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Dissecting the role of cellular senescence in acute myeloid leukaemia immune-surveillance and response to therapy

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Abstract

Cellular senescence is a state of irreversible growth arrest induced by a plethora of endogenous or exogenous stressors, including DNA damage and oncogene activation and tumour suppressor loss in the context of cancer. Interestingly, senescence induction in cancer cells, together with a proliferative arrest, may trigger an immune-mediated cell clearance, thus further contributing to its tumour suppressive function. However, the molecular players involved in the crosstalk between senescent cancer cells and the immune cells are still not fully understood. Hence, this study aims to shed light on the mechanisms involved in such crosstalk, providing more insights into tumour eradication post-treatment and the emergence of therapy-resistant cancer cells.

I carried on my studies exploiting a model of acute myeloid leukaemia (AML), a type of cancer that involves hematopoietic cells and for which the current standard of care is represented by high-dose chemotherapy, typically achieved with administration of Cytarabine (ARA-C). In senescent cells, cell cycle arrest is generally mediated by activation of the p53/p21 and p16INK4A/pRB tumour suppressor pathways. The establishment of senescence rather than acute activation of the DNA damage response (DDR) correlates with the increased expression levels of Human Leukocyte Antigens (HLA) class I and II.

Exploiting the mixed lymphocyte culture reaction (MLR), I found an enhanced T cell activation and proliferation upon senescence establishment, reflecting higher immunogenicity of senescent blasts and confirming the beneficial effect of the senescence program on immune system activation.

The results obtained so far represent a steppingstone to clarify two main aspects of cancer cell response to therapy: the molecular mechanisms underlying the higher HLA molecules expression and the efficacy of the increased immunogenicity of AML blasts in promoting immune-based clearance of tumour cells.

Overall, our findings identify senescence as a positive anti-cancer mechanism that leads to increased immunogenicity, improves T cells activation and, thus, may favour tumour eradication. It remains to be further elucidated how DNA damage response cascade activation affects AML cells features in this context and how different immune cell types interact with senescent AML blasts.

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Acronyms and abbreviations

ALL = Acute lymphoblastic leukemia
Allo-HSCT = Allogeneic hematopoietic stem cell transplant
AML = Acute myeloid leukaemia
ARA-C = Cytarabine, cytosine arabinoside
ATR = ataxia telangiectasia and Rad3 related
ATM = ataxia telangiectasia mutated
ATRA = All-trans retinoid acid
BER = Base excision repair
BM = Bone marrow
BrdU = 5-bromodeoxyuridine
CCF = Cytosolic chromatin fragments
cDNA = Complementary DNA
CDKIs = Cyclin-dependent kinase inhibitors
CLL = Chronic lymphocytic leukemia
CLP = Common lymphoid progenitor
CMP = Common myeloid progenitor
CR = Complete remission
CTRL = Control
DAPI = DNA dye 4',6-diamidino-2-phenylindole
DDR = DNA damage response
DEG = Differentially expressed gene/s
DNA = Deoxyribonucleic acid
DSBs = Double stranded Breaks
FAB = French-American-British
FACS = Fluorescence-activated cell sorting
FDR = False discovery rate
FLT3L = FMS-like tyrosine kinase 3 ligand
G-CSF = Granulocyte-colony stimulating factor
GSEA = Gene-set enrichment analysis
HD/H.D. = Healthy donors
HR = Homologous recombination
HSC = Hematopoietic stem cells
HSCT = Hematopoietic stem cell transplant
HSPC = Hematopoietic stem and progenitor cells
hTRT= Human telomerase reverse transcriptase
IL = Interleukin
LAIP = Leukaemia-associated immuno-phenotype
LSC = Leukemia stem cells
MMR = Mismatch repair
miR = microRNA
NER = nucleotide excision repair
NGFR = Nerve Growth Factor Receptor
NHEJ = non-homologous end-joining
NR = No response
ns = Not statistically significant
NSG = NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ
NSGW41 = NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ KitW41/W41

OIS = Oncogene-induced cellular senescence
OS = Overall survival
PBMC = Peripheral blood mononuclear cells
PCA = Principal component analysis
PCR = Polymerase chain reaction
PD = Progressive disease
PDX = Patient-derived xenograft
RB = retinoblastoma protein
RNA = Ribonucleic acid
ROS = Reactive oxygen species
RT = Reverse transcription
SAHFs = senescence associated-heterochromatin foci
SASP = Senescence-associated secretory phenotype
SIPS = stress-induced premature cellular senescence
SCF = Stem cell factor
TEs= Transposable elements
TF = Transcription factor
TIS = Therapy-induced senescence
TPO = Thrombopoietin
TTCR = Time to first CR
TTR = Time to first relapse
UTR = Untranslated region
UV = ultraviolet irradiation
WB = Western Blotting
WT/wt = Wild type

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1 Cellular senescence

1.1 General features of cellular senescence

Cellular senescence is a state of growth arrest first described by Hayflick and Moorhead in 1961 (Hayflick and Moorhead, 1961). They reported that human lung fetal fibroblasts lose their ability to divide when passaged serially under standard tissue culture conditions after initial rounds of cell divisions. They also quantified that this replicative limit of normal diploid human fibroblasts was reached after approximately 50 divisions. These observations led these investigators to propose that normal cells have an intrinsic replicative potential limit called Hayflick's limit (d'Adda di Fagagna, 2008). We now know that mitotically competent cells respond to different stresses by undergoing cellular senescence, a permanent cell cycle arrest.

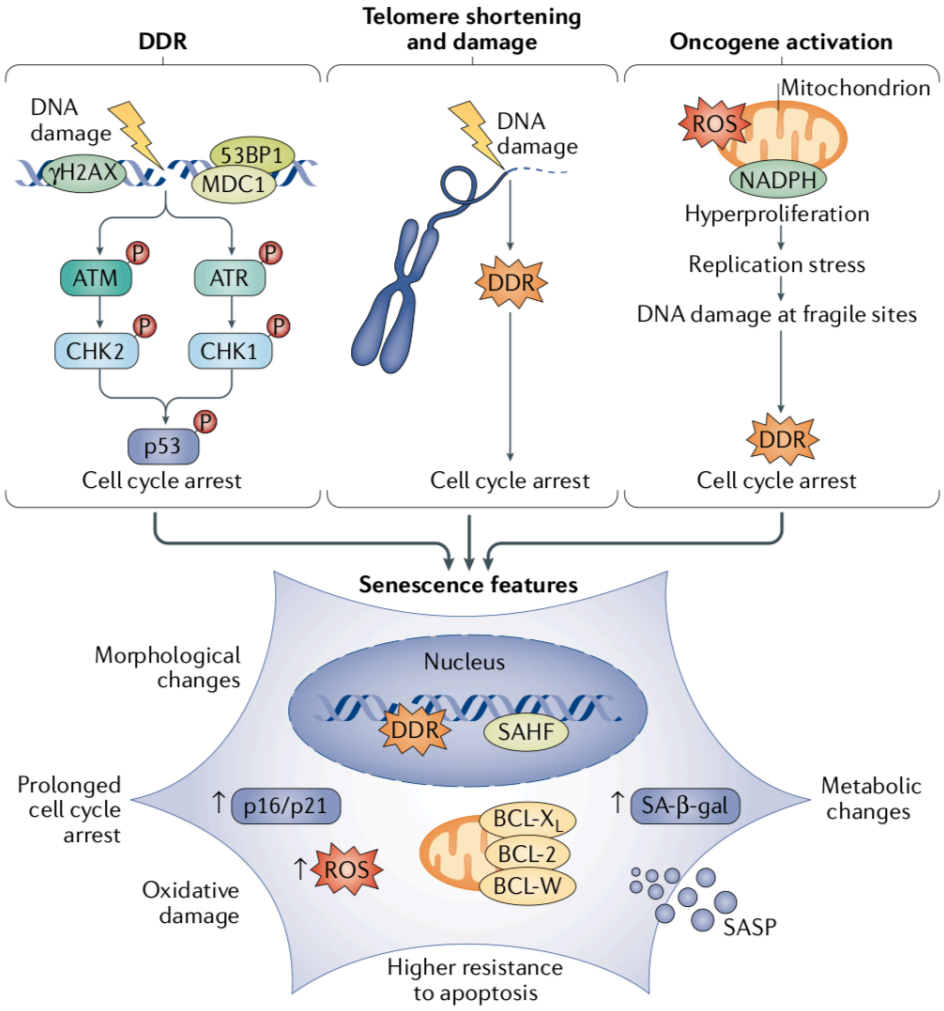


Figure 1. From Di Micco et al. Nature Rev Mol Cell Biol 2021

As presented in Figure 1, some of the possible triggers of senescence are activation of DNA damage response (DDR), the telomer shortening and the oncogene activation. All these types of stressors promote the DDR via activation of one of the two apical kinases: ataxia telangiectasia mutated (ATM) (activated with double-stranded break) or ataxia telangiectasia and Rad3 related (ATR) (activated with single-stranded break) (Ovadya and Krizhanovsky, 2018) (Jackson and Bartek, 2009). As result of this activated cascade cells arrest their cell cycle to repair the DNA lesions; however a persistent DDR due to unrepaired damage promotes the activation of the senescence program (Amant *et al.*, 2019) (Ferini-Strambi *et al.*, 2017). The

different stressors that trigger senescence programs will be better explained in the paragraph below.

Cells can also exist in a reversible growth-arrested state called quiescence or G0-phase. Quiescent cells can resume proliferation in response to appropriate mitogenic stimuli and space in a cell culture dish. By contrast, senescence is a condition in which cells are irreversibly arrested in the presence of nutrients, growth factors and ample space. Senescent cells in culture display an enlarged and flattened morphology with abnormally large cytoplasm and can remain viable and metabolically active for many weeks, even years. Senescent cells arrest with a DNA content typical of the G1-phase, even if some oncogenes cause a fraction of cells to arrest with an S- or G2-phase DNA content (Zhu *et al.*, 1998; Olsen *et al.*, 2002).

One of the most important peculiarities of senescent cells is their increased resistance to apoptotic death. Apoptosis is a cellular mechanism that prevents the proliferation of damaged cells by promptly eliminating them to avoid the accumulation of potentially harmful cells. Senescent human fibroblasts acquire resistance to apoptosis induced by DNA damage, growth factors deprivation and oxidative stress (Chen, Liu and Merrett, 2000). This could partly explain why senescent cells are so stable in culture. A vital aspect still unresolved is the identification of factors that contribute to the choice between apoptosis and senescence; one determinant may be the cell type: for example, damaged human fibroblasts tend to senesce while damaged lymphocytes tend to undergo apoptosis, and this could be related to the physiological turnover in the body of different cell types (Lechel *et al.*, 2005). The intensity, duration and nature of the damage may also explain the choice between senescence and apoptosis. Anyway, senescence and apoptosis programs share some cellular components, notably the activation of the p53 pathway. Indeed, in some senescent cells, resistance to apoptosis can be due to p53 preferential transactivation of genes that mediate proliferation arrest instead of those that facilitate cell death (Jackson and Pereira-Smith, 2006).

Senescent cells also display an altered gene expression pattern, including alterations in known cell-cycle inhibitors. For instance, cycling-dependent kinase inhibitor 1 (p21) and cyclin-dependent kinase inhibitor 2A (p16) are two cyclin-dependent kinase inhibitors (CDKIs), part of the central tumours suppressors pathways governed by p53 and retinoblastoma protein (RB) respectively, often accumulate in senescent cells. Senescent cells also stably repress proliferative genes encoding for DNA replication proteins such as PCNA or cyclin A. This is likely due to an RB-dependent inactivation of E2F transcription factors (Stein *et al.*, 1991; Narita *et al.*, 2003). In addition, senescent cells can secrete proteins that alter the tissue microenvironment and are at the same time involved in enforcing the senescence state; collectively, these proteins are called 'senescence-associated secretory phenotype (SASP) (Acosta *et al.*, 2008; Kuilman *et al.*, 2008), and this process will be extensively described later in the introduction.

1.2 Senescence markers

To date, there are no markers exclusive to the senescent state. One feature used to identify senescent cells is the lack of DNA replication, typically detected by incorporating 5-bromodeoxyuridine (BrdU) or H^3-thymidine, or by immunostaining for proliferation markers, such as PCNA and Ki-67. Unfortunately, these markers are not exclusive to senescent cells: indeed, all non-proliferating cells present the same profile making it impossible to distinguish between senescent, quiescent or differentiated post-mitotic cells. The senescence-associated β-galactosidase (SA-β-gal) was the first marker identified for the specific identification of senescent cells. SA-β-Gal is a lysosomal enzyme active at pH 6.0, specifically in senescent cells (Dimri *et al.*, 1995). To identify senescent cells by this marker, in the past was necessary histochemical staining on fixed cells; however, a more sensitive technique based on fluorescence detection increased the sensitivity of the staining, making it possible to assess alive cells. The origin of SA-β-Gal activity was not being conclusively determined for some time, and its function during senescence establishment

remained unknown. Nowadays, we know that SA-β-Gal is encoded by the lysosomal-β-galactosidase gene (designated GLB1), usually active at acidic pH 4.5 (Lee *et al.*, 2006). The levels of lysosomal-β-galactosidase protein increase during senescence because there is an increase of lysosomal content, and SA-β-gal is detectable at the suboptimal pH of 6.0. Importantly, SA-β-gal is not a specific senescence marker per se; in fact, it is a surrogate marker for the increased number and activity of lysosomes.

The cell cycle inhibitor p16, an essential regulator of senescence, is also often used to identify senescent cells (Krishnamurthy *et al.*, 2004). However, p16 is expressed by many, but not all, senescent cell types (Krishnamurthy *et al.*, 2004), and it can also be expressed by some tumour cells, especially those that have lost Rb function. Some senescent cells can also be identified by cytological macro structures known as senescence associated-heterochromatin foci (SAHFs) (Narita *et al.*, 2003). SAHFs are distinct heterochromatic nuclear structures, recognised by the binding of the DNA dye 4',6-diamidino-2-phenylindole (DAPI), and by the presence of certain heterochromatin-associated histone modifications (such as H3K9 trimethylation) and proteins (such as heterochromatin protein-1 (HP1)). SAHFs contain and are known to repress E2F target genes. However, SAHFs are not detectable in all cell types undergoing senescence or in all kinds of senescence. Since pericentromeric foci are much more prominent in mouse cells than in human cells (Bartholdi, 1991; Cerda *et al.*, 1999), they can be mistaken for SAHFs.

DNA damage response (DDR) foci are present in senescent cells in different species, the most characterize are mice and humans. They contain proteins involved in DNA damage repair and checkpoint activation (D'Adda Di Fagagna *et al.*, 2003) (see paragraph *The DNA damage response (DDR)*). The use of primary cells shows that exogenous DNA damage induction may trigger a permanent cell cycle arrest (Di Leonardo *et al.*, 1994). Recently, cytosolic chromatin fragments (CCFs) were reported in senescent cells (Di Leonardo *et al.*, 1994). Cytosolic fragments are DAPI-positive structures enriched for the repressive chromatin marks and DDR

proteins. The presence of these cytosolic structures strongly correlates with the activation of SASP, another key feature of senescent cells (see below).

1.3 Replicative Senescence

Mammalian somatic cell types have a finite proliferative capacity in culture. Cell proliferation inevitably arrests under optimal growth conditions because of an intrinsic "clock". This "clock" is exquisitely sensitive to the number of cell divisions and not to the time in culture, suggesting that a crucial step in the cell cycle progression is "counted". This counting step turned out to be the number of DNA replication rounds. Because standard DNA polymerases cannot wholly replicate DNA ends, cells lose 50-200 base pairs of telomeric DNA during each cell cycle (Harley, Futcher and Greider, 1990). Human telomeres range from a few Kb to 10-15 Kb in length, and this means that before the block of the cell cycle, cells can perform many divisions. Since telomere attrition correlates with proliferative exhaustion, it was proposed to cause senescence establishment (Harley, Futcher and Greider, 1990). However, causality was proven only when the human telomerase reverse transcriptase (hTERT) construct was introduced in normal human cells allowing an unlimited cell proliferation *in vitro* (Bodnar *et al.*, 1998). Replicative senescent cells accumulate with age and contribute to ageing and age-associated pathologies (Van Deursen, 2014).

1.4 Stress-induced premature senescence

When cells grow *in vitro* and *in vivo*, they can be subjected to several cellular stressors like suboptimal cell culture conditions, hydrogen peroxide, ultraviolet radiations, ionizing radiations, and chemotherapeutic agents. After repeated or acute non-lethal doses, these cellular stressors induce a stress-induced premature cellular senescence (SIPS). Ionizing radiations (IRR) create DSBs resulting in the activation of the ATM-p53-p21 pathway within a few hours from treatment. p16 induction and SA-β-gal activity are

observed only several days after IRR treatment, delayed in comparison with the induced p53 pathway (Suzuki *et al.*, 2006). Ionizing radiation-induced DNA damage foci are protein complexes that include DSBs recognition and mediator proteins and mark DNA damage sites in a similar way cells track telomeric damage/shortening.

Many chemotherapy agents used to treat cancer, such as cisplatin and doxorubicin, can induce SIPS without affecting telomere length. However, chronic exposure to low concentrations of several molecules can inhibit DNA-synthesis like hydroxyurea, aphidicolin, or etoposide can also cause replication stress, leading to the activation of the senescence program after multiple DNA replications. During the treatment, some of the stalled forks can also collapse, causing the formation of DSBs with the consequent activation of the ATR/CHK1 pathway. The induction of classic G1-restricted cellular senescence in different cell types and several cancer cell lines following several DNA breaks in the genome *in vitro* shows that the senescence program can still be activated; this aspect is true also for cancer cell lines suggesting that the establishment of a senescence program can still occur. p53 and p16 pathways are both required for senescence induced by chemotherapeutic drugs. Indeed, many conventional anticancer agents cause DNA damage, which activates p53 through an ARF-independent mechanism. BCL2 and BCLXL, induced after senescence program establishment, can inhibit the pro-apoptotic pathways that can be p53-dependent and -independent. Drug-induced senescence is impaired but not wholly lost in cellular models that present a p53 or p16/ARF loss, whereas ARF deficiency alone is not sufficient to disable senescence activation. p16, like p53, can be induced by DNA damaging treatments, and both can cooperate to arrest the cell cycle and activate the senescence program (Schmitt *et al.*, 2002).

1.5 Oncogene-induced cellular senescence (OIS)

Oncogene activation is one step forward to cell transformation and cancer development. Expression of the constitutively activated form of

RAS, a cytoplasmic transducer of extracellular growth stimuli, in rodent cells can lead to their transformation when co-express with other oncogenes (Malumbres and Barbacid, 2003). However, it was observed that the sole activation of RAS results in a prolonged and irreversible cell cycle arrest in several mammalian cells derived from different tissues; several concomitant features linked to senescence were observed (Serrano *et al.*, 1997). This observation suggested that cell-intrinsic mechanisms respond and act as a barrier against oncogene activation. This type of senescence is established even if the telomere length is comparable to non-senescent cells. OIS is a tumour suppressive mechanism that arrests the proliferation of cells bearing activated oncogenes. Cells must override senescence barriers for cancer development, as demonstrated in mouse models and human premalignant lesions (Di Micco *et al.*, 2006) (Di Micco *et al.*, 2021b).

1.6 Therapy induced senescence (TIS)

Cancer cells, even though immortal by definition, can undergo senescence in response to radiation exposure or genotoxic chemotherapeutic agents' treatment; this phenomenon, named therapy-induced senescence (TIS), is triggered, as anticipated, by unrepaired DSBs into the DNA structure or by an accumulation of reactive oxygen species (ROS) and DNA damage, generally in response to mitochondrial damage (Prasanna et al., 2021).

It is well-established that TIS can exert both anti and pro-tumorigenic effects to date. On the one hand, it causes a persistent arrest of cell proliferation, either in the early or advanced phases of tumour development, hampering precancerous cells replication, promoting immune clearance and leading to tissue remodelling. From this point of view, cellular senescence is considered a positive outcome of cancer therapy. On the other hand, tumour development may be favoured by chronic accumulation of senescent cells, which are not removed by the immune system and may even re-enter the cell cycle. Indeed, a recent study showed that TIS cells start showing stem cell-related characteristics that promote escape from cell cycle arrest after

prolonged time in culture, thus prompting tumour relapse or favouring a more aggressive growth (Fitsiou, Soto-Gamez and Demaria, 2021). Under physiological conditions, SASP has beneficial effects triggering senescence surveillance and promoting senescent cells elimination through the immune system cells recruitment; however, in the context of cancer, the adverse impact of TIS cells persistence after the treatment mainly relies on the SASP activation, which, indeed, creates a proinflammatory microenvironment thus favouring angiogenesis, tumour cells proliferation and, eventually, development of neoplastic pathologies (Battram, Bachiller and Martín-Antonio, 2020).

1.7 The DNA Damage Response (DDR)

Any damage introducing a variation in the nuclear DNA double helix causes a cellular response. An evolutionarily conserved signalling pathway senses DNA damage and transmits this information by starting an amplification cascade signal called DNA damage response (DDR) (Shiloh, 2006). DDR activation by inducing cell cycle arrest prevents the inheritance of damaged or aberrant DNA to daughter cells to block the propagation of corrupted genetic information. Moreover, DDR coordinates cellular machinery to restore genome integrity.

Since DNA is the only cellular molecule that is specifically repaired instead of being replaced, it is easy to understand the importance of functional DNA damage sensors and repair systems for genome integrity. To guarantee genome stability, there are several DNA repair pathways. The choice of which system is better to activate depends on the type of lesion and the cell-cycle phase of the cell. DDR is generally categorised into four different types: base excision repair (BER) and nucleotide excision repair (NER), which are involved in the repair of lesions such as modified bases; mismatch repair (MMR), which plays a role when normal bases pair with wrong partners; and double-strand break repair (DBR), which is divided in homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways. BER pathway functions throughout the cell cycle; MMR is

essential to remove mismatches, small insertions, or deletion loops generated by faulty replication.

1.8 The p53 and RB pathways

DNA damaging agents trigger cell senescence via activation of the DDR. Common knowledge is that while mild DNA damage can induce only a transient growth arrest, persistent DNA damage induces cell senescence with a persistent cell cycle arrest. The molecular players that regulate the switch from transient to irreversible growth arrest are not yet fully understood. The senescence program caused by p53-p21 and p16-RB tumour suppressor pathways triggers a growth arrest.

Stimuli that generate DNA damage (for example, ionizing radiation and telomeric dysfunction) induce DDR activation that downstream triggers senescence program primarily through the p53 pathway. Several proteins regulate this pathway at different levels, such as the E3 ubiquitin-protein ligase HDM2 (MDM2 in mice), promoting p53 degradation. In addition, the alternate-reading-frame protein (ARF) controls p53 by inhibiting HDM2 activity (Sherr and McCormick, 2002; Di Micco *et al.*, 2006). p21 is a crucial transcriptional target of p53 and mediator of cell cycle arrest and then p53-dependent senescence (Brown, Wei and Sedivy, 1997). Experimental impairment of p53, p21 or DDR factors (for example, ATM or CHK2) prevents the activation of senescence program in response to telomere shortening or DNA damage establishment (Brown, Wei and Sedivy, 1997; Beauséjour *et al.*, 2003; D'Adda Di Fagagna *et al.*, 2003).

Stimuli that activate DDR can also engage the p16-RB pathway, although this usually occurs after the engagement of the p53 pathway (Jacobs and De Lange, 2004). Nonetheless, some pro-senescence stimuli act primarily enhancing the activation of the p16-RB pathway; this aspect is well described for epithelial cells because this model is more prone than human fibroblasts to induce p16. Another element to keep in mind is the species-specific difference for senescence estabishment: while mouse cells mainly activate the p53 pathway, human cells activate both p53 and p16-RB

pathways (Smogorzewska and De Lange, 2002). Recently, in T cells, the expression of PD-1 was associated with a massive DDR promoting p16-RB pathway activation (Janelle et al., 2021). Another way to activate p16 without activating p53 is to reduce Polycomb INK4a repressor expression such as BMI1 (Itahana *et al.*, 2003; Bracken *et al.*, 2007) and CBX7 (Gil *et al.*, 2004) when cells undergo senescence.

1.9 The senescence-associated secretory phenotype

The senescence pathway is induced when a damaged/stressed cell cannot repair the damage or resolve the stress. Senescent cells present a persistent DDR activation and an increased secretion of growth factors, proteases and pro-inflammatory molecules collectively known as SASP (Coppé *et al.*, 2008, 2010). In a cell-autonomous manner, SASP reinforces senescence. However, SASP can also act in a paracrine manner inducing normal neighbouring cells into senescence. This process promotes premature ageing and decreases tissue regeneration. SASP contributes to many age-related diseases, including type 2 diabetes and atherosclerosis. An important aspect of cellular senescence in ageing and age-related disease is the production of SASP factors, particularly those that promote directly or indirectly chronic inflammation. IL-6 and IL-8 are just some of the SASP factors able to cause an inflamed environment, indeed a variety of MCPs (monocyte chemoattractant proteins) like CCL2, MIPs (macrophage inflammatory proteins) and other factors able to regulate several aspects of inflammation, such as interleukin 1 (IL1α and IL1β) and GM-CSF (granulocyte/macrophage colony-stimulating factor). Chronic inflammation is a cause, or at least an essential contributor to, virtually every significant age-related disease, both degenerative and hyperplastic (Acosta *et al.*, 2013).

At the same time, SASP can also recruit the immune system to recognize senescent cells and efficiently eradicate them. This aspect is crucial, especially in the context of OIS. Indeed, if the immune system does not

clear a senescent cell with an activated oncogene, it may accumulate further mutations that favor escape from senescence and tumour growth.

