The role of neutrophils in telomere induced senescence via bystander effects

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Dedication

I dedicate this thesis to my parents, Cesar and Dominque, my wife Su Yun and my daughter Margot (“little nice monster”) which have always believed and supported me in every action, thank you for your eternal support, you are wonderful.

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To my lovely family: My daughter “little monster” Margot, Mum and Dad, my sisters Elisabeth and Jennifer for their understanding and constant support in whatever I do, you will always be part of my life wherever I go, with all my love.

Proverb:

“Fais ce que tu dois, advienne que pourra et c’est la vie !”

DECLARATION OF AUTHENTICITY

I declare that this dissertation is my original work, gathered and utilised especially to fulfil the purposes and objectives of this study, and has not been previously submitted to any other university for a higher degree.

ABSTRACT

Senescence is classically defined as a state of permanent cell-cycle arrest. Senescence can occur in response to various stresses, which have been shown to act mainly through the activation of a DNA damage response (DDR). Senescence is characterised not solely by a cell-cycle arrest but also by increased production of Reactive Oxygen Species (ROS) and the development of a Senescence-Associated Secretory Phenotype (SASP). SASP components include growth factors, cytokines, chemokines and immune modulators and have been shown to contribute to senescence in an autocrine manner but also impact on the tissue microenvironment trough paracrine effects. Several studies have linked the SASP with immune surveillance suggesting that Natural Killer cells, monocytes and T lymphocytes CD4+ can effectively eliminate senescent cells. However, the interaction between neutrophils (the first innate immune responders to infection or injury) and cellular senescence has not yet been investigated.

In this thesis, I have shown that neutrophils induce premature senescence in human fibroblasts in a telomere-dependent manner. My data indicates that hydrogen peroxide released by neutrophils damages telomeric DNA, thereby accelerating the rate of telomere shortening and contributing to the early onset of senescence. Consistently, pre-treatment with the antioxidant enzyme catalase, prevents neutrophil-induced telomere shortening and premature senescence. In addition, overexpression of the catalytic subunit of telomerase (hTERT), which maintains telomere length in cultured fibroblasts, is able to bypass neutrophil-induced premature senescence.

In accordance with my *in vitro* results, I have shown that following acute liver injury (using CCl_4) which is characterised by neutrophil infiltration, mouse hepatocytes show increased markers of telomere dysfunction, which can be prevented by neutralisation of neutrophils. Importantly, I have found that during the ageing process or after injection with lipopolysaccharide (LPS), mouse livers experience increased neutrophil infiltrations which positively correlate with markers of telomere-dysfunction.

Finally, I have shown that senescent cells secrete factors which act as a neutrophil chemoattractant and that neutrophils preferentially induce cell-death in senescent cells but not young cells. These data suggest for the first time that neutrophils play an important role in the immune clearance of senescent cells.

Altogether, my data propose that neutrophils act as a double-edged sword: on one hand, they can induce senescence by accelerating telomere shortening; on the other hand, they can be recruited to sites where senescent cells are present and accelerate their specific clearance.

ABBREVIATIONS

4′,6-diamidino-2-phenylindole (DAPI)
5-bromo-2-deoxyuridine (BrdU)
5-ethynyl-2’-deoxyuridine (edU)
8-oxo-7,8-dihydro-2‘-deoxyguanosine (8-oxodG)
Activating Natural Killer cell receptor (NKG2D)
Adenosine triphosphate (ATP)
Alcoholic steatohepatitis (ASH)
Amplex Red reagent (AR)
Anaphase-promoting complex/cyclosome (APC/C)
Anti-oxidant butylated hydroxyanisole (BHA)
Ataxia telangiectasia mutated (ATM)
ATM (ataxia-telangiectasia mutated)
ATM- and Rad3-related (ATR)
ATR (ATM and Rad 3 related)
BCL-2 homology domains (BH1-4)
Bovine serum albumin (BSA)
Bromo and extra terminal domain (BET)
Carbon tetrachloride (CCl4)
Checkpoint kinase 1 (CHK1)
Checkpoint kinase 2 (CHK2)
Chemokine growth-regulated oncogene 1 (Gro-1)
Chemokines (CXC)
Dependant kinases (Cdk)
Dibutyltin dichloride (DBCT)
Diethylnitrosamine (DEN)
Dimethyl sulphoxide (DMSO)
DNA double strand break (DSB)
DNA-dependent protein kinase (DNA-PKcs)
Double strands break (DSB)
ECACC (Europe Collection of Cell Culture)
Epithelial-to-mesenchymal transitions (EMT)
Extracellular matrix proteins (ECM)

Foetal bovine serum (FBS)

Hank's Balanced Salt Solution (HBBS)

Hepatic stellate cells (HSC)

Hepatocellular carcinoma (HCC)

High mobility group box 1 (HMGB1)

Histone deacetylase inhibitors (HDACi)

Interacting nuclear factor 1 interacting nuclear factor 2 (TIN2)

Intercellular Adhesion Molecule 1 (ICAM-1)

Interleukin 6 (IL-6)

Intraperitoneal injection (I.P)

MAPK kinase kinase (MAP3Ks)

MAPK kinases (MKK)

Matrix metalloproteinase (MMPs)

Mediator of DNA damage checkpoint protein 1 (MDC1)

Mitochondria outer membrane permeabilisation (MOMP)

Mitochondrial DNA (mtDNA)

Mitogen-activated protein (MAP)

Mitogen-activated protein kinase (MAPK)

Monoamine oxidation A (MAO-A)

Monocyte chemoattractant protein-1 (MCP-1)

Mouse embryonic fibroblast (MEFs)

MRM complex (MRE11-RAD50-NBS1)

Murine hepatocellular carcinomas (HCCs)

Myeloperoxidase (MPO)

Natural killer cells (NK cells)

Neutrophil elastase (NE)

Neutrophil extracellular traps (NETs)

Neutrophil-depleting antibody (Ly6G)

N-Formylmethionyl-leucyl-phenylalanine (fMLP)

N-Nitrosodiethylamine (DEN)

Non-alcoholic steatohepatitis (NASH)

Non-homologous end joining (NHEJ)

Normal goat serum (NGS)

Oncogene induce senescence (OIS)

P53-binding protein-1 (53BP1)

Paraformaldehyde (PFA)

Phenyl-α-tert-butyl nitrone (PBN)

Phosphate-buffered saline (PBS)

Phosphatidylinositol 3-kinase (PI3K)

Phosphorylated form of the histone variant H2AX (γ-H2AX)

PI3K-like protein kinases (PIKK)

Platelet-derived growth factor AA (PDGF-AA)

Poly ADP ribose polymerase (PARP)

Polymorphonuclear leukocytes (PMN)

Population doubling (PD)

Propidium iodide (PI)

Protection of telomeric protein 1 (POT1)

Proteins telomeric repeat-binding factor (TRF)

Reactive oxygen species (ROS)

Repair factor proliferating cell nuclear antigen (PCNA)

Retinoblastoma protein (pRb)

Reverse transciptase (TERT)

Room temperature (RT)

Roswell Park Memorial Institute (RPMI)

Senescence messaging secretome (SMS)

Senescence–associated heterochromatin foci (SAHF)

Senescent associated secretory phenotype (SASP)

Single strand break (SSB)

Stress-induced premature senescence (SIPS)

Superoxide dismutase (SOD)

Superoxide dismutase 1 (Sod1)

Telomere dysfunction-induced DNA damage foci (TIF)

Telomeric associated foci (TAF)

Telomeric repeat-binding factor 2-interacting protein 1 (Rap1)

Threonine (T)

Toll like receptor (TLR4)

Toll-like receptors (TLR)

Trichloromethyl radical (CCl_3^*)

Trichloromethylperoxy radicals (CCl_3OO^*)

Tripeptidyl peptidase 1 (TPP1)

X-ray irradiation (IR)

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1 CHAPTER 1 - INTRODUCTION

1.1 Cellular senescence

1.1.1 Definition

For a long time, it was believed that strains of human cells could be cultivated infinitely *in vitro* under adequate cell culture conditions (Carrel and Ebeling 1921*)*. However, more than 6 decades ago, Hayflick and Moorhead in 1965 described an interesting phenomenon, which redefined this concept by observing human diploid cell strains *in vitro* (Hayflick and Moorhead 1961, Hayflick 1965). In fact, in opposition from previous work, they discovered that human diploid cells have a finite lifetime determined by their potential for cell doublings. This breakthrough observation ended the dogma that every vertebrate cell grown *in vitro* is immortal.

At the time, they proposed to distinguish a “cell strain” which has a limited number of passages from a “cell line” which is immortal by serial sub-cultivations (Hayflick and Moorhead 1961, Hayflick 1965). This discovery was later named the Hayflick limit characterised by the stage when cells can no longer divide in culture after a period of normal cell proliferation and are thought to be play an important role in the aging process in organisms.

From that point, replicative senescence is achieved in culture when cells lose their ability to divide by reaching the maximum of population doubling of a given cell strain. Although senescent cells do not divide, they remain viable for many months, even years (Wang, Lee et al. 1994).

Subsequently, other groups studying cellular senescence have shown that cellular senescence could be triggered prematurely in response to various stresses (stress-induced premature senescence or SIPS) such as H_2O_2, hyperoxia, or tert-butylhydroperoxide (Toussaint, Medrano et al. 2000).

Two possible apparently antagonistic roles have been proposed for cellular senescence: 1) on one hand, senescence could be a mechanism to stop tumorigenesis (Sager 1991) (act as a tumour suppressor mechanism), a feature which seems to be a beneficial

aspect of cellular senescence. 2) Senescence is proposed to impact negatively on tissue regeneration during age. Therefore, senescence is considered a double edge mechanism; on one hand with a protective effect against cancer, but on the other hand a driver of organismal ageing.

These apparently contradictory concepts can be explained by the antagonistic pleiotropy theory proposed by Williams in 1957 (Williams 1957) . This theory proposes that genes can have beneficial effects on young organisms with the purpose of increasing fitness but have deleterious effects at later ages (Seshadri and Campisi 1990, Campisi 2001, Krtolica, Parrinello et al. 2001, Coppé, Kauser et al. 2006). In addition, Kirkwood proposed the disposable soma theory, which suggests a trade-off between reproduction and ageing because organisms put energy resources into reproduction and maintenance of germ cells, while neglecting maintenance of somatic tissues. This ultimately results in somatic tissue degeneration (Kirkwood 2005).

In recent years, data has accumulated suggesting that senescence is a natural powerful anti-cancer mechanism (Serrano, Lin et al. 1997, Campisi 2001, Campisi 2013) and numerous studies implicates senescence (which reduces tissue homeostasis and exhausts their regenerative capacity) in the ageing process and age-related pathology (Krishnamurthy, Torrice et al. 2004, Herbig, Ferreira et al. 2006, Jeyapalan and Sedivy 2008).

Senescent cells have been shown to accumulate with age within a variety of tissues (Dimri, Lee et al. 1995, Jeyapalan, Ferreira et al. 2007); however, this data does not really demonstrates causality. Recently, scientists at the Mayo Clinic (USA) have generated a mouse model where cells expressing the senescence marker p16ink4a could be eliminated by apoptosis (via activation of caspase 8) upon administration of the synthetic drug AP20187. Baker and colleagues showed that clearance of senescent cells can delay age related tissue dysfunction and improve healthspan in a progeroid mouse model (Baker, Wijshake et al. 2011). Furthermore, the same group showed that clearance of senescent cells in wild-type mice led to increased healthspan and lifespan suggesting that senescent cells are contributing to the ageing process (Baker, Childs et al. 2016).

New evidence suggests that cellular senescence has others beneficial effects beyond tumour suppression. Recent studies have demonstrated a role for senescence in directing

wound healing (Demaria, Ohtani et al. 2014) and in mammalian embryonic development (Muñoz-Espín, Cañamero et al. 2013, Storer, Mas et al. 2013).

Recent studies have shown that the immune system is involved in the clearance of senescent cells to prevent their accumulation (Xue, Zender et al. 2007, Krizhanovsky, Yon et al. 2008, Sagiv, Biran et al. 2013). Therapies using pharmacological agents which can directly kill senescent cells (senolytics) were shown to have positive effects during ageing in mice, improving frailty and cardiac function (Zhu, Tchkonia et al. 2015, Chang, Wang et al. 2016, Zhu, Tchkonia et al. 2016).

To conclude, since its discovery, the knowledge on cellular senescence is expanding, and seems to uncover multiple functions with both beneficial and detrimental effects. Understanding the mechanisms that drive senescence is therefore of high interest and will allow the scientific community to resolve questions in the ageing field.

1.1.2 Causes of senescence

Cellular senescence can be triggered by various stresses, which can be divided into two major categories based on the kinetics of senescence induction chronic and acute. Chronic senescence is a consequence of accumulation of cellular stress damage trough time including the gradual replication-induced senescence (which is thought to be driven by gradual telomere attrition). Acute induction of senescence occurs within a short period of time by an extreme stress and seems to be part of an orchestrated biological process which allows a growth arrest in response to certain stimuli known as stress-induced premature senescence (SIPS) (Campisi and d'Adda di Fagagna 2007, Kuilman, Michaloglou et al. 2010) and Oncogene-Induced Senescence (OIS). OIS occurs as a response to unbalanced mitogenic signals and is triggered with a fast kinetics and a defined stimulus (Blagosklonny 2003).

In this section, I will describe some of the key triggers of senescence in detail.

1.1.2.1 Telomere attrition

Telomeres, which were discovered by Elisabeth Blackburn (Blackburn and Gall 1978) are non-coding, specialised complexes at the end of linear natural eukaryotic chromosomes (Blackburn 1991). Telomeres are a highly conserved sequence of nucleotides (5`-

TTAGGG-3` in mammals) repeated in tandem with an average length at birth of 11 kilobases with a 3` single strand G-rich telomeric strand overhang (McElligott and Wellinger 1997). This 3` overhang is believed to invade the duplex part of the telomere to form a DNA displacement loop or D-loop resulting in a change of conformation of telomeric DNA into large duplex loop (t-loop) (Griffith, Comeau et al. 1999), which protects the 3`end from nucleolytic attack (Blackburn 1991, d'Adda di Fagagna, Teo et al. 2004) (Figure 1.1). The function of the t-loop is to protect the chromosome from end-to-end fusions, which may lead to loss of integrity and instability of the chromosomes. The formation of the t-loop involves proteins with DNA binding domain such as TRF2 (Stansel, de Lange et al. 2001) which is implicated in remodelling the telomeres. Later on, other proteins were found to play an addition role in the formation of the t-loop and work as a complex collectively named shelterin. This complex is specific for telomeric repeats TTAGGG and uniquely qualified to recognise telomeres. The shelterin complex is composed of the protein telomeric repeat-binding factor 1 (TRF1), TRF2 and protection of telomeric protein 1 (POT1), TRF1 interacting nuclear factor 1 interacting nuclear factor 2 (TIN2), Tripeptidyl peptidase 1 (TPP1) and telomeric repeat-binding factor 2-interacting protein 1 (Rap1), which protects the telomeres from DNA damage. Such protected telomeres are called “cap” (de Lange 2001, de Lange 2005) (Figure 1.1).

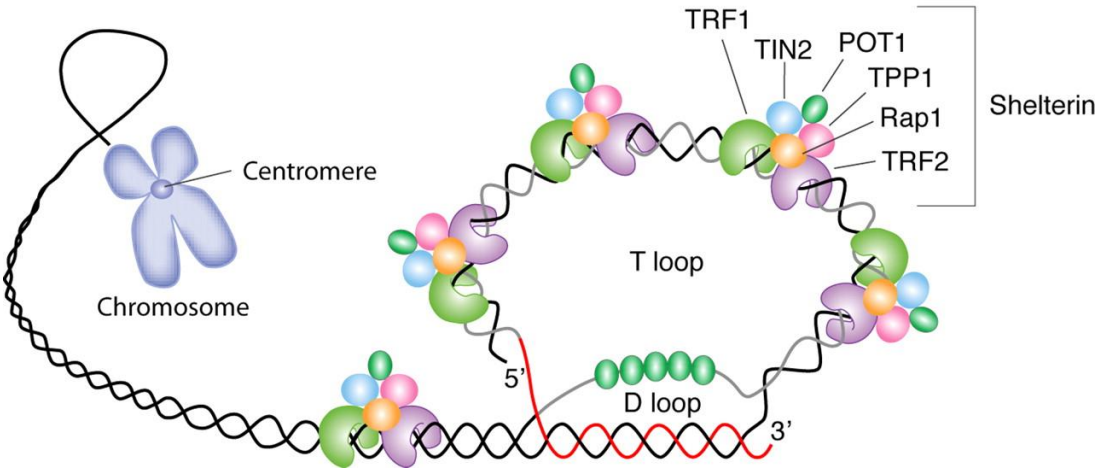


Figure 1.1 Schematic representation of the protected telomere structure (Calado and Young 2008).

Telomeres are at the extremities of chromosome DNA. The telomeric 3′ end terminates as a single-stranded, G-rich overhang able to generate a t-loop, in which the overhang invades the telomeric double helix, remodeling the DNA into a large duplex loop. Telomeres are capped by at least 6 proteins (TRF1, TRF2, TPP1, POT1, TIN2, and Rap1), collectively known as shelterin, that physically shield the DNA (de Lange 2005). TRF1, TRF2, and TPP1 specifically recognize and bind to double-stranded TTAGGG repeats; POT1 binds to the single-stranded telomeric overhang (de Lange 2005, Xin, Liu et al. 2007)19,20; TIN2 and Rap1 are also part of the shelterin complex. The shelterin complex allows discrimination of telomeres from normal double-stranded DNA breaks; lack of shelterin permits telomeres to be identified as double-stranded DNA breaks and triggers DNA-damage response (de Lange 2005).

Like the rest of the genome, telomeric DNA is replicated before the cells divide; however, the DNA polymerases cannot initiate the synthesis of DNA in a 3'–5' without a RNA primer (for the synthesis of Okazaki fragments on the lagging strand). Once the replication is completed, this short RNA is removed and the Okazaki fragments are then connected by DNA ligase to form an unbroken strand. However, at the chromosome end, on the last Okasaki fragment, once the RNA primer is removed it cannot be replaced with DNA because of the absence of the 3'OH end necessary for the DNA polymerase to start the synthesis, leading to the loss of terminal nucleotides (Okazaki, Okazaki et al. 1968, Balakrishnan and Bambara 2013). Hence, 25 to 200 bases of the lagging strand are not copied at each cell cycle. This is known as "the end replication problem", which leads to progressive telomere shortening (Levy, Allsopp et al. 1992) and ultimately to telomere uncapping and supposedly disruption of the t-loop (Levy, Allsopp et al. 1992).

To explain the Hayflick limit, the involvement of telomeres was first hypothesised independently by two scientists (Olovnikov 1971, Watson 1972, Olovnikov 1973). Later,

Harley and colleagues observed for the first time in 1990, using southern blot to measure the length of terminal DNA fragments, a decrease of telomeric DNA in human fibroblasts cultured *in vitro* for several passages. However, it was still unknown if this shortening was causal in replicative senescence (Harley, Futcher et al. 1990).

Later on, Bodnar and colleagues, confirmed that shortening of the telomeres was responsible for cellular senescence by showing that ectopic expression of the catalytic subunit of telomerase leads to cellular immortalisation by preventing telomere shortening and replicative senescence (Bodnar, Ouellette et al. 1998).

Telomerase, discovered by Carol Greider and Elisabeth Blackburn in 1985 (Greider and Blackburn 1985), is a ribonucleoprotein DNA polymerase which elongates and maintains telomeres in eukaryotes. Telomerase contains a telomerase reverse transcriptase (TERT) protein and a telomerase RNA template (TERC) that is specific to the repeated telomere sequence (Greider and Blackburn 1989, Weinrich, Pruzan et al. 1997, Beattie, Zhou et al. 1998).

Studies using immortalised cell lines, primary tumours, adult germlines and normal somatic cell culture show that the telomerase activity is linked to cellular immortality (Kim, Piatyszek et al. 1994). In fact, in somatic culture or benign tumour culture, telomerase activity is repressed, whereas it is detectable in highly proliferative cells (germline and cancer cells) (Wright, Piatyszek et al. 1996).

Hence, based on these studies, it was proposed that telomere shortening was a counting mechanism which determines the number of population doublings of cell culture working like a replication clock (Harley, Futcher et al. 1990, Bodnar, Ouellette et al. 1998). When telomere shortening becomes critical, the t-loop is disrupted and the DNA damage response is triggered to induce senescence (d'Adda di Fagagna, Reaper et al. 2003, Takai, Smogorzewska et al. 2003). However, data from von Zglinicki has shown that, the shortening of telomeres was not only due to the end-replication problem and that additional factors impacted on the rate of shortening of telomeres. Indeed, they showed that the telomere-shortening rate in a cell culture is not constant: it varies from cell to cell in response to oxidative stress and intrinsic heterogeneous anti-oxidant defences (von Zglinicki, Saretzki et al. 1995, Vaziri, West et al. 1997, von Zglinicki, Pilger et al. 2000) (von Zglinicki 2001, von Zglinicki 2002) . In parallel, while oxidative damage in the bulk of

the genome is efficiently repaired, telomeres are less efficiently repaired, accumulating single-stranded DNA breaks. This resulted in accelerated telomere shortening (Petersen, Saretzki et al. 1998). Therefore, the authors hypothesised that telomeres work as sensors for genome instability, and under oxidative stress short telomeres trigger senescence to potentially remove damaged, cancer-prone cells (von Zglinicki 2001, von Zglinicki 2002). Telomere heterogeneity has been observed by quantitative FISH in metaphases from bone marrow cells (Lansdorp 2009). Moreover, it has been shown that cellular senescence is highly heterogeneous, with a fraction of cells spontaneously dropping-out of the cell cycle early on before the Hayflick limit being reached. Cell sorting of these prematurely senescent cells revealed that these have shorter telomeres and show evidence for increased mitochondrial dysfunction and oxidative stress (Passos, Saretzki et al. 2007). This led to the suggestion that random telomere damage induced by oxidative stress could contribute to the heterogeneity observed during cellular senescence (Martin-Ruiz, Saretzki et al. 2004, Lansdorp 2009). Moreover, telomere dysfunction can be initiated and can trigger a senescence response by the presence of individual short telomeres within the cell (Hemann, Strong et al. 2001, Herbig, Ferreira et al. 2006).

Together, these data have highlighted a role for telomeres as protectors of the integrity of DNA. Afterwards, it has been demonstrated that telomere-binding proteins play a role in the stability of telomeres. When telomeres become unprotected due to loss of telomere binding proteins, it has been speculated that this contributes to the loss of the t-loop conformation and the free ends of the telomeres are detected by the cell DNA repair machinery as DNA double strand breaks (DSB) and trigger a DNA damage response (de Lange 2002). Besides, experiments from Stansel and colleagues have shown that TRF2 is involved in the T-loop formation, since impairment of the DNA binding of TRF2 *in vivo* caused cell death via activation of ataxia telangiectasia mutated ATM and p53 (Stansel, de Lange et al. 2001). Further evidence using super-resolution microscopy confirmed that the formation and/or the maintenance of t-loops is dependent on TRF2 since its deletion turns t-loops into linear telomeres (Doksani, Wu et al. 2013). In addition, telomere uncapping through inactivation of the telomeric protein TRF2 engages a senescence response via recruitment of known DNA damage proteins and activates a DDR (Karlseder, Broccoli et al. 1999, Hiroyuki, Agata et al. 2003, Oh, Wang et al. 2003).

This response involves recruitment of DNA repair factors to the site of the damage and the activation of checkpoint pathways, which will impair cell cycle progression (Figure 1.2)

(d'Adda di Fagagna, Reaper et al. 2003, Gire, Roux et al. 2004, Herbig, Jobling et al. 2004). Studies have indicated that DSB can be visualised as foci containing the phosphorylated form of the histone variant H2AX (γ-H2AX). In addition, foci contain as well DNA damage factors such as proteins involved in the recognition, repair and signalling. Such foci are named telomere dysfunction induced foci (TIF) (d'Adda di Fagagna, Reaper et al. 2003, Hiroyuki, Agata et al. 2003, Herbig, Jobling et al. 2004).

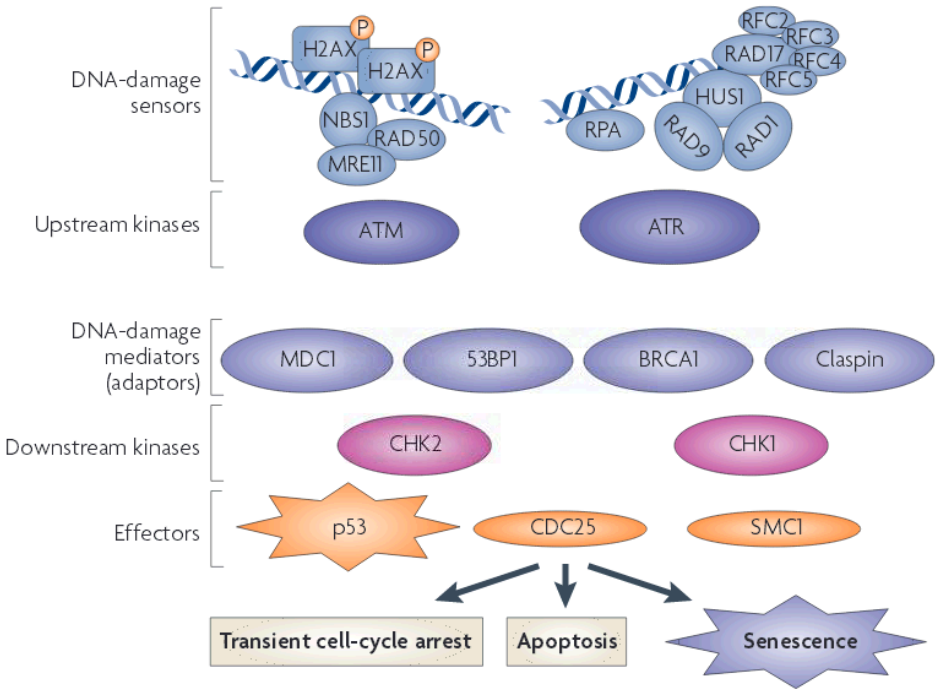


Figure 1.2 The DNA-damage response (Campisi and d'Adda di Fagagna 2007).

When critically short telomeres or DNA double-stranded breaks occurs, they are sensed by protein complexes such as replication protein A (RPA) and replication factor C (RFC) but also by the MRN complex (MRE11-RAD50-NBS1). Then, upstream protein kinases like ataxia telangiectasia mutated (ATM) and Rad-3 related (ATR) become activated leading to phosphorylation of the histone variant H2AX. Then, a series of proteins are recruited in order to transduce the signal and optimise repair activities by other proteins through adaptors like 53BP1 and others DNA-damage response proteins. The signal is transduced by downstream kinases such as checkpoint 1 (CHK1) and CHK2 culminating in the activation of effector molecules like transcription factor p53. P53 transcribes p21, a cyclin kinase inhibitor, which contributes to cell cycle arrest. The duration of the arrest will depend on the nature of the damage: if it is minor, the DNA is repaired and the cell-cycle can continue but if cells are not able to cope with the damage this may lead to a permanent cycle arrest (or senescence) or apoptosis (d'Adda di Fagagna, Reaper et al. 2003, Hiroyuki, Agata et al. 2003, Gire, Roux et al. 2004, Herbig, Jobling et al. 2004, Nakamura, Chiang et al. 2008).

Telomere dysfunction can be triggered by mechanisms other than replicative exhaustion or loss of telomere-binding proteins; in fact, our group and others have shown that telomeres are sensitive to genotoxic and oxidative stress inducing an unresolved DSB leading to a permanent DDR. Such foci located at the telomeres are called telomere associated foci (TAF). Data indicate that these are persistent (long-lived), can occur independently of telomerase activity and expression of telomere-binding protein TRF2 and increase with age irrespective of telomere length (Fumagalli, Rossiello et al. 2012, Hewitt, Jurk et al. 2012). Mechanistically, it has been shown that presence of TRF2 inhibits ligase IV, preventing non-homologous end joining (NHEJ), thereby eliciting a persistent DNA damage response (Fumagalli, Rossiello et al. 2012).

Consistent with this notion, conditional overexpression or deletion of TRF2 results to ligase IV-dependent telomere fusions (van Steensel, Smogorzewska et al. 1998, Celli and de Lange 2005). In budding yeast, when damage is induced at the telomeric regions, inhibition of DNA repair is due to suppression of the recruitment of the ligase IV to mediate non homologous end joining (NHEJ) (Fumagalli, Rossiello et al. 2012).

Since these discoveries, TAF have been found to occur during ageing in liver and intestinal crypts (Hewitt et al. 2012), lung (Birch et al. 2015), in the aorta from aged and hypercholesterolemic mice (Roos, Zhang et al. 2016) and to be induced as a result of low-grade chronic inflammation driven by NF-kB (Jurk, Wilson et al. 2014). Furthermore, treatment with drugs known to eliminate senescent cells reduced the frequency of TAF positive cells (Roos, Zhang et al. 2016) indicating that TAF are a marker of cellular senescence.

Altogether, this data suggest that telomere dysfunction is a key player in the initiation of the senescence program. Furthermore, telomere dysfunction can be induced by shortening or by induction of DSBs in telomere repeats occurring irrespectively of telomere length. The mechanisms mediating the DDR will be described in detail later on.

1.1.2.2 Non-telomeric DNA damage senescence

DNA damage is not restricted to the telomeric regions; it occurs over the whole genome. In response to damage, specialised sensor molecules come to the site and communicate the information via signalling pathways to trigger a DDR (Shiloh 2006).

γH2AX foci have been reported on mitotic chromosomes before and after the cell cycle in human cells, suggesting an adaptation mechanism and the possibility that cells are able to divide with unrepaired damage (Syljuåsen, Jensen et al. 2006). An additional study supports this idea and has shown that persistent DSB accumulate over time; When a threshold level is reached (10 to 20 DSBs), the DDR is fully activated and triggers senescence (Deckbar, Birraux et al. 2007). Additionally, a study by Nakamura *et al*. has shown that when cells are close to senescence they contain similar numbers of foci across the entire genome (Nakamura, Chiang et al. 2008).

When the DNA damage such as DSBs overcome the DNA repair mechanisms, cells trigger the senescence program leading them to a prolonged cell cycle arrest (Di Leonardo, Linke et al. 1994).

Evidence from SIPS studies have shown that critical DNA damage through the entire genome can be caused by different agents such as UV, ethanol, gamma irradiation and genotoxic drugs, which will generate SSBs and DSBs and then trigger a permanent cell cycle arrest (Toussaint, Remacle et al. 2002).

However, regarding the actions of these agents, which induce DNA damage to the entire bulk of the genome, it is likely that both non-telomeric and telomeric DNA damage contribute to the senescent phenotype. Indeed, DSB molecular markers have been shown to share similar DNA damage response whether they localise at the telomeres or on the rest of the genome (d'Adda di Fagagna, Reaper et al. 2003).

It is worth considering that the location of the DNA damage may be the limiting factor: for instance, studies have indicated that only 1 dysfunctional telomere is sufficient to induce cellular senescence (d'Adda di Fagagna, Reaper et al. 2003, Gire, Roux et al. 2004, Sedelnikova, Horikawa et al. 2004, Zou, Sfeir et al. 2004). More recently it has been suggested that activation of a DDR at 5 telomeres is necessary to induce senescence (Kaul, Cesare et al. 2012).

In addition, studies from our group and others have shown that DNA damage foci (detected by γH2AX) can be found in replicative senescent cells as well as stress-induced senescent cells in the entire genome (telomeric and non-telomeric regions). Using live cell imaging to track the focus lifespan, our group has reported that short-lived focus number is relatively constant during senescence (Passos, Nelson et al. 2010,

Hewitt, Jurk et al. 2012). Besides, it was shown that these short-lived foci were mostly due to an increased reactive oxygen species (ROS) production during senescence (since antioxidants are able to prevent them) and are believed to contribute to develop and stabilise the senescent phenotype (Passos, Nelson et al. 2010).

Overall, DSB are generated following high levels of DNA damage both at telomeric regions and within the rest of the genome. If this damage is not repaired correctly, then lesions lead to irreparable DNA damage, which will engage a persistent DNA damage response and eventually trigger the onset of senescence.

1.1.2.3 Mitochondrial dysfunction, oxidative stress and reactive oxygen species (ROS) induce senescence

Oxygen was discovered in the 18th century by Karl W Schelle and Joseph Priestley. Initially, they described it as a gas which triggers combustion but later realised that it was involved in respiration and essential for survival. Later still, Lavoisier named this gas oxygen, which means “acid producer”, and conducted experiments showing that disproportionate oxygen levels were toxic to life.

The mechanism mediating oxygen toxicity was first discovered by Rebeca Gerschman and colleagues in 1954. They showed that oxygen toxicity shared the same mechanism of action with ionizing radiation (X-rays) : the formation of oxidising radicals (free radicals) (Gerschman, Gilbert et al. 1954). Afterwards, work from Dehnam Harman linked free radicals (eg. •OH and $O_2^{\cdot -}$), which are highly reactive molecules bearing an impaired electron, with ageing. He proposed that free radicals were produced by cells through oxygen metabolism by the respiratory enzymes containing metals such as iron, manganese and cobalt (Harman 1956). Only later, the theory was reinforced by the discovery of superoxide dismutase (SOD) from bovine erythrocytes, an enzyme which catalyses the superoxide anion ($O_2^{-\cdot}$) into water and hydrogen peroxide (McCord and Fridovich 1969). This work demonstrated that a superoxide anion was produced in living cells, since an enzyme existed which detoxified it. In 1972, Harman updated his free radical theory of ageing and proposed that mitochondria have a major role in ageing since they are the major sources of oxygen free radicals, contain their own DNA (mtDNA), which is found in close proximity to the electron transport chain (where oxygen free radicals are generated) and is relatively unprotected when compared to the nuclear

genome (Harman 1972). Oxidative stress is the result of an imbalance between free radical production and expression of antioxidant defences (Cadenas and Davies 2000).

Mitochondria are the organelles responsible for energy production in the form of ATP, which is derived from utilisation of oxygen in eukaryotes. Generation of ROS in mitochondria occurs in the electron transport chain, resulting from electron leakage and reaction with oxygen, which forms a superoxide anion (Hanukoglu, Rapoport et al. 1993, Barja 1999). Consequently, the superoxide anion can be converted into hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase enzyme (SOD2). Hydrogen peroxide can diffuse past the outer mitochondrial membrane and lead to damage in other cellular compartments such as the cytoplasm and the nucleus. (Cadenas and Davies 2000).

To maintain functional mitochondria, cells require multiple processes, such as mitochondrial fusion and fission, mtDNA repair and clearance of damaged mitochondria via autophagy (mitophagy) (Youle and van der Bliek 2012, Randow and Youle 2014, Scheibye-Knudsen, Fang et al. 2015). Due to their prokaryotic origin, mitochondria contain their own mitochondrial DNA (mtDNA) which resides inside the mitochondria and encodes for components of the electron transport chain (Prachar 2010). Mitochondrial DNA is considered to be a major target of the oxidative environment and may be subjected to mutations which can cause over time mitochondrial dysfunction (Golden and Melov 2001, Park and Larsson 2011). In fact, mtDNA mutations have shown to contribute to numerous human diseases such as neurodegenerative diseases, muscle atrophy, cardiomyopathy, renal failure and premature ageing (Park and Larsson 2011).

Early studies have shown a causal link between oxidative stress and replicative senescence. Depending on the levels of oxygen in the atmosphere, senescence could be modulated: under low oxygen conditions the replicative lifespan of human fibroblasts is extended (Packer and Fuehr 1977, Chen, Fischer et al. 1995) whereas under high levels of oxygen, cells can enter premature senescence (von Zglinicki, Saretzki et al. 1995). In addition, treatment with exogenous H_2O_2, or inhibition of antioxidant enzymes such as the copper-zinc-containing SOD (SOD1) induces senescence (Chen and Ames 1994, Chen, Bartholomew et al. 1998, Blander, de Oliveira et al. 2003). One explanation could be that oxidative stress can damage several cell components such as DNA, proteins and lipids (Chen, Bartholomew et al. 1998, Sitte, Merker et al. 2000). ROS, especially the hydroxyl

radical (•OH), oxidises DNA bases, leading to single and double strands breaks (SSB and DSB) (Cooke, Evans et al. 2003). Highly cytotoxic lesions such as double stranded breaks trigger a DNA damage response which activate cell-cycle checkpoints, stopping the cell cycle and finally inducing the senescence program if the damage is persistent (Khanna and Jackson 2001, van Gent, Hoeijmakers et al. 2001, Jackson 2002). Furthermore, telomeres can be targeted by ROS leading to accelerated telomere loss and ultimately to irreversible growth arrest (von Zglinicki 2002). Telomeres contain guanine triplets which are very susceptible to oxidative modification leading to the formation of 8-oxo-7,8-dihydro-2‘-deoxyguanosine (8-oxodG) (Henle, Han et al. 1999, Oikawa, Tada-Oikawa et al. 2001). Moreover, studies indicate that the repair of SSBs at the telomeres is less efficient than that in the rest of the genome (Petersen, Saretzki et al. 1998). Accumulation of SSBs at telomeres can then contribute to accelerated telomere shortening upon cell division. Furthermore, exposition of hyperoxia induces DNA strand breaks with similar effects caused by H_2O_2 (Cacciuttolo, Trinh et al. 1993) which has been shown to induce SSBs and DSBs with a slow repair rate (Driessens, Versteyhe et al. 2009).

Data from our group and others have shown that mitochondrial dysfunction, ROS and damage are linked together to establish the growth arrest observed during cellular senescence. Indeed, following a DDR, the activation of signalling pathway involving p53/p21CIP1/WAF1 contributes to mitochondrial dysfunction and increased ROS (Macip, Igarashi et al. 2002). Then ROS will enter in a positive feedback loop leading to more short-lived DNA damage foci and maintain a persistent DNA damage response (Macip, Igarashi et al. 2002, Passos, Nelson et al. 2010). Consistent with a role for ROS in the maintenance and stability of senescence, treatment with antioxidants such as N-acetyl cysteine (NAC) and phenyl-α-tert-butyl nitrone (PBN) was able to partially prevent the cell-cycle arrest (Macip, Igarashi et al. 2002, Macip, Igarashi et al. 2003, Passos, Nelson et al. 2010).

