In contrast, TLR-4, -5 and -9 were overexpressed in these cells after LPS treatment (Figure 33B to D), which could explain, in part, the increase in pro-inflammatory cytokines expression shown in long-period cultures of BMDM.

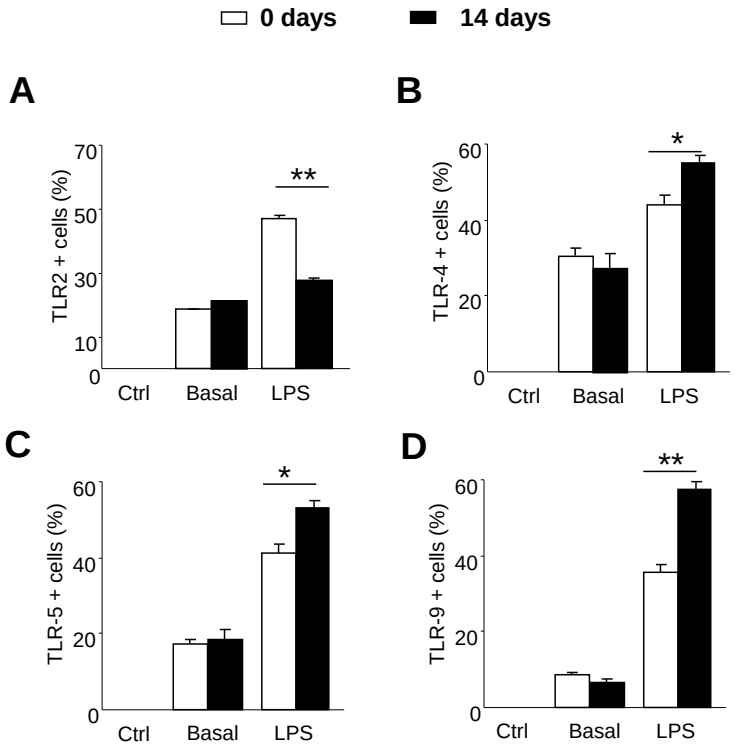


Figure 33. The expression of TLR-4, -5 and -9 are increased in long-period BMDM cultures. (A to D) TLRs surface expression was measured by flow cytometry. BMDM were stimulated with LPS (10 ng ml^{-1}) for 24 h and stained with TLR-2, 4, 5, 9-FITC antibodies. Data were expressed as percentage of positive cells. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Regarding to the alternatively activated macrophage functions, first we evaluated the M2 most important marker: the expression and activity of arginase-1 (Gordon, 2003). Normal and 14 days BMDM were stimulated with IL-4 for 8 or 24h in order to induce the M2 macrophage switching. As we expected, normal BMDM treated with IL-4 presented high mRNA levels of *arginase-1* (*arg1*) (Figure 34A). However, *arginase-1*

expression was significantly reduced in 14 days BMDM (Figure 34A). Additionally, we also measured the enzyme activity through a colorimetric method based on the reaction produced between arginase and the aminoacid L-arginine. Arginase activity was declined in 14 days BMDM after IL-4 treatment (Figure 34B), indicating that tissue repair activity is impaired in long-term cultures of BMDM.

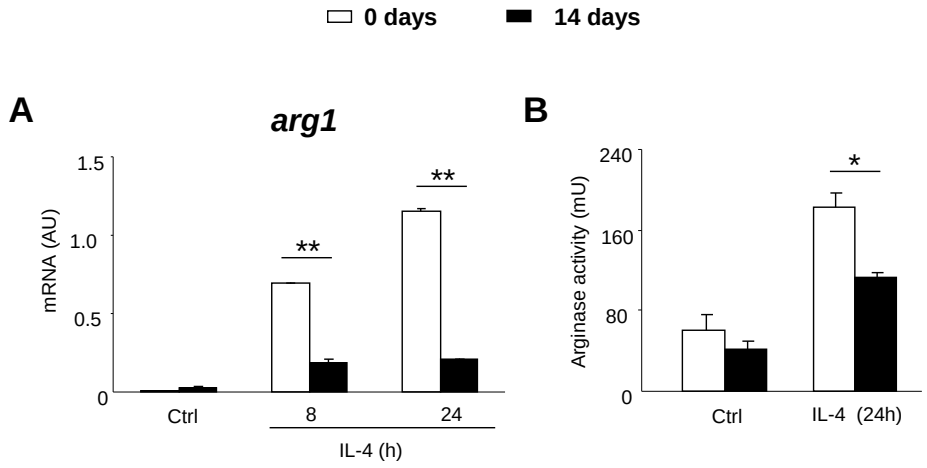


Figure 34. Arginase activity is reduced in long-period BMDM cultures. (A) The levels of *argI* was measured by qRT-PCR in starved BMDM treated with IL-4 (10 ng ml^{-1}) for the times indicated. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. (B) BMDM were stimulated with IL-4 (10 ng ml^{-1}) for 24 h and arginase activity was measured by absorbance. Data were expressed as enzymatic activity (mU). Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Moreover, we analyzed some transcription factor or genes involved in M2 phenotype after IL-4 stimulation. In the case of *mgl1* and *fizz1* were also reduced in 14 days BMDM (Figure 35A and B) confirming few data concerning how aging may affect the alternative activation of macrophages (Smith et al., 2001). However, M2 genes such as *mrc1* and *tgfβ* were increased and not affected, respectively (Figure 35C and D) indicating that some, but not all, M2 genes contribute to the altered long-lasting cultures of BMDM M2 functions.