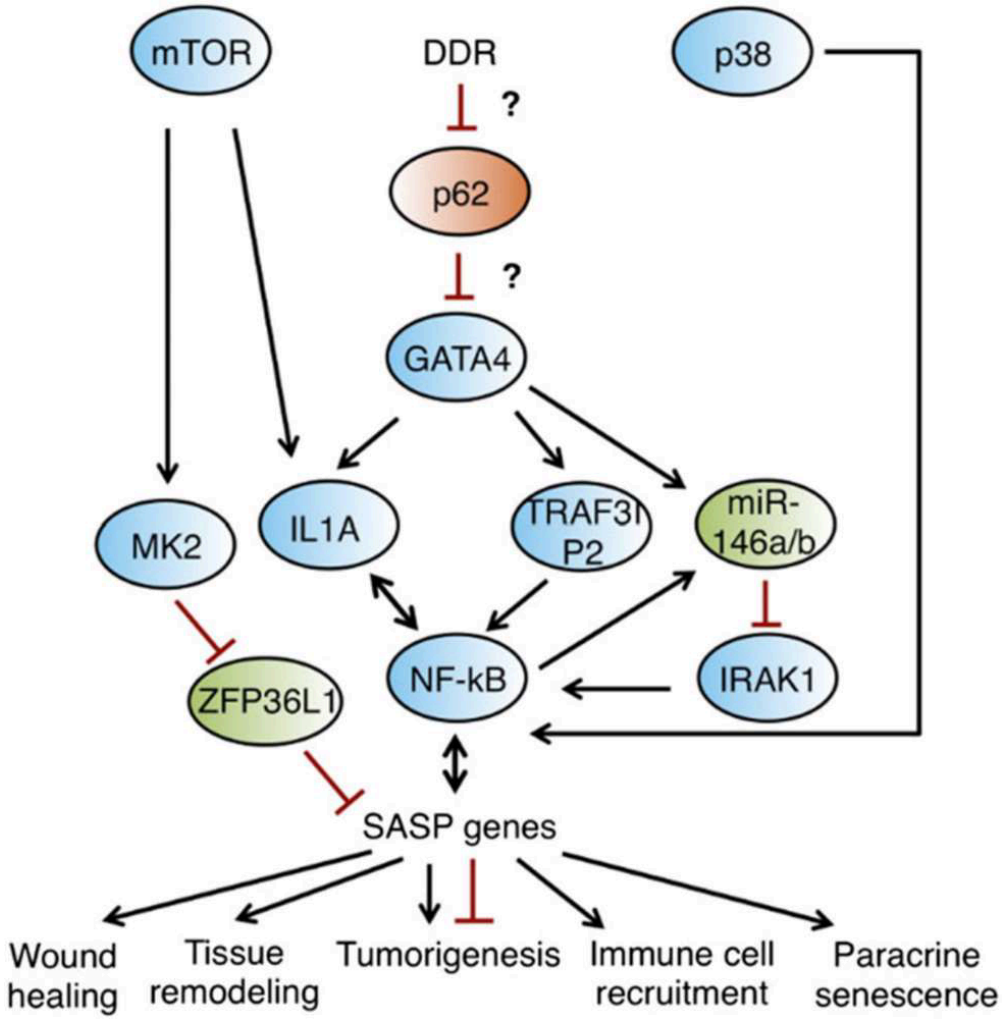


Figure 2. Senescence-associated secretory phenotype (SASP) regulation. *(from Lujambo, BioEssays vol.38, 2016)*

The molecular regulators of SASP and the function of specific SASP effectors are a matter of intense investigation. We now know that secretion of SASP factors in senescent cells appears to be regulated through p38MAPK (Freund, Patil and Campisi, 2011) and NFκB pathways (Chien *et al.*, 2011a). A few years ago, the epigenetic reader BRD4 was identified as a new master regulator of SASP (Tasdemir *et al.*, 2016a). It was reported that BRD4 specifically binds clusters of regulatory elements at SASP genes, and its genetic or chemical inhibition reduces SASP expression. Senescence immune clearance strongly depends on BRD4 as its genetic inhibition in oncogene-expressing hepatocytes is sufficient to impair senescence

immune-surveillance. Although the mechanisms underlying SASP composition and generation are currently not understood, it is well assessed that the key regulators of SASP activation are the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the CCAAT/enhancer-binding protein (C/EBPβ). C/EBPβ regulates different SASP composing cytokines, among which the IL-1β, IL-8, IL-6, GROα and NAP2; moreover, it has been demonstrated that this factor is required to induce senescence, indeed, during OIS, it is upregulated and acts by binding to the promoter of IL6. Concerning NF- κB, in the senescence context, its expression relies mainly on p38MAPK and GATA4; the first acts by increasing NF-κB binding to the DNA, while the second promotes the production of IL-1A, which, in turn, activates NF-κB (Paciência *et al.*, 2019). Under normal conditions, GATA4 binds p62 (also called sequestrosome), leading to its degradation via autophagy, while in senescent cells, this interaction is reduced, thus increasing GATA4 expression. Therefore, GATA4 presence leads to the tumour necrosis factor receptor-associated factor interacting protein 2 (TRAF3IP2) and the IL1A, promoting NF-κB activation, as shown in the schematic representation in Figure 4. The induction of the GATA4 pathway mainly relies on ATM and ATR and thus primarily correlates with the DDR activation; however, to date, it is still unknown the mechanism by which they act on p62 (Lujambio, 2016).

Moreover, SASP regulation also depends on the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, the central cytokines-signalling pathway (Birch and Gil, 2020). Indeed, a decrease in senescence and SASP expression has been demonstrated by inhibiting this signalling pathway, thus preventing the ageing process (Chen *et al.*, 2020).

Recently, work in breast cancer reported a different SASP production due to a different pro-senescence stimulus (Wang *et al.*, 2021a). Senescent cells could not produce cytokines via the NFkB pathway without DNA damage than those treated via Doxorubicin administration. Moreover, when this treatment was done in a mouse model, mice undergoing doxorubicin

treatment showed a pro-inflammatory phenotype driving persistent weight loss, anaemia, and decreased survival.

Finally, the SASP is a plastic phenotype; in fact, SASP factors differ depending on two main aspects: the cell types and the insult that triggered the senescence response. Nevertheless, there is substantial overlap among SASP. The damaging effects of SASP have motivated researchers to develop chemicals that limit SASP to counteract age-associated disorders.

1.10 Cytosolic Chromatin in Senescence

A recent link between DDR, SASP and DNA innate sensing was established (Chen, Sun and Chen, 2016; Dou *et al.*, 2017; Yang *et al.*, 2017). Indeed, it was reported that in senescent fibroblasts, cytosolic chromatin fragments (CCFs) are released upon DNA damage, and these fragments are enriched in γH2AX (a mark of DNA double-strand breaks (DSBs)) and characterized by the two repressive chromatin markers histone H3 lysine-27 trimethyl and histone H3 lysine-9 trimethyl.

The mammalian innate DNA sensing machinery is defined as the first defense line to degrade the microbial and viral DNA accumulated during infections. The sensor of this machinery is cyclic GMP–AMP (cGAMP) synthase (cGAS) and recognize the non-self double-stranded DNA. After the activation of cGAS, we can observe a massive production of 2',3'-cGAMP; this is known to be its second messenger and thanks to it, cGAS activates the stimulator of IFN genes (STING) to induce a type I IFN response. The cGAS–STING pathway may also recognize self- or tumour-derived dsDNA molecules released in the cell's cytosol. The cGAS–STING pathway recognizes CCFs and activates the senescence-associated pro-inflammatory program devoted to cell-autonomous (autocrine) and paracrine senescence functions. The release of CCFs was linked to reduced integrity of the nuclear membrane caused by the reduction of lamin B1 that has been reported in senescent cells (Adams *et al.*, 2013). To support these data, it was reported that lamin B1 depletion in human fibroblast cell lines increased the production of CCFs, while lamin B1 over-expression impaired

their generation. At the same time, lamin B1 upregulation correlated with a downregulation of the SASP factors even if cells were subjected to DNA damage; this experiment showed the link between CCFs release from the nucleus and the cGAS/STING pathway triggering SASP production.

1.11 Transposable elements

For a long time, transposable elements (TEs) were considered "junk DNA" observed after several viral infections and randomly integrating into the genome. TEs can generate genomic instability (Ayarpadikannan and Kim, 2014), so, to prevent this, cells have developed strategies to control their expression and their retrotransposition. For instance, epigenetic repression via DNA methylation (Moolhuijzen *et al.*, 2010) or histone modifications (Varshney *et al.*, 2015) and post-transcriptional mechanisms are key players to keep TEs under control.

TE retrotransposition involves the transcription by cellular RNA polymerases (Ayarpadikannan and Kim, 2014), and the three prominent TE families are LINE-1 elements (L1), Endogenous Retroviruses (ERVs) and *Alu. Alu* is dividend in other subfamilies: the older *Alu* J, the younger *Alu* Y and the intermediate *Alu* S subfamilies (Conti *et al.*, 2016). As expected, most L1, ERVs and *Alu* elements (*Alus*) are transcriptionally inactive because of a tightly controlled epigenetic program involving either DNA methylation or histone modifications.

TE retrotransposition and aberrant expression are common features of neoplastic transformation (Cordaux and Batzer, 2009); however, TE also become transcriptionally active in specific stages of embryogenesis.

Expression of retroelements has been detected by RNA-sequencing (RNA-seq) approaches in human cells upon genotoxic stimuli and cancer cell lines compared to the normal counterpart (Conti *et al.*, 2015).

Retrotransposons expression is activated in response to different stressors, like DNA damage, heat shock or treatment with genotoxic agents (Zhang, Chen and Zhao, 2015a) and senescence-inducing stimuli. Indeed, Alu induction was reported *in vitro* in replicative senescence (Wang,

Beauchemin and Bertrand, 2012), with senescence bypass observed after stable suppression of *Alu* transcription. Moreover, the transcriptional reactivation of the sole ERVs family following epigenetic therapy has been conversely linked to immune cell activation in solid tumours (Grundy, Diab and Chiappinelli, 2022), also suggesting a beneficial role of TE reactivation to eradicate cancer cells.

A few examples of DNA hypomethylation at retroelements loci were reported in some cancers (Carreira et al., *Febs j* 2012); however, changes in DNA methylation patterns cannot fully account for altered TE expression in cancer, pointing towards a regulation that involves histone post-translational modifications for TEs reactivation.

More recently, a work described increased L1 expression in senescent fibroblasts; in particular, it was reported that upregulation of L1 represent an upstream mediator of secretion of SASP and type-I interferon (IFN-α, IFN-β) (De Cecco *et al.*, 2019), through recognition by the cGAS/STING pathway of L1 DNA in the cytosol of senescent cells.

1.12 Beneficial and detrimental effects of cellular senescence

Cellular senescence is associated with beneficial and detrimental effects achieved through cell-autonomous and non-cell-autonomous mechanisms, as shown in Figure 3. On one hand, it represents an evolutionarily conserved mechanism that plays a central role in tissue homeostasis, embryonic development, and tumour suppression; on the other, its chronic inflammatory phenotype promotes many diseases such as atherosclerosis, neurodegeneration, diabetes, and cancer itself (Rodier and Campisi, 2011; Muñoz-Espín and Serrano, 2014).

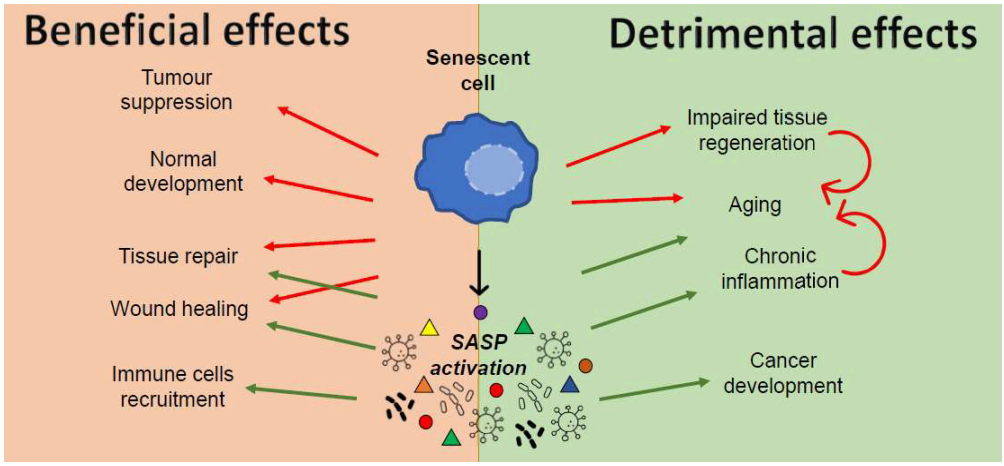


Figure 3. Beneficial and detrimental effects of cellular senescence *(Adapted from Lecot P. et al., Br J Cancer 114, 2016)*

Beneficial aspects

To date, it is well established that cellular senescence can act as a tumour suppressor mechanism by arresting the proliferation of premalignant cells. Indeed, during the early stages of tumorigenesis, oncogenic signalling leads to p16INK4A and p53 pathways activation, thus preventing tumour growth and avoiding the accumulation of new mutations that may confer additional advantages to cancer development.

However, senescence has been associated with other beneficial effects over the years. First, different studies have demonstrated that senescence promotes tissue repair and wound healing. Indeed, even though the production of extracellular matrix (ECM) aids the repair of the damage, in the presence of an excessive deposition, it promotes tissue fibrosis, therefore avoiding the appropriate repair (Amor *et al.*, 2020). Senescence induction, in turn, can limit the excessive fibrotic tissue formation; however, accumulation of senescence cells may promote fibrosis as well through the secretion of different SASP factors, suggesting that depending on the duration of their presence and their level of activation, senescent cells may have a different impact in the process (Rhinn, Ritschka and Keyes, 2019).

In addition, during embryonic development, senescence favours tissues and organs remodelling in the absence of detectable DNA damage and DDR markers (Muñoz-Espín *et al.*, 2013; Storer *et al.*, 2013). Specifically, in this

setting, senescent cells express high levels of SA-β-Gal activity, increased heterochromatin markers (as H3K9me3) and increased expression of the cell cycle inhibitors p21 and p27; however, interestingly, p16INK4A and p53 are absent, suggesting that senescence has a different function between embryonic and adult life (Storer *et al.*, 2013).

Moreover, another relevant positive effect of cellular senescence is achieved through the SASP activation and, specifically, via its proinflammatory functions. Indeed, senescent cells can favour the recruitment of cells of the immune system; specifically, SASP secreted factors induce natural killer (NK) cells activation, which, in turn, remove oncogene-expressing cells (either OIS or senescence-escaped ones) and promote the clearance of damage-induced senescent cells (Lecot et al., 2016). These phenomena can be driven by T-helper cells, which directly recognize senescent cells, or by chemokines and cytokine secretion, which leads to the recruitment of inflammatory phagocytes or the activation of T-cell mediate adaptive immunity (Campisi, 2013; Lecot *et al.*, 2016). Therefore, cellular senescence can also exert its tumour suppressor function *in vivo* in a non-cell-autonomous manner, inducing the immune system cells to target premalignant or malignant cells. This aspect will be described in detail in Chapter 3.

Detrimental effects of cellular senescence

Despite the positive aspects of senescence program activation, it is currently well-established that cellular senescence via cell-autonomous and non-cell-autonomous processes may also lead to deleterious effects. Thereby, cellular senescence may hamper tissue repair or cause tissue and organismal ageing through senescent cells accumulation or reduction of stem and progenitor cells. The deleterious effects are mainly driven by the SASP (Kumari and Jat, 2021). Throughout the physiological ageing process, SASP activation upon senescence induction favours the secretion of an array of proinflammatory molecules, thus prompting the development of a condition termed “inflammageing”. This phenomenon may trigger different

chronic age-associated diseases, such as neurodegenerative disorders, cardiovascular disorders, atherosclerosis, frailty, and even cancer (Di Micco *et al.*, 2021a). For example, it was shown that senescent astrocytes and their secreted SASP factors have a role in Alzheimer's and Parkinson's pathogenesis (Bitto *et al.*, 2010; Salminen *et al.*, 2011). Likewise, the presence of SASP-producing senescent chondrocytes, which are prominent in age-related osteoarthritic joints, seems to contribute to this disease's aetiology and promotion (Roberts *et al.*, 2006; Shane Anderson and Loeser, 2010).

Moreover, it was reported that age-associated inflammation and SASP-factor accumulation might influence stem cells to function either in a cell-autonomous or paracrine manner (Di Micco *et al.*, 2021b). Even though senescence was firstly associated with a tumour suppressive role, to date, it has been demonstrated that again, mainly via SASP activation, senescence may exert protumorigenic functions. First, senescent cancer cells accumulation by the SASP correlates with more aggressive behaviour of cancer cells (Biavasco *et al.*, 2021) (Krtolica *et al.*, 2001). Moreover, SASP promotes the formation of a proinflammatory microenvironment, thus creating a supportive niche for cancer development. Indeed, the secretion of different proteases, part of the SASP factors, leads to ECM degradation, promoting tissue remodelling and either tumour cells invasion or metastasis. SASP factors can also favour tumour development via increased angiogenesis, thus prompting vascularisation (Lecot *et al.*, 2016). Last, SASP factors create an immunosuppressive niche via the secretion of cytokines in charge of recruiting myeloid-derived suppressor cells, therefore hampering the T cells-mediate tumour cells death (Toso *et al.*, 2014) (Figure 4).

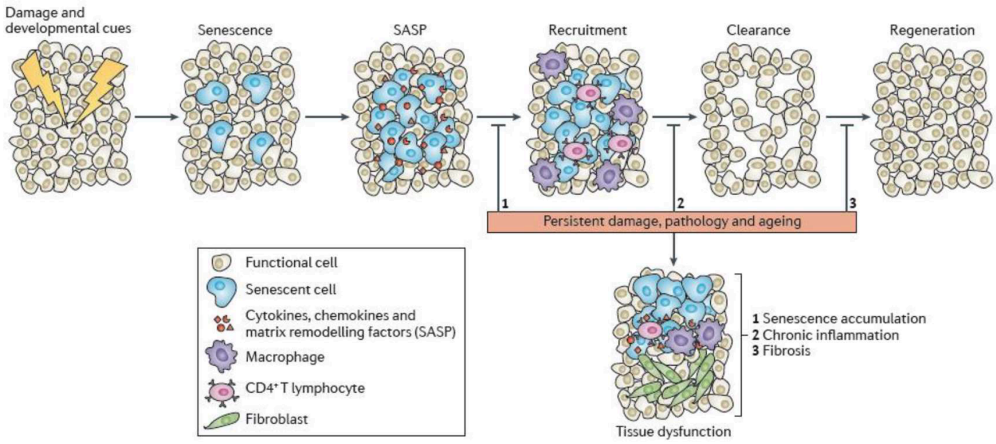


Figure 4. A unique model of cellular senescence program, including its positive and negative effects. *After damage and developmental cues, the senescence program recruits immune cells through the senescence-associate secretory phenotype (SASP). Macrophages and T lymphocytes clear senescence cells while progenitor cells contribute to the repopulation and regeneration of damaged tissues. Upon persistent damage, chronic inflammation and ageing, clearance and regeneration may be compromised with an accumulation of senescent cells and fibrosis* (Muñoz-Espín and Serrano, 2014).

1.13 Senescence as a therapeutic target

Both pro-senescent and anti-senescent approaches can be desirable depending on the therapeutic context. In cancer, for example, TIS can be used as a therapeutic goal. In this context, senescence can be achieved by applying systemic pro-senescent stressors (Sabin and Anderson, 2011). One example is the treatment for acute promyelocytic leukaemia; in this leukaemia, the senescence program is an essential component of therapy and is induced by the administration of arsenic trioxide–retinoic acid (ATRA). ATRA blocks an oncogenic fusion protein activating pro-senescent p53, signalling (Ablain *et al.*, 2014).

Another example is cyclin-dependent kinases 4/6 (CDK 4/6) inhibitors, small molecules considered as p16 mimetics, used for the first time to treat both types of hormone receptor and HER2 positive breast cancers. CDK4/6 inhibitors act blocking the interaction between Cyclin D1 and CDK4/6. Hence, cells block their progression in the cell cycle: in this case, Cyclin D1 is involved in the G1 phase, so cells are arrested in this phase when treated with this inhibitor. Specifically, it has been demonstrated that there is

senescence induction in both melanoma and breast cancer cells upon Palbociclib treatment, a specific CDK 4/6 inhibitor (Fitsiou, Soto-Gamez and Demaria, 2021). Aurora-A kinase inhibitors are other compounds used in cancer therapy. Indeed, the Aurora kinase family comprises serine/threonine kinases involved in mitosis regulation, frequently overexpressed in tumours (Katayama and Sen, 2010). Different studies have shown that diverse Aurora A kinase inhibitors can induce senescence in the context of different types of tumours (Fitsiou, Soto-Gamez and Demaria, 2021). As previously presented, both haematological and solid tumours benefit from senescence-inducing therapies.

The inactivation of oncogenes like c-Myc (Wu *et al.*, 2007) or Shp2 (Serrano, 2015) or restoring tumour-suppressive signalling such as PTEN (Toso *et al.*, 2014) are crucial goals for cancer cells to respond to intrinsic damage and activate senescence program usually.

Therefore, standard chemotherapeutics and new generation drugs can trigger cellular senescence in different cancer models and limit tumour growth; however, the mechanisms involved in this phenomenon are different and, in some contexts, not fully understood. In the context of cancer, an essential aspect in TIS to keep in mind is the possibility of eventually removing senescent cells, thus preventing their accumulation and hampering the pro-tumorigenic effects of SASP as described previously (Sun, Coppé and Lam, 2018a). Indeed, while senescence induction has a beneficial impact on tumour development in the short term, it is a pro-survival mechanism allowing the persistence of cancer cells. This persistence may favour tumour progression in a cell-autonomous manner promoting reprogramming into stem-like, more aggressive cancer cells (Milanovic *et al.*, 2018). For these reasons, over the years, new agents and strategies have been developed to specifically target senescent cells, favouring their elimination and therefore preventing and/or treating age-related diseases and cancers. Specifically, a new class of small molecules, termed senolytic drugs, has been introduced.

First, senescent cells do not divide and express high levels of the anti-apoptotic proteins BCL-2 and BCL-XL (Chang *et al.*, 2016a), leading to apoptosis resistance; these data suggested that senescent cells succumb to apoptosis by inhibiting these factors. Therefore, the first attempts were to inhibit these molecules from inducing apoptosis in senescent cells (Zhu *et al.*, 2015).

One of the most characterized senolytic drugs is the ABT263 molecule (Navitoclax), which binds to BCL-XL preventing its binding to BIM; this compound sequesters proapoptotic molecules, triggering apoptosis (Yosef *et al.*, 2016a; Mezei *et al.*, 2021). Researchers developed a p16-3MR transgenic mouse reporter to identify, monitor, and selectively target senescent cells to test the effects of senescence eradication. These mice carry a trimodal reporter protein (3MR) under the control of the p16 promoter (Figure 5). The 3MR-encoding transgene encodes for a fusion protein consisting of Renilla luciferase (for bioluminescent imaging), for a monomeric red fluorescent protein (mRFP, for sorting and fluorescence microscopy) and for herpes simplex virus thymidine kinase (HSV-TK, which converts ganciclovir (GCV) into a toxic DNA chain terminator to selectively kill HSV-TK–expressing senescent cells) (mouse model description is taken from Demaria et al., 2014). Taking advantage of this model system, researchers could compare the effects of Ganciclovir and the impact of ABT263 and reported a similar clearance of senescent cells in response to DNA damaging agents and physiological ageing.

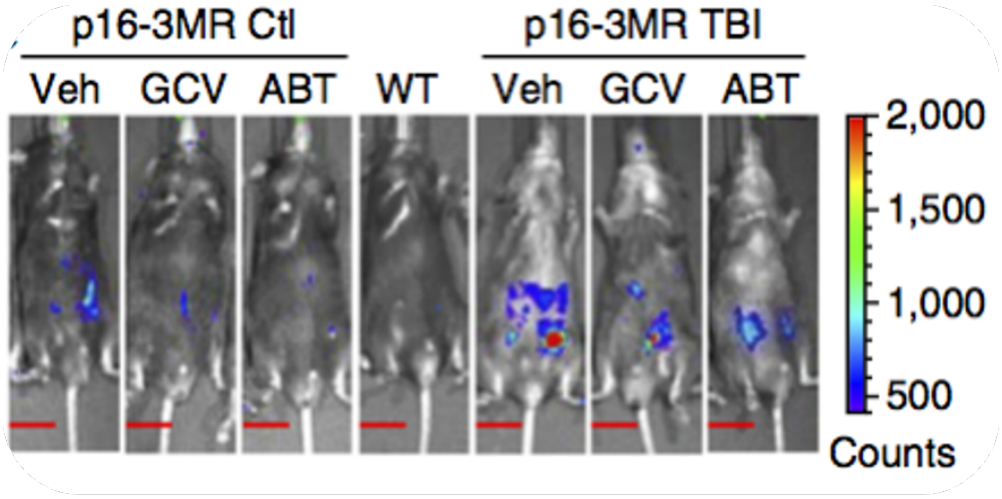


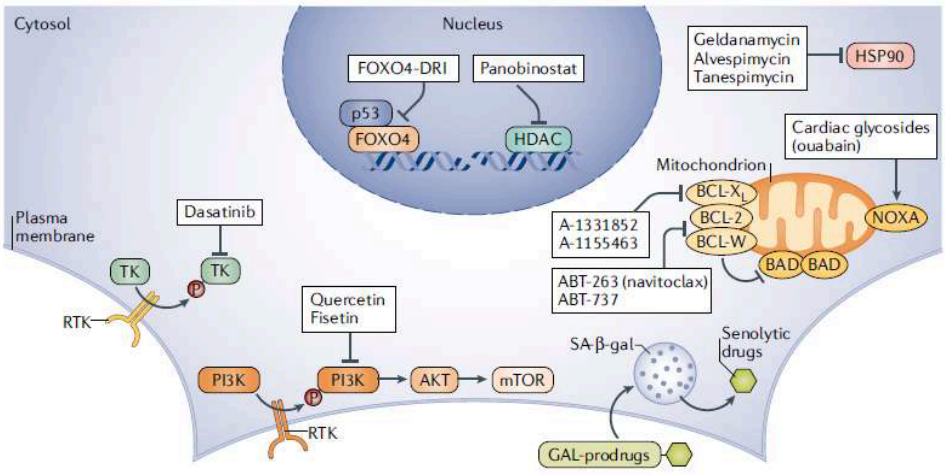
Figure 5. Bioluminescence assay of senescent cells *taken from* (Chang *et al.*, 2016b)

Other well-characterized senolytic compounds are ABT737, a BH3 small-molecule inhibitor that binds to and inhibits BCL-2, BCL-XL and BCL-w and proxofim, a peptide that promotes the apoptosis of senescent cells by blocking the binding between p53 and the forkhead box protein O4 (FOXO4), removes p53 from the nucleus, which, in turn, triggers cytochrome c release. Last, other compounds reported as senolytics are HSP90 inhibitors, piperlongumine (a small natural molecule) analogues and some antibiotics, which have been shown to act on DNA damage-induced senescent cells via metabolic changes (Di Micco *et al.*, 2021b).

Other two senolytic agents discovered are Dasatinib, an inhibitor of multiple tyrosine kinases used for treating cancers, and Quercetin, a natural flavonoid inhibiting PI3K and serpines. Senolytic flavonoids inhibit BCL-2 family members or influence other SCAP factors (Kirkland and Tchkonia, 2020). Notably, the potential beneficial effect of a cocktail of senolytic drugs, Dasatinib plus Quercetin already in clinical trial (Hickson *et al.*, 2019), was reported to selectively eliminate senescent cells and attenuate secretion of frailty-related inflammatory cytokines in both senescent cells cell-transplanted young mice and physiologically aged mice (Xu *et al.*, 2018).

Furthermore, controlling the induction of SASP factors is also a promising approach to counteract chronic inflammation and prevent the

adverse effects of senescence. Among these compounds, named "senomorphic drugs", there are several blockers of the function of crucial inflammatory mediators such as IL-1 (Anakinra; blocking IL1 receptor 1), IL-6 (Tocilizumab; monoclonal antibody against IL-6R), TNF-alfa (Infliximab; chimeric antibody against TNF) (Laberge *et al.*, 2015; Prattichizzo *et al.*, 2016) or inhibitors of the SASP transcriptional network, such as NF-kB (Chien *et al.*, 2011b) mTOR signalling (Herranz *et al.*, 2015; Laberge *et al.*, 2015) or BRD4 (Tasdemir *et al.*, 2016b) inhibitors (Figure 6). Moreover, the upregulation of non-canonical HLA-E molecule (HLA-I) on the surface of senescent cells was reported to inhibit NK and $CD8^+$ T cells. The upregulation of this inhibitory molecule is promoted by SASP production (Pereira *et al.*, 2019). In this work, researchers found that this ligand is also overexpressed in human skin from old subjects compared to young ones.