Finally, very recent data from our group highlighted the involvement of mitochondria in the development of pro-inflammatory and pro-oxidant features of senescence. Eliminating mitochondria within the cells via an artificial system (involving overexpression of Parkin and widespread mitophagy) resulted in the suppression of certain senescent phenotype features of s while maintaining a permanent cell cycle arrest (Correia-Melo, Marques et al. 2016). Moreover, a new pathway has been described linking an activation of a DDR and

mitochondrial dysfunction and ROS generation via the mTORC1 signalling cascade (Correia-Melo, Marques et al. 2016).

However, recent data have put into question the idea that ROS is a driver of ageing and has shown a conflicting role for ROS in the ageing process (Hekimi, Lapointe et al. 2011). For example, increased ROS production by manipulation of antioxidant molecules extends lifespan in yeast and *Caenorhabditis elegans* (Doonan, McElwee et al. 2008, Van Raamsdonk and Hekimi 2009, Mesquita, Weinberger et al. 2010). In mice, genetic manipulations to increase ROS and oxidative damage or increase antioxidant molecules did not affect neither the mean nor the maximum lifespan (Van Remmen, Ikeno et al. 2003, Pérez, Van Remmen et al. 2009). These results led researchers to rethink the free radical theory of ageing initially proposed by Harman.

Nevertheless, as previously mentioned, mitochondrial dysfunction is observed during cellular senescence, in response to several stimuli such as telomere dysfunction, genotoxic stress, and oncogene activation (Hutter, Unterluggauer et al. 2002, Passos, Saretzki et al. 2007, Lu and Finkel 2008). During senescence, mitochondrial dysfunction is reflected by increased ROS, increased mitochondrial mass and a decreased mitochondrial membrane potential (Saretzki, Murphy et al. 2003, Passos, Saretzki et al. 2007). In addition, recent work has shown that induction of mitochondrial dysfunction using mitochondrial inhibiting drugs or downregulation of mitochondrial sirtuins can cause a distinct type of senescence-associated secretory phenotype which was termed by the authors as MiDAS (mitochondria dysfunction–associated senescence). This secretory phenotype did not depend on the IL-1α/NF-κB arm that regulates the “traditional” SASP (Wiley, Velarde et al. 2016).

Finally, since the updated theory by Harman (Harman 1972)**,** a large number of studies have shown that mitochondrial dysfunction (characterised by increased ROS production and low adenosine triphosphate (ATP) (Green, Galluzzi et al. 2011)) is a hallmark of ageing (López-Otín, Blasco et al. 2013).

1.1.2.4 Oncogene -induced senescence

As described briefly above, senescence is an anti-tumour mechanism triggered upon aberrant activation of proliferative pathways involving oncogenes in normal cells.

Oncogene activation occurs when cells are transformed following mutations and become unregulated with aberrant mitotic signals.

It has been reported that a senescence response can be triggered by oncogene activation termed oncogene-induced senescence (OIS). The first evidence of OIS was demonstrated by Serrano et al. in 1997. The authors suggested that following oncogene activation such as overexpression of *ras*, a premature senescence response is triggered involving downstream proteins of the DDR leading to a permanent growth arrest in a p53 and p16 dependent manner. Thus, OIS has been proposed as a major safeguard against transformation (neoplasia) (Serrano, Lin et al. 1997). Later, other oncogenes were described to be involved in OIS, such as RAF, BRAF, MOS, MEK and RAC1 (d'Adda di Fagagna 2008). Once activated, these oncogenes trigger a constitutive mitogen-activated protein (MAP) kinase-signalling pathway leading to abnormal transcriptional activation and expression of growth promoting genes like the c-Myc transcription factor (Sears and Nevins 2002). Consequently, these hyper-replication rates of DNA are associated with DNA replication stress, generate DSBs and activate a DDR and cell cycle arrest (Bartkova, Rezaei et al. 2006, Di Micco, Fumagalli et al. 2006).

In addition, work by Peeper has highlighted that OIS occurred in naevi (Michaloglou, Vredeveld et al. 2005). Moles are non-malignant tumours including melanocytes with oncogenic mutations in BRAF (V600E). Overexpression of the latter in human melanocytes induces a senescence response with a cell cycle arrest and display hallmarks of senescence (especially p16). In this study, authors have reported that the senescence response was not due to telomere attrition since they did not observe any telomere shortening *in situ* (comparing naevi to surrounding tissues) (Michaloglou, Vredeveld et al. 2005). However, later it was reported that the majority of the cells in naevi contained dysfunctional telomeres with the presence of telomere dysfunction-induced DNA damage foci (TIF) and expression of a persistent DDR (Suram, Kaplunov et al. 2012).

Besides, Pospelova et al. using microscopy to detect foci together with comet assay have reported that treatment with sodium byturate in fibroblasts overexpressing *ras* engaged a DDR without actual physical DNA damage. This phenomenon is called pseudo-DNA damage, and could be a marker of hyper-proliferation and activated stress signalling pathways (Pospelova, Demidenko et al. 2009). Another mechanism for OIS has been

reported using an activated *ras* gene (V12*ras*) through oxidative damage caused by endogenous H_2O_2 produced by mitochondria which are rescued under low oxygen conditions (Lee, Fenster et al. 1999). As reported, high levels of ROS induce oxidative damage of abasic DNA sites, created SSBs and DSBs which ultimately trigger senescence (Passos, Saretzki et al. 2007).

Therefore, OIS is a mechanism of response to oncogene activation, which leads to a deregulation of DNA replication, replication stress, activation of a DDR and eventually a permanent cell cycle arrest.

1.1.2.5 Epigenetic modifications

Senescence occurs with drastic phenotypic changes and altered regulation of genes. Genes are under the regulation of epigenetic mechanisms including modifications of histones, DNA methylation, and alterations of chromatin structure. It has been reported, that chromatin regulation and structural changes could be involved during ageing and later on senescence (Ryan and Cristofalo 1972, Narita, Nũnez et al. 2003) with the presence of altered chromatin in the form of senescence–associated heterochromatin foci (SAHF). SAHF contain different markers of heterochromatin such as hypoacetylated histones, increased HP1, and are depleted from the histones found in euchromatin (K9Ac-H3 and K4M-H3) and exhibit high levels of macroH2A (Zhang, Poustovoitov et al. 2005, Funayama, Saito et al. 2006). Moreover, macroH2A, HIRA, ASF1 and UBN1 have the ability to silence gene expression (Adams 2007, Changolkar, Singh et al. 2008).

SAHF formation relies on an intact Retinoblastoma protein (pRb) pathway, which may control the nucleation of heterochromatin at specific sites followed by histone methyltransferases and recruitment of HP1 on the histones of E2F target promoter genes. Then the E2F promoters are methylated on lysine 9 to form a “lock”, preventing the transcription machinery to access the site (Dillon and Festenstein 2002, Trimarchi and Lees 2002). Hence, the E2F responsive genes upon senescence undergo chromatin changes from euchromatin to heterochromatin and may contribute to the irreversibility of the cell cycle arrest (Narita, Nũnez et al. 2003). Heterochromatin instability caused by histone deacetylase inhibitors (HDACi) in cells has been shown to induce a senescent-like phenotype independent of telomere shortening. This data suggests that chromatin

modifications can trigger a premature permanent cell cycle arrest (Ogryzko, Hirai et al. 1996).

1.2 Signalling pathways of senescence

Several studies indicate that the DDR is a central initiating factor in cellular senescence, irrespectively of the inducing stimuli. The DDR has been shown to be responsible for stress-induced (Passos, Nelson et al. 2010) oncogene-induced (Suram, Kaplunov et al. 2012) and replicative senescence (d'Adda di Fagagna, Reaper et al. 2003).

Our genome is constantly under attack from endogenous and exogenous insults (approx. 100000 per cell per day) which can be detrimental to cells (Hoeijmakers 2009). Hence, the cells have developed mechanisms for sensing and repairing those breaks to maintain the genome integrity and avoid uncontrolled transformation (neoplasia). If the damage is fixed, then the cells are able to resume proliferation, however, if the DNA repair machinery fails, then the cells can undergo apoptosis (programmed cell death) or trigger the senescence response program. Hence, following DSB, a DNA damage response arises which acts rapidly and precisely through a set of regulated mechanisms. In the first place, sensor proteins are recruited to the site of the lesions and transduce the signal through mediators (Sousa, Matuo et al. 2012) to finally induce the cell cycle via DNA damage "checkpoints" which act as safeguards to ensure integrity of the genome (Abraham 2001).

Cells transduce DNA damage breaks through a family of proteins containing a serine/threonine kinase activity. All members contain a catalytic domain typical of the lipid kinase phosphatidylinositol 3-kinase domain (PI3K) at their carboxyl termini, and for that reason are named PI3K-like protein kinases (PIKK). Among the PIKK proteins family, in mammalian cells, ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) play a critical role in the amplification of the signal. ATM is mutated in the human autosomal recessive disorder ataxia-telangiectasia (A-T) causing genome instability (Savitsky, Bar-Shira et al. 1995, Derheimer and Kastan 2010). Later on, ATR was discovered using a database searching for proteins which contain PI3K domains (Cimprich, Shin et al. 1996). Upon DSB, ATM is mainly associated with DNA-dependent protein kinase (DNA-PKcs) which is involved in the repair of DSB upon genotoxic stress or in V(D)J recombination in non-homologous end-joining (NHEJ) (Dobbs, Tainer et al. 2010, Nagasawa, Little et al. 2011). ATR is mainly activated by SSB and has been

proposed to function in coordination with ATM during the process of DSB resection following the recruitment of RPA and ATRIP and in replication stress (collapse forks replication) (Shiotani and Zou 2009, Flynn and Zou 2011).

After a DSB, ATM is rapidly recruited and becomes activated at the site of the damage (Andegeko, Moyal et al. 2001). Activated ATM is detected by the presence of autophosphorylation at Ser1981 that causes formation of two active monomers and provides ATM with its kinase activity (Bakkenist and Kastan 2003). Once activated, ATM will start to phosphorylate different substrates, one of the most potent is the phosphorylation of the histone H2AX (γH2A.X) on the serine 139 residue (Rogakou, Boon et al. 1999). ATM also interacts with the sensor protein MDC1 (mediator of DNA damage checkpoint protein 1), which binds to γH2A.X and allows a positive feedback loop, expanding the phosphorylation of further γH2A.X. This can then spread from the initial break and form foci (Rogakou, Boon et al. 1999, Stucki, Clapperton et al. 2005, Lou, Minter-Dykhouse et al. 2006, Meier, Fiegler et al. 2007, Savic, Yin et al. 2009).

In addition, γH2A.X has been reported to be a substrate for ATR in response to single-stranded DNA breaks and replication stress (Ward and Chen 2001, Ward, Minn et al. 2004). Moreover, DNA-PK has been reported to be able to phosphorylate H2AX during DNA fragmentation or under hypertonic conditions after irradiation (Reitsema, Klokov et al. 2005, Mukherjee, Kessinger et al. 2006). Generally, phosphorylation of H2AX is a major player in the DDR and has been reported to be activated after ionising radiation by all players cited above (Wang, Wang et al. 2005).

Apart from γH2A.X, ATM interacts with other key substrates, for example the MRN complex (MRE11-RAD50-NBS1) which is a sensor of DSB, and is involved in the recruitment of ATM to the site of damage (Dupre, Boyer-Chatenet et al. 2006). ATM is also involved in the phosphorylation of mediator proteins which amplify the signal such as p53-binding protein-1 (53BP1) (under the regulation of γH2A.X) to accumulate into foci (Fernandez-Capetillo, Chen et al. 2002). MDC1 is another central mediator phosphorylated by ATM and binds similarly to γH2A.X, then recruits other proteins to the site of the lesion (eg. BRCA1 and 53BP1) (Coster and Goldberg 2010).

To ensure repair upon DSB, ATM and ATR activate cell cycle checkpoints to stop or slow down the cell cycle progression via two principal signalling axes: ATM / checkpoint kinase

2 (CHK2) and ATR/ checkpoint kinase 1(CHK1) pathways (Bartek and Lukas 2003). CHK2 is phosphorylated by ATM on threonine (T) T68, then homodimerised, autophosphorylated and becomes activated (Ahn, Li et al. 2002, Oliver, Paul et al. 2006). Once activated, CHK2 dissociates and phosphorylates many targets implicated in the cell cycle in order to stop it, including the p53 tumour suppressor (Chehab, Malikzay et al. 2000, Shieh, Ahn et al. 2000), Cdc25 family phosphatases (Blasina, de Weyer et al. 1999), E2F1 (Stevens, Smith et al. 2003) and BRCA1 (Lee, Collins et al. 2000). Chk2 is mainly responsible in the activation of a S/G1 arrest (Matsuoka, Huang et al. 1998, Blasina, de Weyer et al. 1999, Chehab, Malikzay et al. 2000). CHK1 is phosphorylated by ATR under the control of two mediator proteins TopBP1 and Claspin (Lee, Kumagai et al. 2003, Delacroix, Wagner et al. 2007). Activated CHK1 inhibits members of the Cdc25 family phosphatases by phosphorylation (Blasina, de Weyer et al. 1999, Falck, Petrini et al. 2002) and cyclin-B–Cdk1(Kramer, Mailand et al. 2004) inducing a G2/M cycle arrest (Liu, Guntuku et al. 2000). Both axes converge to inhibit the Cdc25 family phosphatases causing a rapid arrest, followed later by the activation of the p53-p21 pathway that will stabilise the cell cycle arrest. This two-step mechanism is believed to ensure genomic stability by preventing additional damage while generating a stable cell cycle arrest by p21 (Mailand, Falck et al. 2000, Turenne, Paul et al. 2001).

In the following section, I will describe the three main pathways involved in the cell cycle arrest involving activation of cyclin kinase inhibitors.

1.2.1 p53 –p21pathway

Eukaryotic cells respond to DNA damage by halting their cell cycle progression at G_1, S, and/or G_2 phase. This results from activation of cell cycle checkpoints, which are signal transduction pathways that generate signals to inhibit key cell cycle regulators, most notably the cyclin-dependent kinase (Cdk) complexes that govern cell cycle progression (Elledge 1996, Bartek and Lukas 2001).

TP53 has been characterised as the “master gene” or “guardian of the genome” because of its key involvement in various functions such as cell cycle arrest, DNA repair, apoptosis and cellular senescence (Zilfou and Lowe 2009). TP53 encodes the protein p53 which contains among its various domains a DNA binding domain and acts as a transcription factor which can bind to many different targets (Yee and Vousden 2005). Inactivation of

p53 and/or mutation in the TP53 gene is present in various types of cancer resulting in accumulation of damaged cells and cancer promotion (Petitjean, Mathe et al. 2007).

ATM and ATR upon DNA damage can directly interact with p53 in response to ionising radiation and UV light, respectively (Kastan, Zhan et al. 1992, Khanna and Lavin 1993, Tibbetts, Brumbaugh et al. 1999). It has been reported that, p53 is activated through phosphorylation by ATM on Ser-15 and ATR on both Ser-15 and Ser-37 (Banin, Moyal et al. 1998, Khanna, Keating et al. 1998). Once p53 is activated, p53 is stabilised by a further phosphorylation by CHK1 and/or CHK2 on Ser-20, which engages the dissociation of the complex p53-Mdm2; Mdm2 is a protein involved in the degradation of p53 by the proteasome (Haupt, Maya et al. 1997, Chehab, Malikzay et al. 1999, Shieh, Ahn et al. 2000).

Once fully activated, p53 will induce the expression of the cyclin-dependent kinase (Cdk) inhibitor p21 $^{Waf1/Cip1/Sdi1}$ (El-Deiry, Tokino et al. 1993, el-Deiry, Harper et al. 1994), which belongs to the Cip/Kip family which regulates the activity of the Cdks including p27 ^{Kip1}and p57 ^{Kip2} (Sherr and Roberts 1999). Cdks are mainly responsible for the control of cell cycle transitions and are composed of a catalytic subunit (Cdk) and a regulatory cyclin which activates Cdk (Pavletich 1999). In non-transformed cells, p21 is a common inhibitor of cyclin kinases and is part of a quaternary complex with a cyclin, a Cdk and the repair factor proliferating cell nuclear antigen (PCNA) (Xiong, Zhang et al. 1992, Zhang, Xiong et al. 1993). Among its plethora of targets, p21 inhibits the cyclin E/Cdk2 complex resulting in a G1 arrest (Brugarolas, Chandrasekaran et al. 1995). Furthermore, the complex p21/cyclin E/Cdk2 inhibits and activates the anti-tumour pRb which binds the transcription factor E2F. E2F is a critical checkpoint of the G1/S transition and regulates the transcription of genes involved in DNA replication (Ohtani, DeGregori et al. 1995, Geng, Eaton et al. 1996).

In addition, it has been shown that inactivation of pRb can be triggered by cyclin D /Cdk4 (Resnitzky and Reed 1995, Connell-Crowley, Harper et al. 1997) and cyclin D/Cdk6 under the regulation of p21 (Sherr 1994, LaBaer, Garrett et al. 1997, Medema, Klompmaker et al. 1998, Alt, Gladden et al. 2002)

1.2.2 P16-pRb pathway

A second barrier preventing cell growth following DNA damage or dysfunctional telomeres has been elucidated with the discovery of $p16^{INK4a}$ in budding yeast by Manuel Serrano. P16 acts as an inhibitor of Cdk4/6 /cyclin D, which can also activate pRb by maintaining its hypophosphorylated state (Serrano, Hannon et al. 1993, Alcorta, Xiong et al. 1996, Hara, Smith et al. 1996, Stein, Drullinger et al. 1999).

Later other members of the p16 family where identified: $p16^{INK4a}$ (MTS1, CDK4I and CDKN2), p15INK4b (MTS2), $p18^{INK4c}$, and $p19^{INK4d}$ (Roussel 1999). However, the role of p16 in senescence is still debated, since studies have shown that telomere dysfunction during senescence does not engage the p16 pathway (Herbig, Jobling et al. 2004). However, the latter aspect is still being debated: Firstly, p16 levels have been reported to increase with telomere shortening and could be reversed by telomerase expression suggesting that p16 is activated by telomere dysfunction (Alcorta, Xiong et al. 1996, Hara, Smith et al. 1996, Beauséjour, Krtolica et al. 2003). Secondly, using a dominant negative system of TRF2 which uncaps telomeres, p16 levels are increased but with a slow kinetics compared to induction of p53 and p21.This study suggests that p16 could be a backup mechanism if the arrest fails from the p53/p21 pathway (Jacobs and de Lange 2005).

A different study reinforced that idea; by showing, that p16 could act in a p53 independent manner since cells deficient of p53 still expressed p16 (Macip, Igarashi et al. 2002, Beauséjour, Krtolica et al. 2003). The authors demonstrated that p16 could trigger a cell cycle arrest in the absence of p21/p53 pathway. In addition, they proposed that the senescence response triggered by telomere dysfunction is reversible and depends on the p53 status. However, when p16 is expressed, it acts as “point of no return” and ensures the irreversibility of cell proliferation even if pRB is inactivated (Beauséjour, Krtolica et al. 2003, Mirzayans, Andrais et al. 2010). In addition, Alcorta *et al* proposed that once dephosphorylated by CDK2/4/6 through p21, pRb could interact with transcription factors allowing the transcription and the increase of p16. Hence, the growth arrest would be a two-step process with a fast p21 increase followed by a decrease upon senescence and a late increase of p16 which can remain highly expressed for months (Alcorta, Xiong et al. 1996, Hara, Smith et al. 1996, Mirzayans, Andrais et al. 2010). Others proposed that p16 could represent a hallmark of a differentiation program that is switch on in senescent

cells, since p16 expression is up regulated at late stages of senescence and p16 is increased in terminal differentiation of neuronal cells (Lois, Cooper et al. 1995, Stein, Drullinger et al. 1999).

In addition, depending on the cell type and the species from which the cells derive, the p16 response varies. For example, in IMR90 fibroblasts the pathway p16-pRb is strongly involved regarding the cell cycle arrest, whereas in BJ (neonatal foreskin) fibroblasts or in in mouse embryonic fibroblast (MEFs), the p16-pRb pathway is poorly used or dispensable (Serrano, Lin et al. 1997, Beauséjour, Krtolica et al. 2003, Lowe and Sherr 2003). It has been reported that an intact Rb pathway is required for SAHF formation since inactivation of p16 or Rb counteracts its formation (Narita, Nũnez et al. 2003). SAHF, as mentioned previously, is implicated in the chromatin silencing of E2F target genes involved in proliferation (Lowe and Sherr 2003, Narita, Nũnez et al. 2003). In addition, upon expression of oncogenic *ras,* p16 and p53 have been reported to induce a cell cycle arrest in G1 in human and primary murine fibroblasts (Serrano, Lin et al. 1997). Therefore, p21 and p16 are both upregulated in human senescent cell but the mechanisms by which they are triggered may be independent and occur with different kinetics (Figure 1.3).

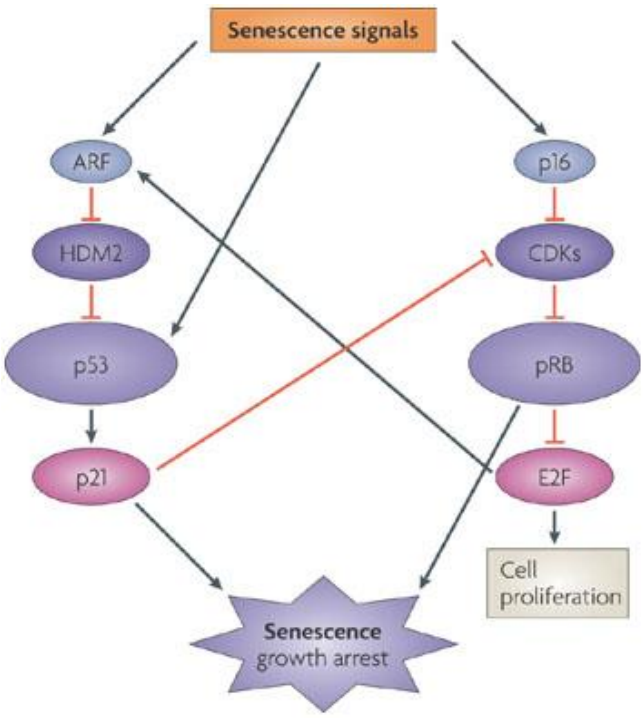


Figure 1.3 Senescence controlled by the p53 and p16–pRb pathways (Campisi and d'Adda di Fagagna 2007).

Senescence inducing signals due to stress usually engage either the p53 or the p16–retinoblastoma protein (pRb) tumour suppressor pathways. Some signals, such as oncogenic RAS engage both pathways. p53 is negatively regulated by the E3 ubiquitin-protein ligase HDM2 (MDM2 in mice), which facilitates its degradation, and HDM2 is negatively regulated by the alternate-reading-frame protein (ARF). Active p53 establishes the senescence growth arrest in part by inducing the expression of p21, a cyclin-dependent kinase (CDK) inhibitor that, among other activities, suppresses the phosphorylation therefore the inactivation of pRb. Senescence signals that engage the p16–pRb pathway generally do so by inducing the expression of p16, a CDK inhibitor that prevents pRb phosphorylation and inactivation. pRb halts cell proliferation by suppressing the activity of E2F, a transcription factor that stimulates the expression of genes that are required for cell-cycle progression. E2F can also curtail proliferation by inducing ARF expression, which engages the p53 pathway. Therefore, there is reciprocal regulation between the p53 and p16–pRb pathways.

1.2.3 p38 (MAPK) pathway

The MAPKs kinases are a family of proteins including p38 mitogen-activated protein kinase (MAPK), ERK (extracellular signal-regulated kinases) and JNK (c-Jun-N-terminal kinases). They are implicated in various signalling events upon exposure to extracellular stimuli. P38 and JNK are mostly involved in response to the activation of the innate and adaptive immune system, genotoxic compounds, UV light and ionizing irradiation, whereas ERK is mostly induced by mitogens (Kyriakis and Avruch 1996, Herrlich, Karin et al. 2008, Junttila, Li et al. 2008).

Once triggered, the MAPK cascade signals through MAPK kinase kinase (MAP3Ks) then MAPK kinases (MKK) like MKK3, MKK4 and MKK6 which can phosphorylate and activate directly p38 (Ashwell 2006). Once activated, p38 has been shown to have a role in cell proliferation and can trigger a cell cycle arrest in G1-S in response to telomere shortening, ROS and ras expression (Lin, Barradas et al. 1998, Deschênes-Simard, Gaumont-Leclerc et al. 2013).

P38 was reported to be activated in a model of oncogenic *ras* induced senescence in primary BJ fibroblast (Wang, Chen et al. 2002). Moreover, overexpression of active MMK3 and MMK4 leading to the constitutive activation of p38 is able to induce senescence, and inactivation of the latter alleviates *ras* induced senescence in this model. The authors suggested that p38 activation via the MEK-ERK pathway is required for *ras* induced senescence (Wang, Chen et al. 2002). P38 has been shown to be activated in response to telomere shortening in human senescent fibroblasts (since expression of TERT

suppressed its activation) as well as “culture shock” due to inadequate culture conditions in a telomere independent manner in MEFs. Using a dominant negative of the upstream kinase MKK6, authors observed an increase of population doublings compared to controls, suggesting that p38 is required for telomere dependent senescence (Iwasa, Han et al. 2003). Additionally, upon treatment with H_2O_2, indirect immediate p38 activation was observed and p38 level could stay elevated for up to 8 days after treatment, under the regulation of MKK3 and MKK6. In these conditions, the authors proposed that p38 activation induces senescence as a result of the accumulation of damage caused by ROS-induced damage (Kurata 2000, Iwasa, Han et al. 2003). In addition, further work has shown that p38 activation induces a cell cycle arrest dependent on pRb but not p53, since senescence can still occur in p53 deficient cells. However, induction of p53, p21 and p16 has been observed in ras induced senescence (Ferbeyre, de Stanchina et al. 2000) in response to p38 activation as p38 can phosphorylate p53 directly (Bulavin, Demidov et al. 2002, Iwasa, Han et al. 2003).

Consequently, the p38 MAPK pathway is a third known mechanism involved in the regulation of the cell cycle, which can lead to cellular senescence in response to certain stimuli.

1.3 The senescence phenotype

While senescent cells cannot respond to mitogenic stimuli (Cristofalo, Phillips et al. 1985), they remain viable and stable for a long period *in vitro* and *in vivo*. Hence, they develop specific features over time, which will be summarised in the following section.

1.3.1 Growth arrest and morphological changes

The most obvious change that occurs when cells are close to senescence is the reduction of the rate of cell division over serial passages until finally they stop dividing in a mostly G1 or rarely G2 permanent cell cycle arrest (Di Leonardo, Linke et al. 1994, Herbig, Jobling et al. 2004, Wada, Joza et al. 2004).

Along with the absence of cell division, morphological changes occur which can be seen directly under the microscope: cells become larger, flatter, increase their nuclear size, RNA, protein, lipids and lysosomes, whereas synthesis of DNA and motility are significantly reduced (Cristofalo, Doggett et al. 1989, Cristofalo and Pignolo 1993).

Senescent cells become also multi-nucleated, polyploid and vacuolated (Comings and Okada 1970, Matsumura 1980, Cristofalo and Pignolo 1993). In addition, senescent cells exhibit an increased sensitivity to cell contact in culture, thereby limiting their saturation density and reaching a stable density which remains for long period of time (Pignolo, Rotenberg et al. 1994).

1.3.2 Resistance to apoptosis

The term apoptosis describes a morphological form of cell death that is known under the name of “programmed cell death” and was first studied in the nematode *Caenorhabditis elegans (Horvitz 1999).* Apoptosis is an essential component of various processes including embryonic development, physiological cell turnover, immune reaction and is a powerful anti-cancer mechanism occurring following extreme stress (Norbury and Hickson 2001, Green and Evan 2002). Apoptosis is mainly driven by caspases such as caspase-3, caspase-6 and caspase-7 which engage the cleavage of substrates like poly ADP ribose polymerase (PARP) and nuclear proteins, causing drastic morphological and biochemical changes leading to cell death (Slee, Adrain et al. 2001).

The last step of apoptosis relies on neighbouring macrophages that will rapidly engulf and digest cellular debris without inflammatory reaction therefore preventing a secondary necrosis (Kurosaka, Takahashi et al. 2003). Senescent cells are usually referred to apoptosis resistant against genotoxic stress such as serum deprivation, UV irradiation and actinomycin D (Wang 1995, Seluanov, Gorbunova et al. 2001). A study by Wang, using serum deprivation in senescent fibroblasts, has reported neither DNA fragmentation nor loss of cell viability after 1 month of culture, whereas young cells became apoptotic after 24h. This resistance was linked to the presence of *bcl2* (B-cell lymphoma 2) which remained expressed in senescent cells contributing to the survival after long term serum deprivation (Wang 1995). Bcl2 is an anti-apoptotic membrane protein expressed in the outer membrane of mitochondria and belongs to a protein family containing anti-apoptotic and pro-apoptotic members classified by the presence of BCL-2 homology domains (BH1-4) (Nguyen, Millar et al. 1993, Kuwana and Newmeyer 2003, Chipuk, Bouchier-Hayes et al. 2006). Members of the pro-apoptotic group such as BAX and BAK are constitutively expressed but only active when apoptosis is triggered and play a role in mitochondria outer membrane permeabilisation (MOMP) leading to the release of cytochrome c and caspases (Von Ahsen, Waterhouse et al. 2000).

It has been shown more recently that the apoptotic resistance is dependent on the stabilisation of p53, which is impaired in senescent cells in response to DNA damage. (Seluanov, Gorbunova et al. 2001). This lack of stabilisation could be a result of an impairment of upstream regulators of p53 such as DNA-PK and PARP, which are downregulated during senescence (Salminen, Helenius et al. 1997). In addition, it was reported that although overlapping, p53 posttranslational modification patterns vary in response to DNA damage and senescence (Webley, Bond et al. 2000). Consequently, senescent cells switch the death pathway from apoptosis toward necrosis which is a passive, unprogrammed cell deathwhich can increase inflammation in the milieu (Moallem and Hales 1998, Fiers, Beyaert et al. 1999). In parallel, a study using UV-induced senescence in BJ fibroblasts has shown similar results regarding apoptosis resistance (Yeo, Hwang et al. 2000).

Apoptosis and senescence are the two main mechanisms involved in cell fate upon extreme stress; however, it is still unclear what drives one way or the other.

1.3.3 Senescence associated secretory phenotype (SASP)

Although senescent cells can no longer divide through the cell cycle, they are still metabolically active. In fact, the senescent phenotype is associated with drastic changes at the transcriptome and secretome levels, developing the so called senescence messaging secretome (SMS) or senescence associated secretory phenotype (SASP) (Coppé, Patil et al. 2008, Kuilman, Michaloglou et al. 2008, Rodier, Coppe et al. 2009). The SASP components are composed of soluble and insoluble factors secreted by the senescent cells such as inflammatory, growth and remodelling factors that can alter the extracellular environment. The SASP is thought to have evolved as a way to activate and recruit immune cells, potentially contributing to the clearance of senescent cells (Xue, Zender et al. 2007, Krizhanovsky, Yon et al. 2008, Kang, Yevsa et al. 2011, Hoenicke and Zender 2012). Recent data also indicates that senescent cells present at sites of wound-healing are important for wound repair due to secretion of platelet-derived growth factor AA (PDGF-AA) (Demaria, Ohtani et al. 2014). However, it has been reported that chronic exposure to the SASP can result in induction of senescence in neighbouring cells by bystander effect *in vitro* and *in vivo* (Nelson, Wordsworth et al. 2012, Acosta, Banito et al. 2013) and promote local tumorigenesis (Krtolica, Parrinello et al. 2001, Parrinello, Coppe

et al. 2005, Liu and Hornsby 2007, Coppé, Patil et al. 2008, Kuilman, Michaloglou et al. 2008).

Additionally, chronic inflammation is associated with normal ageing with an increase of pro-inflammatory mediators detected in the blood of old individuals (Bruunsgaard 2006, Maggio, Guralnik et al. 2006, Freund, Orjalo et al. 2010). SASP factors, such as interleukin 6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinases (MMPs) increase in tissues with ageing and are linked with systemic inflammation and tissue dysfunction (Brod 2000, Freund, Orjalo et al. 2010). Furthermore, the inflammatory response from senescent cells upon upregulation of p53 has been shown to have a role in insulin resistance (Minamino, Orimo et al. 2009). In addition, IL-8 and IL-6 have been shown to induce epithelial-to-mesenchymal transitions (EMTs), which increase cells motility, angiogenesis and promote tumour growth (Tamm, Kikuchi et al. 1994, Sparmann and Bar-Sagi 2004).

On the other hand, the SASP has beneficial effects: IL-8, IL-6, and the chemokine growth-regulated oncogene 1 (Gro-1) have been reported to be involved in an autocrine feedback loop which stabilises the permanent cell cycle arrest (in a p53 dependent manner) (Yang, Rosen et al. 2006, Acosta, O'Loghlen et al. 2008, Kuilman, Michaloglou et al. 2008) .

Another major role of the SASP is to communicate with the immune system through factors such as IL-8 and IL-6 which drive infiltration of immune cells involved in the clearance of senescent cells (Xue, Zender et al. 2007, Krizhanovsky, Yon et al. 2008, Kang, Yevsa et al. 2011). This area of research will be explained in more detail later on.

Mechanistically, the SASP is dependent on persistent DDR that can occur upon several stimuli, especially for the two principal SASP factors IL6 and IL-8 (Rodier, Coppe et al. 2009). Moreover, they have shown that ATM, NSB1 and CHK2 are required but not p53 or pRb suggesting that the induction of the SASP is not dependent on the growth arrest *per se* but on the signalling of the DDR (Rodier, Coppe et al. 2009). In agreement with these results, overexpression of p21 or p16 triggers senescence without a DDR and a SASP (Coppe, Rodier et al. 2011). Nevertheless, in some cases and depending on the stimuli, senescence is triggered by a SASP independently of DDR (Muñoz-Espín, Cañamero et al. 2013, Storer, Mas et al. 2013).

Importantly, regarding the large catalogue of SASP factors, the SASP is not universal and the composition varies depending on the cell type and on specific senescence stimuli (Coppé, Patil et al. 2008).

The NF-κB family transcription factors have been reported to be important in the regulation of secretory components in senescence (Hardy, Mansfield et al. 2005, Acosta, O'Loghlen et al. 2008, Kuilman, Michaloglou et al. 2008, Orjalo, Bhaumik et al. 2009). NF-κB regulates many inflammatory genes such as Toll-like receptors (TLR) and cytokines (Vallabhapurapu and Karin 2009). Moreover, several studies have involved the NF-κB pathway with the development of the SASP and reinforce the role of this transcription factor family in the ageing process (Passos, Nelson et al. 2010, Osorio, Bárcena et al. 2012, Jurk, Wilson et al. 2014). Activation of p38MAPK has been shown to be involved in the development of the SASP without a DDR via NF-κB (Freund, Patil et al. 2011).

Overall, the SASP can have both positive and negative effects. It is involved in tissue repair and removal of senescent cells, but can also induce tissue dysfunction. In order to counteract its negative effects, strategies to eliminate senescent cells or reduce the SASP are being developed with the purpose of delaying or preventing age related diseases (Childs, Durik et al. 2015).

1.3.4 Markers of senescence

Regarding the complexity of the cellular senescence response, which can be triggered by different stimuli, and considering the various types of cellular senescence, nowadays, no universal marker that unequivocally detects senescent cells has been identified. Hence, a combination of different markers is usually used to detect the presence of senescent cells. These will be detailed below.

Senescent cells are blocked in a permanent cell cycle arrest, meaning they are incapable of synthesising DNA. Therefore, assays to measure DNA synthesis have been developed such as 5-bromo-2-deoxyuridine (BrdU) or 3H-thymidine incorporation, 5-ethynyl-2'-deoxyuridine (edU) or by immunostaining for proteins like Ki-67 and PCNA (Lawless, Wang et al. 2010). However, these markers are not able to distinguish between senescent cells and quiescent cells and/or postmitotic cells.

Senescence-Associated Beta-Galactosidase (SA-β-Gal) is a histochemical staining marker which is detectable in most senescent cells at PH 6.0 (Dimri, Lee et al. 1995) and reflects the increased lysosomal biogenesis observed in senescent cells (Lee, Han et al. 2006). However, upon long term culture at high confluence, false positive SA-β-Gal can be detected (Severino 2000). Furthermore, it can be detected in conditions of serum starvation and in immortalised cultures (Cristofalo 2005). This method requires fresh tissues, which limits its applicability. Its detection in tissues is technically challenging and has generated contradictory results. Despite its limitations, this marker is probably the most commonly used in the senescence field.

The CDKi p16 and p21 involved in the pathways stopping the cell cycle are commonly used to identify senescent cells (McConnell, Starborg et al. 1998, Chang, Watanabe et al. 2000, Krishnamurthy, Torrice et al. 2004, Collado, Gil et al. 2005). P16 is believed to be a robust marker of mammalian ageing and senescence since it is widely expressed in human cells, mouse cells and in a variety of tissues (Krishnamurthy, Torrice et al. 2004). Nevertheless, in some cases, p16 levels vary depending on the cell type and have been detected in pre- senescent cells (Beauséjour, Krtolica et al. 2003, Itahana, Zou et al. 2003).

Senescent cells through the regulated transcription factor E2F complex (by pRb and CDK) is silencing genes involved in DNA replication, resulting in intense chromatin remodelling. Such modifications in the nucleus show a dot-like pattern (SAHF) which can be detected using immunofluorescence with 4′,6-diamidino-2-phenylindole (DAPI) (Narita, Nũnez et al. 2003). However, this feature is species and cell type dependent, and has not been observed universally (Narita, Nũnez et al. 2003).

Senescence is mainly triggered by a permanent DDR (Di Leonardo, Linke et al. 1994, d'Adda di Fagagna 2008), therefore markers of DNA damage are good indicators of DNA lesions such as γ-H2AX and p53-binding protein-1 (53BP1) (Fernandez-Capetillo, Chen et al. 2002). However, it has been reported that senescence can occur without a DDR via the P38MAPK pathway (Freund, Patil et al. 2011) which is thus used as a marker of senescence (Debacq-Chainiaux, Boilan et al. 2010).

Another robust marker of senescence is the presence of telomere-associated foci (TAF) which can be detected by co-localisation between DNA damage response proteins such

as γ-H2AX and 53BP1 and telomeres. TAF have been shown to increase in various tissues with age in humans and mice (d'Adda di Fagagna, Reaper et al. 2003, Hiroyuki, Agata et al. 2003, Herbig, Jobling et al. 2004, Hewitt, Jurk et al. 2012, Jurk, Wilson et al. 2014, Birch, Anderson et al. 2015, Wilson, Jurk et al. 2015).