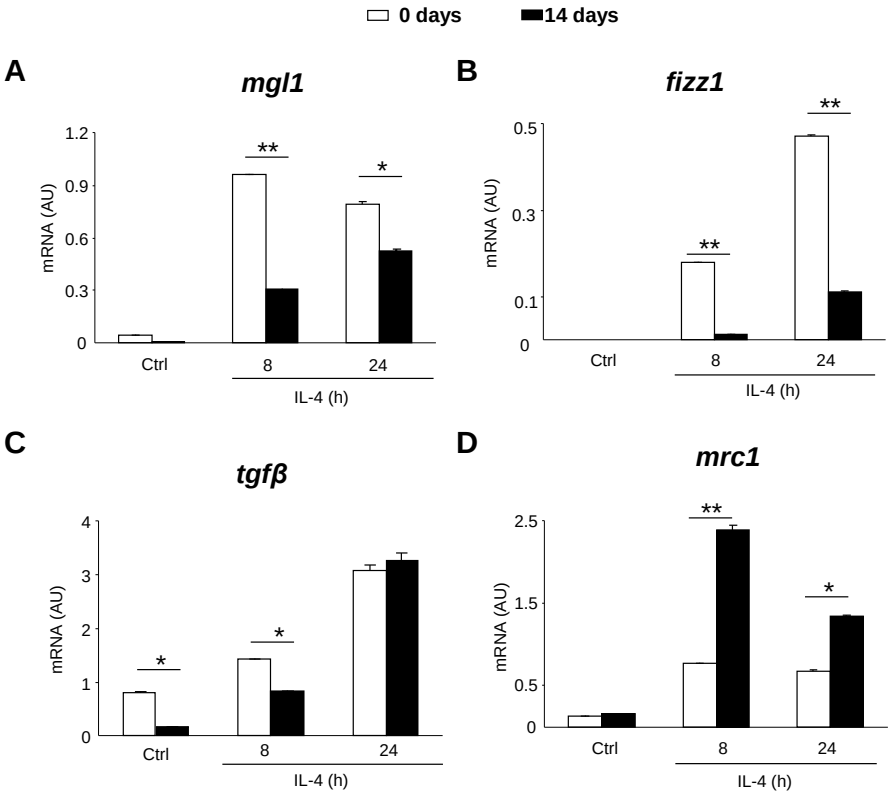


Figure 35. Some genes involved in the alternative activation are differently expressed in long-period BMDM cultures. (A to D) The levels of *mgl1, fizz1, tgfβ* and *mrc1* were measured by qRT-PCR in starved BMDM treated with IL-4 (10 ng ml^{-1}) for the times indicated. Data were expressed as relative mRNA levels, arbitrary units (AU), normalized to the *Hprt1* expression level in each sample. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. *p<0.05; **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Finally, to complete the functionality study of long-term cultures of BMDM, we evaluated the phagocytic activity, one of the most important actions that macrophages carry out.

Phagocytosis by macrophages is critical for uptake and degradation of infectious agents and senescent or apoptotic cells, and it participates in development, tissue remodeling, immune response and inflammation (Aderem and Underhill, 1999). Phagocytosis is extremely complex and no single model can fully account for the diverse structures and outcomes associated with particle internalization. This complexity is in part due to the diversity of receptors capable to stimulate phagocytosis and also due to the capacity of a variety of microbes to influence their fate as they are internalized (Aderem and Underhill, 1999).

In this sense, we measured the ability of senescent macrophages derived from our cellular model to engulf apoptotic cells. For this purpose, we used MDA-MB-231/GFP (MDA-GFP) cell line, green-fluorescence epithelial cells which stably express GFP. To obtain apoptotic MDA-GFP cells, we treated them with actinomycin D (Figure 36A). Phagocytosis was analyzed by microscopy putting in contact normal BMDM with apoptotic MDA-GFP cells in a proportion of 1:10.

We could observe that normal BMDM were able to engulf apoptotic epithelial cells since we detected green fluorescence in BMDM samples (Figure 36B). In addition, phagocytosis activity in senescent macrophages was quantified by flow cytometry method. As negative control we used a pre-treatment with cytochalasin D, a phagocytosis inhibitor, and low temperature (4ºC), to prevent the engulfment of apoptotic bodies. In normal BMDM the phagocytic activity was increased after apoptotic cells incubation, however, this activity was significantly suppressed in 14 days BMDM (Figure 36C), indicating that macrophage phagocytic activity is impaired in long-period cultures of BMDM.

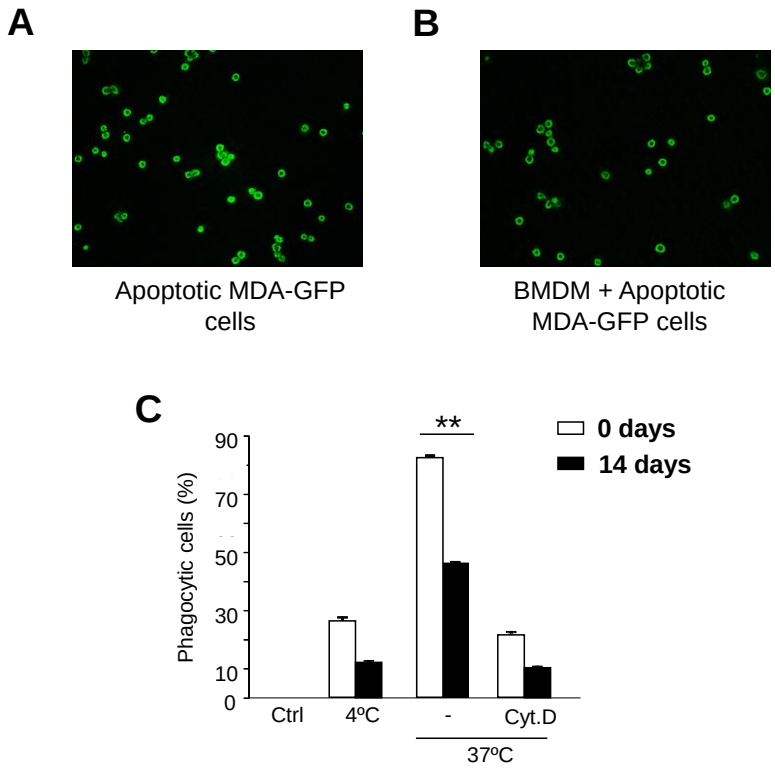


Figure 36. Phagocytic capacity is impaired in long-period BMDM cultures. (A) Representative image of apoptotic MDA-MB-231/GFP cells. Cells were treated with Actinomycin D (5 µg ml^{-1}) for 6 h. (B) Representative image of MDA-MB-231/GFP cells phagocyted by BMDM. BMDM were treated in a proportion of 10:1 with apoptotic MDA-MB-231/GFP cells for 1 h. Images were acquired with a fluorescent inverted microscope. (C) BMDM, at 4ºC or 37ºC, were treated with the presence or absence of a pre-stimulation of cytochalasin D (Cyt.D, 2 µg ml^{-1}) in a proportion of 10:1 with apoptotic

MDA-MB-231/GFP cells for 1 h. GFP positive cells were analyzed by flow cytometry and data were expressed as percentage of phagocytic cells. Each point was analyzed in triplicate and the results are shown as mean ± SD. All assays are representative for at least 3 independent experiments. **p<0.01 correspond to the statistical significance between indicated pair comparisons.

Thus, taking into account the innate immune functionalities of macrophages studied in this work, we could determine that long-period BMDM cultures presented a great number of altered macrophage activities that could contribute to some aspects of the impaired immune response observed in aging.

Discussion

Macrophages are white blood cells localized within tissues, arising from the differentiation of monocytes. These cells play a critical role in the development of the innate immune response since they act as sentinels carrying out different functions in tissues (Xu and Gordon, 2003). They are specialized to phagocyte and eliminate foreign particles (bacteria, viruses, parasites, macromolecules and damaged cells), limiting the anti- inflammatory process. Moreover, macrophages can also participate in the systemic immune modulation through the production of cytokines and chemokines, antigen presentation and the regulation of T cell activation and differentiation, linking the innate immunity to the adaptive (Bowdish et al., 2007).

Although, it is well documented that innate and adaptive immunity are deteriorated progressively with advancing age (Hu et al., 1993; Linton et al., 1996; Linton et al., 1997; Beverly and Grubeck-Loebenstein, 2000; Franceschi et al., 2000; Johnson and Cambier, 2004), the specific impact on macrophages has to be resolved. The inappropriate macrophage function produced by aging contribute significantly to decreased pathogens clearance and decreased responsiveness of the adaptive immune system, participating in the immunosenescence process (Plackett et al., 2004).

We show here that our novel murine senescent macrophage model, based on long-period cultures of BMDM, present altered macrophage functions similarly to those observed in macrophages obtained from old mice. Interestingly, this cellular model also show numerous senescent characteristics which demonstrate that cellular senescence induced in long-term cultures of BMDM may be a good tool to evaluate the impaired macrophage functions produced during the aging process, MacrophAging.