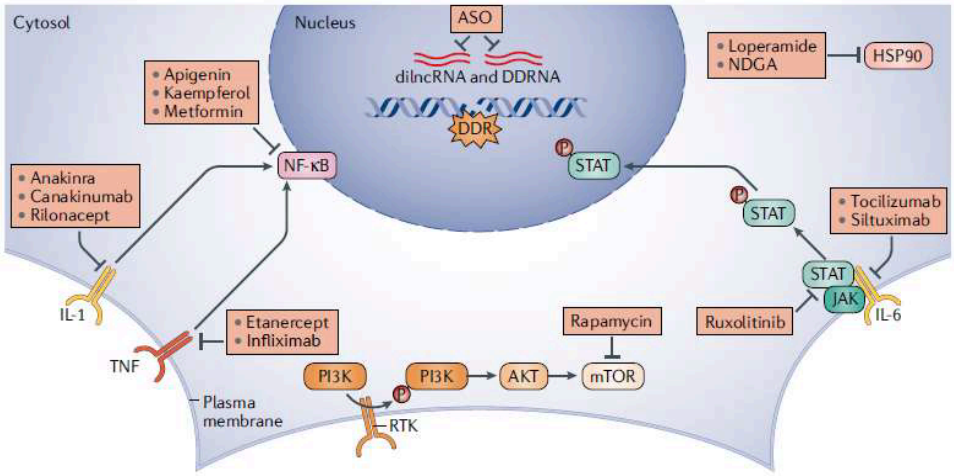


Figure 6. Different senolytics and senomorphics therapeutic interventions (Di Micco *et al.*, 2021b)

One crucial aspect of senolytic therapy is its possible systemic side effect (i.e., off-target toxicity); indeed, so far, only the combination of Dasatinib and Quercetin has been introduced in a clinical trial. To overcome senolytics toxicity, a new drug delivery system was developed based on nanoparticles technology (Muñoz-Espín *et al.*, 2018; González-Gualda *et al.*, 2020). These particles can enter any cell and the specificity for senescent cells consists in the drug release: the particles are indeed made by Gal-oligomer that are processed by SA-β-GAL only in senescent cells. After particle degradation, the senolytic compound is released to eliminate senescent cells.

2 Acute Myeloid Leukaemia

2.1 Leukemia Initiation

Blood cells are produced in the bone marrow, the cancellous tissue found inside bones. Blood-forming stem cells called hematopoietic stem cells (HSCs) divide to produce either more HSCs or cells that become mature over time. An HSC may commit towards the myeloid or lymphoid differentiation. The majority of HSCs divide in an orderly way when pushed into differentiation. At the same time, they die or are cleared by the immune system when they are damaged, and new cells take their place. However, HSPCs can accumulate mutations that confer a proliferative advantage over time, leading to malignant transformation.

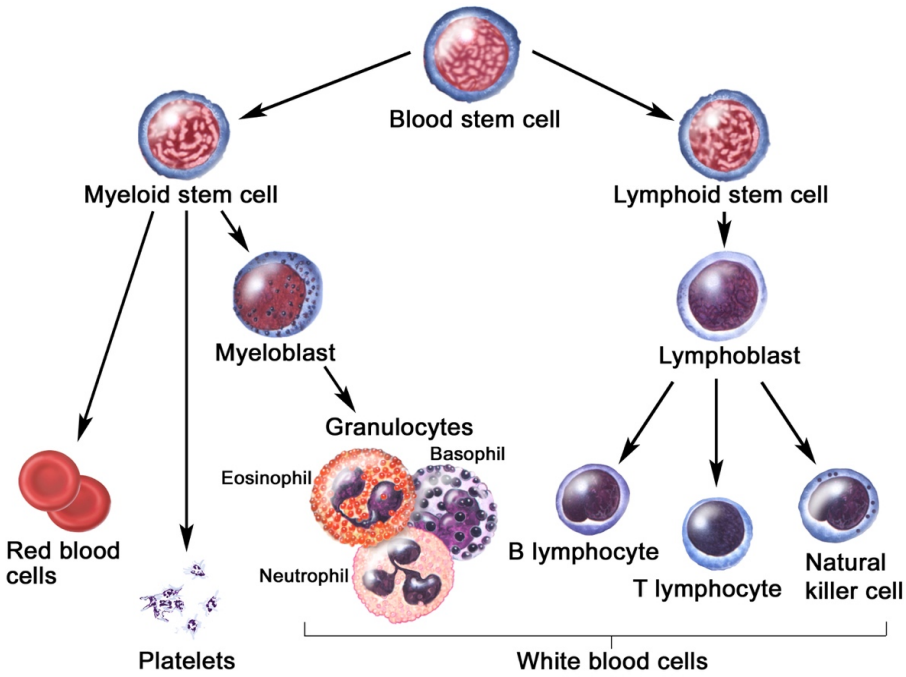


Figure 7: Hematopoiesis and differentiation of blood cell types *from Terese Winslow et al.*

AML is a hematologic malignancy characterised by the expansion of aberrant myeloid cells infiltrating the bone marrow, peripheral blood and

occasionally other tissues. Leukemic cells take over the healthy hematopoietic system by uncontrolled proliferation resulting in multilineage cytopenia. Predominant disease symptoms arise because of the lack of normal blood cells with bleeding and haemorrhage, associated with thrombocytopenia, fatigue and dyspnoea, linked to anaemia, and susceptibility to infections from neutropenia. Aberrant leukemic cells infiltrating extra-medullary organs by impairing their normal functioning can give rise to various associated clinical manifestations. AML is the most common acute leukaemia in adults. Its incidence increases with age, ranging from 1 to 2 cases per 100'000 within the younger population and reaching a maximum of 12 cases per 100'000 in the over 65 years age group. It is most frequently diagnosed amongst the elderly with a median age at diagnosis of 68 years (seer.cancer.gov/statfacts/html/amyl.html).

A common way to identify minimally differentiated cells consists in analyzing surface markers. We can distinguish between AML and acute lymphoblastic leukaemia (ALL) by flow cytometry. Leukaemias are also classified by how the disease progresses as acute or chronic leukaemias. Acute and chronic do not give the patient's prognosis but just tell us how rapidly it goes. Acute leukaemia progresses quickly unless effectively treated; however, it can often be cured taking advantage of standard treatments, such as chemotherapy or bone marrow transplantation, the second one especially in younger and/or fitter patients. A different situation occurs in chronic leukaemia: the disease progresses slowly, but it is not usually possible to cure chronic leukaemia with standard treatments.

It is known that within the bulk of cancer, there is a small population of stem cell-like cells responsible for disease initiation and progression. In the context of leukaemia, leukemic stem cells (LSCs) seem to be placed at the top of the leukemic hierarchy, therefore giving rise to more differentiated leukemic blasts displaying a high proliferative capacity, a block in terminal differentiation and defects in the apoptotic or senescence pathways; this promotes blast accumulation and fuels the disease. Like all leukaemia blasts, markers that describe LSCs are shared with primitive HSPCs, and

present hCD45^{+}/dim, CD34^{+} positivity and absence of CD38 (Hanekamp, Cloos and Schuurhuis, 2017).

Since self-renewing HSCs persist for a prolonged time without being removed, these cells are generally characterised by genomic instability, promoting malignant transformation. Therefore, it is believed that HSCs serve as cancer-initiating cells (cell-of-origin) for LSCs.

2.2 Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a malignant disease characterized by an accumulation of immature myeloid blast cells in the bone marrow. In a second time, these blasts spread to the peripheral blood. The clonal expansion of myeloid precursor cells in AML interferes with normal myelopoiesis. It results in impaired function of normal blood cells, which leads to AML associated symptoms, including fatigue, bleedings and severe infections, some of which are lethal. In most AML cases, there is no apparent underlying cause, but some risk factors correlate with disease initiation and progression (De Kouchkovsky and Abdul-Hay, 2016). Age is the first risk factor; in fact, AML is more common in older people, particularly males.

The diagnosis of acute leukaemia is established by 20% or more blasts in the bone marrow or peripheral blood (Lowenberg, Downing and Burnett, 1999). AML blasts have a variable size ranging from slightly more prominent than lymphocytes to the size of monocytes; moreover, they display larger nuclei with aberrant shapes. AML blasts are characterized by the expression of antigens also shared by the healthy immature myeloid counterpart, including common differentiation (CD) markers CD13, CD33 and CD34 (Saultz and Garzon, 2016). AML is a highly heterogeneous disease with a low mutational burden; the first attempt to classify this disease in several subgroups was based on morphological criteria. This classification is the French-American-British (FAB) and can generate nine subgroups (Figure 8).

FAB Subtype	Common Name (% of Cases)	Results of Staining			Associated Translocations and Rearrangements (% of Cases)	Genes Involved
		Myeloperoxidase	Sudan Black	Nonspecific Esterase		
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	−	−	−*	inv(3q26) and t(3;3) (1%)	*EVI1*
M1	Acute myeloblastic leukemia without maturation (15–20%)	+	+	−		
M2	Acute myeloblastic leukemia with maturation (25–30%)	+	+	−	t(8;21) (40%), t(6;9) (1%)	*AML1-ETO, DEK-CAN*
M3	Acute promyelocytic leukemia (5–10%)	+	+	−	t(15;17) (98%), t(11;17) (1%), t(5;17) (1%)	*PML-RARα, PLZF-RARα, NPM RARα*
M4	Acute myelomonocytic leukemia (20%)	+	+	+	11q23 (20%), inv(3q26) and t(3;3) (3%), t(6;9) (1%)	*MLL, DEK-CAN, EVI1*
M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils (5–10%)	+	+	+	inv(16), t(16;16) (80%)	*CBFβ-MYH11*
M5	Acute monocytic leukemia (2–9%)	−	−	+	11q23 (20%), t(8;16) (2%)	*MLL, MOZ-CBP*
M6	Erythroleukemia (3–5%)	+	+	−		
M7	Acute megakaryocytic leukemia (3–12%)	−	−	+†	t(1;22) (5%)	Unknown

*Cells are positive for myeloid antigen (e.g., CD13 and CD33).

†Cells are positive for α-naphthylacetate and platelet glycoprotein IIb/IIIa or factor VIII–related antigen and negative for naphthylbutyrate.

Figure 8. The French-American-British (FAB) Classification of AML and the associated genetic abnormalities (Lowenberg, Downing and Burnett, 1999)

Unfortunately, this way of classification does not give information regarding prognosis or the best way to treat the disease; for this reason, in 2010, a new type of classification was introduced by the European Leukaemia Net (ELN). This classification is not considered the morphology of blasts but the cytogenetic and molecular data. Patients are classified into favourable, intermediate and adverse (Röllig *et al.*, 2011) (Figure 9). Another advantage of this classification is the link between mutations and prognosis, giving a prognostic significance to the groups.

Risk category*	Genetic abnormality
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}†
	Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD^{high}†
	Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}† (without adverse-risk genetic lesions)
	t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*‡
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214*
	t(v;11q23.3); *KMT2A* rearranged
	t(9;22)(q34.1;q11.2); *BCR-ABL1*
	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2,MECOM(EVI1)*
	−5 or del(5q); −7; −17/abn(17p)
	Complex karyotype,§ monosomal karyotype‖
	Wild-type *NPM1* and *FLT3*-ITD^{high}†
	Mutated *RUNX1*¶
	Mutated *ASXL1*¶
	Mutated *TP53*#

Figure 9: 2017 ELN risk stratification by genetics (Döhner *et al.*, 2017)

2.3 AML mutational landscape

Emerging data obtained using innovative genomic technologies provide a unique view of the spectrum and frequency of AML mutations. Studying distinct mutual exclusive pathways, clonal architecture and the epigenetic landscape, eight different classes of AML mutations were obtained (Figure 10)

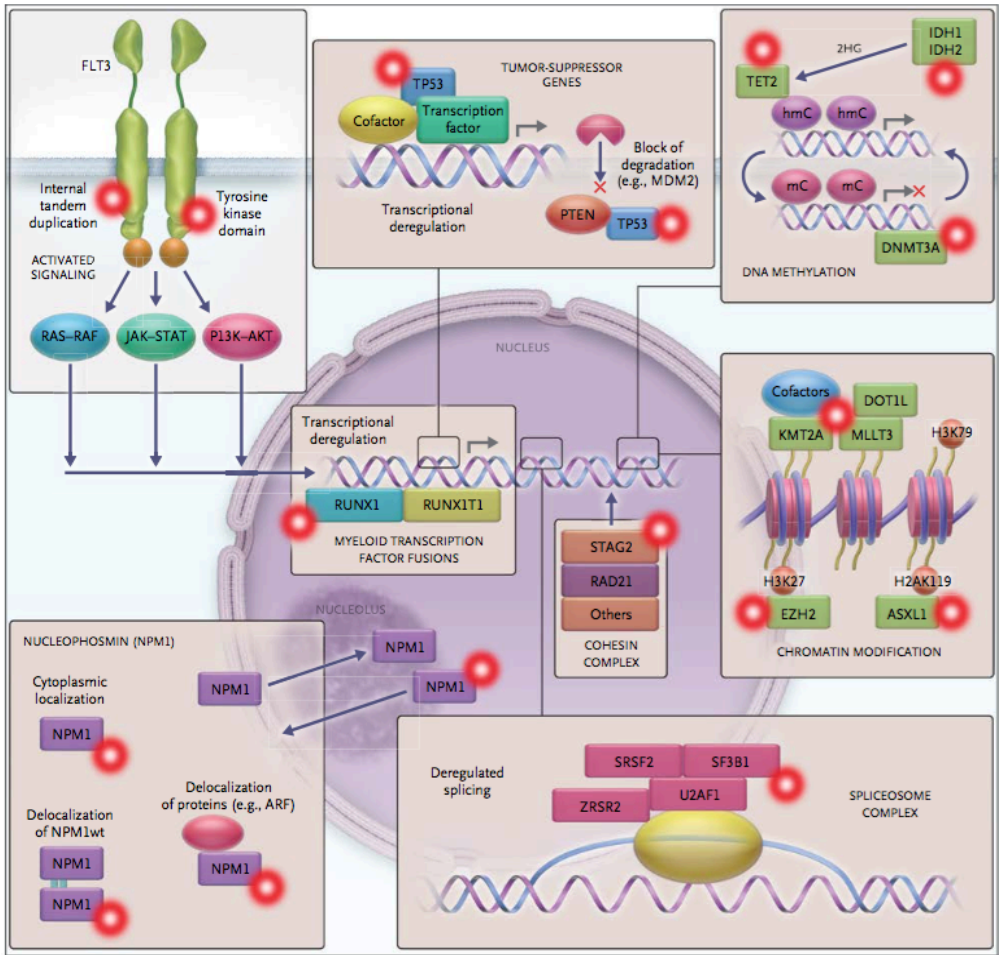


Figure 10. Eight Functional Categories of Genes Commonly Mutated in AML (Döhner *et al.*, 2017)

First of all, mutations in tumour-suppressor genes such as *TP53* (mutated in approximately 8% of patients) can cause a downregulation at the RNA level and impaired degradation through the double mouse minute 2 homologue (MDM2) and the phosphatase and tensin homologue (PTEN). Mutations in myeloid transcription factors such as *RUNX1* (mutated in 5-15% of patients) and the fusions of transcription factors due to chromosomal rearrangements lead to transcriptional deregulation and impaired hematopoietic differentiation. The nucleophosmin *(NPM1)* gene (mutated in between 25-35% of patients) encodes for a multifunctional nucleocytoplasmic shuttling protein that, after the accumulation of several mutations, cannot work properly resulting in wrong protein localization but

also a wrong position of the target proteins. Mutations in other genes like *ASXL1* (expected in 5-17% of patients) and *EZH2* can lead to deregulation of chromatin modifications (e.g., methylation of histones H3 and H2A on lysine residues K79, K27, and K119, respectively) due to the role of these proteins. *DNMT3A* (mutated between 18-22% of patients) and *TET2* mutations (mutated between 7-25% of patients), as well as *IDH1* and *IDH2* (mutated respectively in 7-14% and 8-19% of patients) mutations can lead to the deregulation of DNA methylation (hmC denotes 5-hydroxymethyl-cytosine, and mC 5-methylcytosine). Finally, mutations in genes involved in signalling pathways like the class III tyrosine kinase receptor *FLT3* (mutated in approximately 20% of patients) give a proliferative advantage through the RAS–RAF JAK-STAT and PI3K–AKT signalling pathways.

The mutational pattern is important because it may indicate whether AML was newly diagnosed as a primary or secondary disorder after an antecedent myeloid disorder such as myelodysplastic syndrome. Indeed, recently was demonstrated that AML with mutations in *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR*, or *STAG2* define a stratification in a genetic subtype of AML; each subtype describes a prognosis and the best therapy for that patient (TJ *et al.*, 2013; Giordano, 2014).

A graphical representation of the different gene mutations in AML is reported in Figure 11.

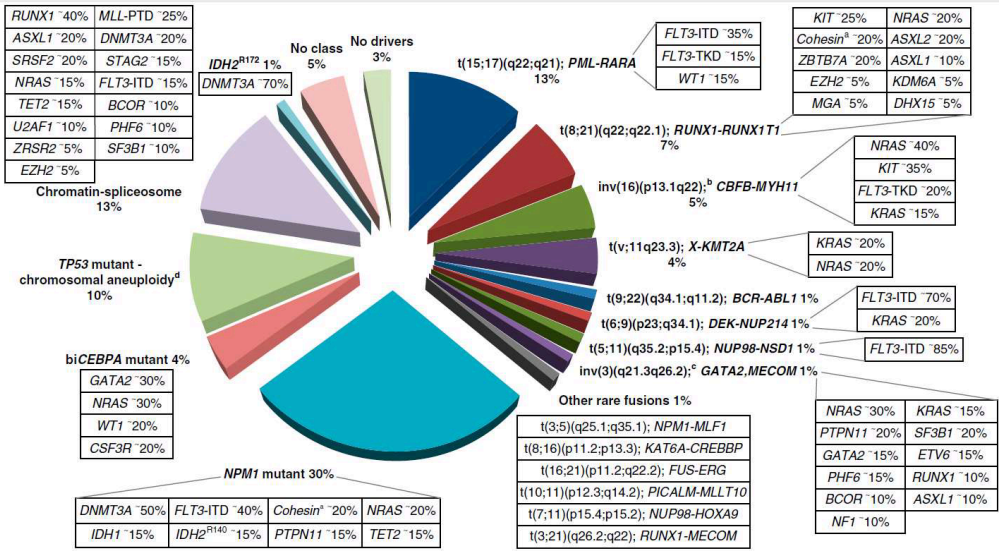


Figure 11. Molecular classes of AML and concurrent gene mutations in adult patients (Döhner *et al.*, 2017)

2.4 AML therapy

First-line treatment of AML patients consists of high dose chemotherapy. This kind of therapy is divided into two steps, and the goal is to achieve complete remission. The first step is called induction therapy; in this phase, the drugs eliminate the leukemic cells to an undetectable level. After this step, consolidation therapy aims to eliminate any residual undetectable disease and perform a cure for (Giordano, 2014). HSC transplantation is increasingly considered if induction chemotherapy fails or after insurgence of relapse but for old patients is often not possible. Transplantation is sometimes used as front-line therapy for people with high-risk diseases (De Kouchkovsky and Abdul-Hay, 2016); the mutational profile usually defines this. Chemotherapy consists of genotoxic drugs that stop the growth of cancer cells, either by killing them (via apoptosis) or by stopping them from dividing. For AML treatment, chemotherapy is usually given by vein injection to reach leukaemia cells in the bone marrow. Intrathecal chemotherapy may treat adult AML that has spread to the brain and spinal cord. Gold standard therapy for AML involves combination chemotherapy with cytosine arabinoside (ARA-C) combined with an anthracycline

(generally Daunorubicin). This protocol is called the 7 + 3 regimen because it consists of the daily administration of ARA-C (for 7 days) and the infusion of anthracycline drug on each of the first 3 days.

Despite the initial response to treatment, relapse formation is very common in AML patients: around 50% of patients develop a recurrence within 3 years after initial therapy. In some patients, the timing to relapse is shorter and this is due to several aspects, the most important are: adverse genetic factors, prior allogeneic transplantation, older age, and poor general health status.

Usually, for people with relapsed AML, the only proven potentially curative treatment is HSPCs transplantation.

2.5 Senescence in cancer therapy

Because of the rapid turnover of blood cells, scientists believed that hematopoietic cells do not enter in senescence but prefer to undergo apoptosis in response to stressors. AML treatments are based on drugs that induce DNA damage and cancer instability. In theory, this would mean the elimination of AML cells, but only recently it was reported that leukemic cells could activate a senescence program in response to chemotherapy(Duy *et al.*, 2021). Moreover, LEE011, a novel inhibitor of CDK4 and CDK6, was shown to induce G1 cell cycle arrest in leukaemia cells. This arrest is enough to activate SA-β-Gal in treated cells (Tao *et al.*, 2017). However, later on, another study highlighted an unexpected role of cell senescence in the treatment of haematological malignancies; specifically, it was demonstrated that, after an initial senescence response to high doses of chemotherapy in lymphomas and leukemic cells, senescent cells acquire features of stem cells and become more aggressive when transplanted in mice (Milanovic *et al.*, 2018). They also show that the Wnt signalling pathway is essential for reprogramming senescent blood cells. This work suggested for the first time that while induction of senescence is a crucial anti-neoplastic strategy, in the long-term senescent cells may contribute to tumour relapse in a cell-intrinsic way. Despite these recent studies, the

molecular mechanisms of senescence induction in AML and its role in response to therapy remain largely unexplored.

3 Senescent cancer cells and the immune cell crosstalk

3.1 Antitumoral response

Immune cells are an essential defense against many agents like bacteria and viruses. In detail, immune cells recognize these adverse agents (non-self) from the healthy tissue (self).

In the contest of senescence, it is reported that TIS mainly correlates with a high burden of DDR activation (Ewald *et al.*, 2010) in solid tumours, promoting a response by innate immunity that contribute to preparing the adaptive immune system for its activation (Burton and Stolzing, 2018). As explained in the first chapter, an extensive DDR leads to senescence activation and then the SASP production. SASP can then recruit and attract innate immune cells (macrophages, granulocytes and NK cells), promoting their activation and the consequent senescent cancer cells eradication (Lee *et al.*, 2019).

A mouse model reported tumour cell elimination by NK cells and neutrophils after senescence activation caused by the restoration of p53 function in liver carcinoma cells (Xue *et al.*, 2007). Then several works said NK cells as crucial players against senescent cells preventing fibrosis formation and promoting tissue regeneration, preserving the function of several tissues (Burton and Stolzing, 2018). In humans, NK cells primarily act against cells that downregulate the major histocompatibility complex class I (MHC-I) HLA-I; these cells express the family killer-cell immunoglobulin-like receptors (KIR). KIR recognise HLA-I molecules shutting down the cytotoxic activity of NK cells. Usually, HLA-I molecules are downregulated in cancer cells promoting NK cells activation; their action intensifies when cancer cells become senescent because they start to express activatory ligands for NK cells (Sagiv *et al.*, 2013, 2016). In addition to NK cells, other studies demonstrated that also macrophages are involved in the immune surveillance of senescent cells (Mevorach *et al.*, 2010; Egashira *et al.*, 2017). Furthermore, upon DDR evoked by oncogenes, macrophages were shown to have a prosenescent role via the secretion of

TGF-β cytokine (Reimann *et al.*, 2010). Therefore, their ability to induce senescence in tumour cells may be used as a possible anti-cancer strategy.

The adaptive immune system is the one that can respond against a precise target. The two leading players for adaptive responses are T and B cells. B cells are not reported to have an essential role against cancer cells elimination. Instead, T cells can contribute to immune surveillance of senescent cells. T cells get activated when recognising an antigen presented through HLA-I or HLA-II. While HLA-I is expressed virtually by all cells, presenting short peptides (8-11 aminoacids), HLA-II is expressed by immune cells defined as antigen-presenting cells (APC), and it can present longer peptides (30+ amino acids). T cells are divided into two main subsets: $CD4^+$ and $CD8^+$; the first subset interacts with HLA-II thanks to a site present in the β2 chain, while the second interacts with HLA-I.

In 2010, a study demonstrated that in a mouse model of T cell acute lymphoblastic lymphoma (T-ALL), genetic inactivation of the proto-oncogene c-Myc correlates with tumour regression. Interestingly, $CD4^+$ T-cells emerged as the critical host effector population for contributing to this sustained tumour regression upon Myc inactivation; mechanistically, $CD4^+$ T-cells keep cancer cells under control by inducing cellular senescence and blocking angiogenesis (Rakhra *et al.*, 2010).

In a model of OIS in the liver, senescent cells increase HLA-II expression, which triggers $CD4^+$ T cells activation. This work has described the importance of the adaptive immune system because the depletion of monocyte and macrophages in mice results in impaired senescence cells eradication (Kang *et al.*, 2011). Another study reported that melanocytes display increased MHC II expression upon OIS, which correlates with T cell proliferation *in vivo* and *in vitro* (van Tuyn *et al.*, 2017). Overall, senescent cells immunosurveillance ensures the clearance of premalignant and malignant cells, reinforcing the functional role of cellular senescence in hampering cancer progression and is reported the importance of both immune systems (adaptive and innate). However, to date, the mechanisms

underlying the crosstalk between the immune cells and senescent cancer cells in the context of AML are unclear.

3.2 T cell compartment in AML

As reported in the previous paragraph, the clinical outcome of AML patients upon chemotherapy remains suboptimal, suggesting that more efforts should be made to identify new therapeutic rationales.

T cells can present distinct differentiation states caused by their activation against the target. With the use of multiparametric flow cytometry, we can distinguish T cells that do not activate against the target (defined as naïve), T stem cell memory (TSCM) that once recognized the target became primed to proliferate and get activated, T central memory cells that recognize the target but are not activated and T effector memory and T terminal effector cells that are fully activated against the target (Gattinoni *et al.*, 2017a) (Figure 12). TSCM present a different metabolism with increased mitochondrial biomass resulting in a more efficient respiratory capacity. Moreover, these cells are ready to proliferate and differentiate after recalling (van der Windt *et al.*, 2012; Van Der Windt *et al.*, 2013).

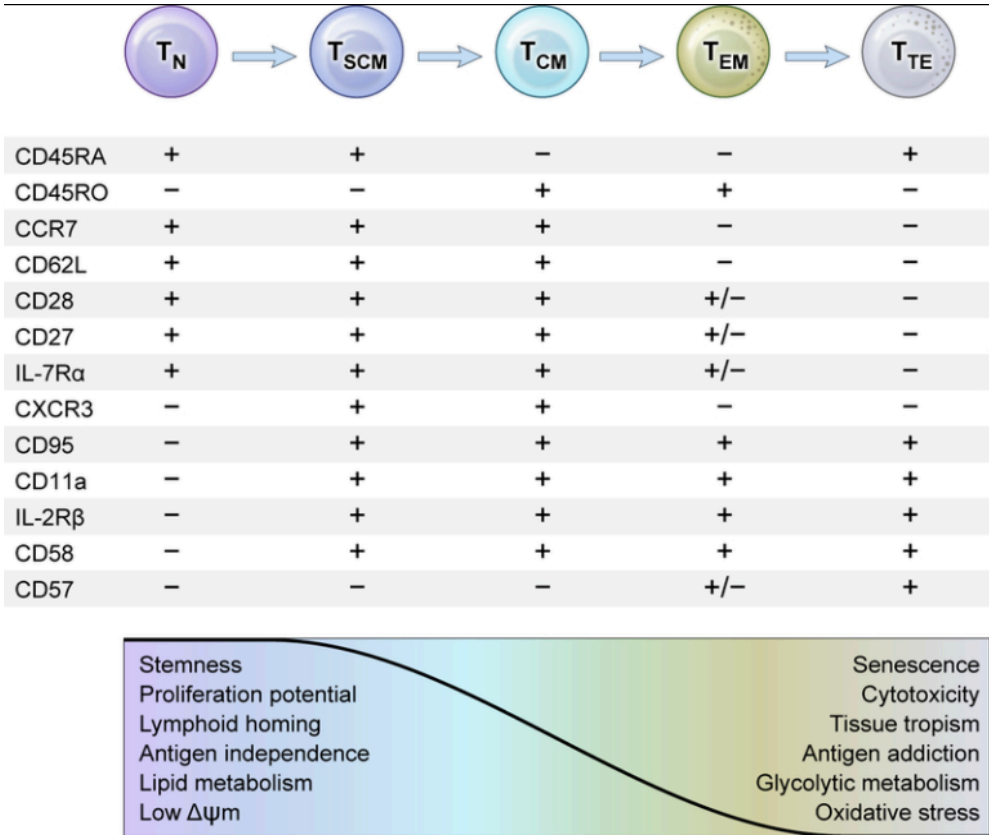


Figure 12 T h subpopulation cells subsets (Gattinoni *et al.*, 2017b)

In preclinical models it was reported an advantage by transferring tumour-reacting T cells $CD62L^+$ (Gattinoni *et al.*, 2009, 2011; Sommermeyer *et al.*, 2016); however, in clinical trials, tumour infiltrating lymphocytes (TILs) are composed mainly by terminally differentiating T cells making the isolation of early memory T-cell subset impossible (Baitsch *et al.*, 2011; Gros *et al.*, 2014).