Because senescent cells develop a SASP over time, cytokines such as IL-6 and IL-8 are recently been used as markers which reflect the last stage of the senescence program (Acosta, O'Loghlen et al. 2008, Coppé, Patil et al. 2008, Kuilman, Michaloglou et al. 2008, Rodier, Coppe et al. 2009).

Furthermore, ROS measurements are used as a marker of mitochondrial dysfunction which is involved in senescence (Chen and Ames 1994, Chen, Bartholomew et al. 1998, Lee, Fenster et al. 1999, Passos, Nelson et al. 2010).

Moreover, new markers have been identified following proteomic screening of plasma membrane-associated proteins expressed in senescent cells. Authors have identified more than 100 proteins, 10 of which were validated *in vitro* and *in vivo.* However further studies are required in order to understand the function of these new markers and validate them (Althubiti, Lezina et al. 2014).

As mentioned, depending on the stimuli that trigger the cell cycle arrest, the species and cell types, the senescent phenotype is plastic to some extent. Hence, to date there is not a universal marker to detect senescent cells but several need to be used together to identify such cells.

1.4 The role of senescence *in vivo*

Since the discovery by Hayflick in 1965, work has been done to demonstrate that cellular senescence is not an *in vitro* artefact but physiologically relevant. Hayflick hypothesised that senescence could be a reflection of ageing and later on, it was shown by others to be implicated in oncogenesis. It was confirmed later with the demonstration of OIS and the fact that in cancer p16 and p53 are among the most mutated proteins. Hence, as it was suggested that senescence is a major tumour suppressor mechanism, but recent data have shown that senescence can have other physiological roles and participate in several other processes such as development, wound healing, fibrosis and ageing (Krizhanovsky, Yon et al. 2008, Jun and Lau 2010, Muñoz-Espín, Cañamero et al. 2013, Storer, Mas et

al. 2013, Demaria, Ohtani et al. 2014). These different roles of senescence *in vivo* will be examined in the next subchapters.

1.4.1 The role of senescence in cancer

Senescence is considered as a potent tumour barrier responding to mutations of oncogenes and tumour suppressor genes. Indeed, a number of studies on cancer has provided evidence for OIS *in vivo* (Braig, Lee et al. 2005, Chen, Trotman et al. 2005, Collado, Gil et al. 2005, Michaloglou, Vredeveld et al. 2005). In these studies, OIS was important to prevent lymphoma development involving chromatin modification via the histone methyl transferase Suv39h1 on histone H3 lysine 9 (H3K9) (Braig, Lee et al. 2005). Another study using an inducible mouse model of K-ras has shown that senescent cells were detected mainly in premalignant cells but not once the tumour was formed (Collado, Gil et al. 2005). Human naevi (moles) are benign tumours found in the skin and are composed of senescent melanocytes due to oncogenic mutation of BRAF (V600E) which overexpressed p16 and stain positive for SA-β-Gal (Michaloglou, Vredeveld et al. 2005). A complementary study has shown that OIS in naevi is caused by telomere dysfunction driven by replication stress (Suram, Kaplunov et al. 2012). In addition, it has been shown that loss of the tumour suppressor gene *Pten* can induce OIS in the prostate under the control of p53 (Chen, Trotman et al. 2005).

On the other hand, senescent cells develop a SASP, which has the ability to modify the surrounding microenvironment, enhance cellular proliferation and cancer in neighbouring cells (Krtolica, Parrinello et al. 2001, Parrinello, Coppe et al. 2005, Bavik, Coleman et al. 2006, Liu and Hornsby 2007). The first evidence was demonstrated by Krtolica which has shown that senescent fibroblasts co-cultured in direct or indirect contact induced a hyperproliferation of mutated epithelial cells for p53 and RAS. Dual injection of senescent fibroblasts and preneoplastic epithelial cells has been shown *in vivo* to cause neoplastic progression and tumorigenesis. The authors reported for the first time, that the microenvironment created by the senescent cells via the SASP is cancer-prone (Krtolica, Parrinello et al. 2001).

Moreover, a very recent study in cancer using mice exposed to chemotherapeutic drugs followed by elimination of senescent cells has shown a reduction of several short- and long-term effects of the drugs, including bone marrow suppression, cardiac dysfunction

and cancer recurrence with an increased physical activity and strength. This study highlights the relationship between senescent cells and chemotherapy treatment (Demaria, O'Leary et al. 2017).

Overall, these studies demonstrate that senescence acts as a powerful anti-tumour mechanism *in vivo* but can also promote tumorigenesis potentially through the SASP.

1.4.2 The role of senescence in ageing and age-related diseases

The first observed evidence that senescent cells are driving tissue ageing was provided by a study using a progeroid genetic mouse model of accelerated ageing (BubR1crossed with inducible ink-attac) where p16ink4a positive cells were cleared from tissues following administration of a drug. BubR1 is a key player of the spindle assembly checkpoint protein regulating the accurate segregation of the chromosome during mitosis by inhibiting Cdc20 from activating the anaphase-promoting complex/cyclosome (APC/C) (Malureanu, Jeganathan et al. 2009). This study showed that p16 positive cells were contributing to premature ageing in tissues such as skeletal muscle, eyes and fat. While in this mouse model elimination of p16 positive cells improved several features of health span, it did not increase lifespan (Baker, Wijshake et al. 2011). However, very recent data from the same group using the same system to clear senescent cells from tissues but in WT mice, has shown a beneficial effect on healthspan and lifespan extension. They observed upon clearance, an improvement of age-dependent fat tissue dysfunction and a reduction of inflammation in skeletal muscle and kidney (Baker, Childs et al. 2016).

Several other studies implicate cellular senescence in the ageing process. For example, a recent study has shown that muscle satellite cells which are quiescent at a young age, can undergo senescence in a p16-dependent manner at older age. Upon injury, p16-positive satellite cells cannot regenerate and silencing of p16 in satellite cells rescues their regenerative capacity (Sousa-Victor, Gutarra et al. 2014). This study demonstrated that senescence plays a role in sarcopenia, a feature of ageing characterised by loss of muscle mass and function (Renault, Thorne et al. 2002, Arthur and Cooley 2012).

Furthermore, data from studies on adipocytes has linked senescence and obesity. It was reported that adipose tissue from obese subjects present hallmarks of senescence *in vivo* in both human and mice (Minamino, Orimo et al. 2009, Tchkonia, Morbeck et al. 2010). Also, a study on diabetes, using a high fat diet in an adipocyte-specific p53 deficiency

mouse model has shown evidence that senescent adipocytes lead to insulin resistance (Minamino, Orimo et al. 2009).

In addition, senescent cells have been linked with several age-related diseases such as glaucoma, osteoarthritis and atherosclerosis (Martin, Brown et al. 2004, Liton, Challa et al. 2005, Zhou, Perez et al. 2006).

Additionally, p16-dependent senescence was shown to play a role in another stem cell compartment: the hematopoietic system. Using a mouse knock out for p16, the authors have observed an improvement in the regenerative capacity and in the proliferation of hematopoietic stem cells (HSCs) (Janzen, Forkert et al. 2006). Furthermore, p16 was shown to increase with age in HSCs, regulate the HSC pool (homing) and increase apoptosis under stress conditions.

Altogether, these studies demonstrate that p16 induced senescence in both stem and progenitor cells and contributes to a decline in regenerative capacity during ageing.

Another feature of ageing is chronic low grade inflammation or “inflammaging” which occurs in absence of infection named sterile inflammation which is a high risk of death in the elderly (Franceschi and Campisi 2014). Chronic Inflammation has been reported in most age-related diseases and one of the explanation could be due to the presence of senescent cells and the SASP, and notably the production of pro-inflammatory cytokines such as IL-6 (Rodier, Coppe et al. 2009, Coppé, Desprez et al. 2010). Indeed, IL-6 is one of the major cytokines secreted in age-related pathologies, engaging a potent chronic immune response and linked to morbidity (Maggio, Guralnik et al. 2006). Furthermore, telomere dysfunction and cell senescence have been shown to be increased with age in a mouse model of low grade chronic inflammation ($nfkb1^{-/-}$). Moreover treatment with anti-inflammatory compounds reduces the levels of senescent cells indicating that inflammation is driving cellular senescence (Jurk, Wilson et al. 2014).

Taken altogether, these data indicate that accumulation of senescent cells within the tissue is closely involved with tissue dysfunction and multiple age-related disorders.

1.4.3 The role of senescence during development

Recently, a new surprising role for cellular senescence has been revealed: senescent cells were detected during mammalian development (Muñoz-Espín, Cañamero et al. 2013, Storer, Mas et al. 2013). These two studies came with a change in the way to define senescence. The classical view of senescence is called chronic senescence whereas the one observed during development and wound healing is called acute. Acute senescence seems to be a programmed process whereas chronic senescence is more stochastic and could be a response to the accumulation of various stresses in the cells (van Deursen 2014).

In the study from Muñoz-Espín and colleagues, senescent cells were observed in the mesonephros and the endolymphatic sac of the inner ear between day 12.5 to 14.5 with a transient increase in cells positive for SA-β-Gal, and SAHF and negative for the proliferation marker Ki-67, which disappeared at day 15. This development program was shown to be p21 dependent but independent of p53 and DNA damage. Furthermore, they observed abundant infiltration of macrophages at day 14.5 which correlated with the presence of senescent cells suggesting that senescence preceded macrophage recruitment (Muñoz-Espín, Cañamero et al. 2013). Macrophages have been reported to be involved in the clearance of OIS cells (Xue, Zender et al. 2007, Kang, Yevsa et al. 2011), however the process leading to their recruitment is not completely understood and it has been speculated that secreted factors from senescent cells are likely to be involved (Xue, Zender et al. 2007).

The other study from Storer et al. focusing on the apical ectodermal ridge during limb formation, led to similar observations at day 11.5 of development. The presence of SA-β-Gal activity, high levels of p21 and SAHF but neither p16 nor DNA damage were detected. Moreover, associated with the presence of these acute senescent cells, was the detection of macrophages, which could potentially be involved in the clearance of senescent cells. To note, although genes involved in the regulation of the SASP where detected, neither IL-6 nor IL-8 were upregulated suggesting a primitive version of senescence intended to act during particular development stages contrasting with the long lasting chronic senescent state (Storer, Mas et al. 2013).

These new data opened a new landmark in the senescence field and challenged the view that senescence evolved solely as a tumour suppressive mechanism.

1.4.4 The role of senescence in wound healing /injury repair

Tissue repair of wounding and injury is a complex process whose role is to maintain homeostasis and provide defence against pathogens while being repaired. Wound healing is a coordinated process including four different phases composed by hemostasis, inflammation, proliferation and remodeling. Upon injury, along with inflammation and infiltration of immune cells, fibroblasts differentiate to myofibroblasts which proliferate and secrete ECM proteins to support tissue repair (Li and Wang 2011). However, if the secretion of ECM is not correctly regulated then this can turn to chronic wound healing followed by fibrosis which is observed in lungs, liver, hearts and kidneys (Bataller and Brenner 2005, Stramer, Mori et al. 2007, Wynn 2008).

Fibrosis is involved in multiple chronic age related pathologies and may result in organ failure (Ziesche, Golec et al. 2013). The lungs are subject to fibrosis with age with the most aggressive case called Idiopathic pulmonary fibrosis (IPF) (Leung, Cho et al. 2015). Recent evidence has shown that in response to injury, the myofibroblasts (the major effectors of fibrosis (Hinz, Phan et al. 2012)) undergo cellular senescence and persist in the fibrotic regions of aged mice. These persistent senescent cells have a major role in the pathogenesis of IPF by blocking the fibrosis resolution via elevated ROS production, impairment of antioxidant defences and acquisition of an apoptosis–resistant phenotype (Hecker, Logsdon et al. 2014).

In addition, fibroblasts derived from IPF human patients were found to be predisposed to senescence and have been shown to accumulate at the wound repair site and be involved in the blockade of fibrosis resolution in older individuals (Raghu, Weycker et al. 2006, Yanai, Shteinberg et al. 2015).

On the other hand, two independent studies have shown that in order to limit fibrosis, stellate cells differentiate into myofibroblasts, proliferate, then undergo senescence and accumulate at the site of tissue repair (Krizhanovsky, Yon et al. 2008, Fitzner, Muller et al. 2012). Krizhanovsky et al showed that in mice upon injection of the damaging agent carbon tetrachloride (CCl_4) in the liver, hepatic stellate cells (HSC) become activated, differentiate into myofibroblasts, proliferate and secreted ECM to create a fibrotic scar.

Then, in response to the high proliferation, a senescence response is triggered resulting in the accumulation of senescent HSCs at the site of damage. These senescent HSC secrete some SASP components involved in the upregulation of MMPs (ECM degrading enzymes) and down regulation of ECM genes therefore limit fibrosis (Krizhanovsky, Yon et al. 2008).

Furthermore, in order to facilitate the resolution of fibrosis, these senescent cells upregulate genes involved in immune surveillance. During fibrosis, immune cells infiltrate at the site of damage (fibrotic scar) and are found in close proximity with senescent cells, especially natural killer cells (NK cells) which are involved in their clearance (Krizhanovsky, Yon et al. 2008).

Moreover, another study using a model of dibutyltin dichloride (DBCT) induced chronic pancreatitis in rat, has shown that cellular senescence is involved in pancreatic fibrogenesis. In this study, Fitzner and colleagues have shown that upon injury, pancreatic stellate cells become activated and proliferate, then triggered the senescence program to limit fibrosis and are finally cleared by lymphocytes to resolve the inflammation (Fitzner, Muller et al. 2012).

To note, senescent cells are not required for the healing *per se* since it occurs in mice null for p53 and p16 however with lower kinetics (Krizhanovsky, Yon et al. 2008, Jun and Lau 2010).

This data suggests that senescent cells could be beneficial to start the remodelling process by limiting the proliferation of activated HSCs but if they become persistent, they could develop acute fibrosis and promote the development of chronic diseases (Krizhanovsky, Yon et al. 2008, Yanai, Shteinberg et al. 2015).

Another study in skin wound healing has shown similar results. Authors have reported that upon injury, fibroblasts differentiate into myofibroblasts, then secrete ECM to repair the damaged tissue. Once repaired, these cells trigger a senescence program in order to limit the fibrosis response (Jun and Lau 2010). Senescence in that particular case is due to a matricellular protein CCN1 (CYR61) expressed at sites of injuries. CCN1 induces senescence by direct binding to the integrin α6β1 and to cell surface heparin sulfate proteoglycans resulting via downstream signals to increase ROS production and a DDR, activation of p53 and p16. Knock in mice for a mutated version of CCN1 are unable to

induce senescence and injection of CCN1 rescues the senescence phenotype and resolution of the fibrosis (Jun and Lau 2010).

In addition, a new study using an inducible genetic mouse model (p16-3MR) where p16 positive cells can be removed upon treatment has shown that indeed senescent cells were involved in wound healing. These senescent cells trough the SASP are secreting PDGF-A which promotes myofibroblast differentiation to ensure a fast closure of the wound. When the mice where fed with the drug to remove senescent cells, the kinetics of wound closure was slower, and fibrosis occurred (Demaria, Ohtani et al. 2014).

1.5 The immune system

1.5.1 Introduction

The immune system is a complex interactive network of lymphoid organs, cells, humoral and inflammatory factors. The main function of the immune system is to protect our tissues against infections from pathogenic microorganisms. Hence, the resolution of an infection is a multistep process and starts with the recognition of “foreign” molecules and distinction from the organism’s own molecules. Once identified, an immune response is triggered to remove the “foreign” molecules.

The immune system is divided in two parts determined by their order of action and specificity. The first line of defence, named the innate immunity, provides a physical, chemical and microbiological barrier. This first step involves a large number of specialised cells such as neutrophils, monocytes, and macrophages. The innate system is evolutionary conserved among species and is essential for survival (Buchmann 2014). The second line of defence, the adaptive immunity, is only present in higher species. This response involves specialised cells, the lymphocytes which provide a memory for subsequent exposure to insure a rapid response against a repeated threat (Ahmed and Gray 1996).

In the early stages of infection, activated macrophages release cytokines into the blood stream that will go into the bone marrow and trigger the release of millions of neutrophils into the circulation. Then, neutrophils, attracted by pro-inflammatory molecules, transit to the site of infection in order to kill the micro-organisms, then undergoe apoptosis to be cleared by macrophages (Kolaczkowska and Kubes 2013). Additional cells have the

potential to kill: natural killer cells. These cells have the morphology of lymphocytes but do not need a specific antigen to kill via perforins and granzymes to induce apoptosis in the targets (Vivier, Tomasello et al. 2008).

1.5.2 Immune clearance of senescent cells

Several recent studies suggest that the SASP has an important role in immunosurveillance. It has been suggested that the SASP contributes to the recruitment and activation of immune cells, leading to clearance of senescent cells (Gasser, Orsulic et al. 2005, Xue, Zender et al. 2007, Krizhanovsky, Yon et al. 2008, Hoenicke and Zender 2012).

A study using human and mouse non-tumour cell lines have shown that following DNA damage and activation of a DDR, NKG2D ligands are upregulated. NKG2D ligands are recognised by NK cells and activated $CD8^{+}$T cells. Furthermore, they have shown that inhibition of ATM using siRNA exhibited reduced NKG2D ligand expression suggesting an ATM dependency (Gasser, Orsulic et al. 2005).

Later-on, a study from Lowe *et al* have shown using transplantation in an immune deficient nude mice of hepatoblasts transduced with *ras* GFP (*HrasV12*) with a p53 inducible system, that reactivation of p53 induced a senescence phenotype in these cells associated with an up regulation of inflammatory cytokines (Xue, Zender et al. 2007). Among them, cytokines such as CSF1, MCP1, CXCL1 and IL-15 can attract macrophages, neutrophils and NK cells. In addition, they noticed an up-regulation of adhesion molecules which facilitated immune recognition and removal of senescent cells and tumours (Xue, Zender et al. 2007). This study suggests that the immune system is activated by senescent cells leading to clearance of potentially tumorigenic cells.

A recent study has shown that induction of OIS in hepatocytes by overexpression of oncogenic $Nras^{G12V}$, led to induction of a SASP, which activated an immune response (Kang, Yevsa et al. 2011). The authors then demonstrated that impaired immune surveillance of senescent hepatocytes resulted in the development of murine hepatocellular carcinomas (HCCs), thus demonstrating that senescence surveillance is important for tumour suppression *in vivo*. This study determined that $CD4^{+}$ T-cells as well as macrophages but not Kupffer cells (which are the resident macrophages in the liver) were involved in the clearance of senescent hepatocytes (Kang, Yevsa et al. 2011).

Furthermore, Lowe and colleagues have proposed that during chronic liver damage and fibrosis, hepatic stellate cells undergo senescence in order to restrict fibrosis progression (Krizhanovsky, Yon et al. 2008). These cells were found in close proximity with NK cells and expressed components for NK cell recognition (CD58, ICAM1 and NK cell receptors ligands (MICA, ULBP2 and PVR)) and IL-8. The authors have demonstrated that NK cells are responsible for clearance of the senescent activated stellate cells via granule exocytosis (Krizhanovsky, Yon et al. 2008, Sagiv, Biran et al. 2013).

A new study has shown that cells undergoing OIS experience massive modifications of histone H3K27Ac enhancers along with SASP genes to promote their expression (Tasdemir, Banito et al. 2016). This occurs under the regulation of cofactors such as the bromo and extra terminal domain (BET) proteins in this particular case the transcriptional coactivator BRD4. Further, they have shown that using ChIP-Seq and gene ontology analysis for BRD4 there is a significant enrichment for genes related to NFκB including SASP key components such as IL8, IL1α, IL1β and several MMPs. Importantly, when they inhibited BRD4 with a pharmacological drug, the SASP was suppressed and immune clearance was impaired but not the other features of senescence (Tasdemir, Banito et al. 2016).

Altogether, these studies suggest that specialised cells from the immune system are involved in clearance of senescent cells in cases of pre-malignant cells and non-cancer pathologies.

Although senescent cells can be cleared by the immune system, they accumulate with age within the tissues. Hence, one explanation could be that senescent cells have developed strategies to evade the immune system via secretion of anti-inflammatory molecules or up/down regulation of receptors (Coppé, Desprez et al. 2010, Freund, Orjalo et al. 2010). This can be coupled with a rate of accumulation of senescent cells which is greater than the removal rate by the immune system. Moreover it has been reported that the immune system becomes impaired with age (Nikolich-Zugich 2008, Wang, Geiger et al. 2011) and immune cells themselves undergo senescence (Effros and Pawelec 1997, Rajagopalan and Long 2012). Taking together, these changes could explain why senescent cells remain and accumulate with age.

1.5.3 The role of neutrophils in ageing and disease

1.5.3.1 Neutrophils and immune response

Multicellular organisms are constantly challenged to survive against micro-organisms like bacteria and fungi causing infections. Phagocytes are specialised cells able to engulf and kill invading pathogens to protect the host. They constitute the first line of defence of the innate immune system. In humans, neutrophils have a segmented nucleus and contain granules and secretory vesicles (Borregaard 2010). They count for 50 to 70% of all circulating leukocytes and are continuously generated in the bone marrow from myeloid precursor lineages from hematopoietic stem cells (Manz and Boettcher 2014). The process is controlled by granulocyte colony stimulating factor (GCSF) (Lieschke, Grail et al. 1994), under the control of interleukin-17A (IL-17A) (Ley, Smith et al. 2006) which is regulated by interleukin IL-23 (IL-23) produced by resident macrophages and dendritic cells. During inflammation, terminally differentiated neutrophils infiltrate the tissue, kill pathogens, undergo apoptosis and are finally removed by macrophages and dendritic cells (Ortega-Gómez, Perretti et al. 2013).

Neutrophils possess 3 types of granules containing pro-inflammatory proteins formed during maturation from the bone marrow to the blood stream (Borregaard 2010, Häger, Cowland et al. 2010, Galli, Borregaard et al. 2011, Lefort, Rossaint et al. 2012). The primary azurophilic granules contain myeloperoxidase (MPO), neutrophil elastase (HNE), antimicrobial factors, lysosomal-associated membrane protein 3 (LAMP3) and CD63. The secondary granules (specific) contain lactoferrin, MMP-9, CD66b and the adhesion molecule CD11b (or Mac-1). The gelatinase granules (secretory) contain matrix metalloproteinase 9 (MMP9), gelatinase, plasma proteins, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), CD11b and CD35 (CR1) (Borregaard, Sehested et al. 1995, Borregaard and Cowland 1997).

The distinction between the granules relies on their content composition (Lominadze, Powell et al. 2005) and their mobilisation upon activation (Borregaard and Cowland 1997).

1.5.3.2 Neutrophil activation mechanism

In sites of inflammation, both bacterial and host cells produce large amounts of inflammatory signals stimulating endothelial cells near the site of inflammation. Stimulants

like bacterial derived lipopolysaccharides (LPS) and the peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) as well as host derived chemoattractants, cytokines such as IL-8 or tumour necrosis factor (TNF-α), interleukin 1β (IL-1β) and IL -17 activate endothelial cells to produce adhesion molecules such as E-selectins and β2-integrins (Borregaard 2010). This contributes to the recruitment of circulating neutrophils via interaction with their surface proteins: P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin (Kansas 1996, McEver and Cummings 1997). These interactions, combined with blood flow leads to neutrophil rolling onto the surface of activated endothelial cells. This is followed by stronger attachment via ICAM and β2-integrins expressed by endothelial cells, leading to neutrophil arrest and transmigration through the blood vessel (see Figure 1.4) (Campbell, Hedrick et al. 1998, Ley, Laudanna et al. 2007). Once arrived to the site of inflammation or infection, neutrophils can then initiate their oxidative burst, by releasing reactive oxygen species onto the environment (Baggiolini and Clark-Lewis 1992, Goldfinger, Han et al. 2003, Xu, Wang et al. 2003).

The concentration of the chemoattractant present has a critical role in the recruitment and activation of neutrophils. For example, even low concentration of the most abundant chemokines such as IL-8 stimulates L-selectin and integrins, a medium concentration can initiate the oxidative burst and the highest one can induce degranulation of neutrophils (Figure 1.4) (Ley 2002). The concept of neutrophil priming was then introduced, where neutrophils are exposed to low concentrations of chemoattractants which prepare neutrophils to be ready to respond quickly to a second insult, such as LPS or fMLP (Guthrie, McPhail et al. 1984).

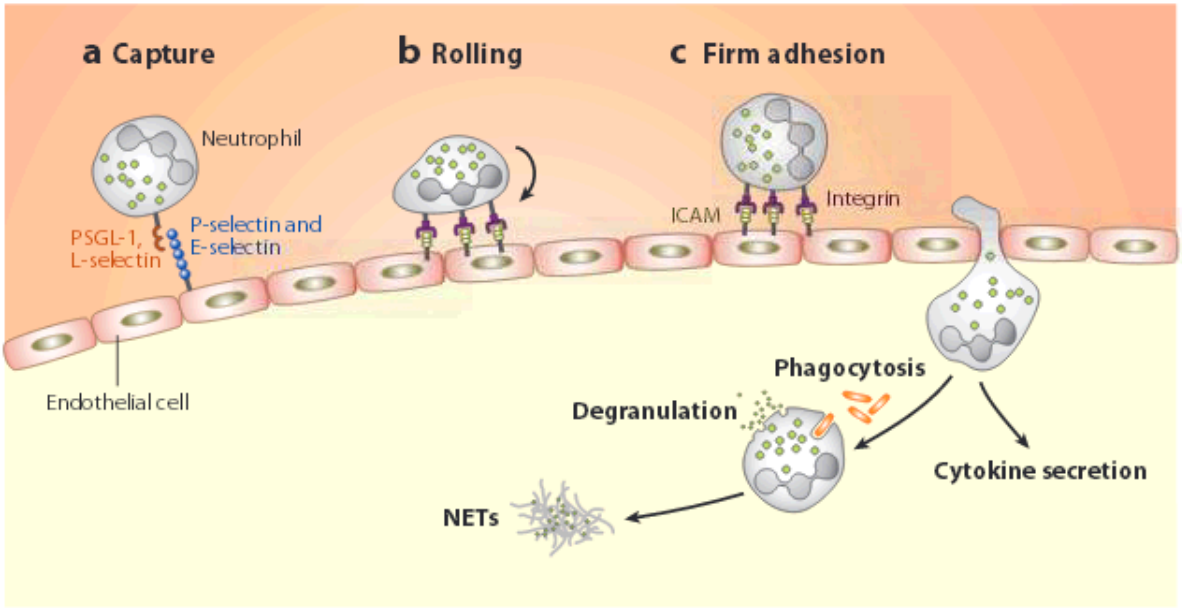


Figure 1.4 Adapted from (Amulic, Cazalet et al. 2012): Neutrophil recruitment to sites of inflammation.

The circulating neutrophil recognises signs of inflammation and migrates to areas where its antimicrobial arsenal is needed for the elimination of infection. (a) Close to the inflammatory sites, stimulated endothelial cells expose a class of molecules, the selectins, which serve to capture circulating neutrophils and tether them to the endothelium; (b) Selectin- mediated rolling along chemoattractant gradients then ensues, followed by (c) integrin- mediated firm adhesion. Subsequently, the neutrophil traverses through the endothelium and arrives at the site of inflammation. Here, the neutrophil releases cytokines that recruit other immune cells and it begins to implement its antimicrobial agenda. Among the processes employed are engulfment of microbes via receptor- mediated phagocytosis, release of granular antimicrobial molecules through degranulation, and formation of neutrophil extracellular traps (NETs).

Neutrophils can eliminate pathogens by three known mechanisms: 1) They can phagocytose microorganisms by encapsulation in phagosomes: then the killing occurs by ROS or antibacterial proteins present in the granules after fusion of these granules with the phagosomes (Borregaard 2010); 2) The antibacterial proteins can also be released in the extracellular milieu (degranulation) (Lacy 2006); 3) Finally, neutrophils can release neutrophil extracellular traps (NETs) when they are strongly activated. These NETs are composed of a mix of chromatin, proteins (histones) and enzymes such as MPO and neutrophil elastase which can immobilise pathogens by trapping them, thereby preventing spreading of the infection (Brinkmann, Reichard et al. 2004).

After activation and elimination of pathogens, neutrophils increase the expression of CXCR4 which triggers their relocation back to the bone marrow to be eliminated (Suratt,

Petty et al. 2004). Furthermore, they can also undergo senescence or apoptosis within the vasculature and be removed by resident macrophages (Shi, Gilbert et al. 2001, Hong, Kidani et al. 2012).

1.5.3.3 Neutrophils and cancer

A recent study where a hepatocellular carcinoma (HCC) was induced by diethylnitrosamine (DEN) in a mouse model of low grade inflammation and accelerated ageing (Jurk, Wilson et al. 2014, Wilson, Jurk et al. 2015) (nfkb1 -/-) demonstrated that neutrophils are involved in HCC by inducing telomere dysfunction. HCC develops on the background of cirrhosis caused by fibrosis and subsequently chronic inflammation with released cytokines / chemokines such as IL-6 (Schutte, Bornschein et al. 2009, Taniguchi and Karin 2014). Along with the disease, neutrophils and macrophages infiltrate the tissue in order to resolve the inflammation and protect surrounding cells from ROS secreted by neutrophils (Shi, Gilbert et al. 2001, McDonald and Kubes 2012).

Hence, chronic inflammation is associated with persistent recruitment of neutrophils which has been shown to induce DNA damage in hepatocytes via ROS, thus leading to the development of HCC (Wilson, Jurk et al. 2015). Furthermore, treatment with an anti-oxidant butylated hydroxyanisole (BHA) or using a neutrophil-depleting antibody (Ly6G in a mouse model) has been shown to stop the progression of HCC (Wilson, Jurk et al. 2015).

Another study, using repetitive UV radiation in mice to initiate primary cutaneous melanomas, has shown that neutrophils promote tumour progression by facilitating metastatic progression (Bald, Quast et al. 2014). Neutrophil infiltration was triggered by high mobility group box 1 (HMGB1) released from damaged keratinocytes and driven by Toll like receptor (TLR4). Indeed, the inflammatory response is known to stimulate angiogenesis and promote evasion of melanoma cells towards endothelial cells leading to perivascular invasion (Bald, Quast et al. 2014).

Interestingly, HMGB1 belongs to the alarmin family which functions normally intracellularly but is also secreted in response to cellular stress or damage resulting in an innate immune response (Wang, Bloom et al. 1999, Bianchi 2007). In the context of cellular senescence, a recent study has shown that HMGB1 is secreted rapidly after a variety of

senescence inducing-stimuli and is essential for optimal secretion of SASP components such as IL-6 and MMP-3 (Davalos, Kawahara et al. 2013).

While neutrophils have been implicated in cancer, not much is known about their role in ageing and their connection to cellular senescence. In this work, I hypothesised that neutrophils can accelerate the onset of cellular senescence and potentially contribute to ageing. This hypothesis will be explored through the following aims:

1.6 Aims

Different stressors have been described to induce a premature senescence response mainly due to DNA damage, dependent and independent of telomeres. This response is predominantly mediated by a DDR and its effectors, which will eventually lead to a persistent DSB and trigger a permanent cell cycle arrest. Recent data has shown the connection between cells from the immune system and senescent cells, mainly involved in their clearance. Additional data from cancer studies in mice have highlighted the implication of neutrophil infiltration in the development of HCC. However, the role of neutrophil in senescence is largely unknown; therefore, the main aim of the study was to study the relationship between neutrophils infiltration and bystander damage in surrounding healthy cells.

- The first aim of the study was to determine whether short-term co-culture of young proliferating human fibroblasts with neutrophils led to cellular senescence and investigate the mechanisms involved.
- The second goal was to understand if this phenomenon occurred *in vivo* following immune infiltration in response to the stress induced by a damaging agent in the liver.
- The last aim was to understand whether senescent cells through the SASP can communicate with neutrophils and determine if they are involved in their clearance.

2 CHAPTER 2 – MATERIAL AND METHODS

2.1 Chemicals and Reagents

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

2.2 Buffers and Solutions

PGB-Triton	0.5% Triton™ X-100, 0.5% BSA, 0.25% fish skin gelatin in PBS.
Hybridization buffer including the telomere probe	70% deionised formamide, 25mM $MgCl_2$, 1 M Tris pH 7.2, 5% blocking reagent (Roche,Welwyn, UK), 4ng/µl Tel-Cy-3 telomere specific labelled peptide nuclei acid probe (CCCTAA) (Panagene, F1002-5), in distilled H_2O
FISH wash buffer	70% formamide in 2 X SSC
Citrate buffer pH 6.0	29.41g of trisodium citrate, 1L of distilled H_2O (pH 6.0 adjust with HCL)
2 X SSC	17.53g of NaCl, 8.82g of sodium citrate, 1L of H_2O (pH7.0 adjust with HCl)
RIPA	150mM NaCl, 1% Triton™ X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50mM Tris pH8.0.

Haematoxylin	5g haematoxylin, 40ml glacial acetic acid, 0.5g sodium iodate, 300 ml glycerin, 50g aluminium potassium sulphate, in 700 ml H_2O.
TBS-Triton	10mM Tris, 150mM NaCl and 0.25% Triton™ X-100, (pH 7.0 adjusted with HCl)

Table 1 Buffers and solutions

2.3 Cell Culture

Human Cell Lines

Human embryonic lung MRC5 fibroblasts were obtained from ECACC (Europe Collection of Cell Culture) (Salisbury, UK). MRC5 fibroblasts were used for molecular and cellular biology analysis at a population doubling (PD) range of 16-25 for stress-induced senescence or as a starting point for reaching replicative exhaustion (replicative senescence). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat inactivated foetal bovine serum (FBS) (Biosera, Ringmer, UK), 100µg ml^{-1} streptomycin, 100 units ml^{-1} penicillin and 2mM L-glutamine, incubated in a humidified atmosphere at 37°C with 95% air and 5% CO2 (complete medium, classic conditions).

To note, when the cells were co-cultured with neutrophils, they were incubated in a humidified atmosphere at 37 °C with 3% (v/v) O2 and 5% (v/v) CO2.

IMR90 (gift from Peter Adams.)

IMR90 cells (Coriell) were grown in DMEM, high glucose supplemented with 20% FBS, 2 mM L-glutamine and 100 µg ml^{-1} penicillin–streptomycin and incubated at 37 °C with 3% (v/v) O2 and 5% (v/v) CO2. IMR90 fibroblasts were used for molecular and cellular biology analysis at a population doubling (PD) range of 25-35 for stress-induced senescence.

Reporter cell line (gift from Glyn Nelson)

MRC5 fibroblasts stable reporter cell line was generated retro-virally using plasmid pG-AcGFP-53BP1c. In all experiments, cells were used at a PD range between 25-32. Cells were cultured in a complete medium and classic conditions.

MRC5 fibroblasts hTERT (gift from Gabriele Saretzky).

MRC5 cells were transfected retro-virally at PD 30 with the human catalytic subunit (*TERT*) of the enzyme telomerase.

In this study, cells were used at two different PDs, an early PD (65) and a more advanced one (PD 154). Cells were cultured in complete medium and classical conditions.

Mouse cell lines

Mouse Neutrophil Isolation:

Briefly, bone marrow was extracted from the femur and tibia of C57BL/6 (WT) and $nfkb1^{-/-}$ mice by flushing with HBBS $–Ca2^{+}$ with 5% serum. Neutrophils were isolated by percoll gradient (62%) and purity was established by Ly6G and CD11b (BD biosciences) flow cytometry (BD FACS cantoII).

Hepatocyte Isolation:

Hepatocytes were isolated from the livers of WT and $nfkb1^{-/-}$ mice by digestion with collagenase clostridium histolyticum then filtered through a 70µm cell strainer.

Cells were collected by centrifugation (500 rpm for 3 minutes), washed 3 times in Krebs Ringer buffer and then re-suspended in Williams medium E with 10% serum (WME,

Gibco) and plated onto collagen coated plates (type I 17 collagen, BD Biosciences). After 4 hours, medium was removed and cells were cultured in fresh 10% or 0.5% Williams Medium E.

Hepatocyte and neutrophil co-culture and ROS production:

WT and nfkb1$^{-/-}$ hepatocytes were plated on collagen coated transwell plates then co-cultured with WT or nfkb1$^{-/-}$ neutrophils +/- a 3µm transwell insert (ThinCert, Griener) at a 1:1 ratio for 18 hours. Intracellular ROS of hepatocytes and isolated neutrophils ws measured following incubation with 10µM difluorofluorescein diacetate (h2DCFDA) (FITC 488 mm), 5µM dihydroehidium (DHE) (Rhodamine, 594nm) or 5µM Cell Rox Orange for 30 min at 37°C. Median fluorescence intensity was measured by flow cytometry. Up to 10,000 events were analysed on FACSan/ FACS Canto II (BD, Oxford, UK) using Flowjo software (FlowJo, Inc).

2.3.1 Cryogenic storage

Exponentially growing adherent cells were trypsinised (when at 70-80% confluence) with Trypsin-EDTA (Sigma, Cat.Number T3924). Trypsin was neutralized with the addition of pre-warmed media and cells centrifuged at 150g for 5 minutes at room temperature. The supernatant was removed and cells were re-suspended in foetal calf serum (FCS) containing 10% (v/v) dimethyl sulfoxide (DMSO) at a density of 1x10^{6} cells/ml. One mL aliquots of cell suspension was immediately transferred to cryovials and placed in a NalgeneTM Cryo freezing container filled with isopropanol (Thermo Scientific #5100-0001) . Cells were placed at -80°C for 24 hours to allow gradual freezing, before being transferred to liquid nitrogen for long-term storage.

2.3.2 Resuscitation of frozen cells

Cryovials were removed from the liquid nitrogen storage bank and immediately thawed for 1-2 minutes at 37°C. Cells were then seeded into a 75 cm^{2} flask with 20 mL pre-warmed growth medium. After 24 hours, the growth medium was exchanged to remove DMSO and cell debris.

2.3.3 Calculating cell density and population doublings

Cell concentration was calculated at each passage (at confluence 70-80%) using a Fuchs Rosenthal haemocytometer (VWR international, UK) with 20 µl of suspended cell solution. Using an optical standard microscope (DMIL, Leica Microsystems, UK), the average of three random counts of 8 adjacent squares was then determined which corresponds to the number of cells in $x10^4$/ml. Then, the total number of cells is calculated by multiplying the density by the total volume of suspension (ml).