Long-term cultures of BMDM are senescent macrophages

Very few data are available regarding the cellular and molecular mechanisms involved in macrophage aging and senescence (Pinke et al., 2013; Pinto et al., 2014; Stranks et al., 2015). Most studies use BMDM from young (6 week-old) and aged mice (24 month-old) grown and differentiated *in vitro* to prevent the influence of other cell types that may be affected by the aging process (Herrero et al., 2002; Sebastián et al., 2009). However, working with old mice carries many problems. For instance, researchers must wait 24 months to get aged mice, long time and many economic costs to initiate the investigation. In addition, during this waiting period may occur different uncontrollable complications: some mice can die by natural death or even they can get sick due to the natural aging program.

To resolve this, some studies from our research group used the first and third generation of mice lacking the gene encoding the telomerase RNA component (*Terc*$^{-/-}$) (Sebastián et al., 2009). These mice show progressive telomere shortening in successive generations with signs of premature aging (Samper et al., 2002). However, for this work we decided to design an innovative cellular model of senescence to study aging on macrophages. Actually, the use of replicative life span of cell cultures as a model for aging has been accepted because *in vitro* life span correlates with species maximum life span potential (Demetrius, 2006) and, most importantly, cultures of normal human and mice cells have been reported to exhibit frequently a negative correlation between proliferative capacity and the age of the donor from whom the culture was established (Cristofalo et al., 1998; Ksiazek et al., 2007). However, even in rodents, the relationship between donor age and proliferative potential is not completely clear (Bruce and Deamond, 1991; Deamond and Bruce, 1991). Although the loss of proliferative potential *in vitro* may not directly reflect changes in replicative capacity that occur *in vivo* during organismal aging, cell cultures remain a powerful tool for a variety of aging-related studies such as stress effects, loss of cellular functions and basic mechanisms of aging.

Our senescent macrophage model was established by sequential passaging until BMDM growth ceased and failed to divide, similarly to other *in vitro* senescent cellular models (Effros et al., 2003; Ksiazek et al., 2007; Phipps et al., 2007), maintaining the specific macrophage marker Cd11b and their particular size and complexity. The results found show that long-period cultures of BMDM reduces their proliferative potential in response to two macrophage specific growth factors, M-CSF and GM-CSF. In this regard, we evaluated macrophage proliferation by different approaches in order to demonstrate the most important and indispensable senescent feature, the irreversible growth arrest. Radioactive thymidine incorporation and cellular counting are decreased in long-term cultures of BMDM. In addition, these BMDM cultures present less BrdU incorporation and a cell cycle blockage at G1 phase measured by cell cycle analysis based on PI staining. However, previous studies from our research group showed that although GM-CSF-dependent proliferation was significantly reduced in aged BMDM obtained from old mice, the proliferative capacity in response to M-CSF treatment was similar to young BMDM (Sebastián et al., 2009).

Furthermore, our results demonstrate that 14 days BMDM, obtained through serial divisions, are positive for diverse senescent markers. As reported in this thesis, replicative senescence is associated with widespread and persistent changes in long-period cultures of BMDM that were consistent with the induction of a senescent

phenotype. Such changes include high activity of SA-β-gal and morphological changes. It has been demonstrated that senescent cells raise SA-β-gal activity reflecting the increase in lysosomal mass (Lee et al., 2006). Additionally, senescent cells increase in size, sometimes enlarging more than twofold relative to the size of non-senescent counterparts (Hayflick, 1965). However, we could only detect that long-period BMDM cultures are more rounded and have less dendritic morphology than normal BMDM.

Previous macrophage aging studies showed that aged BMDM were unable to maintain telomeres at a sufficient length to prevent DDR resulting in impaired proliferation (Harley et al., 1990; Wright et al., 1996; Masutomi et al., 2003; Sebastián et al., 2009). In fact, hematopoietic stem cells show telomere shortening during *in vitro* culture and *in vivo* aging (Vaziri et al., 1994; Engelhardt et al., 1997; Zimmermann et al., 2004) similarly to several mouse stem cell compartments, including skin, small intestine, cornea, testis and brain (Flores et al., 2008). This work demonstrate that long-term cultures of BMDM present shorter telomeres which correlates with signs of persistent activation of a DDR, as evidenced by γ-H2AX-positive.

In addition to these features, we have shown that our senescent cellular model suffers an enhanced oxidative stress which is related with telomeres shortening (von Zglinicki et al., 2002; Tchirkov and Lansdorp, 2003). This higher production of intracellular ROS associates with the mRNA expression of senescent markers connected with oxidative stress (de Haan et al., 1996), such as higher *catalase* and *SOD2* expression, while *SOD1* seems not to be affected by the induction of senescence. However, results published regarding *SOD* homologues expression are controversial. While, some reports indicate that both, *SOD1* and *SOD2*, are upregulated at mRNA level in senescent cells (de Haan et al., 1996), other studies confirm that *SOD1* mRNA expression was overexpressed, and SOD2 was not affected (Kawamura and Sunanaga, 2013). Even so, in agreement with these previous reports, SOD activity is reduced in long-term cultures of BMDM, contributing to the redox imbalance.

Therefore, the results obtained confirm that long-period cultures of BMDM display a senescent phenotype characterized, mainly, by irreversible exit of the cell cycle and by changes in macrophage shape, increased SA-β-gal activity, telomeres shortening, enhanced oxidative stress and persistent DNA damage.

We have also investigated the molecular mechanism involved in the cell cycle blockage induced in long-term cultures of BMDM. The cell cycle arrest at G1 phase has been correlated with the origin of cellular senescence (Magalhaes, 2004). According to the published data, long-period cultures of BMDM increases the expression of G1 CDKIs

such as *p16* and *p27* which are able to inactivate G1 proteins such as CDK4/cycD and CDK2/cycE complexes (Kuilman et al., 2010; Rodier and Campisi, 2011). It has been established that *p16* expression increases with age in mice and humans (Zindy et al., 1997; Nielsen et al., 1999; Krishnamurthy et al., 2004; Ressler et al., 2006; Liu et al., 2009), and its activity has been functionally linked to the reduction in progenitor cell number that occurs in multiple tissues during aging (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). However, although commonly high levels of *p21* are required for the induction of senescence-related growth arrest (Sherr and Roberts, 1999; Chang et al., 2000; Romanov et al., 2010), other studies demonstrate that *p21* overexpression is not crucial (Medcalf et al., 1996; Brown et al., 1997). In this sense, in our senescent macrophage model *p21* mRNA levels are not affected in response to M-CSF and GM-CSF stimulation. Moreover, in concordance with previous studies, we also proved that the expression of *cycD* and *cycE* as well as *Cdk2* and *Cdk4* are reduced in these macrophages (Kuilman et al., 2010; Blagosklonny, 2011).