In several AML patients, a change in the composition between T cell subsets was observed with upregulation of several inhibitory/exhausted molecules after hematopoietic stem cells transplantation (HSCT) (Noviello et al., 2019). This and other works described that the expression of several inhibitory markers TIM-3, LAG-3, PD-1, and KLRG1 are bad prognostic markers for AML eradication after allogeneic haematopoietic stem cells transplantation (allo-HSCT) (Kong *et al.*, 2015; Schade *et al.*, 2016). Also, AML blasts are characterized by the ability to evade the host immune system via different mechanisms; immune-evasion can be acquired through intrinsic mechanisms, therefore directly conveying inhibitory signals to an

immune cell or via extrinsic ones meaning that cancer immunomodulation is exerted through the tumour microenvironment (Figure 13).

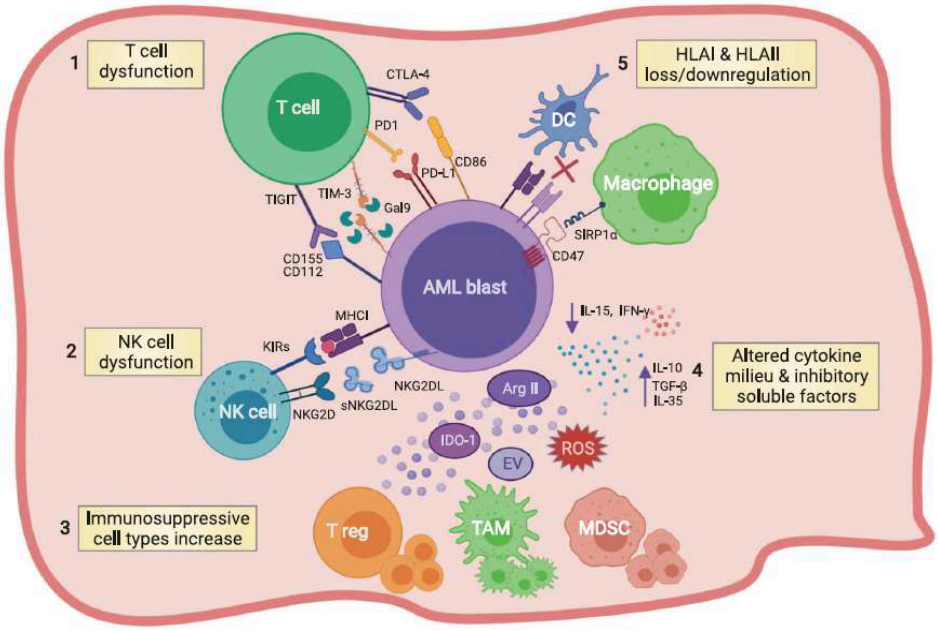


Figure 13: AML blast's immune escaping strategies (Tettamanti *et al.*, 2022)

In the context of allo-HSCT HLA expression on the surface of leukemic cells is fundamental for the Graft versus Leukaemia (GvL) effect mediated by donor-derived T cells; to boost this effect, donor's T cells present a mismatch between TCR and the patient's HLA molecules. Of note, leukaemic blasts show impressive immunoediting capabilities under the selective immune pressure that occurs after allo-HSCT; indeed, AML blasts are characterized by defects in antigen presentation, achieved via genomic HLA loss (Vago *et al*., 2009) or epigenetic downregulation of HLA class II (in up to 40% of post-transplantation relapses (Toffalori *et al.*, 2019) (Gambacorta et al. 2022) and hamper proper blasts eradication via $CD8^+$ and $CD4^+$ T cells (Tettamanti *et al.*, 2022). Another immune evasion mechanism adopted by AML blasts is the altered expression of ligands for immune checkpoints (ICs) molecules present on T cells surface for avoiding autoimmunity phenomena. Among the different ICs, programmed-cell-death ligand 1 (PD-L1) and T-cell immunoglobulin and mucin domain, 3 (TIM-3) are best characterized. After its binding with the PD-1 receptor exposed on

T cells, PD-L1 functions as a co-inhibitory molecule leading to T cells premature exhaustion and to an increased presence of regulatory T cells (Tregs), ultimately blocking $CD8^+$ T cells functionality. TIM-3, in turn, interacts with galectin-9 molecules present on leukemic cells, leading to lower secretion of pro-inflammatory cytokines and promoting T cell and NK cell dysfunctions. The presence of other two receptors, cytotoxic T lymphocyte-associated protein 4 (CTLA4) and lymphocyte activating-3 (LAG-3), correlates with poor prognosis (Zeng *et al.*, 2020). Tumour-extrinsic mechanisms, in turn, comprehend all those strategies mounted by the tumour cell to generate a favourable microenvironment so immunosuppressive to promote tumour growth and inhibit the activation of the immune system by releasing anti-inflammatory molecules. For instance, leukemic blasts have been shown to produce specific enzymes, among which indoleamine 2,3-dioxygenase-1 (IDO1), arginases, CD39 and CD73, able to produce immunosuppressive metabolites (Serra *et al.*, 2011; Mussai *et al.*, 2013; Dulphy *et al.*, 2014; Folgiero *et al.*, 2014). The increased production of anti-inflammatory cytokines with the consequent reduction of pro-inflammatory ones can make leukaemic cells less immunogenic, thus hampering immune responses; this occurs, for example, in the context of chronic myeloid leukaemia (CML), where cells release immune-suppressive cytokines, such as TGFβ and IL-4 (Naka *et al.*, 2010; Tarafdar *et al.*, 2017). These cytokines can antagonize HLA-II and the expression of its master regulator CIITA, rendering leukemic cells invisible to the immune system. Higher IL-4 and IL-10 levels were also detected in blasts, and bone marrow-derived T cells of patients affected by AML (Park et al., 2006). On the contrary, the release of pro-inflammatory cytokines such as granulocyte colony-stimulating factor (G-CSF), IL-15 and IFNs, which myeloid progenitors generally produce, is suppressed. Notably, the IFN-γ signalling pathway has a fundamental role in immune surveillance and adaptive immunity activation. Among its pleiotropic effects, IFNs induces upregulation of HLA molecules. Moreover, in some tumour types, IFN-γ regulates the HLA-II transactivator CIITA, causing its upregulations and

leading to MHC-II expression (Castro *et al.*, 2018). As already anticipated, AML blasts are characterized by immunoediting capabilities such as loss of mismatched HLAs (haploidentical transplants context) or epigenetic downregulation of HLA-II molecules (different donor transplant settings) (Vago and Gojo, 2020). In this scenario, IFN-γ administration restored the HLA expression and T cells' subsequent recognition of AML blasts (Toffalori *et al.*, 2019). Hence, the balance among anti- and pro-inflammatory cytokines for the first ones could be the main payer in AML immune escape and may represent a vital cancer cell vulnerability to be exploited therapeutically.

4. Aim of the work

Cellular senescence is a state of irreversible growth arrest induced by a plethora of endogenous or exogenous stressors, including genomic damage and oncogene activation and tumour suppressor loss in the context of cancer. Interestingly, senescent cells can trigger an immune-mediated clearance of cancer cells, further corroborating the tumour suppressive role of senescence in cancer therapy. However, the molecular interplay between senescent and immune cells is not fully understood. Hence, this study aims to shed light on the mechanisms involved, providing more insights into how these cells interact.

I carried on my studies exploiting a model of acute myeloid leukaemia (AML), a type of cancer that involves hematopoietic cells. The current standard of care is high-dose chemotherapy, typically achieved with Cytarabine (ARA-C) administration. ARA-C is a DNA intercalating agent incorporated into the genome during DNA replication, causing DNA damage and triggering the DNA damage response (DDR). Data obtained in collaboration with Schmitt's lab show the induction of senescence in AML blasts upon *ex-vivo* treatment with ARA-C (Therapy Induced Senescence or TIS) and its positive correlation with patient outcomes. Interestingly, senescence-induced cell cycle arrest is associated with increased expression of human leukocyte antigen (HLA) molecules, which are cell surface molecules specialised in presenting antigenic peptides to T cells, suggesting a causal link between TIS and immunogenicity. However, after five years from treatment, the overall survival rate of AML patients is still as low as 30%. Indeed, failure in AML treatment is frequent and mainly due to the development of chemo-resistant tumours. Within this framework, my thesis project delves deeper into the mechanisms underlying TIS to provide more insights to understand the role of TIS in tumour eradication and immune surveillance and its relationship with the emergence of therapy-resistant cancer cells.

5. Results

5.1 DNA damaging agents cause the activation of the senescent program in leukemic cell lines

To model the response to DNA damage in human AML, I treated two monocytic leukemic cell lines (THP1 and OCI-AML3) with the clinically-relevant chemotherapy agent ARA-C, a pyrimidine analogue that, when incorporated into DNA, interferes with DNA synthesis causing DNA damage during the S phase of the cell cycle. I first conducted titration experiments in erythro-leukemic cells K562 to identify dose and time response to induce cell cycle arrest without significant toxicity. By treating K562 cells for one week in ARA-C at the final concentration of 100nM and 250nM, refreshing the drug every day, I observed a dose-dependent cell cycle arrest at day 7 post-treatment (Figure 14A). I then went on with the 250nM dose and treated THP1 and OCI-AML3 cells, two AML cell lines with respectively an inactive (deletion) or active (wt p53) p53 pathway (Figure 14B). I detected a block in cell proliferation at 72 hours post-treatment that was persistent at 7 days post-treatment in both cell lines (Figure 14C), while THP1 cells showed very low toxicity upon ARA-C treatment OCI-AML3 presented a modest although significant induction of apoptosis over time (Figure 14D). Immunoblot analyses indicate that while in OCI-AML3 cells, the observed cell cycle arrest post-ARA-C treatment was associated with increased phosphorylation of p53 and consequent accumulation of p21 in THP1 cells, the cell cycle arrest was associated with a p53-independent induction of p21 (Figure 14E).

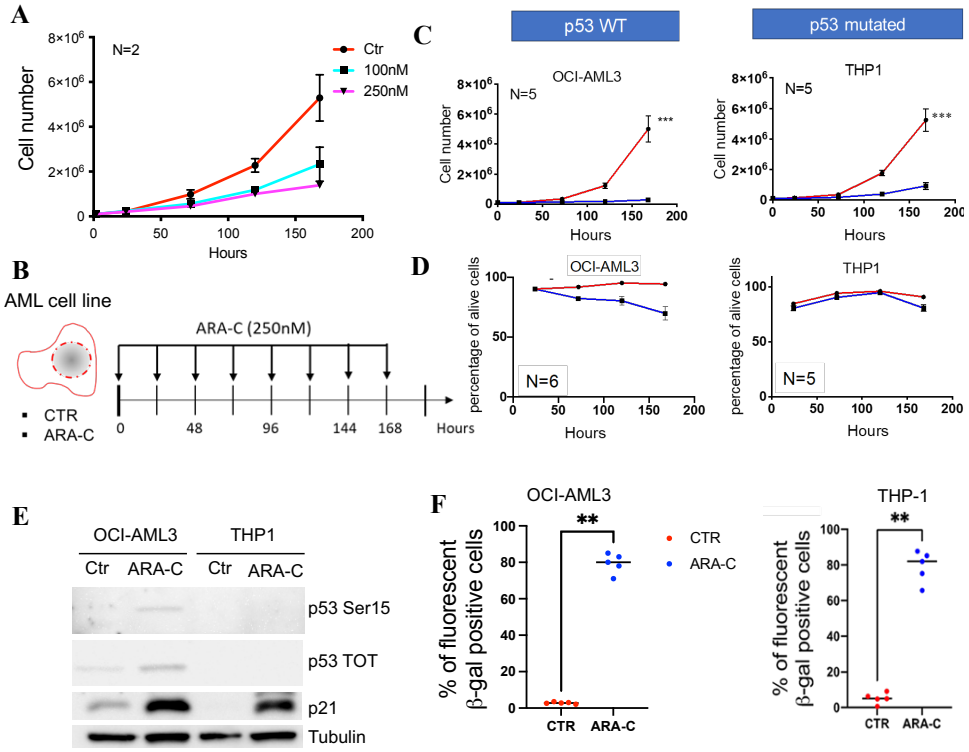


Figure 24. ARA-C induces cell cycle arrest without affecting cell viability in THP-1 cells. *(A)Growth curve of K562 cells treated with two different doses of ARA-C (100nM or 250nM) or untreated. 100.000 cells were seeded at starting point of growth curve. (B) Experimental design, each arrow represents a single ARA-C administration (every 24 hours). (C) Growth curve of OCI-AML3 (left) and THP-1(right) cells either untreated or treated with ARA-C. 100.000 cells were seeded at the starting point of the growth curve. (D) Vitality assay of untreated and treated THP-1 (right) and OCI-AML3 (left) cells by AnnexinV/7AAD staining. Curves represent the percentage of alive (double negative) cells over time. Both graphs: untreated cells (CTR) in red, ARA-C treated cells (ARAC) in blue. (E) Western Blot analysis for p53 pSer15 and p21 in untreated (CTR) or chemotherapy treated (ARA-C) THP-1 and OCI-AML3 cell. Total p53 and vinculin were used as a loading control. (F) Percentage of fluorescent SA-β-gal positive cells as detected by FACS analysis in untreated (CTR, in red) and treated (ARA-C, in blue) THP-1 (right) and OCI-AML3 (left) cells. Biological replicates n=5. The statistical test used was the Mann-Whitney test: '**' $p < 0.01$.*

I then analysed ARA-C treated THP1 and OCI-AML3 cells for senescence induction by using the well-established marker senescence-associated β-galactosidase activity (SA-β-gal). I took advantage of a fluorescent variant of this assay (C12FDG) and the ImageStreamX technology. This imaging cytometer combines the quantitative power of a

flow cytometer (allowing to analyse of cells in suspension) with quantitative imaging (allowing the concomitant study of cell morphology and cellular markers). In detail, I combined measurements of the AnnexinV signal to eliminate dead cells from the analysis, the incorporation of 5-ethynyl-2′-deoxyuridine (EdU) to evaluate proliferation and the fluorescent SA-β-gal activity for senescence evaluation. I considered senescent cells double negative for EdU and AnnexinV signals and positive for fluorescent SA-β-gal activity.

I observed an increase in the percentage of fluorescent SA-β-gal-positive cells after 7 days of treatment (Figure 14F), with approximately 80% of SA-β-Gal positive cells in ARA-C treated THP1 and 90% of fluorescent SA-β-gal positive cells in ARA-C treated OCI-AML3 compared to untreated controls.

5.2 Primary AML cells activate senescence program upon *ex-vivo* ARA-C treatment

To evaluate if induction of senescence also occurs in primary AML blasts, I developed, in collaboration with the laboratory of Dr. Clemens Schmitt, a protocol to preserve and culture AML primary samples *ex-vivo*. More in detail, total PBMCs from AML patients at diagnosis (obtained from the San Raffaele Hospital leukaemia biobank) were seeded for 3 days on a layer of irradiated stromal cells in a media enriched in cytokines (IL6, TPO, IL3, FLT3 and SCF) and supplemented with conditioned media enriched in factors secreted by irradiated 5637 cell line. AML primary blasts from 5 patients with different mutational backgrounds were purified by $CD34^+$-, or, $CD33^+$-based magnetic cell separation, and a dose of ARA-C of 410nM (100 ng/mL) for 5 consecutive days was chosen to treat primary blasts and induce cell cycle arrest while preserving blasts viability (Figure 15A, B). Despite the expected inter-patient variability, all primary samples upon ARA-C treatment showed no signs of proliferation (by EdU incorporation), indicating a DNA replication block (Figure 15C). Consistent with this, all primary AML samples display increased levels of SA-β-Gal after a 5-day

ex-vivo exposure to ARA-C (Figure 15D), a phenomenon also described as therapy-induced senescence (TIS).

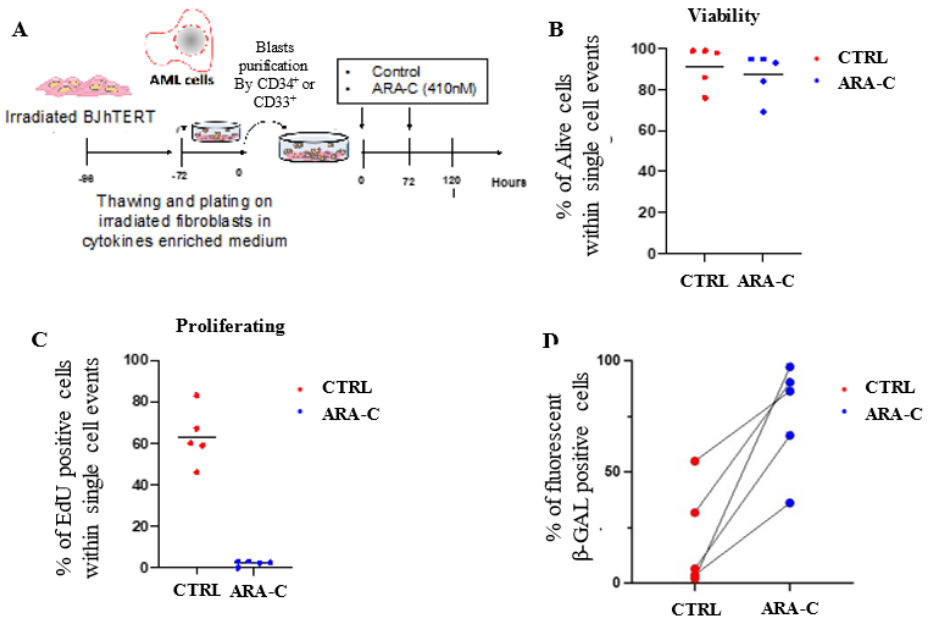


Figure 15. Ex-Vivo chemotherapy induces senescence in primary AML samples *(A) Experimental design. Primary human AML samples are cultured on a layer of irradiated human immortalised fibroblasts (BJhTERT) in 5637 cell line (human bladder carcinoma) conditioned and cytokine-supplemented medium. After 3 days of pre-culturing, blast purification is performed according to leukaemia-associated immunophenotype (CD34 or CD33). During culture, ARAC was administered twice (at times 0 and 72 hours). After 5 days of culture (120 hours), viability, proliferation and SA-β-GAL activity were analysed by ImageStreamX. (B) Viability assay of untreated (CTR) or treated (ARA-C) primary blasts measured by ImagestreamX. AnnexinV negative cells were considered alive, and the percentage was plotted. (C) Proliferation assay of untreated (CTR) or treated (ARA-C) primary blasts measured by ImagestreamX. EdU was used to distinguish cycling (positive) from non-cycling (negative) cells. The percentage of cycling cells was plotted among conditions. (D) Differential fluorescent SA-β-GAL activity between matched*

untreated (CTRL, red) and ARA-C (ARA-C, blue) exposed primary AML blast samples.

To characterise the molecular changes occurring in AML blasts in response to ARA-C, I performed gene expression profiles by bulk RNA sequencing (RNA-seq) in 4 AML samples untreated or treated *ex-vivo* with ARA-C. We found 57 differentially expressed genes that belong to gene categories of the p53 DNA damage response, cell proliferation and apoptosis, consistent with the induction of a senescence program in drug-exposed AML samples. Interestingly, gene set enrichment analysis (GSEA) revealed that, together with gene categories of the DNA repair and p53-dependent DDR pathway, two main

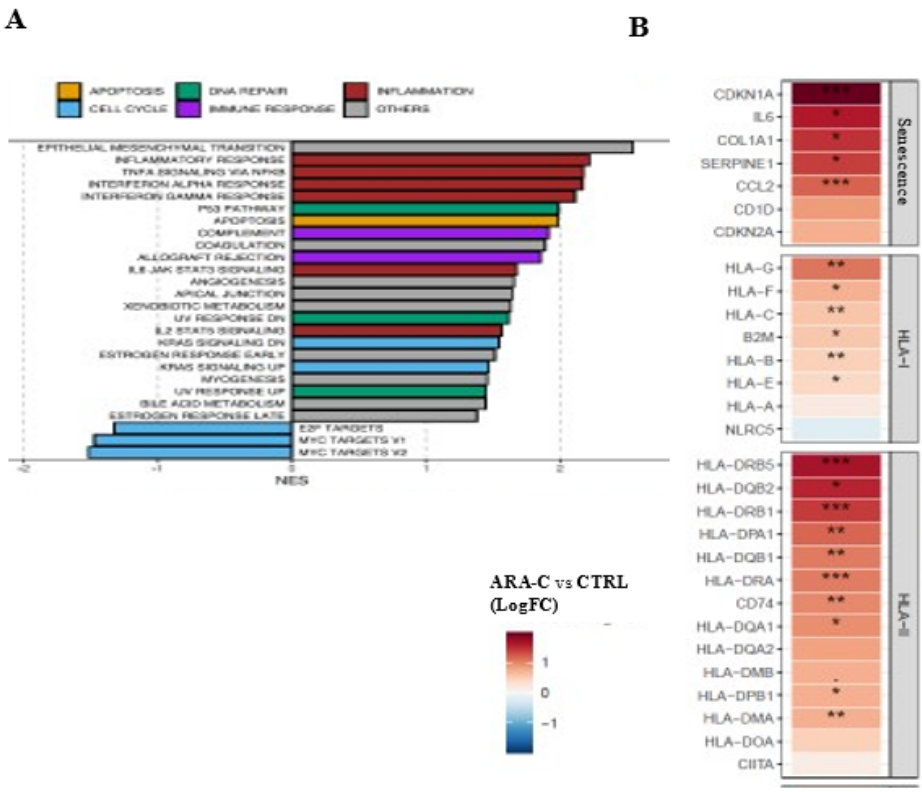


Figure 16. RNA-Seq analyses on ex-vivo treated AML blasts *(A) Gene Set Enrichment Analysis showing significant hallmark categories, colour legend shown on the top (False Discovery Rate <= 0,05), the Normalised Enrichment Score (NES) is reported on the x-axis; all treated samples were ranked by Logarithmic Fold Change value (LogFC) after comparison to untreated samples. (B) Heatmap showing the LogFC of senescence genes HLA class I and HLA class II molecules of the comparison ARA vs CTRL. Colour code on the left (from red to blue) FDR threshold: '***' p < 0.001, '**' p < 0.01, '*'p < 0.05.*

senescence-associate features, the hallmark categories "inflammation" and "immune response", were significantly upregulated (FDR ≤ 0.05) in ARA-C treated samples compared to controls (Figure 16A). Specifically, interferon-alpha and gamma response and TNFa signalling via NF-kB pathways were found within the most significantly upregulated categories, suggesting a possible link between TIS, SASP and blasts immunogenicity. In support of this hypothesis, further exploration of the RNA-seq datasets revealed upregulation of senescence-associated genes (including cell cycle inhibitors CDKN1A (p21), CDKN2A (p16) and SASP factors IL6 and CCL2, and induction of several HLA class I and class II molecules in chemotherapy-treated samples compared to untreated ones (Figure 16B).

5.3 ARA-C treated AML cell lines and primary samples upregulate HLA molecules

To validate RNA-seq findings, I analyzed the expression of HLA at the protein level first in ARA-C treated THP1 and OCI-AML3 cells via FACS, using antibodies broadly recognising the canonical members of HLA class I (HLA-ABC) and of HLA class II (HLA-DR). For this analysis, cells treated

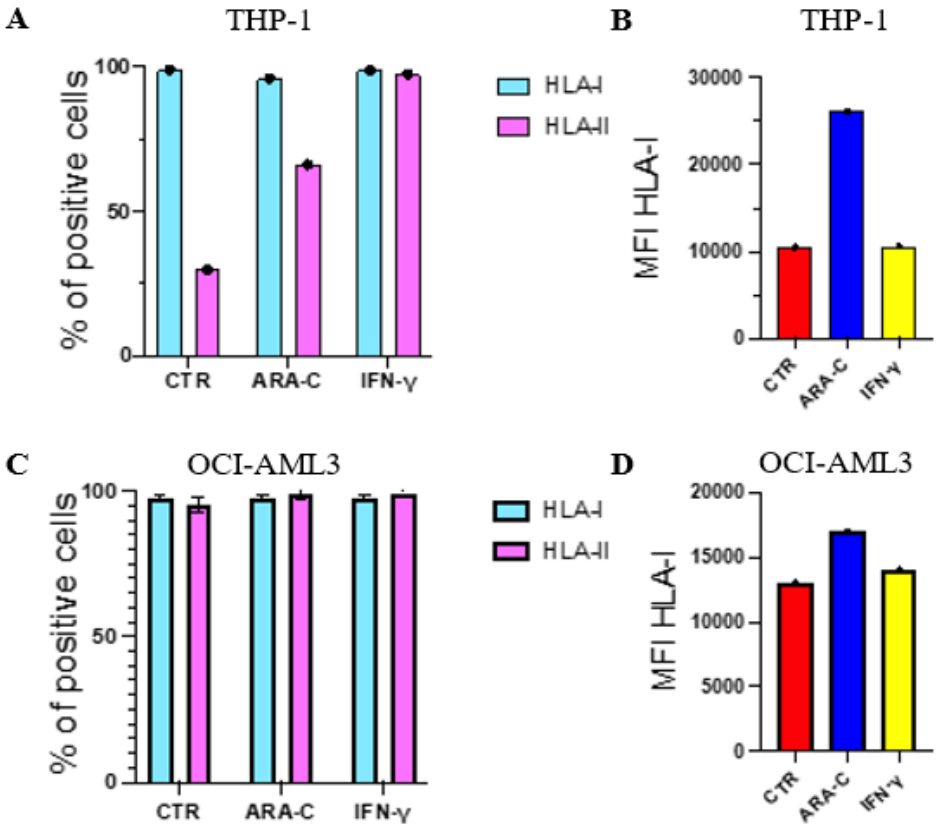


Figure 17. Expression levels of HLA class I and II molecules increase upon ARA-C treatment in THP-1 cells. *(A) Percentage of HLA class I (light blue) and class II (pink) in THP1 untreated (CTR) or treated with either ARA-C and IFN- γ. (B) Mean Fluorescence Intensity (MFI) of HLA class I (left) in THP1 either untreated (CTR, red) or treated with either ARA-C (blue) or IFN-γ (yellow). (C) Percentage of HLA class I (light blue) and class II (pink) in OCI-AML3 untreated (CTR) or treated with either ARA-C and IFN- γ. (E) Mean Fluorescence Intensity (MFI) of HLA class I (left) in OCI-AML3 cells either untreated (CTR, red) or treated with either ARA-C (blue) or IFN-γ (yellow).*

with IFN-ɣ 48h before the analysis was added given its role as a well-characterized positive regulator of HLA class II molecules. FACS analyses for HLA class I and class II were performed at day 7 (168 hours) post-ARA-C treatment (Figure 17A, C). When analyzing HLA-II, I observed two different situations comparing OCI-AML3 and THP-1 cells. In THP-1 cells

at the basal level, I observed that only 30% of cells were HLA-II positive, and this percentage increased up to 60% upon ARA-C treatment. In untreated OCI-AML3 cells were all HLA-II positive, and the percentage of positive cells remained high after ARA-C treatment (Figure 17C). As already reported in the literature, THP-1 and OCI-AML3 cells are both 100% positive for HLA-I expression; however, when analysing the Median Fluorescent Intensity (MFI), I found higher MFI values for ARA-C treated cells compared to both CTRL and IFN- ɣ treated, meaning that the expression of HLA-I at the single-cell level is enhanced in TIS cells (Figure 17B, D). Together, these findings confirm that THP-1 cells recapitulate the induction of HLA class I and class II molecules observed in the gene expression of AML primary samples upon chemotherapy treatment, representing a good model for further functional assays.