To obtain the PD, both the number of cells harvested, and the number of cells seeded is necessary. The following equation is then applied: PD = X + (ln(N1 / N2))/ln2

PD = Population Doubling

X = Previous PD

N1 = Number of cells harvested

N2 = Number of cells seeded

2.4 Induction of DNA damage

2.4.1 X-Ray irradiation

MRC5 fibroblasts at early PD (20-30), IMR90 PD (30-35) and MRC5 mCherry-53BP1c (25-30) reporter cells were seeded onto 6/12 well plates (Corning) 24 hours prior to irradiation. Cells were then exposed to X-ray irradiation (IR) (X-Rad 225, Precision X-Ray INC, N-BRANFORD, CT USA) with different doses (10Gy for IMR90 or 20Gy for MRC5). Following treatment, the medium was changed immediately to prevent further damage from residual compounds generated by IR.

2.4.2 Chemical-induced senescence

Stress-induced senescence was also induced by treating cells with 400µM hydrogen peroxide (H_2O_2) (SIGMA, H1009) for 1 hour in serum free medium, followed by washes of PBS (2 X 5 minutes) at 37°C, then re-incubated in complete classic growth medium.

2.4.3 Replicative senescence

Replicative senescence was reached through replication exhaustion and confirmed by >80% of cells being positive for Sen-β-Gal, negative for replication marker (Ki67) and less than 0.5 Population doublings for at least 4 weeks.

2.5 Treatments

2.5.1 During co-culture (hepatocytes and fibroblasts cells)

2.5.1.1 Catalase

In order to assess the impact of H2O2 released by neutrophils on neighboring cells, we administrated during co-culture the H_2O_2-specific scavenger catalase at 100UI/ml. Following the 3 days co-incubation time, the catalase and the neutrophils were removed and the MRC5 fibroblasts were exponentially cultured until replicative senescence.

2.5.1.2 Recombinant elastase

Mice hepatocytes and neutrophils isolated from the bone marrow were co-cultured in the presence of 40 nM recombinant elastase for 24 hours in Williams Medium E (Natural human Neutrophil Elastase protein (ab91099)).

2.5.1.3 Neutrophils

2.5.1.4 Lipopolysaccharide (LPS)

Depending on the experiment, the desired concentration of freshly isolated neutrophils from venous blood (from middle-aged healthy donors) were primed with LPS (derived from Escherichia coli serotype O26:B6), at 100 ng ml^{-1} for 1 hour at 37°C in the dark at 3% O_2.

2.5.2 Inhibitors used on replicative senescent cells

2.5.2.1 Inhibition of mTORC1

In order to inhibit mTORC1, replicative senescent MRC5 fibroblasts were treated with 100nM rapamycin for 10 days before being harvested for analysis. Growth medium was changed 3 times a week. Cells were cultured in serum-free medium for 24 hours before collection of conditioned media. Control cells were treated with the same volume of DMSO.

2.5.2.2 P38 MAPK inhibitor

In order to suppress the activation of MAPKAP kinase-2 and the phosphorylation of heat shock protein (HSP) 27 in response to IL-1, replicative senescent MRC5 fibroblasts were treated with 10μM SB203580 for 10 days before being harvested for analysis. Growth media was changed 3 times a week. Cells were cultured in serum-free medium for 24 hours before collection of conditioned media. Control cells were treated with the same volume of DMSO.

2.5.2.3 Clorgyline, MAO-A inhibitor

In order to inhibit monoamine oxidation A (MAO-A), replicative senescent MRC5 fibroblasts were treated with 1μM clorgyline (Eli Lilly, UK) for 10 days before being harvested for analysis. Growth media was changed 3 times a week. Cells were cultured in serum-free medium for 24 hours before collection of conditioned media. Control cells were treated with the same volume of deionized water.

2.6 Co-culture experimental procedure

All co-culture experiments were done using MRC5 fibroblasts at an early PD (16-26). 24 hours before the experiment, cells were harvested, counted and 2 $x10^5$ cells were seeded in a T25 flask (Corning). Cells were cultured in classical conditions. On the first day of the co-culture, freshly isolated neutrophils were primed with 100 ng/ml LPS for 1 hour at 37°C, 3% O_2 in the dark. After 1 hour incubation, primed neutrophils were centrifuged at 1000 RPM to wash remaining LPS and $1x10^6$ neutrophils were mixed with the MRC5 fibroblasts on a ratio of 1:5 fibroblasts:neutrophils. In some conditions, catalase (100 UI/ml) was added to the suspension for the duration of the co-culture. As controls, MRC5 fibroblasts were cultured with either catalase alone or with neutrophils not primed with LPS. Every 24 hours, neutrophils were removed by aspiration and fibroblasts were washed once with pre-warmed media prior to the addition of fresh neutrophils from a different middle-aged healthy donor for a further 24 hours of co-incubation. This procedure was repeated once or for 3 consecutive days. On the last day, neutrophils were removed, and the fibroblasts were passaged and transferred onto new T150 flasks until they reached replicative senescence.

For the duration of the experiment (1 or 3 consecutive days), the co-cultured cells were incubated at 3% O_2 at 37°C. Subsequent to neutrophil removal, fibroblast were returned to normal and classical culture conditions.

2.7 Live cell imaging

For live-cell time-lapse microscopy, $10x10^4$ MRC5 fibroblasts, IMR90 or MRC5 mCherry 53bp1c cells were seeded into a 6-well plate glass coverslip bottomed dishes (Mattek), and incubated for 24 hours to let the cells adhere to the glass substratum. Then, depending on conditions cells, were IR with 20Gy and imaged 10 days post IR. Non irradiated, early PD cells were used as controls.

Images were taken every 10 minutes for 24 hours using a 20X objective apochromat air (NA=0.8). A 2 X 2 tile scan was set up to follow cells over a larger area using Z-stacks

over 10 µm. Acquisition was done using a Zeiss Spinning Disk confocal microscope equipped with a QuantEM 5125C camera and Axiovision software (Zeiss). Cells were incubated at 37°C in a humidified environment with 3% O_2 and 5% CO_2.

To assess the viability of the cells in the clearance assay during co-culture with neutrophils, PI was added to the cell suspension (0,2mg/mL). To detect the PI by fluorescence, an argon laser 561 with a Cy3 filter was used.

DNA damage foci (DDF) from live-cell time-lapse microscopy were counted manually using ImageJ. Cells positive for PI were counted manually for each field using ImageJ.

2.8 Measurement of DNA damage: Comet assay

$10x10^4$ cells were trypsinised and centrifuged at 1600rpm, supernatant was discarded and cells were resuspended in 500µl 10% DMSO in FBS. Cells were then frozen at -80°C in a Cryo freezing container filled with isopropanol (Thermo Scientific #5100-0001). On the day of the experiment, cells were defrosted on ice then washed with cold PBS and centrifuged at 4°C at 700g for 5 minutes. The supernatant was removed and cells were resuspended in 0.7% LMP agarose at 37°C to a final concentration of 2 x 10^5 cells/ml. 70µl of cell/agarose mix was placed on slides coated in 1% agarose between a cover-slip in duplicate for each condition, care was taken to avoid bubbles. Slides were then placed at 4°C for 10 mins to allow the gel to set. Coverslips were removed and the slides were placed in lysis buffer (2.5M NaCl, 100nM EDTA, 10nM Tris, 250nM NaOH 10% DMSO, 1% Triton X-100) for 1 hour at 4°C. Slides were then washed twice in cold PBS.

Alkaline buffer

Slides were places for 40 minutes in alkaline buffer (300mM NaOH, 1mM EDTA) at 4°C to denature DNA. Samples were subjected to electrophoresis for 30 minutes at 25V at 4°C in the alkaline buffer.

Neutral buffer

Samples were subjected to electrophoresis for 30 minutes at 25V at 4°C in Tris-borate EDTA (TBE) buffer.

Visualisation

After electrophoresis, slides were washed twice in cold PBS, 500µl of 1000x Sybr Gold (Invitrogen) in tris-borate EDTA (TBE) buffer was added to each gel and slides were incubated for 40 mins in a dark humid chamber. Slides were washed twice in MilliQ water and allowed to dry. Samples were imaged using an Olympus BX51 widefield microscope with Olympus UPlanFL 20x/0.50 air objective. Comets were scored using Comet assay IV (Perceptive Instruments Ltd., Haverhill, Suffolk, United Kingdom). Each experiment was run in duplicate, on two separate gels and an average reading for the two gels was quantified.

2.9 Metaphase generation

Metaphase spreads from MRC5 fibroblasts were prepared at different time points (0, 4, 8 and 16 days after co-culture) by treatment of subconfluent cells with 10µg ml−1 colcemid for 24 hours at 37 °C, followed by addition of 60mM KCl for 15min at RT and fixation in ethanol:acetic acid (3:1) as described (Passos, Saretzki et al. 2007). Slides were air-dried and stored in boxes in the dark at RT.

2.10 Senescence associated β Galactosidase activity assay

Senescence-Associated β Galactosidase assay is a common marker used to detect senescent cells as described previously (Dimri et al., 1995). Briefly, cells on coverslips were washed with PBS and fixed with 2% paraformaldehyde and 0.2% glutaraldehyde solution for 5 minutes at room temperature. Following PBS washes (twice for 5 minutes), cells were incubated in SA-β-Gal staining solution (150mM NaCl, 2mM $MgCl_2$, 40mM citric acid, 12mM $NaPO_3$, 400 µg/mL X-gal, 2.1mg/mL potassium hexacyanoferrat (II) trihydrate and 1.65mg/mL potassium hexacyanoferrat (III) trihydrate), pH 6.0 (for human cells) at 37°C in the dark overnight.

Cells were then washed three times for 5 minutes in PBS and then mounted onto glass microscope slides with ProLong® Gold Antifade Mountant with DAPI (Invitrogen, P36935).

Using a Leica DM5500B microscope and a Leica DFC420 camera using LASAF software, random fields (20X objective) were imaged and cells were scored either positive (dark blue staining) or negative for SA-β Gal staining.

2.11 Flow cytometry

Prior to any measurement and to ensure the optimum performance and reproducibility of the flow cytometer (Partec, http://www.partec.com), a calibration was performed using fluorescent microbeads. Each measurement was assessed in duplicate or triplicate and $1x10^4$ cells (events) were analysed per measurement.

2.11.1 Measurement of reactive oxygen species

2.11.1.1 Dihydroethidium

Dihydroethidium (DHE) is a blue fluorescent dye that when oxidised through binding to superoxide anions forms a red fluorescent product which intercalates with DNA.
Cellular superoxide intensities were determined by harvesting and incubating ~ 2.5 $X10^5$ live cells with 10µM of DHE (Invitrogen, Cat. Number D1168) in serum-free DMEM for 30min at 37°C in the dark. Cells were centrifuged at 1600 RPM for 2 minutes, the supernatant was discarded, and cell pellet was re-suspended in 2ml serum-free DMEM. Measurements of the median fluorescence intensity were determined via flow cytometry using the red fluorescence channel (FL3 channel).

2.11.1.2 MitoSOX

Mitochondrial superoxide levels were as well determined using the same procedure as for DHE staining, however live cells were incubated with 5 µM of MitoSOX™ Red (Life Technologies-Invitrogen, 50 µg vial) for 10 minutes. Median fluorescence intensity was determined using the same red fluorescence channel.

2.11.2 Measurement of cell death

2.11.2.1 Propidium iodide/Annexin V

Cell viability was determined after 24 hours of co-culture using Propidium iodide (PI) in conjunction with Annexin V to establish if cells are viable, apoptotic, or necrotic through differences in plasma membrane integrity and permeability. Annexin V will specifically stain early apoptotic cells, whereas PI will stain late apoptotic and necrotic cells.

According to the manufacturer's recommendations (eBiosciences), supernatants and corresponding cells were collected for each condition. Cells were then resuspended in 100µl of Annexin V buffer (eBiosciences (Hatfield, UK)), and 1 µl of FITC-Annexin V was added to the cells. After 10 minutes of incubation in the darkness at room temperature (RT), 1 µl/ml of PI was added to the cell suspension and immediately analysed by flow cytometry. Measurements of the median fluorescence intensity were determined via flow cytometry using the red fluorescence channel (FL3 channel) for PI and green fluorescence channel FL1 for Annexin V.

2.12 Immunology

2.12.1 Extraction of polymorphonulcear leukocytes and monocytes from whole blood

2.12.1.1 Isolation of peripheral neutrophils

(Neutrophils were obtained from Marie-Helene Ruchaud-Sparagano expertise, John Simpson's group)

Neutrophils from healthy volunteers were isolated from whole blood by means of dextran sedimentation and fractionation over discontinuous Percoll gradients as follows. Briefly, leukocytes were isolated from citrated whole blood obtained by venipuncture of healthy volunteers. After centrifugation of blood at 300g with no brake for 20 minutes and removal of platelet-rich plasma, leukocytes were separated from erythrocytes by dextran sedimentation by using 6% dextran T500 (Dextran T500 from Pharmacosmos). The leukocyte-rich upper layer was then fractionated by using isotonic Percoll Plus (GE

Healthcare, 17-5445-01), and polymorphonuclear leukocytes (PMN) and mononuclear leukocytes were harvested from the 70 to 81% and 55 to 70% interfaces. PMN were washed in HBSS without $Ca2^{+}/Mg2^{+}$ by centrifugation at 200g for 5 minutes and counted then adjusted to 1 million/ml.

Only samples yielding 95% or greater neutrophil purity (assessed by means of morphological analysis using a cytospin and Giemsa staining) and 95% or greater viability (assessed by means of trypan blue exclusion) were used.

2.12.1.2 Monocyte Isolation & Culture

Mononulear cells from the 55/70 layer of the Percoll density gradient were washed and counted. Then cells were resuspended at $4x10^{6}$/ml in RPMI and plated in a 6 well plate at 37°C/ 5% CO_2 for 1 hour. Cells were then washed thoroughly and fresh RPMI containing 10% autologous serum was added then left to mature over 6-7 days in the incubator until they were fully differentiated.

2.12.1.3 Viability of cells

The viability of the neutrophils was assessed by Trypan Blue (Gibco, Life Technologies, 0.4%) staining, which allows the discrimination between viable and non-viable cells. Immediately after extraction of the PMN, 100 µL of cell suspension was mixed with 5 µL of trypan blue solution and applied to a haemocytometer. Then, the cells were counted and discriminated between white and blue cells (live cells with intact cell membranes are not coloured). The viability is sorted by calculating the ratio between live and dead cells. In all experiments, 95% or greater viability was used.

This procedure was also used to determine viability of neutrophils after different incubation periods at various oxygen levels (3% and 20 %) and treatments.

2.12.1.4 Control of neutrophil preparation purity

The control of purity of the preparation is done using a cytospin (Shandon, cytospin 3). 150 µl of cell suspension (~500,000 cells/ml) was loaded into a cytospin chamber and centrifuged for 3 minutes at 300g. Slides were allowed to dry, and were then fixed for 10 minutes with acetone followed by Giemsa solution staining for another 10 minutes.

Following two washes with water, slides were air dried and mounted with DPX. Cell purity was assessed by counting 100 cells under the microscope by means of morphological analysis. In all experiments, 95% or greater purity was used.

2.12.2 Chemotaxis experiments

The method used to measure the chemotactic response of the PMN is based upon migration of cells under agarose gel described by (Nelson, Quie et al. 1975). This technique allows both chemotaxis and spontaneous migration toward a chemoattractant.

Slides were prepared and coated with 0.5% gelatin then 5 ml of mix composed by agarose, gelatin, 25% BSA and DMEM was pour onto the slides. Once dried, the slides were stored at 4° C. On the day of the experiment, 3 wells were cut onto the slide: fresh PMN was resuspended at $2.5x10^6$/ml in DMEM-1%BSA and 10µl was transferred to each central well. Then, chemoattractant or media alone was placed on the other well. After 2 hours of incubation at 37° C for 2 hours, the slides were fixed in 2.5% paraformaldehyde overnight, stained with Giemsa for 10 minutes and mounted in DPX. Slides were analysed under a computer image analysis (Image-Pro software, Image-Pro Software, Rockville, MD, USA). Neutrophil chemotaxis was determined by measuring the distance between the origin and the leading edge of cells migrating towards the chemoattractant, and subtracting the distance of migration between the origin and the leading edge of cells migrating towards medium alone (corresponding to random migration).

2.12.3 Measurement of superoxide anion release by neutrophils

The release of superoxide anions by freshly isolated neutrophils in response to fMLP (10^{-7} M) was measured using cytochrome c reduction assay as previously described (Ruchaud-Sparagano, Walker et al. 2000). Cells were pre-incubated with LPS, PMA or HBSS containing Ca^{2+} and Mg^{+2} , or platelet-activating factor (positive control) for 60 minutes, in the presence of 1% autologous serum, before stimulation with fMLP (100 nM). For each sample, a control with superoxide dismutase (200 U) was included to verify the specificity of cytochrome c reduction by superoxide anions. After 15 minutes incubation at 37°C, the reaction was stopped immediately by placing the cells on ice then centrifuged at 10,000 g for 3 minutes. The superoxide dismutase reduction of cytochrome c was then determined for each supernatant by measuring the absorbance at 550 nm using a BMG Fluostar

Optima plate reader (BMG Labtech Ltd, Aylesbury, UK). Results are expressed as nmole of $O2^-$ per million neutrophils, using the extinction coefficient of 21 x 10^3 M^{-1} cm^{-1}.

2.12.4 Amplex red measurement

Reactions containing 50 µM Amplex Red reagent (AR), 0.1 U/ml HRP, H_2O_2, and conditioned media, were prepared in the dark. 40ul of HRP working stock (10 U/ ml) and 10ul of AR working stock (1 mM) were pipetted to each well in triplicate (or media for the blank + LPS (100ng/ml)) of a 96-well black bottom microplate containing freshly isolated neutrophils at different concentrations. Fluorescence was measured in a fluorescence microplate reader (FLUOstar Omega, BMG Labtech) using excitation in the range of 530–560 nm and emission detection ~590 nm at 37°C for 2h.

2.13 Mice

2.13.1 Mouse groups and treatments

2.13.1.1 Ageing Colony

A long established colony of inbred C57Bl/6 (ICRFa) mice (n=10/group) were fed *ad libidum*, with constant access to water, and culled at 3, 15 or 30 months of age, as described in (Wang *et al.*, 2009; Cameron *et al.*, 2011).

2.13.1.2 Mice and models of liver injury using N-Nitrosodiethylamine (DEN) provided by Derek Mann

Experiments on C57BL/6 (WT) and $nfkb1^{-/-}$ mice were performed under approval from the Newcastle ethical review committee and a UK Home Office licence. $Nfkb1^{-/-}$ mice were a gift from Prof J.Caamano (UK). 15 days old mice were given 30mg/kg DEN in 0.9% saline by intraperitoneal injection (I.P) to induce liver cancer. For acute DEN treatment, 8-week-old mice were given 100mg/kg by I.P to induce liver DNA damage.

2.13.1.3 Models of rodent acute liver injury using carbon tetrachloride (CCl_4) provided by Derek Mann (Moles, Murphy et al. 2014)

Wt, *tlr2*$^{-/-}$ (Takeuchi, Hoshino et al. 1999, Moles, Murphy et al. 2014), *tlr4*$^{-/-}$ (Hoshino, Takeuchi et al. 1999), and *s100a9*$^{-/-}$ (Manitz, Horst et al. 2003) mice were provided by Prof M. Karin, Prof E. Seki and Prof N. Hogg. Single intraperitoneal injection of CCl_4 at a dose of 2 µl (CCl_4: olive oil, 1:1 [v:v])/g body weight was administered for 8, 24, 48, and 72 hours to 8–10 week old male littermates. Mice were pre-treated with Ly-6G or IgG control antibody for 12 h before CCl_4 injection. Animals were culled at 48 hours post-CCl4 injection. At least five animals were used per treatment group.

2.13.1.4 Models of rodent liver sublethal LPS injury provided by Derek Mann (Moles, Sanchez et al. 2013)

Mice (8-10 weeks old; C57BL/6) were purchased from Harlan Laboratories (Indianapolis, Indiana). Pure LPS (InvivoGen, San Diego, CA) was administered by intraperitoneal injection injection at a dose of 300 µg/animal for 24 hours.

2.13.2 Housing of mice

All animal husbandry and experimental procedures were performed in compliance with the Animals (Scientific Procedures) Act 1986 (ASPA). Mice were housed in a temperature-regulated environment (20±2˚C) with a 12 hour light/dark cycle, with lights turned on at 7 am.

2.13.3 Mouse tissue collection and preparation

All tissues were placed in 10% neutral buffered formalin (VWR, 9713.9010) immediately after dissection. After fixation, tissues were dehydrated through a series of graded ethanol baths, before being treated with xylene, followed by paraffin embedding.

2.14 Immunostainings

2.14.1 Immunofluorescence staining on fixed cells

Fixation

Cells were grown on sterile coverslips until they reached 80% confluency, washed with PBS and fixed with 2 % paraformaldehyde (PFA) (VWR, 9713.9010) for 5 minutes at RT. Once fixed, cells were washed twice for 5 minutes in PBS and immediately stored at -80°C for long term preservation.

Permeabilisation

Fixed cells were incubated for 45 minutes in PBG-Triton (Table 1) at RT, whilst shaking on an orbital shaker at 80 RPM.

Immunofluorescent staining

Cells were incubated with a primary antibody (Table 2) diluted in PBG-Triton in a humidified chamber overnight at 4°C. The next day, cells were washed three times for 5 minutes in PBG-Triton, and then incubated with a secondary antibody (Table 2) for 45 minutes to 1 hour at RT in the dark (using aluminium foil to wrap the plates). Finally, cells were washed three times for 5 minutes in PBS before mounting the coverslips onto glass microscope slides with ProLong® Gold Antifade Mountant with DAPI (Invitrogen, P36935). Coverslips were analysed using a Leica DM5500B microscope and images were captured with a DFC360FX camera using LASAF software (Leica) or on a Leica DMi8 wide field fluorescence (inverted) with different objectives depending on the experiment using LASX software. In some cases, z stacks were taken.

2.14.1.1.1.1 TABLE 2: PRIMARY ANTIBODIES FOR IMMUNOFLUORESCENCE ON CELLS

Protein	Host	Specificity	Dilution
Anti-Ki67 (Abcam, ab15580)	Rabbit polyclonal	Human Mouse	1:250

Anti-phospho-histone γH2A.X (Ser139) (Millipore, 05-636)	Mouse monoclonal	Human	1:200
anti-53BP1 antibody 4 μg ml^{-1} (NB100-904 Novus Biologicals)	rabbit polyclonal	Human Mouse	1:250

Table 2 Primary antibodies for Immunofluorescence on cells

2.14.1.1.1.2 Table 3: Secondary antibodies for Immunofluorescence on cells

Protein	Host	Specificity	Dilution
Anti-rabbit Fluorescein-conjugated secondary antibody AlexaFluor 594 (Invitrogen, A21213)	Goat	Rabbit	1:2000
Anti-mouse Fluorescein-conjugated secondary antibody AlexaFluor 488 (Invitrogen, A21042)	Goat	Mouse	1:2000

Table 3 Secondary antibodies for Immunofluorescence on cells

N.B: All wash steps were carried out with gentle shaking on an orbital shaker at 70RPM and all antibody incubations steps carried out in a dense plastic humidifier chamber.

2.14.2 Telomere-FISH on fixed Cells following immunofluorescence

Fluorescence in situ hybridisation (FISH) is a technique that uses fluorescent probes to detect specific parts of chromosomes. In this case, the probe used is design to bind to the telomeric repeats (TelC-Cy5, Panagene, Korea).

After immunofluorescence described above using γH2A.X and the corresponding secondary antibody, cells were washed twice for 5 minutes in PBS and FISH was applied.

Cells were fixed for 30 minutes with a mix of methanol: acetic acid (3:1) then dehydrated through graded cold ethanol solutions (70, 90, and 100%) for 3 minutes each in a humidify chamber and finally air-dried. Cells were incubated in pre-warmed PBS at 37°C for 5 minutes and fixed again with pre-warmed 4% PFA for 2 minutes. Samples were washed twice for 5 minutes in PBS and dehydrated in the same ethanol gradient mentioned previously. The coverslips were allowed to air dry and were then denaturated in a hybridization buffer including the telomere probe (Table 1) at 80°C for 10 minutes, placed into a humidifier chamber and incubated for a minimum of 2 hours at RT.

Coverslips were washed three times for 10 minutes with FISH wash buffer (table 1) followed by three washes of 5 minutes in 0.05% TBS-Tween. Finally, coverslips were dehydrated again in cold ethanol series, air-dried and cells were mounted using ProLong® Gold Antifade Mountant with DAPI (Invitrogen, P36935).

Coverslips were analysed with a 63X objective (oil, NA=1.4) using a Leica DM5500B microscope and images were captured with a DFC360FX camera using LASAF software (Leica) or on a Leica DMi8 wide field fluorescence (Inverted) using LASX software. A Z stack of 12 µm was acquired (with a minimum of 50 optical slices) per image. Quantification of DNA damage foci and telomere-associated foci (i.e. co-localisation between γH2AX and telomeres) was performed blinded using ImageJ software.

For the metaphase spreads, cells were rehydrated twice for 5 minutes in PSB then dehydrated with the ethanol series mentioned above. Slides were allowed to air dry before

the hybridisation buffer was applied. All subsequent steps are the same as in the procedure mentioned above

Acquisition was done on a 100X (NA=1.4) oil objective, Z stacks of 5 µm through the entire chromosomes of each cell was performed using a Leica DMi8 wide field fluorescence (inverted) equipped with a Hamamatsu C11440-22 camera.

2.14.3 Immunostainings on paraffin embedded tissues

2.14.3.1 Using rabbit primary antibodies

Tissue sections of 3µm were cut and placed onto slides coated with 4% APES, then incubated overnight at 37°C. Sections were deparaffinised in Histoclear (National diagnostics, USA) twice for 5 minutes followed by hydration series of a gradient of ethanol (100, 90, 70%, twice for 5 min, 5minutes and 5minutes, respectively) then wash twice for 5 minutes in distilled H_2O. Then, an antigen retrieval step was performed by incubating the sections in 0.01M citrate buffer pH 6.0 (Table 1) for 4 minutes in a microwave oven at 800W, followed by 10 minutes at 400W. Sections were cooled down on ice for 20 minutes and washed twice with distilled water. Sections were then incubated for 30 minutes in blocking reagent (Normal goat serum (NGS) (Vector labs, VECTASTAIN Elite ABC HRP Kit (Peroxidase, Rabbit IgG)) (diluted 1:60 in 0.1% bovine serum albumin (BSA) in PBS) followed by incubation with the primary antibody (table 4) (diluted in the blocking reagent) at 4°C in a humidified chamber. Sections were washed in PBS three times for 5 minutes and incubated with secondary antibody diluted in the blocking solution (table 5) for 45 minutes. For anti-γH2A.X antibody, sections were incubated with a biotinylated secondary antibody (1:200) in blocking solution (goat anti-rabbit biotinylated (Vector labs, VECTASTAIN Elite ABC HRP Kit (Peroxidase, Rabbit IgG)) for 30 minutes at RT. Sections were washed three times for 5 minutes in PBS then incubated with avidin DCS (Vector Lab, A-2011) (1:500 in PBS) for 30 minutes. Sections were finally washed three times for 5 minutes in PBS and mounted with ProLong® Gold Antifade Mountant with DAPI (Invitrogen, P36935). Acquisition was done using a Zeiss Spinning Disk confocal microscope equipped with a QuantEM 5125C camera and Axiovision software (Zeiss).

2.14.4 Protocol for mouse primary antibodies

Immunostainings using primary mouse antibodies on mouse tissue were done with the Mouse on mouse kit (M.O.M basic kit (Vector Lab, BMK-2202)) according to the manufacturer instructions. Briefly, tissues were blocked in the M.O.M blocking reagent diluted in TBS-Triton for 1 hour at RT. Sections were then washed twice for 5 minutes in PBS and then incubated with the primary antibody (Table 4) in the M.O.M diluent in TBS-Triton overnight at 4°C. Then sections were washed in PBS three times for 5 minutes and incubated with an Alexa Fluor conjugated secondary antibody (table 5) (1:1000). Sections were finally washed three times for 5 minutes in PBS and mounted with ProLong® Gold Antifade Mountant with DAPI (Invitrogen, P36935). Acquisition was done using a Zeiss Spinning Disk confocal microscope equipped with a QuantEM 5125C camera and Axiovision software (Zeiss).

2.14.5 ImmunoFISH on tissues (γH2A.X-TeloFISH) staining on paraffin embedded tissues

In some conditions, immunofluorescence staining was coupled with FISH. After incubation with avidin DCS, sections were washed three times for 5 minutes in PBS then crosslinked with a solution of 4% PFA in PBS for 20 minutes at RT followed by three PBS washes for 5 minutes. Sections were then dehydrated using a graded cold ethanol solutions (70, 90, 100%) for 3 minutes each in a humidified chamber and finally air-dried. Then sections were denaturated in a hybridisation buffer including the telomere probe (Table 1) at 80°C for 10 minutes, placed into a humidified chamber and incubated for a minimum of 2 hours at RT. Slides were washed three times for 10 minutes with FISH wash buffer (table 1) followed by 10 minutes wash with 2 X SSC (table 1) and three PBS washes for 10 minutes. Finally, sections were mounted using ProLong® Gold Antifade Mountant with DAPI (Invitrogen, P36935).

Sections were analysed with a 63X objective (oil, NA=1.4) using a Leica DM5500B microscope and images were captured with a DFC360FX camera using LASAF software (Leica) or on a Leica DMi8 wide field fluorescence (Inverted) using LASX software equipped with a Hamamatsu C11440-22 camera. A Z stack of 12 µm was acquired (with a minimum of 50 optical slices) per image. Quantification of DNA damage foci and

telomere-associated foci (i.e co-localisation between γH2AX foci and telomeres) were analysed blinded using ImageJ software.

2.14.5.1.1.1 TABLE 4: PRIMARY ANTIBODIES FOR IMMUNOFLUORESCENCE ON TISSUE

Protein	Host	Specificity	Dilution
Phospho-Histone H2A.X (serine 139) (Cell Signalling, #9718)	Rabbit monoclonal	Human Mouse Rat Monkey	1:200
Anti-PCNA antibody [PC10] (ab29)	Mouse	Human	1:1000

Table 4 Primary antibodies for Immunofluorescence on tissue

2.14.5.1.1.2 TABLE 5: SECONDARY ANTIBODIES FOR IMMUNOFLUORESCENCE ON TISSUE

Protein	Host	Specificity	Dilution
Anti-Mouse IgG (H+L), Alexa Fluor® 647 Invitrogen, A-21235	Goat Polycloncal	Mouse	1:1000
Biotinylated anti-Rabbit IgG (H+L) (VECTASTAIN Elite ABC Kit) PK-6101 – Vector Laboratories	Goat	Rabbit	1:200

Fluorescein-labelled Avidin DCS (Vector Laboratories, A-2011)	N/A	1:500

Table 5 Secondary antibodies for Immunofluorescence on tissue

2.14.6 Q-FISH

Q-FISH analysis of telomere-FISH was performed on fixed cells after chromosome preparation (see above for procedure to generate metaphases) in order to distinguish individual chromosomes. Acquisition was done on a 100X (NA=1.4) oil objective, Z stacks of 5 µm through the entire chromosomes of each cell was performed using a Leica DMi8 wide field fluorescence (inverted) equipped with a Hamamatsu C11440-22 camera. For each individual cell, Z projections were created using imageJ software and the oval tool was used to create a circle to measure the integrated density of individual telomeres signal. A minimum of 10 metaphase spreads were analysed for each condition.

2.15 Immunohistochemistry

2.15.1 IHC on paraffin embedded tissue

Tissue sections of 3µm were cut and placed onto slides coated with 4% APES, then incubated overnight at 37°C. Sections were deparaffinised in Histoclear (National diagnostics, USA) twice for 5 minutes followed by hydration series in a gradient of ethanol (100, 70%) twice for 5 minutes, then endogenous peroxidase was blocked in a solution of Methanol/ 2% hydrogen peroxide for 15 minutes then tissues sections were washed for 5 minutes in PBS. Antigen retrieval was performed using Proteinase K (20µg/ml) in a humidified atmosphere at 37°C, followed by one wash in PBS for 5 minutes. Then, slides were mounted in sequenza and avidin / biotin (ref: SP-2001, Vector laboratories) were blocked for 20 minutes each followed by a blocking solution incubation for 20 minutes with swine serum in PBS (1:5). Sections were incubated with the primary antibody Rat anti-mouse monoclonal NIMP-R14 (ref: ab2557-50 Abcam) (1:200) overnight at 4°C. The next

day, slides were washed in PBS three times for 5 minutes followed by secondary antibody incubation for 90 minutes at RT with goat anti-rat biotin conjugated Ac 1:200 (ref: STAR 80B, Serotec). Then, three PBS washes were performed for 5 minutes each and 3 drops of Vector ABC tertiary (ref: PK 7100 Vector laboratories) was applied for 30 minutes followed by 5 minutes wash in PBS. Detection was performed with DAB mix (ref: SK 4100 Vector laboratories) according to the manufacturer's instructions for 5-10 minutes (visual detection). Sections were then briefly washed with PBS and counterstained with Meyers haematoxylin for 10 seconds and washed in tap water for 10 seconds. Finally, tissue sections were rehydrated in ethanol graded series for 5 minutes each (50, 70,100%) and twice for 5 minutes in Histoclear then mounted in Pertex (ref: 00811, Histolab).

Slides were analysed with a Nikon E800 Wide field (upright) microscope and images were captured with a Leica DFC420 camera using the LAS software (Leica) on a 20X objective (air, NA=0.5). Levels of positive staining were determined by quantifying the number of cells stained per field and generating an average from 10 randomly captured images for each animal.

2.16 Statistical analysis

Data are expressed as the mean ± SD, mean ± S.E.M or median ± range, where appropriate. Where data were normally distributed, statistically significant differences between two groups were evaluated using an independent samples two-tailed t-test and significant differences between groups were assessed using ANOVA. Where data were not normally distributed, statistically significant differences between two groups were evaluated using the Mann-Whitney U test and the Gehan-Breslow test. P values < 0.05 were considered significant. Data were analysed using GraphPad Prism version 7.0, GraphPad Software, San Diego California USA, www.graphpad.com and IBM SPSS statistics version 19 and using Sigma plot version 12.5 from Systat Software, Inc., San Jose California USA, www.systatsoftware.com.

2.17 Ethics statement

All work complied with the guiding principles for the care and use of laboratory animals. The project was approved by the Faculty of Medical Sciences Ethical Review Committee, Newcastle University. Project license number 60/4102.

3 CHAPTER 3 - Neutrophils induce senescence in human fibroblasts and mouse hepatocytes in a ROS dependent manner

3.1 Effects of Neutrophil exposure to LPS and survival in culture

Neutrophils are the main actors involved in microbial killing during infections and for that purpose they produce large amounts of reactive oxygen species such as hydrogen peroxide and superoxide anion, which are very harmful to host tissues (Botha, Moore et al. 1995, Partrick, Moore et al. 1996). Hence, activation of neutrophils is tightly regulated with exogenous and endogenous mediators released during infection (Ferrante, Kowanko et al. 1992, Serhan, Chiang et al. 2008). Furthermore, LPS (lipopolysaccharides) which is a bacterial endotoxin has been shown to prime neutrophils resulting in enhanced respiratory burst in response to a second stimulus such as N-Formylmethionyl-leucyl-phenylalanine (fMLP) or opsonised zymosan. The range of LPS used is from 0.01 to 100 ng/ml, with a maximum response after one hour incubation (Doerfler, Danner et al.).

In this chapter, I aimed to investigate the role of neutrophils as potential inducers of cellular senescence. In order to conduct these investigations, I first needed to establish the best concentration of LPS to prime neutrophils.

For that reason, I tested a range of LPS doses from 0.1 ng/ml to 100 ng/ml (physiological range) following stimulation, to determine which concentration was the best at inducing superoxide release for our future experiments. We measured the superoxide release using the cytochrome c reduction assay in adherent neutrophils upon pre-incubation with a LPS dose of 0.1 to 100 ng/ml for one hour followed by exposure to a second stimulus (zymosan). Figure 3.1 a shows that the best response was obtained with priming the neutrophils for 1h with 100 ng/ml LPS. I therefore decided to use 100 ng/ml LPS to prime the neutrophils for future experiments.

To further confirm the release of ROS produced by human neutrophils upon treatment with LPS for 1 hour, I performed the Amplex Red (AR) assay (Molecular Probes). AR is a fluorescent probe that reacts with hydrogen peroxide and can be used to determine the rate of hydrogen peroxide release by neutrophils. Data indicates that neutrophils primed

for 1h with LPS show a sharp increase in Amplex Red fluorescence, indicative of increased hydrogen peroxide release (Figure 3.1 b).

Upon exposure to LPS, neutrophils have been shown to activate Toll like receptor 4 (TLR4), a pattern recognition receptor involved in innate immune activation. TLR4 activation has been shown to result in NF-κB activation (Sabroe, Jones et al. 2002) and the generation of ROS (Sabroe, Prince et al. 2003). Hence, we measured using flow cytometry the surface expression of TLR4 on neutrophils following exposure to 100 ng/ml LPS and found a significant increased compare to the untreated cells (Figure 3.1 c). As expected, exposure of neutrophils to 100 ng/ml LPS for 1h led to the production and release of IL-8 (a pro-inflammatory cytokine and NF-κB target) measured by ELISA, as shown in Figure 3.1 d and as previously described (Baggiolini and Clark-Lewis 1992).

Following the characterisation of neutrophils to LPS, I evaluated the viability of neutrophils in culture in order to establish the best conditions for future co-culture experiments.

It has been previously reported that neutrophils undergo spontaneous apoptosis under 20% O_2 (ambient oxygen) and that their survival was improved under low oxygen conditions (Walmsley, Print et al. 2005). Consistent with this, we observed that neutrophils survival was significantly improved using low oxygen conditions (Figure 3.1 e).

All these data taken together show that neutrophils primed with 100 ng/ml LPS have an enhanced response (oxygen burst) after exposure to a second stimulus and low oxygen preserves their viability. For these reasons, we decided to use these conditions for our further experiments.