Next, we decided to study the signaling involved in the cell cycle arrest induced in long-period cultures of BMDM. M-CSF and GM-CSF induce macrophage proliferation by stimulating a complex array of signal transduction pathways, including MAPKs (Pixley and Stanley, 2004). Three different MAPKs (ERK1/2, JNK and p38) are activated in response to M-CSF and GM-CSF treatments (Jaworowski et al., 1999; Valledor et al., 2000; Comalada et al., 2004; Sánchez-Tilló et al., 2007). Paradoxically it has been demonstrated that prolongation in ERK1/2 phosphorylation triggers cell cycle arrest involved in cellular senescence since correlates with high levels of *p16* (Valledor et al., 2008; Deschenes-Simard et al., 2013). Similarly, high p38 MAPK activity drives senescence through inhibition of telomerase activity, upregulation of *p16* mRNA and by induction of DDR signaling in some immune cells such as T cells (Lanna et al., 2014). In contrast, loss of JNK activity has been related with the activation of Bcl-2/ROS/DDR signaling cascade that ultimately leads to premature senescence (Lee et al., 2010; Spallarossa et al., 2010). In this regard, we evaluated the activity of these MAPKs in response to M-CSF and GM-CSF by western blot.

The prolongation of ERK and p38 MAPK phosphorylation was detected in long-period cultures of BMDM. However, normal and senescent macrophages present similar JNK activity levels. These results suggest that both, ERK and p38 MAPK, are implicated in the induction of growth arrest in BMDM, while JNK activity seems to be irrelevant. Nevertheless, further research is required to ensure that ERK and p38 MAPK activities are crucial for macrophage senescence since no differences in MAPKs phosphorylation were detected in BMDM from old mice (Sebastián et al., 2009). For

example, it would be significant perform senescent biomarkers assays inhibiting specifically both kinases in long-term cultures of BMDM or using ERK and p38 MAPK knockout macrophages.

Additionally, GM-CSF-dependent proliferation also induces the activation of STAT5 signaling pathway which is involved in the growth of different cells such as macrophages. Long-period cultures of BMDM show a decrease in STAT5 phosphorylation in response to GM-CSF stimulation. Corroborating these results, previous studies from our research group detected a reduction in STAT5 activity in BMDM obtained from old mice (Sebastián et al., 2009).

Taken together, these effects on mRNA transcription of cell cycle regulators genes, the elongation of ERK1/2 and p38 MAPK activities and the reduction in STAT5 phosphorylation may prevent transition from G1 to S phase, blocking the cell cycle and contributing to the induction of macrophage senescence program (Figure 37).

However, cell cycle arrest is not a synonym of senescence. Non-senescent cell cycle blockage can be caused by the absence of growth factors which drives to cellular quiescence (Blagosklonny et al., 2011) or by terminal differentiation (Buttitta and Edgar, 2007). Thus, since senescence, quiescence and terminal differentiation are all described by the accumulation of cells at G1 phase of the cell cycle through high CDKIs activity (Lowe and Sherr, 2003; Pajalunga et al., 2007), it is difficult to distinguish these three processes in a cellular culture. Nevertheless, while quiescence is characterized by reversible cell cycle arrest, terminal differentiation is usually coupled with permanent exit from the cell cycle and represents the most common cellular state in adult animals (Liu et al., 2004). In this sense, quiescence seems not to be involved in the growth arrest presented in long-period cultures of BMDM since proliferative assays were performed with M-CSF and GM-CSF treatments. In addition, both processes, quiescence and terminal differentiation, can be ruled out due to the numerous senescent biomarkers shown in long-term cultures of BMDM which are absent in non-senescent phenotypes.

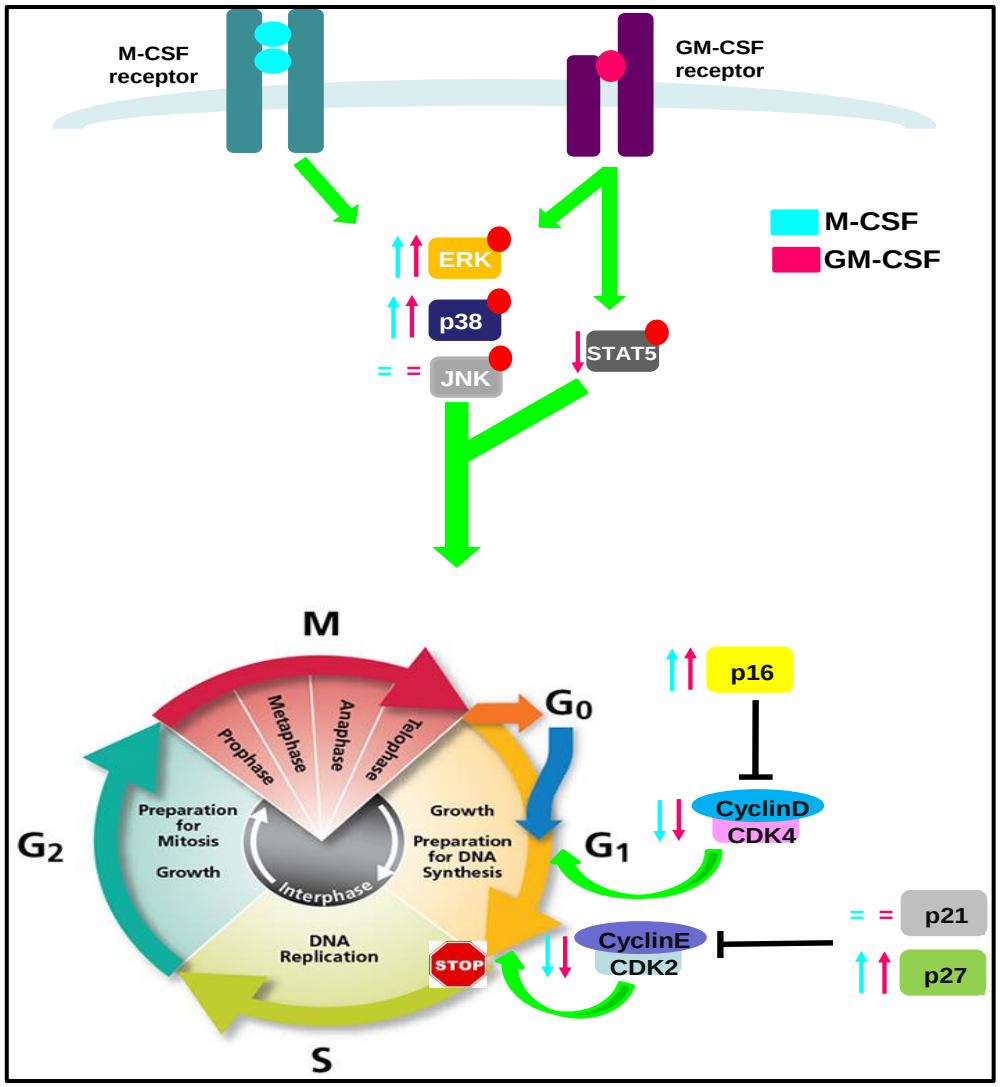


Figure 37. Molecular mechanism involved in cell cycle arrest observed in long-period BMDM cultures. Schematic model for the cell cycle blockage presented in long-period cultures of BMDM comparing the expression or activation of the main components and regulators with normal BMDM. Blue and pink arrows indicate the up- or downregulation of the corresponding genes and the increase or reduction in MAPKs and STAT5 phosphorylation in M-CSF- or GM-CSF-dependent macrophage division, respectively, whereas black flattened arrows represent inhibitions of CDK/cyclin complexes. Equal signs indicate no differences between long-period and normal BMDM. Red spheres indicate phosphorylation, and blue and pink spheres represent M-CSF and GM-CSF growth factors, respectively.