I next measured HLA expression at the protein level in ARA-C ex-vivo treated primary AML blasts via FACS, using antibodies broadly recognising members of HLA class I and of HLA class II. Results confirmed a significant increase in the percentage of HLA class II positive cells (Figure 18A) and a modest, although a not significant, increase in the MFI of HLA class I (Figure 18B) in senescence-competent primary AML samples. To evaluate the link between senescence and HLA induction, I expanded the analyses of the expression of HLA molecules in 5 additional AML patients that were not able to activate the senescence program upon ARA-C treatment (senescence-non competent). Interestingly, a significant positive correlation was found between the percentage of senescent blasts and the HLA class II and class I upregulation upon ARA-C treatment (Figures 18C and 18D), indicating that a senescence program, induced by ARA-C, correlates with the increase of HLA molecules (both class I and class II) on the surface of AML cells.

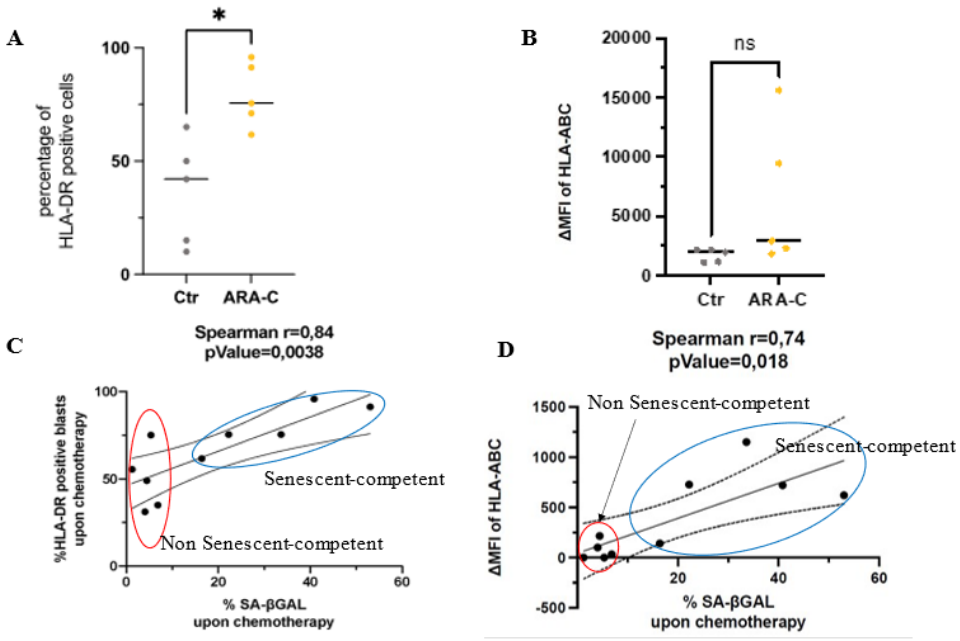


Figure 18. Expression levels of HLA class I and II molecules upon chemotherapy treatment in AML primary samples*. (A) Percentage of HLA-DR (class II) positive cells as detected by FACS analysis in untreated (CTRL, in grey) and treated (ARA-C, in yellow) primary AML blasts. (B) Mean fluorescence intensity of HLA class I as detected by FACS analysis in untreated (CTRL, in red) and treated (ARA-C, in blue) primary AML blasts. (C) Correlation between percentages of SA-β-gal (x-axis) and HLA-DR (class II, y-axis) positive cells, each dot represents a primary AML sample (D) Correlation between percentages of SA-β-gal (x-axis) and HLA-ABC (class I, y-axis) positive cells, each dot represents a primary AML sample. Statistical test: Kruskal-Wallis with Dunn's multiple comparisons. '*'p < 0.05.*

5.4 Senescence induction rather than acute DNA damage induces blasts immunogenicity

To delve deeper into the molecular mechanisms and functional consequences of the senescent program on AML blasts, I then decided to induce senescence without DNA damage to evaluate its effects on HLA molecules by treating THP-1 cells with a CDK4/6 inhibitor (CDK4/6i), Palbociclib. This small molecule blocks cell cycle progression from G1 to S phase by inhibiting the activity of cyclin D-dependent kinases 4 and 6, thus inducing a senescence-like cell cycle arrest in the absence of physical DNA damage. THP-1 cells were therefore treated with CDK4/6i (every other day) or with ARA-C (every day) for 7 days (168 hours), and, on day 7, cells were collected for FACS analysis (Figure 19A).

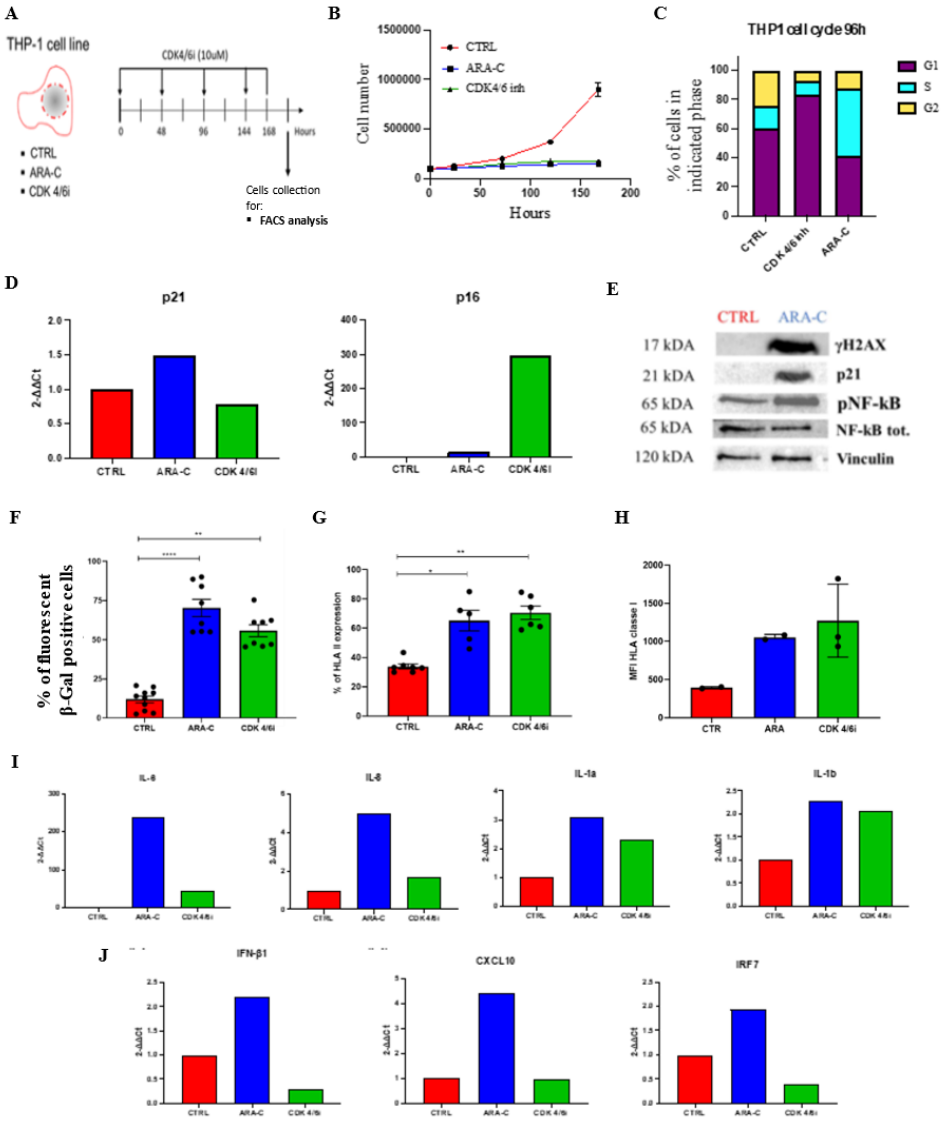


Figure 19. CDK 4/6 inhibitor triggers senescence in THP-1 cells and increases HLA class I and II levels without inducing SASP production. *(A) Schematic representation of the treatment protocol, each arrow indicates a single administration, blue for ARA-C and green for CDK 4/6i. (B) Growth curve of THP-1 cells either untreated or treated with ARA-C or CDK4/6i. 100.000 cells were seeded at the starting point of the growth curve. (C) Cell cycle evaluation of THP-1 cells untreated or treated with ARA-C or CDK4/6i after 3 days from seeding. The cell cycle was measured by combining EdU with Hoechst. (D) Gene expression analysis of p16 and p21 cell cycle inhibitors in CTRL, ARA-C or CDK4/6i treated THP-1 cells samples after 7 days from treatment; the value represents an absolute*

*expression considering GUS-B as housekeeper gene. (E) Western Blotting analysis of CTRL, ARA-C or CDK4/6i treated THP-1 cells searching for p21, phosphoNFkB (ser536), total NFkB as a loading control. (F) Percentage of fluorescent SA-β-gal (C12FDG) positive cells. Untreated (CTRL, in red) or ARA-C (blue) and CDK 4/6i (green) treatments. Individual dots represent biological replicates. Statistical test: Kruskal-Wallis with Dunn's multiple comparisons. '**'p < 0.005; '****'p < 0.0001 (G) Percentage of HLA-II positive THP-1 cells either untreated (CTRL, in red) or upon ARA-C (blue) and CDK 4/6i (green) treatment. Individual dots indicate biological replicates. (H) Mean Fluorescent Intensity (MFI) of HLA class I positive THP-1 cells either untreated (CTRL, in red) or upon ARA-C (blue) and CDK 4/6i (green) treatment. Individual dots indicate technical replicates. (I) Gene expression analysis of general SASP genes (IL-6, IL-8, IL-1α and IL1β) in CTRL, ARA-C or CDK4/6i treated THP-1 cells samples after 7 days from treatment the value represents an absolute expression considering GUS-B as housekeeper gene; the value represents an absolute expression. (J) Gene expression analysis of interferon genes (IRFβ1, CXCL10 and IRF7) in CTRL, ARA-C or CDK4/6i treated THP-1 cells samples after 7 days from treatment the value represents relative expression considering GUS-B as housekeeper gene; the value represents an absolute expression considering. Statistical test: Kruskal-Wallis with Dunn's multiple comparisons. '*'p < 0.05; '**'p < 0.005.*

As expected, I observed a block in THP-1 cell proliferation upon both treatments compared to control untreated cells (Figure 19B). I also evaluated the cell cycle distribution to understand which cell cycle phase cells were blocked. I observed an accumulation in the S phase for THP-1 cells treated upon ARA-C while CDK4/6i-treated cells arrest in the G1 phase of the cell cycle (Figure 19C). To identify the two players able to block the cell cycle, I measured the induction of cell cycle inhibitors p16 and p21 by qPCR. As expected, p16 was upregulated by CDK4/6i treatment while p21 was induced by ARA-C (Figure 19D), and this was confirmed by

WB analysis for p21 and the DDR markers yH2AX (Figure 19E). Then, fluorescent SA-β-Gal staining was applied to evaluate the extent of senescence induction. As a result, CDK 4/6 inhibitor-treated cells showed a significant percentage of positive senescence cells compared to untreated cells (CTRL), reaching an average of 55% after 7 days of treatment, vs 70% of positivity upon ARA-C treatment (Figure 19F).

Moreover, FACS analyses showed that HLA II expression in CDK 4/6i treated cells is significantly higher when compared to control cells and is almost comparable to that observed in ARA-C treated senescent cells (Figure 19G). Then, I quantified the MFI for HLA class I upon CDK 4/6i treatment. Accordingly to what was observed for HLA-II, a higher MFI was observed for CDK 4/6i-treated cells, similarly to that already described for the ARA-C treatment group (Figure 19H). Taken together, these data suggest that cell cycle arrest in the absence of physical DNA damage via CDK4/6 inhibition induces a senescence-like growth arrest and up-regulation of HLA molecules. I next evaluated SASP expression in CDK4/6i, or ARA-C treated AML samples. Immunoblot analysis (Figure 19E) revealed a strong activation of the NFkB pathway in ARA-C treated samples. I then measured the levels of several SASP transcripts (IL-6, IL-8, IL-1α and IL1β) via RT-qPCR and found a consistent induction when cells were induced into senescence by ARA-C compared to controls.

Interestingly, induction of senescence by CDK4/6i led to a minor upregulation of SASP transcripts (Figure 19I). I also measured the expression levels of several interferon genes (IFNβ1, CXCL10 and IRF7) (Figure 19J). I found that senescence-induction by ARA-C treatment could induce their expression while CDK4/6i treatment had almost no effect. These preliminary findings suggest that CDK4/6i and ARA-C treatment may induce distinct SASP responses in AML cells despite a similar senescence-like growth arrest.

I next assessed whether acute DNA damage or transient inhibition of the cell cycle would impact the expression of HLA molecules. To this aim, I administered both ARA-C and CDK4/6i for only 24 hours to AML cells in

contrast to the 7 consecutive days of treatment administered so far (Figure 20A).

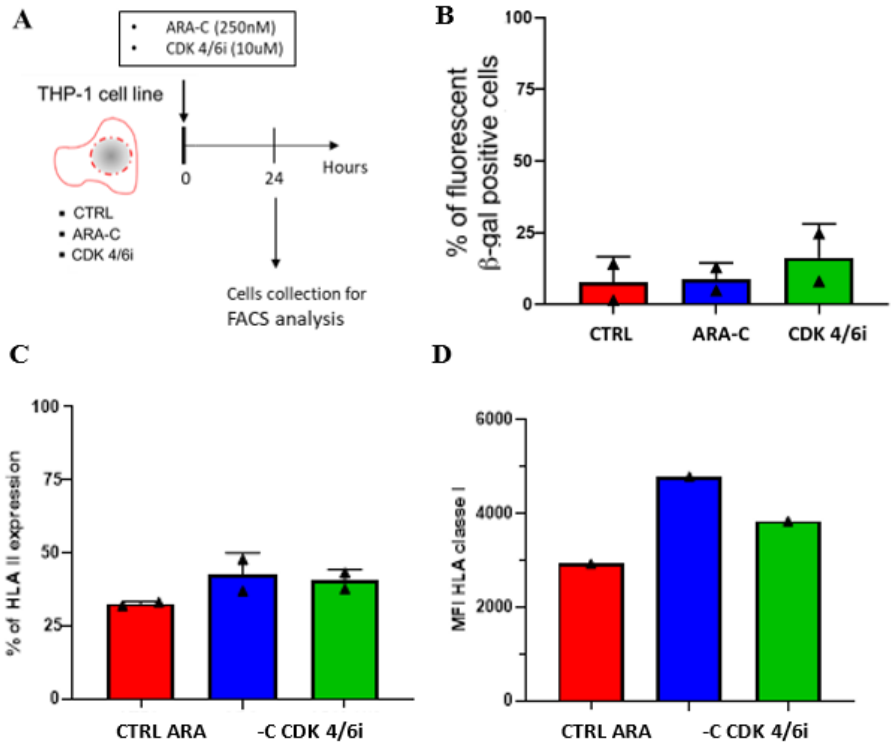


Figure 20. Acute damage is not sufficient to induce senescence and HLA upregulation in THP-1 cells. *(A) Schematic representation of THP-1 cells treatment, arrow indicates single administration. (B) Percentage of fluorescent SA-β-gal (C12FDG) positive cells or (C) percentage of HLA-II positive cells or (D) Mean Fluorescent Intensity (MFI) of HLA class I positive cells. Dots represent technical replicates. Bars represent cells untreated (CTRL, in red) or upon ARA-C (blue) and CDK 4/6i (green) treatment.*

No induction of senescence was observed in the acute treatment conditions compared to controls, suggesting that 24 hours treatment with a genotoxic agent (ARA-C) or with CDK4/6i is insufficient to induce senescence (Figure 20B). Consistently, only a modest increase in the percentage of HLA-II positive cells upon ARA-C and CDK 4/6i treatments was found (Figure 20C), and, when quantifying the MFI of HLA class I, I

observed only a modest increase for both treated groups compared to controls, although more remarkable for the ARA-C treated cells (Figure 20D). These data suggest that complete execution of a senescent program is required to modulate the expression of HLA molecules independently from the initial senescence trigger (e.g., DNA damage (ARA-C) or cell cycle inhibition (CDK4/6i)).

5.5 Transposable elements activate SASP factors in AML response to therapy

Recent literature reported an exciting novel link between SASP and transposable elements (TE) reactivation. Given the upregulation of many SASP transcripts upon induction of AML cells into senescence by ARA-C, I tested the hypothesis that TE reactivation could be the upstream regulator of widespread inflammation in TIS cells. I first analysed the expression levels of retroelements in the RNA-seq of the 4 senescence-competent matched pairs of ARA-C exposed vs untreated AML primary blasts and observed a significant upregulation of most TE families, including AluS, AluJ, ERVs and SVA (Figure 21A). I then treated THP-1 cells induced into senescence by ARA-C with a combination of two reverse transcriptase inhibitors (3TC and D4T) at 2 doses (10 or 100uM), defined as TEi, for 3 days (Figure 21B).

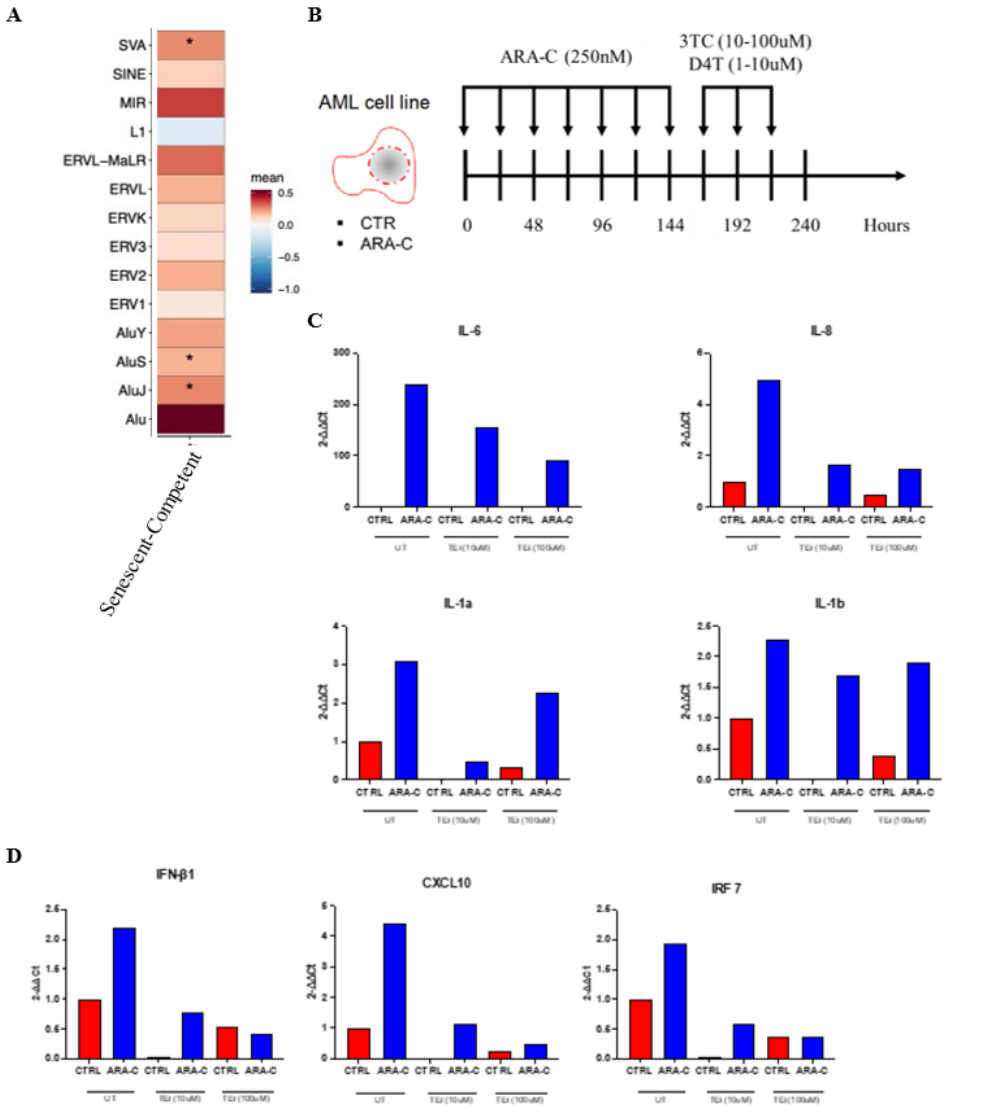


Figure 21. TIS correlates with the induction of TE expression *(A) Heatmap showing the median expression value of each member of the evaluated family after ARA-C (in vitro) Mean threshold: '***' p < 0.001, '**' p < 0.01, '*'p < 0.05. (B) Schematic representation of THP-1 cells treatment, arrow indicates single administration. (C) Gene expression analysis of canonical SASP genes (IL-6, IL-8, IL-1α and IL1β) in CTRL or ARA-C with or without TEi. Values represent an absolute expression considering GUS-B as a housekeeping gene. (D) Gene expression analysis of interferon genes (IRFβ1, CXCL10 and IRF7) in CTRL or ARA-C with or*

without TEi treated THP-1 cells. Values represent an absolute expression considering GUS-B as a housekeeping gene

After 7 days of treatment, I extracted the RNA and measured the levels of several SASP transcripts by RT-qPCR (control and treatment group are the same as in Figure 19I) (Figure 21B). Interestingly, I observed a dose-dependent down-regulation of ARA-C induced SASP genes (especially IL6 and IL8) upon administration of TEi. At the same time, IL1B levels remained unchanged and IL1a levels show a reduction only upon the low dose of administration of TEi (Figure 21C). Importantly, TEi also abolished the ARA-C induced upregulation of genes of the IFN pathway in a dose-dependent manner. This preliminary evidence suggests a key role for TE expression in SASP regulation in TIS AML cells.

5.6 ARA-C treated AML cell lines promotes an increased immunogenicity

To evaluate if the observed up-regulation of HLA class I and class II molecules observed in AML TIS cells triggers an enhanced stimulation of T cells, potentially leading to better immune-mediated clearance of senescent cancer cells, I took advantage of the Mixed Lymphocyte culture Reaction (MLR) assay, where a generalised stimulation/activation of T cells against a desired antigen can be induced. Specifically, T cells from a third party recognise an antigen-presenting cell (APC) through a T cell receptor that interacts with the HLA on the surface of the APC and are activated and proliferate. Since T cells are obtained from an unrelated healthy donor, the T cell receptor recognises a mismatched HLA, provoking an immune response. To specifically address the role of increased expression of HLA class II on TIS cells, only $CD4^+$ T cells obtained from 2 healthy donors were used. Before starting the assay, T cells were stimulated by co-culturing them with THP-1 cells in a 1:1 ratio (Figure 22A).

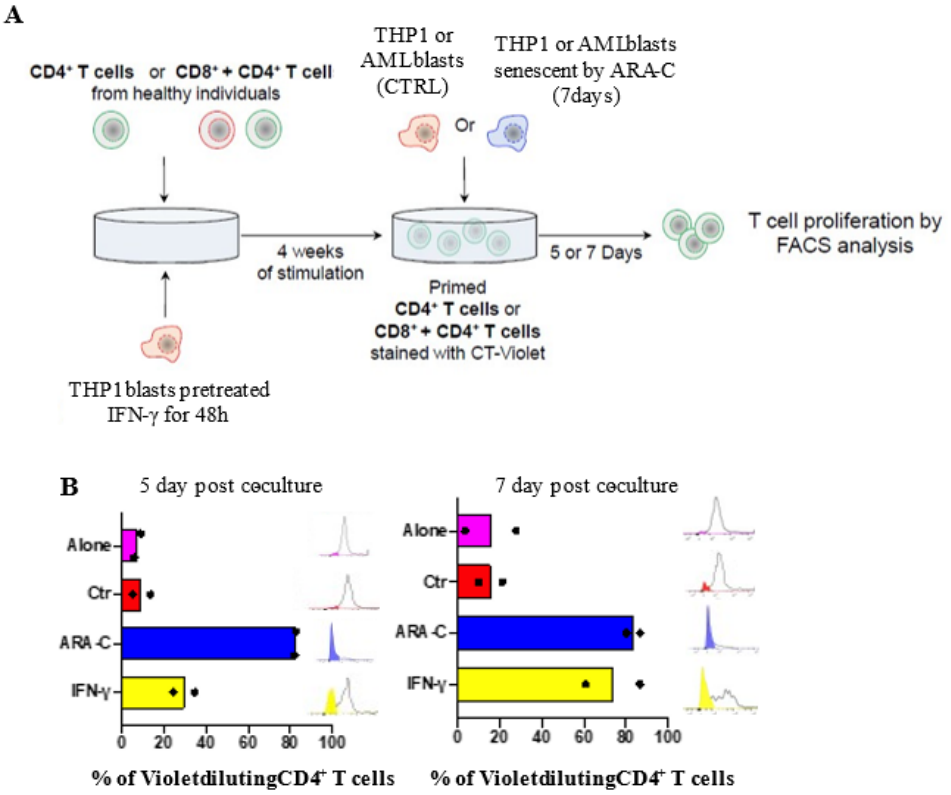


Figure 22. TIS-mediated HLA-II induction triggers T cell activation. *(A) Experimental design of a Mixed Lymphocyte Reaction (MLR) assay. After an initial 2 weeks priming of CD4+ T cells by co-culture with 48h-IFN-γ-treated THP-1 cells, T cells are co-cultured in a ratio of 1:1 with THP-1 cells either untreated or pre-treated with ARAC (7 days). T cells proliferation is assessed at day 5 and day 7 post-co-culture. (B) Percentage of proliferating (CTV negative) CD4+ T cells after 5-and 7-days post in vitro culture alone (pink) or in vitro co-culture with THP-1 cells either untreated (CTRL, in red) or treated with ARA-C (blue) or IFN-γ (yellow).*

To enhance HLA-II surface expression, THP-1 cells were pre-treated for 48h with IFN- ɣ, a known inducer of HLA-II, before co-culture. After one week, the same stimulation process was repeated. Finally, primed $CD4^+$ T cells are labelled with a vital dye (CellTrace Violet, CTV) that is diluted proportionally to their proliferation rate: the more the cells proliferate, the more diluted will be the dye. Labelled T cells were then co-cultured for 7 days with THP-1 cells either untreated (CTRL) or cells induced into-

senescence by ARA-C treatment. T cell proliferation rate was assessed at days 5 and 7 by measuring the percentage of CTV positive cells and calculating the ratio of CTV diluting cells. As a result, already at 5 days after co-culture, T cells co-cultured with ARA-C treated cells showed an increased percentage of CTV diluting $CD4^+$ T cells when compared to CTRL cells; consistently, this effect was maintained 7 days after co-culture (Figure 22B). Interestingly, T cells were more activated when co-cultured with ARA-C treated senescent cells than with those interferon-treated, suggesting that senescence establishment triggers an immune response that cannot simply be explained by the up-regulation of HLA class II molecules, which was shown to be higher upon IFN- ɣ treatment.