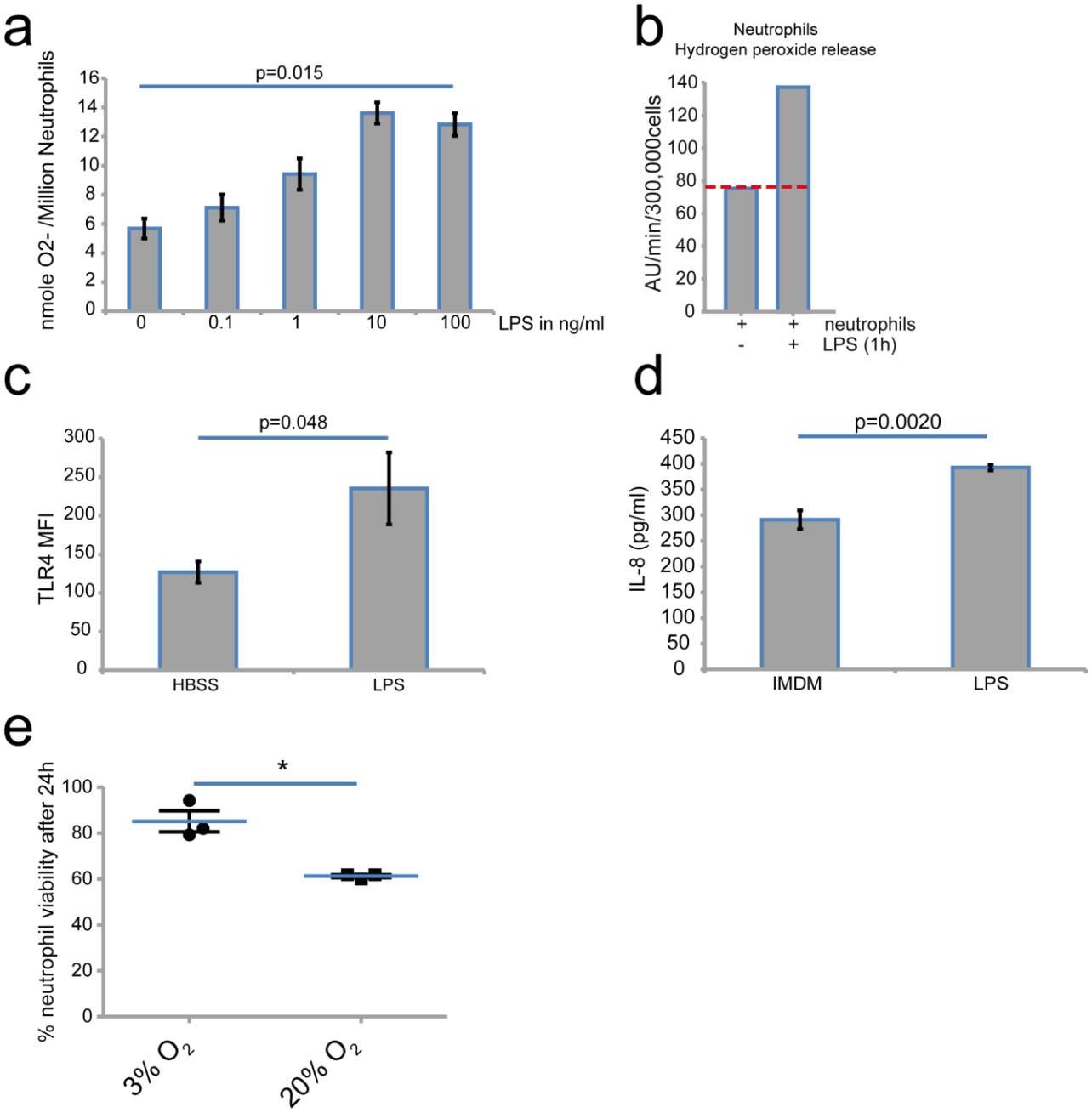


Figure 3.1 Neutrophils release superoxide and IL-8 upon LPS exposure. In collaboration with Dr M-H Ruchaud-Sparagano.

(a) Neutrophils were left to adhere into 24-well plates for 30 min before exposure to various concentrations of LPS. Cells were then stimulated with autologous serum-opsonised zymosan in the presence of cytochrome c and the superoxide dismutase-inhibitable reduction of cytochrome c was then determined as described earlier. Values are mean±SEM from seven separate experiments. *P<0.05 using Tukey's multiple comparison test. (b) Graphs show means release of H_2O_2 using Amplex red, au/min/300 000 neutrophils (n=1) after LPS treatment for 1h at 37°C. (c) Freshly isolated neutrophils were preincubated with LPS (100 ng ml−1) before labelling with PE-anti-TLR-4 antibodies or PE-isotype control antibodies. Gating was

performed using side and forward scatters. Mean fluorescence intensity (MFI) obtained with each isotype control was subtracted from the mean fluorescence intensity obtained with each corresponding antibody. Results are expressed as net mean fluorescence intensity (MFI) from four independent experiments, each performed in triplicates, with error bars representing SEM. *P<0.05 by Tukey's analysis. (d) Undiluted cell supernatants were taken after incubation of neutrophils with LPS (100 ng ml−1) for 20 h and the amount of IL-8 was measured by ELISA. Values are mean±SEM of five independent experiments. Statistical test performed using Tukey's multiple comparison test. (e) Mean number ± SEM of % neutrophils viability cultured at 3% O_2 an 20% O_2 for 24h counted by trypan blue exclusion method (N=3).Statistical test performed using a two tailed test (*P ≤0.05).

3.2 Neutrophils induce a DDR in human fibroblasts

Inflammation is an active well-regulated mechanism which acts in host defences against infectious agents and injury. The inflammatory response is essential for the maintenance of tissue homeostasis and initiated by the release of specific molecular patterns which will result in recruitment of immune cells which subsequently infiltrate tissues (Nathan 2002). Neutrophils, which are part of the innate immunity, are among the first responders to be recruited in order to neutralise the deleterious agents and phagocyte cellular debris. Once the agents are eliminated, the resolution of inflammation is initiated by downgrading the pro-inflammatory factors, which results in a reduction to basal levels of infiltrating immune cells and in the occurrence of tissue remodelling (Serhan, Brain et al. 2007).

In order to kill pathogens, immune cells possess a range of "weapons", such as the production of ROS and potent proteases, which can damage and kill surrounding cells. For this reason, the inflammatory response is normally acute in order to protect the tissue against unwanted collateral side effects.

Indeed, using a mouse model of low-grade chronic inflammation, it has been shown that an elevated, chronic, inflammatory phenotype is detrimental, accelerating ageing (Jurk, Wilson et al. 2014). These mice (NF-κB1$^{-/-}$) develop telomere dysfunction, increased cellular senescence in a variety of tissues and have a decreased tissue regeneration, which importantly can be rescued by the anti-inflammatory drug ibuprofen. Besides, the authors have reported an increase of a plethora of pro-inflammatory cytokine such as IL-6

as well as recruitment of immune cells such as T-cells and neutrophils in the liver, lung and kidneys. Elevated inflammatory factors have been linked to sterile inflammation which results in increased recruitment of immune cells into the tissue without actual infection and is linked to many diseases such as gout, arthritis, pulmonary fibrosis, atherosclerosis and Alzheimer's (Rock, Latz et al. 2010).

In addition, we have shown using the same mouse model that upon injection of a carcinogen, neutrophils rapidly infiltrate the liver and promote the development of hepatocellular carcinoma by increasing ROS in hepatocytes and DNA damage (Wilson, Jurk et al. 2015).

Therefore, we sought to ask whether human neutrophils could induce DNA damage in cells and in the long-run contribute to premature cell senescence. Hence, I designed an experiment using young fibroblasts and human neutrophils freshly isolated from the blood of middle-aged healthy volunteers.

Young MRC5 fibroblasts were co-cultured in direct contact with neutrophils for 24 hours in hypoxic conditions (3% O_2) using the ratio 1:5 (1 fibroblast for 5 neutrophils). The reason behind keeping neutrophils at 3% O_2 is because i) 3% O_2 is an oxygen tension closer to physiological oxygen levels *in vivo*; ii) it has been shown that neutrophil viability is enhanced for longer period of time compared to normoxic conditions (Sharon, Kathryn et al. 2000, Walmsley, Print et al. 2005). 3%O_2 has been described to decrease significantly neutrophil apoptosis after 20h in culture (Sharon, Kathryn et al. 2000, Walmsley, Print et al. 2005). In order to mimic what happens physiologically, prior to the start of the co-culture, neutrophils were primed with LPS (100 ng/mL) for 1 hour at 37°C. Priming neutrophils is known to enhance the respiratory burst in response to a second stimulus such as the formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol myristate acetate (PMA) (Guthrie, McPhail et al. 1984). Finally, the rationale behind using a 1:5 ratio is because we observed that clusters of 4-7 neutrophils could be detected surrounding individual hepatocytes in the liver of aged mice and after LPS injection (see chapter 4). Also, previous work examining the role of neutrophils in elimination of cancer cells had used similar ratios (Yan, Kloecker et al. 2014).

I first observed a significant increase in 53BP1 foci, a marker of the DDR associated with double stranded breaks in MRC5 fibroblasts following neutrophil co-culture when compared to the controls (Figure 3.2 a,b,d).

Then, to evaluate if the presence of neutrophils impacted on cellular proliferation of fibroblasts, I immunostained the fibroblasts with the proliferation marker Ki67 (Figure 3.2 a,c,d). Results show that, upon co-culture there is no significant difference in the % of Ki67 positive cells when compared to the controls (Figure 3.2 a,c,d). This suggests that neutrophils do not impact on fibroblast proliferation.

Previous work from our lab has shown that senescence is characterised by persistent DNA damage foci (Passos, Nelson et al. 2010). Live-cell imaging analysis of senescent MRC5 fibroblasts expressing a reporter for DDR protein 53BP1 has shown that at least 50% of foci are longed-lived lasting for more than eight hours. In order to determine whether neutrophils can impact on focus lifespan, I conducted under the same conditions as previously described, live-cell imaging using MRC5 cells expressing the fusion protein pG-Ac-53BP1c-mcherry which allows us to track the formation of DNA damage foci over time (Hewitt, Jurk et al. 2012).

I found that human fibroblasts which had been cultured with neutrophils for 24h (with 1h LPS priming) show a significant increase in focus lifespan per cell per hour when compared to MRC5 cells alone (Figure 3.2 e,f). These results suggest that an unknown factor potentially secreted by the neutrophils in enhancing the formation rate of DDR foci.

In summary, these results indicate that 24h co-culture with neutrophils is sufficient to induce a DNA damage response in young fibroblasts, with an increased formation rate. However, this small increase in DDR does not seem sufficient to induce a cell-cycle arrest- since there was no significant difference in the % of Ki67 positive cells.

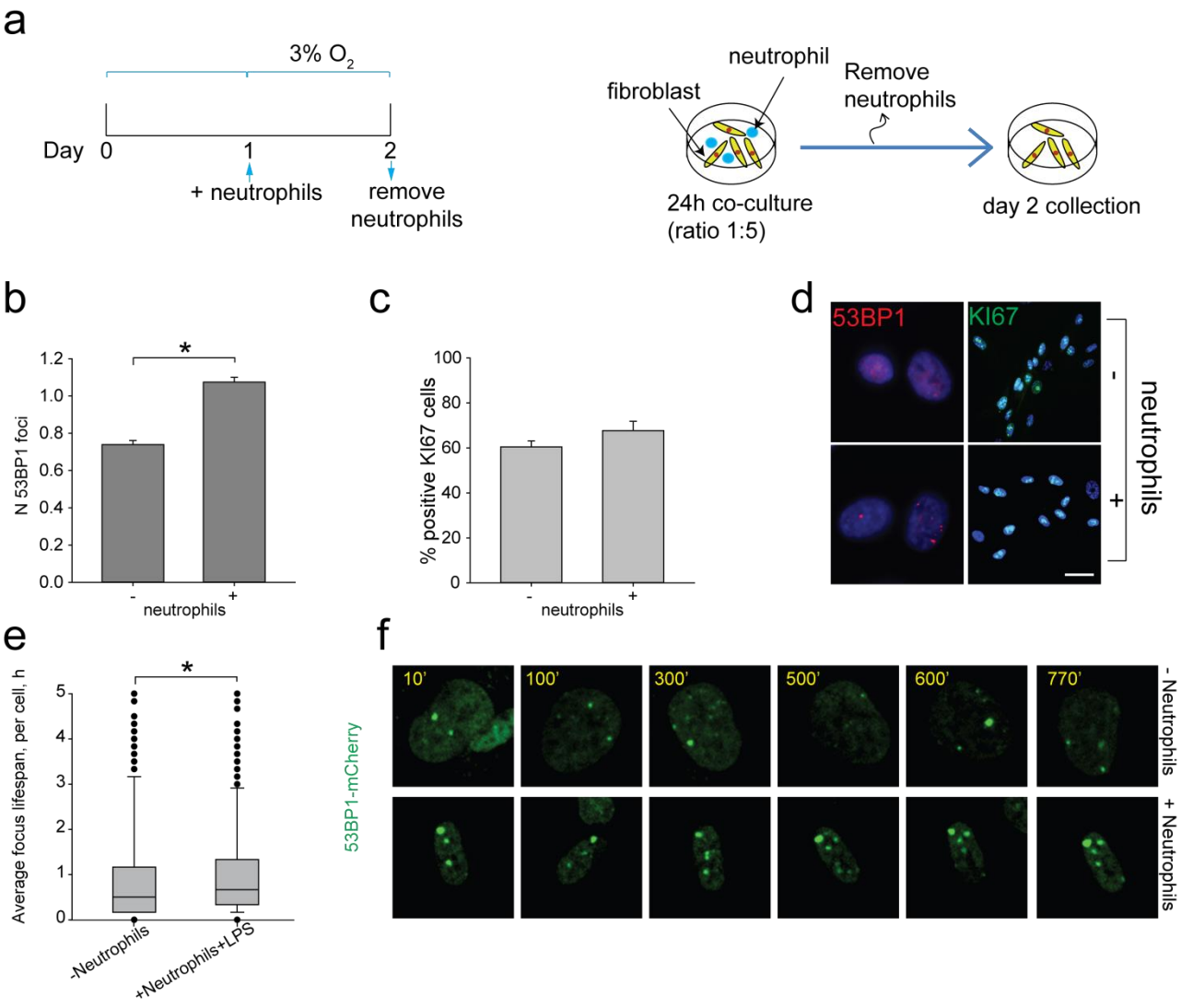


Figure 3.2 Neutrophils induce a DDR in human fibroblasts.

Young MRC5 cells were co- cultured in vitro for 24h at 3% 0_2 with isolated human neutrophils from young healthy volunteer (average age 40). (a) Representation of direct co-culture between human fibroblasts and neutrophils. (b) Mean number ± SEM of 53BP1 foci per nucleus of control and co-culture (*=P ≤0.001 using a two-tailed t-test; N=3). (c) Percentage of Ki-67 positive nuclei in human fibroblasts 24h after co-culture with neutrophils, data are mean ± standard deviation;10 images taken from random planes.; N=1. (d) Representative images of 53BP1 400x (nucleus: DAPi, 53BP1: red) and Ki-67 200x (nucleus: DAPi, Ki67: green; images are from a single Z plane). (e) Mean number ± SEM of 53BP1 foci formation rates in bystander MRC5-mcherry- 53BP1 cells. MRC5 were cultured alone (control) or with primed neutrophils with LPS (1h) (co-culture) for 24 hours prior to imaging. Box plots indicate median, upper and lower quartiles (boxes), upper and lower centiles (whiskers) and outliers (dots). Significant difference between co-culture and control is (*=P ≤0.001, N=22 for control and N=36 for co-culture). (f) Representative images of MRC5-mcherry- 53BP1 reporter cells fluorescence in young MRC5 cell nucleus, control are above and co-culture cells are in the bottom. Time is expressed in minutes. Images are compressed z stacks over 4.5 µm to capture the entire nuclear volume. Statistical analysis performed using One Way ANOVA or two tailed test.

3.3 Extracellular hydrogen peroxide impacts on neutrophil induced DDR

Since neutrophils are able to produce superoxide and H_2O_2 following appropriate activation, mostly driven by NADPH oxidase (Robinson 2008), we decided to test whether ROS are involved in the induction of the DDR observed previously.

To test this hypothesis, I performed a direct co-culture of human fibroblasts and isolated neutrophils for 24h in the presence of a recombinant catalase (an extra-cellular H_2O_2 scavenger) (Figure 3.3 a).

I found that catalase is able to completely rescue the increase in 53BP1 in human fibroblasts co-cultured with primed neutrophils (Figure 3.3 a,b,d). Then, to evaluate if co-culture impacted on cellular proliferation, I conducted immunofluorescence for the proliferation marker Ki67 (Figure 3.3 a,c,e). Preliminary data shows no effects of neutrophils on % of Ki67, but surprisingly, a slight decrease in the % of Ki67 positive cells upon treatment with catalase; however, this will have to be confirmed independently since only 1 experiment was performed.

To sum up, these results suggest that the extracellular ROS produced by neutrophils contributes to the DDR observed in young fibroblasts. However, it is still unclear whether catalase is quenching extracellular ROS generated by neutrophils, or if it is having other effects in fibroblasts.

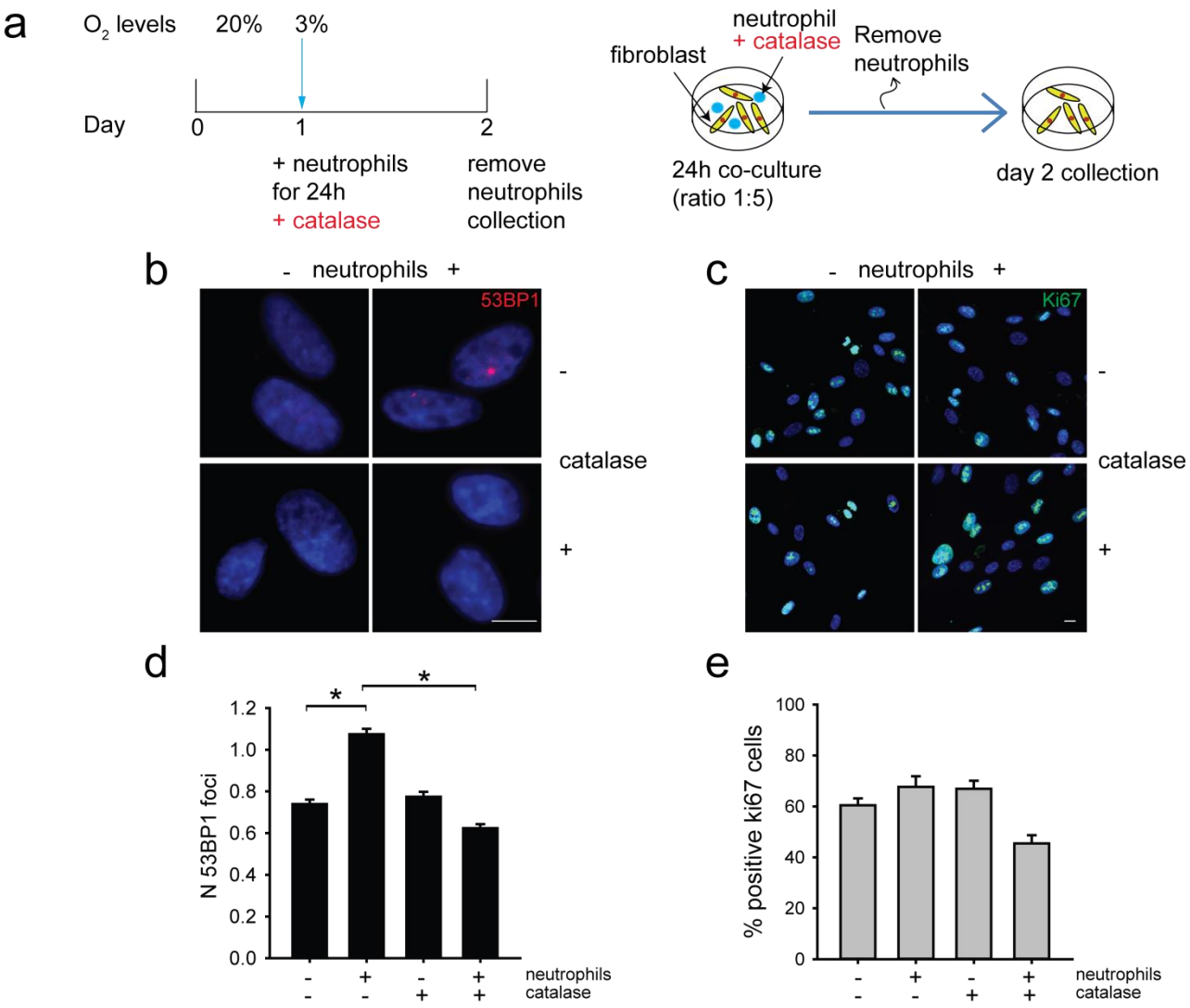


Figure 3.3 ROS may impact on human neutrophil induced DDR on fibroblasts.

Young MRC5 cells were co- cultured in vitro for 24h at 3% 0_2 with isolated human neutrophils from young healthy volunteer (average age 40). (a) Representation of direct co-culture between human fibroblasts and neutrophils using extracellular catalase (100 UI/ml) for 24h. (b) Representative images of 53BP1 400x following 24h co-culture using catalase (nucleus: DAPi, 53BP1: red). (c) Representative images of Ki67 200x following 24h co-culture using catalase (nucleus: DAPi, Ki67: green; images are from a single Z plane). (d) Mean number ± SEM of 53BP1 foci per nucleus of young MRC5 control, co-cultured, control + catalase and co-cultured +catalase (*=P ≤0.001; N=3, Mann-Whitney Rank Sum Test). (e) Percentage of Ki-67 positive nuclei in human fibroblasts 24h after co-culture with neutrophils and catalase (100UI/ml), data are mean ± SD of 10 planes; N=1 (variation comes from 3 technical repeats). Scale bar represent 10 µm.

3.4 Neutrophil elastase contributes to a DDR

Another possible contributor to the neutrophil-mediated bystander effects is the secretion of proteases, particularly neutrophil elastase (NE). Evidence has shown that NE is linked to fibrosis and contributes to extracellular matrix damage in skin (Chua, Dunsmore et al. 2007, Takeuchi, Gomi et al. 2010). Furthermore, studies in lungs have implicated NR in the pathophysiology of acute lung injury, since it can destroy a large variety of extracellular matrix and plasma proteins which can be attenuated by using the inhibitor sivelestat (Kawabata, Hagio et al. 2002).

In order to test if neutrophil elastase could be involved in the induction of DDR in human fibroblasts, I exposed early PD human MRC5 fibroblasts with isolated human neutrophils in the presence or absence of 40nM NE inhibitor sivelestat (Figure 3.4 a) (Kazuhito, Mayumi et al. 1991).

I observed that treatment with the neutrophil elastase inhibitor during the co-culture reduced the number of 53BP1 foci when compared to the co-culture without treatment. (Figure 3.4 b, d) Then, to evaluate if these co-culture conditions impacted on cellular proliferation, I conducted immunofluorescence against proliferation marker Ki-67 (Figure 3.4 c, e). Results indicate that upon co-culture with neutrophils there is no difference in the % of Ki67 positive cells when compared to the controls.

Interestingly, our results indicate that other mechanisms apart from ROS released by the neutrophils could be involved in the generation of DNA damage in young fibroblasts. However, our results are limited, since I did not evaluate if the inhibitor is impacting on the activity of NE, or if NE is being secreted by neutrophils in these conditions. Furthermore, it is possible that the inhibitor is having effects on ROS generation by neutrophils. These hypotheses will have to be experimentally tested before any definitive conclusions can be reached.

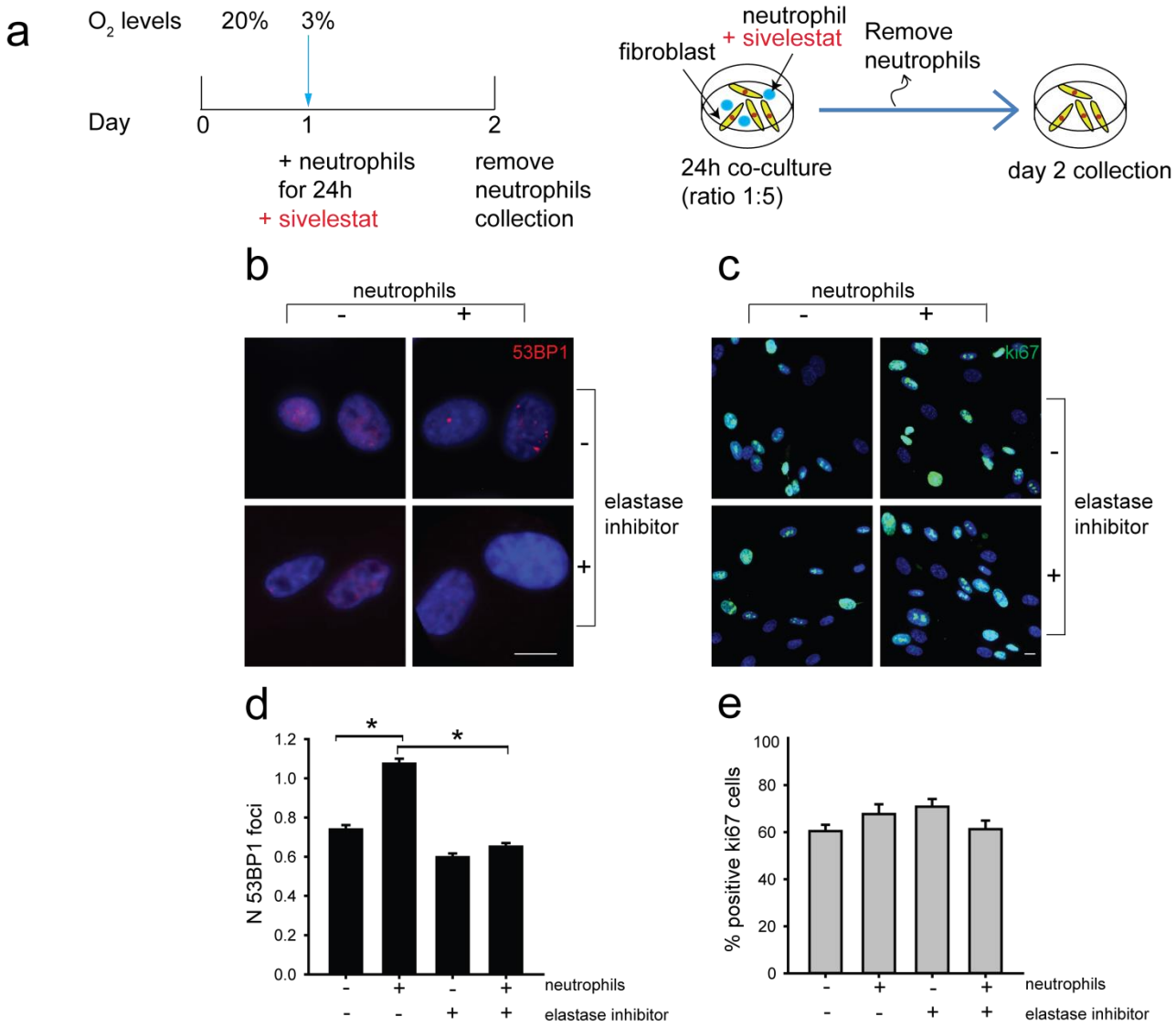


Figure 3.4 Human neutrophil elastase contributes to the DDR.

Young MRC5 cells were co- cultured in vitro for 24h at 3% 0_2 with isolated human neutrophils from young healthy volunteer (average age 40) (a) Scheme of fibroblasts + 40nM per well elastase inhibitor (sivelestat) co-cultured in direct contact with neutrophils. (b) Representative images of 53BP1 400x following 24h co-culture using catalase (nucleus: DAPI, 53BP1: red). (c) Representative images of Ki67 200x following 24h co-culture using catalase (nucleus: DAPI, Ki67: green; images are from a single Z plane). (d) Mean number ± SEM of 53BP1 foci per nucleus of young MRC5 control, co-cultured, control + sivelestat and co-cultured +sivelestat (*=P ≤0.001; N=3, Mann-Whitney Rank Sum Test). (e) Percentage of Ki-67 positive nuclei in human fibroblasts 24h after co-culture with neutrophils and sivelestat, data are mean ± SD of 10 planes; N=1 (variation comes from 3 technical repeats). Scale bar represent 10 µm.

3.5 Co-culture with MRC5 and neutrophils for 3 days is sufficient to induce premature senescence

Previous data has shown that direct co-culture of MRC5 fibroblasts with activated neutrophils lead to increased 53BP1 foci and a small but significant increase in 53BP1 formation rate without affecting cell proliferation. This led me to speculate whether neutrophil-induced DNA damage would be sufficient, at later stages, to induce premature senescence. Previous data indicated that upon injection of carbon tetrachloride (CCl_4), which induces liver damage and subsequent neutrophil infiltration, neutrophils are usually cleared from tissues 72h after acute damage (Moles, Murphy et al. 2014). For that reason, I decided to mimic these conditions *in vitro* by co-culturing fibroblasts with neutrophils for 3 consecutive days. To avoid neutrophil cell-death (which occurs in culture conditions after 24h), I replaced neutrophils every day with freshly isolated neutrophils from similarly middle-aged healthy donors. Furthermore, in order to better maintain neutrophil viability, I cultured neutrophils at 3% O_2. Finally, in order to test the role of ROS in the process, neutrophils and fibroblasts were co-cultured in the presence or absence of extracellular catalase.

After three days of co-culture the neutrophils were removed (and the extracellular catalase) and the MRC5 fibroblasts were cultured until replicative senescence at 20% O_2. At each cell passage cell numbers were recorded in order to calculate the population doublings (Figure 3.5 b).

Results from the growth curves show that upon co-culture with neutrophils (primed and non-primed), MRC5 fibroblasts enter senescence prematurely when compared to the untreated controls. However, when neutrophils were LPS-primed, the effects on the replicative lifespan were more pronounced (Figure 3.5 b,c). Importantly, pre-treatment with the enzyme catalase prevented this effect, suggesting that it is mediated via hydrogen peroxide release by the neutrophils (Figure 3.5 b,c).

Since these were independent cultures and cells were passaged at different time points, it was not feasible to determine the variability between experiments at each time point. However, I calculated the % of change in replicative lifespan for each experiment. Results

show that co-culture with neutrophils primed with LPS resulted in around 12% decrease in the replicative lifespan of human fibroblasts, which could be rescued by extracellular catalase. Non-primed neutrophils had a milder, albeit significant, effect (5%) (Figure 3,5c).

Since I used neutrophils from different middle-aged donors, I made sure that similarly aged donors were used in all the experiments (Figure 3.5 d, Table 1).

In summary, these data indicate that short-term co-culture with neutrophils for 3 days is able to reduce significantly the lifespan of MRC5 fibroblasts in a ROS dependent manner.

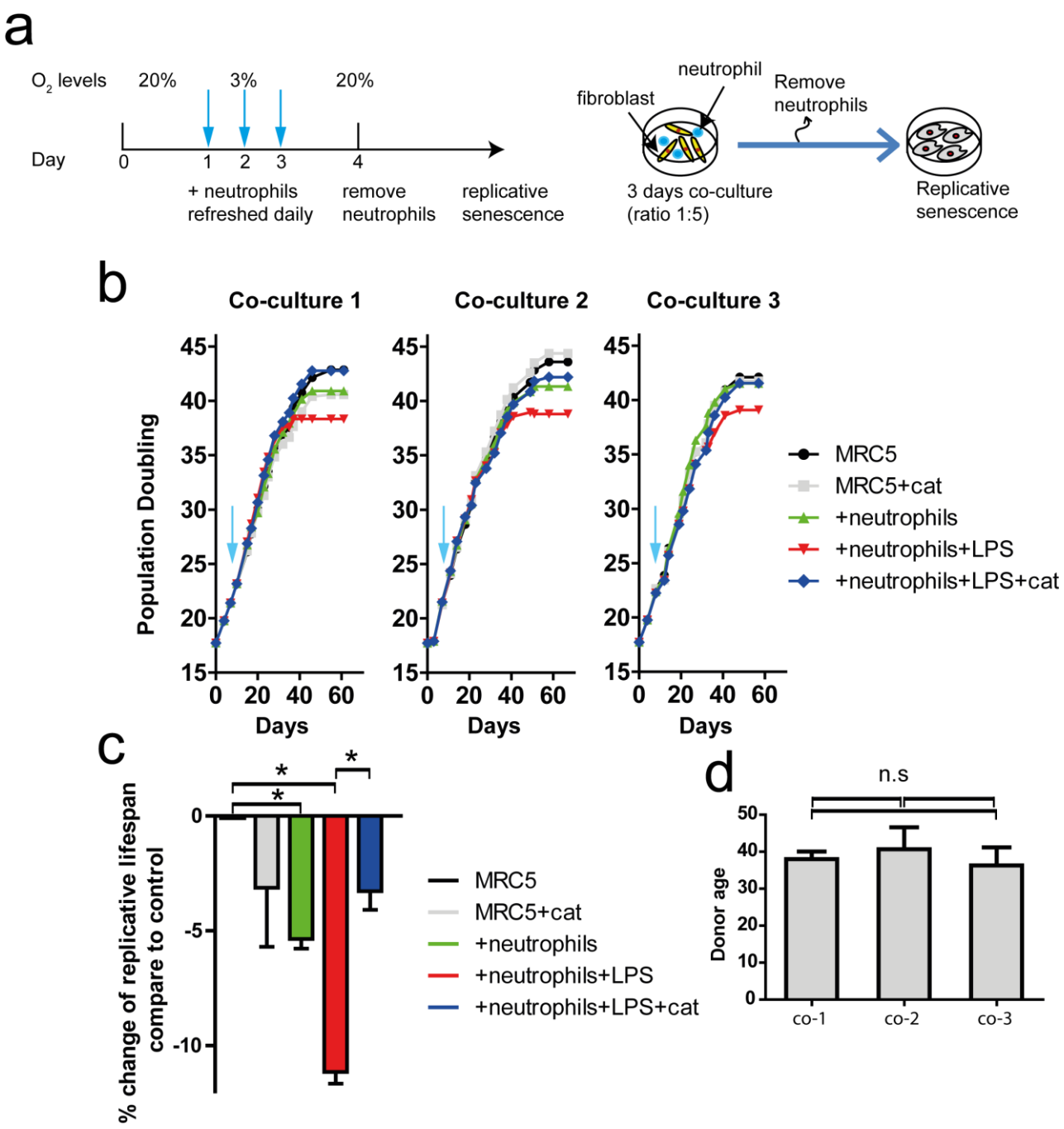


Figure 3.5 Co-culture of human neutrophils and fibroblast for 3 days is sufficient to induce premature senescence in fibroblasts in a ROS-dependent manner.

(a) Representation of in vitro direct co-culture between young MRC5 with fresh isolated 1h LPS primed human neutrophils replaced every 24h from different middle-aged donor for 3 days at 3% 0_2 with or without catalase, then the neutrophils and the catalase were removed and MRC5 fibroblasts cells were cultured until replicative senescence at 20% 0^2. (b) MRC5 Fibroblasts were expanded until the cells reached replicative senescence with different conditions: black line represents control cells, grey line represents control cells + catalase (100UI/ml), green line represents fibroblasts treated with nonprimed neutrophils, red line represents the fibroblasts treated with neutrophils activated for 1h with 100ng/ml LPS, blue line represents the fibroblasts treated with neutrophils activated for 1h with 100ng/ml LPS + catalase (100UI/ml) for 72h. Cumulative population doubling curves of each independent experiment is represented. Each cell

passage is indicated by a point with the corresponding number of cumulative population doubling (N=3). Blue arrow indicates the last day of the co-culture (day 3). (c) Graph expressed in % change of replicative lifespan normalised to the control (control are considered 0) using the last population doubling point for each condition, colour legend is indicated on the left side of the graph. Statistical analysis performed using One Way ANOVA; *P<0.05. (d) Graph represent the mean of the 3 different donors age for each independent co-culture (co-); Statistical analysis performed using One Way ANOVA; n.s (non-significant) P>0.05.

Date	Gender	Age	Neutrophil purity (%)	co-culture
16/02/2016	f	42	98.1	1
17/02/2016	f	35	98.6	1
18/02/2016	f	37	97.3	1
09/02/2016	f	38	98.5	2
10/02/2016	m	52	96.3	2
11/02/2016	m	32	97.9	2
02/02/2016	f	32	97.4	3
03/02/2016	f	46	96.8	3
04/02/2016	f	31	97	3

Table 6 Neutrophil quality after extraction from the venous blood from middle-aged healthy donors

3.6 Senescent markers are upregulated after short-term neutrophil exposure

In order to confirm that neutrophils can induce premature senescence in MRC5 fibroblasts I used well-described markers to characterise the phenotype (Lawless, Wang et al. 2010).

In accordance with the growth curves, MRC5 fibroblasts that were co-cultured with neutrophils experienced a significant decrease in the proliferation marker (Ki-67) at 22 days (Figure 3.6 a,b) . This was observed both with primed neutrophils as well as non-primed neutrophils. Importantly, the phenotype was rescued to the level of controls when catalase was added (Figure 3.6 a,b).

Next, I performed senescence-associated β galactosidase activity (Sen-β-Gal) at pH 6 staining at several time points (8, 15 and 22 days) in MRC5 fibroblasts after co-culture. Data shows a tendency for an increase 15 days after co-culture and a significant increase 22 days after co-culture with neutrophils primed with LPS (Figure 3.6 a,c). Importantly, catalase significantly reduced the effect (Figure 3.6 a,c).

Increased ROS is a hallmark of cellular senescence (Macip, Igarashi et al. 2002, Macip, Igarashi et al. 2003, Passos, Saretzki et al. 2007, Passos, Nelson et al. 2010). In order to determine the impact of neutrophils on ROS generation in MRC5 fibroblasts, I measured ROS using flow cytometry in MRC5 fibroblasts 22 days after co-culture using ROS-indicator probes MitoSOX and DHE. Both fluorescent probes detect superoxide anions in living cells; however the MitoSOX probe detects superoxide within the mitochondrial matrix. MitoSOX data indicates that three days co-culture with LPS primed neutrophils leads to a significant increase in ROS production of MRC5 fibroblasts (Figure 3.6 d). To note, there is a tendency for an increase with un-primed neutrophils when compared to the controls (Figure 3.6 d) and catalase treatment significantly rescues the increase. DHE measurements indicate a tendency for an increase in ROS production in both conditions where fibroblasts were co-cultured with primed or un-primed neutrophils. Interestingly, using DHE measurement, catalase was also found to significantly decrease the level of ROS when compared to three days co-culture with primed neutrophils (Figure 3.6 e). The differences between dyes may be a reflection of the fact that ROS in senescent MRC5

fibroblasts has been shown to be of mitochondrial origin (Correia-Melo, Marques et al. 2016).