As it has been mentioned before, long-period cultures of BMDM express high levels of *p16*. Most senescent cells increase the expression of *p16*, which is not commonly expressed by quiescent or terminally differentiated cells (Alcorta et al., 1996; Hara et al., 1996; Serrano et al., 1997; Brenner et al., 1998; Stein et al., 1999). It is long known that *p16* levels accumulate and cause growth arrest when cells approach their replicative life span (Itahana et al., 2003). Moreover, increased *p16* expression is also linked to OIS and premature senescence (Serrano et al., 1997; Zhu et al., 1998; Lin et

al., 1998). The capacity of different oncogenes to trigger senescence seems to depend on cell and tissue type, perhaps reflecting the integrity of tumor suppressor networks. Therefore, we also investigated the profile expression of the most important senescence-related oncogenes in long-term cultures of BMDM. In this regard, these senescent macrophages overexpress *c-Myc* and *Raf* oncogenes as well as *E2F1*.

Although, some reports have described that development of senescence in different cell types is *c-Myc*-independent (He et al., 2008) or even could be associated with reduction in *c-Myc* expression (Guney et al., 2006; Mallette et al., 2007; Guo et al., 2007), in general, overexpression of *c-Myc* has been related to apoptosis and senescence as a defense mechanism to control cell growth (Evan and Vousden, 2001; Campaner et al., 2010). Similarly and supporting our current observations, *Raf* and *E2F1* upregulation is presented by senescent cells in a p53-independent mechanisms (Olsen et al., 2002; Chistoffersen et al., 2010).

Numerous reports show convincingly that p53 and its downstream effector p21 or p16-pRB signaling pathway play a key role in the regulation of cellular senescence mechanism (Lowe et al., 2004).

Overexpression of *p53* (Wang et al., 1998) and *p21* (Brown et al., 1997; Stott et al., 1998; Fang et al., 1999; Wang et al., 1999) autonomously induced senescence in human cells and activation of p53 (Wei et al., 2001) induced senescence in a p21-dependent pathway in human diploid fibroblasts (HDF) and human glioblastoma cells, respectively. Moreover, inactivation of p53 prevents senescence in mouse embryonic fibroblasts (MEF) (Harvey and Levine, 1991) and human fibroblast lacking *p21*can bypass the senescence-related cell cycle exit (Hara et al.,1991). However, although long-period BMDM are able to induce p53 activity upon DNA damaging treatments and express *p21* at similar levels as normal BMDM, at basal and growing conditions neither present p53 phosphorylation nor increase *p21* mRNA expression, discarding this mechanism as the inductor of senescence in our macrophage senescent model.

In addition, senescence can also be mediated by p16-pRB-dependent mechanism indicating the redundant role of p16 since it is implicated in the irreversible growth arrest and drives to the hyperphosphorylation and accumulation of pRB which initiates the senescence program. It has been demonstrated that p16-dependent activation of pRB causes formation of senescence-associated heterochromatin foci (SAHF), which silence critical pro-proliferative genes (Narita et al., 2003). Moreover, the importance of *p16* in the induction of senescence is clear as $p16^{-/-}$ cells can be driven into senescence by the sole re-expression of *p16* (Uhrbom et al., 1997). In this sense, the

extraordinary high expression of *p16* in long-term cultures of BMDM makes us suspect that p16-pRB pathway is the mechanism involved in the induction of senescence in these macrophages. Nevertheless, further studies will be required to elucidate the molecular events that regulate the senescence program in long-period cultures of BMDM and whether this mechanism is correlated with the presenting macrophages obtained from old mice.

Consistent with evidences provided in this thesis, *p16* overexpression is related to telomeric or intrachromosomal DNA damage (Brenner et al., 1998; Robles and Adami, 1998; Ramirez et al., 2001; te Poele et al., 2002; Jacobs and de Lange, 2004; Le et al., 2010). Consequently, we decided to study the involvement of telomere attrition in the induction of senescence-associated impaired proliferative capacity in long-period BMDM. We show that the activation of telomerase through TA-65 pre-treatment moderately improves normal macrophage proliferation in senescent macrophages. TA-65 is a drug that is able to activate telomerase and enhance growth *in vitro* as well as *in vivo* senescence-related or aged-related models (Hartwig et al., 2012; Boccardi and Paolisso, 2014). Our results also accord with several studies done on both rodents and humans using telomerase activators (Molgora et al., 2013, Taka et al., 2014). Bernardes de Jesús and co-workers demonstrated that mice treated with TA-65 elongated short telomeres affecting telomerase activity and increasing life span without incidence of cancer (Bernardes de Jesús et al., 2011). Other report describing initial research in aged humans treated with TA-65 provided evidences of the improvement of certain immune parameters that had previously been associated with beneficial health effects (Harley et al., 2011). In this sense, our findings revealed that telomeres shortening presented in long-term cultures of BMDM contributes significantly to the irreversible cell cycle arrest induced in these macrophages.

However, a better understanding of these processes may facilitate new approaches which elucidate the complete mechanism of senescence induction in long-term cultures of BMDM connecting all participators: cell cycle exit, telomeres attrition, enhanced oxidative stress and DNA damage.

M-CSF- and GM-CSF-dependent DNA repair is not affected in long-period cultures of BMDM

To protect the integrity of their DNA, cells need to be able to sense DNA damage and activate response pathways that coordinate cell cycle progression and DNA repair.

H2AX phosphorylation is induced by DSBs and is able to activate the repair machinery. The molecular mechanism involves ATM and ATR kinases which are important DNA damage checkpoints that activate other kinases such as Chk1 (activated by ATR) and Chk2 (activated by ATM). These kinases also phosphorylate and activate the tumor suppressor protein p53. ATM and ATR can additionally enhance p53 activity by directing p53 phosphorylation on Ser15. Activated p53 can halt progression of the cell cycle in the G1 phase, allowing DNA repair, apoptosis or senescence induction, depending on the cellular situation, to prevent the transmission of damaged DNA to the daughter cells (Di Micco et al., 2006; Bartkova et al., 2006).

However, although we are able to detect high levels of ɣ-H2AX after M-CSF and GM-CSF treatments in senescent macrophages, they do not show p53 activation in the same conditions. These results suggest that DDR induced in long-term cultures of BMDM at growing conditions is p53-independent. In contrast, senescent macrophages are able to phosphorylate p53 upon H_2O_2 stimulation, indicating that p53 activation is not impaired. In this regard, while p53 is required for normal induction of cell death following DNA damage, (Yuan et al., 1999; Irwin et al. 2003; Urist et al. 2004; Ozaki and Nakagawara, 2005; Roos and Kaina, 2006), several reports confirm the activation of H2AX independently of p53 phosphorylation (Reinhardt et al., 2007; Sidi et al., 2008; Valentine et al., 2011).