I then decided to evaluate the physical interaction via immunological synapses between senescent AML cell and T cells. This interaction is mediated by molecules involved in T cell activation and eventually leads to T cell-mediated clearance of AML cells. Immunological synapses allow a sustained T cell receptor-mediated signalling and a stabilised adhesion thanks to the actin cytoskeleton, which accumulates on the interface between the target cell and the T cell. I visualised and quantified the immunological synapse formation between T cells and ARA-C treated senescent THP-1 cells by ImageStreamX technology. As explained in the previous paragraph, T cells were co-cultured with THP-1 cells left either untreated (CTRL) or induced into senescence with ARA-C (ARA-C). Before co-culture, THP-1 cells were stained with C12FDG, the substrate used for fluorescent-SA-β-Gal detection (Figure 23A).

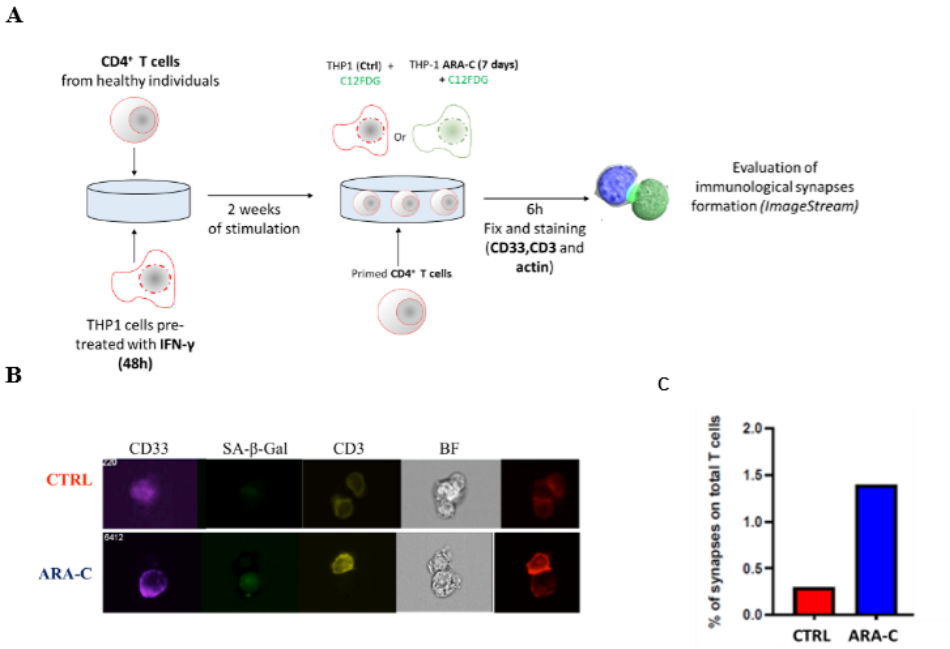


Figure 23. Increased immunological synapses formation upon ARA-C treatment of THP-1 cells. *(A) Experimental design of immunological synapses evaluation protocol. Primed CD4+ T cells are co-cultured in a ratio of 1:1 with THP-1 cells untreated or pre-treated with ARA-C (7 days) previously incubated with C12FDG, the substrate used to evaluate SA-β-gal activity. After 6 hours, cells are fixed and stained then considered with ImageStreamX. (B) Representative images of the events acquired for synapses evaluation in different channels: brightfield (BF), violet for the THP-1 marker CD33, yellow for the T cell marker CD3, red for Actin and green for fluorescent SA-β-gal. (C) Percentage of the immunological synapses number on total T cells co-cultured with either untreated (CTL, in red) or ARA-C treated cells (ARA-C, in blue).*

6 hours later, cells were fixed and stained with an anti-CD3 antibody as a marker for T cells, an anti-CD33 antibody, as a marker for AML cells and phalloidin, which binds specifically at the interface between F-actin subunits and stains actin filaments (Figure 23B). As reported in Figure 22C, ARA-C treated senescent cells showed a higher percentage of synapses on total T cells than untreated controls (CTRL). Given that the increased

percentage of immunological synapses correlates with a higher proliferation rate of T cells and a higher HLA class II expression on senescent blasts, one can speculate that chemotherapy treatment on AML blasts, upon senescence induction, promotes T cells mediated clearance of leukemic cells.

5.7 Primary AML blasts are eliminated by T cells when undergoing senescence by ARA-C treatment

At this point, I decided to perform the MLR assay using as target cells primary AML blasts, either *senescent competent* or *non-senescent competent*.

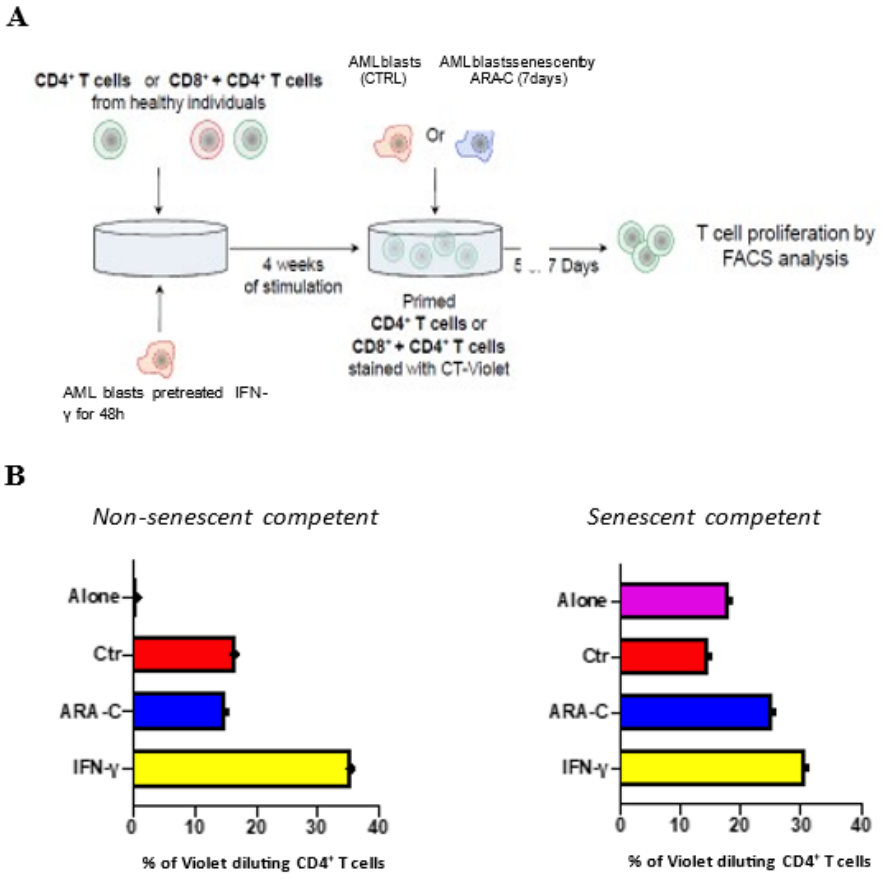


Figure 24. ARA-C treatment promotes increased immunogenicity in primary AML blasts. *(A) Experimental design of a Mixed Lymphocyte Reaction (MLR) assay. After an initial 2 weeks priming of CD4+ T cells by co-culture with 48h-IFN-γ- treated primary AML blasts, T cells are co-*

cultured in a ratio of 1:1 with target cells either untreated or pre-treated with ARAC (7 days). T cells proliferation is assessed at day 7 post-co-culture. (B) Percentage of proliferating (CTV negative) CD4+ T cells after 7-days post in vitro culture alone (pink) or in vitro co-culture with primary AML blasts (left: non-senescent competent, right: senescent competent) either untreated (CTR, in red) or treated with ARA-C (blue) or IFN-γ (yellow).

In AML blasts from a *senescence-competent* patient, I observed a comparable level of T cells expansion when cultured alone or co-cultured with untreated AML blasts while a slight increase (10%) in *non-senescent competent*, suggesting that the immunogenic basal level of primary AML cells is extremely low. On the other hand, T cell proliferation presented the same level of proliferation when ARA-C treated *non-senescent competent* blasts were used compared to the untreated one. A marked increase in proliferation was observed when the targets were *senescent competent* ARA-C or IFN- ɣ treated blasts, used for both patients as a positive control (Figure 24B). These data indicate that T cells are activated and start to proliferate when in the presence of senescent blasts; however, it remains to be determined whether this enhanced activation translates into AML cells being eradicated by T cells. To evaluate the killing activity of activated T cells against primary AML blasts, I repeated the MLR assay staining AML blasts with fluorescent SA-β-gal. Then I added to the media a fluorescent pro-substrate for caspase-3; in this way, when a cell undergoes apoptosis, this compound is cleaved and emits red fluorescence (Figure 25A).

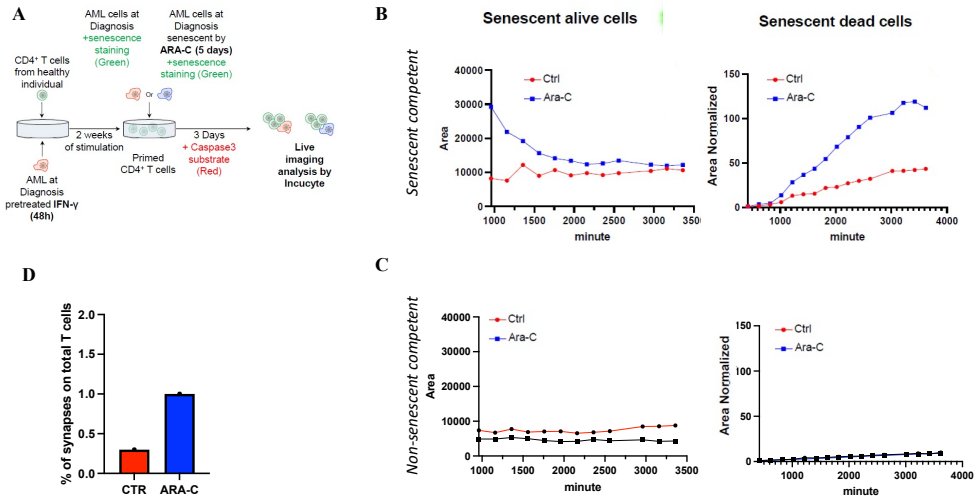


Figure 25. ARA-C treatment promotes an increased clearance of primary AML blasts by T cells. *(A) Experimental design of a Mixed Lymphocyte Reaction (MLR) assay. After an initial 2 weeks of priming of CD4+ T cells by co-culture with 48h-IFN-γ- treated primary AML blasts, T cells were co-cultured in a ratio of 1:1 with target cells either untreated or pre-treated with ARAC (7 days). Senescent alive and senescent dead cell measurements were assessed at day 3 post-co-culture. (B) Quantification of are occupied by senescent live cells is measured over time and plotted in a senescent competent (top) and non-senescent competent (bottom). The same was done for the quantification of senescent dead cells. (C) Percentage of immunological synapses on total T cells co-cultured with either untreated (CTL, in red) or ARA-C treated cells (ARA-C, in blue).*

The co-culture was then followed real-time through the *Incucyte* live imaging system, which allows the acquisition of fluorescent and brightfield images in a temperature, humidity and CO_2 controlled environment, keeping cells alive. *Senescent-competent* AML cells treated with ARA-C start with a wider area occupied by senescent live cells, positive for the green (fluorescent SA-β-gal) signal and negative for the red fluorescence (apoptosis). Over time, this area decreases to a level comparable to that observed in untreated cells (Figure 25B). Conversely, when analyzing the

area occupied by senescent dead cells, which were positive for both the green fluorescence (fluorescent SA-β-gal) and the red fluorescence (apoptosis), I observed that in ARA-C treated cells the area occupied by senescent dead cells increases over time, suggesting a T cell-mediated eradication of senescent cells. By contrast, in the *non-senescent competent* group, the area occupied by ARA-C treated senescent cells is very low and comparable to the CTRL condition, consistent with the absence of senescent cells, and was maintained constant over time (Figure 25C).

I then quantified the immunological synapses events between ARA-C treated or untreated *senescent competent* blasts and observed three times more events in the ARA-C treated condition compared to controls (Figure 25D).

All together these findings suggest that upon TIS, AML blasts show increased immunogenicity promoting their clearance by $CD4^+$ T cells.

5.8 ARA-C treated patients show an increase of senescence program in AML blasts population

Since I found that chemotherapy induce senescence in AML primary blasts when treated *ex-vivo*, I asked whether this was the case also when primary AML blasts receive chemotherapy *in vivo.* I, therefore, studied the induction of senescence in bone marrow (BM) and/or peripheral blood (PB) of AML patients collected at the moment of diagnosis, at day 4 after induction therapy *in vivo* (7+3 chemotherapy protocol), which consists of the administration of ARA-C for 7 days and 3 days of daunorubicin (anthracycline) (SENLEUK clinical protocol; Figure 26A).

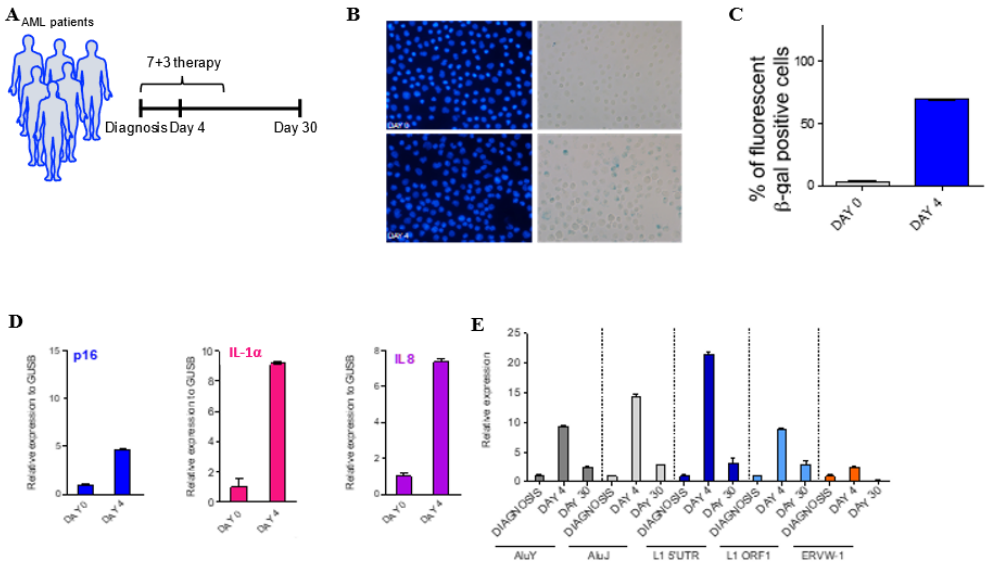


Figure 26. Patients treated with 7+3 protocol show the activation of a senescence program in AML blasts. *(A) Experimental design of the SEN-LEUK clinical protocol. PBMCs were collected from AML patients at three different time points: at diagnosis, at 4 and 30 days post-chemotherapy treatment. (B) Representative images of SA-β-gal in AML samples at diagnosis (top images) and 4 days post-chemotherapy (bottom images). (C) Quantification of SA-β-gal positive cells at diagnosis (Day 0) and 4 days post-chemotherapy (blu). (D) Gene expression analysis of p16, IL1α, IL8 by RT-qPCR on AML blasts purified from one AML patient at diagnosis (day 0) and 4 days post-chemotherapy. (E) Relative gene expression analysis of TEs in AML blasts obtained from the SEN-LEUK protocol. The blasts were analysed at the moment of diagnosis, after 4 or 30 days post-chemotherapy.*

After blasts purification with magnetic beads using CD34 or CD33 as markers of malignancy, I evaluated senescence establishment using the classical SA-β-gal assay and performed gene expression analysis of SASP transcripts and cell cycle inhibitors. I observed induction of senescence in blasts collected at day 4 post-treatment when compared to diagnosis (day 0)

(Figure 26B, C) and reported induction of SASP genes *IL-8* and *IL-1*α and in p16 at day 4 post-treatment (Figure 26D).

I then measured the level of expression of several TE families in a senescent-competent sample obtained by the SEN-LEUK protocol. I observed an upregulation of all measured TE genes 4 days post-chemotherapy treatment compared to the diagnosis (Figure 26E). This upregulation was still detected for the majority of TE transcripts at 30 days post-chemotherapy, although to a lesser extent than day 4 post-chemotherapy. These data suggest that early in response to chemotherapy AML blasts activate TE, pointing to the intriguing possibility that their activation contributes to senescence induction, interferon responses and immune-mediated recognition of senescent cells via viral mimicry.

5.9 T cell subsets differ in senescence-competent and non senescence-competent AML patients post-chemotherapy

To gain more molecular insights into the interplay between senescent cells and T cells *in vivo*, I then characterized the T cell compartment in a senescence-competent AML patient, by evaluating the differentiation, activation and exhaustion status of T cells at the moment of diagnosis and at day 30 post-chemotherapy. Since T cells require between approximately three weeks to activate and respond to a given target, I did not perform the analysis on T cells at day 4 post-treatment.

All T cells generally display 5 stages of differentiation, each of them defined by the expression of three surface markers: CD62L, CD45RA and CD95. Specifically naïve T cells are defined by $CD62L^+$ $CD45RA^+$ and $CD95^-$, stem cell memory T cells are $CD62L^+$ $CD45RA^+$ and $CD95^+$, central memory have $CD62L^+$ and $CD45RA^-$, effector memory $CD62L^-$ and $CD45RA^-$ and effector $CD62L^-$ and $CD45RA^+$ (Figure 27B). I first analyze T cell composition in an AML patient from our SENLEUK cohort of senescence-competent samples at diagnosis and observed that T cells are mainly characterized by central-memory T cells (around 70%) compared to around 30% in the healthy donor (Figure 27C).

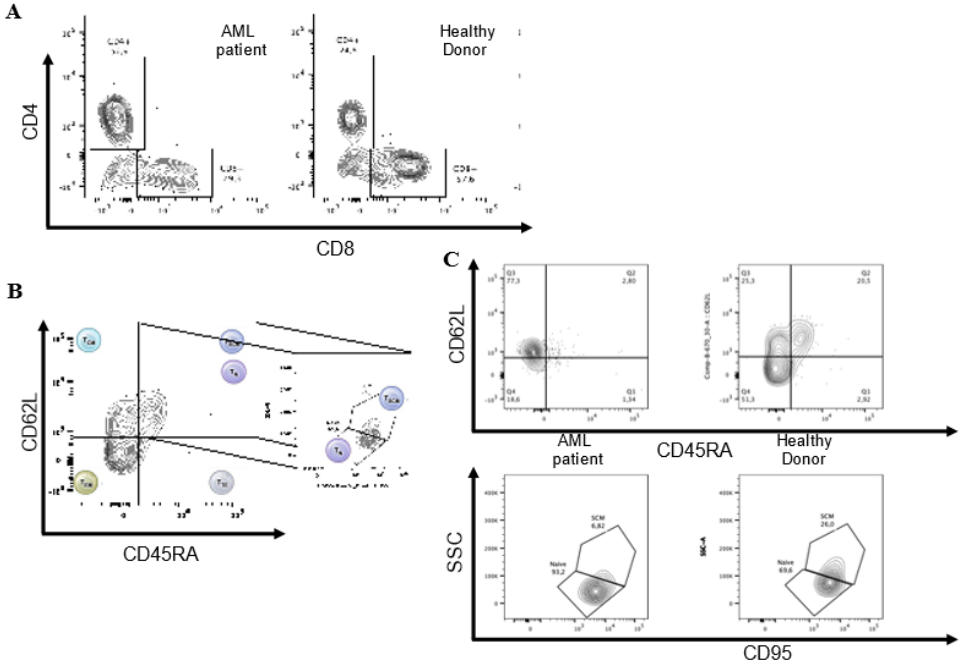


Figure 27. Subsets in T cells are AML patients treated with 7+3 protocol showing the senescent program's activation in AML blasts*(A) T cells distribution among CD4$^+$ and CD8$^+$ cells in an AML patient (left) and the healthy donor (right). (B) Gating strategy to identify T cell subsets. Naive are define as CD45RA$^+$ CD62L$^+$ and CD95$^-$, stem cell memory as CD45RA$^+$ CD62L$^+$ and CD95$^+$, central memory as CD45RA$^-$ CD62L$^+$ and CD95$^-$, effector memory as CD45RA$^-$ CD62L$^-$ and CD95$^-$ and effectors as CD45RA$^+$ CD62L$^-$ and CD95$^-$. (C) T cell subsets composition in an AML patient (left) and healthy donor (right).*

Moreover, the effector memory T cell population was lost entirely in the patient, while in the healthy donor, they were about 50% (Figure 27C). This data suggests an impairment in the patient T cells compartment likely due to the activation of a response against the blasts.

When analyzing the T cell compartment of the same senescence-competent patient at day 30, I observed that the T cells subsets were restored. Indeed, I observed the T effector memory population greatly represented compared to the healthy donor (Figure 28A, B). Moreover, the percentage of the T stem cell memory subset became comparable to the one

observed in healthy control. Interestingly, the vast majority of T cells were $CD4^+$ cells (50%) with a small representation of the $CD8^+$ T cell counterpart (25%) (Figure 28A, B). I also analyzed the T cell phenotype in a non-senescence competent patient at diagnosis and 30 days post-chemotherapy. At the moment of diagnosis, the non-senescence competent T cells present a similar phenotype to senescence-competent patients at diagnosis. After therapy, I observed a slight reduction in the subset of central memory T cells and a slight increase in effector memory and stem cell memory T cells while $CD8^+$ T cells were completely lost (Figure 28A and B).

I next measured exhaustion and activation markers on the surface of T cells in senescence-competent and non-senescent competent AML patients. I considered as exhaustion markers PD-1, TIM-3 and LAG-3, while I measured as activation markers the expression of CD25 and CD69. At the diagnosis, both senescent competent and non-senescent competent display an exhausted T cell compartment (around 90% of T cells are positive for at least TIM-3 and LAG-3) (Figure 28D), with a small proportion of T cells displaying activation markers on their surface, likely reflecting a phenotype induced by high tumour burden. However, after chemotherapy, the T cells in the senescence-competent patient show an increase in the levels of their activation markers (CD25 and CD69) compared to a non-senescent competent patient (Figure 28C) while the surface expression of TIM-3 and LAG-3 markers was reduced. Although the sample size for this analysis was very limited, these preliminary data point to a possible correlation between the induction of senescence in AML blasts and a less exhausted T cell phenotype (Figure 28D). These findings, if validated in a bigger cohort of senescence-competent and non-senescence-competent AML patients, will reinforce the critical role of senescence establishment in AML immunogenicity.

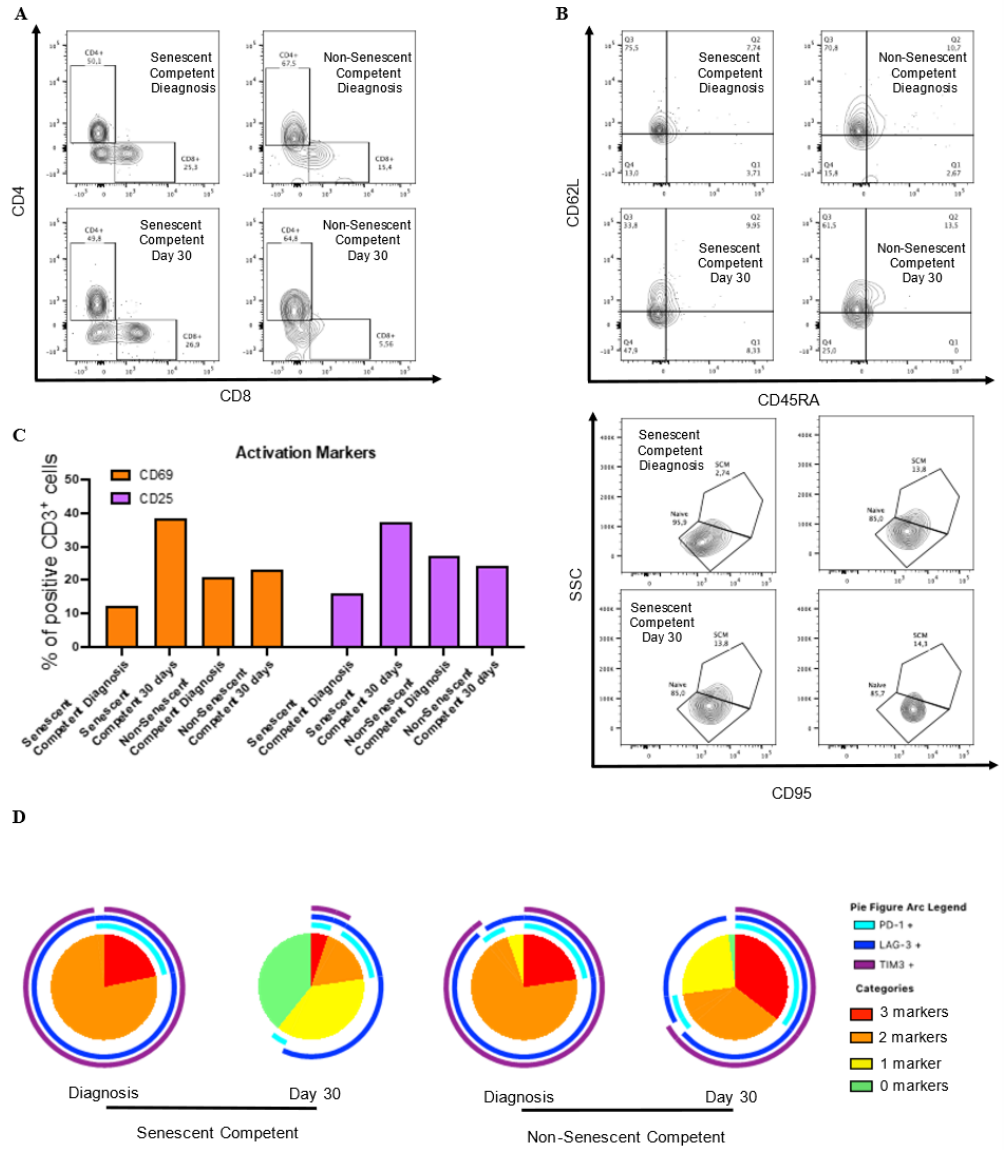


Figure 28. Characterization of T cell subsets in senescence-competent and non-senescence competent AML patients. *(A) T cell distribution among CD4$^+$ and CD8$^+$ cells in two AML patients at the moment of diagnosis and 30 days post-chemotherapy. (B) T cell subsets composition in two AML patients: naive were defined as CD45RA$^+$ CD62L$^+$ and CD95$^-$, stem cell memory as CD45RA$^+$ CD62L$^+$ and CD95$^+$, central memory as CD45RA$^-$ CD62L$^+$ and CD95$^-$, effector memory as CD45RA$^-$ CD62L$^-$ and CD95$^-$ and effectors as CD45RA$^+$ CD62L$^-$ and CD95$^-$. (C) T cell activation markers in two AML patients. (D) T cell exhaustion markers in two different AML*

patients. The pie chart represents the percentage of T cells positive for 3, 2, 1 or 0 markers. The arcs indicate the percentage of cells positive for that specific marker.