Altogether, these results suggest that the hydrogen peroxide released by neutrophils during the three days of co-culture is sufficient to induce senescent markers at later time points.

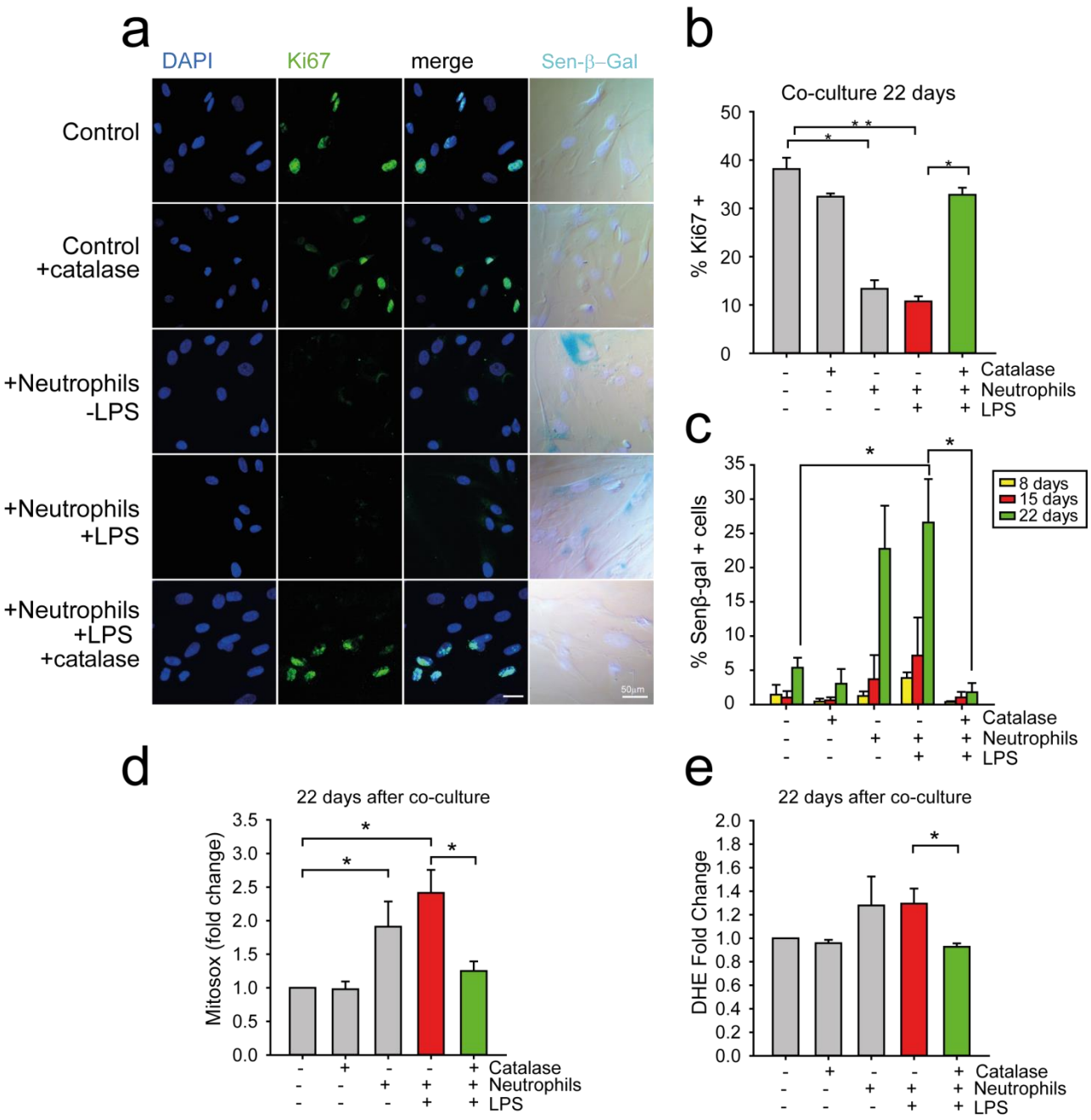


Figure 3.6 Senescent markers are upregulated at 21 days post-culture with neutrophils.

(a) (Right) Representative images of Ki-67 staining in non-treated MRC5 fibroblasts and MRC5 fibroblasts +catalase, MRC5 fibroblasts +neutrophils –LPS, MRC5 fibroblasts + neutrophils +LPS, MRC5 fibroblasts + neutrophils +LPS +catalase 22 days post co-culture. Scale bar: 10 µm (blue:DAPI ; green : Ki67). (Left) Representative images of Sen-β-Gal staining 22 days after co-culture MRC5 fibroblasts +/- neutrophils +/- LPS +/- catalase (light blue-DAPI; darker cytoplasmic blue- Sen-β-Gal) Scale bar: 50 µm. (b) Graph represents % Ki67-positive nuclei for each condition 22 days after co-culture, data are mean±s.e.m (n=3, One Way ANOVA; *=P<0.001). (c) Quantification of mean number of Sen-β-Gal-positive cells (%) for all conditions 8, 15 and 22 days after co-culture, data are mean±s.e.m. (n=3, One Way ANOVA; *=P<0.03). (d,e) MitoSOX and DHE ratio intensities 22 days post co-culture in MRC5 fibroblasts for all conditions

measured by FACS (fold change compare to control (MRC5 non-treated)), mean ± s.e.m., (n=3, One Way ANOVA; *=P<0.02.and using a two-tailed t-test for DHE; N=3*=P ≤0.05).

3.7 Neutrophil co-culture induces DNA damage, TAF and telomere shortening after 8 days in human fibroblasts

Telomere dysfunction, characterised by co-localisation of DDR proteins and telomeres is a feature of cellular senescence (d'Adda di Fagagna, Reaper et al. 2003). Importantly, data indicates that contrary to genomic DNA damage, telomeric DNA damage can't be easily repaired and induces a persistent DDR which can contribute to cellular senescence (Hewitt, Jurk et al. 2012).

In order to investigate if neutrophil co-culture accelerated telomere dysfunction, I analysed by Immuno-FISH co-localisation between DNA damage response protein γH2A.X and telomeres.

I found that human fibroblasts which had been cultured with neutrophils (both with and without LPS priming) show significantly increased telomere-associated foci (TAF) when compared to untreated controls (Figure 3.7 a,b,d). Importantly, pre-treatment with the enzyme catalase prevented this effect, suggesting that the effect is mediated via hydrogen peroxide release (Figure 3.7 a,b,d). Similarly, total DNA damage foci and DNA breaks measured by alkaline COMET assay increased in fibroblasts which had been co-cultured with neutrophils and the effect was rescued by catalase (Figure 3.7 c,e).

In summary, these results suggest that hydrogen peroxide released by neutrophils contributes to general DNA damage, but also to telomere dysfunction.

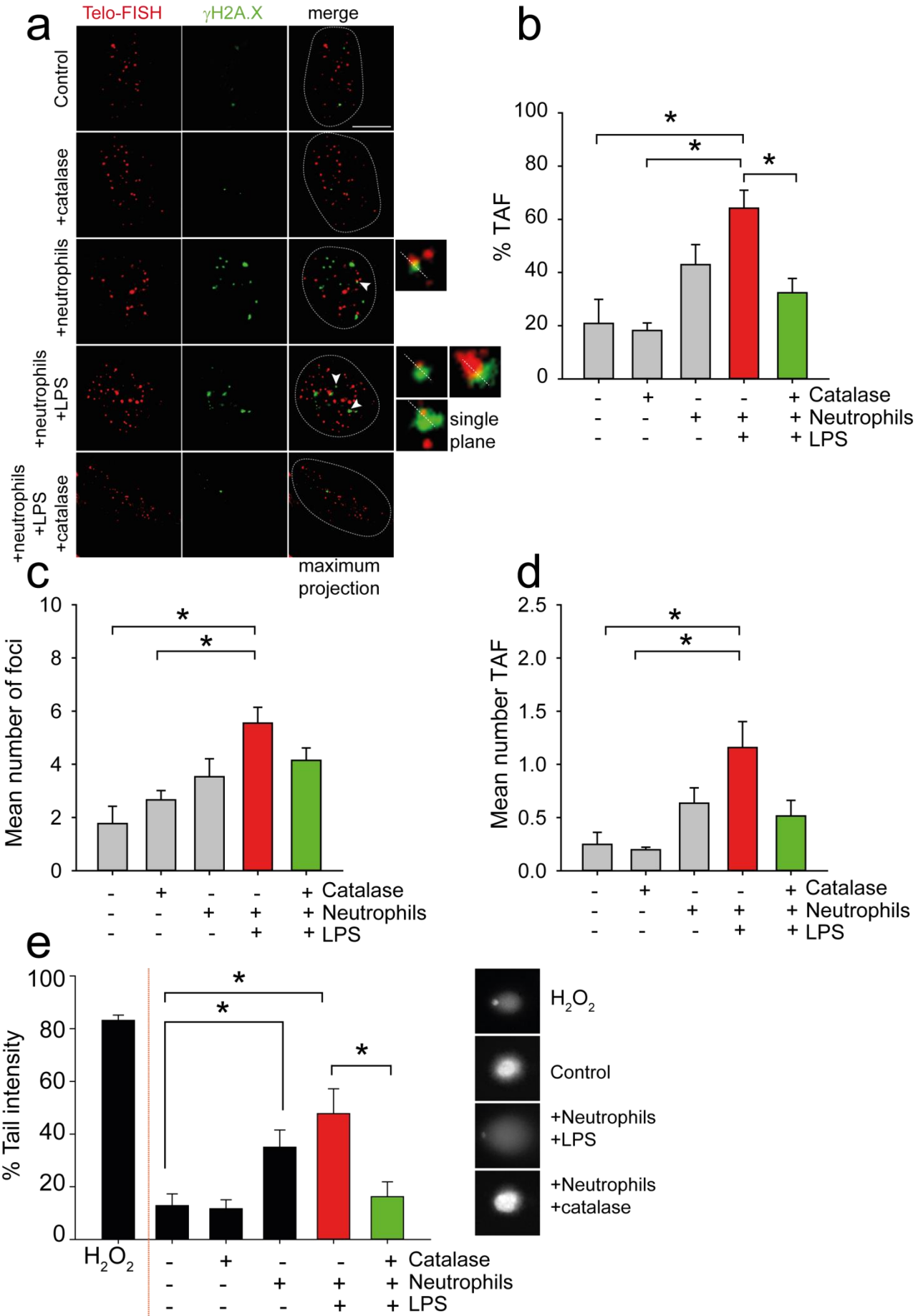

a
Telo-FISH
γH2A.X
merge
Control
+catalase
+neutrophils
+neutrophils +LPS
+neutrophils +LPS +catalase
single plane
maximum projection
b
% TAF
100
80
60
40
20
0
*
*
*
+ Catalase
+ Neutrophils
+ LPS
c
Mean number of foci
10
8
6
4
2
0
*
*
+ Catalase
+ Neutrophils
+ LPS
d
Mean number TAF
2.5
2.0
1.5
1.0
0.5
0.0
*
*
+ Catalase
+ Neutrophils
+ LPS
e
% Tail intensity
100
80
60
40
20
0
*
*
*
H2O2
+ Catalase
+ Neutrophils
+ LPS
H2O2
Control
+Neutrophils +LPS
+Neutrophils +catalase

Figure 3.7 Co-culture of human neutrophils and fibroblasts for 3 days is sufficient to induce DNA damage at telomeres in fibroblasts in a ROS-dependent manner.

(a) Representative images of γH2A.X immuno-FISH in MRC5 fibroblasts 8 days after co-culture with different treatments. Images are Huygens (SVI) deconvolved Z projections of 10-μm stacks taken with a ×63 oil objective. White arrows indicate colocalization, and colocalizing foci are amplified in the right panel (amplified images are from single Z planes where colocalization was found). Scale bar=10 μm (red: telomeres, green: γH2A.X). (b) Percentage of γH2A.X foci colocalizing with telomeres (%TAF) in MRC5 fibroblasts 8 day post co-culture. (c)Total number of γH2A.X foci in human fibroblasts 8 day post co-culture. (d) Total number of γH2A.X foci colocalizing with telomeres (TAF) in MRC5 fibroblasts 8 day post co-culture. (e) Alkaline comet assay results of different parameters 8 days after co-culture. Graph shows % tail intensity, MRC5 treated for 1h with H_2O_2 was used as an internal control. (f) Representative images of comets for each condition. Data are mean ± SEM of n=3. Statistical analysis performed using One Way ANOVA, * P<0.05.

3.8 Neutrophil co-culture induces accelerated telomere shortening in fibroblasts

Previous experiments have shown that fibroblasts co-cultured with neutrophils induce telomeric and non-telomeric DNA damage in fibroblasts. However, it is still unclear whether damage at telomeres is a contributor to cellular senescence in this particular experimental setting. In order to further investigate the role of telomeres in neutrophil-induced senescence, I co-cultured neutrophils and early passage human MRC5 fibroblasts as previously described. I collected cells at different time points following recovery and generated metaphase spreads followed by telomere FISH (Figure 3.8 a). This method allowed me to accurately measure individual telomere length of cells at different points in time.

My results show that co-culture of neutrophils with human fibroblasts accelerates the rate of telomere shortening between days 4 and 16, which could be prevented by the action of catalase (Figure 3.8 b,c,d,e).

These results support a model by which short-term exposure to neutrophils generate single-stranded breaks via ROS in young fibroblasts, which upon cell division results in a accelerate telomere loss ultimately resulting in premature senescence. Similar effects

were shown by others upon exposure to mild-stress (von Zglinicki, Saretzki et al. 1995, Petersen, Saretzki et al. 1998).

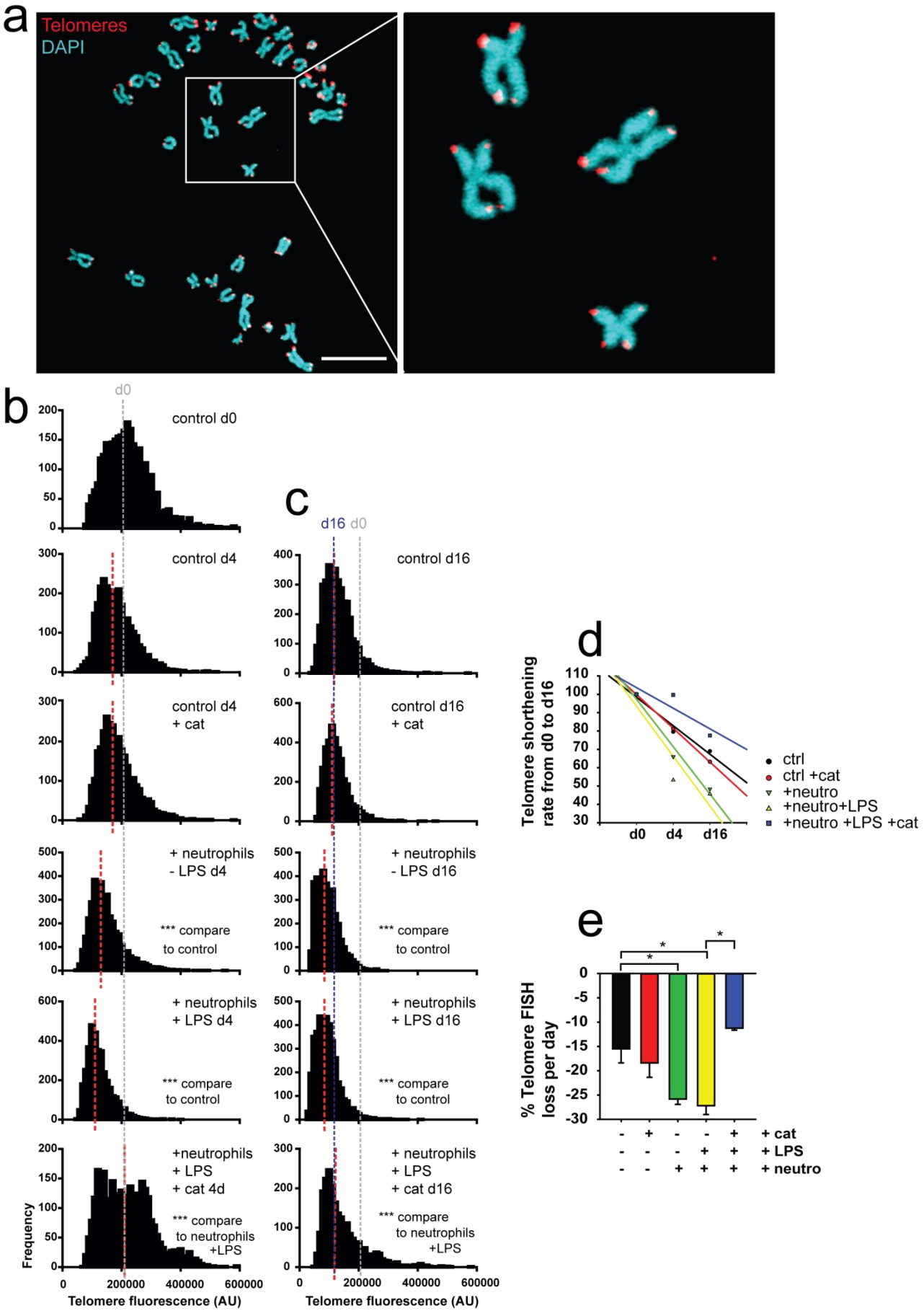

a
Telomeres
DAPI
b
c
d
e
d0
d16
control d0
control d4
control d16
control d4 + cat
control d16 + cat
+ neutrophils - LPS d4
+ neutrophils - LPS d16
+ neutrophils + LPS d4
+ neutrophils + LPS d16
+neutrophils + LPS + cat 4d
+ neutrophils + LPS + cat d16
*** compare to control
*** compare to neutrophils +LPS
Frequency
Telomere fluorescence (AU)
Telomere shortening rate from d0 to d16
ctrl
ctrl +cat
+neutro
+neutro+LPS
+neutro +LPS +cat
% Telomere FISH loss per day
+ cat
+ LPS
+ neutro

Figure 3.8 Co-culture of human neutrophils and fibroblasts for 3 days induces telomere shortening in fibroblasts in a ROS-dependent manner.

(a) Representative images of metaphase spreads hybridized with telomeric immuno-FISH at d0 from MRC5 fibroblasts, images are Z projections of 5-µm stacks taken with a ×100 oil objective. Scale bar=10 µm (red: telomeres, blue: DAPI). (b) Histograms represent individual telomere FISH intensity measured by Image J for each condition 4 day after co-culture; day 0 control (before co-culture) is indicated on the top with a grey dotted line representing the median > 100 telomeres per condition (baseline). Red dotted lines represent median > 100 telomeres per condition. (c) Histograms represent telomere intensity for each condition 16 day after co-culture. Red dotted lines represent median > 100 telomeres per condition; grey dotted line represents median at day 0, blue dotted line represents the median of the control at day 16. Histograms expressed in frequencies of events of telomere fluorescence (AU: arbitrary unit). (d) Graph represents the telomere shortening rate from d0 to d16 for each condition, each line correspond to the linear regression of the medians normalised to the control at day 0 (considered at 100%). (e) Graph represents the % of telomere FISH signal loss per day calculated from the linear equation of each condition (Y=ax+b). Statistical analysis performed using Mann-Whitney tests for the histograms show significant difference in telomere intensity between control and co-culture with neutrophils + and – LPS, and co-culture with neutrophils +LPS and co-culture with neutrophils +LPS +catalase ($P<0.05$) (n=3). Statistical analysis performed using One Way ANOVA, * $P<0.050$.

3.9 Expression of hTERT is able to counteract neutrophil-induced premature senescence

In order to establish if telomere shortening was playing a causal role in the process, I used MRC5 fibroblasts expressing the catalytic subunit of the enzyme telomerase (hTERT), which have a relatively constant telomere lengths irrespectively of cell division (Ahmed, Passos et al. 2008).

In these cells, co-culture with neutrophils as previously described, did not result in any changes in population doublings over a period of 60 days (Figure 3.9 a,b). Interestingly, the results were the same independently of the PD of the hTERT expressing MRC5 fibroblasts (which ranged from PD60-160). Thus, in these particular settings, neutrophils were not able to induce premature senescence in hTERT MRC5 fibroblasts. This indicates that neutrophil-induced senescence seems to be telomere dependent.

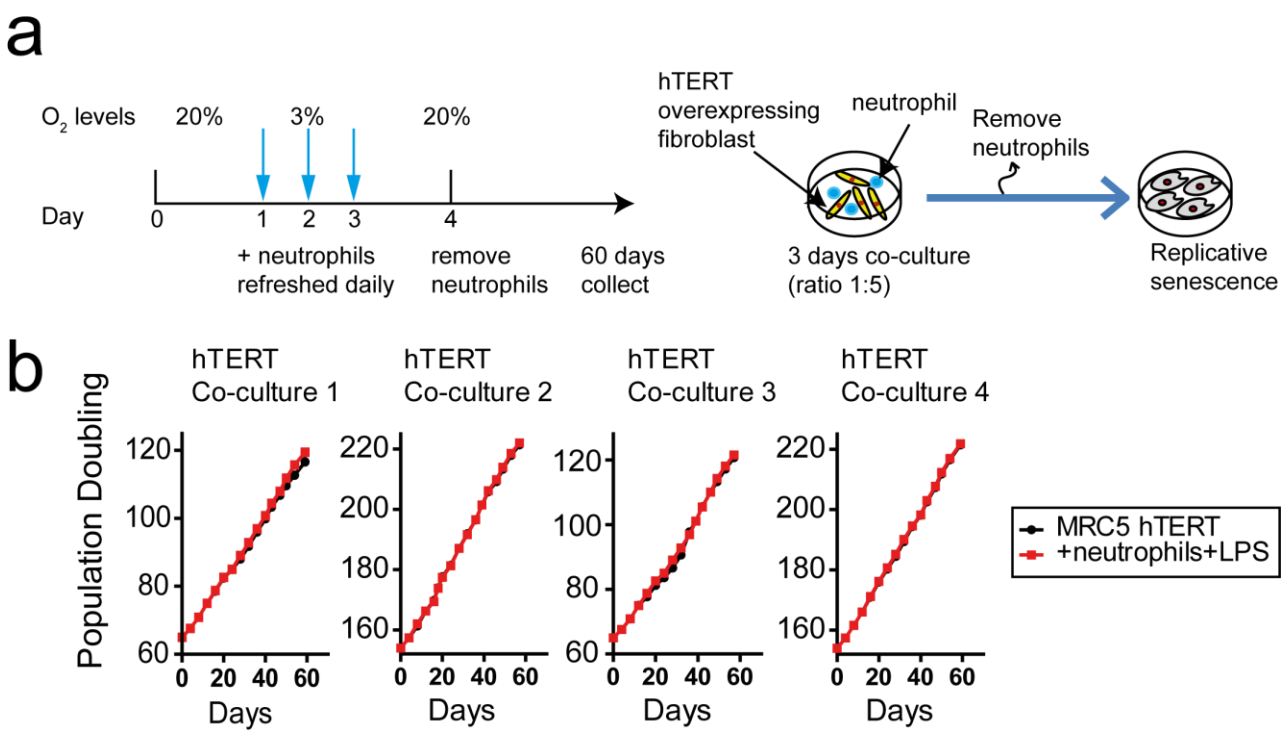


Figure 3.9 Co-culture of human neutrophils and fibroblasts ectopically expressing hTERT does not induce premature senescence.

(a) Representation of in vitro direct co-culture between young MRC5 with fresh isolated human neutrophils replaced every 24h for 3 days at 3% 0_2, then the neutrophils were removed and hTERT MRC5 were cultured for 65 days at 20% 0_2. (b) MRC5 Fibroblasts were expanded until the cells reached the same length of culture as in the conditions with neutrophils (65 days) with 2 different conditions: black line represents the control cells (untreated); red line represents the fibroblasts treated with neutrophils activated for 1h with 100ng/ml LPS for 72h (replaced every day). Each cell passage is indicated by a point and the number of cumulative population doubling (n=4).

3.10 Discussion

Acute inflammation is a well-orchestrated program which results in infiltration of different immune cells. Once inflammation is resolved, immune cells are phagocytosed and homeostasis is resumed (Serhan and Savill 2005).

Neutrophils are a type of polymorphonuclear infiltrating leucocyte which play a major role during infection and resolution of the inflammation via a role in the clearance of extracellular pathogens (Serhan, Chiang et al. 2008, Mayadas, Cullere et al. 2014). Meanwhile, this simple view has evolved, and there is evidence that neutrophils are implied in different functions such as activation and regulation of innate and adaptive immunity via the secretion of a broad repertoire of cytokines (Mantovani, Cassatella et al. 2011). Neutrophils have been shown to be involved in the resolution of inflammation via production of pro-resolving lipid mediators such as leukotriene B4 (LTB4) and resolvins (Serhan, Chiang et al. 2008). In addition, neutrophils are important players in chronic inflammation occurring in the lungs and have been implicated in the persistence of chronic obstructive pulmonary disease (COPD) (Weathington, van Houwelingen et al. 2006). Moreover, neutrophils are involved in "sterile inflammation" which is a type of inflammation which occurs in the absence of infection. This response is part of the wound healing response, in which, upon tissue injury, neutrophils are recruited to clear debris accumulated during the process (Kono and Rock 2008).

Neutrophils, because of their primary functions in the fight against pathogens, contain pore forming molecules, hydrolytic, and oxidative compounds which can cause serious secondary damage to the microenvironment (Segal 2005). Long term inflammation state could lead to the development of chronic inflammation and later-on numerous disorders such as fibrosis and ultimately cancer (Rybinski, Franco-Barraza et al. 2014). Thus, excess recruitment of neutrophils to sites of sterile inflammation can be detrimental and contribute to various diseases (Imaeda, Watanabe et al. , Liu, Han et al. 2006)

Neutrophil infiltration has been shown to contribute to the development of various human cancers including colorectal, renal, gastric, ovarian and hepatocellular carcinoma (Dumitru, Lang et al. 2013, Wilson, Jurk et al. 2015). Furthermore, secretion of pro-inflammatory cytokines by tumour cells such as CXC-chemokines and notably IL-8, are powerful neutrophil chemoattractants (Inoue, Slaton et al. 2000, Venkatakrishnan, Salgia et al. 2000, Huang, Chen et al. 2015). Furthermore, recruitment of neutrophils by hepatocytes upon injury is responsible for the induction of genotoxic stress and telomere lesions in the microenvironment contributing to the development of hepatocellular carcinoma (Farazi, Glickman et al. 2003, Begus-Nahrmann, Hartmann et al. 2012).

While neutrophils have been shown to play a role in disease, not much is known about the role of neutrophils in the ageing process. Chronic inflammation has been shown to induce premature senescence via increased production of ROS and telomere dysfunction (Jurk, Wilson et al. 2014), however, mechanistically it is still unclear how inflammation results in ROS generation. One possibility, which I tested in this project, is that inflammation results in recruitment of neutrophils to tissues causing ROS-mediated telomere-dysfunction. Consistent with this hypothesis, my data shows that mild damage caused by neutrophils (within a period of 3 days) in young cells is sufficient to accelerate the rate of telomere shortening and induce premature senescence. Furthermore, expression of the catalytic subunit of telomerase can rescue the effect, indicating that telomeres are the limiting factor in neutrophil-induced cellular senescence.

My results are consistent with previous studies which have shown that activated neutrophils induce single-stranded breaks in a plamacytoma cancer cell line and induce DNA damage and oxidative DNA lesion 8-hydroxydeoxyguanosine (8-OHdG) in respiratory tract epithelial cells, in a ROS dependent manner both *in vitro* and *in vivo* (Shacter, Beecham et al. 1988, Knaapen, Schins et al. 2002). Nevertheless, in both studies, phorbol myristate acetate (PMA) was used to activate neutrophils. PMA is a direct activator of protein kinase C which elicits a respiratory burst leading to degranulation and release of large amounts of H_2O_2. To avoid this potential oxidative burst caused by the PMA, we decided to only prime the neutrophils with LPS (100 ng/ml) (Figure 3.1). After exposure to a second stimulus (such as opsonized zymosan or phorbol-myristate acetate), primed neutrophils play a critical role in endothelial cell

injury by enhancing the response of ROS release (respiratory burst) (Smedly, Tonnesen et al. 1986). Consistent with our results, priming neutrophils with 100 ng/ml LPS for 1h gives the maximal response with further enhanced activation of TLR4 and IL-8 released (Figure 3.1) (Smedly, Tonnesen et al. 1986, Aida and Pabst 1990).

However, besides ROS, neutrophils contain a multitude of antimicrobial proteins, cytotoxic substances and proteases which can also be involved in the induction of the DDR such as myeloperoxidase (MPO) and neutrophil elastase (NE) (Faurschou and Borregaard 2003). MPO and NE are involved in the formation of NETs composed of chromatin filaments assembled with proteases and proteins. The formation of NETs is a way to kill extracellular pathogens developed by innate immune cells (Papayannopoulos, Metzler et al. 2010, Pinegin, Vorobjeva et al. 2015). Presence of NETs have been linked to several conditions including autoimmune diseases such as Lupus erythematosus and their presence is associated to a bad prognostic (Villanueva, Yalavarthi et al. 2011).

Our data shows that sivelestat, an inhibitor of the protease neutrophil elastase is able to rescue neutrophil-induced DNA damage in MRC5 fibroblasts (Figure 3.3 a,b,d). However, more experiments are required to understand if NETs are generated under co-culture, and how elastase impacts on the DDR. Given that the presence of extracellular catalase also inhibits the DDR, it is possible that elastase inhibition also affects ROS generation, through yet unknown mechanisms.

While all experiments resulted in neutrophil-induced premature senescence, I observed considerable heterogeneity between independent experiments. This may be the result of using donors of different age and gender, despite the fact that I ensured that donors were within the same age range throughout experiments. It is also possible that stronger effects would be observed if I used a higher ratio of neutrophils/recipient cells, however, the use of 1:5 ratio was based on previous published data (Yan, Kloecker et al. 2014) and on our observation that neutrophil (detected with anti-NIMP antibody) cluster around individual hepatocytes at a similar ratio.

The results obtained with catalase (Figure 3.3, 3.5, 3.6, 3.7 and 3.8) suggest that extracellular ROS produced by the neutrophils are responsible for accelerated

telomere shortening and, subsequently, premature senescence. Previous work had shown a relationship between ROS and accelerated telomere shortening (Petersen, Saretzki et al. 1998, Oikawa and Kawanishi 1999, von Zglinicki 2000). Telomeres are thought to be favoured targets of oxidative attack due to their high content of guanine, which is highly susceptible to oxidative modifications (Oikawa and Kawanishi 1999). Studies have shown that mild oxidative stress causes single-stranded breaks to preferentially accumulate at telomeres, which upon cell division result in accelerated telomere shortening (Petersen, Saretzki et al. 1998, von Zglinicki 2000). Moreover, treatments with antioxidants, expression of antioxidant enzymes and therapies targeting ROS specifically produced in mitochondria have been shown to decelerate the rate of telomere shortening (Saretzki, Murphy et al. 2003, Serra, von Zglinicki et al. 2003, Passos, Saretzki et al. 2007).

Additionally, evidence suggests that oxidative damage at telomeres can result in the displacement of shelterin proteins TRF1 and TRF2 from telomere regions. This may be another mechanism by which oxidative stress contributes to telomere dysfunction (Opresko, Fan et al. 2005). Furthermore, telomere regions have been shown to be less well repaired than other regions in the genome, inducing a persistent DNA damage response (Hewitt, Jurk et al. 2012). This may be due to the fact that the shelterin component TRF2 plays a role in the inhibition of Non Homologous End Joining (NHEJ) (Fumagalli, Rossiello et al. 2012). TRF2 and RAP1 have been shown to prevent the action of DNA-PK, a double-stranded break repair complex protein and inhibit ligase-IV-mediated end joining (Bombarde, Boby et al. 2010).

Telomere dysfunction driven by ROS results in the activation of a DNA damage response at telomeres, resulting in cellular senescence (d'Adda di Fagagna, Reaper et al. 2003). Induction of senescence also results in increased intracellular ROS. Previous work has shown that activation of p53 and p21 (Macip, Igarashi et al. 2002, Macip, Igarashi et al. 2003) results in increased intracellular ROS which act as intracellular signals to maintain the cell-cycle arrest. Mitochondrial-derived ROS have been proposed to play a role in the stabilisation of cellular senescence via the generation of further DNA damage as part of a positive feedback loop (Passos, Nelson et al. 2010). Consistently with this concept, we found that fibroblasts exposed to neutrophils had increased mitochondrial ROS generation, as well as increased genomic DNA damage.

Altogether, my data is consistent with a model by which neutrophils produce mild ROS which leads to DNA damage in cells and accumulation of single stranded breaks at telomeres which still allows cellular proliferation (Petersen, Saretzki et al. 1998). With cell division (following neutrophil removal), telomere shortening rates are enhanced, resulting in activation of a DDR at telomeres and premature senescence, via activation of cyclin-kinase inhibitors p21 (Wright and Shay 1992) and later on p16 (Beauséjour, Krtolica et al. 2003, Jacobs and de Lange 2005). When telomerase is expressed, telomeres are better maintained and neutrophil induced senescence is bypassed (Figure 3.10).

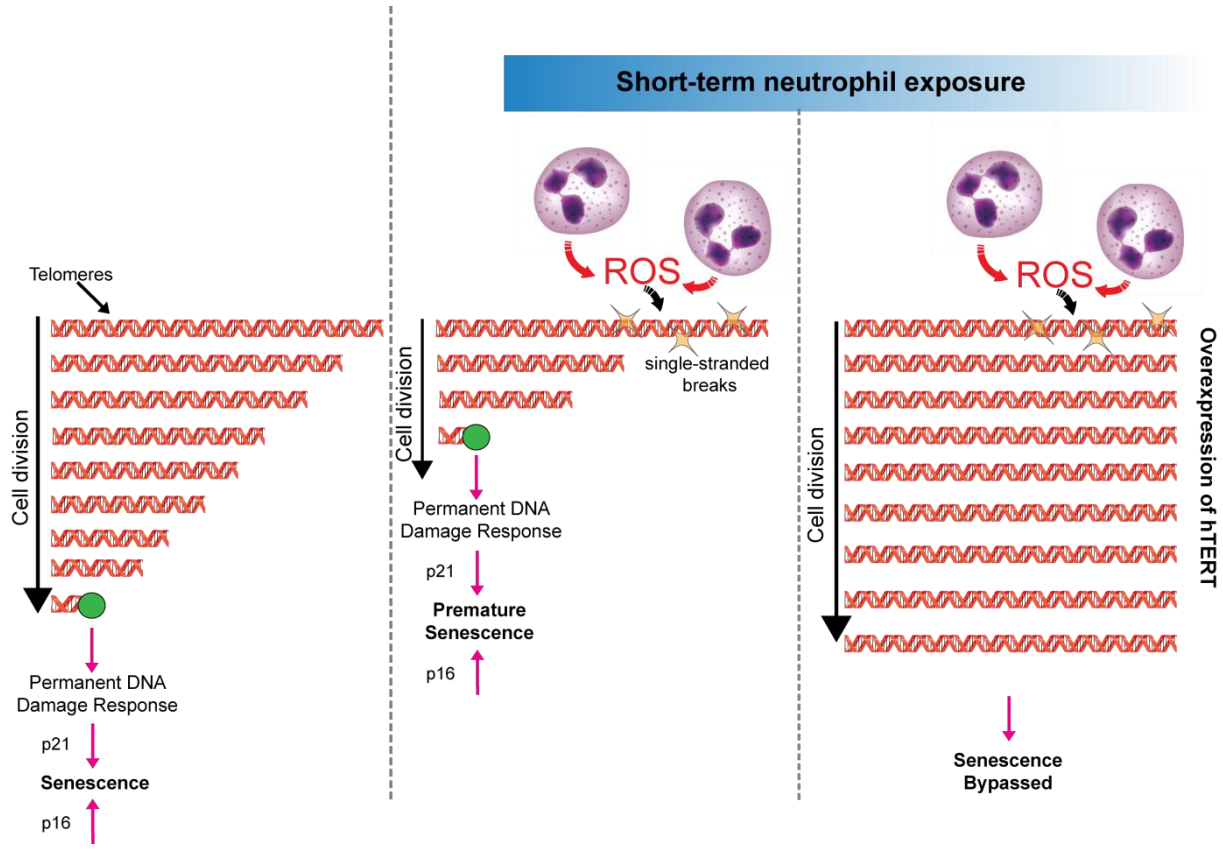


Figure 3.10 Short-term neutrophil exposure with MRC5 fibroblasts triggers premature senescence via telomere shortening in a ROS dependent manner.

(Left) Classical condition of telomere shortening driven by the cellular division and the "end-replication problem" which trigger a permanent DNA damage response and cellular senescence. (Middle) short-term neutrophil exposure accelerates telomere shortening in a ROS-dependent manner via generation of single-stranded DNA breaks at telomere regions. (Right) MRC5 overexpressing catalytic subunit of telomerase (hTERT) upon neutrophil exposure bypass senescence via maintenance of telomere length.

4 CHAPTER 4 - Neutrophil infiltration increases with age and induces telomere dysfunction in mice

In the previous chapter we demonstrated *in vitro* that short-term co-culture with primed LPS neutrophils can induce premature senescence of young MRC5 fibroblasts in a ROS dependent manner. The data suggests that the extracellular ROS produced by the neutrophils can accelerate telomere erosion and shorten replicative lifespan.

Telomere-Associated Foci (TAF) can be easily detected in tissues by immunoFISH and are characterized by co-localisation between DDR proteins such as γH2A.X and telomere regions. TAF have been shown to be a marker of telomere dysfunction and cellular senescence in both human and mouse tissues and have been shown to occur due to exposure to oxidative stress and DNA damaging agents (Hewitt, Jurk et al. 2012). It has been shown that TAF frequencies increase with age in mouse liver hepatocytes, intestinal crypts and in the lung (Hewitt, Jurk et al. 2012, Birch, Anderson et al. 2015). Moreover, recent work from our lab has shown that patients with inflammatory lung diseases such as IPF, Bronchiectasis and COPD have increased number of TAF in airway epithelial cells (Birch, Victorelli et al. 2016). In addition, TAF have been shown to increase in a mouse model of low grade chronic inflammation. This phenomenon was shown to be ROS-dependent since treatment with antioxidants rescued the phenotype (Jurk, Wilson et al. 2014), however, mechanistically it is still unclear how chronic inflammation drives telomere dysfunction. Furthermore, the role of neutrophils in the induction of senescence *in vivo* has not been investigated. For these reasons, I hypothesised that short-term recruitment of neutrophils to liver results in telomere-dysfunction and potentially cellular senescence *in vivo*.