This p53-independent alternative DDR mechanism has been associated with a reduced or delayed apoptosis (McNamee and Brodsky, 2009). Although senescence is considered as tumor-suppressive mechanism that promotes apoptosis of cells, the induction of senescence in certain cell types, such as fibroblasts (Kim et al., 2011) and T-cells (Monti et al., 2000), may result in resistance to apoptosis whose accumulation contribute to the pathogenesis of certain age-related diseases (Raghu et al., 2006; Beltrami et al., 2012). Consistently, apoptosis induced in senescent macrophages is decreased. This resistance to apoptosis has been related with the up-regulation of several anti-apoptotic genes such as *Bcl-2* (Wang, 1995; Ryu et al., 2007) and *survivin* (Al-Khalaf and Aboussekhra, 2012). According to published data, long-period cultures of BMDM also present high levels of *Bcl-2* mRNA expression which contributes to reduce apoptosis.

Although M-CSF and GM-CSF induce different mechanisms for proliferation, they share the same signaling cascade for survival in macrophages which consists in the activation of PI-3K-Akt pathway (Comalada et al., 2004). In this sense, the protective role of M-CSF and GM-CSF to DNA damage has been established in colonic

macrophages and dendritic cells (Galeazzi et al., 2000; Xu et al., 2008; Bernasconi et al., 2010; Egea et al., 2013; Li et al., 2013) and in alveolar macrophages (Bernier et al., 2001; Cakarova et al., 2009). Additionally, the repair machinery induced by DDR produced by oxidative stress is not affected by the senescent program due to the ability of senescent macrophages to reduced H2AX phosphorylation after M-CSF and GM-CSF recovery conditions. These results suggest that the accumulation of DNA damage in long-period cultures of BMDM is not produced by an impaired repair capacity.

Macrophage functions are altered in long-period cultures of BMDM

As the average human life expectancy has increased, the impact of aging and age-related disease has become the focus of many research groups. The appearance of chronic inflammatory pathologies is common in the elderly and it has been linked with an abnormal development of the inflammatory response (Franceschi et al., 2000; Tracy, 2003; Kerr, 2004; Casseta and Gorevic, 2004; Krabbe et al., 2004; Casserly and Topol, 2004; Hakim et al., 2004). Thus, deficiencies in innate and adaptive immunity diminish the efficiency to eliminate pathogens and restore homeostasis in the context of infectious disease and wound healing. Investigation in immune aging centers on the development of treatments which increase the quality of life in the elderly population through immune response modulation. In order to modify immune function, it is essential to understand the mechanisms underlying age-related functional impairments.

As macrophages represent a first line of defense against invading pathogens and a link between innate and adaptive immunity, altered macrophages functions are critical for immunosenescence and inflammaging development. However, the impact of advanced aged on these processes seems to be controversial. For instance, some published reports showed no significant effect on monocytes recruitment while other observed an important delay and reduced number of monocytes (Ashcroft et al., 1998; Swift et al., 1999; Swift et al., 2001; Plackett et al., 2004).

Our work provides further evidences for the impaired classical and alternative activation of senescent macrophages which can contribute to dysregulate the immune response observed in aged individuals.

Regarding to altered M1 functions, we show a declined antigen presentation represented with low levels of class II MHC expression on the surface of senescent macrophages in response to IFN-γ. This data is consistent with previous results from our research group which observed decreased expression of MHC II in macrophages from old mice (Herrero et al., 2002). This reduced antigen presentation has been also reported in aged humans (Effros and Walford, 1984; Seth et al., 1990; Haruna et al., 1995; Garg et al., 1996; Zissel et al., 1999). Moreover, this defective antigen presenting capacities is connected with impaired adaptive immunity since provokes less T cell activation and clonal expansion (Plowden et al., 2004).

Additionally, long-period cultures of BMDM also exhibit reduced microbicidal activity. NOS2, the enzyme that synthetizes NO, mRNA and protein expression is attenuated in this cells. In this regard, it is not surprising that NO production is declined in senescent macrophages comparing with normal BMDM in response to LPS or LPS plus IFN-γ. Since NO is a powerful and crucial toxic molecule which mediates bacterial wall lysis and, therefore, pathogen killing, impaired synthesis of NO may produce deficiencies in pathogen clearance which can lead to increased bacterial burden (Frazier et al., 2009). Our findings are in concordance with *in vitro* and *in vivo* reported results (Tschudii et al., 1996; Cernadas et al., 1998; Matsushita et al., 2001) indicating that macrophage intracellular anti-bacterial activity is impaired in senescent macrophages.

Senescence effects on M1 macrophage activity are not limited to antigen presentation and pathogen elimination, cytokine expression is also affected. The mRNA and protein expression as well as the secretion of pro-inflammatory cytokines such as IL-1β and TNF-α are increased in response to LPS in long-term cultures of BMDM.

In the case of IL-1β, LPS stimulation is not sufficient to induce the release of IL-1β, but the activation of inflammasome is required (Lamkanfi and Dixit, 2012). Inflammasomes are intracellular multiprotein complexes that assemble in response to a diverse range of microbial products and also to endogenous danger signal after specific recognition through NLRs, expressed in innate immune cells (von Moltke et al., 2013). Consequently, we determined IL-1β production activating the most studied inflammasome mechanism: the NLRP3 inflammasome activation through ATP signaling (Halle et al., 2008; Elinav et al., 2013). Although IL-1β is overexpressed and secreted by senescent endothelial cells (Maier et al., 1990), fibroblasts (Kumar et al., 1992; Rinehart et al., 1999), and chemotherapy-induced senescent epithelial cells (Chang et al., 2002), we cannot ruled out the effects of senescence induction on inflammasome compounds and inflammasome signaling pathway since it has been

demonstrated the involvement of MAPKs, transcription factors, ROS production and phagocytosis in inflammasome expression and activation which can also affect IL-1β levels (Cogswell et al., 1994; Baldassare et al., 1999; Stutz et al., 2009; Sheibel et al., 2010; Cai et al., 2012; Fields and Ghorpade, 2012; Qu et al., 2012; Qiao et al., 2013).

Similarly, TNF-α production is increased in senescent macrophages. This is consistent with published data where showed high levels of circulating TNF-α in aged individuals (Bruunsgaard, 2006) as well as increased TNF-α production produced by NK cells (King et al., 2009) and B cells (Ratliff et al., 2013) from aged mice. Moreover, we also evaluated the expression and release of IL-10, a well-known anti-inflammatory cytokine which controls TNF-α and other pro-inflammatory cytokines effects at systemic level. The induction of senescence on macrophages also increases the expression and secretion of IL-10 which can be explained by the enhanced STAT-3 phosphorylation exhibit in long-period cultures of BMDM in response to LPS. This finding is also in concordance with the involvement of STAT-3 activation for premature cytokine-induced senescence development observed in human fibroblasts (Kojima et al., 2013).

This altered cytokine secretion induced in senescent macrophages can be described by the potential SASP development. SASP phenotype consists in differences in the production of several factors that can affect neighboring cells by activating receptors and inducing signal transduction pathways. These factors can be divided in soluble signaling mediators, including cytokines (IL-6, IL-1β, TNF-α), chemokines (MCP-2, CXCL-1, -2, -8) and growth factors (GM-CSF), and in secreted proteases (PAI-1, -2) and ECM components (MMP-1, -3, -10) (Coppé et al., 2014).

In this sense, the results obtained suggest that our macrophage senescent model presents an altered pro- and anti-inflammatory cytokine profile which can participate significantly to the defective immune response against pathogen infection observed in aged organisms.