6. Discussion

Since its first description by L. Hayflick more than 50 years ago, cellular senescence has been extensively studied and shown to contribute to relevant biological processes, including organismal ageing and cancer development. Cellular senescence is a state of irreversible growth arrest triggered by many cellular and external stimuli, such as the induction of the DNA Damage Response (DDR), telomeric shortening, oncogene activation, and the inactivation of tumour suppressors (Di Micco *et al.*, 2006). Even though immortal by definition, cancer cells can still undergo senescence in response to genotoxic agents; in fact, many anticancer therapies have the potential to selectively induce senescence in cancer cells, which may in part contribute to their therapeutic activity. This phenomenon, defined as therapy-inducing senescence (TIS), is generally triggered by DDR activation, resulting in the p53/p21 pathway activation and is considered a positive outcome in cancer therapy, halting the proliferation of oncogenic cells at least in the short-term.

Senescent cells also activate the so-called senescence-associated secretory phenotype (SASP), a plethora of pro- and anti-inflammatory molecules, which allows them to communicate with the surrounding microenvironment. Through SASP, senescent cells can reinforce the senescence state in a cell-autonomous manner promoting stable cell cycle arrest (Coppé *et al.*, 2010; Weiner-Gorzel *et al.*, 2015; Borghesan *et al.*, 2019; Fulzele *et al.*, 2019). More recently, it was reported that senescent cells could stimulate the recruitment of immune cells for their efficient eradication (Burton and Stolzing, 2018). More in detail, inflammatory cytokines and chemotactic molecules released in SASP promote the activation of immune cells, from both innate and adaptive systems, and favour senescent cells eradication pointing to an increased immunogenicity of senescent cells.

The literature reports the beneficial effects of immune-mediated clearance of senescent cells in preventing tumour development (Burton and Stolzing, 2018). The senescence program can also promote the overexpression of NK ligands like MICA and ULBP2 in senescent fibroblast for its receptor NKG2D to mediate NK cytotoxicity against senescent cells. These findings were confirmed even in the context of haematological malignancies; indeed, in multiple myeloma, chemotherapy treatment via doxorubicin administration was found to induce this upregulation triggering an NK-mediated senescent cells clearance.

The involvement of a $CD4^+$ T cell-mediated immune response upon senescence induction was reported in other studies; for example, Kang et al. showed that upon HLA class II expression, $CD4^+$ T helper cells could promote the elimination of premalignant senescent hepatocytes. Overall, even though senescent cell immunosurveillance promotes the clearance of premalignant and malignant cells in both solid and haematological tumours, the molecular players involved in the crosstalk between senescent cancer cells and immune cells are still poorly understood.

An important consideration is that, if not restricted in time or the absence of an efficient immune system, SASP can also potentially enhance the malignant transformation of surrounding cells in a paracrine manner. Indeed, it is reported that SASP factors can promote pre-neoplastic epithelial cells proliferation (Krtolica *et al.*, 2001; Lau and David, 2019).

In the present study, I focused on AML as a working model, an aggressive disease of bone marrow characterised by aberrant and fast proliferation of myeloid cells compartment. First-line treatment for this haematological malignancy is based on high doses of Cytarabine (ARA-C) and Dounrubicine administration (Kantarjian *et al.*, 2021). This protocol is known as the 7+3 regimen because it consists of daily administration of ARA-C (for 7 days) and the short anthracycline infusion on each of the first 3 days. Treatment failure rapidly occurs due primarily to the development of chemoresistant clones (Sekeres *et al.*, 2020), thus highlighting the need to delve deeper into the cellular responses occurring early after therapy.

First, I evaluated the functional consequences of ARA-C on two AML cell lines: one mutated for p53 (THP-1) and the other wild type (OCI-AML3). I observed a reduction upon chemotherapy treatment in cell proliferation with a slight increase in the percentage of apoptotic cells in both systems. This aligns with published literature showing that ARA-C promotes cell cycle arrest in the S phase (Sun, Coppé and Lam, 2018b). Next, by exploiting a novel fluorescent SA-β-galactosidase assay, I found that the growth arrest observed was associated with senescence establishment in both cell lines. As known from the literature, tumour suppressor p53/cell cycle inhibitor p21 and RB/p16 pathways are the main actors involved in cell cycle arrest associated with senescence establishment. We noticed that only OCI-AML3 activate p53 and p21 in response to senescence-inducing stimuli, while in THP-1 cells, the arrest seems independent from p53 activation. Because the INK4A locus encodes for p16 and the cell cycle inhibitor p15 is inactivated by DNA methylation in THP-1 (Guo *et al.*, 2000), the molecular effectors of therapy-induced arrest in these cell lines remain to be investigated. The PTEN-p27 axis plays essential functions in senescence maintenance in human fibroblasts (Flores, Martiń-Caballero and Garciá-Fernández, 2014). P27 protein is frequently downregulated in many human cancers, and its inactivation correlates with a worse prognosis in patients. Therefore, p27 levels may increase in AML cells in response to DNA damage. The induction of heterochromatin has also been associated with the activation of cellular senescence (Prieur *et al.*, 2011), thus suggesting that TIS in AML could be as well activated in an epigenetic manner.

I then established a protocol to treat *ex vivo* primary AML blast at diagnosis on a layer of stromal cells. Upon treatment, I was able to observe senescence induction by RNA-seq and imaging-based evaluation of SA-β-gal in 5 AML patients. Similar data were recently reported from the Melnick lab (Duy *et al.*, 2021), showing that *ex-vivo* cultured primary AML cells upon three days of ARA-C or anthracyclines treatment recapitulates

key features of the senescence program activation, among which increased cell size and SA-β-gal activity.

Surprisingly, in our RNA-seq datasets, we not only detected upregulation of senescence-associated genes in chemotherapy-treated samples but also reported the induction of several HLA class I and II molecules compared to untreated counterparts. I validated RNA-seq findings by measuring the expression levels of HLA molecules on cell surfaces on ARA-C treated AML primary blasts, OCI-AML3 and THP-1 cells. IFN-γ was used as control, given its role as HLA positive regulator (Zhou, 2009). As a result, both HLA class I and II were found upregulated in the treated counterpart in both primary samples and THP-1 cells, even if to a lesser extent compared to IFN-γ treated cells. In OCI-AML3, I could not appreciate a good induction of HLA-II molecules because all cells express these molecules at a steady-state, while in THP-1, where just 30% of cells were positive for HLA class-II, the induction upon chemotherapy almost reached 100%. In addition, a positive correlation between senescence AML blasts and HLA-II upregulation (and HLA class I intensity) was found, suggesting that the two phenomena are strictly interconnected.

To better investigate the molecular mechanisms involved in such upregulation, I focused on the role of DNA damage response activation and cell cycle arrest, which are two primary outcomes of ARA-C treatment. RNA-seq data showed the upregulation of several immune-related gene categories, including interferon-alpha response, interferon-gamma response and inflammatory response in primary blasts upon chemotherapy treatment; these data were then validated via qPCR for 3 interferon-related genes (IRF7, CXCL10 and IFN-β1) and 4 genes of the "classic" SASP (IL-6, IL-8, IL1α and IL1β). To characterise whether the increased HLA expression requires senescence induction or simply the activation of the DDR, I compared side-by-side the effect of ARA-C treatment in THP-1 cells with Palbociclib, a cyclin-dependent kinase (CDK) 4 and 6 inhibitor (CDK4/6i) able to induce senescence and cell cycle arrest in the absence of physical DNA damage. In combination with endocrine agents, CDK4/6i is currently

used to treat metastatic breast cancer, and to date, it is under evaluation for clinical trial implementation in the context of other solid tumours (Schettini *et al.*, 2018). In AML, the use of Palbociclib can promote apoptosis if used in combination with autophagy inhibitors in a synergistic manner (Nakatani *et al.*, 2021). Through the binding with CDK4 and 6 proteins, Palbociclib blocks the progression through the G1 phase of the cell cycle without inducing physical DNA damage working as a p16 mimetic; recently, it has been shown that this growth arrest correlates with senescence induction in different human cell lines (healthy fibroblasts, breast cancer and skin cancer cells) (Jost *et al.*, 2021). Moreover, in 2017, a study reported that different leukaemia cell lines (i.e., MV4-11, HL-60, NB4) treated with a novel CDK 4/6 inhibitor (LEE011 or Ribociclib), underwent cell cycle arrest and cellular senescence induction (Tao *et al.*, 2017).

As expected, CDK 4/6 inhibitor-treated THP1 cells showed a higher percentage of SA-β-Gal positive cells when compared to untreated cells; notably, this treatment led to an increase in HLA molecules expression comparable to that observed in ARA-C treated cells. These findings confirm that senescence establishment leads to HLA up-regulation rather than chemotherapy *per se*, although the mechanisms behind this effect are not yet understood. The literature reports the induction of the canonical HLA class I (HLA-A, HLA-B and HLA-C) in a mouse model of mammary carcinoma and PDX of the same disease with the consequent elimination of the tumour due to activation of the immune system (Goel et al., 2017). In this work, we did not investigate the interaction between senescent AML cells via CDK4/6 inhibitors and the T cells, but it is something I will explore in the future. Indeed, we did not observe in CDK4/6i treated cells upregulation of SASP effectors IL6 and IL8, neither we find induction of Type I and II IFN genes, compared to cells induced into senescence by ARA-C, and we believe that the transcriptional profile of CDK4/6i treated AML may help uncover the cellular pathways linking this type of senescence to increased AML immunogenicity. In literature it was recently reported the link between the expression of Type III IFN genes and the overexpression of

HLA class I genes (Goel et al., 2017); this aspect should be better investigated for both HLA class I and II overexpression and in ARA-C treated ones.

In addition, I assessed whether acute induction of the DDR or prompt cell cycle arrest could impact the expression of HLA molecules by treating cells with ARA-C or CDK4/6i for only 24 hours (without inducing chronic DNA damage or cell cycle arrest). Senescence induction was not found in all the treated conditions, as detected by SA-β-Gal, in line with the fact that senescence takes several days to be established. Notably, only a minor increase in the percentage of HLA-II positive was found in treated cells. No changes in HLA-I levels were reported, suggesting that full activation of a senescence program is required to induce the increased expression of HLA molecules, independently from the initial senescence trigger, DNA damage upon ARA-C treatment or cell cycle inhibition through CDK4/6i treatment.

Given that HLA class II molecules play a central role in the control of adaptive immune responses through the selection of the $CD4^+$ T cells, I analysed whether the observed HLA-II up-regulation in senescent THP-1 cells was correlated with increased immunogenicity, as determined by the enhanced proliferation of $CD4^+$ T-cells likely promoting a stronger senescent cells clearance. Intriguingly, $CD4^+$ T cells were more activated when co-cultured with senescent cells upon ARA-C treatment. This finding supports the notion that senescence may lead to enhanced T cell stimulation. However, other functional assays, such as cytokine release measurement through IFN-γ ELISpot assay, antigen-specific activation (by CD137/41-BB upregulation), or T-cell mediated killing activity, are required to claim that senescent blasts increase T cell-mediated immunogenicity.

In addition to the increased T cell proliferation, ARA-C treated senescent blasts displayed a higher percentage of immunological synapses formation than untreated cells, as detected by ImageStreamX analysis. Since the increased percentage of immunological synapses correlates with a higher proliferation rate of T-cells and a higher HLA-II expression, it is tempting to speculate that these are “positive” synapses (not inhibitory ones) and,

consequently, that ARA-C treatment may promote T-cell activation via senescence engagement. The best way to evaluate the AML immunogenicity should be by co-culturing primary blasts with immune cells from the same patient and evaluate their activation. To aim this, AML blasts will be co-cultured in the presence of highly immunogenic peptides like melanA or WT1. Unfortunately, these tumour antigens are presented by HLA class I and not by class II; the absence of tumour antigens presented by HLA class II is traceable by studies in solid tumours.

We are currently trying to uncover how SASP is activated in TIS AML. It was shown that after DNA damage induction, cytosolic DNA fragments are recognised by cytosolic DNA sensors, among which the cGAS/STING pathway represents the main activators of the SASP program (Loo *et al.*, 2020). Therefore, it would be important to identify cytosolic fragments in AML cells undergoing senescence and correlate them with SASP induction. Significantly, DNA damaging agents could reduce nuclear integrity and degrade Lamin B1, thus allowing the exit of DNA from the nucleus. Consistent with this speculation, Lamin B1 depletion in human fibroblast cell lines increased CCFs formation, while Lamin B1 overexpression impaired their generation (Adams *et al.*, 2013). Upon ARA-C treatment, we observed an upregulation of Type I IFN genes, and this aspect is reported to be a marker of good response to chemotherapy (Sistigu et al., 2014), suggesting a link between viral mimicry and response to therapy. In line with this observation, I found increased transcription of transposable elements (TEs) generally activated in response to different stressors, like DNA damage, heat shock or treatment with genotoxic agents (Zhang, Chen and Zhao, 2015b) and senescence-inducing stimuli. More recently, in several solid cancers it was shown that p53 triggers viral mimicry, ultimately recruiting the immune system and inhibiting immune evasion (Zhou et al., 2021). More in detail, these effects were caused by the expression of endogenous retroviruses (ERVs). Since 2013, the link between senescence and a global epigenetic change promoting transposable elements reactivation was established (De Cecco *et al.*, 2013) and more

recently, the absence of this epigenetic switch was linked to evasion from viral mimicry and an increased immune evasion of breast cancer (Deblois et al., 2020). Acting in the opposite way, by opening the chromatin after inhibition of histone methyl transferase in cancer, researchers reported ERVs and IFN genes overexpression (Chiappinelli et al., 2015). More recently, the L1 TEs family was linked to interferon production in senescent cells promoting an aged-like inflammation (De Cecco *et al.*, 2019).

Interestingly, TEs were upregulated upon chemotherapy in both THP-1 and primary AML samples undergoing senescent. Inhibiting TE expression in TIS cells with two molecules D4T and 3TC, blunted SASP in a dose-dependent manner indicating for the first time that TE reactivation is an upstream mediator of SASP in AML. However, if TEi treated senescent cells show less HLA class I and II upregulation and T cell-mediated immunogenicity remains to be further investigated.

The paracrine effects of SASP on tumour growth remain an area of intense investigation. Indeed, SASP can induce paracrine senescence in human fibroblasts (Coppé *et al.*, 2010) and promote tumour suppression and eradication. Nevertheless, several works reported that SASP factors might promote cancer proliferation (Krtolica *et al.*, 2001; Lau and David, 2019), apoptosis resistance (Terlecki-Zaniewicz *et al.*, 2019) and tissue dysfunctions (Weiner-Gorzel *et al.*, 2015). A publication from C. Schmitt's group reported an unexpected cell-intrinsic link between the senescence program and the acquisition of self-renewing properties, which they postulate serves as a physiological rescue mechanism during development and in tissue homeostasis (Milanovic *et al.*, 2018).

Combining this knowledge, we speculate that the persistence of some senescence cancer cells after chemotherapy treatment may contribute to relapse, suggesting that mitigation of senescence-related detrimental programs after induction therapy may be beneficial to AML patients in the long-term. Selective SASP inhibitors may indeed reduce the negative aspects of senescence on tumour growth. However, SASP inhibitors effects may be transient, and in this context, senolytic compounds will be preferred

to ameliorate chemotherapy-induced side effects in cancer patients and delay or prevent AML relapse. These compounds exploit senescence vulnerabilities and have been shown to eliminate senescent cells in preclinical models (Chang *et al.*, 2016a). Senolytics are still far from being used on humans due to excessive toxicity suggesting the medical need to keep developing new drugs to eliminate senescent cells and inhibit selected SASP factors. Another way to prevent relapse is to further enhance the immune system activation against senescent cancer cells.

I acknowledge that our *in vitro* system is reductionist compared to the complex situation of TIS induced *in vivo* in leukemic patients. Cancer cells undergoing senescence by chemotherapy should be eradicated by the innate and adaptive immune system, thus limiting *in vivo* their persistency. However, given the haematological origin of the tumour under consideration at the advanced age of disease onset, I can speculate that the immune system of AML patients will be compromised, particularly in the T cell compartment, as described for the exhausted phenotype of $CD8^+$ T cells, that could be only partly reverted upon chemotherapy (Knaus *et al.*, 2018). However, the exciting observation in patients that activate a senescence program (senescence-competent) that the T cell compartment appears less exhausted upon chemotherapy and more activated is in line with the increased immunogenicity observed *ex-vivo* when performing co-culture experiments between TIS cells and T cells. This aspect was not observed in patients that did not activate a senescence program upon therapy (non senescent-competent) still in line with the *ex-vivo* experiments. I also observed a switch in T cell distribution between $CD4^+$ and $CD8^+$ in AML patients at the moment of diagnosis: in healthy donors, more than 50% of T cells are $CD8^+$ and just 25-30% are $CD4^+$; in AML patients these percentages are inverted. It would be interesting to investigate if this perturbation is caused by a reduction of $CD8^+$ or an expansion of $CD4^+$ maybe with a T_{reg} phenotype able to inhibit T cell activation. Another important aspect will be to define whether senescence activation correlates with the response to therapy in primary AML patients. I was able to stratify

10 AML patients between responder and non-responder based on the capability to activate the senescence program. AML is a very heterogeneous disease due to different genetic mutations. Unfortunately, in our small cohort, we were not able to evaluate the effect of senescence on patients bearing distinct genetic background. However, this aspect of my thesis is part of a more extensive work performed in collaboration with the lab C. Schmitt, where more than 200 patients were analysed and induction of senescence was associated with a better clinical outcome upon chemotherapy irrespectively of their genetic make-up.

7. Conclusions and future perspectives

Overall, in this PhD thesis I present the senescence program as a key player for tumour suppression in AML. I convincingly show that senescence induction arrest cell cycle attest of leukemic cells and increases blasts immunogenicity promoting their eradication by T cells. Even if several aspects have been touched on in this work, others need to be further investigated.

Delving deeper into the molecular mechanisms, in the context of ARA-C-induced senescence, it remains to be further elucidated how the activation of the DNA damage response cascade leads to HLA upregulation in AML cells. In the future, we envision assessing the role of the upstream mediators of the DNA damage cascade by exploiting chemical inhibitors (such as ATMi, ATRi and DNAPKi). Moreover, it is our main scientific interest to elucidate how senescence affects the activation of other immune cell types of the innate system (including NK cells, macrophages and DCs), besides T cells.

In the beginning, I presented the clinically relevant chemotherapy agent cytarabine (ARA-C) able to activate the senescence program in two AML cell lines (THP-1 and OCI-AML3) and in 5 primary patients samples. This senescence program activation is independent of p53 status and genetic background. Analysing RNA-seq from AML primary blasts senescent by ARA-C administration, I observed induction of several HLA molecules, cell cycle inhibitors and SASP cytokines. These data were mostly recapitulated in THP-1 cells: the induction of SASP genes and cell cycle inhibitors were indeed confirmed by qPCR and WB analysis. Moreover, the induction of HLA molecules both I (HLA-ABC) and II (HLA-DR) molecules by FACS analysis correlated in primary samples with the activation of a senescence program. Correlation does not mean causality therefore in the future, I will

assess whether genetic inactivation of p16 or p21 cell cycle inhibitors affects the levels of HLA molecules upon chemotherapy.

To elucidate the contribution of DDR in senescence establishment and SASP production in the AML model, I treated THP1 with CDK4/6 inhibitor Palbociclib, a drug that is used against breast cancer to induce G1 cell cycle arrest. From these experiments, we can elicit that DDR is not required for senescence establishment and CDK4/6i treated AML displays upregulation of HLA molecules. Conversely, SASP genes were less induced when AML cells were treated upon CDK4/6i. This phenotype is clear for both inflammatory cytokines and type I and II interferon genes.

TIS cells may favour tumour progression or relapse through the proliferation of more aggressive or reprogrammed stem cell-like cancer cells (Milanovic *et al.*, 2018; Fitsiou, Soto-Gamez and Demaria, 2021). In the future, I plan to identify SASP factors in TIS AML by protein arrays and investigate the pro-tumorigenic role of SASP on untreated AML blasts. Interestingly, I observed the induction of several transposable elements (TEs) when primary blasts and THP-1 cells were treated induced into senescence. In THP-1 cells I was able to revert the phenotype of SASP induction treating TIS with TEi, 3TC and D4T. This discovery suggests a link between SASP genes and TEs reactivation in AML upon chemotherapy. In the future, I will also analyse whether TEs are upstream mediators of the observed increased expression of HLA molecules and TIS immunogenicity and dissect the molecular networks of transcription factors activated downstream of TEs.

Of note, induction of TIS in AML triggers increased $CD4^+$ T cell activation, immunological synapses and blasts killing in mixed lymphocyte reaction (MLR) assays. Combining all these data, it is becoming clear that senescence establishment promotes T cell-mediated senescence eradication. MLR experiments have several biases, the most important is the fact that T cells are from an unrelated donor and when cultured with primary blasts mimic the effect of bone marrow transplantation. In the future, I will perform MLR experiments with T cells collected from the same AML

patient undergoing senescence via chemotherapy. I will culture AML cells in presence with highly immunogenic peptides (like melanA or WT1) and then measure T cell activation, senescent blast killing and immunological synapses formation (Galloway et al. 2019).

Another important implication of my work will be to test TIS immunogenicity in preclinical mouse models of AML. In collaboration with the laboratory of Dr L. Vago at San Raffaele, we established patient-derived xenografts (PDX) from primary AML and we plan to perform *in vivo* chemotherapy and combinatorial approaches with senolytics, SASP inhibitors or T cells injection for complete AML eradication. We will monitor AML growth in mice by the bioluminescence of leukemic blasts transduced with a luciferase-expressing construct already available in Di Micco's lab. A significant challenge of PDX models for human AML is the absence of an immune system, limiting our ability to investigate the interaction between senescent leukemic blasts and the immune microenvironment. A solution to such a problem may be the co-infusion of the patient or healthy donors derived T cells, a procedure that still requires thorough optimization. Complementary approaches will be to make use of the well-established NRasG12D/MLL-AF9-driven (NMA) mouse model of AML (Milanovic *et al.*, 2018) or to engineer murine HSCs with MLL-AF9 translocation (Milne, 2017) to be transplanted into syngeneic recipients in the absence of myeloablative procedures. These systems allow the preservation of the immune system and provide a suitable model to study the crosstalk between leukemic cells and the immune microenvironment.

Furthermore, by crossing NRasG12D/MLL-AF9-driven (NMA) mouse model with a senescence mouse model such as p16- (Wang *et al.*, 2021b) or p21-Cre (Demaria *et al.*, 2014), we could generate a system where we can track the expression of senescence markers in leukemic cells upon chemotherapy and evaluate their role on the immune system before and after the genetic ablation of senescent cells. In this setting, we aim to adopt high-throughput imaging techniques coupled with proteomics and/or transcriptomics to identify immune niches surrounding senescent/non-

senescent AML cells to understand the composition and mechanisms of interaction. This elegant system will deliver novel tools for the study of TIS in AML and significantly deepen our understanding of immune responses in AML, providing candidates to be validated in human samples.

To increase the clinical relevance of our findings, we also plan to measure senescence and its immunogenicity in response to other regimens used in the clinic for the treatment of AML patients. Specifically, since intensive chemotherapy may not be suitable for elderly patients, alternative therapeutic options are available, given the frequent presence of comorbidities. In last years the administration of azacytidine (AZA), a chemical cytidine analogue, was found to be effective for the treatment of myelodysplastic syndromes (Raj and Mufti, 2006) and AML (Schuh *et al.*, 2017); however, given in monotherapy, AZA treatment has still low response rates and requires 3 to 4 months to achieve best responses (which, are still no curative) (Dombret *et al.*, 2015).

Therefore, recently, the combination of AZA and Venetoclax, a Bcl-2-selective inhibitor, has shown effectiveness in AML elderly patients (DiNardo *et al.*, 2019). Venetoclax (ABT-199) was found to induce apoptosis in haematological tumour cell lines since their survival relies on Bcl-2 (Souers *et al.*, 2013). Additionally, Venetoclax treatment led to tumour regression in mouse xenograft chronic lymphocytic leukaemia (CLL) models and a clinal trial on patients with refractory CLL (Souers *et al.*, 2013). More importantly, Venetoclax is a molecule derived from Navitoclax, a drug developed to eliminate selectively senescent cells. Indeed, Venetoclax treatment was found to impact senescent fibroblasts (IMR-90 cell line) viability (in a dose-dependent manner) in the context of OIS (Yosef *et al.*, 2016b). Integrating these findings with our data we can speculate that the good response of patients to AZA + Venetoclax, is due to a synergistic effect: first AZA will induce senescence in AML blasts and then Venetoclax will eradicate senescent blasts, ensuring better clinical outcomes in the long-term.

In summary, this thesis provides evidence that senescence is a key actor in the response of AML cells to ARA-C treatment, mediating their increased recognition by the adaptive immune system. Our findings set the stage for future therapeutic approaches based on senescence modulation and or eradication for leukaemia treatment.

8. Materials and methods

Cell culture

OCI-AML3 cells were maintained in IMDM medium supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria), 2% Penicillin/Streptomycin (P/S) and 1% Glutamine at 5% CO2 and 37°C. THP1 cells (from ATCC) were maintained in RPMI medium supplemented with 10% FBS, 2% P/S and 1% glutamine at 5% CO2 and 37°C.

Patients Samples

Our study is based on a test cohort of 10 patients admitted to the IRCCS San Raffaele Scientific Institute, Milan, Italy, between March 2012 and September 2019 as newly diagnosed AML. Bone marrow aspirates and peripheral blood samples were obtained upon informed patient consent, and the local ethics commission specifically approved the use of primary samples for research.

Patient Characteristics of the Milan cohort	
Age at study entry – yr	59.7±12.6
Male sex – n° (%)	8 (80)
Bone marrow blasts at diagnosis – %	67.5±23.4
Normal cytogenetics – n°/total n° (%)	6/10 (60)
White-cell count at diagnosis –	103,300±117,000

n°/mm³	
CD33$^+$	10/10 (100)
CD34$^+$	4/10 (40)
Mutation – n°./total n°. (%)	
NPM1	3/10 (30)
FLT3	5/10 (50)
DNMT3A	0/6 (0)

T cell purification (healthy donor and patients)

Whole blood samples were processed from healthy donors (HD) and peripheral blood mononuclear cells (PBMCs) were obtained through density gradient centrifugation using Lymphoprep™. To enrich for CD4$^+$ T lymphocytes, subsequent magnetic separation with CD4$^+$ T Cell Isolation Kit (Miltenyi) was performed using the manufacturer's protocol. After the magnetic separation, viable samples consisted of >90% CD4$^+$ cells.

Cell treatments

To find the best dose of ARA-C treatment to induce cellular senescence in AML cell lines, a titration was performed with 100 and 250nM of ARA-C for seven consecutive days. Cell were plated at 1x10^5 cells/mL in a 6 multiwell plate in the presence of 250nM ARA-C. To induce cellular senescence by Palbociclib (CDK 4/6i) treatment, cells were plated at 1x10^5 cells/mL in a 6 multiwell plate in the presence of 10uM CDK 4/6i. The drug was added every other day to the cell medium.