4.1 Mouse neutrophils induce a DDR in isolated hepatocytes

In order to investigate the role of neutrophils in liver senescence, I first conducted *ex-vivo* experiments. I isolated neutrophils and hepatocytes from young wild-type mice and co-cultured them for 24 hours. Furthermore, I tested whether direct contact was required by co-culturing the cells in the same plate or by using transwells (Figure 4.1 a).

I found that short-term co-culture of neutrophils and hepatocytes resulted in increased 53BP1 foci in hepatocytes (Figure 4.1 b,e). Furthermore, I found that hepatocytes co-cultured with neutrophils had increased ROS (measured by fluorescent probes DHE and Cell ROX) when compared to untreated controls (Figure 4.1 c,d). This effect occurred irrespectively of cells being in the same plate or using transwells, suggesting that soluble factors derived from neutrophils drive DDR and ROS in hepatocytes.

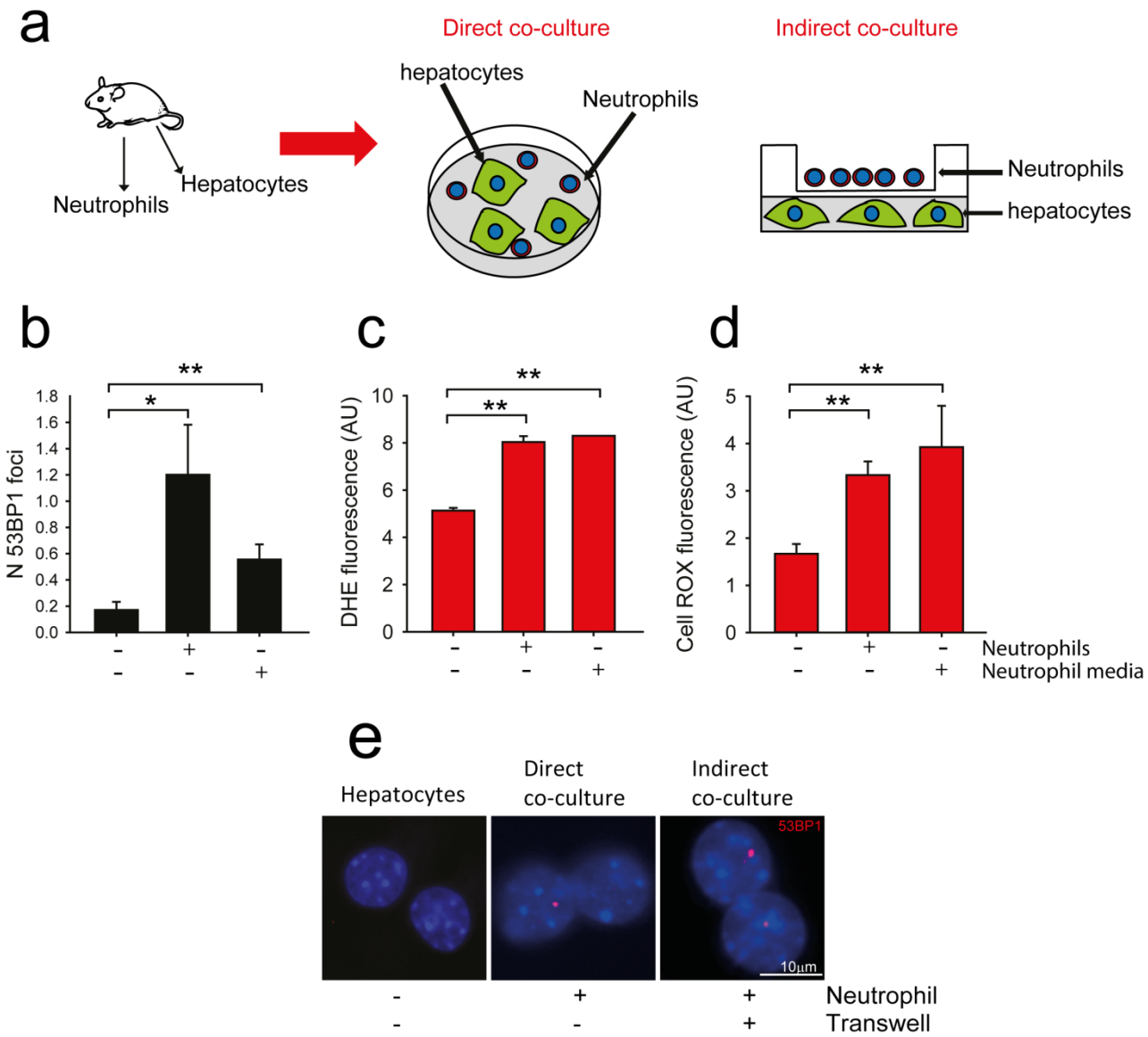


Figure 4.1 Mouse neutrophils induce a DDR and increased ROS in isolated adult mouse hepatocytes.

(a) Scheme showing direct and indirect contact using transwells for co-culture of isolated hepatocytes and neutrophils isolated from mouse' bone marrow (b) Mean number ± SEM of 53BP1 foci per nucleus of control

and co-culture with and without transwells (P ≤0.01; n=3). (c,d) Graphs showing mean fluorescence intensity ± SD (n=3) of ROX and RHE (measure of ROS production) in hepatocytes co-cultured with neutrophils (direct and indirect contact). (e) Representative images of 53bp1 (nucleus: DAPi, 53bp1: red). Statistical significance was determined using One Way ANOVA, *P<0.05, **P<0.01 compared to control.

4.2 Mouse recombinant neutrophil elastase induce a DDR in isolated hepatocytes

According to our previous results showing that the DDR in co-culture with neutrophils using an elastase inhibitor (sivelestat) is significantly decreased, we next tested whether neutrophil elastase could contribute to the DDR in hepatocytes. For that purpose, primary hepatocytes were first isolated from young wild-type mice and cultured in the presence or absence of mouse recombinant elastase (40 nM per well which has been shown previously to promote lung tumor growth (Houghton, Rzymkiewicz et al. 2010)) for 24h. Data shows that following culture with mouse recombinant elastase there is a significant increase in 53BP1 foci compared to the controls (P ≤0.05) (Figure 4.2).

Taken together with the previous findings using the elastase inhibitor sivelestat, these results indicate that elastase may be also involved in bystander effects mediated by neutrophils. However, further investigations need to be conducted to determine how elastase impacts on hepatocytes and the physiological relevance of these results.

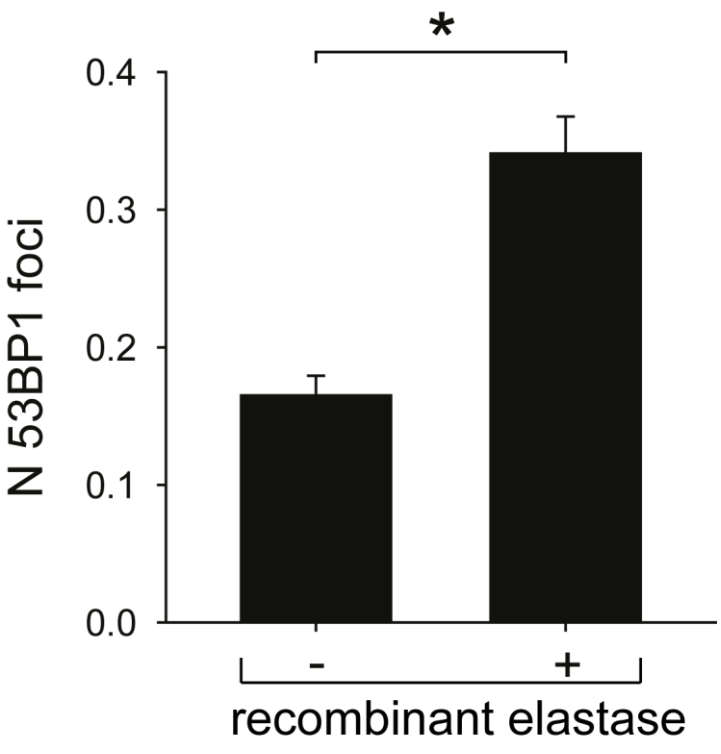


Figure 4.2 Recombinant mouse neutrophil elastase induces a DDR in isolated adult mouse hepatocytes.

Graph shows the mean number ± SEM of 53BP1 foci per nucleus of control and recombinant elastase (40nM per well) in mouse primary hepatocytes. Statistical significance was determined using an unpaired t-test, *P<0.05, n=3.

4.3 Neutrophil infiltration increases with age in the liver

Based on my *in vitro* results showing that three day co-culture with primed neutrophils induce a premature senescence in MRC5 fibroblasts when compared to the controls, I next sought to understand if neutrophils could be involved in ageing *in vivo*. One of the most common features of ageing is inflammation (López-Otín, Blasco et al. 2013). Neutrophils are the main actors of the innate immune system, and communicate via cytokines and chemokines (Borregaard 2010) which have been shown to be increased in old tissues. Hence, I measured the levels of neutrophil infiltration in the liver of young (3 months) and old wild-type mice (24 months) using immunohistochemistry staining for Nimp-R14, which binds to Ly6G anchored protein present at the surface of neutrophils. I found that with age, the number of neutrophils significantly increases in the liver (Figure 4.3 a, b). Interestingly, I observed that neutrophils formed clusters surrounding individual hepatocytes.

Based on the fact that senescent cells increase with age in liver (Wang et al. 2009; Hewitt et al. 2012) and have been shown to produce neutrophil chemoattractants, it is possible that senescent cells are involved in the recruitment of neutrophils. However, it is also a possibility that other stimuli unrelated to senescence result in neutrophil recruitment and subsequently induce senescence as we have demonstrated *in vitro*. In order to test the latter hypothesis, I investigated the relationship between liver injury, neutrophil infiltration and telomere dysfunction using different mouse models. These results will be described in the next subchapters.

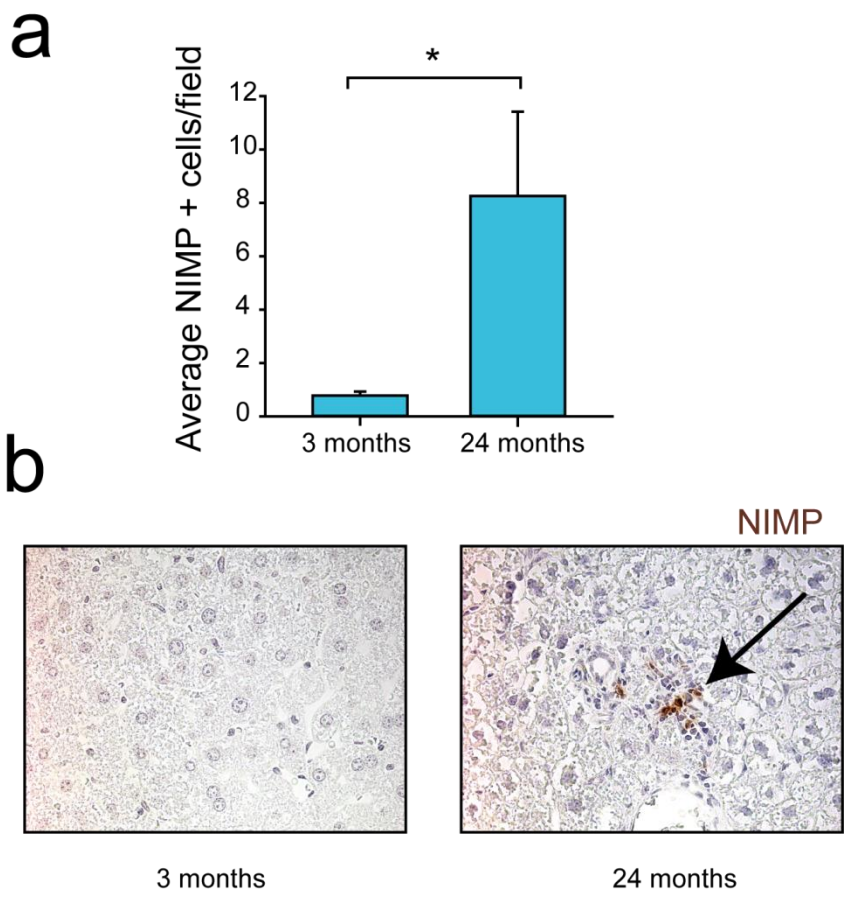


Figure 4.3 Neutrophil infiltrations increase with age in the liver of C57Bl/6 mice.

(a) Graph shows average of NIMP-1+ cells in the liver per field of WT mice 3-24 months of age. (b) Representative images at 200× magnification show liver neutrophil infiltration in 3-24 months of WT mice, black arrow denotes Nimp-1+ cell (neutrophil). Data are mean number ± SEM*=P ≤0.02 using an unpaired two-tailed t-test; n=5).

4.4 LPS injection induces TAF and neutrophil infiltration in the liver

Having shown that co-culture of neutrophils with hepatocytes *in vitro* increases DNA damage foci and ROS in the latter, I next wanted to investigate if induction of liver inflammation and subsequent neutrophil infiltration was associated with an increased DDR. In order to test this, young wild-type mice (8-10 weeks old; C57BL/6) were injected with LPS at a dose of 300 μg/animal and culled 24 hours later. Furthermore, livers were collected and analysed for the presence of neutrophils, γH2A.X and TAF.

I first found that upon injection of LPS, there was a significant increase in the mean number of neutrophils per field of vision in liver tissue compared to untreated control mice (Figure 4.4 a,b,c). Then, I performed immuno-FISH staining for TAF in association with PCNA (a proliferation marker), on hepatocytes following LPS injection (24h later). It has been reported that upon infection, following an acute immune response, fibrosis and remodelling occurs in the liver (Tanaka and Miyajima 2016) . Thus, I counted the TAF and γH2A.X foci only in PCNA negative cells in order to avoid dividing cells, which are positive for γH2A.X (Hudson, Kovalchuk et al. 2011). To note, injection of LPS in these conditions did not trigger cellular proliferation and I observed a very low number of cells positive for PCNA (data not shown). I found a significant increase in the mean number of TAF per nucleus and % of cell positive for TAF in PCNA negative hepatocytes following injection with LPS compared to the control (untreated mice) (Figure 4.4 e,f). Interestingly, the mean number of total γH2A.X foci per hepatocyte in mice injected with LPS compared to controls did not change significantly (Figure 4.4 g) suggesting that telomeres are potentially more sensitive to LPS induced injury. Altogether, this data indicates that 24 hours after injection of LPS, neutrophil infiltrations in the liver are increased as well as telomere-dysfunction in non-proliferating hepatocytes.

However, at this point we cannot establish causality between neutrophil recruitment and telomere-damage, since LPS results in the recruitment of several innate immune cells and is not exclusive to neutrophils (Steinmüller, Srivastava et al. 2006, Chakraborty, Zawieja et al. 2013, Movita, van de Garde et al. 2015). Consequently, further experiments are needed to establish the role of the neutrophils in the induction of telomere dysfunction *in vivo*.

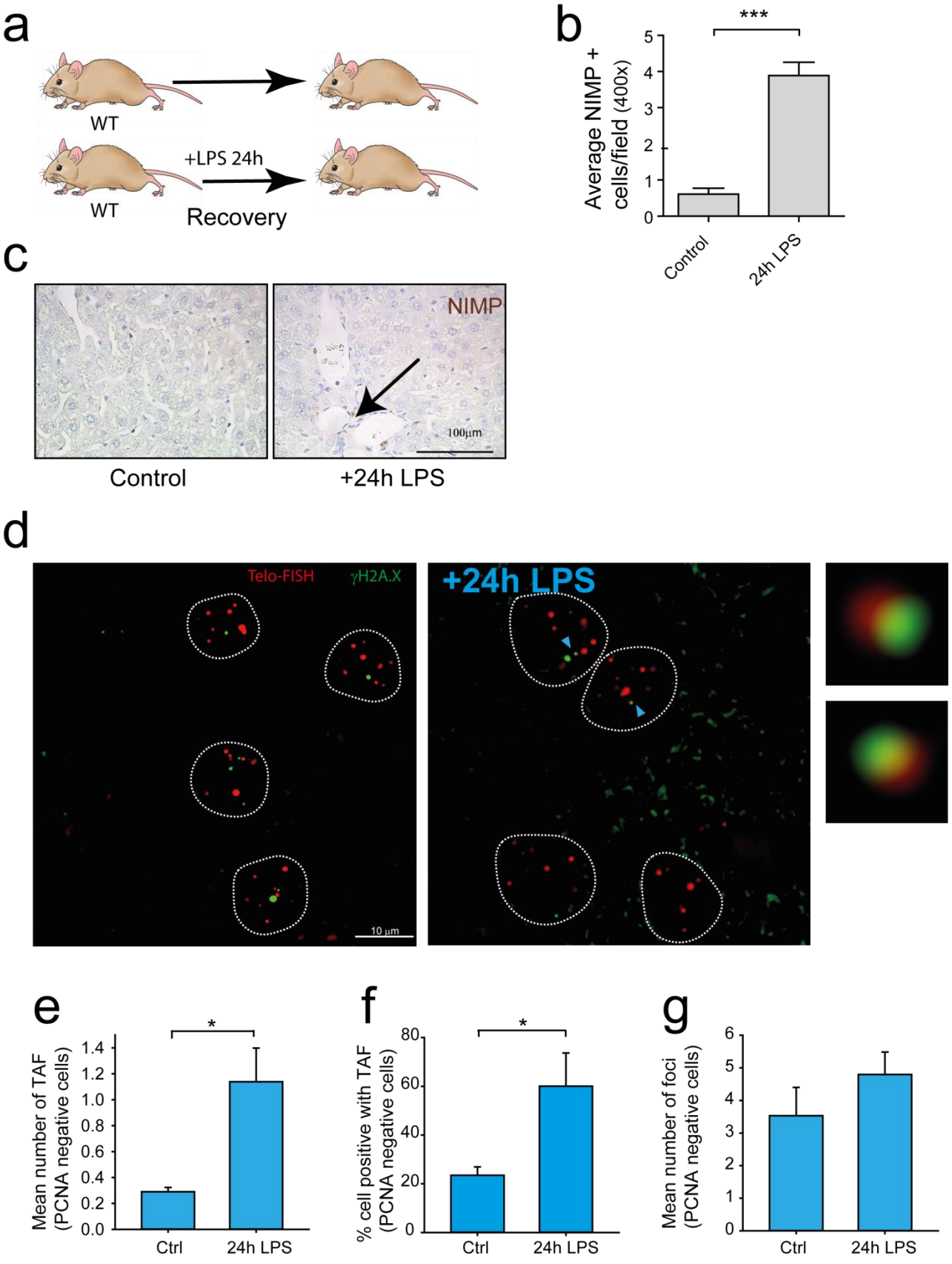

a
WT
+LPS 24h
WT
Recovery
b
***
Average NIMP + cells/field (400x)
5
4
3
2
1
0
Control
24h LPS
c
NIMP
100µm
Control
+24h LPS
d
Telo-FISH
γH2A.X
+24h LPS
10 µm
e
*
Mean number of TAF (PCNA negative cells)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
24h LPS
f
*
% cell positive with TAF (PCNA negative cells)
80
60
40
20
0
Ctrl
24h LPS
g
Mean number of foci (PCNA negative cells)
6
5
4
3
2
1
0
Ctrl
24h LPS

Figure 4.4 24h LPS injection in young wild type mice increases neutrophil infiltration in the liver and TAF in hepatocytes.

(a) Scheme of the experimental conditions of WT mice injected with LPS and sacrificed after 24h. (b) Average of NIMP-1+ cells field (x400) in mouse liver; black arrow represents a neutrophil (data are mean±s.e.m n=4/6, ***=P<0.001 using a two-tailed unpaired t-test). (c) Representative pictures at 400× (liver); Scale bar=100 μm. (d) Representative images of γH2A.X immuno-FISH in hepatocytes 24h after injection with LPS. Images are Huygens (SVI) deconvolved Z projections of 10-μm stacks taken with a ×63 oil objective and colocalizing foci are amplified in the right panel, blue arrows indicate the TAF (amplified images are from single Z planes where colocalization was found). Scale bar=10 μm (red: telomeres, green: γH2A.X). (e) Total number of γH2A.X foci colocalizing with telomeres (TAF) in in mouse hepatocytes PCNA negative cells 24h after injection with LPS (data are mean±s.e.m n=4/6, *=P<0.05 using a two-tailed unpaired t-test).(f) Percentage of γH2A.X foci colocalizing with telomeres (%TAF) in mouse hepatocytes PCNA negative cells 24h after injection with LPS (data are mean±s.e.m n=4/6, *=P<0.05 using a two-tailed unpaired t-test). (g) Total number of γH2A.X foci in mouse hepatocytes PCNA negative cells 24h after injection with LPS; data are mean±s.e.m. of n=4/6.

4.5 Impairment of neutrophil recruitment following carbon tetrachloride (CCl4) injection reduces TAF

4.5.1 Using tlr2 -/- mice model

Having shown that 24h after LPS injection neutrophils infiltrate the liver and this correlates with a significant increase in damaged hepatocytes, we next designed an experiment with the purpose of investigating the specific role of neutrophils in induction of senescence during liver injury.

The first strategy used to impair neutrophil recruitment was the *tlr2*$^{-/-}$ mouse model (Moles, Murphy et al. 2014). Previous work had demonstrated a specific role for TLR2 in the recruitment of neutrophils after CCl_4-induced liver injury. *Tlr2*$^{-/-}$ mice show significantly reduced neutrophil infiltrations in the liver 24h after injury, since TLR2 was shown to be required for the chemokine gradient generated by resident macrophages to recruit neutrophil to the site of injury (Moles, Murphy et al. 2014).

In this experiment, I used the well-established model of induction of liver fibrosis via the drug carbon tetrachloride (CCl_4), which induces lipid peroxidation and ultimately necrosis of the cells (de Toranzo, Gomez et al. 1978).

Both wild-type (WT) and *tlr2 -/-* mice were injected with CCl_4 and mice were culled at 8h, 48h and 72h post injection (Figure 4.5 a).

Following injury, I observed for WT and *tlr2 -/-* mice a tendency for an increase of PCNA positive hepatocytes at 48 and 72h, indicating increased compensatory proliferation and remodelling (Figure 4.5 b,d).

Then, I found that the mean number of TAF and the % of hepatocytes positive for TAF (in PCNA negative cells) had a tendency for an increase in WT mice following injection with CCl_4 (from 8 to 72h) (Figure 4.5 b,c,e,g). However, in *tlr2 -/-* mice the mean number of TAF (Figure 4.5 c,e) and the % of hepatocytes containing TAF (Figure 4.5 c,g) increased from 0 to 48h but remained the same at 72h, suggesting that the presence of neutrophils may be involved in the induction of telomeric DNA damage in surrounding hepatocytes. Similar patterns were observed for γH2A.X alone upon CCL_4 injury, however, no differences were seen between wild-type and *tlr2*$^{-/-}$.

Neutrophil-mediated bystander effects may be involved in the generation of TAF in hepatocytes upon exposure to the damaging agent CCl_4.

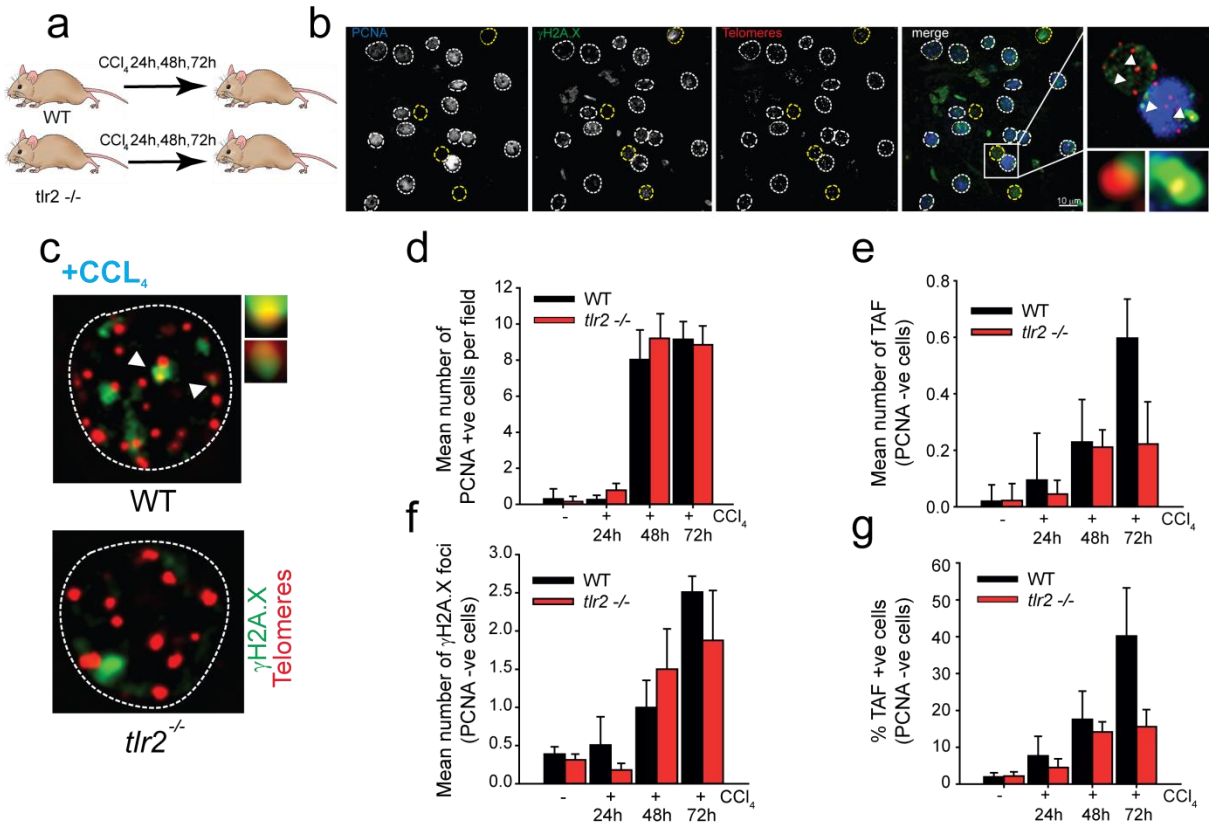


Figure 4.5 TLR2 deletion decreases TAF and total gH2A.X foci in mouse hepatocytes following CCl4 injection (a model of liver fibrosis).

(a) Scheme of the experimental conditions of WT and tlr2 -/- mice injected with CCl_4 and culled after 8, 24 and 72h. (b) Representative images of γH2A.X immuno-FISH in hepatocytes 72h after injection with CCl_4. Images are Huygens (SVI) deconvolved Z projections of 10-µm stacks taken with a ×63 oil objective. Scale bar=10 µm (blue: PCNA, red: telomeres, green: γH2A.X). (c) Amplified colocalizing foci in WT and $tlr2^{-/-}$, white arrows indicate the TAF (amplified images are from single Z planes where colocalization was found). Scale bar=10 µm (red: telomeres, green: γH2A.X). (d) Average of PCNA + hepatocytes per field (x630) in mouse liver. (e) Total number of γH2A.X foci colocalizing with telomeres (TAF) in in mouse hepatocytes PCNA negative cells 24, 48 and 72h after injection with CCl_4. Data are mean±s.e.m. of n=3. (f) Total number of γH2A.X foci in mouse hepatocytes PCNA negative cells 24, 48 and 72h after injection with CCl_4; data are mean number ±s.e.m. of n=3. (g) Percentage of γH2A.X foci colocalizing with telomeres (%TAF) in mouse hepatocytes PCNA negative cells 24, 48 and 72h after injection with CCl_4. All data are mean±s.e.m. of n=3.

4.5.2 Using a Ly6G neutralizing antibody

Having shown that impairment of neutrophil chemotaxis following CCl_4 injection in *tlr2 -/-* mice leads to a decrease in telomere dysfunction in hepatocytes, I then used another more specific strategy to impair neutrophil recruitment to the site of damage.

Using a similar experimental design with the toxic drug CCl_4, we used the neutralising neutrophil antibody Ly6G, which allows a depletion of circulating neutrophils (Moles, Murphy et al. 2014).

In this scenario, mice were pre-treated with Ly6G for 12h before CCl_4 injection (Single intraperitoneal injection of CCl_4 at a dose of 2 µl/g body weight), and mice were sacrificed 8h and 48h after injection (Figure 4.6 a).

Following injury, I observed a tendency for an increase in PCNA positive hepatocytes at 8 and 48h for both mice treated with Ly6G and mice treated with isotype control (IgG), indicating increased compensatory proliferation and remodelling (Figure 4.6 b). The mean number of γH2A.X foci per hepatocyte increased after injury but was significantly reduced upon neutrophil neutralisation (Figure 4.6 c,d).

Then, I found that the mean number of TAF and the % of hepatocytes positive for TAF increased after injury in IgG-treated mice but was significantly reduced in the mice depleted from neutrophils (Figure 4.6 c,e,f). For the reasons stated previously, only PCNA negative cells were quantified.

Hence, inhibition of neutrophil infiltration using Ly6G neutralising antibody in response to CCl_4 treatment reduces both general DNA damage and telomere-specific DNA damage in surrounding hepatocytes while maintaining a normal remodelling response.

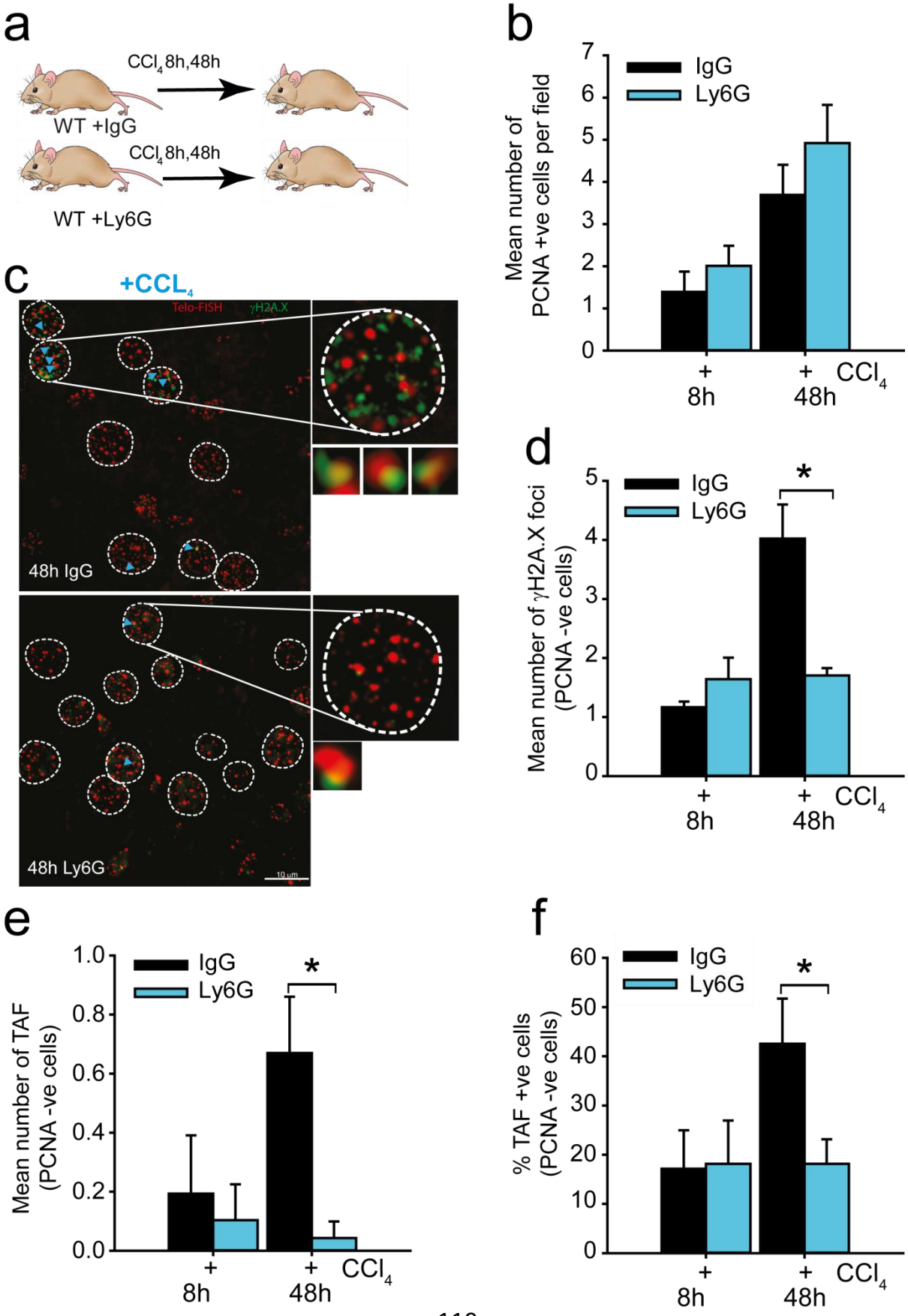

a
CCl4 8h,48h
WT +IgG
CCl4 8h,48h
WT +Ly6G
b
Mean number of PCNA +ve cells per field
IgG
Ly6G
0
1
2
3
4
5
6
7
+ 8h
+ 48h
CCl4
c
+CCL4
Telo-FISH
γH2A.X
48h IgG
48h Ly6G
10 μm
d
Mean number of γH2A.X foci (PCNA -ve cells)
IgG
Ly6G
*
0
1
2
3
4
5
+ 8h
+ 48h
CCl4
e
Mean number of TAF (PCNA -ve cells)
IgG
Ly6G
*
0.0
0.2
0.4
0.6
0.8
1.0
+ 8h
+ 48h
CCl4
f
% TAF +ve cells (PCNA -ve cells)
IgG
Ly6G
*
0
10
20
30
40
50
60
+ 8h
+ 48h
CCl4

Figure 4.6 Neutralisation of neutrophil infiltration decreases TAF and total gH2A.X foci in mouse hepatocytes following CCl4 injection (a model of liver fibrosis).

(a) Scheme of the experimental conditions of WT injected with CCl4 +/- Ly6G and culled after 8, 48h. (b) Average of PCNA + cells field (x630) in mouse liver after injection with CCl4 +/- Ly6G at 8 and 48h. (c) Representative images of γH2A.X immuno-FISH in hepatocytes 48h after injection with CCl4 +/- Ly6G. Images are Huygens (SVI) deconvolved Z projections of 10-μm stacks taken with a ×63 oil objective. Scale bar=10 μm (red: telomeres, green: γH2A.X). (d) Total number of γH2A.X foci in mouse hepatocytes PCNA negative cells 8 and 48h after injection with CCl4 +/- Ly6G; data are mean±s.e.m of n=3 (*=p<0.05 using a two-tailed unpaired t-test). (e) Total number of γH2A.X foci colocalizing with telomeres (TAF) in mouse hepatocytes PCNA negative cells 8 and 48h after injection with CCl4 +/- Ly6G. Data are mean±s.e.m. of n=3 (*=p<0.05 using a two-tailed unpaired t-test). (f) Percentage of γH2A.X foci colocalizing with telomeres (%TAF) in mouse hepatocytes PCNA negative cells 8 and 48h after injection with CCl4 +/- Ly6G. Data are mean±s.e.m. of n=3.

4.6 Discussion

In response to pathogenic attacks and tissue injury, the organism has developed a mechanism named inflammation which involves a large number of steps and cell types in order to repair tissue and restore homeostasis (Shaw and Martin 2009). One of the major actions is the recruitment and accumulation of innate immune cells namely neutrophils and macrophages (Shaw and Martin 2009). Due to neutrophils primary function to eliminate pathogens, they generate various ROS including superoxide ($O2^{\cdot-}$, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and nitric oxide ($NO^{\cdot}$) (Forman and Torres 2002, Winterbourn and Kettle 2013). Hence, the trafficking of these cells is tightly controlled; however repeated tissue damage and regeneration may result in an augmentation of macrophages and neutrophils recruitment delaying their clearance, thus leading to an increase of ROS production (Bian, Guo et al. 2012). On the long term, this leads to chronic inflammation and results in tissue damage. Presence of unwanted neutrophils in the tissue is linked with tissue dysfunction, development of cancer and fibrosis in the lungs and liver (Houghton 2013, Wilson, Jurk et al. 2015).

The liver is an essential central organ connecting the guts and the circulatory system. It is exposed continuously to bacteria, toxins and food derived antigens. The main function of the liver is insured by hepatocytes which represent 70 to 85 % of the cell liver population. Hepatocytes are mainly responsible for protein synthesis, glycogen storage, detoxification and secretion of bile. Because of its vital functions, the liver is somehow vulnerable to diseases and pathogenesis such as fatty liver, viral hepatitis, cirrhosis and hepatocellular carcinoma (Starley, Calcagno et al. 2010). Those diseases are generally associated with liver inflammation and necrosis which imply neutrophil infiltration and poor prognostic.

With ageing, organisms tend to develop a chronic inflammation, which increases risk factors for morbidity and mortality in the elderly (Alafuzoff, Helisalmi et al. 2000). This kind of Inflammation can be partially explained by the accumulation in the tissue of senescent cells which have been shown to produce pro-inflammatory factors

collectively known as the “SASP” which have been show to induce tissue damage (Coppé, Desprez et al. 2010). In some cases, senescent cells communicate with immune cells and up-regulate their receptors to recruit them in order to be cleared (Burton and Krizhanovsky 2014). These studies are in agreement with our findings that neutrophil infiltration increases with age in the liver of WT mice. This suggests two possible scenarios which are not mutually exclusive. Firstly, as senescent cells accumulate with age within tissues (Wang, Jurk et al. 2009, Hewitt, Jurk et al. 2012) neutrophils could be recruited to clear them. In fact, previous data has shown that other immune cells such as natural killer cells, T-cells and macrophages are involved in the clearance of senescent cells (Gasser, Orsulic et al. 2005, Xue, Zender et al. 2007, Krizhanovsky, Yon et al. 2008, Hoenicke and Zender 2012). However, it is also possible that with ageing, a decline in the immune system, results in impaired clearance of senescent cells by the immune cells, resulting in both the accumulation of senescent cells and enhanced recruitment of immune cells by components of the SASP.