Next, we explored the potential role of TLRs in altered M1 functions observed in long-term cultures of BMDM. Activation of TLRs in macrophages usually drives to the transcriptional regulation of pro-inflammatory and antiviral cytokines and chemokines that modulate the innate immune response. Therefore, impaired TLRs signaling can be determinant for the acquisition of SASP phenotype which leads to inflammation imbalance (Olivieri et al., 2013). In this regard, the expression of TLRs and the signaling pathway involved has been investigated in immune cells of aged/senescent murine and human models resulting in important changes in TLRs expression that affect signal transduction.

To address this question, we measured the surface protein expression of TLR-2, -4 and -5 and the intracellular protein expression of TLR-9 in normal and senescent macrophages at basal and activated conditions. We selected these four TLRs due to the reported evidences of different expression levels in aged murine and human models whereas rest of TLRs seem not to be affected by aging (Sun et al., 2010; Sun et al., 2012). Our work indicates that long-term cultures of BMDM up-regulate TLR-4, -5 and -9 whereas TLR-2 protein expression is down-regulated in response to LPS, indicating a putative link between altered TLRs expression and inflammaging in our macrophage senescent model. However, in absence of stimulation normal and senescent macrophages express similar TLRs levels, as it has been previously published in neutrophils and macrophages (Bohemer et al., 2004; Sun et al., 2012).

TLR-2 together with TLR-1 or TLR-6 detects various bacterial components including peptidoglycan, lipopeptide and lipoprotein of gram-positive bacteria. The down-regulation of TLR-2 observed in senescent macrophages may be responsible to the aged-related decline of the immune response to gram positive bacteria observed *in vivo* (Boyd et al., 2012). However, it has been also published a slightly increased in TLR-2 expression in alveolar macrophages, suggesting also defects in signal transduction (Boyd et al., 2012). TLR-4 and TLR-5 recognize LPS and bacterial flagellin, respectively, inducing a signaling cascade which results in increasing the secretion of pro-inflammatory cytokines such as TNF-α and IL-1β. Both receptors are overexpressed in long-term cultures of BMDM that can amplify the signaling transduction and explain the increase in TNF-α and IL-1β production observed in senescent macrophages. Additionally, TLR-4 and TLR-5 expression was reported to be higher in old rhesus macaques compared to younger animals although in humans are unaltered (Fulop et al., 2004; Murciano et al., 2007; Asquith et al., 2012). In the case of TLR9, which detects viral and bacterial DNA and although published results showed unclear results, it has been reported that TLR-9 knockout lymphocytes induces earlier senescence in these mice produced by pro-inflammatory properties (Abu-Tair et al., 2013). In contrast, our results indicate an overexpression of TLR-9 in senescent macrophages. However, we do not have data about levels of IFN type I expression or secretion. In this sense, further investigations are required to connect TLRs expression and signal transduction with altered pro-inflammatory profile observed in senescent macrophages.

Regarding to M2 altered functions, our data indicate clearly a suppressed alternative activation in long-period cultures of BMDM in response to IL-4. Using established markers for M2 phenotype, our results show that a*rginase-1* expression and activity is

significantly reduced as well as *fizz1* expression, a crucial transcription factor for M2 differentiation, and *mgl1*, a receptor for C-type lectins that recognizes galactose residues expressed in pathogens (Roszer, 2015). In contrast, *tgfb* and *mrc1* mRNA levels are not affected or even increase, respectively, in senescent macrophages. This may be explained due to *tgfb* and *mrc1* expression is mediated by STAT-3 signaling pathway (Sica and Mantovani, 2012) which is overactivated in long-term cultures of BMDM, as we mentioned before. Moreover, although *tgfb* and *mrc1* are considered M2 markers, since they are overexpressed during parasite infection and in allergic asthma (Roszer, 2015), their expression is not restricted to M2 macrophages (Barros et al., 2013)

At endothelial level there are evidences that relate high arginase activity with aging. It has been reported that high arginase activity, low NO production and increased ROS levels are presented in old rats which result in promoted fibrosis (Kim et al., 2009). In fact, knocking down *arginase-1* (White et al., 2006) restores aging-related endothelial senescence and improves vascular function (Bivalacqua et al., 2007). Nevertheless, consistently with our data Mahbub and co-workers demonstrated that IL-4-dependent *arginase-1* and *fizz1* mRNA expression was reduced in splenic macrophages and in BMDM from aged mice (Mahbub et al., 2012). Additionally, for the first time, herein we show altered *mgl1* mRNA expression associated with cellular senescence in macrophages.

Therefore, taking into account the critical role of *arginase1*, *fizz1* and *mgl1* in control the resolution of inflammation and in parasitic infection, the results obtained indicate the inability of senescent macrophages to repair tissues and to orchestrate an appropriate M2 response during parasite invasion resulting in inflammatory imbalance and in susceptibility to Th2-pathologies (Hesse et al., 2001; Loke et al., 2007; Nair et al., 2009; Pesce et al., 2009).

Furthermore, we also investigated the phagocytic ability of senescent macrophages to eliminate apoptotic cells. The evidence for impaired phagocytic capacity of long-term cultures of BMDM was evaluated through fluorescence microscopy and by flow cytometry experiments using apoptotic GFP-epithelial cells. Whereas 80% of normal macrophages are positive for green fluorescence after apoptotic fibroblast challenge, only 40% of senescent macrophages are able to engulf apoptotic fibroblasts. On the other hand, although we have some preliminary results showing that pathogen clearance by macrophage phagocytosis is also suppressed in long-term cultures of BMDM, we were not able to determine whether the defects in both mechanisms are

common or whether are produced by signal transduction deficiencies. In contrast to pathogen elimination, apoptotic cells are immunosuppressive. Apoptotic cells clearance by phagocytes induce the inhibition of the pro- inflammatory cytokines TNF-α, GM-CSF, IL-12, IL-1β and IL-18 through the promotion of anti-inflammatory cytokines IL-10 and TGF-β (Birge and Ucker, 2008). Moreover, phagocytes present antigens from apoptotic cells to T cells maintaining the peripheral immune tolerance (Steinman and Nussenzweig, 2002; Steinman et al., 2003). In this sense, defective or delayed phagocytosis of apoptotic cells may result in the development of autoimmune diseases such as systemic lupus erythematosus (SLE) or cystic fibrosis (Liu and Mohan, 2006).

However, research works regarding the influence of aging on macrophage phagocytic function are controversial. De La Fuente and co-workers demonstrated a decrease in macrophage phagocytosis in old mice compared with young mice (De La Fuente, 1985). Aprahamian and collaborators demonstrated an age-related reduction in phagocytosis of apoptotic cell debris in mice *in vivo* but not *in vitro* (Aprahamian et al., 2008). Furthermore, human studies have reported impaired phagocytosis by macrophages from older individuals (Hearps et al., 2012), while earlier studies reported no effect of aging on phagocytic function in humans (Fietta et al., 1994).

Therefore, this work provides relevant evidences of damaged M1 and M2 activities (summarized in Figure 38) in our novel senescent macrophage model that correlate with that observed in macrophages from aged mice as it was previously demonstrated (Herrero et al., 2002; Sebastián et al., 2009; Mahbub et al., 2012).