Blasts purification from patients and ex vivo treatment

Primary human AML samples were cryopreserved, thawed and cultured on irradiated BJhTERT feeder layers in 5637 cell line-conditioned and cytokine-supplemented media. Blast purification was achieved by a CD34-

or CD33-based magnetic-activated cell separation system after 3 days of pre-culturing. AnnexinV signal was used to analyse cell viability and density after 5 days of treatment; fluorescence-based detection of SA-β-Gal (fluorescence SA-β-Gal) was performed as described below. Immunofluorescence image-based flow cytometry was conducted on an Amnis ImageStreamX Mark II Imaging Flow Cytometer (Luminex). Recorded immunofluorescence intensities were normalised by cell size to minimise interference by differences in autofluorescences between conditions for individual samples. The treatment-induced SA-β-Gal ratio (*i.e.* the fraction of SA-β-Gal reactive viable cells under treatment divided by SA-β-Gal-reactive viable untreated cells at the same time point) was calculated to determine the TIS capacity of individual AML samples.

Growth curves

For the growth curve, $1x10^5$cells of interest were plated in a 6 multiwell plate with 1mL of medium (which medium is described in cell culture). For each condition, a technical triplicate was prepared. Every other day, starting 24h after seeding, cells were counted with trypan blue solution using counting slides.

Immunofluorescence analysis

Multitest slides were treated for 20 min with Poly-L-Lysine solution (Sigma-Aldrich) at 1mg/mL concentration. After two washes with DPBS solution, approximately $1.5x10^5$ cells were seeded on covers for 20 min and fixed with 4% paraformaldehyde for 20 min. Next, cells were permeabilised with 0.2% Triton X100 and probed with the indicated primary antibodies after blocking with 0.5% BSA and 0.2% fish gelatin in PBS. After primary antibodies incubation, we performed three washes with DPBS and incubated with Alexa 488-, 568- and/or 647-labelled secondary antibodies (Invitrogen). Nuclear DNA was stained with DAPI (Sigma Aldrich), and covers were mounted with Aqua-Poly/Mount solution (Polysciences. Inc.)

on glass slides. Fluorescent images were acquired using Widefields Zeiss Axio Observer.Z1 microscope. (M&M from Biavasco et al., 2021).

SA-β-Gal staining

Multitest slides were treated for 20 min with Poly-L-Lysine solution (Sigma-Aldrich) at 1mg/mL concentration. After two washes with DPBS solution, approximately $1.5x10^5$ cells were seeded on covers for 20 minutes and fixed for 20 minutes with 4% paraformaldehyde. After two washes with PBS covers were incubated overnight at 37°C in the buffer solution (pH 6.0; Cell signalling technology Kit #9860) containing 1mg/ml 5- Bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal). Positive cells display blue signals in their cytosol. Images were acquired with Widefields Zeiss Axio Observer.Z1 microscope.

Fluorescent-SA-β-Galactosidase staining

Leukemic cells were seeded at the $1x10^5$ cells/mL concentration and incubated with Chloroquine at a final working concentration of 150 μM for 1h at 37°C. Then, cells were stained with 16,66 nM C12FDG for 30 min at 37°C. After washing in PBS, fluorescence was quickly acquired at FACS Canto, and the collected data were analysed using the FlowJo software. An example of the gating strategy used is reported in Figure 29.

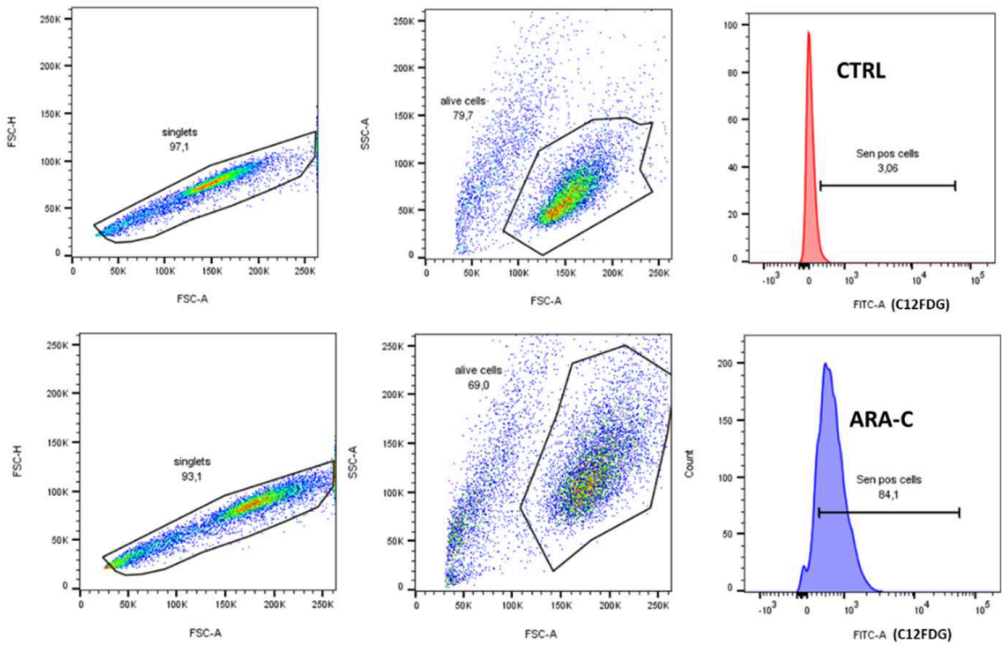


Figure 29. Gating strategy for the fluorescent-β-gal (C12FDG). *Analysis shown are representative flow cytometry plots with the sequential gates. According to physical parameters, live cells are gated upon the inclusion of singlets only. The gate for assessing the percentage of senescent positive cells is made on the control (CTRL).*

ImagestreamX technology for senescence evaluation

Immunofluorescence image-based flow cytometry was conducted on an Amnis ImageStreamX Mark II Imaging Flow Cytometer (Luminex).

The ImageStream system integrates the features of flow cytometry and fluorescence microscopy combined with a modern methodology for image analysis. It specifically allows the study of many cells based on their fluorescence features and provides statistical analysis of these features. It also allows detailed morphometric cellular analysis based on acquired cellular images integrating various morphometric and photometric characteristics of the examined cells.

Alive cells were first stained for fluorescent-SA-β-gal and AnnexinV, then fixed, permeabilised and stained for EdU (Click-it). After the staining, cells were analysed using the gating strategy reported in Figure 31. The events

considered in the analysis were those with a gradient value higher than 60 based on bright-field (BF) images; this gate keeps only the events in focus. Then a second gate was used to obtain just single-cell events; this gate was done on a dot plot with the area in the X-axis and the aspect ratio in the Y-axis. The aspect ratio is used to evaluate the circularity of an event: if the value is near 1, the event is circular; below 0,6 means is something asymmetric. I considered the alive cells (negative for AnnexinV) within the single-cell gate. Finally, I evaluated the percentage of cycling cells by the EdU signal and the senescent cells by the fluorescent-SA- β-gal.

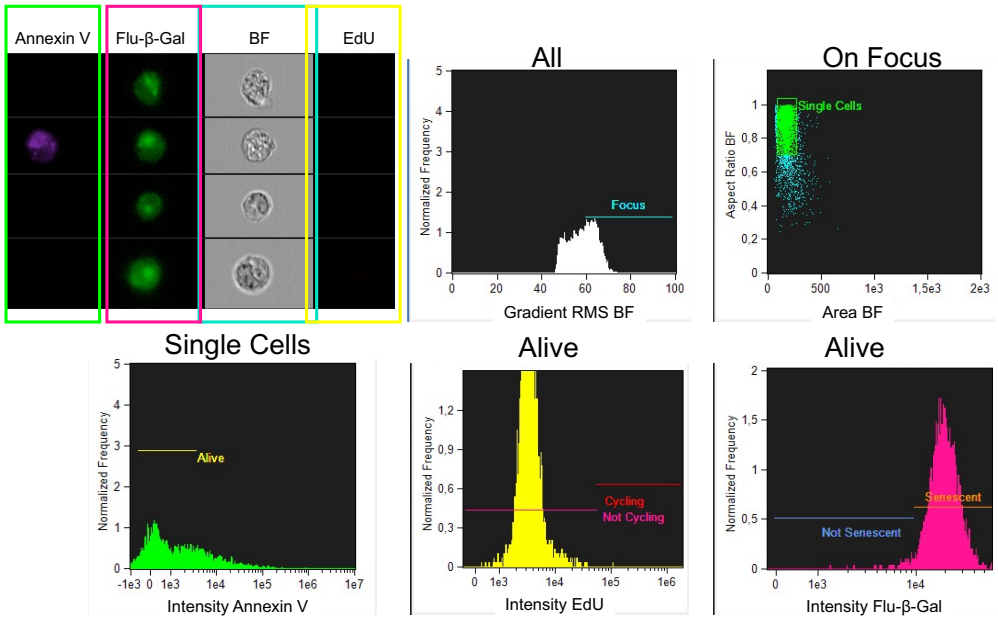


Figure 30. Gating strategy for senescence and proliferation evaluation.

For each marker is possible to measure the intensity and the area occupied in each picture. As described in the introduction, senescent cells become bigger in size and this cause an increase in the basal autofluorescent of the cells. I optimise the staining using senescent K562 leukemic cells after ARA-C treatment to control for autofluorescence of senescent cells. Recorded immunofluorescence intensities were normalised by cell size to minimise interference by differences in autofluorescences between conditions for individual samples, as shown in Figure 31.

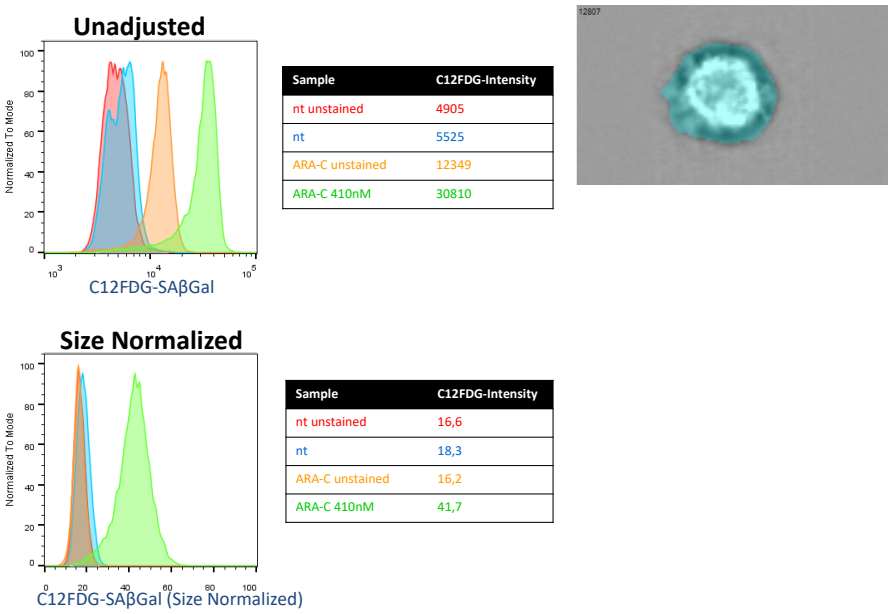


Figure 31. Size normalisation. *The top panel indicate a specific signal in the unstained (without C12FDG) senescent cells. The fluorescent-SA- β-gal signal intensity was divided per cell size measured on the brightfield signal; as reported in the bottom panel, this was sufficient to eliminate the autofluorescence bias.*

Gene expression analysis

Total RNA was extracted from live cells with QIAzol reagent (QIAGEN); after adding chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase. RNA is precipitated from the aqueous phase by adding isopropanol and put into miRNeasy Mini column (QIAGEN) from miRNeasy Mini Kit. After several washes, a DNAse treatment was performed to degrade DNA. The elution was performed with RNAse-free water (30µL/sample). 500 ng of total RNA (as quantified with Nanodrop 8000 device (Thermo Scientific)) was used for retrotranscription using

iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions.

Reaction protocol:

- Priming= 5min at 25°C
- Reverse transcription= 20 min at 46°C
- RT inactivation= 1 min at 95°C
- Hold at 4°C

RT-minus reactions (without reverse transcriptase enzyme) were also prepared. Estimated 25ng of cDNA in 20µl reaction volume were analysed in triplicate by quantitative RT-PCR amplification on a Viia7 Real-time PCR applied biosystem (Lifetechnologist) using SYBR Green assay (Quanti Fast SYBR Green PCR Kit, Qiagen) according to manufacturer's instructions and for 40 cycles. CT-values were obtained by calculating the second derivative using Quant studios RT-PCR software and normalising samples against a housekeeping gene GUSB. RT-minus preparations proved to be negative. Following forward and reverse primers (FP and RP) were designed with Metabion online software against *Homo Sapiens*.

A primer efficiency analysis was performed for all primers to define the correct concentration, according to the Leivac distribution. (M&M from Biavasco et al., 2021).

Target	Optimal concentration of primers	Sequenza fw		Sequenza rev
GUSB	350 nM	CTGACACCTCCAAGTATCCCAAG		GTCGTGTACAGAAGTACAGACCGC
IL1β	100 nM	GCTCAAGTGTCTGAAGCAGCC		CAGCTTCAAAGAACAAGTCATCCT
IL 8	100 nM	CATCTCACTGTGTGTAAACATGAC		CCTTGGCAAAACTGCACCTTCAC
IL1α	120 nM	GGTTGAGTTTAAGCCAATCCA		TGCTGACCTAGGCTTGATGA
IL6	130 nM	GATTCAATGAGGAGACTTGCCTGG		CTCACTACTCTCAAATCTGTTCTGG
CCL2	150 nM	CTGTGATCTTCAAGACCATTGTG		AGTTTGGGTTTGCTTGTCCAG

Apoptosis assay

Leukemic cells were seeded in IMDM or RPMI medium and 10% FBS in the presence of Palbociclib (10µM) or Ara-C (250nM) at 37°C (5% CO2).

At each time point, cells were subjected to flow cytometry experiments after live staining with Annexin V/7AAD staining. This staining was performed using $1x10^5$ cells in 100µL of binding buffer 1x (obtained diluting the stock 10x), adding 5µL of Annexin V and 1µL of 7AAD. After FACS analysis, cells were divided into four populations: alive cells are the double negative population, necrotic cells are 7AAD positive, but Annexin V negative, Annexin V positive cells but 7AAD are in early apoptosis, and the population double positive is composed by cells in late apoptosis. The gating strategy was performed using FMO controls for Annexin V and 7AAD with a sample containing a mix of control and treated cells. Stained cells were analysed at the CANTO flow cytometer, and data were analysed using the FlowJo program.

Multiparametric Flow Cytometry

For immunophenotypic analysis, a maximum of $0.5x10^5$ cells per tube were stained in 100 µl of 1X PBS, 2% FBS, plus the mix of antibodies. Staining was performed at RT for 15 minutes, followed by washing with 2 ml of 1X PBS and 2% FBS before the analysis. Here, the complete list of antibodies: human CD45 PE-Cy7, CD33 BV510, HLA-ABC Pacific Blue (Clone W6/32), HLA-DR FITC (Clone L243), Annexin V PB (640918), 7-AAD (420403) CD4 PE (RPA-T4), all from Biolegend (San Diego, California, US); Annexin V PE (556422), CD3 PE (UCHT) from BD medical (Franklin Lakes, New Jersey, U.S.). CellTrace™ Violet (CTV) Cell Proliferation Kit from Thermo Fisher Scientific (Waltham, MA, USA) was also used to track T cell proliferation. All antibodies were tested on cell lines reported to be positive for the markers of interest and titrated for optimal on-target/off-target activity. At least 200.000 events in the live cell gate were acquired per sample. The effects of ARA-C and CDK 4/6i treatment on senescence induction and the expression of HLA-ABC/DR on leukemic cells and T cell proliferation were assessed using Canto flow cytometer (BD Biosciences). Data were processed using FlowJo version 10.8.1 (Tree Star). The gating

strategy used to assess the cell surface expression of HLA-DR/HLA-ABC is reported in Figure 32.

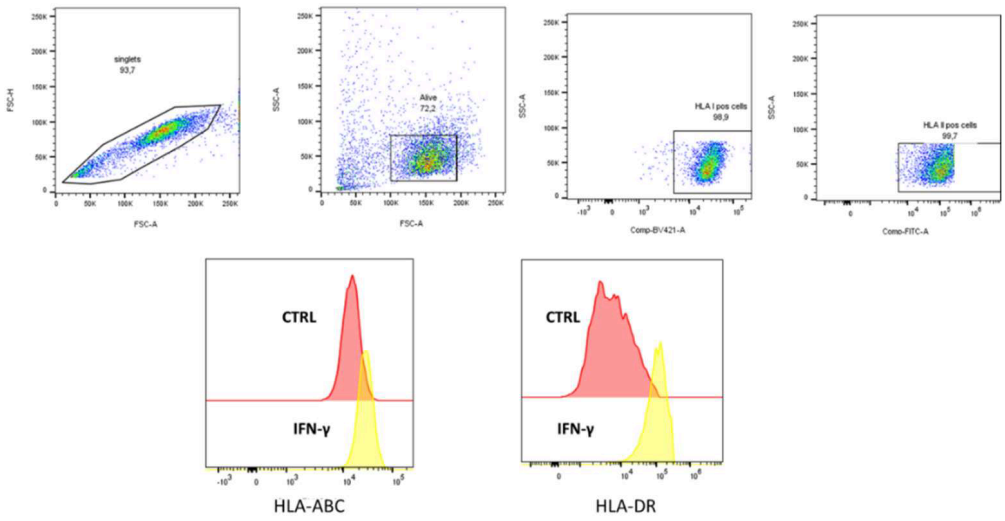


Figure 32. . Gating strategy for the immunophenotypic analysis of the expression of HLA-ABC/DR. *The Figure shows representative flow cytometry plots with the sequential logical gates. According to physical parameters, live cells are gated upon the inclusion of singlets only.*

Cell lysate preparation

Cells were collected by trypsinisation and were rinsed once in PBS. Pellets were lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 % Triton X-100 and protease inhibitors described above) and incubated for 30 min on ice. Whole-cell extracts were clarified by centrifugation at 12000 rcf for 10 min at 4 °C in a benchtop centrifuge. The protein concentration of the clarified lysates was estimated using the BCA method (Pierce Thermofisher, 23225). An equal amount of proteins was used for western blot analysis, as explained below.

Western blotting analysis

Cells were collected, and total proteins extraction was performed by homogenising and lysing in sample buffer (0,1 M Tris pH 6.8, 20% Glycerol, 2% SDS). The homogenates were then passed through a syringe and boiled at 95°C for 5min twice. After protein extraction, 25µg of whole-cell lysate was boiled and, after adding 4x Laemli Sample Buffer completed with β-mercaptoethanol, were resolved by SDS-PAGE. Resolved proteins were then transferred to nitrocellulose membranes (Amersham) using Biorad electrophoresis systems. After transfer, membranes were incubated with skimmed milk (Regilait) at 5% for 1 hour. Membranes were probed with primary antibodies in 5% or 2,5% skimmed milk (Regilait). The following primary antibodies were used:

α-Target	Optimal Dilution	animal	Vendor	catalog number	Residue
γH2AX	1:2000	mouse	Biolegend	613402	ser139
TOT H3	1:1000	rabbit	abcam	ab1791	
TOT p53	1:200	mouse	santa cruz	sc-126	
p21	1:1000	mouse	BD PharmingenTM	556430	
NFκB	1:1000	rabbit	Cell Signalling Inc.	#8242	
pNFκB	1:1000	rabbit	Cell Signalling Inc.	#3033	ser536

After incubation with primary antibodies, three washes with TBS-T were performed. Incubation with HRP-coupled secondary antibodies (Sigma-Aldrich) was carried out for 1 hour. After the other three washes with TBS-T, ECL PlusTM Western Blotting Detection Reagents was added for 5 min. After 5 minutes, the Chemidoc instrument (Biorad) was used for signal detection. ImageJ program was used to quantify Figures obtained from the Chemidoc instrument.

Mixed lymphocyte culture reaction (MLR)

Through magnetic beads separation, $CD4^+$ T cells were selected from peripheral blood mononuclear cells of HLA-disparate healthy individuals. We used the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec GmbH) to obtain pure, untouched T cells. Then, $CD4^+$ T cells were obtained by further depleting $CD8^+$ T cells, taking advantage of the anti-human $CD8^+$ microbeads kit (Miltenyi Biotec GmbH). Purified T cells were stimulated

with leukemic blasts at diagnosis at an effector: target ratio of 1:2. Cells were cultured in IMDM supplemented with 1% L-glutamine, 1% penicillin/streptomycin (P/S), 10% HS (Euroclone) and IL-2 (Novartis) at a final concentration of 150 UI/ml. IL-2 was replaced every 3–4 days, and responders were re-stimulated every 7 days for a total of 14 days. After the second round of stimulation, $CD4^+$ T cells from MLR responders were tested against targets of choice for proliferation, as reported below. (M&M from Gambacorta et al., 2022).

Functional assays for T cell proliferation evaluation

T cell proliferation was measured by dilution of Cell Trace Violet (Thermo Fisher). This fluorescent dye readily crosses the plasma membrane and covalently binds inside cells, stably marking them. Effector cells isolated from healthy donors were resuspended at the concentration of 1×10^6 cells/mL in 1× PBS and stained with Cell Trace Violet for 20 minutes at 37°C. At the end of incubation, cells were washed with pre-heated IMDM added with 10% FBS, 1% L-glutamine and 1% pen/strep (P/S). Cell Trace Violet staining was checked via flow cytometry. For the evaluation of T cell proliferation against target cells, stained $CD4^+$ T cells were co-cultured with untreated target cells, pre-treated for 7 days with ARA-C (250nM), and pre-treated for 48h with IFN-ɣ (200 U.I./mL) at an effector to target ratio of 1:1 in IMDM added with 10% FBS, 1% L-glutamine and 1% pen/strep (P/S) and IL2 at the final concentration of 60 UI/mL. In addition, primed $CD4^+$ T cells from MLR were used as a positive control. After 7 days, cells were collected and stained for flow cytometry.

An example of the gating strategy used to assess T cell proliferation is shown below. (M&M from Gambacorta et al., 2022).

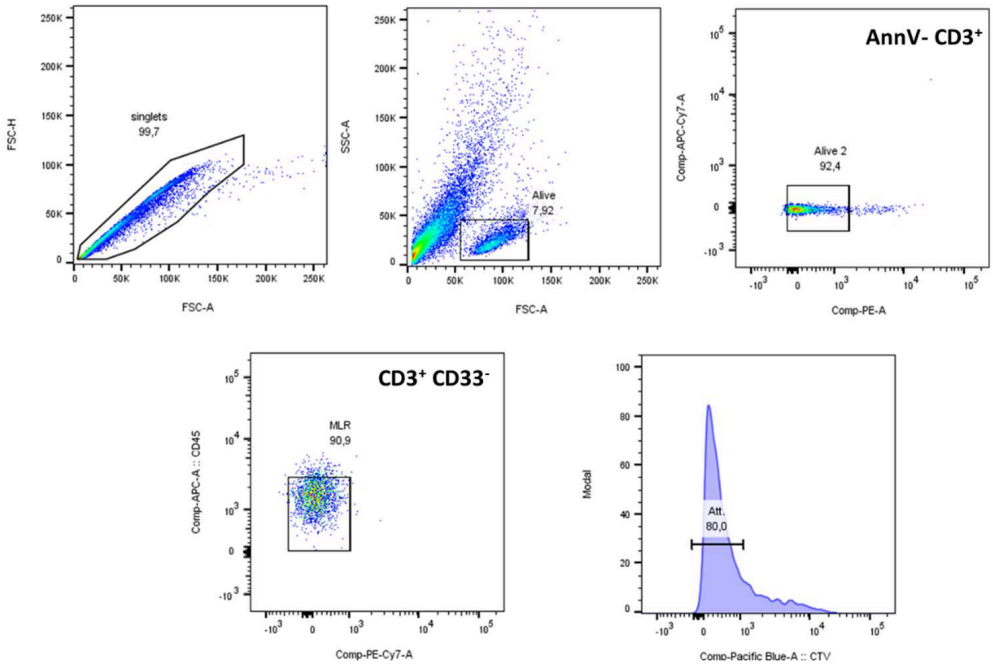


Figure 33. Gating strategy for T cell proliferation. *Shown are representative flow cytometry plots with the sequential gates. According to physical parameters, live cells are gated upon the inclusion of singlets only. T lymphocytes were defined as the AnnV-CD3+CD33- population. Negative control with only T cells was used to determine the autofluorescence of the cell surface markers of interest.*

Immunological synapses identification

After 4h from the start of the co-culture between T cells and leukemic cells, C12FDG staining was performed. In addition, anti-$CD3^+$ and anti-$CD33^+$ were added to distinguish leukemic cells from T cells. Cell were then fixed in PFA 2% and stained with phalloidin to detect actin filaments. Data acquisition (10.000 cells per sample) was performed with an ImageStream system (Amnis), and data were analysed with IDEAS 3.0 software.

To find the contact zone between blasts and T cells, total events were gated on true leukemic cell/ T-cell pairs (doublets positive for both CD33 and CD3 signals). Single colour controls were collected and used to calculate a spectral crosstalk compensation matrix. The area of the immunological synapse was then identified in the two-cell conjugates using two

independent algorithmic functions. The Valley function identifies the dimmest area between two nuclei, and the Interface function identifies the point of contact between two cells and selects only the ($CD3^+$) T cell portion of the T cell/blast synapse (Ahmed *et al.*, 2009). Then, a mask to detect the enrichment of actin signal in the interface of two cells was designed.

RNA sequencing analysis

High-quality reads obtained after quality inspection and adapter trimming with Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) were aligned to the human genome (Gencode hg38) with STAR release 2.7.6a (Dobin, 2013). Gene expression quantification was obtained with featureCounts v2.0.1 (Liao, Smyth and Shi, 2014). Raw reads were normalised by library size, and the differential expression (DE) analysis was performed on the normalised count matrix with DESeq package (Love, Huber and Anders, 2014), testing for the contrast treatment vs control (ARA-C vs CTRL), and taking into account the paired design of the experiment (*i.e.,* PZ1_CTRL, PZ1_ARA-C). Finally, adjusted *P* values for multiple hypothesis corrections were used to retain significant genes (FDR < 0.05). The Gene Set Enrichment Analysis (GSEA) was performed for the functional annotation using the cluster profile package (Wu *et al.*, 2021) Genes were ranked according to the LogFC. The analysis was performed taking into account the MSigDB database (Hallmark signatures lists) and retaining results with FDR < 0.05. We performed a multi mapping approach to detect HLA transcripts (class I and II), considering the following parameters for the alignment with STAR '--winAnchorMultimapNmax 100 --outFilterMultimapNmax 100', and for the expression quantification with featureCounts '-M --fraction'. The differential expression analysis was performed with DESeq as above. RNA-seq analysis of TE families was performed on high-quality reads, with SalmonTE (Jeong *et al.*, 2018), which adopts Repbase as an annotation file. SalmonTE was used with the option counts for the quantification and the

stranded library parameter. The logFC for TEs were obtained using the DESeq package in R. Deregulated TE families were identified calculating an average logFC from TE copies within a family, computing a one-sample t-test, and considering a p-value <= 0.05.

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