The second scenario is that age-dependent changes in tissues or increased infections result in the release of chemoattractant factors which lead to the recruitment of neutrophils or other immune cells and these contribute to senescence as a collateral damage. In fact, my results so far both *in vitro* and *in vivo* are more supportive of the latter scenario, since limiting recruitment of neutrophils in liver fibrosis models reduces telomere-dysfunction- a known marker and inducer of cellular senescence. However, all my experiments were conducted in young animals which have a fully functional immune system. Future experiments should investigate the impact of neutrophil clearance in ageing animals.

Our data using ex-vivo hepatocytes and neutrophils extracted from the bone marrow indicates that co-culture induces a DDR with an enhanced response with direct contact compared to indirect contact (Figure 4.1). Thus, we can speculate that factors secreted from neutrophils are linked to this process since in our experiment neutrophils with indirect contact are still able to induce a DDR in co-culture hepatocytes.

The impact of senescent cells in the recruitment of neutrophils or their potential involvement in the clearance of senescent cells has also not been fully investigated. However, data indicates a potential role in the process of apoptosis. Data shows that apoptotic hepatocytes can attract neutrophils via the secretion of CXC chemokines (Faouzi, Burckhardt et al. 2001). Once neutrophils are recruited, they may adhere via their surface molecules such as LFA-1 and Mac-1 to ICAM-1 on the hepatocyte (Ito, Abril et al. 2006). This attachment will lead to degranulation and neutrophil oxidative burst (Shappell, Toman et al. 1990). In addition, a plethora of molecules released during inflammation such as IL-6, IL-8, TNFα, IL-1α are involved in the simulation of the oxidative burst. They could also enhance the response to N-formyl-peptides (Elbim, Bailly et al. 1994).

In this work, I used two models to neutralise neutrophil migration:

The Ly6G neutralising antibody has been shown to be specific to neutrophils (Daley, Thomay et al. 2008) with little effect on macrophages and CD3+ lymphocytes (Wilson, Jurk et al. 2015). Consistent with our results, in a cancer study using a mouse model of low-grade inflammation nfkb1$^{-/-}$, it was demonstrated that neutrophils promote the induction of TAF in hepatocytes which were rescued by limiting neutrophil infiltrations using Ly6G (Wilson, Jurk et al. 2015).

The *Tlr2*$^{-/-}$ mouse model failed to recruit neutrophils upon injury due to the defective chemokine CXCL-2 expressed by resident hepatic macrophages (Moles, Murphy et al. 2014). However, we cannot exclude that other roles of TLR2 may impact on neutrophil or hepatocyte functions relevant for the induction of senescence. Interestingly, our data show that neutrophil depletion (via Ly6G or deletion of Tlr2) has no effect on the proliferative response after liver injury (Figure 4.5 and 4.6) in agreement with previous published work (Moles, Murphy et al. 2014). In the *Tlr2*$^{-/-}$ mouse model, I only observed trends but no statistically significant differences, which is probably a consequence of the low number of animals used (n=3). However, I am planning as part of future work to increase the number of animals per group, in order to reach more reliable conclusions.

CCl_4 can be very pathogenic, because once metabolized in the liver it is transformed into trichloromethyl radical (CCl3*) which reacts with DNA, proteins and lipids and will ultimately trigger mutations to develop HCC. Then, CCl3* is oxygenated to form trichloromethylperoxy radicals (CCl3OO*) which initiate lipid peroxidation and abolition of membrane permeability leading to mitochondrial dysfunction and further cell damage (Weber, Boll et al. 2003).

My data shows that neutrophil infiltration increases in the liver with ageing in mice. This could be attributed to the chronic inflammation occurring with age, leading to increased secretion of pro-inflammatory factors such as IL-6 and IL-8 which may attract neutrophils (Franceschi and Campisi 2014). Infiltrating neutrophils might further damage cells with ROS and other secreted compounds leading to generation of increased DNA damage and ultimately, senescence. However, others have proposed that the SASP and recruitment of immune cells could have beneficial effects. For instance, it was shown that during chronic liver damage, fibrosis was restricted by NK cells clearing stellate senescent cells (Krizhanovsky, Yon et al. 2008). In addition, in mice where cancer is induced using oncogenic $Nras^{G12V}$, CD4 T cells and monocytes/macrophages were responsible for clearance of pre-malignant tumour cells (Xue, Zender et al. 2007, Kang, Yevsa et al. 2011). In these studies it was reported that senescent activated stellate cells downregulate extracellular matrix genes and upregulated genes involved in immune recognition for NK cells such as NK cell receptor ligands (MICA, ULBP2 and PVR), adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and cytokines such as IL-6, IL-11 and IL-8. Neutrophils express receptors such as LFA-1 which is known to bind to ICAM-1 (Dustin and Springer 1988) and IL-8 which belongs to the CXC chemokines which have been shown to attract and activate neutrophils (Baggiolini, Dewald et al. 1994, Hammond, Lapointe et al. 1995).

Therefore, it is possible that senescent cells can also attract neutrophils within the tissue and could be involved in their clearance, however, evidence is lacking. Actually, neutrophils by their primary functions to eradicate pathogens are able to perform phagocytosis; degranulation and activate oxygen burst and release NETs

(Kolaczkowska and Kubes 2013). These functions could be many ways that can be involved in the process of clearance of senescent cells. In the next chapter, I investigated the impact of senescent cells in the recruitment of neutrophils as well as their potential role in clearance of senescent cells.

5 CHAPTER 5 - Neutrophils are recruited by senescent cells and selectively kill them

5.1 Neutrophil chemotaxis assay using young and senescent conditioned media

Chemotaxis of neutrophils is regulated by molecules called chemokines through a gradient from the blood to the tissues (Kolaczkowska and Kubes 2013).

Once cells become senescent, they display with time a SASP including secretion of several cytokines and chemokines (Coppé, Desprez et al. 2010). Other groups have shown that macrophages and natural killer cells are found *in vivo* in close proximity with senescent cells and are involved in their clearance (Burton and Krizhanovsky 2014), however, the role of neutrophils in the process has not been explored.

Hence, I hypothesised that senescent cells are able to recruit neutrophils via SASP components. In order to investigate this hypothesis, I collected conditioned media from young (early PD) and replicatively senescent fibroblasts and performed an assay to measure the chemotaxis of neutrophils called “the sub-agarose method” (Ruchaud-Sparagano, Drost et al. 1998) (Figure 5.1 a).

Figure 5.1 b,c shows the migration of human neutrophils towards the chemoattractant fMLP which recruits neutrophils to sites of bacterial infection and contributes to their subsequent activation in tissue. This positive control indicates that the robustness of the technique. Then, data from comparison between senescent cells and young cells shows that neutrophils migrate preferentially towards senescent cells (Figure 5.1 b). These results indicate that secreted factors from senescent cells act as a chemoattractant for neutrophils.

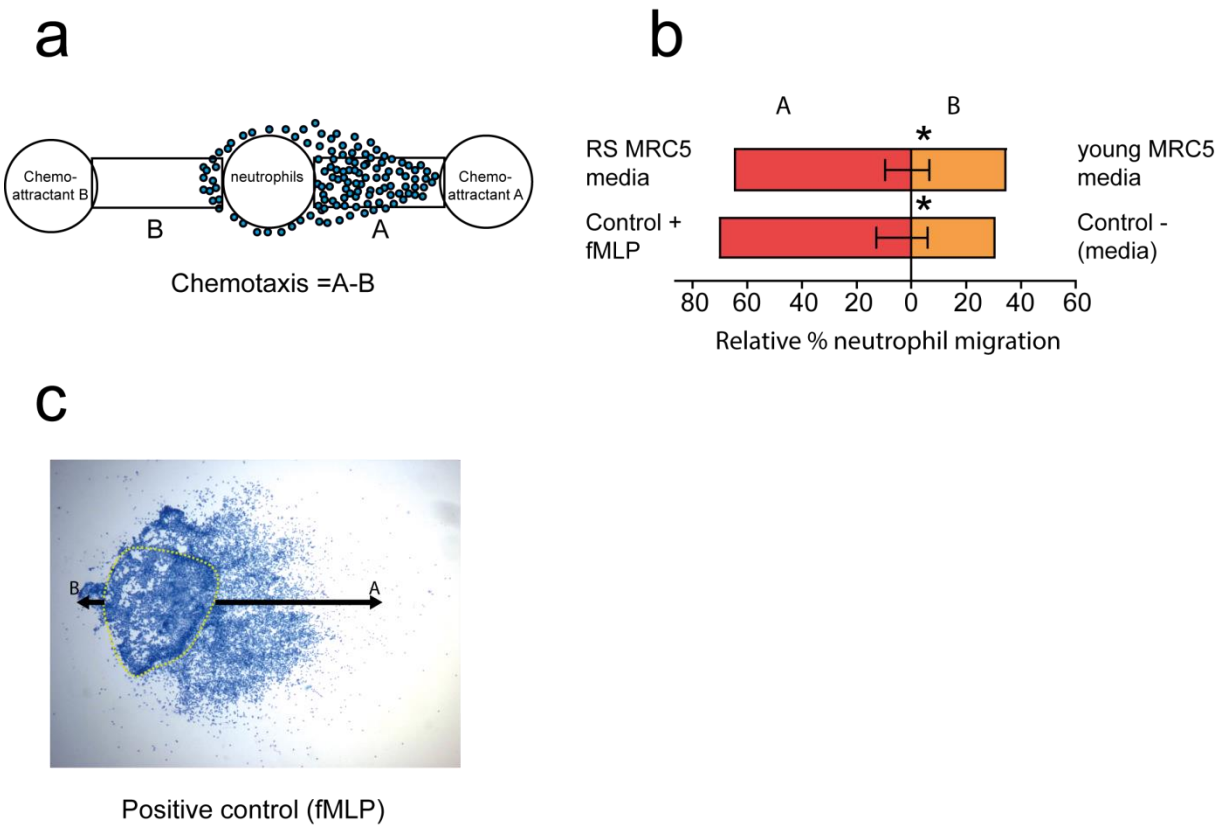


Figure 5.1 Neutrophils are attracted by conditioned media from senescent cells in a chemotaxis assay.

(a) Scheme of neutrophil migration sub-agarose technique assay. Holes of a defined diameter and distance are punched out of agarose gels; in one of these holes neutrophils are seeded, which migrate underneath the agarose layer toward a chemoattractant, whereas migration toward medium alone, conditioned media from young cells or untreated conditioned media from senescent cells serve as control. The migration distance is expressed in µm toward indicated target. (b) Neutrophil chemotaxis towards fMLP (100nM), conditioned media from young cells or replicative senescent cells and media alone. Untreated freshly isolated human neutrophils were added to each central well, incubated for 2h (data are mean±s.e.m, n=12; *=P<0.001 using Mann-Whitney rank sum test). (c) Representative image of the positive control (fMLP) and HBSS media following 2h incubation, cells are stained with Giemsa (x20).

5.2 Chemotaxis using senescent conditioned media treated with SASP inhibitors with neutrophils

Then, we used well-described drugs which have been shown to reduce the levels of molecules composing the SASP such as rapamycin and SB 203580 (MAPK p38 inhibitor) (Freund, Patil et al. 2011, Marina, Lixin et al. 2012, Herranz, Gallage et al. 2015, Laberge, Sun et al. 2015, Correia-Melo, Marques et al. 2016). After 10 days treatment of replicatively senescent MRC5 cells with rapamycin and p38 inhibitor, we collected the conditioned media. Data shows that neutrophil migration towards conditioned media from senescent MRC5 cells treated with rapamycin or SB 203580 is reduced around 80% and 60% respectively (n=3 and n=2) compared to untreated senescent MRC5 cells (Figure 5.2). This data suggests that components from the SASP released by senescent MRC5 cells are involved in the attraction and recruitment of neutrophils.

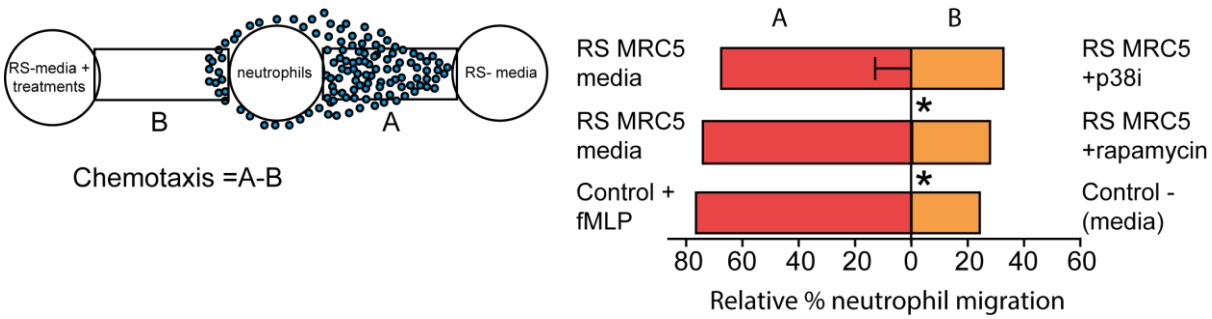


Figure 5.2 Treatment of senescent cells with SASP inhibitors prevents the migration of neutrophils using a chemotaxis assay.

Neutrophil chemotaxis towards fMLP (100nM), conditioned media from replicative senescent cells +/- treatment with p38 inhibitor (SB203580) and rapamycin for 10 days. Untreated freshly isolated human neutrophils were added to each central well, incubated for 2h, % of neutrophil migration is represented toward each condition (data are mean±s.e.m of n=3 (*=p<0.001 using a two tail unpaired t-test).

5.3 Live-cell imaging of co-cultures of senescent cells and neutrophils

Having found that neutrophils migrate preferentially towards senescent cells, I investigated the hypothesis that neutrophils could be involved in clearance of senescent cells, similarly to what has been demonstrated for other innate immune cells such as natural killer cells and macrophages (Krizhanovsky, Yon et al. 2008, Muñoz-Espín, Cañamero et al. 2013, Storer, Mas et al. 2013).

In order to study this phenomenon, I co-cultured freshly isolated neutrophils and MRC5 / IMR90 irradiated with 20Gy, with a minimum of 10 days post-irradiation, and performed live-cell imaging using a wide field microscope to determine whether neutrophils can clear senescent cells. After 24h of co-culture, I did not observe any clearance *per se* however; this could be because cells moved considerably being difficult to track them over 24 hours. Nonetheless, I made two interesting observations: i) the neutrophils seem to cluster around senescent cells and ii) senescent cells cultured in the presence of neutrophils experience detachment from the surface of the dish (Figure 5.3 a). Quantification of the mean number of detaching cells over a period of 24h in both young and senescent fibroblasts (using fibroblasts lines MRC5 and IMR90) confirms the initial observation (Figure 5.3. b,c). Interestingly, I observed higher frequencies of detaching cells upon co-culture with neutrophils in the senescent IMR90 when compared to senescent MRC5 fibroblasts. I speculate that this difference could be due to different levels of SASP factors secreted by these two fibroblast cell lines since unpublished data from our lab shows that IMR90 senescent fibroblasts secrete 10 times more IL-6 and IL-8 (which are main SASP factors) than MRC5 fibroblasts.

Then I repeated the co-culture with freshly isolated neutrophils and senescent IMR90 (induced by X-ray irradiation) and applied to the co-culture propidium iodide (PI),

which is an intercalating agent binding to the DNA. PI can only enter into cells with compromised membranes undergoing cell death, allowing the visualisation of dying cells. Using this technique with live cell imaging, we observed that neutrophils were able to kill specifically senescent IMR90 but not early passage cells during 24h co-culture (Figure 5.3 d, e).

In summary, using live cell imaging my preliminary data shows that neutrophils specifically kill senescent cells but not young cells. However, there were several technical challenges to this methodology, particularly the fact that fibroblasts move very quickly when in culture, making it difficult to track individual cells over a period of 24h by live-cell imaging. Therefore, I needed to apply a different methodology to obtain quantitative measurements of cell-death due to neutrophils in both young and senescent cell populations.

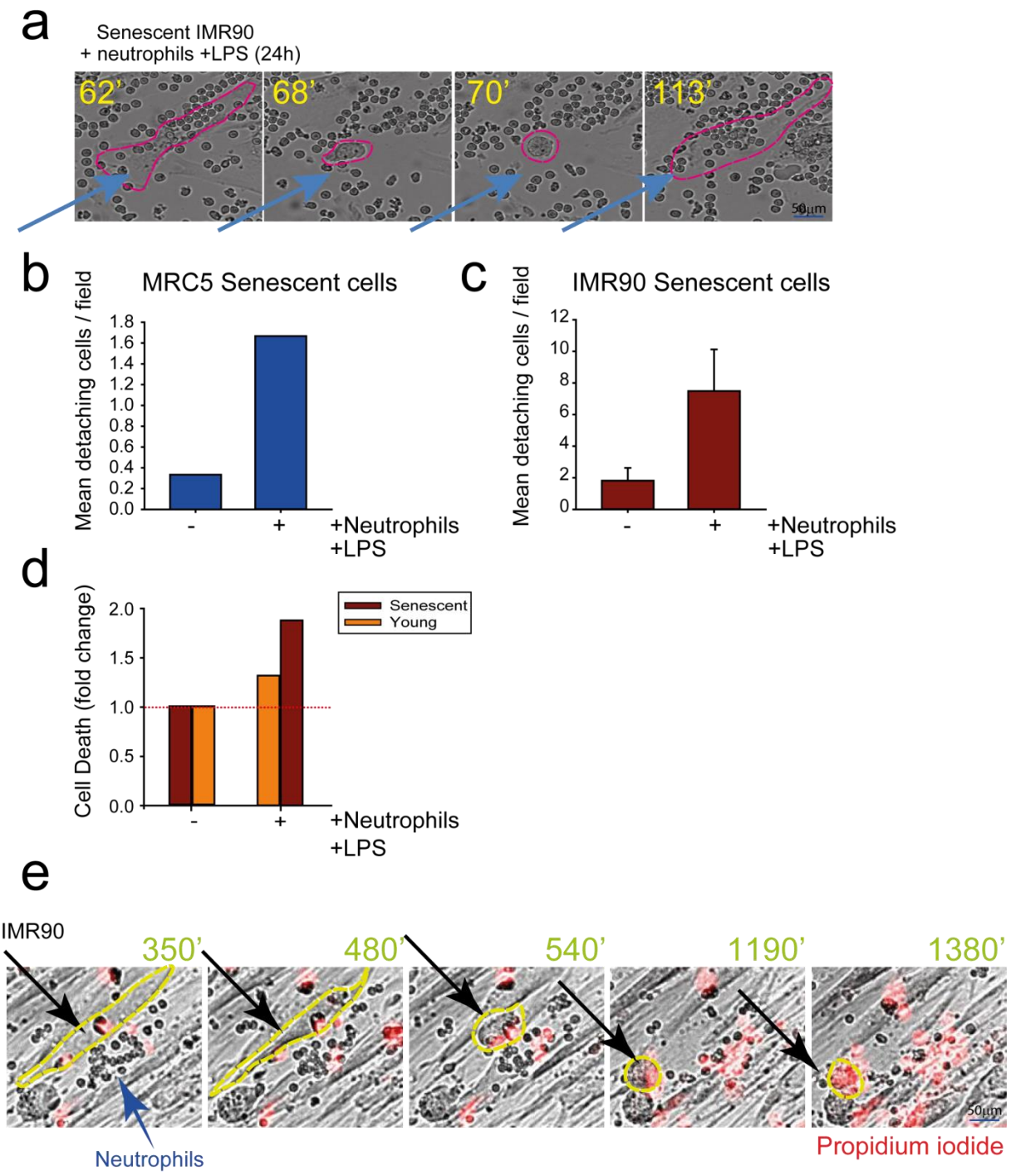


Figure 5.3 Live-cell imaging indicates that neutrophils induce specific detachment and cell-death of senescent cells.

(a) Representative images of an IMR90 fibroblast detaching upon co-culture with human neutrophils over time (time in yellow expressed in minutes). (b) Graph showing the mean of detaching cells per field (x100) upon 24h co-culture with irradiated MRC5 with 20gy (20 days post irradiation); n=1. (c)

Graph showing the mean of detaching cells per field (x100) upon 24h co-culture with irradiated IMR90 with 20gy (20 days post irradiation); n=2. (d) Graph showing the fold change of cell death normalised to the controls of young cells and irradiated IMR90 (20 days post irradiation) following 24h with LPS primed neutrophils co-culture using live cell microscopy with propidium iodide. (e) Representative images of cleared senescent 20gy irradiated IMR90 cell (20 days post irradiation) upon co-culture with human neutrophils overtime (time expressed in minutes; red: propidium iodide).

5.4 Assessment of cell viability of young vs senescent fibroblasts following co-culture with neutrophils

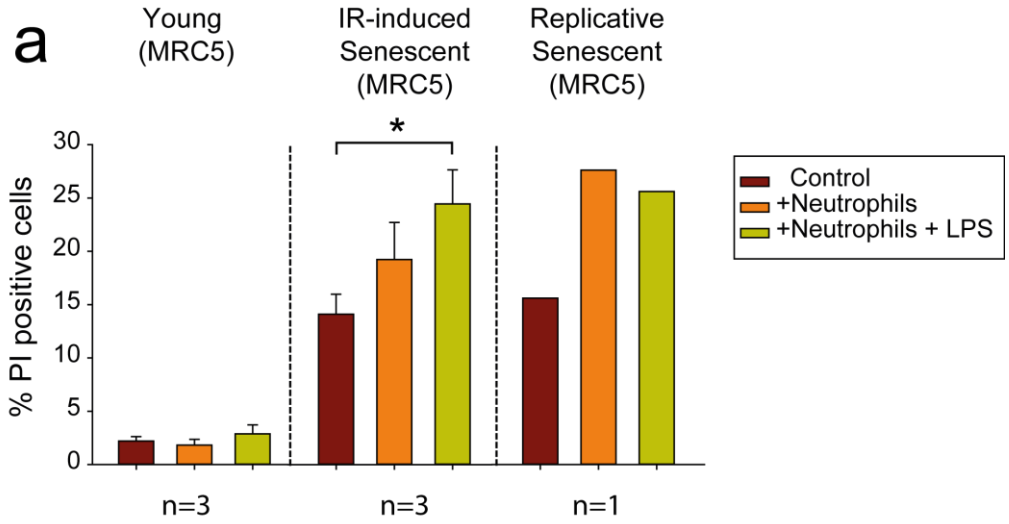
In order to obtain quantitative data of cell-death following co-culture of young (early PD) and senescent fibroblasts, we used flow cytometry, which allows the analysis of larger cell populations than live-cell imaging.

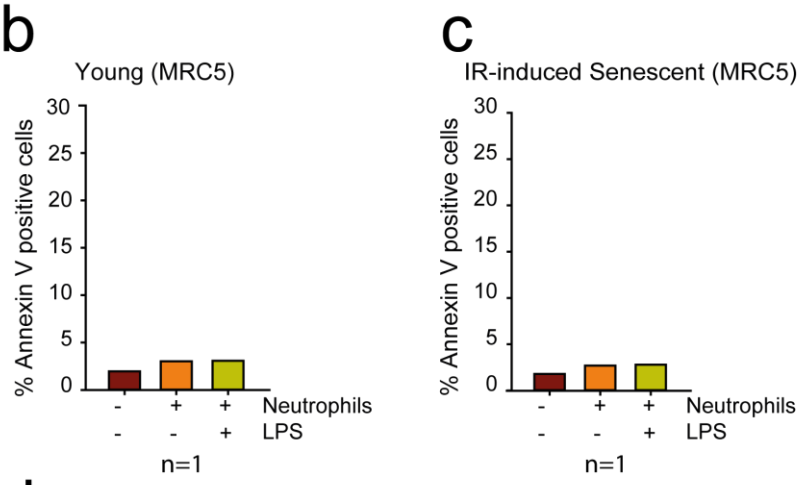
Cell viability is classically measured by flow cytometry using fluorescent dyes PI and Annexin V, which are able to determine if cells are viable, apoptotic or necrotic via differences in the integrity and permeability of the plasma membrane (Vermes, Haanen et al. 2000). Annexin V binds to phosphatidylserine (PS) in a calcium-dependent manner. PS is normally found on the intracellular side of healthy cells, but translocates to the external side during apoptosis. Annexin V alone cannot differentiate between apoptotic and necrotic cells, but PI will stain late apoptotic and a necrotic cell, as it goes to the nucleus where it binds to DNA. Annexin V will specifically stain early apoptotic cells. Therefore, using this system I should be able to distinguish if MRC5 fibroblasts are being killed and in which manner (apoptosis/necrosis) when co-cultured with neutrophils.

Hence, I cultured primed or non-primed neutrophils with young or senescent MRC5 cells for 24h and then measured the fibroblast viability using PI and Annexin V by flow cytometry. Results show no effects of neutrophils on cell-death in young

fibroblasts. (Figure 5.4 a,b,d). In contrast, results indicate that co-culture of senescent MRC5 with primed neutrophils leads to a significant increase in PI positive cells compared to the control (untreated cells) (Figure 5.4 a,d). However, we observe negligible differences with Annexin V (Figure 5.4 c,d). These results indicate that neutrophils can trigger cell-death in senescent cells by necrosis or late apoptosis (since we detect very low number of Annexin V staining) upon 24h co-culture with neutrophils primed with LPS at 3%O_2. Then we tested whether replicatively senescent MRC5 obtained by serial passages in culture (>60 days) could be killed by neutrophils as we have seen with MRC5 cells irradiated with 20gy. Upon 24h co-culture at 3%O_2, we saw an increase in the % PI positive cells in both conditions (Figure 5.4 a,d), however these results will need to be confirmed independently.

Altogether, this data indicates that activated neutrophils can trigger cell-death in senescent cells. Furthermore, the results indicate that most likely necrosis is preferred toward apoptosis.


a
Young (MRC5)
IR-induced Senescent (MRC5)
Replicative Senescent (MRC5)
*
% PI positive cells
30
25
20
15
10
5
0
n=3
n=3
n=1
Control
+Neutrophils
+Neutrophils + LPS


b
Young (MRC5)
% Annexin V positive cells
30
25
20
15
10
5
0
- + + Neutrophils
- - + LPS
n=1
c
IR-induced Senescent (MRC5)
% Annexin V positive cells
30
25
20
15
10
5
0
- + + Neutrophils
- - + LPS
n=1

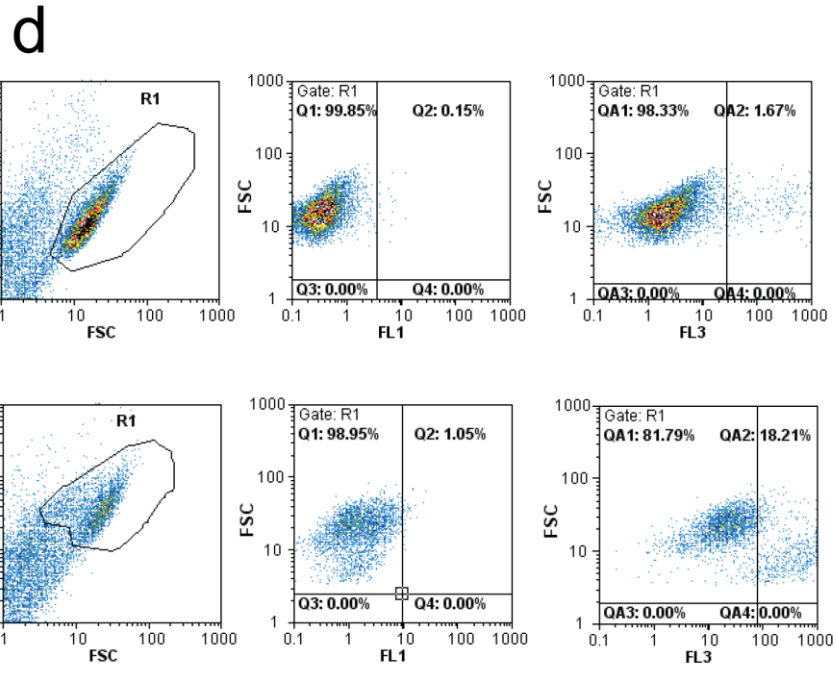

d
Young (MRC5)
IR-induced Senescent (MRC5)
R1
SSC
FSC
Gate: R1
Q1: 99.85%
Q2: 0.15%
Q3: 0.00%
Q4: 0.00%
FL1
QA1: 98.33%
QA2: 1.67%
QA3: 0.00%
QA4: 0.00%
FL3
Q1: 98.95%
Q2: 1.05%
QA1: 81.79%
QA2: 18.21%

Figure 5.4 Human neutrophils are involved in the clearance of senescent cell but not young cells.

(a) (Left) Flow cytometry analysis of propidium iodide (PI) staining of young apoptotic MRC5; n=3, (Middle) 10 days post irradiation with 20Gy (senescent) apoptotic MRC5 fibroblasts; n=3 *p<0.05 using a two tailed unpaired t-test, (Right) replicative senescent apoptotic MRC5 fibroblasts; n=1; following 24h co-culture at 3% O2 +/- neutrophils +/-LPS depending on condition, expressed in % PI positive cells. (b) Flow cytometry analysis of Annexin-V staining of young apoptotic MRC5 fibroblasts following 24h co-culture at 3% O2 +/- neutrophils +/-LPS depending on condition, expressed in % PI positive cells; n=1. (c) Flow cytometry analysis of annexin-V staining of 10 days post irradiation (senescent) apoptotic MRC5 fibroblasts following 24h co-culture at 3% O2 +/- neutrophils +/-LPS depending on condition, expressed in % PI positive cells; n=1. (d) MRC5 cells were gated using side and forward scatters, then using FL1 to measure Annexin V and FL3 to measure PI staining.

5.5 Discussion

Senescent cells develop with time a SASP which may induce alteration of the microenvironment, and promote the development of cancer (Krtolica, Parrinello et al. 2001, Acosta, O'Loghlen et al. 2008, Kuilman, Michaloglou et al. 2008).The SASP from senescent cells contain a large range of pro-inflammatory molecules including chemokines, cytokines, adhesion molecules and immune modulators which have the abilities to communicate with immune cells (Freund, Orjalo et al. 2010). To note, the SASP is cell type specific, depends on the context and the senescence stimuli (Coppé, Patil et al. 2008). To date, it has been reported that senescent cells are able to attract NK cells, monocytes/macrophages, and T cells which are involved in the clearance mechanisms. Indeed, two studies demonstrated that senescent hepatocytes and hepatic stellate cells where found in close proximity with NK cells which recognised and eliminated them (Xue, Zender et al. 2007, Krizhanovsky, Yon et al. 2008, Sagiv, Biran et al. 2013). These studies have provided evidence suggesting that senescent cells upregulate receptors involve in the recognition of NK cells (activating Natural Killer cell receptor (NKG2D), MICA and ULBP2) (Sagiv, Biran et al. 2013). Furthermore it was shown that NK cells were able to eliminate senescent cells by granule exocytosis through perforin and granzyme which will induce cell death (Sagiv, Biran et al. 2013) .However, regarding the plethora of molecules composing the SASP, it is not surprising that NK cells are not the only cells to be found in the vicinity of senescent cells. In fact, molecules such as IL-8 (CXCL8), IL-6, GROα (CXCL1), GROβ (CXCL2), GROγ (CXCL3), MCP-1 (CCL2), MCP-4 (CCL13), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), I-309 (CCL1), and MCP-3 (CCL7) are also secreted (Coppé, Patil et al. 2008, Freund, Orjalo et al. 2010) and can contribute to recruitment of monocytes/macrophages and T cells (Kang, Yevsa et al. 2011, Lujambio, Akkari et al. 2013) .

According to my *in vivo* data showing that there is an increase of neutrophil infiltration within the liver with age, I speculated that perhaps senescent cells can attract neutrophils.

In fact, one of the most prominent expressed chemokine from the SASP is IL-8 (CXCL8) which is a strong chemoattractant for neutrophils (Mahalingam and Karupiah 1999).

Our chemotaxis assay using conditioned media from senescent cells and young cells indicates that neutrophils migrate preferentially toward the media from senescent cells (Figure 5.1). This indicates that compounds secreted from senescent cells are implicated in the recruitment of neutrophils. Additionally, our data indicates that upon treatment of replicative senescent cells for 10 days with SB203580 (p38 inhibitor) and rapamycin (mTOR inhibitor) the migration toward conditioned media from senescent cells is blunted (Figure 5.2). These two inhibitors are well described to decrease the SASP components: rapamycin inhibits the mammalian TORC1 complex which in turn will reduce IL1α and NF-κB transcriptional activity resulting in a decrease of the SASP components such as IL-6 and IL-8 (Laberge, Sun et al. 2015 1416). SB203580 (p38 inhibitor) has been shown to block the production of IL-1, TNFα and IL-8 (Freund, Patil et al. 2011).

In addition, our data indicates that neutrophils seem to cluster around senescent fibroblasts which results in detachment from the dish and eventually cell-death (Figure 5.3). We also observed that IMR90 are more prone to cell death compared to MRC5 fibroblasts. It is possible that recruitment of neutrophils by senescent cells is cell-type and tissue specific. Accordingly, SASP components have been shown to be cell-type specific, depending on the senescence induction stimuli and the expression levels of pro-inflammatory molecules (Coppé, Patil et al. 2008). In addition, unpublished data from our lab has shown that senescent IMR90 fibroblasts secrete increased abundance of pro-inflammatory cytokines IL-6 and IL-8 when compared to senescent MRC5 fibroblasts.

I report for the first time that neutrophils are able to specifically kill MRC5 senescent cells with very little effects on young cells (Figure 5.4). The mechanism of clearance indicates that necrosis is preponderant toward apoptosis since we detect very little staining for Annexin V. This result is consistent with a study using a mouse model co-expressing oncogenic Ras and inducible p53 where reactivation of p53 in the liver was followed by infiltration of neutrophils and NK cells resulting by killing senescent cells by necrosis but not apoptosis (Xue, Zender et al. 2007). However, it will be necessary to perform a time course in order to determine between apoptosis and necrosis since in our experiment we only observed after 24h co-culture.

We only observed a small increase or no effect with primed neutrophils using LPS for 1 h at 37 °C compared to untreated cells. This suggests that either during the isolation of the neutrophils they were already activated, or the SASP compounds are concentrated enough to fully activate the neutrophils (Figure 5.4). Upon extraction from the whole blood, neutrophils can be primed due to the isolation protocol resulting in changes in antigen expression and altered response to stimuli (Watson, Robinson et al. 1992). In addition, we can speculate that IL-8 plays a role in the killing because this chemokine has been previously shown to recruit and activate neutrophils (Hammond, Lapointe et al. 1995). However, we cannot exclude that other SASP components are also involved and/or senescent cells upregulate receptors involve in recognition of the neutrophils to clear them.

Altogether, our results demonstrate for the first time that neutrophils are involved in the clearance of senescent cells, however further work is needed to understand by which mechanism the killing occurs and if this phenomenon can be observed *in vivo.*

6 CHAPTER 6 – CONCLUSIONS

Over the years, a large number of data has been produced regarding cellular senescence. Since its discovery *in vitro*, the implications of this process have been widened from the merely “telomere clock” limiting the life of mitotic cells. New evidence has shown that senescence is implicated in embryonic development, tumour suppression, tissue repair and wound healing suggesting that senescence has beneficial effects.

Recent data has demonstrated a role for the SASP in communicating with the immune system in order to clear senescent cells. Several reports have involved NK cells, macrophages, and LT4 cells in the senescence clearance process but surprisingly, neutrophils, which are major components of the innate immune system, have not been investigated. Neutrophils are the first immune cells to infiltrate the site of damage after stress or infection. Neutrophils have been implicated in the resolution of fibrosis and the induction of cancer; however their implication in the induction of senescence has not been investigated. In addition, following acute stress in tissues, neutrophils have been observed to infiltrate at sites of damage. Neutrophils because of their nature possess a large plethora of “weapons” in order to kill pathogens and remove cellular debris. Among them, neutrophils secreted a large amount of ROS (namely h_2O_2) which are known from previous work to induce premature senescence.

Therefore, in this thesis, I hypothesised that neutrophils could induce cellular senescence via bystander effects. I have shown that short-term co-culture for 3 days between young fibroblasts and human neutrophils from middle-aged healthy donors leads to a significant reduction in the replicative lifespan of human fibroblasts grown *in vitro*. This occurred together with induction of several markers of cellular senescence and induction of DNA damage. Furthermore, I observed that telomere-shortening rates were enhanced and that expression of telomerase was able to counteract the effect. Mechanistically, I have found that extra-cellular catalase was

able to reduce both telomere shortening and DNA damage, suggesting that hydrogen peroxide secreted by the neutrophils is responsible for the observed phenotype.

This was in accordance with the idea that short-term exposure to neutrophils generates single-stranded breaks via ROS in young fibroblasts, which upon cell division experience a more rapid telomere loss ultimately triggering premature senescence. Similar effects were shown by others upon exposure to mild-stress (von Zglinicki, Saretzki et al. 1995, Petersen, Saretzki et al. 1998).

I cannot exclude that other factors secreted by the neutrophils can play a role in this effect since the use of an inhibitor against neutrophil elastase or the addition of recombinant elastase was able to reduce or enhance DDR foci respectively. Nonetheless, the mechanisms involved are not yet elucidated.

Consistent with a role for neutrophils in telomere dependent senescence, I found an association between neutrophil infiltrations and telomere dysfunction in ageing mice and following LPS injection. Additionally, in a model of acute liver fibrosis through injection of CCl_4, I found that inhibition of neutrophil recruitment reduced telomere-associated DNA damage.

Altogether, my results suggest that neutrophils may inadvertently induce collateral DNA damage which will trigger gradually a premature senescence in young cells via oxidative stress-mediated telomere dysfunction.

Finally, I asked the question if neutrophils are somehow involved in the clearance of senescent cells similarly to what was observed with NK cells, CDT4 cells and macrophages. My data indicates that neutrophils are preferentially recruited by factors secreted by senescent cells and can induce cytotoxicity in senescent cells but not in young cells. This suggests that neutrophils may be involved in the clearance of senescent cells; however, the same mechanism can lead to the induction of damage in young surrounding cells which may become prematurely senescent.

Given the fact that the immune system is less efficient with age, it is also possible that as we grow old, neutrophils are recruited to the site of where senescent cells are present, but are not effective in eliminating them. As future work, I propose to investigate neutrophils obtained from donors of different ages in terms of their ability to induce cell-death in senescent cells.

To conclude, my PhD work unravelled a dual role of neutrophils in the process of senescence. While hydrogen peroxide released by neutrophils can accelerate telomere-dependent senescence, neutrophils may also be involved in the specific elimination of senescent cells, through yet unclear mechanisms.

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