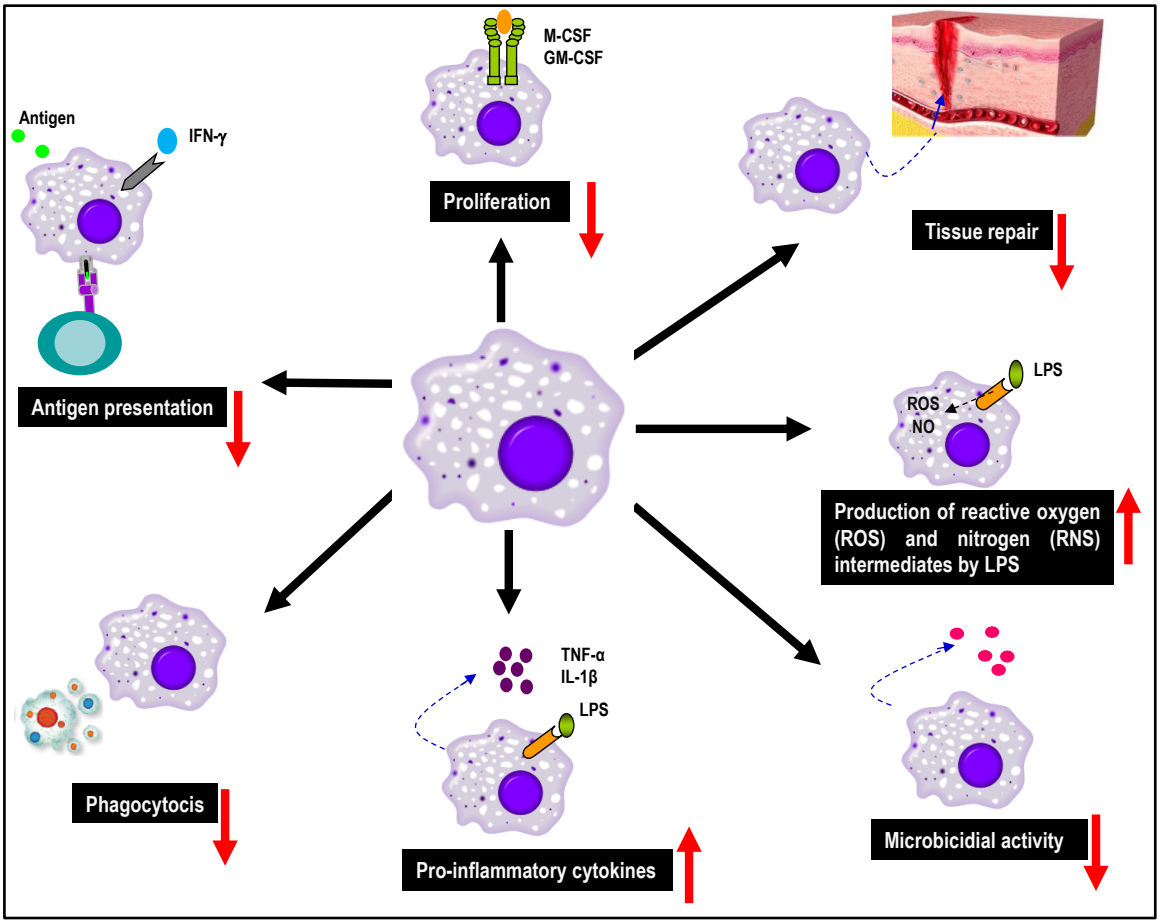


Figure 38. Impaired functional activities in long-term cultures of BMDM. Schematic representation of altered classical and alternative macrophage phenotypes of senescent macrophages. Proliferation in response to M-CSF and GM-CSF, antigen presentation, phagocytosis of apoptotic cells, microbicidal activity as well as tissue repair capacities are reduced in long-term cultures of BMDM comparing with normal macrophages. On the contrary, pro-inflammatory cytokines, such as TNF-α and IL-1β, and ROS production are increased in these macrophages. Red arrows indicate the reduction or increase in macrophage capabilities comparing with normal BMDM.

Long-term cultures of BMDM should be considered as a good cellular model for macrophage age-related studies

Although there are two main theories which try to explain age-related impairments in macrophages, the intrinsic defects hypothesis (Yoon et al., 2004) and the microenvironment effects hypothesis (Chen et al., 1996; Mahbub et al., 2012), based on our data, this thesis clearly demonstrate senescence-associated macrophage alterations without the influence of neighboring cells or other factors. However, we

believe that due to the high plasticity of macrophages and their rapid adaptation to environmental changes, the microenvironment also plays a key role in macrophage function participating and contributing to the intrinsic macrophage dysfunction.

The effect of aging on macrophages appears to be multifaceted, affecting almost every aspect of their normal cellular function (Murciano et al., 2007; Pott et al., 2012; Olivieri et al., 2012; Qian et al., 2012). We have demonstrated that long-term cultures of BMDM present a wide range of senescent characteristics that allow identify these cells as senescent macrophages. Additionally, the impaired proliferative capacity featured by cell cycle arrest at G1 phase, the suppressed M1 and M2 functionality and the damaged phagocytosis of apoptotic cells observed in our novel senescent macrophage model is in concordance with bibliographic data.

To our knowledge, this is the first time that has been achieved a senescent cellular model of macrophages that resembles macrophage deficiencies from aged mice. Therefore, we demonstrate that long-term cultures of BMDM are a relevant cellular model to study different aging-related processes on macrophages including proliferation, senescence mechanisms, classical and alternative macrophage phenotypes and phagocytosis.

Conclusions

Long-term cultures of BMDM are senescent macrophages and the molecular mechanism involved in cell cycle arrest has been provided

1. Long-term cultures of BMDM are positive for SA-β gal activity and present reduced proliferative capacity in response to M-CSF and GM-CSF, altered morphology, telomeres shortening, high DNA damage, increased ROS production and apoptosis resistance.

2. Cell cycle blockage at G1 phase observed in long-term cultures of BMDM is induced by an overexpression of *p16* and *p27* as well as by suppressed expression of *cyclinD1*, *cyclinE*, *Cdk2* and *Cdk4*. In addition, upregulation *of c-Myc*, *Raf* and *E2F1* also contribute to the impaired cell cycle progression. The signaling pathway involved in cell cycle arrest observed implicate sustained ERK and p38 MAPK activation and reduced STAT5 phosphorylation.

3. Accumulation of DNA damage shown in long-term cultures of BMDM is not produced by impaired DNA repair machinery.

4. p16 pathway and telomere shortening are involved in the induction mechanism of senescence in long-term cultures of BMDM.

Classical and alternative macrophage functions are compromised in long-term cultures of BMDM

1. Long-term cultures of BMDM exhibit suppressed antigen presentation capacity and microbicidal activity.

2. TLR-2, -4, -5 and -9 profile expression is altered in long-term cultures of BMDM which is associated with the increased secretion of IL-1β, TNF-α and IL-10.

3. *Arginase-1*, *fizz1* and *mgl1* mRNA expression is reduced in M2 senescent macrophages whereas other M2 markers (*tgfb* and *mrc1*) are not affected.

4. Arginase activity and phagocytosis are impaired in long-term cultures of BMDM.

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